Drug resistance and drug action in

Trypanosoma congolense

by

Andrew S. Peregrine

Submitted to The University of Glasgow

August 1996

For the Degree of

Doctor of Veterinary Medicine

in the

Faculty of Veterinary Medicine

Dedication

To my Mother and Father for their love and support
during many years of work in Africa.

Declaration

I declare that the work reported in this thesis was carried out by myself except for contributions by the under-mentioned people. The extent of their contributions is indicated.

Chapter 1

All the work described in this chapter was carried out by myself.

Chapter 2

The data analysis methodology described in Sections 2.1 and 2.3 for obtaining an estimate of the prevalence of drug-resistant infections in cattle under field conditions was developed by Dr. G.J. Rowlands and myself. Interpretation of field results described in these two Sections was carried out by Dr. G.J. Rowlands and myself.

Experiments described in Sections 2.2, 2.4 and 2.5, and the laboratory work described in Section 2.3, were designed, organised and supervised by myself, with technical assistance provided by Messrs. V. Codjia, W. Mulugeta, G. Ndoutamia and V. Codjia, respectively. Molecular karyotype analyses described in Sections 2.2 and 2.4 were supervised by Dr. P.A.O. Majiwa. Isometamidium-uptake experiments described in Section 2.4 were supervised by Dr. J. Wilkes. Interpretation of all laboratory work was carried out by myself, with input from Drs. Majiwa and Wilkes for the work they supervised.

The papers given in Sections 2.2, 2.3, 2.4 and 2.5 were written by myself. The paper in Section 2.1 was written primarily by Dr. Rowlands, with input from myself.

Chapter 3

All experiments described in Sections 3.1, 3.2 and 3.3 were designed, organised and supervised by myself, with technical assistance provided by Mr. M. Mamman. The cannulation method described in Section 3.1, for repeated sampling of cerebrospinal fluid in goats, was developed by myself. For all three publications, I interpreted the results and wrote the paper.

Chapter 4

Experimental work described in Sections 4.1, 4.2, 4.3, 4.4 and 4.5 was designed, organised and supervised by myself. Technical assistance was provided for Section 4.1 by Dr. R.S. Silayo; for Sections 4.2, 4.3 and 4.4 by Mr. M. Mamman; and for

Section 4.5 by Mr. E.M.E. Burudi. Characterisation of anti-trypanosome antibody responses in Sections 4.2 and 4.3 was supervised by Mr. J. Katende and Dr. D.J.L. Williams, respectively. Molecular biology techniques described in Section 4.5 were supervised by Dr. N.B. Murphy. Modelling supervision for Section 4.4 was provided by Prof. G. Gettinby. Interpretation of data was carried out by myself, with input from Mr. Katende, Drs. Williams and Murphy, and Prof. Gettinby for the work they supervised. All five papers were written by myself with input from the aforementioned scientists that provided supervision.

Chapter 5

All experiments described in Section 5.1 were designed, organised and carried out by myself, with technical assistance provided by Ms. A.I. Ibitayo. I interpreted the results and wrote the paper.

Section 5.2 describes a series of experiments that used the autofluorescent property of isometamidium to characterise the interaction of the compound with a drug-sensitive clone of *T. congolense*. The work was designed and carried out by Dr. D. Zilberstein, with supervision from myself. The paper was written by Dr. Zilberstein and myself.

Section 5.3 describes the production and characterisation of anti-isometamidium monoclonal antibodies. This work was originally designed, organised and supervised by myself. The first set of monoclonal antibodies to isometamidium was produced by myself, with technical assistance from Messrs. J. Katende and L.D.B. Kinabo. The second set of described monoclonal antibodies was produced by Ms. E. Gault with technical assistance from Dr. J.N. Flynn and Mr. M. Eisler. Characterisation of all the antibodies and interpretation of the data was carried out by myself and Mr. Eisler. The paper was written by myself, with input from Mr. Eisler.

The original ideas leading to the work in Section 5.4 were conceptualised by myself. All experiments described in Section 5.4 were supervised by myself, but jointly designed and carried out with Mr. C. Wells. Interpretation of the results and writing of the paper was carried out jointly with Mr. C. Wells.

The preparation of this thesis, in its entire drafting, typing, composition and printing, was carried out by myself on a Gateway 2000 (P4D-501) Personal Computer using Word 6.0.

Andrew S. Peregrine

Statement on conduct of the experimental work

All of the laboratory work described in this thesis, except for part of the work described in Section 5.3, was carried out at the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya: renamed the International Livestock Research Institute (ILRI) in January 1995. Section 5.3, in part, describes work that was carried out in the Departments of Veterinary Physiology and Veterinary Pharmacology at The University of Glasgow Veterinary School, Glasgow, Scotland.

All the experimental work in this thesis was carried out as part of my duties as an employee of ILRAD and ILRI between 1988 and 1995. The objectives of these studies were to improve the control of trypanosomiasis in domestic livestock by more rational use of chemotherapy and chemoprophylaxis, and to determine the molecular basis of drug action and drug resistance in *Trypanosoma congolense* in order to potentially develop a rapid, biochemical-based, diagnostic for drug resistance.

Acknowledgements

I wish to acknowledge the help and support I have received from the following people that were associated with the submission of this thesis:

- Professor Max Murray of the Department of Veterinary Medicine, The University of Glasgow, for willingly taking on the responsibilities of being my D.V.M. Advisor.
- Dr. A.R. Gray, Director of the International Laboratory for Research on Animal Diseases, for encouraging my interest and research on chemotherapy and drug resistance in pathogenic African trypanosomes.
- Drs. Noel Murphy and Jon Wilkes of the International Livestock Research Institute, Nairobi, and Professor George Gettinby of the Department of Statistics and Modelling Science, University of Strathclyde, for discussion and encouragement.
- The following higher-degree students who have been a pleasure to supervise:
  (a) Dr. Mohammed Mamman, Ahmadu Bello University, Zaria, Nigeria.
  (b) Dr. Victor Codjia, Division Trypanosomiase Animale, Cotonou, Benin.
  (c) Dr. Guelmbaye Ndoutamia, Laboratoire de Farcha, Ndjamena, Tchad.
  (d) Dr. Wubet Mulugeta, Addis Ababa University, Ethiopia.
  (e) Dr. Mboya Burudi, University of Nairobi, Kenya.
- Mr. Clive Wells for all the technical assistance provided with electron microscopy techniques to identify the localisation of isometamidium in *Trypanosoma congolense*.
- Mr. Sammy Kemei for his technical assistance throughout almost all of the studies.
- Mr. David Elsworth for the provision of graphics assistance.

Contents

	Page
Dedication	ii
Declaration	iii
Statement on conduct of the experimental work	v
Acknowledgements	vi
Contents	vii
Abbreviations	x
Summary	xi
Chapter 1: Overview of the current status of chemotherapy and chemoprophylaxis for trypanosomiasis in domestic livestock	1
1.0 Introduction	2
1.1 Peregrine, A.S. (1994) Chemotherapy and delivery systems: haemoparasites. *Veterinary Parasitology* 54, 223-248.	4
1.2 Discussion	31
Chapter 2: Studies on resistance to anti-trypanosomal compounds in trypanosome populations in the field, and the contribution of cross-resistance to the resistance phenotype	37
2.0 Introduction	38
2.1 Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M. and Peregrine, A.S. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. *Acta Tropica* 53, 135-150.	42
2.2 Codjia, V., Mulatu, W., Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authié, E., d'Ieteren, G.D.M. and Peregrine, A.S. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. *Acta Tropica* 53, 151-163.	59

2.3 Peregrine, A.S., Mulatu, W., Leak, S.G.A. and Rowlands, G.J. (1994) Epidemiology of bovine trypanosomiasis in the Ghibe valley, Ethiopia: multiple-drug resistance and its effective control. In *Proceedings of the 7th International Symposium on Veterinary Epidemiology and Economics*, 15th-19th August, 1994, Nairobi, Kenya. *The Kenya Veterinarian* 18, 369-371. 73

2.4 Mulugeta, W., Wilkes, J., Mulatu, W., Majiwa, P.A.O., Masake, R. and Peregrine, A.S. Long-term occurrence of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium in cattle at Ghibe, Ethiopia. Submitted to *Acta Tropica.* 77

2.5 Ndoutamia, G., Moloo, S.K., Murphy, N.B. and Peregrine, A.S. (1993) Derivation and characterization of a quinapyramine-resistant clone of *Trypanosoma congolense*. *Antimicrobial Agents and Chemotherapy* 37, 1163-1166. 104

2.6 Discussion 109

Chapter 3: Role of invasion of the central nervous system in expression of resistance to diminazene 120

3.0 Introduction 121

3.1 Peregrine, A.S. and Mamman, M. (1994) A simple method for repeated sampling of lumbar cerebrospinal fluid in goats. *Laboratory Animals* 28, 391-396. 126

3.2 Mamman, M., Moloo, S.K. and Peregrine, A.S. (1994) Relapse of *Trypanosoma congolense* infection in goats after diminazene aceturate is not a result of invasion of the central nervous system. *Annals of Tropical Medicine and Parasitology* 88, 87-88. 133

3.3 Mamman, M. and Peregrine, A.S. (1994) Pharmacokinetics of diminazene in plasma and cerebrospinal fluid of goats. *Research in Veterinary Science* 57, 253-255. 136

3.4 Discussion 140

Chapter 4: Effect of duration of infection and size of trypanosome population on the sensitivity of trypanosomes to diminazene *in vivo* 146

4.0 Introduction 147

4.1 Silayo, R.S., Mamman, M., Moloo, S.K., Aliu, Y.O., Gray, M.A. and Peregrine, A.S. (1992) Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate. *Research in Veterinary Science* 53, 98-105. 152

4.2 Mamman, M., Katende, J., Moloo, S.K. and Peregrine, A.S. (1993) Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology* 50, 1-14. 161

4.3 Mamman, M., Williams, D.J.L., Murphy, N.B. and Peregrine, A.S. (1995) Apparent rarity of diminazene-resistant trypanosomes in goats infected with a diminazene-resistant population of *Trypanosoma congolense*. *Research in Veterinary Science* 58, 113-118. 176

4.4 Mamman, M., Gettinby, G., Murphy, N.B., Kemei, S. and Peregrine, A.S.(1995) Frequency of diminazene-resistant trypanosomes in populations of *Trypanosoma congolense* arising in infected animals following treatment with diminazene aceturate. *Antimicrobial Agents and Chemotherapy* 39, 1107-1113. 183

4.5 Burudi, E.M.E., Peregrine, A.S., Majiwa, P.A.O., Mbiuki, S.M. and Murphy, N.B. (1994) Response of diminazene-resistant and diminazene-susceptible *Trypanosoma congolense* to treatment with diminazene when occurring as a mixed infection in goats. *Annals of Tropical Medicine and Parasitology* 88, 595-606. 191

4.6 Discussion 204

Chapter 5: Molecular basis of isometamidium's activity 214

5.0 Introduction 215

5.1 Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* 102, 93-100. 221

5.2 Zilberstein, D., Wilkes, J., Hirumi, H. and Peregrine, A.S. (1993) Fluorescence analysis of the interaction of isometamidium with *Trypanosoma congolense*. *Biochemical Journal* 292, 31-35. 230

5.3 Peregrine, A.S., Eisler, M.C., Katende, J., Flynn, J.N., Gault, E.A., Kinabo, L.D.B. and Holmes, P.H. (1994) Generation of monoclonal antibodies to the anti-trypanosomal drug isometamidium. *Hybridoma* 13, 289-294. 236

5.4 Wells, C., Wilkes, J. and Peregrine, A.S. (1995) Use of fluorescence, immunoelectron-microscopy and autoradiography techniques to localise Samorin® in *Trypanosoma congolense*. In *Proceedings of the 23rd Meeting of the International Scientific Council for Trypanosomiasis Research and Control*, 11th-15th September, 1995, Banjul, The Gambia. OAU/STRC Publication, Abstract P527. 243

5.5 Discussion 261

Chapter 6: General discussion and conclusions 269

Abbreviations

AUC_{0-48h}	area under the concentration-time curve from 0 to 48 hours after treatment
$AUMC_{0-48h}$	area under the first moment of the concentration-time curve from 0 to 48 hours after treatment
bp	base pair
b.w.	body weight
CD_{50}	50% curative dose
cDNA	complementary deoxyribonucleic acid
Cl	total body clearance
C_{max}	maximum drug concentration
CNS	central nervous system
CSF	cerebrospinal fluid
DIC	differential interference contrast microscopy
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
IL	International Livestock Research Institute stabilate number
ILRAD	International Laboratory for Research on Animal Diseases
ILRI	International Livestock Research Institute
kDNA	kinetoplast DNA
kg	kilogram
µg	microgram
M	molar
Mab	monoclonal antibody
mg	milligram
ml	millilitre
mM	millimolar
nm	nanometer
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	packed red-blood cell volume
pg	picogram
t_{max}	time taken to reach C_{max}
U.V.	ultraviolet
$V_{d(ss)}$	apparent volume of distribution at steady state

Summary

Trypanosomiasis occurs in domestic livestock in 37 countries in sub-Saharan Africa, and within most of these countries appears to be the most important disease constraint to livestock production. The disease is also a significant constraint to livestock production in parts of South America, the Middle East and the Far East.

In the absence of a vaccine for trypanosomiasis, administration of anti-trypanosomal compounds to livestock and control of the tsetse-fly vector are the primary methods used to control the disease in sub-Saharan Africa. However, because vector control is limited in application, chemotherapy and chemoprophylaxis are the predominant methods used to control the disease in most countries. At present the salts of isometamidium, homidium and diminazene are the only compounds recommended for use in cattle, sheep and goats. All three compounds have been on the market for at least 35 years and drug resistance appears to be an increasing problem. In contrast, the literature reviewed at the beginning of this thesis indicates that drug resistance does not appear to be a problem associated with the compounds recommended for use against the parasites causing theileriosis, babesiosis, anaplasmosis and cowdriosis, even though some of them have been used for as long as the anti-trypanosomal compounds. Reasons for the difference in incidence of drug resistance associated with these two groups of compounds appear to be differences in drug pressure and pathogenesis of the diseases (Chapter 1).

In work to characterise the epidemiology of drug-resistant trypanosomes, a mathematical model was developed to analyse longitudinal parasitological data from the field for evidence of drug resistance. Application of the model to data from cattle in the Ghibe valley, Ethiopia, indicated that between 1986 and 1989 there was a significant, and increasing, prevalence of diminazene-resistant *Trypanosoma congolense* infections. *In vivo* characterisation of the drug sensitivity of trypanosome isolates collected from cattle at Ghibe in 1989 confirmed this conclusion. In additional work, more than 90% of the same isolates were also shown to be resistant

in cattle to recommended doses of isometamidium chloride and homidium chloride. Furthermore, cloned populations derived from one of these isolates were shown to express the multiple-resistance phenotype. This therefore indicated that there was a high prevalence of multiple-drug resistant infections, and that for at least one population this phenotype was associated with a single infection (Chapter 2).

The origin of the multiple-drug resistance at Ghibe is unclear. However, in light of work described in this thesis, and elsewhere, it appears most likely to be due to cross-resistance associated with the development of resistance to quinapyramine.

In 1993 a second set of trypanosome isolates was collected from cattle at Ghibe. As before, greater than 90% of the isolates (all *T. congolense*) were resistant in cattle to recommended doses of diminazene aceturate, isometamidium chloride and homidium chloride, and a high level of genetic heterogeneity was observed amongst the populations. Thus, the multiple-resistance phenotype appeared to have remained stable over a 4-year period (Chapter 2).

Invasion of the CNS has been shown to result in apparent drug resistance with some populations of *T. b. brucei*, *T. vivax* and *T. simiae*. However, it was unclear whether such a phenomenon was associated with *T. congolense* when it occurred as a single infection in ruminants. A cannulation technique was therefore developed for repeated collection of CSF in goats, in which a catheter was placed surgically in the subarachnoid space between the sixth and seventh lumbar vertebrae. Volumes of CSF in excess of 1.0 ml could then be obtained readily, several times daily, for up to 6 weeks. In further work the technique was used to examine CSF from goats infected with *T. congolense* IL 3274 to determine if the population enters the CNS. *Trypanosoma congolense* IL 3274 is a cloned population that is resistant in goats to recommended doses of diminazene aceturate when administered after the development of parasitaemia; treatment results in a period of aparasitaemia followed by the reappearance of trypanosomes in the blood of all animals after approximately 7 days following administration of diminazene aceturate. Lumbar CSF was collected from seven goats throughout an infection with this clone until 3 days after

trypanosomes reappeared in the bloodstream following treatment with diminazene aceturate. However, neither intact trypanosomes nor trypanosomal antigen were detected in any sample. Thus, reappearance of trypanosomes in the bloodstream following drug treatment did not appear to be associated with invasion of the CNS (Chapter 3). Invasion of trypanosomes into other "drug-inaccessible" sites awaits future evaluation.

Application of the cannulation technique to characterise the pharmacokinetics of diminazene in goat CSF indicated that concentrations of the molecule in CSF were 3-4 times lower than those occurring simultaneously in plasma. Furthermore, the kinetics of the drug in these two fluids differed significantly with respect to C_{max}, t_{max}, $AUC_{0\text{-}48h}$, $AUMC_{0\text{-}48h}$, Cl and $V_{d(ss)}$. Collectively, the data indicate that if trypanosomes did enter CSF they would be less likely to be eliminated by diminazene than trypanosomes present within plasma (Chapter 3).

Some researchers have concluded that multiple administration of anti-trypanosomal compounds may enhance their therapeutic efficacy. To investigate this hypothesis, which could have practical application for controlling drug-resistant infections, goats were infected with *T. congolense* IL 3274 and treated at the first peak of parasitaemia with diminazene aceturate at a dose of 7.2 mg/kg b.w. on two occasions, 8 or 24 hours apart. Since both regimens resulted in only a slight improvement in diminazene's therapeutic efficacy, compared to single treatment with the same dosage, these regimens can not be recommended for use in the field for treating trypanosome infections with the same, or greater, level of resistance as *T. congolense* IL 3274 (Chapter 4).

In further studies that were carried out to characterise the epidemiology of drug-resistant trypanosomes, initial work focused on the stability of diminazene resistance in *T. congolense* in goats during the early stages of tsetse-transmitted infections. Experiments with *T. congolense* IL 3274 indicated that while infections in all animals were susceptible to treatment with diminazene aceturate 24 hours following initiation of infection, all infections were resistant to treatment with the same drug

dosage on day 19 of infection. Additional work showed that the major decrease in drug sensitivity occurred between day 1 and day 4. This apparent difference in drug sensitivity could be explained by differences in the drug sensitivity of the parasite life-cycle stages present in goats on these two days, differences in the pharmacokinetics of diminazene in tissue fluid and lymph, or instability of resistance to diminazene in the parasites. When the same experiment was repeated using tsetse flies infected with a subpopulation of *T. congolense* IL 3274 that reappeared in goats following treatment with diminazene the same result was obtained as before, i.e., infections were fully sensitive to diminazene on day 1 but resistant to the drug on day 19 of infection. Thus, either tsetse transmission resulted in reversion of the trypanosomes' drug sensitivity to that of the parental population or the diminazene sensitivity of the trypanosome population was not affected by application of drug pressure (Chapter 4).

In a follow-up experiment, the frequency with which diminazene-resistant trypanosomes occur in bloodstream parasitaemias before and after treatment with diminazene was evaluated in goats infected with *T. congolense* IL 3274. Surprisingly, the frequency appeared to be less than $1{:}10^3$ parasites, both before and after treatment. Thus, the majority of trypanosomes which reappear in the bloodstream following drug treatment appear to be sensitive to the dosage of diminazene aceturate that was used. Since this observation could be associated with an ability of diminazene-sensitive trypanosomes to survive treatment when occurring simultaneously with diminazene-resistant parasites, an experiment was carried out in which goats were infected simultaneously with *T. congolense* IL 3274 and an unrelated drug-sensitive clone of *T. congolense* (IL 1180). At the first peak of parasitaemia the goats were treated with diminazene aceturate, and the trypanosome population that reappeared in the bloodstream thereafter was examined using a PCR technique that was specific for the drug-sensitive population. On the basis of the sensitivity of this method (a DNA quantity equivalent to 13 trypanosomes), DNA from the drug-sensitive clone could not be detected in any sample collected up to 60 days following treatment. This therefore suggested that the drug-sensitive trypanosomes occurring in trypanosome parasitaemias that develop in animals

following treatment are not present in animals at the time of treatment but arise subsequent to treatment (Chapter 4).

The estimate of the proportion of diminazene-resistant trypanosomes in goats infected with a "drug resistant" trypanosome clone, given above, was a rough approximation since only a relatively small number of animals could be used. In order to obtain an estimate with a higher level of accuracy a mouse model system was developed in which parasitaemia profiles simulated those observed in goats following treatment with diminazene aceturate. As with goats, diminazene-resistant trypanosomes were shown to constitute a very small proportion of the trypanosome parasitaemias that reappeared in mice after treatment. Furthermore, when trypanosomes that reappeared in mice after treatment were characterised for their sensitivity to the same drug dosage, the proportion of trypanosomes resistant to this dosage was not a fixed constant for different population sizes. Instead, maximum likelihood estimates for the proportion of drug-resistant trypanosomes appeared to vary inversely with the population size. Since the susceptibility of cells to diminazene has been shown to be associated with the cells' stage within the cell-division cycle, these results could be associated with different proportions of trypanosomes in different population sizes being in different stages of the division cycle (Chapter 4).

In a final set of experiments, work was undertaken to characterise the molecular basis of isometamidium's action, and resistance to the compound, in *T. congolense*. Initial experiments *in vivo* demonstrated significant variation in resistance to the compound amongst nine clones derived from a stock of *T. congolense*. In addition, significant variation in resistance to isometamidium was also shown amongst nine clones derived from one of these clones. This therefore suggested that at least a component of the genetic basis of resistance to isometamidium is unstable. It also indicated the importance of using cloned populations for studies on the molecular basis of drug action and drug resistance.

In the first of a series of cell biology studies on isometamidium's mode of action, the autofluorescent property of the compound was used to characterise its interaction with drug-sensitive trypanosomes. Changes in the fluorescence of the molecule that occur upon incubation with *T. congolense* IL 1180 were shown to be due to interaction with an intracellular component(s). Furthermore, the effects of digitonin and N-ethylmaleimide on this interaction indicated that the compound is transported into trypanosomes via a plasma membrane-associated protein carrier (Chapter 5).

In order to identify the organellar localisation of isometamidium after uptake into *T. congolense* IL 1180, initial work focused on immunolocalisation of the molecule using an anti-isometamidium Mab that was produced by immunising mice with an isometamidium-human serum albumin conjugate. Sections of *T. congolense* IL 1180 bloodstream forms, produced after incubation of viable trypanosomes with isometamidium, were examined with the Mab in immunoelectron microscopy. High levels of labelling were observed in the kinetoplast and mitochondrion relative to the nucleus. Furthermore, relatively low levels of labelling were observed in endocytic organelles. However, since the technique could only detect drug in trypanosomes after 30 minutes incubation at 37°C, and high background labelling at this time point indicated that the cells were overloaded with isometamidium, definitive conclusions about drug localisation could not be made. As a result, an electron-microscopy autoradiography technique was developed that allowed one to characterise uptake of tritiated Samorin in *T. congolense* IL 1180 bloodstream forms after incubation at 37°C for periods as short as 15 seconds. At incubations lasting between 15 seconds and 5 minutes a signal was only detected within the kinetoplast. This would therefore suggest that isometamidium's primary site of action is within this organelle. Finally, electron-microscopy autoradiography failed to demonstrate an association of the drug with endocytic vesicles, confirming that endocytosis via the flagellar pocket does not play a major role in uptake of isometamidium by trypanosomes (Chapter 5).

The primary objective of research on the molecular basis of action of isometamidium is to use the information, thereafter, to help define the molecular

basis of resistance to the compound. The resultant data may then be used to develop a biochemical-based methodology that can rapidly quantify the level of isometamidium resistance in large numbers of trypanosome isolates. Such a methodology would allow one to more quickly and more accurately determine the prevalence of different drug-resistance phenotypes than is possible with the currently available methods. As a result, the most efficacious anti-trypanosomal compound for a given situation could be identified more quickly than is possible at present. However, in situations where there is a high prevalence of multiple-drug resistant trypanosome populations it was unclear, at the beginning of the work described in this thesis, whether there was an appropriate method for controlling such infections. Thus, because the aforementioned work in cattle at Ghibe demonstrated a very high prevalence of multiple-drug resistant trypanosome infections (Chapter 2), that the multiple-drug resistance was associated, at least in part, with single rather than mixed infections, and that the disease was a constraint to livestock production, the impact of a tsetse-control programme using deltamethrin-impregnated "targets" was evaluated when combined with treatment of individual cattle with diminazene aceturate. Subsequent to implementation of this control programme the relative density of *Glossina pallidipes*, the main vector at Ghibe, decreased from a mean of 1.9 flies/trap/day in the 12-month period preceding establishment of the targets to a mean of 0.4 flies/trap/day in the 12-month period afterwards. In association with the reduced trypanosome challenge the apparent prevalence of *T. congolense* infections in cattle fell from approximately 30% to 5% over the same period. Thus, even when there is a high prevalence of multiple-drug resistant infections, tsetse control integrated with chemotherapy can be used to reduce the apparent prevalence of infections in cattle (Chapter 2).

In summary, drug resistance in trypanosomes appears to be an increasing problem associated with the current anti-trypanosomal compounds recommended for use in domestic livestock. Since it appears unlikely that new compounds will be developed in the near future, the long-term productivity of livestock in trypanosomiasis-endemic areas is dependent, at least in the near future, on maintaining the efficacy of these compounds. In this respect, the research described in this thesis was carried

out, firstly, to develop methods to more accurately determine the prevalence and level of drug resistance in the field than is possible with other techniques. Secondly, much of the described research was carried out to characterise aspects of the epidemiology of drug-resistant infections, including definition of the role of various factors in development, maintenance and abrogation of drug-resistant infections in the field. Finally, a number of potential methods for controlling drug-resistant trypanosome infections were evaluated *in vivo*. In particular, integration of chemotherapy with vector control was shown to be a viable method for controlling multiple-drug resistant infections in cattle.

Chapter 1

Overview of the current status of chemotherapy and chemoprophylaxis for trypanosomiasis in domestic livestock

Section 1.0

- Introduction -

Throughout sub-Saharan Africa, chemotherapy and chemoprophylaxis are the predominant methods that are used to control trypanosomiasis in domestic livestock. Furthermore, in South America, the Middle East and the Far East they constitute the only control method for the disease. Unfortunately, due to the costs associated with the development and registration of new therapeutic compounds, and the current relatively small global expenditure on molecules with anti-trypanosomal activity, the pharmaceutical industry appears increasingly reluctant to develop new compounds for trypanosomiasis (Gutteridge, 1985). As a result, and because alternative, sustainable, control methods are yet to be forthcoming, it is increasingly important to maintain the efficacy of the currently available compounds for as long as possible.

The paper contained within this Chapter gives a summary of the therapeutic and prophylactic attributes of the anti-trypanosomal compounds currently recommended for use in domestic livestock. Reports of resistance to each of the compounds are compared to the situation for the compounds recommended for treatment and/or prophylaxis of theileriosis, babesiosis, anaplasmosis and cowdriosis. Since a number of problems, in addition to drug resistance, are associated with the use of anti-trypanosomal compounds, in contrast to the drugs recommended for tick-borne diseases, the potential factors responsible for the differing situation are evaluated. Lastly, as a result of some of these problems, various alternative delivery systems have been developed for a number of the anti-trypanosomal compounds. The current status, development rationale, and potential future application are therefore given for each delivery system.

Reference:

Gutteridge, W.E. (1985) Existing chemotherapy and its limitations. *British Medical Bulletin* 41, 162-168.

Section 1.1

Peregrine, A.S. (1994) Chemotherapy and delivery systems: haemoparasites. *Veterinary Parasitology* 54, 223-248.

ELSEVIER

Veterinary Parasitology 54 (1994) 223–248

veterinary parasitology

Chemotherapy and delivery systems: haemoparasites

Andrew S. Peregrine

International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya

Abstract

Chemotherapy of haemoparasitic diseases in domestic animals is dependent on a limited number of compounds, many of which are chemically closely related. In this review, a summary is given of each of the drugs currently available for treatment and prophylaxis of trypanosomosis and the tick-borne diseases theileriosis, babesiosis, anaplasmosis and cowdriosis. In contrast to the situation with the drugs used for tick-borne diseases, drug resistance appears to be becoming an increasing problem associated with the compounds used for trypanosomosis. The literature that has been reviewed, therefore, is that which relates to the methods used to identify and quantify drug resistance in trypanosome populations, reports of resistance to trypanocides, and cross-resistance between trypanocides. The possible reason(s) for the apparent lack of development of resistance to the compounds used for treatment of tick-borne diseases is also discussed. Local toxicity at the site of injection is a problem that is particularly associated with many of the trypanocides when used on a long-term basis in individual animals. Various alternative preparations of the currently used trypanocides therefore have been evaluated in an attempt to reduce this toxicity, and are summarised. Finally, future developments in haemoparasitic chemotherapy are considered and, for trypanosomosis, highlight the importance of integrating chemotherapeutic and chemoprophylactic programmes with control of the vector when drug resistance becomes a significant constraint.

Keywords: Chemotherapy; Control methods; *Anaplasma* spp.; *Babesia* spp.; *Cowdria* spp.; *Theileria* spp.; *Trypanosoma* spp.; Drugs, general

1. Introduction

Tsetse-transmitted trypanosomosis, caused primarily by *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei brucei*, is arguably the most important disease of domestic livestock in Africa, with approximately 44 million

0304-4017/94/$07.00
SSDI 0304-4017(94)03086-C

cattle at risk of infection. In addition, the disease renders approximately 7 million km^2 of land in Africa unsuitable for livestock or mixed agriculture, an area that could theoretically support 140 million cattle and equivalent numbers of sheep and goats (FAO/WHO/OIE, 1982). Non-tsetse transmitted trypanosomosis, caused by *Trypanosoma evansi* and *Trypanosoma vivax*, occurs in various forms in South America, Africa and Asia (including China), and is a potential risk to over 500 million cattle, 100 million buffalo and 12 million camels.

In contrast to trypanosomosis, tick-borne diseases of domestic livestock occur on all of the world's major continents, mainly between latitudes 40°N and 32°S. Tropical theileriosis, caused by *Theileria annulata*, is endemic in 11 countries in West Asia and North Africa, nine countries in Asia as well as parts of southern Europe, and at least 280 million cattle are at risk from the disease. Theileriosis due to *Theileria parva* occurs in at least 11 African countries and approximately 24 million cattle are estimated to be at risk (Mukhebi, 1992). Cowdriosis occurs in sub-Saharan Africa and the Caribbean, and approximately 175 million cattle are potentially at risk in Africa (Norval et al., 1992). Finally, at least 500 million cattle are exposed to babesiosis and anaplasmosis (De Vos, 1992). Trypanosomosis, theileriosis, babesiosis, anaplasmosis and cowdriosis therefore each constitute a significant constraint to livestock production in many regions of the world. However, each disease can be controlled effectively, with both chemotherapeutic and chemoprophylactic agents and control of the arthropod vectors being equally important.

2. Compounds currently in use

2.1. Trypanosomosis

Chemotherapy for trypanosomosis in domestic livestock currently depends upon the salts of six compounds, several of which are chemically closely related. Table 1 lists the dosage regimens that should be used for each compound, their spectra of activity, the animal species in which the compounds should be used, and the routes by which the compounds should be administered. Diminazene, homidium and isometamidium are primarily used for treatment and prophylaxis of trypanosomosis in cattle, sheep and goats. Quinapyramine, suramin and melarsomine are primarily used as therapeutic agents for infections with *Trypanosoma evansi*, although quinapyramine is also used for prophylactic purposes. These latter three compounds therefore are generally restricted to use in camels, equidae and buffaloes.

Diminazene aceturate

Diminazene (Jensch, 1958) is an aromatic diamidine and is marketed in combination with phenyldimethyl pyrazolone (antipyrine) (44.5:55.5 w/w), a stabiliser that prolongs the activity of the compound in solution (Fairclough, 1962). Sensitive populations of *Trypanosoma congolense* and *Trypanosoma vivax* are

Table 1
Chemotherapeutic and chemoprophylactic compounds used for animal trypanosomosis

Compound	Trade name	Treatment regimen		Use	Activity in the field	Animal
		Dose (mg kg^{-1})	Route			
Diminazene aceturate	Berenil® Veriben® Ganaseg®	3.5–7.0	i.m.	T	*T. congolense* *T. vivax* (*T. brucei*) (*T. evansi*)	Cattle small ruminants [dogs] [equidae]
Homidium chloride Homidium bromide	Novidium® Ethidium®	1.0	i.m.	T/P[a]	*T. congolense* *T. vivax*	Cattle small ruminants pigs [equidae]
Isometamidium chloride	Samorin® Trypamidium®	0.25–0.5 0.5–1.0	i.m. i.m.	T P	*T. congolense* *T. vivax* *T. brucei* *T. evansi*	Cattle small ruminants equidae camels
Quinapyramine dimethylsulphate	Trypacide sulphate®	3.0–5.0	s.c.	T	*T. congolense* *T. vivax*	Camels equidae
Quinapyramine dimethylsulphate: chloride (3:2 w/w)	Trypacide Pro-salt®	3.0–5.0[b]	s.c.	P	*T. brucei* *T. evansi* *T. equinum* *T. simiae*	pigs dogs
Suramin	Naganol®	7.0–10.0[c]	i.v.	T(P)	*T. evansi*	Camels equidae
Melarsomine	Cymelarsan®	0.25	s.c./i.m.	T	*T. evansi*	Camels

i.m., intramuscular; s.c., subcutaneous; i.v., intravenous; T, therapeutic agent; P, prophylactic agent; ( ), limited activity; [ ], small therapeutic index.
[a]Prophylaxis observed in areas of low tsetse challenge.
[b]Dosage of sulphate.
[c]Grams per animal.

eliminated by intramuscular (i.m.) treatment of the host with diminazene aceturate at a dose of 3.5 mg kg^{-1} body weight (BW). However, higher doses may be required for infections with *Trypanosoma brucei* (Fussgänger and Bauer, 1958). Diminazene is now probably the most commonly used therapeutic agent for trypanosomosis in livestock in sub-Saharan Africa. This is due to a number of factors: activity against trypanosomes that are resistant to most other trypanocides (Williamson, 1970), the low incidence of resistance that has been detected as a result of using the compound (Williamson, 1976), and a higher therapeutic index, in most animal species, than other trypanocides (Fairclough, 1963; Williamson, 1970).

Homidium bromide/chloride

Homidium (Watkins and Woolfe, 1952) belongs to the phenanthridine class of compounds and is manufactured as both the bromide and the chloride salts, which are equally active in vivo (Leach and Roberts, 1981). Both salts are generally recommended for use as therapeutic agents at a dose of 1.0 mg kg^{-1} BW. However, the same dose, in cattle, has been shown to have prophylactic activity varying from 2 to 19 weeks against field challenge (Leach et al., 1955; R.B. Dolan et al., 1990, 1992). Variation in homidium susceptibility, and the level of trypanosome challenge, are thought to be the primary factors determining the duration of prophylaxis (R.B. Dolan et al., 1990, 1992).

Isometamidium chloride

Isometamidium (Berg, 1960) is a phenanthridine-aromatic amidine, formed by combining homidium with the diazotized *p*-aminobenzamide moiety of diminazene, and is marketed as both a therapeutic and prophylactic agent. In the dose range recommended for prophylactic purposes (0.5–1.0 mg kg^{-1} BW), the compound has been used successfully to maintain the productivity of zebu cattle exposed to tsetse challenge in both village and ranch management systems in East Africa (Trail et al., 1985; Moloo et al., 1987). However, considerable variation in prophylactic activity has been observed in that a dose of 1.0 mg kg^{-1} BW has been shown to confer prophylaxis to cattle for 2–22 weeks (Kirkby, 1964; Pinder and Authié, 1984; Whitelaw et al., 1986; Peregrine et al., 1991). Such variation in prophylactic activity appears to be independent of both the level of trypanosome challenge and the presence or absence of infection at the time of treatment (Peregrine et al., 1988). Variation in drug susceptibility between different trypanosome populations appears to be the major factor determining the duration of prophylaxis (Peregrine et al., 1991).

Quinapyramine sulphate/chloride

Quinapyramine (Davey, 1950) is a quinoline pyrimidine and is marketed as the sulphate salt as a therapeutic agent, and as a combination of the sulphate and chloride salts (3:2 w/w) for prophylactic purposes. The compound was widely used in Africa between the 1950s and 1970s as a therapeutic and prophylactic agent in cattle (Fiennes, 1953; Ruchel, 1975). However, it ceased to be manufac-

tured in 1974 (Schillinger and Röttcher, 1986) because of problems with drug toxicity and the ease with which drug resistance appeared to develop (Ndoutamia et al., 1993). However, the withdrawal of the compound particularly compromised control of trypanosomosis in camels. Thus, in 1984 quinapyramine was reintroduced to the market (Schillinger and Röttcher, 1986) but only for use in camels and horses. The compound was no longer recommended for use in cattle because of the previously observed problems with drug resistance and particularly because induction of resistance to quinapyramine appeared to be associated with cross-resistance to each of the other trypanocides that are used in cattle (Whiteside, 1960).

Suramin sodium

Suramin was first marketed in the 1920s and is the oldest of the trypanocides used in domestic livestock. It is a sulphonated naphthylamine and has been the drug of choice for treatment of *Trypanosoma evansi* infections in camels and horses for many years, since many of the other trypanocides are relatively toxic to these animals (Gardiner and Mahmoud, 1992). Suramin has also been used successfully to treat *Trypanosoma evansi* infections in Asian cattle and buffalo at doses of approximately 3 g per animal (Dieleman, 1986). But while the compound is generally active against trypanosomes of the *Trypanozoon* subgenus, it is inactive against *Trypanosoma vivax* and *Trypanosoma congolense* (Leach and Roberts, 1981). In the bloodstream, suramin binds strongly to plasma proteins (Dangerfield et al., 1938) and can be detected for up to 3 months following intravenous treatment, which accounts for its prophylactic activity (Dewey and Wormall, 1946). However, since there is great individual variation in the duration of prophylaxis, routine prophylactic administration of suramin appears to be contraindicated unless there is a high level of veterinary management (Bennett, 1933). Finally, the future availability of suramin is currently in question since manufacture of the compound has recently been discontinued (Otsyula et al., 1992).

Melarsomine

Melarsomine (Raynaud et al., 1989) is a melaminyl thioarsenite and is the most recently introduced of the trypanocides used in domestic livestock. The molecule was patented in 1985 by E. Friedheim and was synthesised by combining melarsen oxide with cysteamine. In addition to its brand name, Cymelarsan®, it is also known as Mel Cy or RM 110. The compound is water soluble and is active against members of the *Trypanozoon* subgenus (Raynaud et al., 1989), particularly *Trypanosoma evansi*. Trials with melarsomine in camels artificially infected with *Trypanosoma evansi* have demonstrated therapeutic activity over the dose range 0.3–1.25 mg kg^{-1} BW (Tager-Kagan et al., 1989; Zelleke et al., 1989). Similarly, efficacy against natural infections with *Trypanosoma evansi* has been demonstrated in camels in Kenya over the dose range 0.2–1.2 mg kg^{-1} BW (Otsyula et al., 1992), and in buffaloes in China over the dose range 0.25–3.0 mg kg^{-1} BW (Lun et al., 1991). Because of satisfactory efficacy within the lower part

of the dose ranges evaluated, and local and systemic toxicity at the higher doses (Lun et al., 1991; Otsyula et al., 1992), it is recommended that a standard dose of 0.25 mg kg^{-1} BW should be used for treatment of acute, subacute and chronic *Trypanosoma evansi* infections in camels (Rhône Mérieux, France). The compound has no prophylactic effect (Raynaud et al., 1989).

2.2. Theileriosis, babesiosis, anaplasmosis and cowdriosis

The compounds that are currently available for treatment and prophylaxis of theileriosis, babesiosis, anaplasmosis and cowdriosis are summarised in Table 2. For each compound, the spectrum of activity and recommended dose regimen is given.

Until the early 1980s, chemotherapeutic control of bovine theileriosis was dependent upon the tetracyclines (Neitz, 1953; Brocklesby and Bailey, 1962). However, their use for the treatment of clinical theileriosis appeared to be limited (Dolan, 1981). Fortunately, three new compounds have been introduced for theileriosis treatment since 1981: parvaquone, buparvaquone and halofuginone. While the therapeutic repertoire for theileriosis has therefore been enhanced in recent years, the opposite appears to have been the case for babesiosis, with the withdrawal of amicarbalide, phenamidine and pentamidine from the market (see Dolan, 1992). Thus, treatment of this disease complex is now dependent upon the tetracyclines, diminazene and imidocarb. In some parts of the world, quinuronium sulphate is also still available. Finally, for anaplasmosis and cowdriosis the chemotherapeutic repertoire is even more limited, with the tetracyclines and imidocarb available for anaplasmosis, and only the tetracyclines available for cowdriosis.

Parvaquone

Parvaquone (McHardy, 1979) is a hydroxynaphthoquinone and is an analogue of menoctone, the first naphthoquinone that was shown to exhibit useful antitheilerial therapeutic levels of activity (McHardy et al., 1976). Parvaquone exhibits therapeutic levels of activity against only *Theileria* spp. and the recommended treatment regimen for such purposes in cattle is two i.m. doses of 10 mg kg^{-1} BW, given 48 h apart. However, additional treatments with the same dose may be used if necessary (McHardy, 1984). Using this standard treatment regimen, parvaquone has been shown to exhibit high levels of therapeutic activity in cattle against artificial infections with cattle-derived and buffalo-derived *Theileria parva* (Dolan et al., 1984). It has also been shown to be active against natural infections with both *Theileria parva* (Chema et al., 1986; Mbwambo et al., 1987) and *Theileria annulata* (Unsuren et al., 1988). However, recovery rates appear to be highest when treatment is administered during the early stages of the disease (Mbwambo et al., 1987).

In experiments to investigate the therapeutic activity of parvaquone, single i.m. treatment with parvaquone at a dose of 20 mg kg^{-1} BW has been shown to result in high levels of recovery in cattle artificially infected with either cattle-derived

Table 2
Chemotherapeutic and chemoprophylactic compounds used for tick-borne diseases

Compound	Trade name	Activity in the field	Animal	Treatment regimen			Use
				Dose (mg kg^{-1})	Repeated at	Route	
Parvaquone	Clexon®	Theileriosis	Cattle	10.0[a]	48 h[b]	i.m.	T
Buparvaquone	Butalex®	Theileriosis	Cattle	2.5[c]	–	i.m.	T
Halofuginone lactate	Terit®	Theileriosis	Cattle	1.2	48 h[b]	p.o.	T
Diminazene aceturate	Berenil®	Babesiosis	Cattle				
	Veriben®		sheep	3.5	–	i.m.	T
	Ganaseg®		Horses	3.5–11.0[d]	–	i.m.	T
			Dogs	2.0–3.5[e]	–	i.m.	T
Imidocarb dipropionate	Imizol®	Babesiosis	Cattle	1.2	–	s.c.	T
				3.0	–	s.c.	P
				2.4	–	s.c.	S
			Sheep	2.0	24 h[b]	s.c.	T
			Horses	2.4	24 h[f]	i.m.	T
				2.4	–	i.m.	P
				2.4	72 h	i.m.	S[g]
				4.8	72 h[h]	i.m.	S[f]
			Dogs	6.0	–	s.c.	T
				2.4	–	s.c.	P
		Anaplasmosis	Cattle	3.0	–	s.c.	T/P
Quinuronium sulphate	Acaprin®	Babesiosis	Cattle				
			sheep	1.0	(24 h)	s.c.	T
Tetracyclines	Short-acting preparations	Theileriosis	Cattle				
		Babesiosis	sheep				
	Long-acting preparations	Anaplasmosis	goats				
		Cowdriosis	horses				
			dogs	5.0–20.0	1–5 days[i]	i.m.	T/I
	Doximplant-S®[j]	Cowdriosis	Goats				
			sheep	7.5–12.7	–	s.c.	I
			Cattle	5.0–18.0	–	s.c.	I

[a]For *Theileria annulata*, single i.m. treatment with 20 mg kg^{-1} BW may be adequate.
[b]In advanced cases, additional treatments at the same intervals may be required.
[c]In advanced cases an additional treatment at the same dose may be required, within 48–72 h of initial injection.
[d]Infections with *B. equi* require a second treatment with the same drug dose after 24 h—additional treatments may also be required.
[e]Infections with *B. gibsoni* require a dose of 10 mg kg^{-1} BW—additional treatments at 48 h intervals may be required.
[f]For *B. equi*.
[g]For *B. caballi*.
[h]On three occasions, i.e. a total of four treatments.
[i]For as many times as necessary.
[j]Implant formulation used for infection and treatment immunisation against *Cowdria ruminantium*.
i.m., intramuscular; p.o., per os; s.c., subcutaneous; T, therapeutic agent; P, for prophylaxis; S, for sterilisation; I, for immunisation; ( ), may not be required.

Theileria parva or *Theileria annulata* (Dolan et al., 1984; Gill et al., 1984). Similar efficacy has also been demonstrated in cattle naturally infected with *Theileria annulata* (Hawa et al., 1988). Thus, although not recommended by the manufacturer, a single treatment regimen may adequately control the disease, particularly if used early in infection.

Buparvaquone

Buparvaquone (Hudson et al., 1985; McHardy et al., 1985) is a 'second generation' hydroxynaphthoquinone, and was identified as a result of research to find potent theileriacides with longer in vivo persistence than parvaquone (Hudson et al., 1986). In studies carried out in vitro, buparvaquone has been shown to be 20 times more active than parvaquone against cattle-derived *Theileria parva*. Furthermore, in cattle infected with the same *Theileria* spp., single i.m. treatment with 2.5 mg buparvaquone kg^{-1} BW appears to have at least the same level of theileriacidal activity as i.m. treatment with 20 mg parvaquone kg^{-1} BW (McHardy et al., 1985). The greater potency of buparvaquone compared with parvaquone is thought to be due to longer persistence of buparvaquone in plasma (Kinabo and Bogan, 1988) which, in turn, is due to the slower metabolism of buparvaquone to a hydroxy derivative (McHardy, 1989a).

In addition to activity against cattle-derived *Theileria parva*, the recommended single i.m. dose of buparvaquone at 2.5 mg kg^{-1} BW has also been shown to have a high level of activity against artificial infections of *Theileria annulata*, *Theileria sergenti* and buffalo-derived *Theileria parva* in cattle (Minami et al., 1985; Dhar et al., 1988; Mutugi et al., 1988; Sharma and Mishra, 1990). Similar levels of efficacy have also been observed in deer artificially infected with *Theileria cervi* (Mitema et al., 1991). Finally, in the field, the same treatment regimen has produced cure rates of approximately 90% for both *Theileria parva* and *Theileria annulata* (McHardy, 1989b, 1991). In contrast, however, T. Dolan et al. (1992) have concluded that a single treatment with buparvaquone results in lower recovery rates than when the drug is administered to cattle on two occasions, 48 h apart.

Because buparvaquone appears to be more active than parvaquone against both *Theileria parva* and *Theileria annulata* (McHardy et al., 1985; Hashemi-Fesharki, 1991), it appears that buparvaquone may be the only hydroxynaphthoquinone that will be marketed in the future as a theileriacide. The compound has no activity against *Anaplasma marginale*, *Cowdria ruminantium*, *Babesia bigemina* and *Cytauxoon felis* (McHardy, 1989a).

Halofuginone

Halofuginone is a quinazolinone that was originally developed as the hydrobromide salt for use as an anticoccidial agent (Ryley and Wilson, 1975). However, as the lactate salt, which is better tolerated by cattle and has a higher level of water solubility, the compound is also active against both *Theileria parva* and *Theileria annulata* infections in cattle when administered orally at doses of 1–2 mg kg^{-1} BW (Schein and Voigt, 1979; Uilenberg et al., 1980). Other routes of administra-

tion should not be used since this results in severe local reactions (Schein and Voigt, 1981). Unlike parvaquone and buparvaquone, which are active against both the schizont and piroplasm stages, halofuginone is only active against the schizont stage. Thus, in field conditions, the compound appears to be most active against early stages of the disease (Njau et al., 1985), although re-administration of the drug, 48 h after the first treatment, improves therapeutic efficacy (Njau and Mkonyi, 1981; Njau et al., 1985). It is therefore recommended that animals should receive two doses of halofuginone, 48 h apart (Table 2), although additional treatments at similar intervals may be given if necessary. Administration of doses in excess of the recommended dose (1.0 mg base kg^{-1} BW) is contraindicated since halofuginone has a narrow therapeutic index (Schein and Voigt, 1981). Finally, when compared with other theileriacides, the in vivo potency of halofuginone generally appears to be less than that of parvaquone. Since the two compounds have the same intrinsic activity against *Theileria parva* (Morgan and McHardy, 1982), this is probably due to differences in drug pharmacokinetics in vivo.

Diminazene aceturate

In addition to activity against *Trypanosoma* spp., diminazene aceturate is active against *Babesia* spp. in cattle, sheep, horses and dogs (Kuttler, 1981). In Table 2, the dose regimens that are recommended for the different *Babesia* spp. in these different animal species are listed. Particular attention should be given to the use of diminazene in dogs since the compound has a narrow therapeutic index in these animals. Thus, while a single i.m. dose of 0.2–3.5 mg kg^{-1} BW has been safely used to control *Babesia canis* infections, toxicity may be encountered at the single dose that may be required for infections with *Babesia gibsoni*, 10 mg kg^{-1} BW (Thibault and Jaussaud, 1987). Such toxicity, however, can be circumvented by using two doses of 3.0 mg kg^{-1} BW, given 24 h apart (Farwell et al., 1982).

Imidocarb dipropionate

Imidocarb dipropionate (Schmidt et al., 1969) is a diamidine of the carbanilide series and is recommended for treatment and prophylaxis of *Babesia* spp., and treatment of *Anaplasma* spp. The dihydrochloride salt is equally active but appears to result in a higher level of local toxicity than the dipropionate salt. Table 2 lists the recommended treatment regimens for the different parasites in different animal species. While a number of compounds are currently available as babesiacides, imidocarb appears to be the drug of choice for infections with *B. bigemina*, *Babesia bovis* and *Babesia divergens* (Kuttler, 1981). Furthermore, unlike the other compounds that are marketed as babesiacides, imidocarb has significant prophylactic activity against *Babesia* spp., especially *B. bigemina*, which generally lasts for 4–6 weeks (Uilenberg et al., 1981). Prophylactic activity for up to 15 weeks has been observed (Todorovic et al., 1973). When used for

treatment of infections with *Anaplasma* sp., the efficacy of imidocarb appears to be greatest when administered during the early stages of infection (McHardy and Simpson, 1974).

Quinuronium sulphate

Until the 1940s, quinuronium sulphate was extensively used as a babesiacide. However, it had a narrow therapeutic index (Kuttler, 1988) and was thereafter generally replaced with compounds such as the aromatic diamidines which had higher therapeutic indices. On most markets it is no longer available. However, in the Middle East, it is still commonly used for ovine babesiosis (see Dolan, 1992).

Tetracyclines

Tetracycline antibiotics, such as chlortetracycline, oxytetracycline and rolitetracycline, have for many years been used to control infections with *Theileria* spp., *Babesia* spp., *Anaplasma* sp. and *Cowdria ruminantium*. However, the activity of these compounds against *Theileria* spp. and *Babesia* spp. generally appears to be restricted to the preclinical stages of infection (Brocklesby and Bailey, 1962; Kuttler, 1981; Uilenberg, 1983; Spooner, 1990). They are therefore commonly used in infection and treatment regimens for inducing immunity to the different parasites, especially *Theileria* spp., since treatment is administered in the early stages of infection (Radley et al., 1975; Dolan, 1987). Prior to the introduction of long-acting tetracycline preparations (e.g., Terramycin/LA®) multiple treatment regimens were usually required for such purposes. However, treatment with long-acting preparations is now commonly practised since this reduces the number of treatments that are required. There may, however, still be a role for short-acting preparations since they are generally cheaper than their long-acting counterparts (Young et al., 1990, 1992). Finally, a potential alternative to the use of long-acting injectable preparations may be the use of slow-release drug implants such as the doxycycline implant (Doximplant-S®); a preparation that appears to be particularly efficacious for immunising goats against cowdriosis in cowdriosis-endemic areas (Norval et al., 1992).

Unfortunately, not all populations of *Theileria* spp. are responsive to treatment with tetracyclines in the early stages of infection. Thus, in an attempt to improve the efficacy, but retain the simplicity, of immunisation protocols for *Theileria* spp., the suitability of parvaquone and buparvaquone for such purposes has been investigated in cattle; parvaquone at a single i.m. dose of 20 mg kg^{-1} BW eliminated infections with *Theileria parva* when administered on days 0 and 4 of infection (Dolan et al., 1988). However, some of the animals remained susceptible to homologous challenge. When treatment was delayed until day 8, solid immunity was conferred to cattle. Thus, cattle would have to be handled on at least two occasions if parvaquone was used for immunisation. In contrast, administration of buparvaquone at a single i.m. dose of 2.5 mg kg^{-1} BW on the day of infection appeared to control infections with both *Theileria parva* and *Theileria annulata*, and can result in satisfactory levels of immunity to homologous challenge (Dhar et al., 1990; Mutugi et al., 1991; Ngumi et al., 1992). Further experiments are

required to confirm these observations and to define the optimal dosage regimens for inducing immunity with buparvaquone. Halofuginone does not appear to have a role in infection-and-treatment immunisation regimens since it is not active against all the life-cycle stages that occur in vivo (Dolan, 1985).

3. Drug resistance

3.1. Trypanosomosis

Diminazene, homidium, isometamidium, quinapyramine and suramin have all been used in the field for over 30 years. In a similar manner to other compounds that have been used for such lengthy periods, the use of each of these five trypanocides has been associated with the development of drug resistance in many field situations. Furthermore, since many of the compounds are chemically closely related, cross-resistance appears to have greatly compounded the problem (Williamson, 1970).

Methods to identify drug resistance

Field data. Cross-sectional studies on the prevalence of trypanosomosis in field situations do not provide informative data about the prevalence of drug resistance. However, data from longitudinal studies can be used for this purpose. For instance, at a site in southwest Ethiopia, a model was applied to parasitological data collected on a monthly basis from cattle treated with only diminazene aceturate. The model allowed one to distinguish the incidence of new infections from recurrent infections, and showed that the mean prevalence of diminazene-resistant infections increased significantly from 6% in 1986 to 14% in 1989 (Rowlands et al., 1993). Codjia et al. (1993) subsequently determined the drug-resistance phenotype of trypanosome isolates from this site and confirmed the occurrence of drug-resistant populations.

Large animals. For many years the drug-resistance phenotype of trypanosome populations has been characterised in small and large ruminants (Gray and Roberts, 1971; Gitatha, 1979; Codjia et al., 1993). Unlike other characterisation systems, the data generated are directly applicable to the field. However, purchase and maintenance of animals for this purpose is expensive. Furthermore, it can take up to 6 months to derive definitive data, and is an impractical system for screening large numbers of isolates.

Laboratory rodents. Because of the expense and logistical problems incurred when using large animals, mice and rats have been used by many workers to characterise the drug resistance of trypanosome populations. Such systems reduce experimental costs and reduce the time taken to characterise a population to approximately 2 months. However, not all field isolates grow in laboratory rodents

(Hawking, 1963). Furthermore, there does not appear to be a consistent relationship between drug sensitivity data obtained in small animals and that obtained in large animals (Sones et al., 1988). Thus, extrapolation of drug sensitivity data from small to large animals is not possible.

In vitro cultivation. The development of in vitro cultivation techniques for trypanosomes (Hill and Hirumi, 1983) has facilitated the development of in vitro systems for characterising the drug sensitivity of trypanosome populations in the absence of the host. Kaminsky (1990) has described the different systems that have been developed for this purpose. Compared to drug-sensitivity studies carried out in vivo, such systems are usually cheaper, and may be quicker. However, logistically, it is not possible to screen large numbers of isolates with such systems. It is also not yet possible to use drug-sensitivity data derived in vitro to predict the drug sensitivity of trypanosome populations in definitive hosts (Kaminsky, 1990). However, Gray et al. (1993) have observed a consistent relationship between the in vitro sensitivity of *Trypanosoma congolense* metacyclic trypanosomes to diminazene, homidium and isometamidium, and the sensitivity of the same populations to these trypanocides in cattle. Further clarification of the relationship between drug sensitivity data obtained in vitro and in vivo will help determine the future application of in vitro characterisation systems.

Reports of drug resistance

Resistance to the trypanocides used in cattle has been reported at sites in west, central, east, and southern Africa. In Table 3, the reports of such resistance, characterised in cattle, are summarised. Reports of resistance to quinapyramine are also included. On the basis of these data, resistance to diminazene, homidium, isometamidium or quinapyramine has been reported in eleven countries in sub-Saharan Africa. Furthermore, in seven of the 11 countries, multiple drug resistance has been described.

The prevalence of suramin resistance is not known because only a limited number of studies have been done. However, Schillinger and Röttcher (1986) concluded that drug resistance is responsible for the widespread decrease in efficacy of suramin that has been observed in camel-rearing areas. Resistance to melarsomine has yet to be reported from the field. However, since resistance to melarsomine has been rapidly induced in *Trypanosoma evansi* in immunosuppressed mice (Osman et al., 1992), there is no reason to believe that melarsomine resistance will develop any more slowly than resistance to the other trypanocides.

Cross-resistance

As a result of studies in the field on the development of resistance to quinapyramine, homidium, metamidium (a mixture of isomers, containing isometamidium; Wragg et al., 1958) and diminazene, Whiteside (1962) concluded that induction of resistance to quinapyramine results in cross-resistance to diminazene, and that reciprocal cross-resistance occurs between quinapyramine, homi-

Table 3
Reports of resistance to standard recommended doses of diminazene, homidium, isometamidium and quinapyramine in cattle

Country	Trypanosome species	Resistance to	Reference
Nigeria	*T. congolense*	h,i	Jones-Davies and Folkers, 1966
Nigeria	*T. vivax*	d	Jones-Davies, 1967
Nigeria	*T. congolense*	d,h,i	Na'Isa, 1967
Nigeria	*T. congolense*	d	MacLennan and Jones-Davies, 1967
Chad	*T. vivax*	d	Graber, 1968
Nigeria	*T. congolense*	d	Jones-Davies, 1968
Nigeria	*T. congolense*	d,h,i,q	Gray and Roberts, 1971
	T. vivax	d,h,q	Gray and Roberts, 1971
Uganda	*T. vivax*	d	Mwambu and Mayende, 1971
Sudan	*T. congolense*	h,i	Abdel Gadir et al., 1972
Zimbabwe	*T. congolense*	i	Lewis and Thomson, 1974
Kenya	*T. congolense*	d,h,i,q	Gitatha, 1979
Nigeria	*T. vivax*	d,h,i	Ilemobade, 1979
Sudan	*T. congolense*	h	Abdel Gadir et al., 1981
	T. brucei/T. vivax	h	Abdel Gadir et al., 1981
Tanzania	*T. congolense*	d	Njau et al., 1981
Côte d'Ivoire	*T. congolense*	h,i	Küpper and Wolters, 1983
	T. vivax	h,i	Küpper and Wolters, 1983
Burkina Faso	*T. congolense*	i	Pinder and Authié, 1984
Kenya	*T. vivax*	d,h,i,q	Röttcher and Schillinger, 1985
Kenya	*T. vivax*	d,h,i,q	Schönefeld et al., 1987
Somalia	*T. vivax*	h,i,q	Schönefeld et al., 1987
Tanzania	*T. congolense*	d	Mbwambo et al., 1988
Somalia	*T. congolense*	d,i	Ainanshe et al., 1992
Burkina Faso	*T. congolense*	d,h,i,q	Clausen et al., 1992
Sudan	*T. congolense*	d,h,i	Mohamed-Ahmed et al., 1992
	T. brucei	d,h,i	Mohamed-Ahmed et al., 1992
	T. vivax	h	Mohamed-Ahmed et al., 1992
Ethiopia	*T. congolense*	d,h,i	Codjia et al., 1993

d, diminazene; h, homidium; i, isometamidium; q, quinapyramine.

dium and metamidium. Thus, as a result of these conclusions, Whiteside (1960) proposed that homidium and diminazene, and isometamidium and diminazene, should be used together as 'sanative' pairs of drugs, since induction of resistance to one drug can be eliminated by use of the other. Sanative combinations of drugs are therefore recommended for use in the field, and have not been associated with the development of drug resistance (Trail et al., 1985).

Amongst the trypanocides that were evaluated by Whiteside (1962), quinapyramine was associated with the greatest problems since induction of resistance to this compound, under field conditions, appeared to result in cross-resistance to homidium, isometamidium and diminazene. These conclusions have subsequently been confirmed in a laboratory study in which resistance to quinapyra-

mine was induced in a clone of *Trypanosoma congolense* (Ndoutamia et al., 1993), and therefore indicate that quinapyramine should not be used in cattle.

Finally, amongst the trypanocides used for *Trypanosoma evansi* infections in camels, cross-resistance generally does not occur between quinapyramine and suramin (Gill, 1971). Furthermore, in vitro, quinapyramine- and suramin-resistant populations of *Trypanosoma evansi* appear to be fully susceptible to treatment with melarsomine (Zweygarth and Kaminsky, 1990). Therefore, there appears to be no cross-resistance amongst quinapyramine, suramin and melarsomine. However, whilst induction of resistance to melarsomine does not result in cross-resistance to suramin, it does appear to result in cross-resistance to diminazene (Osman et al., 1992).

3.2. Theileriosis, babesiosis, anaplasmosis and cowdriosis

Drug resistance has yet to constitute a constraint to the efficacy of the compounds that are currently used for theileriosis, babesiosis, anaplasmosis and cowdriosis. Similarly, the withdrawal of drugs previously used for these diseases has not been due to problems with drug resistance, but because of a decline in demand (see Dolan, 1992).

Differences in susceptibility to parvaquone have been observed amongst isolates of *Theileria* spp. (Dolan, 1981). Similarly, different levels of susceptibility to babesiacides are often observed amongst the *Babesia* spp., with the small babesias (e.g. *B. bovis*, *B. equi*, *B. gibsoni*) being generally more refractory to treatment than their larger relatives (e.g. *B. bigemina*, *Babesia caballi*, *B. canis*) (Kuttler, 1981). However, such variation in apparent resistance does not appear to have compromised the efficacy of any of these compounds.

The relatively short periods during which parvaquone, buparvaquone and halofuginone have been used in the field probably account for the apparent lack of resistance to these compounds. However, resistance has not developed to compounds such as imidocarb and the tetracyclines, which have been used in the field for as long as many of the trypanocides. Since resistance to such drugs can be produced under experimental conditions (Beveridge, 1970; Dalgliesh and Stewart, 1977), the lower intensity of drug pressure in the field for tick-borne diseases, compared with trypanosomosis, may account for the fact that resistance has not been observed with these compounds.

4. Alternative delivery systems

4.1. Trypanosomosis

Local toxicity in domestic livestock is a problem associated with many of the currently used trypanocides, particularly when they are used repeatedly on a long-term basis in individual animals. Various formulations of the currently used trypanocides have therefore been produced and evaluated to determine whether they

ameliorate local toxicity and/or increase the prophylactic activity of the compounds.

Suraminates

In contrast to diminazene, homidium, isometamidium and quinapyramine, which are all cations at physiological pH, suramin is an anion. Williamson (1957) utilised this property to produce suramin complexes of each of these trypanocides, as part of a series of studies to determine if such formulations decreased the toxicity of the parent compounds, and increased their prophylactic activity. Experiments in cattle demonstrated that the prophylactic activity of some of the compounds was extended when administered as a suraminate, and that this was greatest with homidium suraminate (Williamson and Desowitz, 1956). However, since this preparation produced severe local toxicity in cattle (Stephen, 1958) the use of suraminates in cattle was not recommended.

In experiments carried out in pigs, quinapyramine suraminate at a dose of 40 mg base kg^{-1} BW was shown to eliminate infections with *Trypanosoma simiae*, even when administered in the clinical stage of disease (Stephen, 1962). Furthermore, the prophylactic activity of the complex appeared to be similar to that of quinapyramine chloride (Stephen and Gray, 1960; Gray, 1961). Thus, since local toxicity was not associated with the use of quinapyramine suraminate, Stephen (1966) recommended that the complex could be used as a prophylactic agent in all ages of pigs at a dose of 40 mg base kg^{-1} BW.

Dextran complexes

Diminazene, isometamidium and quinapyramine have each been complexed with dextran sulphate (molecular weight 500 000) (James, 1978). This procedure greatly reduced the toxicity of isometamidium for rodents (James, 1978; Aliu and Chineme, 1980). However, since the prophylactic activity of the complexes varied greatly between studies, a definitive conclusion about the effect of complex formation on prophylactic activity cannot be made.

Homidium polymer

In an attempt to increase the prophylactic activity of homidium, De Deken et al. (1989) produced a slow release cylindrical polymeric device, measuring 10 mm × 1.7 mm, that contained 25% (w/w) homidium. Subcutaneous implantation of the device into rabbits resulted in a significantly higher concentration of homidium in the serum compared with non-polymerised homidium. It also greatly extended the prophylactic activity of the compound against challenge with *Trypanosoma congolense*, and was not associated with side effects. Evaluation of the homidium polymer in cattle has not been carried out. However, if the ratio of animal body weight to the device's surface area were to remain constant, the required size of the polymeric device would be prohibitively large.

Liposomal formulations

Fluck and Hopkins (1987) produced large multi-lamellar liposomes that contained diminazene, homidium or isometamidium, and evaluated their prophylactic activity in cattle in the field. Whilst a small increase in prophylactic activity was observed with the liposomal formulations of diminazene and homidium, a similar effect was not apparent with isometamidium. Administration of isometamidium in liposomes did, however, appear to reduce the local toxicity of the molecule. Further evaluation of liposomal formulations appears to have been hampered by problems with standardisation of formulations.

Carrier erythrocytes

In an attempt to prolong the prophylactic activity of homidium, the molecule has been entrapped in bovine red blood cells (RBC) (DeLoach, 1985). Using this methodology, a maximum of 5 mg was encapsulated in 10^{10} RBC. Thus, 20 ml of packed homidium-containing RBC would only contain sufficient drug for a 100 kg animal (DeLoach, 1985). The methodology, as described, would therefore appear to be impractical for field use. Experimental trials in ruminants have not been reported.

Intravenous isometamidium

Administration of isometamidium via the i.v. route is not recommended by the manufacturer (RMB Animal Health Ltd.). However, i.v. doses of 0.6 mg kg^{-1} BW and 1.0 mg kg^{-1} BW have been used to control bovine trypanosomosis in areas of low and high tsetse challenge, respectively, in Kenya, and have circumvented the development of large fibrous lesions that occur as a result of multiple i.m. injections (Dowler et al., 1989; Münstermann et al., 1992). At both of these field sites, administration of isometamidium via the i.v. route appeared to enhance the therapeutic activity of the compound when compared with administration via the i.m. route. However, similar studies in laboratory-maintained cattle have demonstrated that i.m., as opposed to i.v., administration of isometamidium maximises the therapeutic activity of the compound against parasites with either high or low levels of resistance to isometamidium (Sutherland et al., 1991, 1992). Thus, i.v. administration of isometamidium would appear to be contraindicated where resistance to the compound is a significant problem.

4.2. Theileriosis, babesiosis, anaplasmosis and cowdriosis

Local toxicity is not a problem that is generally encountered with the compounds used for treatment and prophylaxis of tick-borne diseases. Furthermore, there is not a requirement for compounds with extended periods of prophylactic activity for sustained effective control of these diseases. Thus, research on the efficacy of alternative formulations of the currently used compounds has received little attention. Tetracycline has been entrapped in carrier-RBC and used to treat anaplasmosis in cattle (DeLoach and Wagner, 1983). However, pharmacokinetic studies demonstrated that this had little effect on the drug's half-life

in vivo (DeLoach and Wagner, 1984), and therefore appeared to have limited potential for increasing the compound's efficacy. In contrast, encapsulation of imidocarb dipropionate into bovine RBC significantly increased the half-life of the molecule compared with free drug, suggesting that this delivery system would allow one to reduce the dose of drug required for prophylactic purposes (DeLoach et al., 1981). Logistical and financial constraints in the field appear to have precluded further evaluation of this delivery system.

5. Future developments

No new therapeutic compounds for trypanosomosis or tick-borne diseases are currently being developed by the pharmaceutical industry (see Dolan, 1992). This is because the cost of developing new compounds for such diseases is now considered prohibitively high unless such compounds have a broad spectrum of activity. Thus, in the future, the identification of new anti-parasitic compounds will be dependent upon research on biochemical pathways that are common amongst, but unique to, parasites. In *Trypanosoma brucei brucei*, for instance, the trypanocidal action of two chemically unrelated compounds has recently been shown to be mediated by interference with biochemical pathways involved in purine metabolism (Byers et al., 1992; Carter and Fairlamb, 1993). Since an inability to synthesise purines de novo is common to trypanosomes and many other haemoparasites (Hassan and Coombs, 1988), research on purine metabolism may be a promising area in the future to identify molecules with broad-spectrum anti-haemoparasitic activity. New anti-parasitic compounds may also be identified as a result of serendipitous activity of compounds developed for other purposes.

5.1. Trypanosomosis

Drug resistance is becoming an increasing constraint to the efficacy of the currently used trypanocides. Furthermore, it appears unlikely that new compounds will be introduced in the near future. Thus, in order to maintain the efficacy of the currently used compounds, it is important that chemotherapeutic and chemoprophylactic regimens are rationalised on the basis of the drug-susceptibility phenotype of trypanosome populations in a given locality. However, such rationalisation is at present not possible because the systems that are currently available to characterise the drug-resistance phenotype of trypanosome populations are not field applicable; limited numbers of field isolates can be characterised, and all of the systems take many months to produce definitive data. There is therefore a requirement for assays that will rapidly quantify the drug-resistance phenotype of large numbers of trypanosome isolates. In an attempt to meet these criteria, Eisler et al. (1993) have described an enzyme-linked immunosorbent assay (ELISA) for isometamidium that will rapidly quantify the concentration of isometamidium in large numbers of serum samples. Thus, when used in combina-

tion with a trypanosomosis diagnostic test, the assay can be used as an indirect assay for drug resistance. Relevance of this assay to the field awaits evaluation. The development of direct assays for drug resistance, that can rapidly characterise the drug-resistance phenotype of large numbers of trypanosome isolates, will almost certainly be dependent upon a detailed understanding of the molecular bases of drug-resistance.

In many field sites, 'sanative' drug combinations have been used to prevent the development of drug resistance. However, such combinations are often not used. Thus, prior to the implementation of methodologies that can be used to rationalise chemotherapeutic and chemoprophylactic regimens on the basis of the resistance phenotype of trypanosome populations, it is important that greater attention is given to the use of sanative drug pairs in the field. The introduction of preparations which prolong the prophylactic activity of the currently used trypanocides would appear to be contraindicated since such formulations are likely to increase the rate of development of drug resistance (De Deken et al., 1989).

Finally, in a number of situations, multiple-drug resistance has been described (Schönefeld et al., 1987; Clausen et al., 1992; Mohamed-Ahmed et al., 1992; Codjia et al., 1993). Thus, chemotherapy per se may no longer be able to control trypanosomosis at these sites, particularly if the multiple resistance is expressed at the level of individual trypanosomes (Codjia et al., 1993). However, successful control in such apparently hopeless circumstances may be achieved by integrating chemotherapeutic regimens with a tsetse control programme (R.B. Dolan et al., 1992; S.G.A. Leak, personal communication, 1994). Furthermore, by combining such control strategies, which has often not been practised in the past, a higher level of sustainable trypanosomosis control can be achieved than is possible with individual control strategies.

5.2. Theileriosis, babesiosis, anaplasmosis and cowdriosis

Drug resistance is currently not a problem associated with any of the compounds used for treatment of tick-borne diseases. However, since the development of drug resistance has been associated, sooner or later, with the use of all other anti-parasitic compounds, it is reasonable to assume that resistance to these drugs will occur in the future. Thus, in order to impede the development of resistance, it is important that compounds belonging to more than one chemical class continue to remain available for each disease. The development of assays for quantifying resistance in the different parasites may also become a necessity in the future.

Acknowledgements

Drs. T.T. Dolan, N. McHardy and A.S. Young are thanked for discussions concerning the content of this review, and for critically evaluating the manuscript.

Miss Maria Mulindi is thanked for secretarial assistance. This is ILRAD Publication No. 1186.

References

Abdel Gadir, F., Abdel Razig, M. El T. and Osman, O.M., 1972. Sensitivity of ethidium bromide resistant *Trypanosoma congolense* strain to Samorin and Berenil. Sudan J. Vet. Sci. Anim. Husb., 13: 68–73.

Abdel Gadir, F., Osman, O.M., Abdalla, H.S. and Abdel Razig, M.T., 1981. Ethidium bromide-resistant trypanosomes in southern Darfur. Sudan J. Vet. Res., 3: 63–65.

Ainanshe, O.A., Jennings, F.W. and Holmes, P.H., 1992. Isolation of drug-resistant strains of *Trypanosoma congolense* from the lower Shabelle region of Southern Somalia. Trop. Anim. Health Prod., 24: 65–73.

Aliu, Y.O. and Chineme, C.N., 1980. Isometamidium–dextran complex: toxicity and activity against *Trypanosoma vivax* and *Trypanosoma congolense* in rats and mice. Toxicol. Appl. Pharmacol., 53: 196–203.

Bennett, S.C.J., 1933. The control of camel trypanosomiasis. J. Comp. Pathol., 46: 174–185.

Berg, S.S., 1960. Structure of isometamidium (M&B 4180A), 7-m-amidinophenyldiazoamino-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride, the red isomer present in metamidium. Nature, 188: 1106–1107.

Beveridge, E., 1970. Drug resistance in *Babesia rodhaini*. Br. J. Pharmacol., 39: 239–240.

Brocklesby, D.W. and Bailey, K.P., 1962. Oxytetracycline hydrochloride in East Coast fever (*Theileria parva* infection). Br. Vet. J., 118: 81–85.

Byers, T.L., Casara, P. and Bitonti, A.J., 1992. Uptake of the antitrypanosomal drug 5′-([(Z)-4-amino-2-butenyl]methylamino)-5′-deoxyadenosine (MDL 73811) by the purine transport system of *Trypanosoma brucei brucei*. Biochem. J., 283: 755–758.

Carter, N.S. and Fairlamb, A.H., 1993. Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature, 361: 173–176.

Chema, S., Waghela, S., James, A.D., Dolan, T.T., Young, A.S., Masiga, W.N., Irvin, A.D., Mulela, G.H.M. and Wekesa, L.S., 1986. Clinical trial of parvaquone for the treatment of East Coast fever in Kenya. Vet. Rec., 118: 588–589.

Clausen, P.-H., Sidibe, I., Kaboré, I. and Bauer, B., 1992. Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse fly challenge in the pastoral zone of Samorogouan, Burkina Faso. Acta Trop., 51: 229–236.

Codjia, V., Mulatu, W., Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authié, E., d'Ieteren, G.D.M. and Peregrine, A.S., 1993. Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. Acta Trop., 53: 151–163.

Dalgliesh, R.J. and Stewart, N.P., 1977. Tolerance to imidocarb induced experimentally in tick-transmitted *Babesia argentina*. Aust. Vet. J., 53: 176–180.

Dangerfield, W.G., Gaunt, W.E. and Wormall, A., 1938. Studies on Bayer 205 (Germanin) and Antrypol. I. The determination of small amounts of Bayer 205 (and Antrypol). II. The persistence of Bayer 205 in the bloodstream after injection into animals. Biochem. J., 32: 59–70.

Davey, D.G., 1950. Experiments with 'Antrycide' in the Sudan and East Africa. Trans. R. Soc. Trop. Med. Hyg., 43: 583–616.

De Deken, R., Geerts, S., Kageruka, P., Ceulemans, F., Brandt, J., Schacht, E., Pascucci, C. and Lootens, C., 1989. Chemoprophylaxis of trypanosomiasis, due to *Trypanosoma* (*Nannomonas*) *congolense*, in rabbits using a slow release device containing homidium bromide. Ann. Soc. Belge Méd. Trop., 69: 291–296.

DeLoach, J.R., 1985. Encapsulation potential of a trypanocidal drug homidium bromide in bovine carrier erythrocytes. Res. Exp. Med., 185: 345–353.

DeLoach, J.R. and Wagner, G.G., 1983. Encapsulation of drugs in bovine carrier erythrocytes for treatment of anaplasmosis. In: Proc. 10th Int. Symposium on Controlled Release of Bioactive Materials. San Francisco, CA, pp. 71–76.

DeLoach, J.R. and Wagner, G.G., 1984. Pharmacokinetics of tetracycline encapsulated in bovine carrier erythrocytes. Am. J. Vet. Res., 45: 640–642.

DeLoach, J.R., Wagner, G.G. and Craig, T.M., 1981. Imidocarb dipropionate encapsulation and binding to resealed carrier bovine erythrocytes for potential babesiasis chemotherapy. J. Appl. Biochem., 3: 254–262.

De Vos, A.J., 1992. Distribution, economic importance and control measures for *Babesia* and *Anaplasma*. In: T.T. Dolan (Editor), Recent Developments in the Control of Anaplasmosis, Babesiosis and Cowdriosis. Proc. of a Workshop, ILRAD, Nairobi, Kenya, 13–15 May 1991, ILRAD, Nairobi, pp. 3–12.

Dewey, H.M. and Wormall, A., 1946. Studies on suramin (antrypol: Bayer 205). 5. The combination of the drug with the plasma and other proteins. Biochem. J., 40: 119–124.

Dhar, S., Malhotra, D.V., Bhushan, C. and Gautam, O.P., 1988. Treatment of experimentally induced *Theileria annulata* infection in cross-bred calves with buparvaquone. Vet. Parasitol., 27: 267–275.

Dhar, S., Malhotra, D.V., Bhushan, C. and Gautam, O.P., 1990. Chemoimmunoprophylaxis against bovine tropical theileriosis in young calves: a comparison between buparvaquone and long-acting oxytetracycline. Res. Vet. Sci., 49: 110–112.

Dieleman, E.F., 1986. Trypanosomiasis in Indonesia. A review of research, 1900–1983. Vet. Q., 8: 250–256.

Dolan, R.B., Okech, G., Alushula, H., Mutugi, M., Stevenson, P., Sayer, P.D. and Njogu, A.R., 1990. Homidium bromide as a chemoprophylactic for cattle trypanosomiasis in Kenya. Acta Trop., 47: 137–144.

Dolan, R.B., Stevenson, P.G.W., Alushula, H. and Okech, G., 1992. Failure of chemoprophylaxis against bovine trypanosomiasis on Galana ranch in Kenya. Acta Trop., 51: 113–121.

Dolan, T.T., 1981. Progress in the chemotherapy of theileriosis. In: A.D. Irvin, M.P. Cunningham and A.S. Young (Editors), Advances in the Control of Theileriosis. Martinus Nijhoff, The Hague, pp. 186–208.

Dolan, T.T., 1985. The choice of drug for infection and treatment immunization against East African theileriosis. In: A.D. Irvin (Editor), Immunization against Theileriosis in Africa. ILRAD, Nairobi, pp. 100–103.

Dolan, T.T., 1987. Immunization to control East Coast Fever. Parasitol. Today, 3: 4–6, 10.

Dolan, T.T., 1992. Therapeutics. In: T.T. Dolan (Editor), Recent Developments in the Control of Anaplasmosis, Babesiosis and Cowdriosis. Proc. of a Workshop, ILRAD, Nairobi, Kenya, 13–15 May 1991, ILRAD, Nairobi, pp. 166–167.

Dolan, T.T., Young, A.S., Leitch, B.L. and Stagg, D.A., 1984. Chemotherapy of East Coast fever: parvaquone treatment of clinical disease induced by isolates of *Theileria parva*. Vet. Parasitol., 15: 103–116.

Dolan, T.T., Linyonyi, A., McHardy, N., Bond, A.L. and Clampitt, R.B., 1988. Chemotherapy of East Coast fever: parvaquone treatment of *Theileria parva parva* at intervals after infection. Res. Vet. Sci., 44: 15–20.

Dolan, T.T., Injairu, R., Gisemba, F., Maina, J.N., Mbadi, G., Mbwiria, S.K., Mulela, G.H.M. and Othieno, D.A.O., 1992. A clinical trial of buparvaquone in the treatment of East Coast fever. Vet. Rec., 130: 536–538.

Dowler, M.E., Schillinger, D. and Connor, R.J., 1989. Notes on the routine intravenous use of isometamidium in the control of bovine trypanosomiasis on the Kenya Coast. Trop. Anim. Health Prod., 21: 4–10.

Eisler, M.C., Gault, E.A., Smith, H.V., Peregrine, A.S. and Holmes, P.H., 1993. Evaluation and improvement of an enzyme-linked immunosorbent assay (ELISA) for the detection of isometamidium in bovine serum. Ther. Drug Monit., 15: 236–242.

Fairclough, R., 1962. A summary of the use of Berenil in Kenya. In: Proc. 9th Meeting of the International Scientific Council for Trypanosomiasis Research, Conakry, Guinea, 1962. Commission for Technical Cooperation in Africa, Lagos, CCTA Publ. No. 88, pp. 81–86.

Fairclough, R., 1963. Observations on the use of Berenil against trypanosomiasis of cattle in Kenya. Vet. Rec., 75: 1107–1113.

FAO/WHO/OIE. 1982. Animal Health Yearbook, 1981. V. Kouba (Editor), FAO, Rome.

Farwell, G.E., LeGrand, E.K. and Cobb, C.C., 1982. Clinical observations on *Babesia gibsoni* and *Babesia canis* infection in dogs. J. Am. Vet. Med. Assoc., 180: 507–511.

Fiennes, R.N.T.W., 1953. The therapeutic and prophylactic properties of antrycide in trypanosomiasis of cattle. Br. Vet. J., 109: 280–295.

Fluck, D.J. and Hopkins, J.S., 1987. Chemotherapy and chemoprophylaxis of bovine trypanosomiasis with liposomal trypanocides. In: Proc. 19th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Lomé, Togo, 1987, OAU/STRC, Nairobi, Publ. No. 114, pp. 302–313.

Fussgänger, R. and Bauer, F., 1958. Berenil: ein neues Chemotherapeuticum in der Veterinärmedizin. Med. Chem., 6: 504–531.

Gardiner, P.R. and Mahmoud, M.M., 1992. Salivarian trypanosomes causing disease in livestock outside sub-Saharan Africa. In: J.P. Kreier and J.R. Baker (Editors), Parasitic Protozoa, Vol. 2. Academic Press, San Diego, CA, pp. 277–314.

Gill, B.S., 1971. Resistance of *Trypanosoma evansi* to quinapyramine, suramin, stilbamidine and tryparsamide and analysis of cross-resistance. Trans. R. Soc. Trop. Med. Hyg., 65: 352–357.

Gill, B.S., Bhattacharyulu, Y., Singh, A. and Kaur, D., 1984. Chemotherapy of bovine tropical theileriosis: *Theileria annulata* infection. Res. Vet. Sci., 37: 247–248.

Gitatha, S.K., 1979. *T. congolense* (Shimba Hills) resistant to various trypanocidal drugs. In: Proc. 16th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yaounde, Cameroon, 1979, Eleza Services, Nairobi, OAU/STRC Publ. No. 111. pp. 257–263.

Graber, M., 1968. Note sur la résistance au Bérénil d'une souche tchadienne de *Trypanosoma vivax*. Rev. Élev. Méd. Vét Pays Trop., 21: 463–466.

Gray, A.R., 1961. Suramin complexes. VIII. A further trial of the prophylactic activity of antrycide–suramin complex and antrycide chloride against *Trypanosoma simiae* in pigs. Ann. Trop. Med. Parasitol., 55: 432–439.

Gray, A.R. and Roberts, C.J., 1971. The cyclical transmission of strains of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs. Parasitology, 63: 67–89.

Gray, M.A., Kimarua, R.W., Peregrine, A.S. and Stevenson, P., 1993. Drug sensitivity screening in vitro of *Trypanosoma congolense* originating from cattle and tsetse flies at Nguruman, Kenya. Acta Trop., 55: 1–9.

Hashemi-Fesharki, R., 1991. Chemotherapeutic value of parvaquone and buparvaquone against *Theileria annulata* infection of cattle. Res. Vet. Sci., 50: 204–207.

Hassan, H.F. and Coombs, G.H., 1988. Purine and pyrimidine metabolism in parasitic protozoa. FEMS Microbiol. Rev., 54: 47–84.

Hawa, N., Rae, D.G., Younis, S.S., Mahadi, W., Ibrahim, R. and Al-Wahab, W., 1988. Efficacy of parvaquone in the treatment of naturally occurring theileriosis in cattle in Iraq. Trop. Anim. Health Prod., 20: 130–136.

Hawking, F., 1963. Chemotherapy of trypanosomiasis. In: R.J. Schnitzer and F. Hawking (Editors), Experimental Chemotherapy, Vol. 1. Academic Press, New York, pp. 129–256.

Hill, G.C. and Hirumi, H., 1983. African trypanosomes. In: J.B. Jensen (Editor), In Vitro Cultivation of Protozoan Parasites. CRC Press, Boca Raton, FL, pp. 193–219.

Hudson, A.T., Randall, A.W., Fry, M., Ginger, C.D., Hill, B., Latter, V.S., McHardy, N. and Williams, R.B., 1985. Novel antimalarial hydroxynaphthoquinones with potent broad spectrum anti-protozoal activity. Parasitology, 90: 45–55.

Hudson, A.T., Pether, M.J., Randall, A.W., Fry, M., Latter, V.S. and McHardy, N., 1986. In vitro activity of 2-cycloalkyl-3-hydroxy-1, 4 naphthoquinones against *Theileria*, *Eimeria* and *Plasmodium* species. Eur. J. Med. Chem., 21: 271–275.

Ilemobade, A.A., 1979. Drug sensitivity of mouse infective *Trypanosoma vivax* isolates in cattle and sheep. In: Proc. 16th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yaounde, Cameroon, 1979, Eleza Services, Nairobi, OAU/STRC Publ. No. 111, pp. 251–253.

James, D.M., 1978. Prophylactic activity in rodents of trypanocides complexed with dextran. Trans. R. Soc. Trop. Med. Hyg., 72: 471–476.

Jensch, H., 1958. Über neue Typen von Guanylverbindungen. Med. Chem., 6: 134–169.

Jones-Davies, W.J., 1967. A Berenil-resistant strain of *Trypanosoma vivax* in cattle. Vet. Rec., 81: 567–568.

Jones-Davies, W.J., 1968. Berenil resistance in naturally occurring *Trypanosoma congolense*. Bull. Epizoot. Dis. Afr., 16: 213–216.

Jones-Davies, W.J. and Folkers, C., 1966. Some observations on cross-resistance to Samorin and Berenil of homidium-resistant field strains of *Trypanosoma congolense* in northern Nigerian cattle. In: Proc. 11th Meeting of the International Scientific Council for Trypanosomiasis Research, Nairobi, Kenya, 1966, Publications Bureau, Lagos, OAU/STRC Publ. No. 100, pp. 35–40.

Kaminsky, R., 1990. In vitro techniques for assessment of drug resistance in trypanosomes. Ag-Biotech News Inf., 2: 205–210.

Kinabo, L.D.B. and Bogan, J.A., 1988. Parvaquone and buparvaquone: HPLC analysis and comparative pharmacokinetics in cattle. Acta Trop., 45: 87–94.

Kirkby, W.W., 1964. Prophylaxis and therapy under continuous exposure to the risk of natural infection with trypanosomiasis by tsetse flies. Bull. Epizoot. Dis. Afr., 12: 321–329.

Küpper, W. and Wolters, M., 1983. Observation on drug resistance of *Trypanosoma* (*Nannomonas*) *congolense* and *Trypanosoma* (*Duttonella*) *vivax* in cattle at a feedlot in the Northern Ivory Coast. Tropenmed. Parasitol., 34: 203–205.

Kuttler, K.L., 1981. Chemotherapy of babesiosis: a review. In: M. Ristic and J.P. Kreier (Editors), Babesiosis. Academic Press, New York, pp. 65–85.

Kuttler, K.L., 1988. Chemotherapy of babesiosis. In: M. Ristic (Editor), Babesiosis of Domestic Animals and Man. CRC press Inc., Boca Raton, Florida, pp. 227–243.

Leach, T.M., Karib, A.A., Ford, E.J.H. and Wilmshurst, E.C., 1955. Studies on ethidium bromide. VI. The prophylactic properties of the drug. J. Comp. Pathol., 65: 130–142.

Leach, T.M. and Roberts, C.J., 1981. Present status of chemotherapy and chemoprophylaxis of animal trypanosomiasis in the eastern hemisphere. Pharmacol. Ther., 13: 91–147.

Lewis, A.R. and Thomson, J.W., 1974. Observations on an isometamidium resistant strain of *Trypanosoma congolense* in Rhodesia. Rhod. Vet. J., 4: 62–67.

Lun, Z.-R., Min, Z.-P., Huang, D., Liang, J.-X., Yang, X.-F. and Huang, Y.-T., 1991. Cymelarsan in the treatment of buffaloes naturally infected with *Trypanosoma evansi* in South China. Acta Trop., 49: 233–236.

MacLennan, K.J.R. and Jones-Davies, W.J., 1967. The occurrence of a Berenil-resistant *Trypanosoma congolense* strain in northern Nigeria. Vet. Rec., 80: 389–390.

Mbwambo, H.A., Mkonyi, P.A. and Chua, R.B., 1987. Field evaluation of parvaquone against naturally occurring East Coast fever. Vet. Parasitol., 23: 161–168.

Mbwambo, H.A., Mella, P.N.P. and Lekaki, K.A., 1988. Berenil (diminazene aceturate)resistant *Trypanosoma congolense* in cattle under natural tsetse challenge at Kibaha, Tanzania. Acta Trop., 45: 239–244.

McHardy, N., 1979. Experimental therapy of theileriosis. J. S. Afr. Vet. Assoc., 50: 321–322.

McHardy, N., 1984. Recent advances in the chemotherapy of theileriosis. In: H.P. Riemann and M.J. Burridge (Editors), Impact of Diseases on Livestock Production in the Tropics. Elsevier, Amsterdam, pp. 179–192.

McHardy, N., 1989a. Buparvaquone, the new antitheilerial: a review of its efficacy and safety. In: T.T. Dolan (Editor), Theileriosis in Eastern, Central and Southern Africa. Proc. of a Workshop on East Coast Fever Immunization, Lilongwe, Malawi, 20–22 September 1988, ILRAD, Nairobi, pp. 158–165.

McHardy, N., 1989b. Multinational research on the use of buparvaquone (Butalex) for the control of theileriosis. In: K. Demirozu, U.G. Nadas, C. Alt i nel, Y. Uysal, J. Turkaslan and H. Alp (Editors), Proc. Int. Symposium on Mycoplasmosis and Theileriosis, 11–13 October 1989, Pendik, Turkey, Pendik Animal Diseases Central Research Institute, Istanbul, pp. 120–126.

McHardy, N., 1991. Integrated strategy to control East Coast Fever. Vet. Rec., 129: 475.

McHardy, N. and Simpson, R.M., 1974. Imidocarb dipropionate therapy in Kenyan anaplasmosis and babesiosis. Trop. Anim. Health Prod., 6: 63–70.

McHardy, N., Haigh, A.J.B. and Dolan, T.T., 1976. Chemotherapy of *Theileria parva* infection. Nature, 261: 698–699.

McHardy, N., Wekesa, L.S., Hudson, A.T. and Randall, A.W., 1985. Antitheilerial activity of BW 720C (buparvaquone): a comparison with parvaquone. Res. Vet. Sci., 39: 29–33.

Minami, T.M., Nakano, T., Shimizu, S., Shimura, K., Fujinaga, T. and Ito, S., 1985. Efficacy of naphthoquinones and imidocarb dipropionate on *Theileria sergenti* infections in splenectomized calves. Jpn. J. Vet. Sci., 47: 297–300.

Mitema, E.S., Kocan, A.A., Mukolwe, S.W., Sangiah, S. and Sherban, D., 1991. Activity of buparvaquone against *Theileria cervi* in white-tailed deer. Vet. Parasitol., 38: 49–53.

Mohamed-Ahmed, M.M., Rahman, A.H. and Abdel Karim, E.I., 1992. Multiple drug-resistant bovine trypanosomes in South Darfur Province, Sudan. Trop. Anim. Health Prod., 24: 179–181.

Moloo, S., Chema, S., Connor, R., Durkin, J., Kimotho, P., Maehl, Mukendi, F., Murray, M., Rarieya, M. and Trail, J., 1987. Efficacy of chemoprophylaxis for East African Zebu cattle exposed to trypanosomiasis in village herds in Kenya. In: Proc. 19th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Lomé, Togo, 1987, OAU/STRC, Nairobi, Publ. No. 114, pp. 282–287.

Morgan, D.W.T. and McHardy, N., 1982. Comparison of the antitheilerial effect of Wellcome 993C and halofuginone. Res. Vet. Sci., 32: 84–88.

Mukhebi, A.W., 1992. Economic impact of theileriosis and its control in Africa. In: R.A.I. Norval, B.D. Perry and A.S. Young (Editors), The Epidemiology of Theileriosis in Africa. Academic Press, London, pp. 379–462.

Münstermann, S., Mbura, R.J., Maloo, S.H. and Löhr, K.-F., 1992. Trypanosomiasis control in Boran cattle in Kenya: a comparison between chemoprophylaxis and a parasite detection and intravenous treatment method using isometamidium chloride. Trop. Anim. Health Prod., 24: 17–27.

Mutugi, J.J., Young, A.S., Maritim, A.C., Linyonyi, A., Mbogo, S.K. and Leitch, B.L., 1988. Immunization of cattle using varying infective doses of *Theileria parva lawrencei* sporozoites derived from an African buffalo (*Syncerus caffer*) and treatment with buparvaquone. Parasitology, 96: 391–402.

Mutugi, J.J., Young, A.S., Kariuki, D.P., Ole Tameno, J.M. and Morzaria, S.P., 1991. Epidemiological observations on theileriosis following field immunisation using infection and treatment. Trop. Anim. Health Prod., 23: 75–82.

Mwambu, P.M. and Mayende, J.S.P., 1971. Berenil resistant *Trypanosoma vivax*, isolated from naturally infected cattle in Teso District, eastern Uganda. Proc. 13th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Lagos, 7–11 September 1971, OAU/STRC, Niamey, Publ. No. 105, pp. 133–138.

Na'Isa, B.K., 1967. Follow-up of a survey on the prevalence of homidium-resistant strains of trypanosomes in cattle in northern Nigeria and drug cross-resistance tests on the strains with Samorin and Berenil. Bull. Epizoot. Dis. Afr., 15: 231–241.

Ndoutamia, G., Moloo, S.K., Murphy, N.B. and Peregrine, A.S., 1993. Derivation and characterization of a quinapyramine-resistant clone of *Trypanosoma congolense*. Antimicrob. Agents Chemother., 37: 1163–1166.

Neitz, W.O., 1953. Aureomycin in *Theileria parva* infection. Nature, 171: 34–35.

Ngumi, P.N., Young, A.S., Lampard, D., Mining, S.K., Ndungu, S.G., Lesan, A.C., Williamson, S.M., Linyonyi, A. and Kariuki, D.P., 1992. Further evaluation of the use of buparvaquone in the infection and treatment method of immunizing cattle against *Theileria parva* derived from African buffalo (*Syncerus caffer*). Vet. Parasitol., 43: 15–24.

Njau, B.C. and Mkonyi, P.M., 1981. Preliminary studies on chemotherapy of bovine theileriosis with halofuginone in Tanzania. In: A.D. Irvin, M.P. Cunningham and A.S. Young (Editors), Advances in the Control of Theileriosis. Martinus Nijhoff, The Hague, pp. 215–217.

Njau, B.C., Mkonyi, P.M. and Kundy, D.J., 1981. Berenil resistant *Trypanosoma congolense* isolated

from naturally infected goats in Tanga Region, Tanzania. In: Proc. 17th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Arusha, Tanzania, 1981, OAU/STRC Publ. No. 112, Eleza Services Ltd., Nairobi, pp. 289–298.

Njau, B.C., Mkonyi, P.M., Maleche, W.C.H., Kitaly, J.I. and Maiseli, N.C., 1985. Treatment of field cases of East Coast fever with halofuginone lactate. Trop. Anim. Health Prod., 17: 193–200.

Norval, R.A.I., Meltzer, M.I. and Burridge, M.J., 1992. Distribution, economic importance and control measures for *Cowdria ruminantium*. In: T.T. Dolan (Editor), Recent Developments in the Control of Anaplasmosis, Babesiosis and Cowdriosis. Proc. of a Workshop, ILRAD, Nairobi, Kenya, 13–15 May, 1991, ILRAD, Nairobi, pp. 13–27.

Osman, A.S., Jennings, F.W. and Holmes, P.H., 1992. The rapid development of drug-resistance by *Trypanosoma evansi* in immunosuppressed mice. Acta Trop., 50: 249–257.

Otsyula, M., Kamar, K., Mutugi, M. and Njogu, A.R., 1992. Preliminary efficacy trial of Cymelarsan, a novel trypanocide, in camels naturally infected with *Trypanosoma evansi* in Kenya. Acta Trop., 50: 271–273.

Peregrine, A.S., Ogunyemi, O., Whitelaw, D.D., Holmes, P.H., Moloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M., 1988. Factors influencing the duration of isometamidium chloride (Samorin) prophylaxis against experimental challenge with metacyclic forms of *Trypanosoma congolense*. Vet. Parasitol., 28: 53–64.

Peregrine, A.S., Moloo, S.K. and Whitelaw, D.D., 1991. Differences in sensitivity of Kenyan *Trypanosoma vivax* populations to the prophylactic and therapeutic actions of isometamidium chloride in Boran cattle. Trop. Anim. Health Prod., 23: 29–38.

Pinder, M. and Authié, E., 1984. The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. Acta Trop., 41: 247–252.

Radley, D.E., Brown, C.G.D., Burridge, M.J., Cunningham, M.P., Kirimi, I.M., Purnell, R.E. and Young, A.S., 1975. East Coast Fever: 1. Chemoprophylactic immunization of cattle against *Theileria parva* (Muguga) and five theilerial strains. Vet. Parasitol., 1: 35–41.

Raynaud, J.P., Sones, K.R. and Friedheim, E.A.H., 1989. A review of cymelarsan®—a new treatment proposed for animal trypanosomiasis due to *T. evansi* and other trypanosomes of the *T. brucei* group. In: Proc. 20th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Mombasa, Kenya, 1989, OAU/STRC, Nairobi, Publ. No. 115, pp. 334–338.

Röttcher, D. and Schillinger, D., 1985. Multiple drug resistance in *Trypanosoma vivax* in the Tana River District of Kenya. Vet. Rec., 117: 557–558.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M. and Peregrine, A.S., 1993. Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. Acta Trop., 53: 135–150.

Ruchel, W.-M., 1975. Chemoprophylaxis of bovine trypanosomiasis. Thesis, University of Göttingen, Germany, 248 pp.

Ryley, J.F. and Wilson, R.G., 1975. Laboratory studies with some recent anticoccidials. Parasitology, 70: 203–222.

Schein, E. and Voigt, W.P., 1979. Chemotherapy of bovine theileriosis with halofuginone. Acta Trop., 36: 391–394.

Schein, E. and Voigt, W.P., 1981. Chemotherapy of theileriosis in cattle. In: A.D. Irvin, M.P. Cunningham and A.S. Young (Editors), Advances in the Control of Theileriosis. Martinus Nijhoff, The Hague, pp. 212–214.

Schillinger, D. and Röttcher, D., 1986. Treatment of camels for *Trypanosoma brucei evansi* infection (surra). World Anim. Rev., 60: 26–32.

Schmidt, G., Hirt, R. and Fischer, R., 1969. Babesicidal effect of basically substituted carbanilides. I. Activity against *Babesia rodhaini* in mice. Res. Vet. Sci., 10: 530–533.

Schönefeld, A., Röttcher, D. and Moloo, S.K., 1987. The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. Trop. Med. Parasitol., 38: 177–180.

Sharma, N.N. and Mishra, A.K., 1990. Treatment of bovine tropical theileriosis with buparvaquone. Trop. Anim. Health Prod., 22: 63–65.

Sones, K.R., Njogu, A.R. and Holmes, P.H., 1988. Assessment of sensitivity of *Trypanosoma congo-*

lense to isometamidium chloride: a comparison of tests using cattle and mice. Acta Trop., 45: 153–164.

Spooner, P.R., 1990. The effects of oxytetracycline on *Theileria parva* in vitro. Parasitology, 100: 11–17.

Stephen, L.E., 1958. Suramin complexes. IV. Ethidium bromide complex: a large-scale laboratory trial of its prophylactic activity in cattle. Ann. Trop. Med. Parasitol., 52: 417–426.

Stephen, L.E., 1962. Suramin complexes. IX. An experiment to test the therapeutic activity of antrycide–suramin complex against *Trypanosoma simiae* in large white pigs. Ann. Trop. Med. Parasitol., 56: 401–405.

Stephen, L.E., 1966. Pig trypanosomiasis in Africa. Commonwealth Agricultural Bureaux, UK. 65 pp.

Stephen, L.E. and Gray, A.R., 1960. Suramin complexes. VI. The prophylactic activity of antrycide–suramin complex and antrycide chloride against *Trypanosoma simiae* in pigs. Ann. Trop. Med. Parasitol., 54: 493–507.

Sutherland, I.A., Moloo, S.K., Holmes, P.H. and Peregrine, A.S., 1991. Therapeutic and prophylactic activity of isometamidium chloride against a tsetse-transmitted drug-resistant clone of *Trypanosoma congolense* in Boran cattle. Acta Trop., 49: 57–64.

Sutherland, I.A., Codjia, V., Moloo, S.K., Holmes, P.H. and Peregrine, A.S., 1992. Therapeutic activity of isometamidium chloride in Boran cattle against a tsetse-transmitted clone of *Trypanosoma congolense* with a low level of drug resistance. Trop. Anim. Health Prod., 24: 157–163.

Tager-Kagan, P., Itard, J. and Clair, M., 1989. Essai de l'efficacité du CymelarsanND sur *Trypanosoma evansi* chez le dromadaire. Rev. Élev. Méd. Vét. Pays Trop., 42: 55–61.

Thibault, J.M. and Jaussaud, P.H., 1987. Le diminazène et son utilisation chez le chien. 1. Aspects pharmacologiques et thérapeutiques. Rev. Méd. Vét., 138: 45–49.

Todorovic, R.A., Vizcaino, O.G., Gonzalez, E.F. and Adams, L.G., 1973. Chemoprophylaxis (Imidocarb) against *Babesia bigemina* and *Babesia argentina* infections. Am. J. Vet. Res., 34: 1153–1161.

Trail, J.C.M., Murray, M., Sones, K., Jibbo, J.M.C., Durkin, J. and Light, D., 1985. Boran cattle maintained by chemoprophylaxis under trypanosomiasis risk. J. Agric. Sci., 105: 147–166.

Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection): current status. Adv. Vet. Sci. Comp. Med., 27: 427–480.

Uilenberg, G., Jongejan, F., Perie, N.M. and Franssen, F.F.J., 1980. Chimiothérapie des theilérioses bovines par un anticoccidien, l'halofuginone. Note préliminaire. Rev. Élev. Méd. Vét. Pays Trop., 33: 33–43.

Uilenberg, G., Verdiesen, P.A.H.M. and Zwart, D., 1981. Imidocarb: a chemoprophylactic experiment with *Babesia canis*. Vet. Q., 3: 118–123.

Unsuren, H., Kurtdede, A. and Goksu, K., 1988. Effectiveness of parvaquone in cattle infected with *Theileria annulata*. Trop. Anim. Health Prod., 20: 256–258.

Watkins, T.I. and Woolfe, G., 1952. Effect of changing the quaternizing group on the trypanocidal activity of dimidium bromide. Nature, 169: 506.

Whitelaw, D.D., Bell, I.R., Holmes, P.H., Moloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M., 1986. Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. Vet. Rec., 118: 722–726.

Whiteside, E.F., 1960. Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In: Proc. 8th Meeting of the International Scientific Committee for Trypanosomiasis Research, Jos, Nigeria, 1960, CCTA Publ. No. 62, Publications Bureau, London, pp. 141–154.

Whiteside, E.F., 1962. Interactions between drugs, trypanosomes and cattle in the field. In: L.G. Goodwin and R.H. Nimmo-Smith (Editors), Drugs, Parasites and Hosts. Churchill, London, pp. 116–141.

Williamson, J., 1957. Suramin complexes. I. Prophylactic activity against *Trypanosoma congolense* in small animals. Ann. Trop. Med. Parasitol., 51: 440–456.

Williamson, J., 1970. Review of chemotherapeutic and chemoprohylactic agents. In: H.W. Mulligan (Editor), The African Trypanosomiases. Allen and Unwin, London, pp. 125–221.

Williamson, J., 1976. Chemotherapy of African trypanosomiasis. Trop. Dis. Bull., 73: 531–542.

Williamson, J. and Desowitz, R.S., 1956. Prophylactic activity of suramin complexes in animal trypanosomiasis. Nature, 177: 1074–1075.

Wragg, W.R., Washbourn, K., Brown, K.N. and Hill, J., 1958. Metamidium: a new trypanocidal drug. Nature, 182: 1005–1006.

Young, A.S., Leitch, B.L., Dolan, T.T., Mbogo, S.K., Ndungu, S.G., Grootenhuis, J.G. and De Castro, J.J., 1990. Evaluation of infection and treatment methods in immunization of improved cattle against theileriosis in an endemic area of Kenya. Vet. Parasitol., 35: 239–257.

Young, A.S., Mutugi, J.J., Kariuki, D.P., Lampard, D., Maritim, A.C., Ngumi, P.N., Linyonyi, A., Leitch, B.L., Ndungu, S.G., Lesan, A.C., Mining, S.K., Grootenhuis, J.G., Orinda, G.O. and Wesonga, D., 1992. Immunisation of cattle against theileriosis in Nakuru District of Kenya by infection and treatment and the introduction of unconventional tick control. Vet. Parasitol., 42: 225–240.

Zelleke, D., Kassa, B. and Abebe, S., 1989. Efficacy of RM110, a novel trypanocide, in the treatment of *Trypanosoma evansi* infections in camels. Trop. Anim. Health Prod., 21: 223–226.

Zweygarth, E. and Kaminsky, R., 1990. Evaluation of an arsenical compound (RM 110, mel Cy, Cymelarsan®) against susceptible and drug-resistant *Trypanosoma brucei brucei* and *T. b. evansi*. Trop. Med. Parasitol., 41: 208–212.

Section 1.2

- Discussion -

In contrast to the therapeutic and prophylactic compounds currently recommended for theileriosis, babesiosis, anaplasmosis and cowdriosis, the literature reviewed in this Chapter indicates that the major problem associated with use of anti-trypanosomal compounds is the development of drug resistance. Furthermore, over the last thirty years the incidence of reports of resistance to anti-trypanosomal compounds has been gradually increasing. Similarly, the frequency of reports of multiple-drug resistance has been proliferating. It is unclear, however, whether the increase in incidence of such reports reflects a true enlargement of the incidence of drug resistance or a growth in interest in drug resistance by scientists in different parts of Africa.

In 1993, at the time of publication of the paper in Section 1.1, publications in international journals indicated that resistance to the minimum recommended dose of diminazene aceturate, isometamidium chloride or the salts of homidium in cattle had been observed in trypanosome populations in eleven countries within sub-Saharan Africa; Burkina Faso, Chad, Côte d'Ivoire, Ethiopia, Kenya, Nigeria, Somalia, Sudan, Tanzania, Uganda and Zimbabwe. Since the first reports of resistance to isometamidium in West, Central, East and Southern Africa all occurred within a 14-year period, between 1966 and 1979, it would appear most likely that the development of resistance in these different regions occurred independently, rather than as a result of spread across the continent. Such an aetiology would be consistent with the suggested mode of development of resistance to anti-malarial compounds in *Plasmodium* spp. across Africa (WHO, 1987).

At present, the factor(s) responsible for the development of resistance to anti-trypanosomal compounds has not been clearly defined. In some parts of Africa, innate resistance (Hayes and Wolf, 1990) may be a contributing factor (Williamson, 1960). Such resistance has recently been suggested to be responsible for the diminazene resistance described in *Trypanosoma vivax* from French Guyana, South America (Desquesnes et al., 1995). However, in light of the widespread use of anti-

trypanosomal compounds in Africa, such an aetiology of drug resistance is now largely impossible to establish within this continent.

In general, the most important factor predisposing to the development of drug resistance appears to be underdosing and the resultant exposure of parasites to sub-therapeutic drug concentrations (Stephen, 1986). In many situations within Africa the availability of anti-trypanosomal compounds is hampered by poor infrastructures and delivery systems (Holmes and Scott, 1982). An additional confounding factor, that also results in inadequate supplies to farmers, has been the inability of some governments to establish revolving funds for the purchase of trypanocides (Connor, 1989). As a result, the lack of adequate quantities of trypanocides, and their relatively high prices, has encouraged both fraudulent marketing and underdosing.

Lastly, as a result of the closely related chemical structures of the different anti-trypanosomal compounds it has been suggested that resistance to many of the compounds may develop in the field as a result of cross-resistance (Whiteside, 1960; Williamson, 1970). However, since such conclusions were based on field studies and not controlled laboratory experiments, some workers have questioned the validity of the conclusions (Rhône Mérieux, personal communication).

As mentioned, definitive information on the factors responsible for development, maintenance and abrogation of drug-resistant trypanosome populations in the field is yet to be derived. The research presented in Chapters 2, 3, 4, and 5 of this thesis was carried out to address this knowledge deficit.

Chapter 2 describes a series of studies that were carried out in the Ghibe valley, Ethiopia, to define the level and prevalence of drug resistance in trypanosomes at this site, and the impact of drug resistance on livestock production. In particular, the development of a model for deriving estimates of the prevalence of drug-resistant infections in cattle at Ghibe from longitudinal parasitological data is described, and verified with laboratory studies. Since a high prevalence of multiple-drug resistant

infections was observed, the long-term persistence of such infections and the impact of various control methods on these infections was investigated. Lastly, the potential contribution of quinapyramine resistance to the resistance phenotype at Ghibe was also evaluated.

Since reappearance of *T. brucei brucei* in animals following treatment may be associated with invasion of the central nervous system (CNS), rather than true drug resistance (Jennings et al., 1977, 1979), Chapter 3 describes work that was carried out to determine whether *T. congolense* can invade the CNS in goats. A CNS-cannulation procedure was developed for this purpose and was used to characterise the pharmacokinetics of diminazene in cerebrospinal fluid of goats: to determine whether the compound would exhibit trypanocidal activity against trypanosomes if they accessed the CNS.

Chapter 4 contains a series of experiments that were carried out *in vivo* to characterise the stability of resistance to diminazene in *T. congolense*. Particular attention was given to determining the effect of duration of infection and trypanosome-population density on the level of diminazene resistance of parasite populations. Finally, since a number of researchers have suggested that the therapeutic activity of diminazene would be enhanced if the drug was given to infected animals on two occasions, rather than one, this hypothesis was evaluated in goats infected with a diminazene-resistant trypanosome population.

As highlighted in Section 1.1, all the methods that are currently available for quantifying the level of drug resistance of trypanosome populations take many weeks or months to derive definitive data. Furthermore, many are impractical for obtaining precise estimates of the true prevalence of drug-resistant infections in the field. Since information on the molecular basis of the action of anti-trypanosomal compounds may lead to the development of biochemical-based diagnostics for rapidly quantifying the level of resistance of trypanosome populations, Chapter 5

describes a series of experiments that were carried out to elucidate the molecular basis of isometamidium's activity.

References:

Connor, R.J. (1989) Final report of the regional trypanosomiasis expert. Regional tsetse and trypanosomiasis control programme, Malawi, Mozambique, Zambia and Zimbabwe. FGU-Kronberg Consulting + Engineering GmbH, Germany, pp. 123.

Desquesnes, M., La Rocque, S. (de) and Peregrine, A.S. (1995) French Guyanan stock of *Trypanosoma vivax* resistant to diminazene aceturate but sensitive to isometamidium chloride. *Acta Tropica* 60, 133-136.

Hayes, J.D. and Wolf, C.R. (1990) Molecular mechanisms of drug resistance. *Biochemical Journal* 272, 281-295.

Holmes, P.H. and Scott, J.M. (1982) Chemotherapy against animal trypanosomiasis. In: *Perspectives in trypanosomiasis research.* Baker, J.R. (ed.), Research Studies Press, pp. 59-69.

Jennings, F.W., Whitelaw, D.D. and Urquhart, G.M. (1977) The relationship between duration of infection with *Trypanosoma brucei* in mice and the efficacy of chemotherapy. *Parasitology* 75, 143-153.

Jennings, F.W., Whitelaw, D.D., Holmes, P.H., Chizyuka, H.G.B. and Urquhart, G.M. (1979) The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal of Parasitology* 9, 381-384.

Stephen, L.E. (1986) Trypanosomiasis: a veterinary perspective. Pergamon Press, pp. 454-479.

Whiteside, E. F. (1960) Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In *Proceedings of the International Scientific Committee for Trypanosomiasis Research, 8th meeting, Jos, Nigeria.* Commission for Technical Co-operation South of the Sahara, London, Publication No. 62, pp. 141-153.

WHO (1987) The epidemiology of drug resistance of malaria parasites: memorandum from a WHO meeting. *Bulletin of the World Health Organisation* 65, 797-816.

Williamson, J. (1960) Some problems in trypanocidal drug resistance. In *Proceedings of the International Scientific Committee for Trypanosomiasis Research, 8th meeting, Jos, Nigeria.* Commission for Technical Co-operation South of the Sahara, London, Publication No. 62, pp. 163-169.

Williamson, J. (1970) Review of chemotherapeutic and chemoprophylactic agents. In *The African trypanosomiases*. Mulligan, H.W. (ed.), Allen and Unwin, Ltd., London, pp. 116-141.

Chapter 2

Studies on resistance to anti-trypanosomal compounds in trypanosome populations in the field, and the contribution of cross-resistance to the resistance phenotype

Section 2.0

- Introduction -

In the literature review in Section 1.1 of this thesis a summary is given of all the reports of resistance to recommended doses of diminazene aceturate, isometamidium chloride and homidium chloride/bromide in livestock that had been published in international scientific journals up to 1993. Unfortunately, in almost all of these publications the reported drug-sensitivity data were only for a limited number of trypanosome populations. Thus, the relationship between the described drug-resistance phenotypes and the prevalence of these phenotypes in the areas the trypanosomes were obtained from is generally not clear. This particular problem was highlighted in work that was carried out in the mid 1980s on isolates of *Trypanosoma vivax* from cattle at Galana Ranch, Kenya. In this work Röttcher and Schillinger (1985) described a cocktail of 11 isolates from cattle on the Ranch that was resistant in cattle to recommended doses of isometamidium chloride, diminazene aceturate, homidium chloride and quinapyramine sulphate. However, subsequent evaluation of the drug sensitivity of the individual isolates that comprised the cocktail failed to identify which isolate(s) the resistance was associated with (Njogu and Heath, 1986). Thus, the inference by Röttcher and Schillinger (1985) that multiple-drug resistance in *T. vivax* was widespread was not confirmed.

Because of the general paucity of information on the prevalence of drug-resistant trypanosome infections in the field, and the associated lack of information on the epidemiology of drug-resistant trypanosomes, the work described in Sections 2.1, 2.2, 2.3 and 2.4 was carried out as part of a long-term study, from 1986 to 1993, in the Ghibe valley, Ethiopia, to define the constraint that drug resistance in trypanosomes poses to livestock production at the site. Section 2.1 describes the application of a mathematical model, using longitudinal parasitological data, to determine the apparent prevalence of drug-resistant infections in cattle at the site. Section 2.2 describes a series of laboratory investigations that were carried out to characterise the drug sensitivity of trypanosome isolates that were collected from cattle in the valley in 1989.

At present, very little information is available on the pathogenicity of drug-resistant trypanosome populations. As a result, their potential threat to livestock production is unclear. In the past, some workers have suggested that drug-resistant trypanosomes are less pathogenic than drug-sensitive populations (Goble et al., 1959; Stephen, 1962). However, as indicated in various Sections of this chapter, such a generalisation can not be applied to the trypanosome populations occurring in cattle at Ghibe during the course of the described studies. In fact, as a result of the significant constraint that trypanosomiasis posed to livestock production at the site (Rowlands et al., 1994a, b), Section 2.3 describes an experiment that was carried out at Ghibe to determine if the pathogenic multiple-drug resistant infections could be adequately controlled. Lastly, because little information was available on the long-term stability of drug resistance in the field, Section 2.4 describes the drug sensitivity of a group of field isolates that were randomly collected from cattle in Ghibe in 1993, and compares them to the isolates described in Section 2.2 that were collected from the same site in 1989.

Sections 2.1, 2.2, 2.3 and 2.4 indicate that there was a high prevalence of multiple-drug resistant infections in cattle at Ghibe in both 1989 and 1993. However, the origin of this resistance was unclear. Since the development of resistance to quinapyramine has been suggested to be associated with a greater spectrum of cross-resistance than development of resistance to any of the other anti-trypanosomal compounds recommended for use in domestic livestock (Whiteside, 1960; Gray and Roberts, 1971), Section 2.5 contains work that was carried out to clarify this matter, by characterising the cross-resistance associated with development of resistance to quinapyramine in a clone of *T. congolense* under controlled laboratory conditions.

References:

Goble, F.C., Fewell, B. and Steiglitz, A.R. (1959) The virulence and drug susceptibility of certain strains of trypanosomes of the Brucei-Evansi group maintained by syringe passage. *Annals of Tropical Medicine and Parasitology* 53, 189-202.

Gray, A.R. and Roberts, C.J. (1971) The cyclical transmission of strains of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* 63, 67-89.

Njogu, A.R. and Heath, B.R. (1986) Multiple drug resistance in *Trypanosoma vivax* in Kenya. *Veterinary Record* 118, 133-134.

Röttcher, D. and Schillinger, D. (1985) Multiple drug resistance in *Trypanosoma vivax* in the Tana river district of Kenya. *Veterinary Record* 117, 557-558.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M. (1994a) Effects of trypanosomiasis on growth and mortality of young East African Zebu cattle exposed to drug-resistant trypanosomes. *Preventive Veterinary Medicine* 21, 87-101.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M. (1994b) Effects of trypanosomiasis on reproduction of East African Zebu cows exposed to drug-resistant trypanosomes. *Preventive Veterinary Medicine* 21, 237-249.

Stephen, L.E. (1962) Some observations on the behaviour of trypanosomes occurring in cattle previously treated with prophylactic drugs. *Annals of Tropical Medicine and Parasitology* 56, 415-421.

Whiteside, E.F. (1960) Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In *Proceedings of the International Scientific Committee for Trypanosomiasis Research, 8th meeting, Jos, Nigeria.* Commission for Technical Co-operation South of the Sahara, London, Publication No. 62, pp. 141-154.

Section 2.1

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M. and Peregrine, A.S. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. *Acta Tropica* 53, 135-150.

Acta Tropica, 53(1993)135–150
Elsevier Science Publishers B.V.

ACTROP 000270

Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections

G.J. Rowlands[a], Woudyalew Mulatu[b], E. Authié[c], G.D.M. d'Ieteren[a], S.G.A. Leak[c], S.M. Nagda[a] and A.S. Peregrine[c]

[a]*International Livestock Centre for Africa, P.O. Box 46847, Nairobi, Kenya,* [b]*International Livestock Centre for Africa, P.O. Box 5689, Addis Ababa, Ethiopia, and* [c]*International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya*

(Received 10 June 1992; accepted 16 December 1992)

An average of 840 East African Zebu cattle from nine herds in the Ghibe valley, southwest Ethiopia were monitored from January 1986 to April 1990. Each month blood samples were collected for analysis of packed red cell volume (PCV) and detection of trypanosomes. Animals found to be parasitaemic and with a PCV less than 26% were treated with diminazene aceturate at a dose of 3.5 mg kg body weight. The majority of infections were associated with *Trypanosoma congolense* (84% of infections in adult cattle and 71% in cattle less than 24 months of age), and the mean percentage of adult animals detected parasitaemic 1 month after treatment of an infection with *T. congolense* was 27%. In order to assess possible existence of drug resistance, a model was applied which allowed monthly incidences of new infections to be distinguished from recurrent infections. This model showed that the monthly incidence of new infections of *T. congolense* in adult cattle increased significantly from 11% in 1986 to 24% in 1989 following a concomitant increase in the tsetse challenge. The corresponding increase in overall prevalence of *T. congolense* was from 17% to 38% and the mean prevalence of recurrent infections increased significantly from 6% to 14%. These findings ruled out the possibility that the high prevalence of trypanosome infections in cattle was due only to a high tsetse challenge and pointed to the existence of *T. congolense* populations which expressed resistance to diminazene. There were variations associated with season, herd, age and sex in the incidence of new infections, prevalence of recurrent infections and relapse to treatment.

Key words: Ethiopia; Trypanosomiasis; Drug resistance; Diminazene aceturate; *Bos indicus*

Introduction

Studies on the health and productivity of East African Zebu cattle under levels of high tsetse challenge began in 1986 in the Ghibe River valley, southwest Ethiopia, as described by Leak et al. (1993). As the project continued it became apparent that

Correspondence to: G.J. Rowlands, International Livestock Centre for Africa, P.O.Box 46847, Nairobi, Kenya.

many animals were repeatedly being detected parasitaemic despite treatment with diminazene aceturate at a dose of 3.5 mg kg body weight (b.w.) (Woudyalew Mulatu et al., 1991), and it appeared that this trypanocidal drug treatment was becoming less effective. *Trypanosoma congolense* was found to be the predominant species (Woudyalew Mulatu et al., 1991). It was also the species that increased in prevalence over the period of study and the species, therefore, that was most likely to be contributing to the problem of recurring infections in the cattle. Rowlands *et al.* (1991, 1993) developed a model to distinguish between new and recurrent infections in order to determine if the high infection rates observed following treatment of *T. congolense* infections were due solely to the tsetse challenge or if they were instead due to relapses of infections following the treatment. This paper has used the same method to describe variations in overall trypanosome prevalence, incidence of new infections, prevalence of recurrent infections and rates of relapse to treatment of new infections with herd, year, season, sex and age. The data indicate that drug resistance is the most likely cause of drug failure.

Materials and Methods

Approximately 840 eartagged East African Zebu cattle from nine herds in the Ghibe valley, southwest Ethiopia were monitored each year from 1986 to 1990 as described by Leak et al. (1993). These cattle were of varying ages, including calves and mature cattle, both female and male, from seven herds at Ghibe itself and two herds at Tolley, approximately 25 km up the Ghibe valley. Each herd comprised cattle belonging to several owners. During the day, cattle in each herd were herded together and looked after by a herdsman. Male cattle over 3 years of age were used as oxen. Animals worked usually during the morning, particularly during the wet season, and grazed the rest of the day. Cattle were returned to their owner's homestead each evening. Herds 1–3 at Ghibe grazed in the same area at the bottom of the valley. Herds 4 and 5 and herds 6 and 7 similarly grazed in close proximity in two different areas 3 to 5 km up the valley. Herds 8 and 9 at Tolley grazed separately from each other. Cattle were weighed monthly from January 1986 to April 1990 and blood samples collected for the analysis of packed red cell volume (PCV) and for the detection of trypanosomes using the phase-contrast, buffy-coat technique (Murray et al., 1977). Animals with a PCV below 26%, and found to be either parasitaemic or exhibiting clinical signs consistent with trypanosomiasis, were treated with diminazene aceturate at a dose of 3.5 mg/kg b.w. Eighty seven per cent of all animals found parasitaemic during the study had a PCV $< 26\%$ and were treated. Ten per cent of the treatments were administered to animals which had a PCV $< 26\%$ and which showed clinical signs but were not detected parasitaemic.

Statistical analysis

The method of logistic regression (Cox, 1970) was used for the analysis of effects of year, season, herd, age and sex, and their interactions, on monthly trypanosome prevalence at Ghibe and Tolley (GLIM System Release 3.77, 1987). Logistic models, $\log \{r_i/(n_i - r_i)\}$, were fitted to the numbers of animals, r_i, detected parasitaemic out of a total of n_i in each subclass i. Data were used from March 1986 to February

1990. When calculating annual means, the January and February values were considered together with the values from March to December of the previous year. Seasonal rainfall patterns were characterised by an early rainy season from March to May and a longer rainy season from June to September. The remainder of each year was primarily dry (Leak et al., 1993). The data were therefore subdivided into the months representing these three seasons. Standard errors calculated by the model were inflated by the extra-binomial variance calculated as the ratio of the residual deviance and its degrees of freedom (Williams, 1982), and these were used in the calculation of 95% confidence intervals of odds ratios. *F* values, calculated as the ratios of mean deviances to the residual mean deviance in the analysis of deviance tables, were used for assessing statistical significance. Deviances, representing variations among years, seasons and their interaction, were compared with the variation among months within years and seasons. Similar models were fitted for PCV by least-squares analysis of variance, which also included the effect of trypanosome infection.

Preliminary analyses demonstrated that at both Ghibe and Tolley trypanosome prevalence increased with age to 2–3 years of age. Five age groups were formed for the analyses of effects of age on trypanosome prevalence and PCV : 0–9, 10–15, 16–24, 25–36 months (m), ≥ 3 years (y) of age. The 0–9m age category was chosen to represent unweaned calves which stayed mainly at the homestead until their dams ceased to be milked (usually at about 8–10 months post-calving) and the 16–24 m and 25–36m categories chosen to represent young cattle who had all joined the herds and were likely to have been exposed to the same levels of tsetse challenge as the older cattle. Dates of birth were not known accurately for animals born before 1st July 1985. Age categories were therefore calculated as follows: 1986 (0–9m, 10–15m > 3y); 1987 (0–9m, 10–15m, 16–24m, >3y); 1988 and 1989 (0–9m, 10–15m, 16–24m, 25–36m, ≥3y). The same age classes were used for the analyses of proportions of infections attributable to *T. congolense*, *T. vivax* and *T. brucei*.

In order to discriminate between new and recurrent *T. congolense* infections, the predominant species affecting these cattle, Rowlands et al. (1991) defined a 'new infection' as an infection that was detected in an animal which was aparasitaemic and had a PCV > 26% in at least the two previous months. This definition was applied and year × season × herd × sex × age tables of proportions were derived restricting the data to those values preceded by at least two 'negative' months, as defined above. Logistic regression models were again used to fit effects of year, season, herd, age and sex to new infection incidence. In some cases the numbers of observations used in the analysis were few; herds therefore were first combined. The statistical analyses of trypanosome prevalence had shown that six of the seven herds at Ghibe could be paired (herds 2, 3; 4, 5; 6, 7), since each pair of herds had similar monthly trypanosome prevalences. Each pair was also located in a different region of the valley. Herd 1, however, was kept separate in the analysis since it was a large herd and the cattle belonged to employees of a state farm; management practices may have been different from the other herds. The two herds at Tolley (herds 8 and 9) were kept separate, having very different monthly trypanosome prevalences from each other. Statistical analyses of the monthly prevalence of *T. congolense* infections were also undertaken but weighted by the numbers of observations used in the calculation of new infection incidence; this was to allow a direct comparison with the results of the statistical analysis of incidence of new infections. The prevalence of recurrent infections, which represent those infections that relapsed following

treatment, was then calculated as the difference between the estimates of monthly prevalence of *T. congolense* infections and the estimates of monthly incidence of new infections.

The proportions of animals which were infected and treated one month, and found to be parasitaemic again the next month, were also analysed, both for all treated infections and for new infections only. In statistical analyses conducted at the same site, Rowlands et al. (1990) found no differences in the prevalence of *T. congolense* infections 1 month following treatment of infections preceded by either one or two negative months. Because of the larger sample size produced, infections preceded by one negative month were added to those preceded by two negative months to calculate relapse rates to treatment of new infections in this study.

Twelve-month rolling means for tsetse challenge (Leak et al., 1993), for the monthly prevalence of *T. congolense* infections in cattle over 3 years of age, for the incidence of new infections and for the percentage of animals detected parasitaemic 1 month following treatment were plotted, pooling the results for herds 1–7.

Results

Effects of year and season on trypanosome prevalence and PCV

Variations from 1986 to 1989 in the annual monthly mean trypanosome prevalence in cattle of 3 years of age and above, when all trypanosome species were considered together, are shown in Table 1 and Fig. 1. This table also includes average raw mean values with which the adjusted values, corrected for variations among herds and between sexes, can be compared. The monthly trypanosome prevalence was significantly higher ($P < 0.001$) in herds at Ghibe in 1988 and 1989 compared with 1986 and 1987, and this pattern was similar for all herds, that is there were no herd × year interactions. At Tolley, the increase in average trypanosome prevalence with year was not as great and the difference in prevalence values between 1986 and 1989 was only significant ($P = 0.06$) when the two herds were considered together. However, there was a significant difference between the two herds (data not shown). Whereas there was little change in trypanosome prevalence in herd 8 over the four years the increase in trypanosome prevalence in herd 9 was similar in magnitude to the increases in prevalences observed in herds at Ghibe.

Averaged over the four years, the mean prevalence of trypanosome infections was slightly higher in herds 4 and 5 (36.5 and 34.0%, respectively) than in herds 1–3 (28.0, 30.8 and 30.4%, respectively) and 6 and 7 (27.1 and 28.9%, respectively) at Ghibe, and twice as high in herd 8 (35.0%) than herd 9 (19.2%) at Tolley ($P < 0.001$). Mean PCVs were lower in the herds with the higher trypanosome prevalences (data not shown).

When averaged over years, the monthly trypanosome prevalence in cattle at Ghibe was significantly higher during the dry season, between October and February, than the remainder of the year ($P < 0.001$) (Table 1). Although there were significant year × season interactions, the pattern of higher mean trypanosome prevalences between October and February than between March and September was consistent from 1987 to 1989. These seasonal variations were similar for each herd. At Tolley, the mean trypanosome prevalence during the dry season was also higher than the

TABLE 1

Annual and seasonal variations in trypanosome prevalence and PCV: logistic regression and analysis of variance of male and female cattle 3 years of age and over, sampled on a monthly basis from 1986 to 1989 and corrected for herd and sex

	Trypanosome prevalence (%)[a]					PCV (%)[c] (mean)
	year					
	1986	1987	1988	1989	mean	
Months (1986–1990)						
Ghibe (297)[b]						
March–May[e]	26.5	17.5	21.6	40.9	25.8 (25.2)[d]	25.2
June–Sept.	22.7	16.9	36.6	35.3	27.0 (26.4)	25.2
Oct.–Feb.	26.1	28.6	51.4	50.3	38.4 (37.4)	24.1
Mean	25.0	21.4	38.2	42.8	31.2 (30.7)	24.7
Tolley (109)[b]						
March–May	25.9	25.3	26.2	33.5	27.6 (29.6)	25.3
June–Sept.	24.1	18.7	28.4	26.5	24.2 (26.2)	25.4
Oct.–Feb.	20.7	30.2	37.4	37.0	30.9 (32.1)	24.4
Mean	23.0	24.8	31.4	32.5	27.7 (29.4)	25.0

[a]Includes *T. congolense*, *T. vivax* and *T. brucei* species.
[b]Average number of animals sampled per month.
[c]Corrected also for trypanosome infections.
[d]Raw means uncorrected for variations in year, herd and sex.
[e]These ranges in months correspond to periods of early rains, main rains and a dry period, respectively.

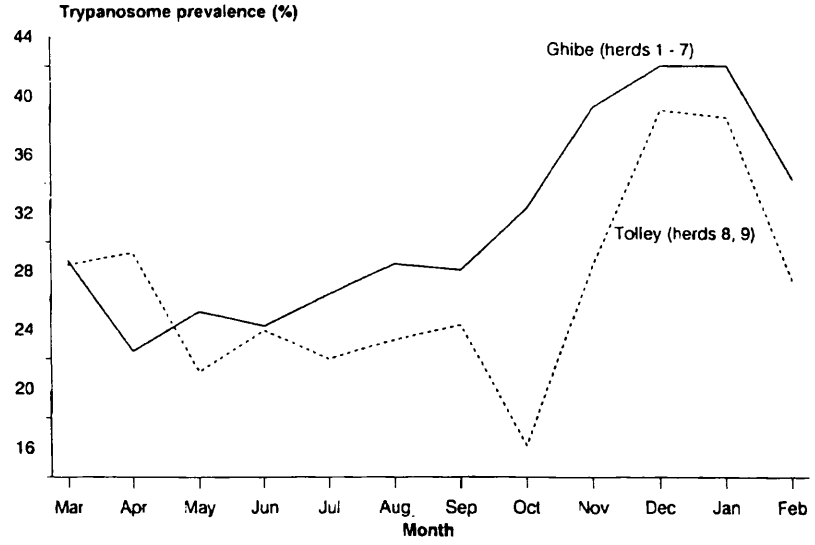


Fig. 1. Mean monthly trypanosome prevalence averaged over 1986, 1987, 1988 and 1989 for herds in Ghibe and Tolley.

corresponding mean trypanosome prevalence during the wet season, but not significantly so. Each year the monthly trypanosome prevalence at Tolley decreased in October to values well below those observed during the remainder of the dry season (Fig. 1).

Packed cell volume, corrected by analysis of variance for the effect of trypanosome infection, was also significantly lower between October and February than the remainder of the year (Table 1). However, there were major year × season interactions in PCV ($P < 0.001$). Mean PCV decreased rapidly during the last quarter of 1988 at Ghibe and remained low during the first quarter of 1989; a mean of 21.7% from October 1988 to February 1989 coincided with a period of high trypanosome prevalence (Table 1). However, in the corresponding period in 1989–1990, when the trypanosome prevalence was similarly high, PCV was not as low (mean of 24.1%). A similar trend occurred at Tolley.

Associations of trypanosome prevalence and PCV with age

Averaged over the period of the study, the mean monthly trypanosome prevalence in cattle increased significantly with age until 25–36 months of age at both Ghibe and Tolley ($P < 0.001$) (Table 2) and these increases were consistent for all herds. For example, the ratio of the odds of a 25–36 month old animal to a 16–24 month old animal being detected parasitaemic in any one month (that is the ratio of those detected parasitaemic to those not detected parasitaemic in the former age category

TABLE 2

Variations in the mean monthly trypanosome prevalence in cattle at Ghibe and Tolley by age and year, including *T. congolense*, *T. vivax* and *T. brucei* species, and corrected by logistic regression for differences among herds, season and sex

Age	Number[a]	Trypanosome prevalence (%)						
		year				mean[b]		
		1986	1987	1988	1989	Tc	Tv	all species
Ghibe								
0–9 m	68	11.0	3.4	6.4	12.0	5.1	1.6	7.5
10–15 m	52	13.5	8.6	13.4	31.8	11.1	3.1	15.9
16–24 m	78	-	12.4	22.6	34.6	16.7	4.7	23.5
25–36 m	77	-	-	35.0	40.8	24.2	3.5	32.3
⩾3 y	296	25.0	21.4	38.2	42.8	26.1	3.6	31.2
Tolley								
0–9 m	15	17.7	3.4	4.5	5.9	4.5	1.7	6.7
10–15 m	11	10.8	14.4	13.7	22.0	10.5	3.1	16.0
16–24 m	17	-	16.5	25.6	22.3	17.7	4.2	24.7
25–36 m	19	-	-	30.4	29.6	26.0	2.5	30.1
⩾3 y	109	23.0	24.8	31.4	32.5	22.9	3.4	27.7

[a] Average number of animals sampled per month.
[b] Means corrected for differences among years.
Tc = *T. congolense*, Tv = *T. vivax*.
–, not available.

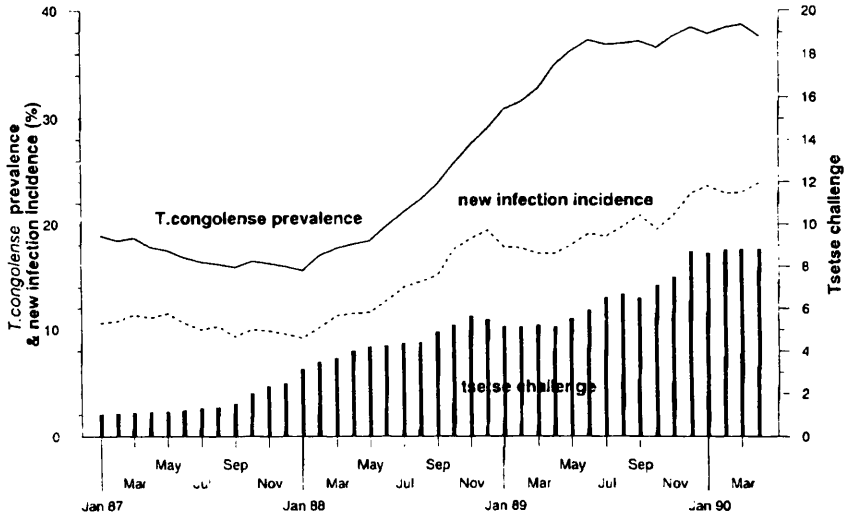


Fig. 2. Rolling 12-month means of tsetse challenge, of prevalence of *T. congolense* infections and of incidence of *T. congolense* infections in cattle over 36 months of age, pooling the results for herds 1-7 at Ghibe, that is ignoring herd effects. Tsetse challenge is calculated as the product of the numbers of flies caught per trap per day and the trypanosome infection rate in those tsetse

divided by the corresponding ratio in the latter age category (Thrusfield, 1986)), was 1.55 (95% confidence limits : 1.55-1.86) at Ghibe. A similar trend occurred at Tolley but, because of the fewer animals studied, the corresponding ratio was not significantly different from 1. Overall, the average increase in monthly trypanosome prevalence at the two sites was from 7.1% to 30.4% when comparing 0-9 and 25-36 month age categories. (This increase may have been partially confounded with management effects - see Discussion.) There were, however, significant differences among years in the magnitude of this increase with age, at both Ghibe ($P < 0.001$) and Tolley ($P < 0.05$). A 1.8-fold difference in the trypanosome prevalence in 1986 between the 0-9 month and $\geqslant 3$ year age groups contrasts with an average 6.6-fold difference in 1987 and 1988 and a 4.5 fold difference in 1989. Table 2 also shows the average mean monthly prevalences of *T. congolense* and *T. vivax* infections. Apart from a slight difference in the prevalence of *T. vivax* infections between 0-9 and 10-15 months of age, the increase in trypanosome prevalence with age was due to *T. congolense*. There was no effect of age on the prevalence of *T. vivax* in animals greater than 9 months of age.

Packed cell volume was significantly higher ($P < 0.001$) in calves under 9 months of age than in older cattle (Table 3). Furthermore, there was a significant age x trypanosome infection interaction; parasitaemia had a greater effect on PCV in animals under 9 months of age (from 29.8% in non parasitaemic samples to 22.7% in parasitaemic samples) than in animals above 3 years of age (from 26.3% to 21.7%).

Associations of trypanosome prevalence and PCV with sex

Corrected monthly trypanosome prevalences were higher in male than female cattle over 3 years of age both at Ghibe ($P < 0.001$) and at Tolley ($P < 0.05$) (Table 3).

142

TABLE 3

Variations in the mean monthly trypanosome prevalence and PCV in cattle at Ghibe and Tolley by age and sex, corrected by logistic regression and analysis of variance respectively for differences among herds, years and seasons

Age	Number[a]		Trypanosome prevalence (%)			PCV (%)[b]		
	male	female	male	female	mean	male	female	mean
Ghibe								
0–9 m	32	36	7.3 (6.2)[c]	7.8 (6.4)[c]	7.5	27.6	28.1	27.8
10–15 m	24	28	17.0 (16.6)	14.8 (14.1)	15.9	26.4	26.8	26.6
16–24 m	32	46	25.3 (22.0)	21.7 (20.7)	23.5	25.7	26.2	26.0
25–36 m	27	50	39.8 (37.3)	25.6 (29.0)	32.3	25.2	26.7	26.0
⩾3 y	97	199	34.3 (35.0)	28.2 (28.6)	31.2	24.4	25.0	24.7
Tolley								
0–9 m	7	8	8.2 (7.5)	5.4 (5.4)	6.7	28.8	28.9	28.9
10–15 m	5	6	18.3 (16.3)	13.9 (14.2)	16.0	26.7	27.0	26.9
16–24 m	8	9	25.9 (20.9)	23.5 (20.2)	24.7	26.2	27.2	26.7
25–36 m	9	10	32.4 (29.3)	27.9 (26.3)	30.1	25.5	26.8	26.7
⩾3 y	56	53	29.8 (34.8)	25.8 (23.9)	27.7	24.7	25.3	25.0

[a]Average number of animals sampled per month.
[b]Corrected by analysis of variance also for trypanosome infections.
[c]Raw means uncorrected for variations in year, herd and season.

The odds of being detected parasitaemic were 1.34 (95% confidence limits: 1.19–1.50) and 1.24 (1.02–1.50) higher in males than females at the two sites, respectively; the difference was consistent in each season. (These differences may have been partially confounded with possible differences between males and females in their exposure to tsetse - see Discussion). Male cattle less than 3 years of age at Ghibe also had higher trypanosome prevalences than females ($P < 0.001$). However, this difference varied significantly with age and was greatest in the 25–36 month age category (Table 3). A similar trend occurred at Tolley, but again, with the fewer numbers of animals studied, sex was not significant. Packed cell volume was significantly higher in female than male cattle at each site ($P < 0.001$). At Ghibe there was a significant age × sex interaction ($P < 0.001$) with the difference in PCV between males and females increasing with age (Table 3).

Proportions of T. congolense, T. vivax and T. brucei infections

The proportions of blood samples containing *T. congolense* and *T. vivax* in each year are in Table 4. *T. congolense* was identified in an average of 81% of blood samples collected from parasitaemic adult cattle in 1986, 1987 and 1988. The percentage increased to 94% in 1989. In contrast, the percentages of samples containing *T. vivax* decreased from a mean of 16% between 1986 and 1988 to 7% in 1989 in the same age group. The ratio of *T. vivax* to *T. congolense* infections was higher in animals younger than 24 months of age than animals older than this age. *T. brucei* was responsible for very few cases of trypanosome infection, on average 4% (data not given). There was no effect of season or herd on the distribution of trypanosome

TABLE 4

Percentages of samples from parasitaemic cattle with single or multiple infections containing *T. congolense* or *T. vivax*, by year and age, and estimated by logistic regression analysis

Age	Year										
	1986		1987		1988		1989		mean		
	Tc[a]	Tv	Tc	Tv	Tc	Tv	Tc	Tv	Tc	Tv	Tc/Tv
0–9 m	71	23	52	45	54	41	94	7	68	29	2.3
10–15 m	79	14	68	25	57	40	82	16	72	24	3.0
16–24 m	-	-	66	27	64	34	85	14	72	25	2.9
25–36 m	-	-	-	-	74	21	93	6	84	14	6.0
⩾3 y	79	18	80	15	83	14	94	7	84	14	6.0

[a]Tc = *T. congolense*; Tv = *T. vivax*.
-, not available.

species. Furthermore, in the majority of cases only one trypanosome species was detected.

Since *T. congolense* accounted for the majority of infections, further analysis was restricted to this species.

New and recurrent T. congolense infections

Table 5 separates the monthly prevalence of *T. congolense* into the monthly incidence of new infections and the monthly prevalence of recurrent infections of *T. congolense* in cattle over 3 years of age for Ghibe and Tolley combined. The incidence of new infections, corrected for herd differences, increased significantly ($P < 0.001$) from an average of 10.5% in 1986 and 1987 to 16.0% in 1988 and to 23.8% in 1989, giving an odds ratio of 2.55 (95% confidence limits : 1.60–4.05) of detecting parasitaemia in 1989 compared with 1986. The prevalence of recurrent infections, derived from the difference between the overall trypanosome prevalence and the incidence of new infections, increased from an average of 6.7% between 1986 and 1987 to 14.0% in 1989. Highest values of new infection incidence occurred during the dry period between October and February and these were higher than in both other periods ($P < 0.01$). The odds of a new infection occurring during the dry period was 2.20 (95% confidence limits : 2.20–3.62) times the odds of a new infection occurring during the early period of rainfall from March to May. In contrast, the prevalences of recurrent infection were similar between these two periods, but each was higher than between June and September (Table 5). The average incidence of new infections was much lower in herd 9 than in each of the other herds ($P < 0.01$). However, the mean prevalence of recurrent infections in this herd was as high as herds 1–3, which had higher incidences of new infections than herd 9. The incidence of new infections, corrected for year and herd, steadily increased with age up to 3 years ($P < 0.001$), whilst the prevalence of recurrent infections appeared to reach its maximum in cattle of 25 months of age and over (Table 6). In all age groups, however, recurrent infections represented a significant proportion of all infections.

144

TABLE 5

Variations with year, season, herd and sex in the mean monthly prevalence of *T. congolense* infections, incidence of new infections, prevalence of recurrent infections and percentage of animals detected positive one month following treatment in cattle at Ghibe and Tolley, 3 years of age and over

	Number of samples[a]	New infection incidence (%)	Prevalence of all *T. congolence* infections (%)	Recurrent infection prevalence[b] (%)	Animals positive one month after treatment of	
					new infections (%)	any infection[c] (%)
Year						
1986	13	10.9	17.4	6.5	19.8	19.3
1987	28	10.1	17.0	6.9	23.1	20.7
1988	11	16.0	28.4	12.4	24.7	33.2
1989	9	23.8	37.8	14.0	33.5	39.2
Months (1986–1989)						
March–May[d]	11	9.2	21.0	11.8	26.5	23.8
June–Sept.	27	15.0	21.0	6.0	17.4	22.5
Oct.–Feb.	24	18.2	29.2	11.0	31.4	34.1
Herds						
1	20	14.2	22.4	8.2	20.0	26.4
2, 3	8	18.9	26.2	7.3	18.6	29.2
4, 5	9	15.4	26.2	10.8	29.3	32.0
6, 7	9	14.7	25.1	10.4	26.3	23.0
8	2	18.2	35.0	16.8	37.0	32.8
9	14	7.6	15.0	7.4	21.7	21.9
Sex						
Male	13	16.6	27.3	10.7	23.6	27.8
Female	49	12.4	21.9	9.5	26.4	27.0

[a]Average number of samples per month used for calculation of new infection incidence.
[b]Calculated by subtraction of new infection incidence from prevalence of all *T. congolense* infections.
[c]Both new and recurrent infections.
[d]See footnote of Table 1.

There was a higher incidence of new infections in adult male than female cattle ($P < 0.05$) (Table 5), but recurrent prevalences were similar.

Rolling 12-month means of tsetse challenge at Ghibe, and of the prevalence of *T. congolense* infections and incidence of new *T. congolense* infections in cattle of 3 years of age and above in herds 1–7, are shown in Fig. 2. The values shown for each month represent the mean of 12 months, i.e., the month itself and each of the preceding 11 months. The figure illustrates the increases in rolling means of both the incidence of new infections and the trypanosome prevalence from January 1987 to April 1990, and shows the two curves diverging from December 1988 onwards. The increases in trypanosome prevalence and incidence of new infections from January 1988 onwards coincided with an increase in tsetse challenge. Data from herds 8 and 9 at Tolley, which had different estimates of tsetse challenge from Ghibe (Leak et al., 1993), were not included in this figure.

TABLE 6

Variations with age in the mean monthly prevalence of *T. congolense* infections, incidence of new infections, prevalence of recurrent infections and the percentage of animals detected positive one month following treatment in cattle at Ghibe and Tolley, corrected by logistic regression for variations among years and herds

Age	Number of samples[a]	New infection incidence (%)	Prevalence of all *T. congolense* infections	Recurrent infection prevalence[b] (%)	Animals positive one month after treatment of	
					new infections	any infection[c]
0–9 m	21	2.9	4.6	1.7	3.8	10.2
10–18 m	15	5.8	9.2	3.4	11.9	16.2
16–24 m	27	9.4	15.1	5.7	18.7	24.1
25–36 m	20	11.5	21.9	10.4	19.0	31.2
⩾3 years	62	14.5	24.2	9.7	25.0	27.3

[a] Average number of samples per month used for calculation of new infection incidence.
[b] Calculated by subtraction of new infection incidence from prevalence of all *T. congolense* infections.
[c] Both new and recurrent infections.

Proportions of animals detected parasitaemic following treatment

Table 5 gives the percentage of animals found positive 1 month following treatment of an infection with *T. congolense*. The percentage increased significantly ($P < 0.001$) from an average of 20.0% in 1986 and 1987 to 39.2% in 1989 (odds ratio 2.58, 95% confidence interval : 2.03–3.25). The percentage also varied with season, being on average higher between October and February than the rest of the year ($P < 0.001$). The mean percentage of adult animals found positive 1 month after treatment, and corrected for effects of year, season and herd, was 27.4% averaged over the entire study, and there was no difference between sexes. Table 5 also demonstrates the percentage of animals found positive 1 month following treatment of a new infection, i.e., excluding recurrent infections. When only these relapses were considered, slightly different results were obtained. For example, the mean relapse rate in 1988 was higher when relapses to all infections (33.2%) were compared to relapses to new infections only (24.7%). In contrast, in 1986 and 1987 mean relapse rates were similar. Between June and September of each year fewer cows were found to be positive following treatment of a new infection when compared with those found positive following treatment of any infection. Over the entire study the mean relapse rate to new infections was 25.0% and was lower than corresponding rates to recurrent infections in some but not all herds. Table 6 shows the effect of age on the percentages of animals found to be positive 1 month following treatment. In cattle less than 9 months of age, 10.2% of all treated animals were found to be infected one month later. This average increased until 16–24 months of age ($P < 0.001$). The mean relapse rate to new infections was lower at all ages than the mean relapse rate to recurrent infections.

Fig. 3 compares rolling 12-month means for the percentages of animals in herds 1–7 found to be parasitaemic 1 month following treatment of a new infection with those found to be parasitaemic following treatment of any infection, i.e. both new and recurrent infections combined. The two curves start to diverge during 1988.

146

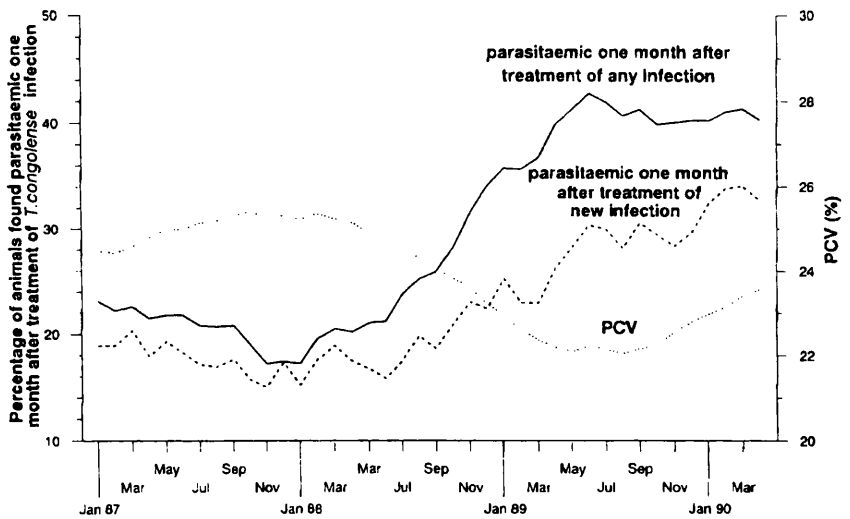


Fig. 3. Rolling 12-month means of percentages of animals detected parasitaemic one month following treatment and of PCV in cattle over 3 years of age in herds 1–7 at Ghibe.

This occurred at a time when mean PCV began to fall. The percentages of animals found positive after treatment of new infections remained around 20% until March 1989.

Discussion

The increase in monthly *T. congolense* prevalence from 1986 to 1989 in cattle at Ghibe and Tolley has been shown in this analysis to be partly attributable to an increase in the incidence of new infections. However, over the same period there was also an increase in the prevalence of recurrent infections; throughout the 4 years over one-third of infections detected in any one month were shown to be recurrent. This is likely to be a minimal estimate. In describing their method for distinguishing between new and recurrent infections, Rowlands et al. (1990) showed that when the numbers of negative months preceding an infection was increased from 1 to 4 in the definition of a new infection, estimates of 'new infection' incidence decreased but reached a plateau at 3 months. The choice of 3 for the number of negative months required to precede a positive sample was, therefore, recommended. However, this resulted in a very small sample size which made further statistical analysis difficult. It was decided, therefore, to use 2, and not 3, preceding negative months to define a new infection. Although the values shown in Tables 5 and 6 probably overestimate and underestimate the levels of true new and recurrent infections, respectively, this approach was justified on the grounds that a regression of trypanosome prevalence against tsetse challenge still provided a better fit when the new infection incidence, and not the overall trypanosome prevalence, was used (Rowlands et al., 1991, 1993).

In view of the significant number of recurrent infections, treatment with diminazene aceturate at a dose of 3.5 mg/kg b.w. was clearly not effective in curing trypanosome infections. A short trial was, therefore, conducted in 1989 to compare

the therapeutic efficacy of diminazene aceturate at doses of 3.5 and 7 mg/kg b.w. in two of the herds at Ghibe. The results showed that the higher dose decreased the proportion of animals which had relapsed by day 20 following treatment (Rowlands et al., 1990). This higher dose also maintained the mean PCV at a significantly higher level. Nevertheless, even at this dose, 25% of animals were detected parasitaemic by day 20 following treatment (compared with 55% at the lower dose) indicating that this drug dosage was not able to cure all infections. These findings, therefore, pointed to the existence of *T. congolense* populations which expressed resistance to diminazene and this has been further substantiated in experimental work conducted by Codjia et al. (1993) with trypanosome isolates collected from cattle in Ghibe in July 1989. This is not the first report of drug resistance in Ethiopia. Nineteen years previously Scott and Pegram (1974) reported cases of resistance to homidium bromide.

The mean prevalence of recurrent infections in 1986 was 6.5%; this accounted for 0.37 of all infections and suggested that the drug was ineffective from the start of the project. Although the prevalence of recurrent infections increased in 1988 and 1989, the proportion of new infections remained similar to that in 1986. Thus, although the changes in 12-month rolling means illustrated in Fig. 1 suggest that the problem of drug resistance may have been exacerbated due to repeated treatment with diminazene aceturate, the increase in prevalence of recurrent infections may more simply be a reflection of the increase in new infection rate. In summary, therefore, resistance to diminazene aceturate at a dose of 3.5 mg/kg b.w. would appear to have been present at the start of the project. However, there is no clear evidence from the data presented that it became a more significant problem as the project progressed.

The annual percentages of adult cattle detected positive 1 month following treatment were as high, or higher, than the trypanosome prevalences over the same period. The former values include both infections that relapsed following treatment and new infections that arose during the month following treatment. The fact that the two values are similar provides further evidence of the inefficacy of the treatment. Had diminazene aceturate provided some protection against further infection, then one would have expected the number of infections detected at 1 month following treatment to be lower. This does not appear to have been the case. Indeed, in younger cattle the percentages of animals found positive 1 month following treatment were much higher than the corresponding trypanosome prevalences.

In 1986 and 1987 similar prevalences of *T. congolense* were found following treatment of both new and recurrent infections. This would indicate that recurrent infections were occurring in all animals at that time, and were not due to the failure of only a few animals to respond to treatment of long-standing infections. During 1988, however, the numbers of animals detected parasitaemic 1 month following treatment of a recurrent infection increased beyond the numbers detected parasitaemic following treatment of a new infection. Packed cell volume began to fall to low levels towards the end of the year. Other factors, therefore, may have been affecting the health of the cattle at this time and, under these stressful conditions, the treatment seemed to control long-standing infections less well than new infections.

As previously mentioned, 12 isolates were collected from cattle at Ghibe in 1989 and characterised under laboratory conditions for their drug-resistance phenotypes. All 12 isolates were found to be resistant in calves to treatment with diminazene

aceturate at a dose of 7 mg kg b.w. (Codjia et al., 1993). At the time of collection of the isolates in July 1989 the trypanosome prevalence in cattle at Ghibe was 37%. With such a high incidence of drug resistance demonstrated under laboratory conditions one might have expected a much higher prevalence of parasitaemic animals in the field. Thus, whilst treatment may not have eliminated infections, it may have helped to limit the trypanosome growth and allowed the cattle to maintain reasonable health and condition. Bourn and Scott (1978) similarly observed that zebu oxen in western Ethiopia remained in good condition through the judicious use of trypanocidal drugs despite the presence of drug-resistant strains of *T. congolense*. The overall productivity calculated on the basis of levels of growth, calving rate and mortality achieved over the 4-year period at Ghibe (data not given) were in the range of values reported in other village production systems across Africa (de Leeuw, 1990). Under this treatment regime, therefore, it appeared that animals were aided in maintaining their parasitaemia at levels that were not always detected by the phase-contrast, buffy-coat technique (Murray et al., 1977). The use of trypanosome antigen-detection enzyme immunoassays (Nantulya and Lindqvist, 1989) may possibly have shown a higher prevalence of cattle harbouring trypanosome infections.

The differences in prevalence of trypanosome infections with age demonstrated the importance of using age as a classification in the statistical analysis of the described data. Although there is some evidence that calves of less than one year may be more resistant to trypanosome infections than adults (Fiennes, 1970; Wellde et al., 1981), the change in prevalence of trypanosome infections in the age classes defined in our study is likely to be a combination of both the increased exposure to tsetse with age and the cumulative exposure to multiple infections brought about by the high levels of tsetse challenge and the inability of the treatment to eliminate all these infections. Calves stayed at the homestead until their dams ceased to be milked. Most will have joined the herd by 10 months of age, though a few may have been a little older. In the youngest age group at least, the exposure to tsetse would have been lower than in older age groups. In all age groups, however, there was evidence of significant levels of recurrent infections.

The higher ratio of *T. congolense* to *T. vivax* infections in older cattle in this study confirms earlier findings (d'Ieteren et al., 1988) and suggests that cattle may more readily develop immunity to *T. vivax* than *T. congolense* (MacLennan, 1970). Similarly, when oxen were first introduced into a tsetse infested area of western Ethiopia in 1972, the majority of infections were *T. vivax*. However, within a year *T. congolense* became the predominant species (Bourn and Scott, 1978).

Since the results from this study indicated that measures other than chemotherapy were needed to control trypanosomiasis, a tsetse control campaign was initiated in May 1990 (Woudyalew Mulatu et al., 1993). Early results have indicated a significant reduction in both the apparent tsetse challenge and the trypanosome prevalence in cattle. Analysis of the relationships between trypanosome prevalence and age of the same cattle described here, but under lower tsetse challenge, may shed further light on the full reasons for the observed increase in *T. congolense* prevalence with age.

The higher incidences of new infections in adult male than female cattle confirm studies in mice in which males were shown to be more susceptible than females to *T. congolense* (Murray, Morrison and Whitelaw, 1982). The differences in trypanosome prevalence between male and female cattle in this study first became significant between 25 and 36 months of age (Table 3). Records showed less than a third of the

bulls had been used as oxen by 3 years of age, so that a possible increase in susceptibility to trypanosomiasis brought about by the stress of work may not have been the only contributing factor. Nevertheless, adult males and females are likely to have been exposed to different levels of tsetse challenge, and the reasons for the differences in new infection incidence between male and female cattle in this environment deserve further investigation.

In conclusion, the results of this paper have allowed a statistical model for the estimation of incidence of new infections to be applied in the evaluation of variations associated with year, season, herd, age and sex in both the incidence of new infections and the prevalence of recurrent infections in cattle at Ghibe, Ethiopia. The statistical analyses ruled out the possibility that the high prevalence of trypanosome infections in cattle was due only to a high tsetse challenge and confirmed earlier suspicions of drug failures (Woudyalew Mulatu et al., 1991).

Acknowledgements

We are grateful to Miss J. M. Rarieya and the late B. Ogara for computer data entry and verification, to Wollega Dufera, Girma Tegegne and Berhanu Elfaged for data collection, blood sampling and analysis, to herd owners and herdsmen at Ghibe and Tolley for their cooperation, and to Mrs. G. Maloba for her patience in typing various drafts of this paper. We also thank Dr. Zeleke Dagnachew, Head, Veterinary Services Department, Ethiopia for permission to publish this work.

References

Bourn, D. and Scott, M. (1978) The successful use of work oxen in agricultural development of tsetse infested land in Ethiopia. Trop. Anim. Hlth Prod. 10, 191–203.

Codjia, V., Woudyalew Mulatu, Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authié, E. and Peregrine, A.S. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. Acta Trop. 53, 151–163.

Cox, D.R. (1970) Analysis of Binary Data, Methuen, London.

Fiennes, R.N.T. (1970) Pathogenesis and pathology of animal trypanosomiases. In The African Trypanosomiases (ed. H.W. Mulligan), George Allen & Unwin, London, pp. 729–750.

d'Ieteren, G.D.M., Awuome, K., Bokovi, K., Chema, S., Colardelle, C., Coulibaly, L., Defly, A., Feron, A., Grundler, G., Handlos, M., Hecker, P., Itty, P., Kakiese, O., Leak, S.G.A., Maehl, J.H.H., Maloo, S.H., Mawuena, K., Minengu, M., Morkramer, G., Mukendi, F., Mulatu, W., Mulungo, M., Nagda, S.M., Nankodaba, G., Ngamuna, S., Ordner, G., Paling, R.W., Pelo, M., Rarieya, J.M., Schuetterle, A., Sheria, M., Thorpe, W., Tikubet, G., Trail, J.C.M. and Yangari, G. (1988) Genetic and environmental factors affecting trypanosome prevalence and parasitaemia in livestock. In: Livestock Production in Tsetse Affected Areas of Africa. ILCA ILRAD, Nairobi, Kenya, pp. 87–99.

Leak, S.G.A., Woudyalew Mulatu, Authié, E., d'Ieteren, G.D.M., Peregrine, A.S., Rowlands, G.J. and Trail, J.C.M. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 1. Tsetse challenge and its relationship to trypanosome prevalence in cattle. Acta Trop., 53, 121–134.

de Leeuw, P.N. (1990) Interactive effects of environment, management and mortality on cattle productivity in livestock systems in Sub-Saharan Africa. Proc. 6th International Conference of Institutes for Tropical Veterinary Medicine, Wageningen, The Netherlands, 1989, pp. 29–38. Faculty of Veterinary Medicine, University of Utrecht.

MacLennan, K.J.R. (1970) The epizootiology of trypanosomiasis in livestock in West Africa. In The African Trypanosomiases (ed. H.W. Mulligan), George Allen & Unwin, London, pp. 756–765.

Murray, M., Morrison, W.I. and Whitelaw, D.D. (1982) Host susceptibility to African Trypanosomiasis: trypanotolerance. Adv. Parasitol. 21, 1–68.

Murray, M., Murray, P.K. and McIntyre, W.I.M. (1977) An improved parasitological technique for the diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 71, 325–326.

Nantulya, V.M. and Lindqvist, K.J. (1989) Antigen-detection enzyme immunoassay for the diagnosis of *Trypanosoma vivax*, *T. congolense* and *T. brucei* infections in cattle. Trop. Med. Parasitol. 40, 267–272.

Rowlands, G.J., Woudyalew Mulatu, Authié, E., d'Ieteren, G.D.M., Leak, S.G.A., Peregrine, A.S. and Trail, J.C.M. (1990) Prevalence of *Trypanosoma congolense* in East African Zebu cattle under high tsetse challenge. Proc. Annual Meeting of Society of Veterinary Epidemiology and Preventive Medicine, 1990, Belfast, N. Ireland, pp. 145–152.

Rowlands, G.J., Authié, E., d'Ieteren, G.D.M., Woudyalew Mulatu, Peregrine, A.S. and Leak, S.G.A. (1991) An epidemiological study of recurrent trypanosome infection in East African Zebu cattle and its association with drug resistance. Proc. 6th International Society of Veterinary Epidemiology and Economics, Ottawa, Canada, 1991, pp. 444–446.

Rowlands, G.J., Woudyalew Mulatu, Authié, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M., Peregrine, A.S. and Rarieya, J.M. (1993) A method for distinguishing new and recurrent trypanosome infections in a field survey of East African Zebu cattle in Ethiopia. Proc. 21st Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yamoussoukro, Côte d'Ivoire, 1991, in press.

Scott, J.M. and Pegram, R.G. (1974) A high incidence of *Trypanosoma congolense* strains resistant to homidium bromide in Ethiopia. Trop. Anim. Hlth. Prod. 6, 215–221.

Thrusfield, M.V. (1986) Veterinary Epidemiology. Butterworth, London.

Wellde, B.T., Hockmayer, W.T., Kovatch, R.M., Bhogal, M.S. and Diggs, C.L. (1981) *Trypanosoma congolense*: Natural and acquired resistance in the bovine. Exp. Parasitol. 52, 219–232.

Williams, D.A. (1982) Extra-binomial variation in logistic linear models. Appl. Statist. 31, 144–148.

Woudyalew Mulatu, d'Ieteren, G.D.M., Duffera, W., Girma, T., Leak, S.G.A., Maehl, J.H.H., Nagda, S.M., Rarieya, J.M., Rowlands, G.J., Thorpe, W., Tikubet, G. and Trail, J.C.M. (1991) Factors affecting trypanosome prevalence in zebu cattle exposed to high trypanosomiasis risk in South-West Ethiopia. Proc. 20th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Mombasa, Kenya, 1989, OAU ISCTRC, Nairobi, pp. 507–510.

Woudyalew Mulatu, Leak, S.G.A., Authié, E., d'Ieteren, G., Peregrine, A.S. and Rowlands, G.J. (1993) Preliminary results of a tsetse control campaign using deltamethrin impregnated targets to alleviate a drug-resistance problem in bovine trypanosomiasis. Proc. 21st Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yamoussoukro, Côte d'Ivoire, 1991 (in press).

Section 2.2

Codjia, V., Mulatu, W., Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authié, E., d'Ieteren, G.D.M. and Peregrine, A.S. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. *Acta Tropica* 53, 151-163.

Acta Tropica, 53(1993)151–163
Elsevier Science Publishers B.V.

ACTROP 00271

Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium

V. Codjia[a], Woudyalew Mulatu[b], P.A.O. Majiwa[a], S.G.A. Leak[a], G.J. Rowlands[c], E. Authié[a], G.D.M. d'Ieteren[c] and A.S. Peregrine[a]

[a]*International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya,* [b]*International Livestock Centre for Africa (ILCA), Addis Ababa, Ethiopia, and* [c]*International Livestock Centre for Africa (ILCA), Nairobi, Kenya*

(Received 10 June 1992; accepted 16 December 1992)

In July 1989, blood samples were collected from parasitaemic cattle in the Ghibe valley, Ethiopia, frozen in liquid nitrogen and transported to Nairobi, Kenya. Twelve of the stabilates were inoculated into individual Boran (*Bos indicus*) calves and characterised for their sensitivity, in turn, to diminazene aceturate (Berenil®), isometamidium chloride (Samorin®) and homidium chloride (Novidium®). All 12 stabilates produced infections which were shown to be *Trypanosoma congolense* and resistant to treatment with diminazene aceturate at a dose of 7.0 mg kg^{-1} body weight (b.w.). Eleven of the infections were also resistant to isometamidium chloride at a dose of 0.5 mg kg^{-1} b.w. and homidium chloride at a dose of 1.0 mg kg^{-1} b.w. The drug-sensitivity phenotypes of three of the same isolates were also determined in goats which were each treated with only one of the three trypanocides: all expressed the same phenotypes as the populations expressed in the aforementioned Boran calves. Five clones were derived from one of the isolates which expressed a high level of resistance to all three trypanocides; each clone expressed high levels of resistance to all three trypanocides when characterised in mice. Thus, the multi-resistance phenotype of the parental isolate was associated with expression of multi-resistance by individual trypanosomes. Finally, molecular karyotypes and electrophoretic variants of six enzymes were determined for seven and eight of the isolates, respectively. Six different karyotypes were observed and all eight of the latter isolates belonged to different zymodemes, indicating that the multi-resistance phenotype at Ghibe was associated with many genetically distinct populations.

Key words: Ethiopia; *Trypanosoma congolense*; Drug resistance; Diminazene aceturate; Isometamidium chloride; Homidium chloride; Molecular karyotype; Isoenzyme

Introduction

In 1985 a project was initiated in the Ghibe valley, Ethiopia, to determine the constraints to livestock production at that site (ILCA, 1986). The valley is situated

Correspondence to: A.S. Peregrine, International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya.

on the border of the Shoa and Kaffa/Illubabor administrative regions (8°N, 37.5°E), and the Ghibe river flows through the valley (Leak et al., 1993). From January 1986 to April 1990, blood from the ear vein was collected on a monthly basis from 840 cattle at this site (Rowlands et al., 1993) and examined for the presence of trypanosomes using the buffy-coat phase contrast technique (Murray et al., 1977). Animals that were detected parasitaemic and had a packed red blood cell volume (PCV) less than 26% were treated intramuscularly (i.m.) with diminazene aceturate (Berenil®) at a dose of 3.5 mg kg^{-1} body weight (b.w.). The monthly prevalence of trypanosome infections in cattle has been presented by Rowlands et al. (1993). These data indicated that the majority of infections were *Trypanosoma congolense* and that over the entire experimental period a significant proportion failed to respond to treatment. Furthermore, the percentage of infections which relapsed following treatment increased from approximately 20% in 1987 to 40% in 1989. In order to confirm these conclusions, jugular blood was collected from parasitaemic cattle at Ghibe in July 1989, frozen in liquid nitrogen and transported to Nairobi, Kenya. In the work presented here, the drug-sensitivity phenotypes of 12 isolates were characterised in Boran (*Bos indicus*) calves. In order to determine the genetic identity of these populations, isolates were also characterised on the basis of their molecular karyotypes and electrophoretic variants of six enzymes.

Materials and Methods

Experimental animals

Cattle

Twelve Boran (*Bos indicus*) calves, aged 3–5 months, were obtained from a tsetse-free area of Kenya. Prior to experimental work all calves were screened for the presence of antibodies to *T. congolense*, *T. vivax* and *T. brucei* (Katende et al., 1987) and found to be negative. One week before infection with field isolates, calves were placed in individual plastic-film negative pressure isolators (ISOTEC, UK). Animals were maintained within the isolators for one month following infection to ensure that the isolates were free from bovine pathogens other than trypanosomes. During such maintenance, animals were supplied with a commercial pelleted ration (Unga Feeds Ltd., Nairobi), and water and hay *ad libitum*. One month after infection, calves were communally housed in loose boxes within fly-proof isolation units and managed as described by Whitelaw et al. (1986).

Goats

Adult male castrated goats (East African Maasai X Galla) were obtained from a region of the Kenyan Rift Valley known to be free from trypanosomiasis. Sera collected from all animals prior to experimental work were shown to be negative for antibodies to *T. congolense*, *T. vivax* and *T. brucei* (Katende et al., 1987). On arrival at ILRAD animals were treated for helminths, nasal bots and coccidiosis as described by Peregrine et al. (1991b) and maintained as described by Whitelaw et al. (1986).

Mice

Outbred Swiss white mice were obtained from the ILRAD breeding colony and maintained on a commercial pelleted ration (Unga Feeds Ltd., Nairobi).

Trypanosome populations

Field isolation

Trypanosome field isolates were collected from male and female East African Zebu cattle at Ghibe in July 1989 into potassium ethylenediamine tetra-acetate (EDTA), and stored in liquid nitrogen (Dar et al. 1972). Thereafter, they were transported to Nairobi, Kenya, for analysis. In the work described here, the term 'field isolate' refers to these stabilates. The number allocated to each field isolate denotes the ear-tag number of the animal from which the isolate was collected. Twelve field isolates were examined in the present study: four were collected from animals less than 3 years of age, eight were collected from animals which ranged from 3 to 9 years of age.

Laboratory procedures

Each field-isolate stabilate was thawed, suspended in phosphate saline glucose, pH 8.0, and inoculated intravenously (i.v.) into the jugular vein of an individual Boran calf, i.e., one isolate per calf. At the first peak of parasitaemia, jugular blood was collected from each animal into potassium EDTA and used to prepare a 'primary isolate' (WHO, 1978) which was stored in liquid nitrogen (Dar et al., 1972). The date when each population was isolated from the field, the designation of each primary isolate, and the ILRAD stabilate number of each primary isolate are given in Table 1.

TABLE 1

Trypanosome populations used

Herd origin	Date of isolation	Primary isolate[a]	ILRAD number[b]
1	20.7.89	Ghibe/89/ILRAD/2	IL3915
2	22.7.89	Ghibe/89/ILRAD/259	IL3916
5	21.7.89	Ghibe/89/ILRAD/811	IL3339
2	22.7.89	Ghibe/89/ILRAD/890	IL3917
2	22.7.89	Ghibe/89/ILRAD/998	IL3918
5	21.7.89	Ghibe/89/ILRAD/1263	IL3919
5	21.7.89	Ghibe/89/ILRAD/1392	IL3330
3	20.7.89	Ghibe/89/ILRAD/1591	IL3920
3	20.7.89	Ghibe/89/ILRAD/1756	IL3921
1	20.7.89	Ghibe/89/ILRAD/1969	IL3922
3	20.7.89	Ghibe/89/ILRAD/1981	IL3923
1	20.7.89	Ghibe/89/ILRAD/2031	IL3924

[a] Nomenclature according to WHO (1978), given, in order, as place of isolation, year of isolation, institute that conducted isolation and animal number from which isolate was collected.
[b] ILRAD number of primary isolate.

154

Administration of trypanocides

Isometamidium chloride (Samorin®, Batch No. DX0092, May & Baker, UK), homidium chloride (Novidium®, Batch No. DX3335, May & Baker, United Kingdom) and diminazene aceturate (Berenil®, Batch No. 093D574, Hoechst, Germany) were used in all studies. All trypanocides were administered intramuscularly to calves and goats, in the middle third of the neck. Drugs were administered intraperitoneally (i.p.) to mice. Cattle and mice were weighed immediately prior to administration of trypanocides for calculation of drug dosages.

Monitoring of animal infections

Jugular blood samples were collected on a daily basis from calves and goats into evacuated tubes containing EDTA. The level of parasitaemia was then estimated using phase-contrast examination of the blood buffy-coat (Murray et al., 1977). Thin blood smears were prepared at the first peak of parasitaemia and stained with Giemsa (Lumsden et al., 1973) to determine the species of trypanosomes present. Animals in which infections failed to relapse following treatment were monitored daily for 180 days before an infection was said to have been eliminated. Mice were monitored twice weekly for 60 days after treatment for the presence of trypanosomes by microscopic examination ($\times 250$) of tail-blood wet films.

Drug-sensitivity tests in Boran calves

All drug sensitivity studies were conducted with the same Boran calves that were used to prepare trypanosome primary isolates. At the first peak of parasitaemia, each calf was treated with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w. If infections relapsed following treatment, the animals were treated with isometamidium chloride at a dose of 0.5 mg kg^{-1} b.w. If a second relapse occurred, animals were treated with homidium chloride at a dose of 1.0 mg kg^{-1} b.w. Time intervals between each treatment are given in Table 2.

Drug-sensitivity tests in goats

Primary isolates IL 3339 and IL 3924 (see Table 1) were inoculated i.v. into groups of 9 goats each. Primary isolate IL 3923 was inoculated i.v. into 7 goats. IL 3339 was chosen for this study since it was the only population in Boran calves that was sensitive to treatment with isometamidium chloride. IL 3923 and IL 3924 were chosen at random. Each group of goats was then subdivided into three groups of 2–3 animals each (Groups A, B and C). When all the goats were detected parasitaemic, animals in each group were treated as follows: Group A with diminazene aceturate at a dose of 3.5 mg kg^{-1} b.w., Group B with isometamidium chloride at a dose of 0.5 mg kg^{-1} b.w. and Group C with homidium chloride at a dose of 1.0 mg kg^{-1} b.w.

TABLE 2

Drug sensitivity tests conducted sequentially in Boran calves

R = Resistant: infection relapsed after treatment.
S = Sensitive: infection considered cured after treatment (i.e., animal not detected parasitaemic following treatment for 180 days).

Field isolate No.	Treatment with diminazene aceturate (7.0 mg kg^{-1} b.w.)	Relapse interval[a] (days)	Interval between treatment with diminazene and isometamidium (days)	Treatment with isometamidium chloride (0.5 mg kg^{-1} b.w.)	Relapse interval[a] (days)	Interval between treatment with isometamidium and homidium (days)	Treatment with homidium chloride (1.0 mg kg^{-1} b.w.)	Relapse interval[a] (days)
2	R	14	16	R	4	16	R	1[b]
259	R	9	22	R	18	30	R	10
811	R	22	30	S	–[c]	-	-	–
890	R	9	15	R	1[b]	13	R	1[b]
998	R	10	15	R	9	13	R	1[b]
1263	R	12	17	R	8	16	R	1[b]
1392	R	6	15	R	10	18	R	1[b]
1591	R	12	15	R	7	12	R	2
1756	R	10	15	R	9	12	R	7
1969	R	12	19	R	11	22	R	1[b]
1981	R	4	18	R	9	19	R	11
2031	R	7	23	R	8	19	R	6

[a] Number of days between treatment and detection of trypanosomes following a period of aparasitaemia.
[b] i.e., not detected aparasitaemic following treatment.
[c] Data not available.

156

Cloning of primary isolate and drug-sensitivity tests in mice

Since many isolates expressed resistance to diminazene, isometamidium and diminazene in ruminants, an experiment was conducted to determine whether this phenotype was expressed at the clonal level. Primary isolate IL 3330 was chosen at random and inoculated i.p. into normal mice and passaged eight times in other mice to increase the level of parasitaemia. After the eighth passage, clones were derived from the population according to the method of Barry and Gathuo (1984). Five of these clones (clones 1, 2, 7, 15 and 20), chosen at random, and IL 3330, were characterised in mice for their sensitivities to diminazene aceturate, isometamidium chloride and homidium chloride according to the method of Peregrine et al. (1991a). In brief, groups of non-irradiated mice (six per group) were inoculated i.p. with 1.0×10^6 trypanosomes derived from populations expanded in vivo, and treated 6 h later with varying doses of diminazene aceturate (20–30 mg kg^{-1} b.w.), isometamidium chloride (0.1–10 mg kg^{-1} b.w.) and homidium chloride (0.1–20 mg kg^{-1} b.w.). The sensitivity was expressed as a 50% curative dose (CD_{50}) value.

Fractionation of chromosome-sized DNA molecules

Seven populations were examined in this study. Primary isolates were expanded in sublethally irradiated (650 rad) mice. At the first peak of parasitaemia, blood was collected into sodium citrate (final concentration 0.03%, w/v) and trypanosomes purified from other cellular components by column chromatography using diethylaminoethyl-cellulose (Lanham and Godfrey, 1970). Chromosome-sized DNA molecules were then prepared from each population as previously described (Van der Ploeg et al., 1984; Majiwa et al., 1985; Masake et al., 1988). Fractionation of chromosome-sized DNA molecules was performed using contour-clamped homogeneous field gel-electrophoresis (CHEF) (Chu et al., 1986). Electrophoresis was carried out at 120 V by applying 20 s pulses over an 18 h period.

Isoenzyme analysis

Eight populations were examined, in addition to one reference control, *T. congolense* MID 1034 (Knowles et al., 1988). Trypanosomes were obtained from each primary isolate as described above. Electrophoresis of trypanosome enzymes was carried out as described by Knowles et al. (1988). The enzymes studied were EC 1.1.1.37, malate dehydrogenase (MDH); EC 2.7.5.1, phosphoglucomutase (PGM); EC 5.3.1.9, glucose phosphate isomerase (GPI); EC 1.2.1.12, glyceraldehyde phosphate dehydrogenase (GAPDH); the peptidase activity which hydrolyses the substrate L-leucylglycylglycine (PEP 1); the peptidase activity which hydrolyses the substrate L-leucyl-L-alanine (PEP 2).

Results

Drug-sensitivity tests in Boran calves

After inoculation of 12 Boran calves with field isolates all the animals were detected parasitaemic within 4–22 days. Stained blood smears, prepared from each animal

at the first peak of parasitaemia, indicated that on the basis of morphology all trypanosomes were *T. congolense*. Following treatment with 7.0 mg diminazene aceturate kg^{-1} b.w., trypanosomes reappeared in all 12 calves within 4–22 days (Table 2). Subsequent to detection of parasitaemia, all 12 animals were treated with 0.5 mg isometamidium chloride kg^{-1} b.w.: infections in 11 of the animals relapsed, within 1–18 days. In contrast, the infection in one animal (field isolate 811) was eliminated. Homidium chloride at a dose of 1.0 mg kg^{-1} b.w. was subsequently administered to all the animals in which infections relapsed following treatment with isometamidium chloride: infections in all 11 animals relapsed, within 1–11 days following treatment (Table 2).

Drug-sensitivity tests in goats

All infections in goats due to IL 3339, IL 3923 or IL 3924 relapsed within 6–25 days following treatment with diminazene aceturate (Group A, Table 3). Similarly, infections in all goats infected with the same populations relapsed within 1–8 days following treatment with homidium chloride (Group C, Table 3). Responses to treatment with isometamidium chloride were more variable (Group B, Table 3): two animals infected with IL 3339 (a derivative of field isolate 811) and one animal infected with IL 3923 were cured. Infections in all three animals infected with IL 3924 relapsed within 15–17 days following treatment with isometamidium chloride.

Drug-sensitivity tests in mice

IL 3330 and five clones derived from this population were characterised in mice for their sensitivity to diminazene, isometamidium and homidium. All six populations had diminazene aceturate and isometamidium chloride CD_{50} values in excess of 30 mg kg^{-1} b.w. and 10 mg kg^{-1} b.w., respectively. The homidium chloride CD_{50} values for all six populations were either equal to, or in excess of, 20 mg kg^{-1} b.w.

Fractionation of chromosome-sized DNA molecules

The molecular karyotypes of the seven populations that were examined are shown in Fig. 1. Two populations (IL 3918 and IL 3921) had karyotypes which appeared to be identical. The other five populations had karyotypes which differed from each other and were also different from that of IL 3918 and IL 3921. A comparison of the profiles with those of other trypanosomes whose molecular karyotypes have been similarly analysed, indicated that the overall pattern resembled that observed among the Savannah-type *T. congolense* (Majiwa et al., 1985). This was confirmed by hybridisation of DNA from these trypanosomes with the repetitive DNA probe pgNRE-372 (Majiwa et al., 1985), which is specific for Savannah-type *T. congolense* (data not shown).

Isoenzyme analysis

Electrophoretic variants for six enzymes were determined for eight trypanosome populations (Fig. 2). On the basis of these six enzymes all eight populations belonged to different zymodemes. However, all zymodemes were similar to those described

TABLE 3

Drug sensitivity tests conducted in goats using single treatment

Primary isolate No.	Group A Treatment with 3.5 mg diminazene aceturate kg^{-1} b.w.			Group B Treatment with 0.5 mg isometamidium chloride kg^{-1} b.w.			Group C Treatment with 1.0 mg homidium chloride kg^{-1} b.w.		
	No. treated	No. relapsed	Relapse interval[a] (days)	No. treated	No. relapsed	Relapse interval[a] (days)	No. treated	No. relapsed	Relapse interval[a] (days)
IL3339	3	3	12, 15, 25	3	1	41	3	3	1[b], 1[b], 2
IL3923	3	3	7, 15, 18	2	1	25	2	2	4, 4
IL3924	3	3	6, 6, 6	3	3	15, 15, 17	3	3	1[b], 1[b], 8

[a] Number of days between treatment and detection of trypanosomes following a period of aparasitaemia.
[b] i.e., not detected aparasitaemic following treatment.

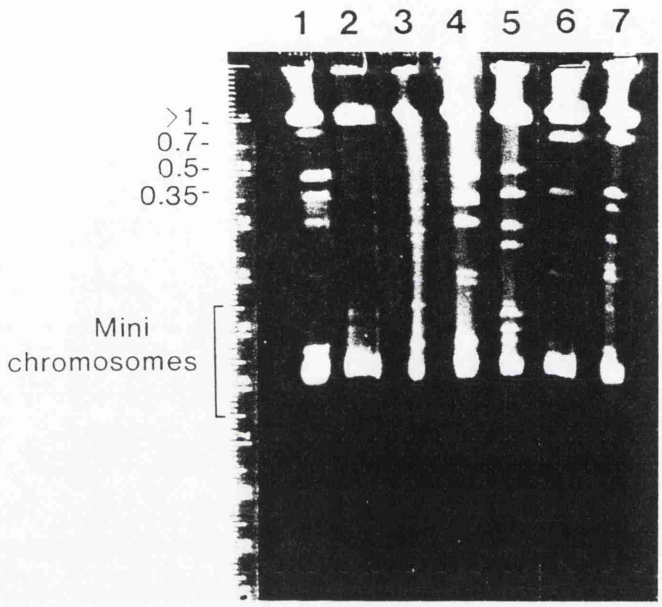


Fig. 1. Comparative molecular karyotypes of Ghibe isolates. Chromosome-sized DNA molecules from seven isolates were subjected to CHEF electrophoresis for 17 h to reveal DNA molecules less than 1 Mb. Lanes contain DNA from the following isolates: 1: IL 3916; 2: IL 3918; 3: IL 3919; 4: IL 3330; 5: IL 3921; 6: IL 3922; 7: IL 3924. Sizes were estimated by comparison with concatamers of phage-lambda DNA. Approximate sizes of the chromosomes are indicated, in Mb pairs, on the side of the photograph.

by Young and Godfrey (1983) and Gashumba et al. (1988) for Savannah-type *T. congolense* stocks from West and East Africa.

Discussion

This study has examined 12 isolates of *T. congolense* that were collected in July 1989 from cattle in Ghibe, Ethiopia. In initial studies to determine the approximate prevalence of drug resistance at that site, each isolate was characterised in individual Boran calves: all 12 populations were resistant to diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w.; eleven populations were also resistant to isometamidium chloride at a dose of 0.5 mg kg^{-1} b.w. and to homidium chloride at a dose of 1.0 mg kg^{-1} b.w. In this work, experimental calves were treated sequentially with diminazene aceturate, isometamidium chloride and homidium chloride. The potential risk of such a treatment regime resides in selection of trypanosomes resistant to each trypanocide. Thus, data concerning the level of resistance to isometamidium and homidium may not reflect the level of resistance of the parental population since the animals were first treated with diminazene aceturate. As a result of field studies conducted in Kenya, Whiteside (1958) concluded that induction of resistance to diminazene does not result in cross-resistance to metamidium (a mixture of isomers, one of which was isometamidium [Wragg et al., 1958]), but that induction of resistance to metamidium results in cross-resistance to homidium. This would therefore indicate that, while the aforementioned protocol should not bias the isometam-

160

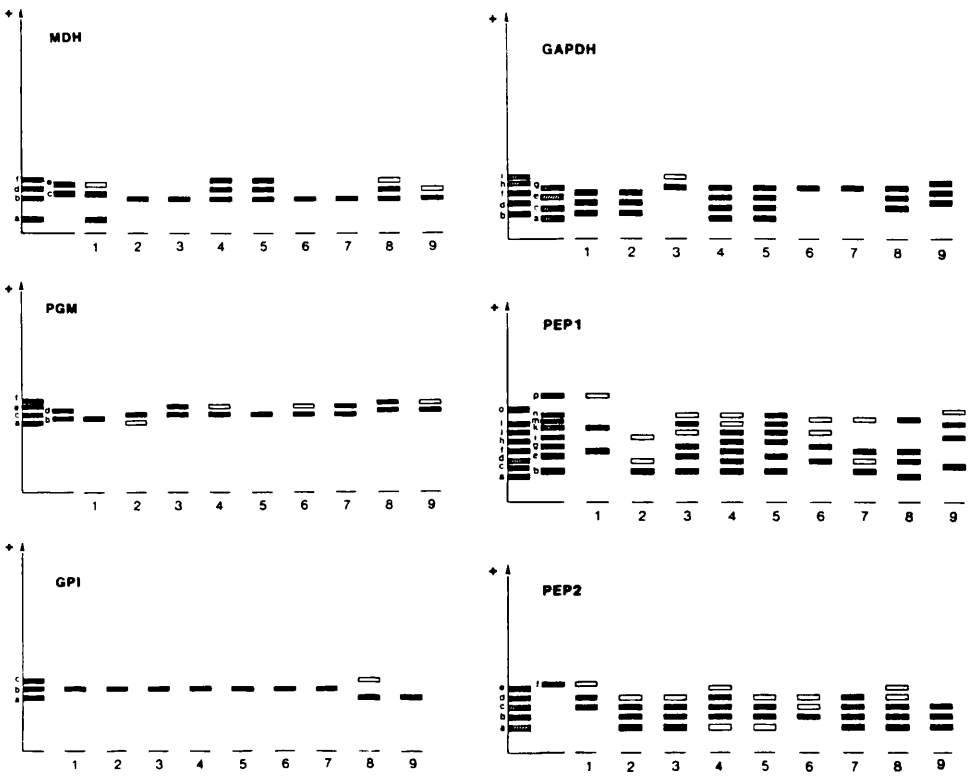


Fig. 2. Electrophoretic enzyme patterns of trypanosome isolates from Ghibe. Isolates were loaded in lanes as follows: 1, MID 1034; 2, IL 3915; 3, IL 3916; 4, IL 3918; 5, IL 3919; 6, IL 3330; 7, IL 3920; 8, IL 3921; 9, IL 3924. A filled bar indicates a strong band and an open one a weak band. MDH, malate dehydrogenase; PGM, phosphoglucomutase; GPI, glucose phosphate isomerase; GAPDH, glyceraldehyde phosphate dehydrogenase; PEP1, peptidase 1, substrate L-leucylglycylglycine; PEP2, peptidase 2; substrate L-leucyl-L-alanine.

idium-sensitivity of a trypanosome population, it may bias data concerning the sensitivity of a population to homidium.

In order to ascertain whether the above protocol did bias the data, and to increase the number of animals in which isolates were characterised, the drug-resistance phenotypes of three populations were also determined in groups of 2–3 goats which were infected with a primary isolate and treated with only one trypanocide. The response to treatment was similar to that observed in Boran calves. Thus, diminazene at a dose of 7.0 mg kg^{-1} b.w. failed to eliminate infections in all animals to which the drug was administered. Furthermore, infections with IL 3339 were eliminated in 2 out of 3 animals when treated with isometamidium chloride at a dose of 0.5 mg kg^{-1} b.w. The same drug dosage eliminated IL 3339 in one Boran calf. These data would therefore indicate that in July 1989 there was a high prevalence of trypanosome populations in cattle at Ghibe that were resistant to diminazene, isometamidium and homidium at the aforementioned doses. Since 37% of all cattle infections at Ghibe in 1986 appeared to be refractory to treatment with diminazene (Rowlands et al., 1993), this would suggest that drug-resistant trypanosomes were

present in Ghibe at the time this project was initiated. The origin of resistance at that time is unclear.

Expression of resistance to diminazene and a phenanthridine (i.e., isometamidium or homidium) has previously been described for populations of *T. congolense* from the Central African Republic (Finelle and Yvore, 1962), Nigeria (Gray and Roberts, 1971), Kenya (Gitatha, 1979), Ivory Coast (Küpper and Wolters, 1983) and Burkina Faso (Authié, 1984; Pinder and Authié, 1984), and for populations of *T. vivax* from Ivory Coast (Küpper and Wolters, 1983). Since diminazene and either isometamidium or homidium are the only sanative combinations for bovine trypanosomiasis (Whiteside, 1958), it is important to determine whether the multi-resistance phenotype is expressed at the clonal level; if it is not, the sanative combination would eliminate the population. In the work described here, five out of five clones derived from one of the multi-resistant populations were shown to have diminazene aceturate, isometamidium chloride and homidium chloride CD_{50} values in excess of 30 mg kg^{-1} b.w., 10 mg kg^{-1} b.w. and 20 mg kg^{-1} b.w., respectively. Thus, compared to highly sensitive populations of *T. congolense* (Peregrine et al., 1991a), all five clones expressed high levels of resistance to all three trypanocides. Furthermore, the multi-resistance phenotype of the population was expressed at clonal level. If such resistance at the clonal level was highly prevalent at Ghibe this would indicate that chemotherapy per se would not control trypanosomiasis at that site.

Molecular karyotypes of seven of the 12 isolates were determined. Eight populations were also characterised on the basis of electrophoretic variants of six enzymes. Six of the seven populations examined had different karyotypes and all eight of the respective isolates belonged to different zymodemes. The results therefore indicated that the multi-resistance phenotype expressed by populations of *T. congolense* from Ghibe was associated with many genetically distinct populations rather than a single genotype.

At present the prevalence of drug resistance in trypanosomes across Ethiopia is unknown. Scott and Pegram (1974) described the occurrence of homidium-resistant populations of *T. congolense* in Didessa and Angar valleys in Wollega province. Dagnatchew et al. (1983) described the occurrence of *T. congolense* populations in Debeka and Miridicha, Sidamo province, and Ghimbi and Ekoloko, Wollega province. However, whether drug resistance still occurs at these sites is not known.

Characterising the drug sensitivity of trypanosome populations is currently carried out by using in vitro cultivation (Kaminsky, 1990), mice (Scott and Pegram, 1974) or cattle. The reason that cattle were used in this study was so that the data could have direct relevance to the field; this does not appear to be the case with data obtained in mice or in vitro since there is not a consistent correlation between the sensitivity expressed in these systems and that expressed in cattle (Sones et al., 1988; Kaminsky, 1990). Because of the potential threat that drug resistance in trypanosomes poses to livestock production in Africa, and the logistical problems and cost of using cattle to determine the true prevalence and level of drug resistance in an area, there is a need to develop new systems that will rapidly screen the drug-resistance phenotypes of trypanosomes in large numbers of animals. In the mean time, Rowlands et al. (1993) have described methods for analysing field data which provide estimates of the proportion of infections failing to respond to treatment. Using this technique to analyse data from cattle at Ghibe they demonstrated a high prevalence of trypanosome infections that failed to respond to treatment with dimina-

zene. This conclusion was consistent with the data reported herein. As a result of these findings it was concluded that chemotherapeutic agents would not control trypanosomiasis at Ghibe. Thus, an integrated control programme, involving both tsetse-fly control and chemotherapeutic agents, was implemented in 1990 to control trypanosomiasis at the site.

Acknowledgements

We are grateful to Dr. Zeleke Dagnachew, Head, Veterinary Services Department, Ethiopia, for permission to publish this work. The authors wish to thank Mr. S. Kemei for technical support. RMB Animal Health Ltd., UK, kindly donated the isometamidium chloride and homidium chloride used in this study.

This is ILRAD publication number 1044.

References

Authié, E. (1984) Mise en évidence d'une résistance aux trypanocides parmi des souches de *Trypanosoma congolense* récemment isolées au Burkina. Rev. Elev. Méd. vét. Pays trop. 37 (No. spécial), 219–235.

Barry, J.D. and Gathuo, H. (1984) Antigenic variation in *Trypanosoma vivax*: isolation of a serodeme. Parasitology 89, 49–58.

Chu, G., Vollrath, D. and Davis, R.W. (1986) Separation of large DNA molecules by contour-clamped homogeneous electric field. Science 234, 1582–1585.

Dagnatchew, Z., Lemecha, H. and Mulatu, W. (1983) Trypanocidal drug resistance problem in certain parts of Ethiopia. Trypanosomiasis Control Services, Ministry of Agriculture, Annual Report 1982–1983, pp. 52–57.

Dar, F.K., Ligthart, G.S. and Wilson, A.J. (1972) Cryopreservation of pathogenic African trypanosomes *in situ*: metacyclic and bloodstream forms. J. Protozool. 19, 494–497.

Finelle, P. and Yvore, P. (1962) Quelques observations sur la chimiorésistance. In International Scientific Council for Trypanosomiasis Research, 9th meeting, Conakry, Guinea, CCTA publication No. 88, pp. 107–110.

Gashumba, J.K., Baker, R.D. and Godfrey, D.G. (1988) *Trypanosoma congolense*: the distribution of enzymic variants in East and West Africa. Parasitology 96, 475–486.

Gitatha, S.K. (1979) *T. congolense* (Shimba hills) resistant to various trypanocidal drugs. In International Scientific Council for Trypanosomiasis Research and Control, 16th meeting, Yaoundé, Cameroon, OAU STRC publication No. 111, pp. 257–263.

Gray, A.R. and Roberts, C.J. (1971) The cyclical transmission of strains of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs. Parasitology 63, 67–89.

ILCA (1986) The ILCA-ILRAD trypanotolerance network. Situation Report, December 1985. Proceedings of a network meeting held at ILCA, Nairobi, June, 1986. International Livestock Centre for Africa, Addis Ababa, Ethiopia, pp. 69–70.

Kaminsky, R. (1990) *In vitro* techniques for assessment of drug resistance in trypanosomes. AgBiotechnol News 2, 205–210.

Katende, J.M., Musoke, A.J., Nantulya, V.M. and Goddeeris, B.M. (1987) A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescent antibody test for diagnosis of bovine trypanosomiasis. Trop. Med. Parasitol. 38, 41–44.

Knowles, G., Betschart, B., Kukla, B.A., Scott, J.R. and Majiwa, P.A.O. (1988) Genetically discrete populations of *Trypanosoma congolense* from livestock on the Kenyan coast. Parasitology 96, 461–474.

Küpper, W. and Wolters, M. (1983) Observation on drug resistance of *Trypanosoma (Nannomonas) congolense* and *Trypanosoma (Duttonella) vivax* in cattle at a feedlot in the northern Ivory Coast. Tropenmed. Parasitol. 34, 203–205.

Lanham, S.M. and Godfrey, D.G. (1970) Isolation of Salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. 28, 521–534.

Leak, S.G.A., Woudyalew Mulatu, Authié, E., d'Ieteren, G.D.M., Peregrine, A.S., Rowlands, G.J. and Trail, J.C.M. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 1. Tsetse challenge and its relationship to trypanosome prevalence in cattle. Acta Trop. 53, 121–134.

Lumsden, W.H.R., Herbert, W.J. and McNeillage, G.J.C. (1973) Techniques with trypanosomes. Churchill Livingstone, London, pp. 122–124.

Majiwa, P.A.O., Masake, R.A., Nantulya, V.M., Hamers, R. and Matthyssens, G. (1985) *Trypanosoma (Nannomonas) congolense*: identification of two karyotypic groups. EMBO J. 4, 3307–3313.

Masake, R.A., Nyambati, V.M., Nantulya, V.M., Majiwa, P.A.O., Moloo, S.K. and Musoke, A.J. (1988) The chromosome profiles of *Trypanosoma congolense* isolates from Kilifi, Kenya and their relationship to serodeme identity. Mol. Biochem. Parasitol. 30, 105–112.

Murray, M., Murray, P.K. and McIntyre, W.I.M. (1977) Improved parasitological technique for the diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 71, 325–326.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991a) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. Parasitology 102, 93–100.

Peregrine, A.S., Moloo, S.K. and Whitelaw, D.D. (1991b) Differences in sensitivity of Kenyan *Trypanosoma vivax* populations to the prophylactic and therapeutic actions of isometamidium chloride in Boran cattle. Trop. Anim. Hlth. Prod. 23, 29–38.

Pinder, M. and Authié, E. (1984) The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. Acta Trop. 41, 247–252.

Rowlands, G.J., Woudyalew Mulatu, Authié, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M. and Peregrine, A.S. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. Acta Trop. 53, 135–150.

Scott, J.M. and Pegram, R.G. (1974) A high incidence of *Trypanosoma congolense* strains resistant to homidium bromide in Ethiopia. Trop. Anim. Hlth. Prod. 6, 215–221.

Sones, K.R., Njogu, A.R. and Holmes, P.H. (1988) Assessment of sensitivity of *Trypanosoma congolense* to isometamidium chloride: a comparison of tests using cattle and mice. Acta Trop. 45, 153–164.

Van der Ploeg, L.H.T., Cornellissen, C.A., Michels, P.A.M. and Borst, P. (1984) Chromosome rearrangement in *Trypanosoma brucei*. Cell 39, 213–221.

Whitelaw, D.D., Bell, I.R., Holmes, P.H., Moloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M. (1986) Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. Vet. Rec. 118, 722–726.

Whiteside, E.F. (1958) The maintenance of cattle in tsetse-infested country. A summary of four years' experience in Kenya. In International Scientific Council for Trypanosomiasis Research, 7th meeting, Bruxelles, Belgium. CCTA Publication No. 41, pp. 83–90.

WHO (1978) Proposals for the nomenclature of Salivarian trypanosomes and for the maintenance of reference collections. Bull. WHO 56, 467–480.

Wragg, W.R., Washbourn, K., Brown, K.N. and Hill, J. (1958) Metamidium: A new trypanocidal drug. Nature 182, 1005–1006.

Young, C.J. and Godfrey, D.G. (1983) Enzyme polymorphism and the distribution of *Trypanosoma congolense* isolates. Ann. Trop. Med. Parasitol. 77, 467–481.

Section 2.3

Peregrine, A.S., Mulatu, W., Leak, S.G.A. and Rowlands, G.J. (1994) Epidemiology of bovine trypanosomiasis in the Ghibe valley, Ethiopia: multiple-drug resistance and its effective control. In *Proceedings of the 7th International Symposium on Veterinary Epidemiology and Economics*, 15th-19th August, 1994, Nairobi, Kenya. *The Kenya Veterinarian* 18, 369-371.

The Kenya Veterinarian 18(2) (1994) 369-371

- 10.6 -

EPIDEMIOLOGY OF BOVINE TRYPANOSOMIASIS IN THE GHIBE VALLEY, ETHIOPIA: MULTIPLE-DRUG RESISTANCE AND ITS EFFECTIVE CONTROL

PEREGRINE, A.S.[a], WOUDYALEW MULATU[b], LEAK, S.G.A.[a], ROWLANDS, G.J.[c]

In 1986 a project was set up to determine the constraints to production of Zebu cattle under trypanosomiasis risk in the Ghibe valley, southwest Ethiopia. The Ghibe valley is situated 183 km southwest of Addis Ababa, and lies at the border of the Shoa and Kaffa/Illubabor administrative regions at 8°N,37.5E. The geography, vegetation and terrain of the valley have been described by Leak et al. (1993). The valley has a pronounced dry season from November to February and rainfall occurs between March and October.

Approximately 1800 East African Zebu cattle were resident within the Ghibe valley during the experimental period described here (1986-1992) and were maintained under traditional village management (Rowlands et al., 1993). Tsetse populations were surveyed using unbaited blue biconical traps from 1986 to April 1990, and traps baited with acetone and cow urine from April 1990 to 1992. Trapping was carried out for five consecutive days per month in the same trapping sites in the tsetse habitat along the Ghibe river and its tributaries. The cages from these traps were emptied daily. Live tsetse were dissected and their trypanosome infection rates were determined. When the guts of tsetse were found to contain undigested blood, the contents were preserved on filter paper to determine the source of the bloodmeal (Leak et al., 1993).

Approximately 600 eartagged East African Zebu cattle from seven herds in the Ghibe valley were monitored and weighed on a monthly basis throughout the study. These cattle were of varying ages, including calves and mature cattle, both female and male. Jugular blood was collected into potassium ethylenediamine tetra-acetate and examined for the presence of trypanosomes using the phase-contrast, buffy-coat technique. Packed cell volumes (PCV) were also determined on a monthly basis. Throughout the entire experimental period parasitaemic animals with a PCV below 26% were treated by intramuscular injection with diminazene aceturate (Berenil, Hoechst AG, Germany) at a dose of 3.5 mg/kg bodyweight (b.w.). A similar drug dosage was administered to cattle which exhibited clinical signs of trypanosomiasis despite the absence of trypanosomes in their blood. Eighty seven per cent of all animals found parasitaemic had a PCV < 26% and were treated. Ten per cent of all treatments during this period were administered to animals which exhibited clinical signs but were not detected parasitaemic.

Three species of tsetse fly were detected in the valley between March 1986 and November 1992; *Glossina pallidipes*, *G. fuscipes* and *G. morsitans submorsitans*. *Glossina pallidipes* was the predominant tsetse species detected. The overall mature trypanosome infection rates in dissected tsetse were 3.6% in *G. pallidipes* and 1.2% in *G. fuscipes*. Approximately 76% of feeds of *G. pallidipes* were identified as having originated from cattle; man, monkeys and warthog appeared to be the next most important hosts for this species. For *G. fuscipes*, cattle, again, appeared to have been the most important source of feeds (63%). Such figures could not be derived for *G. m. submorsitans* due to the low numbers of this species which were caught. The tsetse challenge to cattle, due to *G. pallidipes*, increased approximately three-fold from January 1987 to April 1990. Throughout this period, the majority of infections in cattle were associated with *Trypanosoma congolense*. Furthermore, the monthly incidence of new infections of *T. congolense* in adult cattle increased from approximately 10% at the beginning of 1987 to 24% by April 1990. The overall prevalence of *T. congolense* infections in these adult cattle rose from 17% to 38% over this same period. Also, the prevalence of recurrent infections (i.e., refractory to treatment with diminazene aceturate), derived from the difference between the overall trypanosome-prevalence and the incidence of new infections,

[a]International Laboratory for Research on Animal Diseases (ILRAD), P. O. Box 30709, Nairobi, Kenya.
[b]International Livestock Centre for Africa (ILCA), P. O. Box 5689, Addis Ababa, Ethiopia.
[c]International Livestock Centre for Africa (ILCA), P. O. Box 46847, Nairobi, Kenya.

increased from an average of 7% between 1986 and 1987 to 14% in 1989. In all age groups, recurrent infections represented a significant proportion of all infections.

In order to determine the drug-resistance phenotype of trypanosome populations in cattle at Ghibe, blood samples were collected from parasitaemic cattle in July 1989. Twelve of the resultant stabilates were then inoculated into individual Boran (*Bos indicus*) calves, under fly-proof conditions, and characterised for their sensitivity, in turn, to diminazene aceturate (Berenil), isometamidium chloride (Samorin) and homidium chloride (Novidium). All 12 stabilates produced infections which were shown to be *T. congolense* that were resistant to treatment with diminazene aceturate at a dose of 7.0 mg/kg. b.w. Eleven of the infections were also resistant to isometamidium chloride at a dose of 0.5 mg/kg b.w. and homidium chloride at a dose of 1.0 mg/kg b.w. Five clones were derived from one of the isolates which expressed a high level of resistance to all three trypanocides; each clone expressed high levels of resistance to all three trypanocides (Codjia et al., 1993). Thus, the multiple-drug resistance phenotype of the parental isolate was associated with expression of multiple-drug resistance by individual trypanosomes. Finally, molecular karyotypes and electrophoretic variants of six enzymes were determined for seven and eight of the aforementioned isolates, respectively. Six different karyotypes were observed and all eight of the latter isolates belonged to different zymodemes, indicating that the multiple-drug resistance phenotype at Ghibe was associated with many genetically distinct populations of trypanosomes.

Because of the high level of multiple-drug resistant infections, and because this appeared to be expressed at the level of individual trypanosomes, it was concluded that chemotherapeutic agents *per se* would not control trypanosomiasis at Ghibe on a long-term basis. Thus, an integrated control programme, involving both tsetse-fly control and chemotherapeutic agents, was implemented in April 1990 in an attempt to control trypanosomiasis at the site; black-cotton cloth targets, measuring 1.0 x 2.0m, were assembled using locally available materials. Subsequently, they were erected throughout the valley at an average density of 4/km^2, depending on the suitability of vegetation for tsetse flies. Every target was impregnated with the synthetic pyrethroid deltamethrin (Glossinex; Wellcome Ltd., U.K.); once a month during the rainy season and once every three months during the dry season. Initial impregnation was carried out at a concentration of 0.1% of active ingredient. Re-impregnation was carried out at half the original concentration. All targets were used in conjunction with acetone and cattle urine, in separate containers, as attractants.

Subsequent to initiation of the target-control methodology, the relative density of the main vector at Ghibe, *G. pallidipes*, fell from a mean of 1.9 flies/trap/day in the 12 months prior to the introduction of tsetse control to a mean of 0.4 flies/trap/day in the 12 months, May 1990 to April 1991, during tsetse control. In the first quarter of 1991 it was a constant 0.09 flies/trap/day. In association with the decline in apparent tsetse density, the prevalence of *T. congolense* infections in cattle fell from approximately 30% before the tsetse control programme began to a mean of approximately 5% in the first quarter of 1991. Finally, the prevalence of diminazene-resistant infections, calculated as above, decreased by approximately 75% in the first 12 months following initiation of the tsetse control programme. Thus, despite a high prevalence of multiple-drug resistant infections in cattle, a decline in the tsetse populations was associated with a substantial decrease in the apparent prevalence of these drug-resistant infections.

One of the most important criteria affecting the long-term effectiveness of any disease control strategy is the sustainability of the given methodology. Prior to June 1991, thefts of targets and attractant bottles had not been a significant problem at Ghibe. However, in June 1991, Ethiopia experienced a period of political instability. In association with this instability, substantial numbers of targets and attractant bottles were stolen, such that less than 40% of the targets and attractant bottles in place at the beginning of 1991 remained in place during the 12-month period following June 1991. In association with the removal of targets and attractant bottles, a very rapid increase in the apparent tsetse challenge due to *G. pallidipes* was observed. Similarly, an increase in both the overall prevalence and the monthly incidence of trypanosome infections in cattle was also observed. Thus, the demise of the target control strategy was associated with a rapid reinvasion of tsetse flies into the Ghibe valley, and a concomitant increase in both the incidence and prevalence of trypanosome infections in cattle at that site. While the use of impregnated target technology has therefore been shown to be effective at Ghibe, and elsewhere, in controlling certain tsetse species, the sustainability of this control methodology is therefore problematical. In an attempt to circumvent some of the problems associated with this methodology the ability of synthetic pyrethroid 'pour-on' insecticides

(Thompson et al., 1991), applied to cattle, is therefore under investigation at a neighbouring site.

In conclusion, improved control of multiple-drug resistant trypanosome infections in cattle can be achieved if one integrates chemotherapy and tsetse control strategies. The use of such an integrated approach to trypanosomiasis control, however, depends on the tsetse control strategy being sustainable. The particular circumstances at Ghibe were not conducive to sustainability of the "target" methodology. However, results obtained in the application of a 'pour-on' at a neighbouring site offer greater promise.

ACKNOWLEDGEMENTS

We are grateful to Dr. Zeleke Dagnachew, former Head, Veterinary Services Department, Ethiopia, for permission to publish this work. RMB Animal Health Ltd., U.K., kindly donated the isometamidium chloride and homidium chloride used in this study.

REFERENCES

Codjia, V., Mulatu, W., Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authié, E., d'Ieteren, G.D.M. and Peregrine, A.S., 1993. Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. Acta Trop., 53: 151-163.

Leak, S.G.A., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Peregrine, A.S., Rowlands, G.J. and Trail, J.C.M., 1993. Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 1. Tsetse challenge and its relationship to trypanosome prevalence in cattle. Acta Trop., 53: 121-134.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M. and Peregrine, A.S., 1993. Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. Acta Trop., 53: 135-150.

Thompson, J.W., Mitchell, M., Rees, R.B., Shereni, W., Schoenfeld, A.H. and Wilson, A., 1991. Studies on the efficacy of deltamethrin applied to cattle for the control of tsetse flies (*Glossina* spp.) in southern Africa. Trop. Anim. Hlth. Prod., 23: 221-226.

Section 2.4

Mulugeta, W., Wilkes, J., Mulatu, W., Majiwa, P.A.O., Masake, R. and Peregrine, A.S. Long-term occurrence of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium in cattle at Ghibe, Ethiopia. Submitted to *Acta Tropica*.

Long-term occurrence of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium in cattle at Ghibe, Ethiopia

Wubet Mulugeta[1], Jon Wilkes[1], Woudyalew Mulatu[2], Phelix A.O. Majiwa[1], Rachael Masake[1] and Andrew S. Peregrine[1]

[1] *International Livestock Research Institute (ILRI), P.O. Box 30709, Nairobi, Kenya*

[2] *International Livestock Research Institute (ILRI), P.O. Box 5689, Addis Ababa, Ethiopia*

keywords trypanosomosis, *Trypanosoma congolense*, drug resistance, diminazene, isometamidium, homidium, cattle, Ethiopia

correspondence Dr Andrew S. Peregrine, International Livestock Research Institute, P.O. Box 30709, Nairobi, Kenya

Summary

Ten trypanosome isolates were collected at random from cattle at Ghibe, Ethiopia, in February 1993 and all shown to be savannah-type *Trypanosoma congolense*. When inoculated into naïve Boran (*Bos indicus*) calves, all 10 isolates were resistant to diminazene aceturate (Berenil®), isometamidium chloride (Samorin®) and homidium chloride (Novidium®) at doses of 7.0 mg/kg body weight (b.w.), 0.5 mg/kg b.w. and 1.0 mg/kg b.w., respectively. In order to determine whether this multiple-drug resistance was expressed by individual trypanosomes, clones were derived from 2 of the isolates and characterised in mice for their sensitivity to the 3 compounds; by comparison to drug-sensitive populations, the 2 clones expressed high levels of resistance to all 3 trypanocides. In experiments to characterise the uptake kinetics of [^{14}C]-Samorin, the maximal rates of uptake (V_{max}) for 4 Ghibe isolates ranged from 9.2 to 15.0 ng/10^8 trypanosomes/minute. In contrast, V_{max} for the isometamidium-sensitive clone *T. congolense* IL 1180 was 86.7 ± 8.6 ng/10^8 trypanosomes/minute. Lastly, molecular karyotypes were determined for 8 isolates; 7 different chromosome profiles were observed. These data indicate that in February 1993 there was a high prevalence of drug-resistant trypanosome populations with different chromosome profiles in cattle at Ghibe. Since a similar situation existed at the same site in July 1989, this suggests that the drug-resistance phenotype of trypanosomes at Ghibe had not altered over a 4-year period.

Introduction

Studies to determine the constraints to production of East African Zebu cattle under trypanosomiasis risk in the Ghibe valley, south-west Ethiopia, were initiated in 1986 and have been described by Leak *et al.* (1993) and Rowlands *et al.* (1993). Between January 1986 and April 1990 the predominant trypanosome species observed in cattle at this site was *Trypanosoma congolense* (Rowlands *et al.* 1994). Furthermore, the apparent prevalence of such infections increased from 17% to 38% over the same period, despite administration of the anti-trypanosomal compound diminazene aceturate (Berenil®) to infected animals. This increase in prevalence was shown to be partly associated with an increase in trypanosome challenge. However, on the basis of parasitological data obtained in the field, it was also thought to be associated with expression of resistance to diminazene by trypanosome populations (Rowlands *et al.* 1993). This hypothesis was confirmed when 12 isolates, collected from cattle at Ghibe in July 1989, were shown to be resistant to the maximum recommended dose of diminazene aceturate: 7.0 mg/kg body weight (b.w.) (Codjia *et al.* 1993). It was also found that 11 of the 12 isolates were resistant in cattle to recommended doses of the other 2 anti-trypanosomal compounds recommended for use in cattle: isometamidium (Samorin®) and homidium (Novidium®) (Codjia *et al.* 1993). There therefore appeared to be a high prevalence of multiple-drug resistant infections in cattle at Ghibe in July 1989. Furthermore, since the multiple-drug resistance was expressed by individual trypanosomes (Codjia *et al.* 1993) it was concluded that chemotherapy *per se* would not control trypanosomiasis at that site. As a result, a tsetse-control programme was initiated in April 1990 in which deltamethrin-impregnated targets were erected across the Ghibe valley at an average density of 4/km^2. This was associated with a significant reduction in the apparent density of tsetse flies in the following 12-month period. Furthermore, despite administration of diminazene aceturate to infected animals as before, the apparent prevalence of *T. congolense* infections in cattle fell from approximately 30% before the tsetse control programme began to a mean of approximately 5% in the first 3 months of 1991 (Peregrine *et al.* 1994). A decrease in the rate of relapse of infections in cattle after treatment with diminazene aceturate was also observed

(Mulatu *et al.* 1991). In order to determine whether the latter observation was associated with an alteration in the drug-resistance phenotype of trypanosome populations, a second set of trypanosome isolates was collected from cattle at Ghibe in February 1993. This paper describes the characterisation of these populations.

Material and methods

Experiment animals

Ten Boran (*Bos indicus*) calves, aged 3-6 months, were obtained from an area of Kenya that is free of tsetse flies. Preinfection sera were collected from the animals and screened for the presence of *T. congolense*, *T. vivax* and *T. brucei*, using species-specific antigen-detection ELISAs (Nantulya & Lindqvist 1989). All were found to be negative. Animals were kept within fly-proof isolation units throughout the experiment and were managed as described by Whitelaw *et al.* (1986).

Outbred adult Swiss mice and Sprague Dawley rats were obtained from the ILRI's breeding colony. All animals were maintained on a commercially pelleted ration (Unga Feeds Ltd., Nairobi) provided *ad libitum*.

Trypanosomes

Blood samples were collected at random on 16th February 1993 from cattle that were detected parasitaemic at Ghibe. Stabilates were then prepared (Dar *et al.* 1972), stored in liquid nitrogen, and transported to Nairobi for analysis. Ten field isolates were examined in the present study (see Table 1). Each isolate was numbered according to the methodology of WHO (1978); the herd of origin for each population is listed in Table 1. *Trypanosoma congolense* IL 3918 and IL 3922 are stocks that were isolated from cattle at Ghibe in 1989 (Codjia *et al.* 1993).

Trypanosoma congolense IL 1180 is a cloned derivative of an isolate from the Serengeti National Park, Tanzania (Nantulya *et al.* 1984), and was used as a reference drug-sensitive population (Peregrine *et al.* 1991) for Samorin-uptake studies.

Anti-trypanosomal compounds

Diminazene aceturate (Berenil®, Lot No. BN 371A748; Hoechst, Germany), isometamidium chloride (Samorin®, Lot No. U6127B; RMB Animal Health Ltd.,

Table 1

Origin of Ghibe trypanosome populations and their infectivity for rodents

Herd origin	Field isolate	ILRI number*	Infectivity for mice	Infectivity for rats
3	Ghibe/93/IL/3437	IL 4009	+	+
1	Ghibe/93/IL/958	IL 4010	+	+
1	Ghibe/93/IL/1745	IL 4011	-	-
3	Ghibe/93/IL/3599	IL 4012	+	-
1	Ghibe/93/IL/48	IL 4013	+	-
1	Ghibe/93/IL/3555	IL 4014	-	-
1	Ghibe/93/IL/3195	IL 4015	+	+
3	Ghibe/93/IL/3349	IL 4016	+	-
3	Ghibe/93/IL/3529	IL 4017	+	-
3	Ghibe/93/IL/3348	IL 4018	+	-

*, ILRI number of primary isolate; +, infective; -, non-infective.

U.K.) and homidium chloride (Novidium®, Lot No. 7HH; RMB Animal Health Ltd., U.K.) were used for drug-sensitivity studies. Each trypanocide was either administered intramuscularly to calves in the middle third of the neck (as recommended by the manufacturers), or intraperitoneally to mice. [^{14}C]-labelled Samorin (Batch No. GHS-511; RMB Animal Health Ltd., U.K.) was used for studies to characterise uptake of the drug by trypanosomes.

Monitoring of infections

Jugular blood samples were collected daily from calves into evacuated tubes containing potassium-ethylenediamine tetra-acetate (EDTA). Thereafter, the buffy-coat was examined for the presence of parasites using the technique of Murray *et al.* (1977). Identification of the trypanosome species was achieved on the basis of morphology, by examination of thin-blood smears stained with Giemsa (Lumsden *et al.* 1973).

The presence of trypanosomes in mice was determined by examining wet films of tail blood, 3-times weekly, for 60 days following infection.

Drug-sensitivity tests in Boran calves

Each field isolate was thawed at room temperature, diluted in phosphate-buffered saline-glucose, pH 8, and injected into the jugular vein of individual Boran calves, i.e., 1 isolate per animal. At the first peak of parasitaemia, jugular blood was collected from each animal into evacuated tubes containing potassium-EDTA. Primary isolates were then prepared according to Dar *et al.* (1972) (see Table 1). Thereafter, each Boran calf was treated with diminazene aceturate at a dose of 7 mg/kg b.w. If animals were subsequently detected parasitaemic they were treated with isometamidium chloride at a dose of 0.5 mg/kg b.w. If a second relapse occurred, the animals were treated with homidium chloride at a dose of 1.0 mg/kg b.w. The time interval between each treatment, for each animal, is given in Table 2.

Table 2

Drug sensitivity of Ghibe isolates carried out sequentially in Boran calves

Field isolate number	Treatment with diminazene aceturate (7.0 mg/kg b.w.)	Relapse* interval (days)	Interval between treatment with diminazene and isometamidium (days)	Treatment with isometamidium chloride (0.5 mg/kg b.w.)	Relapse* interval (days)	Interval between treatment with isometamidium and homidium (days)	Treatment with homidium chloride (1.0 mg/kg b.w.)	Relapse* interval (days)
48	R	13	28	R	6	33	R	7
958	R	10	32	R	5	9	R	8
1745	R	12	28	R	26	33	R	7
3195	R	7	27	R	9	18	R	4
3348	R	9	9	R	8	11	R	6
3349	R	6	20	R	8	18	R	12
3437	R	6	23	R	4	33	R	7
3529	R	5	20	R	10	18	R	5
3555	R	16	32	R	9	33	R	7
3599	R	15	38	R	7	33	R	7

R, resistant, i.e., trypanosomes reappeared after treatment; *, number of days between the day of treatment and the day that trypanosomes were first detected following a period of aparasitaemia.

Infectivity for mice and rats

At the first peak of parasitaemia, jugular blood samples were collected from each of the 10 Boran calves into potassium-EDTA. Thereafter, for each sample, 0.3 ml was inoculated intraperitoneally into each of 5 irradiated (600 rad) mice, and 1.0 ml was inoculated intraperitoneally into each of 2 irradiated (600 rad) rats.

Drug-sensitivity tests in mice

In order to determine the drug-resistance phenotype of cloned trypanosome populations, 2 primary isolates (IL 4009 and IL 4015) were chosen at random and inoculated intraperitoneally into non-irradiated mice. When the level of parasitaemia was greater than 10^8/ml, clones were derived from each population according to the method of Barry and Gathuo (1984). Two resultant clones (1 per isolate) were then characterised in mice for their sensitivities to diminazene aceturate, isometamidium chloride and homidium chloride as described by Peregrine *et al.* (1991); the range of drug doses investigated was 10-50 mg/kg b.w. for diminazene aceturate, and 1-20 mg/kg b.w. for isometamidium chloride and homidium chloride. Responses to treatment were expressed as a 50% curative dose (CD_{50}), using standard logit analyses.

Uptake of Samorin

Uptake of [^{14}C]-Samorin was determined in 4 primary isolates (IL 4009, IL 4010, IL 4012 and IL 4015) and compared with *T. congolense* IL 1180. The methodology used was essentially as described by Zilberstein *et al.* (1993). In brief, trypanosomes were separated from rat blood using diethylaminoethyl (DEAE)-cellulose (Lanham & Godfrey 1970). Thereafter, they were suspended at 2 x 10^8 cells/ml in Dulbecco's phosphate-buffered saline, containing 5 mM glucose and 100 μM hypoxanthine (PSGH). A 0.1 ml aliquot of this suspension was then incubated for 10 minutes at 37°C with 0.6 ml of [^{14}C]-Samorin in PSGH (final concentration ranging from 0.05 to 2.25 μg/ml). The reaction mixture was subsequently transferred to an Eppendorf

microcentrifuge tube, onto a 200 µl layer of silicone oil (Aldrich Chemical Co. Ltd., U.K.: 1.05 g/ml). The silicone oil itself lay on top of 50 µl of 12% perchloric acid. Uptake was terminated by centrifugation at 14,000 g for 2 minutes. Transfer into the perchloric acid was essentially complete within 20 seconds (J. Wilkes, unpublished observation). The level of [^{14}C] radioactivity in aliquots of perchloric acid lysate was then measured by liquid-scintillation counting. Kinetic parameters were determined using the ENZFIT program (Elsevier Biosoft, U.K.), in which data were fitted to appropriate models.

Fractionation of chromosome-sized DNA molecules

Eight primary isolates were examined. Each population was expanded in sublethally irradiated (600 rad) rats, then separated from rat blood using DEAE-cellulose (Lanham & Godfrey 1970). Chromosome-sized DNA molecules were then prepared from each population as described previously (Van der Ploeg *et al.* 1984; Majiwa *et al.* 1985; Masake *et al.* 1988). Fractionation of chromosome-sized DNA molecules was then performed using contour-clamped homogeneous field gel-electrophoresis (CHEF; Chu *et al.* 1986) using 150 volts and 60 second pulse intervals over a 46-hour period. In addition to the 8 populations examined, 2 isolates which were collected from Ghibe in 1989 and analysed by Codjia *et al.* (1993) were included for comparative purposes.

DNA-probe hybridisation

After fractionation, the chromosome-sized DNA molecules in the gel were transferred to a nitrocellulose filter paper which was then hybridised with radiolabelled pgNRE-372; a repetitive sequence of DNA common to savannah-type *T. (Nannomonas) congolense* (Majiwa *et al.* 1985).

Results

Drug-sensitivity tests in Boran calves

Following inoculation with trypanosome isolates, all 10 Boran calves were detected parasitaemic within 7-33 days. On the basis of morphology, the parasites occurring in each animal were identified as *T. congolense*. Subsequent to treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w., trypanosomes reappeared in all 10 calves within 5-16 days (Table 2). When the same 10 animals were thereafter treated with 0.5 mg isometamidium chloride/kg b.w., trypanosomes reappeared in all animals within 4-26 days. When homidium chloride was administered to the 10 animals at a dose of 1.0 mg/kg b.w., trypanosomes reappeared in all the animals within 4-12 days following treatment (Table 2).

Drug-sensitivity tests in mice

Cloned populations were derived from *T. congolense* IL 4009 and *T. congolense* IL 4015 (IL 4009.1 and IL 4015.1, respectively) and characterised in mice for their drug sensitivity. Clones IL 4009.1 and IL 4015.1 had diminazene aceturate CD_{50} values of 45.9 ± 0.13 (S.D.) mg/kg b.w. and 30.0 ± 0.25 mg/kg b.w., respectively. Both clones had homidium chloride CD_{50} values that were in excess of 20 mg/kg b.w. Finally, the isometamidium chloride CD_{50} values for the 2 clones were 12.5 ± 0.21 mg/kg b.w. and 9.8 ± 0.16 mg/kg b.w., respectively.

Samorin uptake

The kinetic parameters for uptake of Samorin by the 4 *T. congolense* populations from Ghibe were compared with those of the drug-sensitive *T. congolense* clone IL 1180 (see Table 3). The maximal rates of uptake (V_{max}) of Samorin by the 4 populations ranged from 9.2 to 15.0 ng/10^8 trypanosomes/minute. In contrast, V_{max} for IL 1180 was at least 6 times higher. For all populations the uptake process was

Table 3

Kinetics of Samorin uptake by *Trypanosoma congolense* populations

T. congolense population	V_{max} (ng/10^8 tryps./min)	K_m (μg/ml)
IL 1180	86.73 ± 8.60	0.47 ± 0.16
IL 4009	10.55 ± 1.20	0.35 ± 0.30
IL 4010	15.00 ± 2.30	1.05 ± 0.44
IL 4012	12.70 ± 1.40	0.25 ± 0.17
IL 4015	9.20 ± 1.80	1.23 ± 0.48

data = mean ± standard deviation; tryps. = trypanosomes.

saturable within 10 minutes. K_m for the Ghibe populations ranged from 0.25 to 1.23 μg/ml. The value for IL 1180 was 0.47 μg/ml.

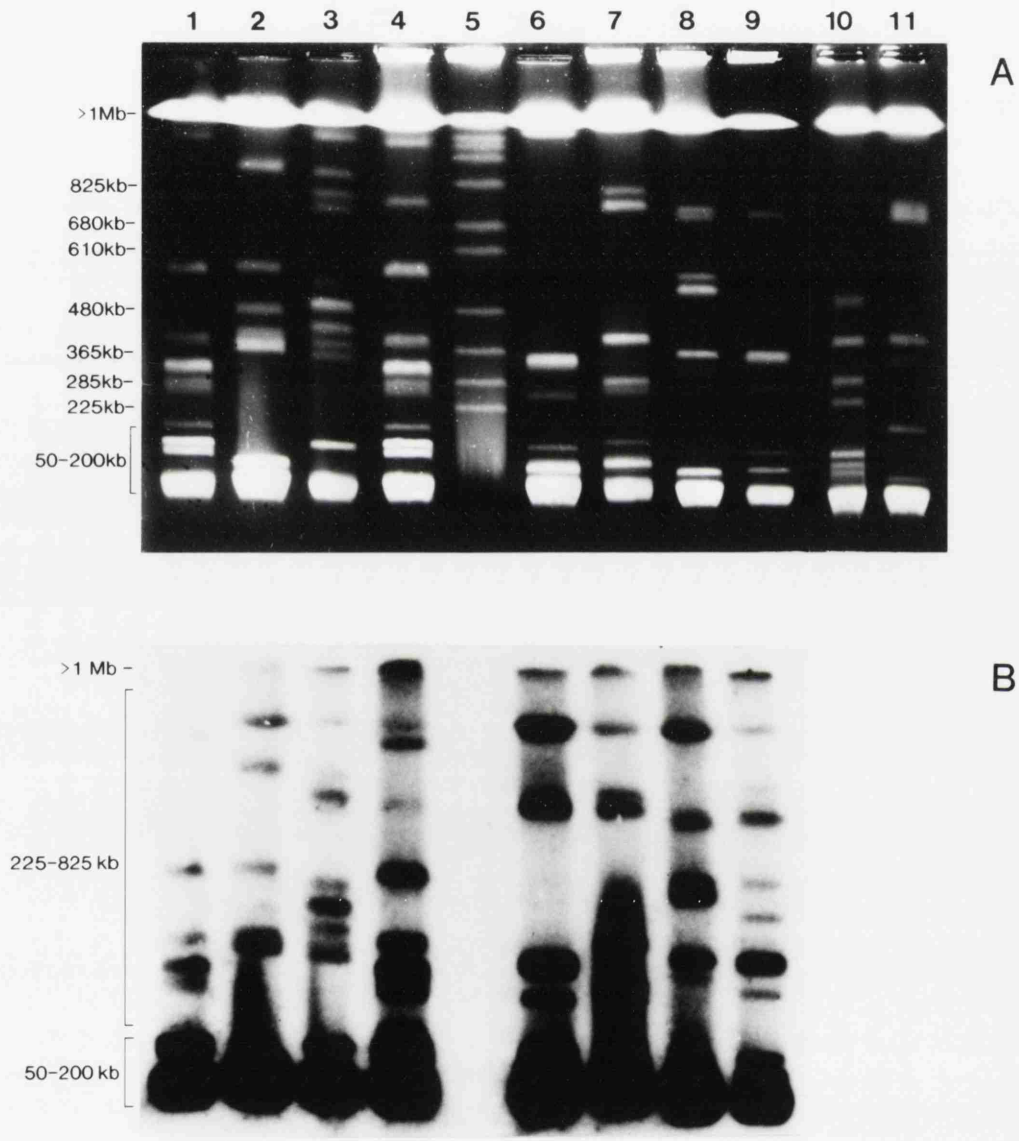
Fractionation of chromosome-sized DNA molecules

Analysis of the chromosome profiles of the Ghibe isolates revealed distinct differences in both size and number of chromosome-sized DNA molecules displayed by each trypanosome population. Amongst the 8 populations examined 7 different molecular karyotypes were observed (see Figure 1A). Two isolates (IL 4009 and IL 4013; Figure 1A, lanes 1 and 4, respectively) displayed the same karyotypes. The chromosome profiles of the 8 isolates collected in 1993 were also different from those which were collected in 1989 (Codjia *et al.* 1993). Despite these differences, however, the molecular karyotypes of all the populations were consistent with that of savannah-type *T. congolense* (Majiwa *et al.* 1985). This was further confirmed by hybridisation of DNA probe pgNRE-372 (Majiwa *et al.* 1985) to chromosome-sized bands of each population (see Figure 1B).

Figure 1

Figure 1A Comparative chromosome profiles of Ghibe isolates. Chromosome-sized DNA molecules from 8 isolates were subjected to CHEF electrophoresis for 46 hours at a pulse frequency of 60 seconds to reveal DNA molecules less than 1 Mb. Lanes contain DNA from the following isolates: lane 1, IL 4009; lane 2, IL 4010; lane 3, IL 4012; lane 4, IL 4013; lane 5, yeast chromosome-size markers; lane 6, IL 4015; lane 7, IL 4016; lane 8, IL 4017; lane 9, IL 4018; lane 10, IL 3918; lane 11, IL 3922.

Figure 1B An autoradiogram of the chromosome-sized DNA molecules in the gel shown in Figure 1A (lanes 1-9). Chromosome-sized DNA molecules were transferred to a nitrocellulose filter paper and hybridised with radiolabelled pgNRE-372 under standard conditions.


1
2
3
4
5
6
7
8
9
10
11
A
>1Mb-
825kb-
680kb-
610kb-
480kb-
365kb-
285kb-
225kb-
50-200kb
>1 Mb -
225-825 kb
50-200 kb
B

Discussion

In the work described here, 10 trypanosome isolates were collected in February 1993 from parasitaemic cattle at Ghibe, Ethiopia, inoculated into naïve Boran cattle and characterised for their sensitivity to recommended doses of diminazene aceturate, isometamidium chloride and homidium chloride. All 10 isolates were resistant to these compounds at doses of 7.0 mg/kg b.w., 0.5 mg/kg b.w. and 1.0 mg/kg b.w., respectively. Therefore, multiple-drug resistant infections appeared to be highly prevalent in cattle at Ghibe at the time these isolates were collected.

In order to determine whether the multiple-drug resistance phenotype was associated with trypanosomes that were heterogeneous or homogeneous in their drug-resistance phenotype, 2 clones (IL 4009.1 and IL 4015.1) were derived from 2 of the isolates and characterised in mice for their sensitivity to diminazene, isometamidium and homidium. By comparison to other trypanosome populations that have been characterised in a similar manner (Peregrine *et al.* 1991; Codjia *et al.* 1993), both clones expressed high levels of resistance to all 3 compounds. Thus, the multiple-drug resistance of the parental isolates was expressed at the level of individual trypanosomes.

On the basis of hybridisation of trypanosome DNA to the DNA probe pgNRE-372, all 8 isolates that were examined appeared to be savannah-type *T. congolense*. This was further confirmed by molecular karyotype analysis (Majiwa *et al.* 1985). However, the latter technique indicated that 7 of the 8 populations had different chromosome profiles. Thus, the multiple-drug resistance phenotype of the isolates was associated with trypanosome populations that are of different molecular karyotypes.

This is not the first time that multiple resistance to anti-trypanosomal compounds has been reported at Ghibe. Codjia *et al.* (1993) described 12 isolates of *T. congolense* that were collected from cattle at the site in 1989; 11 of the 12 populations expressed high levels of resistance to diminazene, isometamidium and homidium. Furthermore, cloned populations derived from an isolate were shown to express resistance to all 3 compounds. Finally, a high level of heterogeneity in chromosome profiles was also demonstrated amongst the isolates. Thus, by

comparison to the work described here, there does not appear to have been an alteration in the drug-resistance phenotype of trypanosome populations at Ghibe between 1989 and 1993. In light of the high prevalence of such infections in cattle at Ghibe in 1989, this finding is perhaps not surprising, although a number of workers have demonstrated instability of resistance to anti-trypanosomal compounds in trypanosome populations (Whiteside 1963; Peregrine *et al.* 1991; Mamman *et al.* 1993).

The occurrence of multiple-drug resistance in trypanosome populations does not necessarily indicate that anti-trypanosomal compounds will not eliminate infections. For instance, if the multiple resistance is associated with a mixed infection, administration of the compounds that the different sub-populations are sensitive to will eliminate the entire trypanosome population. However, chemotherapy is not a viable control option if the multiple-drug resistance is expressed by individual trypanosomes (Codjia *et al.* 1993). Since this appeared to be the case at Ghibe in 1989, a tsetse control programme was initiated in 1990 which was subsequently associated with a substantial reduction in the apparent prevalence of parasitaemic cattle (Peregrine *et al.* 1994). However, the work described here has shown that, despite these changes, multiple-drug resistant infections were still present in cattle at the site 4 years later.

In addition to Ethiopia, the simultaneous occurrence of diminazene-, isometamidium- and homidium-resistance in trypanosome populations has been reported in Nigeria (Ilemobade 1979), Kenya (Gitatha 1979; Schönefeld *et al.* 1987), Burkina Faso (Clausen *et al.* 1992), and Sudan (Mohamed-Ahmed *et al.* 1992). However, for each of these reports, it is unclear whether the multiple resistance was associated with mixed or single infections.

At present, cattle, mice and *in vitro*-cultivation methodologies are used to characterise the drug sensitivity of trypanosome populations. However, while each method has certain advantages (Peregrine 1994), none of the methods can be used to rapidly quantify the drug-resistance phenotype of large numbers of isolates. In recent work, uptake of Samorin has been examined in populations of *T. congolense* that vary in sensitivity to Samorin, using fluorescence and radiolabelled methodologies (Sutherland *et al.* 1991, 1992). While both described techniques indicated that drug

uptake was more rapid, and greater, in Samorin-sensitive, as compared to Samorin-resistant, populations, neither technique was sufficiently robust to allow one to determine V_{max} and K_m values for all the trypanosome populations examined. Since such methodologies might be used to rapidly quantify the Samorin resistance of trypanosome populations, a silicone oil-cushion centrifugation methodology was used in the work described here to characterise uptake of [^{14}C]-labelled Samorin (Zilberstein *et al.* 1993) since it facilitated determination of V_{max} and K_m values for every population. Uptake of [^{14}C]-labelled Samorin was determined for 4 isolates from Ghibe and compared to the Samorin-sensitive clone *T. congolense* IL 1180 (Table 3). The maximal rates of uptake (V_{max}) of Samorin for the Ghibe isolates were similar to each other, but were approximately one sixth of the value for *T. congolense* IL 1180. Zilberstein *et al.* (1993) have demonstrated that Samorin is transported across the plasma membrane of *T. congolense* IL 1180 via a transport system sensitive to -SH reactive inhibitors, indicative of a protein carrier. One possible explanation for the reduced V_{max} values in the 4 Samorin-resistant isolates from Ghibe is that the total number of Samorin transporters in the plasma membrane is lower than in *T. congolense* IL 1180. Alternatively, while the total number of transporters may be unaltered, their turn-over rate for Samorin may be significantly reduced. Work is currently underway in our laboratory to fully characterise the Samorin transporter(s) and to determine the role it plays in expression of resistance. If the same resistance mechanism is used by all trypanosome populations then such information may be employed in the development of a field-applicable methodology for rapidly quantifying the Samorin resistance of trypanosome populations.

Acknowledgements

RMB Animal Health Limited is thanked for providing the Samorin (radiolabelled and non-labelled) and homidium chloride. Dr. Abebe Wondimu, Head, Veterinary Services Division, Ethiopia, is thanked for permission to publish this work. We are also indebted to Messrs. S. Kemei, J. Makau, and Mrs. M. Maina for technical assistance. This is ILRI publication No. 1482.

References

Barry JD & Gathuo H (1984) Antigenic variation in *Trypanosoma vivax*: isolation of a serodeme. *Parasitology* **89,** 49-58.

Chu G, Vollrath D & Davis RW (1986) Separation of large DNA molecules by contour-clamped homogeneous electric field. *Science* **234,** 1582-1585.

Clausen P-H, Sidibe I, Kaboré I & Bauer B (1992) Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse fly challenge in the pastoral zone of Samorogouan, Burkina Faso. *Acta Tropica* **51,** 229-236.

Codjia V, Mulatu W, Majiwa PAO *et al.* (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. *Acta Tropica* **53,** 151-163.

Dar FK, Ligthart GS & Wilson AJ (1972) Cryopreservation of pathogenic African trypanosomes *in situ*: metacyclic and bloodstream forms. *Journal of Protozoology* **19,** 494-497.

Gitatha SK (1979) *T. congolense* (Shimba Hills) resistant to various trypanocidal drugs. In *Proceedings, International Scientific Council for Trypanosomiasis Research and Control, 16th Meeting, Yaoundé, Cameroon.* OAU/STRC, Nairobi, pp 257-263.

Ilemobade AA (1979). Drug sensitivity of mouse infective *Trypanosoma vivax* isolates in cattle and sheep. In *Proceedings, International Scientific Council for Trypanosomiasis Research and Control, 16th Meeting, Yaoundé, Cameroon.* OAU/STRC, Nairobi, pp 251-253.

Lanham SM & Godfrey DG (1970) Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Experimental Parasitology* **28,** 521-534.

Leak SGA, Mulatu W, Authié E *et al.* (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 1. Tsetse challenge and its relationship to trypanosome prevalence in cattle. *Acta Tropica* **53,** 121-134.

Lumsden WHR, Herbert WJ & McNeillage GJC (1973) Techniques with trypanosomes. Churchill Livingstone, London, pp 122-124.

Majiwa PAO, Masake RA, Nantulya VM, Hamers R & Matthyssens G (1985) *Trypanosoma (Nannomonas) congolense*: identification of two karyotypic groups. *EMBO Journal* **4,** 3307-3313.

Mamman M, Katende J, Moloo SK & Peregrine AS (1993) Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology* **50,** 1-14.

Masake RA, Nyambati VM, Nantulya VM, Majiwa PAO, Moloo SK & Musoke AJ (1988) The chromosome profiles of *Trypanosoma congolense* isolates from Kilifi, Kenya and their relationship to serodeme identity. *Molecular and Biochemical Parasitology* **30,** 105-112.

Mohamed-Ahmed MM, Rahman AH & Abdel Karim EI (1992) Multiple drug-resistant bovine trypanosomes in South Darfur Province, Sudan. *Tropical Animal Health and Production* **24,** 179-181.

Mulatu W, Leak SGA, Authié E, d'Ieteren G, Peregrine AS & Rowlands GJ (1991) Preliminary results of a tsetse control campaign using deltamethrin impregnated targets to alleviate a drug-resistance problem in bovine trypanosomiasis. In *Proceedings, International Scientific Council for Trypanosomiasis Research and Control, 21st Meeting, Yamoussoukro, Côte d'Ivoire.* OAU/STRC, Nairobi, p 203.

Murray M, Murray PK & McIntyre WIM (1977) Improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **71,** 325-326.

Nantulya VM & Lindqvist KJ (1989) Antigen-detection enzyme immunoassay for the diagnosis of *Trypanosoma vivax*, *T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology* **40,** 267-272.

Nantulya VM, Musoke AJ, Rurangirwa FR & Moloo SK (1984) Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe passaged infections. *Infection and Immunity* **43,** 735-738.

Peregrine AS (1994) Chemotherapy and delivery systems: haemoparasites. *Veterinary Parasitology* **54,** 223-248.

Peregrine AS, Knowles G, Ibitayo AI, Scott JR, Moloo SK & Murphy NB (1991) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* **102,** 93-100.

Peregrine AS, Mulatu W, Leak SGA & Rowlands GJ (1994) Epidemiology of bovine trypanosomiasis in the Ghibe valley, Ethiopia: multiple-drug resistance and its effective control. *Kenya Veterinarian* **18,** 369-371.

Rowlands GJ, Mulatu W, Authié E, d'Ieteren GDM, Leak SGA & Nagda SM (1994) Effects of trypanosomiasis on growth and mortality of young East African Zebu cattle exposed to drug-resistant trypanosomes. *Preventive Veterinary Medicine* **21,** 87-101.

Rowlands GJ, Mulatu W, Authié E *et al.* (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. *Acta Tropica* **53,** 135-150.

Schönefeld A, Röttcher D & Moloo SK (1987) The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. *Tropical Medicine and Parasitology* **38,** 177-180.

Sutherland IA, Mounsey A & Holmes PH (1992) Transport of isometamidium (Samorin) by drug-resistant and drug-sensitive *Trypanosoma congolense*. *Parasitology* **104,** 461-467.

Sutherland IA, Peregrine AS, Lonsdale-Eccles JD & Holmes PH (1991) Reduced accumulation of isometamidium by drug-resistant *Trypanosoma congolense*. *Parasitology* **103,** 245-251.

Van der Ploeg LHT, Cornelissen AW, Michels PAM & Borst P (1984) Chromosome rearrangements in *Trypanosoma brucei*. *Cell* **39,** 213-221.

Whitelaw DD, Bell IR, Holmes PH *et al.* (1986) Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. *Veterinary Record* **118,** 722-726.

Whiteside EF (1963) A strain of *Trypanosoma congolense* directly resistant to berenil. *Journal of Comparative Pathology* **73,** 167-175.

WHO (1978) Proposals for the nomenclature of salivarian trypanosomes and for the maintenance of reference collections. *Bulletin of the World Health Organization* **56,** 467-480.

Zilberstein D, Wilkes J, Hirumi H & Peregrine AS (1993) Fluorescence analysis of the interaction of isometamidium with *Trypanosoma congolense*. *Biochemical Journal* **292,** 31-35.

Section 2.5

Ndoutamia, G., Moloo, S.K., Murphy, N.B. and Peregrine, A.S. (1993) Derivation and characterization of a quinapyramine-resistant clone of *Trypanosoma congolense*. *Antimicrobial Agents and Chemotherapy* 37, 1163-1166.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1993, p. 1163–1166
0066-4804/93/051163-04$02.00/0

Vol. 37, No. 5

Derivation and Characterization of a Quinapyramine-Resistant Clone of *Trypanosoma congolense*†

GUELMBAYE NDOUTAMIA, SHAMSHUDEEN K. MOLOO, NOEL B. MURPHY, AND ANDREW S. PEREGRINE*

International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya

Received 9 November 1992/Accepted 5 March 1993

Over a period of 208 days a quinapyramine-resistant population was derived in vivo from a quinapyramine-susceptible clone of *Trypanosoma congolense*: IL 1180. While the dose of quinapyramine sulfate required to cure 50% of mice infected with the parental clone was 0.23 mg/kg of body weight, the 50% curative dose for the resistant derivative, IL 1180/Stabilate 12, was greater than 9.6 mg/kg. This approximately 40-fold increase in resistance to quinapyramine was shown to be associated with an 8-fold increase in resistance to isometamidium, a 28-fold increase in resistance to homidium, and a 5.5-fold increase in resistance to diminazene. Cross-resistance to homidium and diminazene was also demonstrated in goats. Two clones derived from the drug-resistant derivative underwent cyclical development in *Glossina morsitans centralis*, producing mature infection rates of 39.6 and 23.9%. Thus, induction of resistance to quinapyramine in *T. congolense* IL 1180 was associated with cross-resistance to isometamidium, homidium, and diminazene and did not compromise the population's ability to undergo full cyclical development in tsetse flies.

One of the most important diseases reducing productivity of domestic livestock in sub-Saharan Africa is trypanosomiasis (3). The trypanosome species responsible for this disease complex in cattle, sheep, and goats include *Trypanosoma congolense*, *T. vivax*, and *T. brucei* (5). Methods to control trypanosomiasis include control of tsetse populations, the exploitation of trypanosome-tolerant livestock, and administration of antitrypanosomal drugs (16).

Chemotherapy of trypanosomiasis in domestic livestock is at present dependent upon the salts of a relatively small number of synthetic compounds: homidium, isometamidium, diminazene, and quinapyramine (Fig. 1). All four compounds have been on the market for at least 30 years, and there are now reports of drug resistance in *T. congolense* and *T. vivax* in many parts of Africa (14). Furthermore, because of the close chemical relationships between the compounds, the development of resistance to individual trypanocides often appears to be associated with cross-resistance to others (31, 33).

Between the 1950s and 1970s quinapyramine was widely used in Africa as a therapeutic and prophylactic agent in cattle (8, 26). Resistance to the compound appeared to develop rapidly (28, 34) and was often associated with concomitant cross-resistance to isometamidium, homidium, and diminazene (18, 31). However, it was impossible to ascertain whether the apparent cross-resistance actually was cross-resistance or reflected difficulties in interpreting field-derived data. In 1976, quinapyramine ceased to be manufactured (13) because of the ease with which resistance appeared to develop (8, 14, 22) and because of problems with drug toxicity (7). However, in 1984 the compound was reintroduced to the market, but only for use in camels (27). Despite the problems that appeared to occur when quinapyramine was previously used in cattle, the compound has recently been reintroduced in some African countries for use as a trypanocide in this livestock species. In light of the aforementioned concerns, the study described here was undertaken, under controlled laboratory conditions, to determine the ease with which resistance to quinapyramine could be induced in a clone of *T. congolense* and the cross-resistance phenotype of such a quinapyramine-resistant population.

The study used a doubly cloned derivative of a *T. congolense* Savannah-type isolate collected from a lion in the Serengeti, Tanzania: *T. congolense* IL 1180 (9, 15, 19). In mice, the isometamidium chloride and diminazene aceturate 50% curative doses (CD_{50}s) are 0.018 and 2.3 mg/kg of body weight (b.w.), respectively (25).

In an attempt to induce resistance to quinapyramine in IL 1180, 10 nonirradiated outbred Swiss white mice (group 1) were infected intraperitoneally with a stabilate of IL 1180 which had been suspended in phosphate-buffered saline–glucose, pH 8.0. Subsequently, the mice were monitored three times a week by examining wet-blood films of tail blood at ×250 magnification. When the level of parasitemia in one or more animals had attained 50 to 100 trypanosomes per field, all the mice were treated with quinapyramine sulfate (Trypacide sulphate; May and Baker Ltd., Dagenham, United Kingdom) at a dose of 0.005 mg/kg of b.w.; within 2 days the mice became aparasitemic. When trypanosomes reappeared and attained a level of parasitemia similar to that immediately prior to treatment, the same 10 mice were retreated with quinapyramine sulfate at double the dose. When the parasites subsequently reappeared following a period of aparasitemia, the animals were anesthetized and exsanguinated into sodium citrate (final concentration, 3% [wt/vol]). Blood from each animal was then pooled, and a volume of approximately 0.5 ml was frozen in liquid nitrogen after 10% (vol/vol) glycerol was added (6). The remainder of the trypanosomes were then used to infect a second group of 10 naive mice (group 2). A further 10 groups of mice were infected and treated in the same manner: every group was treated on two occasions with quinapyramine

* Corresponding author.

† International Laboratory for Research on Animal Diseases publication 1129.

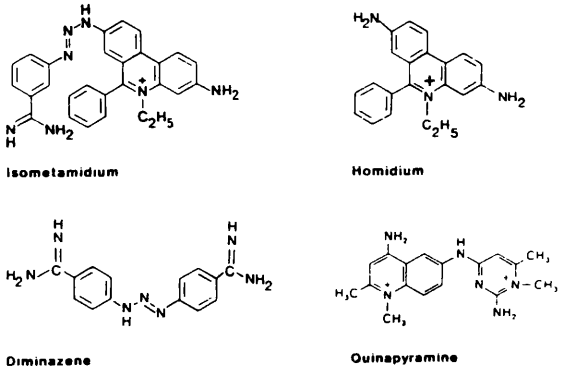


FIG. 1. Chemical structures of different trypanocides.

sulfate; the first dose was the same as that used for the second treatment of the previous group of mice; the second dose was greater than that used for the first treatment. Trypanosomes that reappeared in mice following the second treatment were used to infect the next group of naive mice. At each stage that a trypanosome population was passaged, a stabilate was prepared in liquid nitrogen. The final population that arose in group 12 was termed Stabilate 12.

Stabilate 12 was inoculated intraperitoneally into sublethally irradiated (650 rad) mice. Seven days later, when the animals were parasitemic, five clones were derived as described by Barry and Gathuo (2).

In order to characterize the drug sensitivities of IL 1180 and various derivatives in mice, stabilates of each population were expanded in sublethally irradiated (650 rad) mice and characterized as described by Peregrine et al. (25). Standard logit analyses were used to express the drug susceptibility of each population to each of the different trypanocides as a CD_{50}. The quinapyramine sulfate CD_{50} for the parental clone, IL 1180, was shown to be 0.23 mg/kg of b.w. (Table 1). In mice, the quinapyramine sulfate CD_{50} for IL 1180/Stabilate 12 was greater than 9.6 mg/kg of b.w.; a CD_{50} could not be determined since it was in excess of the maximum tolerated dose. To determine whether selection for resistance to quinapyramine resulted in cross-resistance to isometamidium (Samorin; May and Baker Ltd.), homidium (Novidium; May and Baker Ltd.), and diminazene (Berenil; Hoechst, Frankfurt, Germany), IL 1180 and IL 1180/Stabilate 12 were characterized for their susceptibilities to these three trypanocides in mice. The CD_{50}s for IL 1180 were 0.018, 0.37, and 2.3 mg/kg of b.w., respectively. In contrast, the same values for IL 1180/Stabilate 12 were 0.10, 10.35, and 12.74 mg/kg of b.w., respectively (Table 1). All five clones that were derived from IL 1180/Stabilate 12 had quinapyramine sulfate CD_{50}s in excess of 12.0 mg/kg of b.w. (Table 1; data for clones 2 to 5 not given), the maximum tolerated dose in these studies. Since clone 1 appeared to express a higher level of resistance than the other clones, on the basis of the rate of relapse following treatment, this clone was also characterized in mice for its susceptibility to isometamidium chloride, homidium chloride, and diminazene aceturate; CD_{50}s were 0.12, 6.83, and 8.34 mg/kg of b.w., respectively (Table 1). The clone therefore expressed a lower level of resistance to homidium and diminazene than did the population from which it was derived. However, the level of resistance to isometamidium was not significantly different.

In order to determine whether IL 1180/Stabilate 12/clone 1 expressed resistance to quinapyramine in a definitive host, the drug susceptibility of the population was also characterized in adult East African Maasai X Galla goats, maintained as described by Whitelaw et al. (30). Subsequent to expansion of IL 1180/Stabilate 12/clone 1 in sublethally irradiated (650 rad) mice, aliquots of 1.0×10^6 trypanosomes were inoculated intravenously into each of 22 goats (see below). The animals were then divided into four groups of five animals each (groups A1, B1, C1, and D1) and one group of two animals (group E1). Following inoculation of trypanosomes, jugular blood was collected from each animal three times a week and centrifuged, and the buffy coat was examined microscopically for the presence of trypanosomes (17). On day 3 following infection, when all animals were parasitemic, animals in groups A1, B1, C1, and D1 were treated with 3.0 mg of quinapyramine sulfate per kg of b.w., 0.25 mg of isometamidium chloride per kg of b.w., 1.0 mg of homidium chloride per kg of b.w., and 3.5 mg of diminazene aceturate per kg of b.w., respectively (the minimum recommended field doses for each compound). Animals in group E1 served as nontreated controls. In a second part to the experiment a further 10 goats were infected with IL 1180 in a manner identical to that used for animals in groups A1 to E1. After infection, the animals were divided into five groups of two animals each (groups A2, B2, C2, D2, and E2) and treated with the four aforementioned trypanocides as described above. Animals in group E2 served as nontreated controls. Following treatment, jugular blood was collected from all experimental animals three times a week for 150 days and examined for the presence of trypanosomes (17).

This experiment showed that IL 1180 was fully susceptible to each of the four drug doses in goats, with no trypanosomes detected in the treated infected goats. In contrast, infections with IL 1180/Stabilate 12/clone 1 relapsed in five of five goats after treatment with quinapyramine sulfate, in two of five goats after treatment with homidium chloride, and in three of five goats after treatment with diminazene aceturate. However, none of the five goats relapsed after treatment with isometamidium chloride. Thus, while IL 1180 was fully susceptible to the minimum recommended dose for each of the four trypanocides, IL 1180/Stabilate 12/clone 1 expressed resistance to quinapyramine sulfate, homidium chloride, and diminazene aceturate.

TABLE 1. Susceptibility of *T. congolense* IL 1180 populations to trypanocides in mice

Trypanosome	CD_{50} [mg/kg (95% confidence interval)] of:			
	Quinapyramine sulfate	Isometamidium chloride	Homidium chloride	Diminazene aceturate
IL 1180	0.23 (0.15–0.34)	0.018 (0.013–0.025)	0.37 (0.3–0.4)	2.3 (2.0–2.6)
IL 1180/Stabilate 12	>9.6	0.10 (0.09–0.1)	10.35 (9.7–10.9)	12.74 (11.7–13.7)
IL 1180/Stabilate 12/clone 1	>12	0.12 (0.11–0.13)	6.83 (6.5–7.1)	8.34 (7.6–9.0)

TABLE 2. Infectivity of *T. congolense* IL 1180 populations for *G. m. centralis*

Animal no.	*T. congolense* clone	No. of flies dissected[a]	Infection rate (%) in:		
			Midgut	Labrum	Hypopharynx
B 189	IL 1180	157	21.02	17.83	17.83
CJ 153	IL 1180/Stabilate 12/clone 1	169	56.80	41.42	39.64
CJ 154	IL 1180/Stabilate 12/clone 3	176	28.41	24.43	23.86

[a] The flies, all male, were first fed on animals 10 days after trypanosomes were first detected. Dissections were carried out 26 and 27 days later.

In a final experiment to determine whether induction of resistance to quinapyramine altered the ability of IL 1180 to undergo cyclical development, the tsetse transmissibility of IL 1180/Stabilate 12/clones 1 and 3 was ascertained in teneral male *Glossina morsitans centralis*, obtained from the International Laboratory for Research on Animal Diseases-bred colony, by standard procedures. Flies that extruded metacyclic trypanosomes in their salivary probes (4) were then fed on mice on a daily basis to determine the transmission rate of the trypanosome population. All the flies were then dissected to determine the infection rates. Both clones 1 and 3 were shown to undergo cyclical development, producing hypopharyngeal infection rates of 39.64 and 23.86%, respectively (Table 2). The transmission rates of the same clones were 100 and 90%, respectively. Thus, induction of resistance did not appear to affect the populations' ability to undergo full cyclical development in tsetse flies.

This study has therefore shown that the level of resistance of IL 1180 to quinapyramine was increased at least 40-fold by repeated subcurative treatment of infected mice over a period of 208 days. Furthermore, experiments to characterize the cross-resistance phenotype of the quinapyramine-resistant derivative in mice and goats indicated that induction of resistance to quinapyramine was associated with significant levels of cross-resistance to homidium, diminazene, and isometamidium.

While induction of resistance to quinapyramine in the clone of *T. congolense* described here was not difficult to achieve, attempts to induce resistance to quinapyramine in trypanosomes by other workers have met with various degrees of success. Using a similar procedure, Fiennes (8) failed to induce resistance in a stock of *T. congolense*. In contrast, a similar protocol successfully induced resistance to quinapyramine in *Trypanosoma equiperdum* (11, 24), *T. congolense* (12), and *Trypanosoma evansi* (10).

The cross-resistance phenotypes of trypanosome populations selected for resistance to quinapyramine, reported by different authors, have varied. A quinapyramine-resistant *T. equiperdum* population produced by Ormerod (24) expressed a high level of cross-resistance to dimidium and stilbamidine. Similarly, a quinapyramine-resistant stock of *T. congolense* generated by Hawking (12) was shown to be cross-resistant to diminazene. However, while a similar population of *T. evansi* was cross resistant to metamidium (a mixture of isomers, one of which was isometamidium [35]), the same population exhibited no cross-resistance to either stilbamidine or the organic arsenical tryparsamide (10). Finally, a population of *T. equiperdum* in which resistance to quinapyramine was induced appeared to be more susceptible to diminazene than the parental population (11). As a result of these data and other data concerning the drug resistance phenotypes of trypanosome populations isolated from field situations where quinapyramine was used, Whiteside (32) and Hawking (12) concluded that induction of resistance to quinapyramine leads to cross-resistance to phenanthridine compounds but does not always result in cross-resistance to diminazene, suggesting the induction of different mechanisms of resistance in different trypanosome populations. In the experiments described here, induction of resistance to quinapyramine in a cloned population of *T. congolense* resulted in significant cross-resistance to isometamidium, homidium, and diminazene. Since the salts of isometamidium, homidium, and diminazene are the only trypanocides currently marketed for use in cattle and the development of new trypanocides for use in cattle is now prohibitively expensive (33), the body of data generated to date would therefore indicate that use of quinapyramine in cattle as a trypanocide is contraindicated.

While there is a limited amount of information concerning the mechanism of action of quinapyramine in trypanosomes (1, 20, 21, 23, 29), we are unaware of data pertaining to the molecular basis for resistance to the compound. The trypanosome populations generated in this study represent ideal material for such work and are therefore currently being used in studies to determine the genetic and molecular bases of resistance to quinapyramine in *T. congolense*.

Rhône Poulenc is thanked for providing the isometamidium chloride, homidium chloride, and quinapyramine sulfate used in this work. The staff of the International Laboratory for Research on Animal Diseases tsetse vector laboratory are thanked for their contribution to this study. Maria Mulindi is thanked for typing the manuscript.

REFERENCES

1. **Bacchi, C. J., H. C. Nathan, S. H. Hutner, D. S. Duch, and C. A. Nichol.** 1979. Negation of trypanocidal drug cures by polyamines, p. 1119–1121. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease. American Society for Microbiology, Washington, D.C.
2. **Barry, J. D., and H. Gathuo.** 1984. Antigenic variation in *Trypanosoma vivax*: isolation of a serodeme. Parasitology **89:** 49–58.
3. **Brady, J.** 1991. Seeing flies from space. Nature (London) **351:**695.
4. **Burtt, E.** 1946. Salivation by *Glossina morsitans* on to glass slides: a technique for isolating infected flies. Ann. Trop. Med. Parasitol. **40:**141–144.
5. **Curd, F. H. S., and D. G. Davey.** 1950. "Antrycide"—a new trypanocidal drug. Br. J. Pharmacol. **5:**25–32.
6. **Dar, F. K., G. S. Ligthart, and A. J. Wilson.** 1972. Cryopreservation of pathogenic African trypanosomes *in situ*: metacyclic and bloodstream forms. J. Protozool. **19:**494–497.
7. **Davey, D. G.** 1957. The chemotherapy of animal trypanosomiasis with particular reference to the trypanosomal disease of domestic animals in Africa. Vet. Rev. Annot. **3:**15–36.
8. **Fiennes, R. N. T. W.** 1953. The therapeutic and prophylactic properties of antrycide in trypanosomiasis of cattle. Br. Vet. J. **109:**280–295.
9. **Geigy, R., and M. Kauffmann.** 1973. Sleeping sickness survey in the Serengeti area (Tanzania), 1971. I. Examination of large mammals for trypanosomes. Acta Trop. **3:**12–23.
10. **Gill, B. S.** 1971. Resistance of *Trypanosoma evansi* to quinapy-

ramine, suramin, stilbamidine and tryparsamide and analysis of cross-resistance. Trans. R. Soc. Trop. Med. Hyg. **65**:352–357.
11. **Hawking, F.** 1958. The action of Berenil on trypanosomes including strains resistant to antrycide and stilbamidine. J. Comp. Pathol. **68**:295–297.
12. **Hawking, F.** 1963. Drug-resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, Berenil and other compounds in mice. Ann. Trop. Med. Parasitol. **57**:262–282.
13. **Holmes, P. H., and J. M. Scott.** 1982. Chemotherapy against animal trypanosomiasis, p. 59–69. *In* J. R. Baker (ed.), Perspectives in trypanosomiasis research. Research Studies Press, New York.
14. **Leach, T. M., and C. J. Roberts.** 1981. Present status of chemotherapy and chemoprophylaxis of animal trypanosomiasis in the eastern hemisphere. Pharmacol. Ther. **13**:91–147.
15. **Majiwa, P. A. O., R. A. Masake, V. M. Nantulya, R. Hamers, and G. Matthyssens.** 1985. *Trypanosoma (Nannomonas) congolense*: identification of two karyotypic groups. EMBO J. **4**:3307–3313.
16. **Morrison, W. I., M. Murray, and W. I. M. McIntyre.** 1981. Bovine trypanosomiasis, p. 469–497. *In* M. Ristic and I. McIntyre (ed.), Diseases of cattle in the tropics. Martinus Nijhoff Publishers, The Hague, The Netherlands.
17. **Murray, M., P. K. Murray, and W. I. M. McIntyre.** 1977. Improved parasitological technique for the diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. **71**:325–326.
18. **Mwambu, P. M., and J. S. P. Mayende.** 1971. Occurrence of berenil resistant strains of *T. vivax*. Trans. R. Soc. Trop. Med. Hyg. **65**:254–255.
19. **Nantulya, V. M., A. J. Musoke, F. R. Rurangirwa, and S. K. Moloo.** 1984. Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe-passaged infections. Infect. Immun. **43**:735–738.
20. **Newton, B. A.** 1962. Inactivation of ribosomes by antrycide. Biochem. J. **84**:109.
21. **Newton, B. A.** 1963. Interaction of antrycide with isolated ribosomes. Biochem. J. **89**:93–94.
22. **Newton, B. A.** 1964. Mechanisms of action of phenanthridines and aminoquinaldine trypanocides. Adv. Chemother. **1**:35–83.
23. **Newton, B. A.** 1966. Effects of antrycide on nucleic acid synthesis and function. Symp. Soc. Gen. Microbiol. **16**:213–234.
24. **Ormerod, W. E.** 1952. A study of resistance to antrycide in a strain of *Trypanosoma equiperdum*. Br. J. Pharmacol. **7**:674–684.
25. **Peregrine, A. S., G. Knowles, A. I. Ibitayo, J. R. Scott, S. K. Moloo, and N. B. Murphy.** 1991. Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. Parasitology **102**:93–100.
26. **Ruchel, W.-M.** 1975. Chemoprophylaxis of bovine trypanosomiasis. Thesis, p. 248. University of Göttingen, Göttingen, Germany.
27. **Schillinger, D., and D. Rottcher.** 1986. Treatment of camels for *Trypanosoma brucei evansi* infection (surra). World Anim. Rev. **60**:26–32.
28. **Unsworth, K.** 1954. Observations on antrycide-fast strains of *Trypanosoma congolense* and *T. vivax*. Ann. Trop. Med. Parasitol. **48**:178–182.
29. **Waring, M. J.** 1965. The effects of antimicrobial agents on ribonucleic acid polymerase. Mol. Pharmacol. **1**:1–13.
30. **Whitelaw, D. D., I. R. Bell, P. H. Holmes, S. K. Moloo, H. Hirumi, G. M. Urquhart, and M. Murray.** 1986. Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. Vet. Rec. **118**:722–726.
31. **Whiteside, E. F.** 1960. Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis, p. 141–153. *In* International Scientific Council for Trypanosomiasis Research, 8th meeting, Jos, Nigeria. Publication 62. Commission for Technical Co-operation South of the Sahara, London.
32. **Whiteside, E. F.** 1962. Interactions between drugs, trypanosomes and cattle in the field, p. 116–141. *In* L. G. Goodwin and R. M. Nimmo-Smith (ed.), Drugs, parasites and hosts. J. and A. Churchill, Londo[illegible]
33. **Williamson, J.** 19[illegible]. Review of chemotherapeutic and chemoprophylactic agents, p. 116–141. *In* H. W. Mulligan (ed.), The African trypanosomiases. Allen and Unwin Ltd., London.
34. **Wilson, S. G.** 1949. Drug-resistance shown by trypanosomes following "Antrycide" treatment. Nature (London) **163**:873–874.
35. **Wragg, W. R., K. Washbourn, K. N. Brown, and J. Hill.** 1958. Metamidium: a new trypanocidal drug. Nature (London) **182**:1005–1006.

Section 2.6

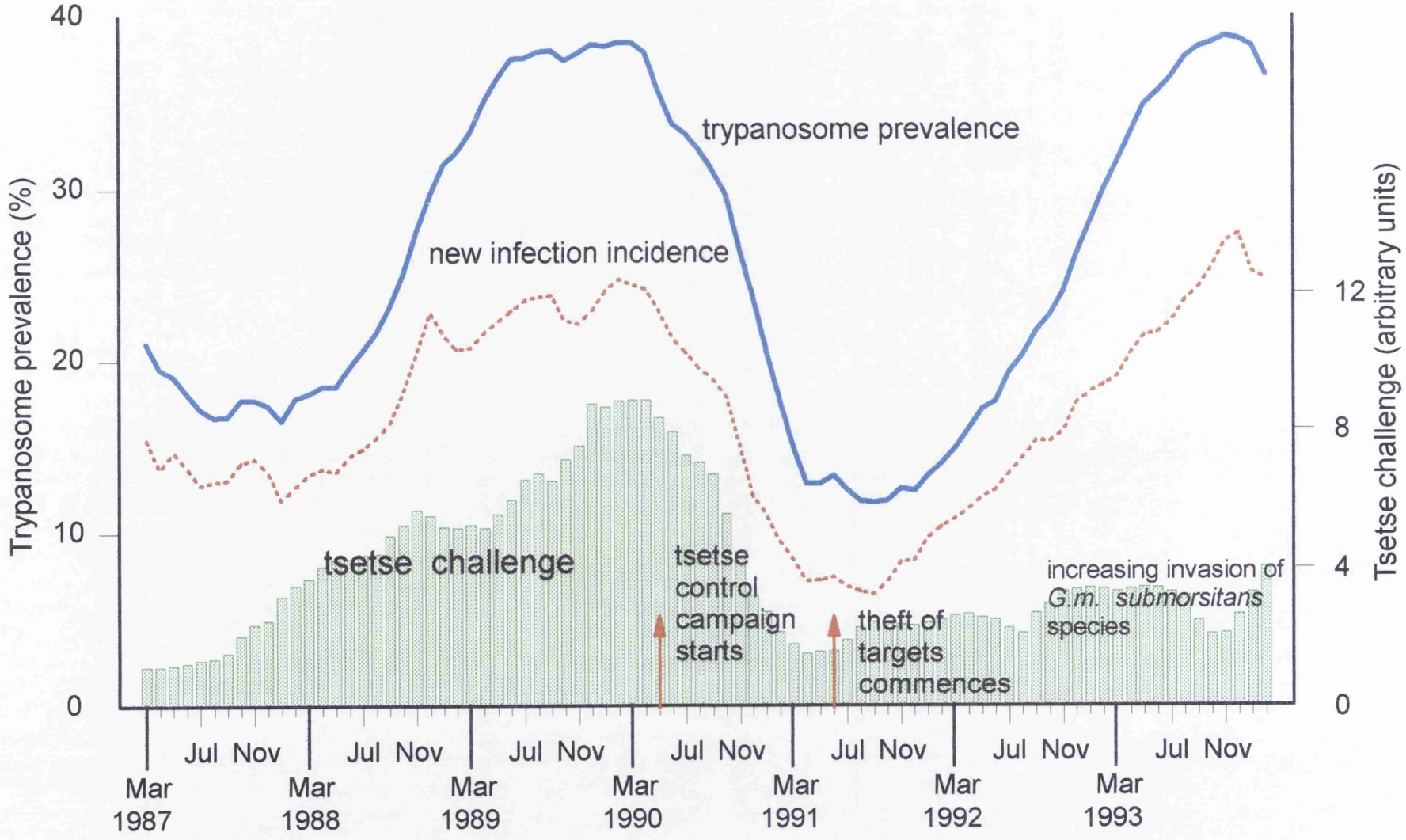
- Discussion -

The papers given in this Chapter describe a number of studies that were carried out to characterise the epidemiology of trypanosomiasis in the Ghibe valley, southwest Ethiopia. From January 1986 to April 1990 a total of approximately 840 cattle in the valley (40-50% of the total cattle population) were monitored on a monthly basis for the presence of trypanosome infections (Section 2.1). Throughout this period the only anti-trypanosomal compound officially given to the cattle was diminazene aceturate; animals were only treated if they were detected parasitaemic and had a packed red cell volume (PCV) less than 26%. Despite this treatment regimen, however, the overall prevalence of infections (primarily *T. congolense*) increased by over two-fold during the experimental period, to approximately 38% by the end of 1989 (see Figure 1). Since there was also an increase in the tsetse challenge over this period (Figure 1; Leak et al., 1993), it was not clear whether the increase in prevalence of infections was just due to an increase in tsetse challenge or was associated with both an increase in the challenge and the presence of drug-resistant trypanosome infections. Section 2.1 describes a mathematical model that was developed for analysing longitudinal parasitological data from animals at Ghibe, that allows one to determine the monthly incidence of infections. Since 87% of all animals detected parasitaemic during the study had a PCV less than 26%, and were therefore treated the first time they were detected parasitaemic (Section 2.1), the difference between the monthly incidence and monthly prevalence of infections was used as an estimate of the prevalence of diminazene-resistant infections. The data indicated that *T. congolense* populations existed at Ghibe which expressed significant levels of resistance to diminazene, and that the prevalence of such infections increased from 6% to 14% between 1986 and 1990: demonstrated by the divergence in the lines representing "trypanosome prevalence" and "new infection incidence" in Figure 1. However, since approximately 70% of the tsetse population at Ghibe were feeding on cattle between 1986 and 1990 (Leak et al., 1993), the increase in prevalence of diminazene-resistant infections in the cattle was most likely associated with the increase in tsetse challenge.

In order to confirm that cattle at Ghibe were infected with diminazene-resistant trypanosomes, Section 2.2 describes a series of laboratory studies that were carried

Figure 1

Rolling 12-month means of *T. congolense* prevalence and new infection incidence in cattle, and tsetse challenge, at Ghibe (March 1987 - February 1994)


Trypanosome prevalence (%)
40
30
20
10
0
trypanosome prevalence
new infection incidence
tsetse challenge
tsetse control campaign starts
theft of targets commences
increasing invasion of G.m. submorsitans species
12
8
4
0
Tsetse challenge (arbitrary units)
Mar 1987
Jul Nov
Mar 1988
Jul Nov
Mar 1989
Jul Nov
Mar 1990
Jul Nov
Mar 1991
Jul Nov
Mar 1992
Jul Nov
Mar 1993
Jul Nov

out to characterise 12 trypanosome isolates collected at random from cattle at the site in 1989. All 12 isolates, which were all savannah-type *T. congolense*, were inoculated into individual naïve cattle and shown to be resistant to diminazene aceturate at a dose of 7.0 mg/kg b.w. Furthermore, 11 of the isolates were also resistant to isometamidium chloride at a dose of 0.5 mg/kg b.w. and homidium chloride at a dose of 1.0 mg/kg b.w. Thus, the prevalence of multiple-drug resistant infections amongst infected cattle at Ghibe appeared to be in excess of 91%, and the prevalence of diminazene-resistant infections in infected cattle appeared to be approximately 100%. This latter figure is considerably greater than the estimate of 6-14% that was made from field data (Section 2.1). However, it should be noted that the laboratory studies used cattle that had never been previously exposed to trypanosome infections. In contrast, in light of the medium-to-high trypanosome challenge to which cattle were exposed at Ghibe (Leak et al., 1993), and the associated multiple infections that cattle would have experienced during the course of their life, it is most probable that expression of acquired resistance by the cattle to trypanosome infections (Cunningham, 1966; Wellde et al., 1981; Nantulya et al., 1984; Akol and Murray, 1985) resulted in an apparent underestimate of the true prevalence of diminazene-resistant infections.

Characterisation of clones derived from one of the isolates collected from cattle at Ghibe in 1989 indicated that the multiple-drug resistance was expressed by cloned populations. Furthermore, molecular karyotype and isoenzyme analyses indicated that all 12 isolates were generally distinct from one another. This therefore suggested that instead of the drug resistance developing in one, or a very limited number, of trypanosome populations at Ghibe, as observed with *Trypanosoma evansi* in northern Kenya (Waitumbi et al., 1994), the resistance developed either simultaneously or independently in a relatively large number of different trypanosome populations. Heterogeneity in molecular karyotypes and zymodemes also suggests that unless the resistance developed very recently, there should be little variation in growth characteristics of the different trypanosome populations (Doyle et al., 1980) in either definitive hosts or vectors at the site.

Because of the high prevalence of multiple-drug resistant infections in cattle at Ghibe, and because this phenotype appeared to be expressed by single rather than mixed infections, it was concluded that anti-trypanosomal compounds by themselves would not adequately control trypanosome infections at the site. However, in light of the wide variation in pathogenicity of trypanosome infections for domestic livestock (Hudson, 1944; Maxie et al., 1979; Nantulya et al., 1984), control of the infections could only be justified in financial terms if they were a significant constraint to livestock productivity. Thus, since trypanosomiasis was shown to have a significant detrimental effect on the productivity of both young and adult cattle at Ghibe (Section 2.1; Rowlands et al., 1994a, b), and excessive use of anti-trypanosomal compounds can result in problems with drug toxicity (Dolan et al., 1992; Stevenson et al., 1993), a tsetse control programme was initiated in April 1990 (see Section 2.3) to see if it would have any impact on the prevalence of trypanosome infections in the cattle. Throughout this work, treatment of cattle with diminazene aceturate continued exactly as before, i.e., animals were only treated if they were detected parasitaemic and had a PCV less than 26%. Figure 1 shows the impact of the tsetse-control method (odour-baited deltamethrin-impregnated targets placed across the entire valley at an average density of 4/km^2) on the tsetse challenge to cattle, and on both the incidence and prevalence of trypanosome infections in cattle. In summary, as a result of setting up tsetse targets across the site, the relative density of the main vector, *Glossina pallidipes*, fell from a mean of 1.9 flies/trap/day in the 12 months prior to introduction to 0.09 flies/trap/day in the first 3 months of 1991. Furthermore, the apparent prevalence of *T. congolense* infections in cattle fell from approximately 30% before the tsetse control programme began to a mean of approximately 5% in the first 3 months of 1991. Lastly, on the basis of the data-analysis method described in Section 2.1, the prevalence of diminazene-resistant infections appeared to decrease by approximately 75% in the first 12 months following initiation of the tsetse-control programme (Section 2.3). Thus, despite a high prevalence of multiple-drug resistant infections in cattle at Ghibe at the time the tsetse control programme was initiated, a decrease in the tsetse population was associated with a large reduction in the apparent prevalence of trypanosome infections in cattle. Similar results have been obtained in Burkina Faso (Bauer et al.,

1995). Unfortunately, as indicated in Figure 1, thefts of targets compromised the sustainability of this control method.

In order to determine whether the reduction in apparent prevalence of trypanosome infections in cattle was associated with an increase in the drug sensitivity of trypanosome populations, Section 2.4 describes the characterisation of 10 trypanosome isolates that were collected at random from cattle at Ghibe in February 1993. In a similar manner to the isolates collected 4 years earlier, all 10 isolates were savannah-type *T. congolense*, and all expressed high levels of resistance to diminazene, homidium and isometamidium in cattle. Thus, the drug-resistance phenotype of trypanosome populations at Ghibe did not appear to have changed over a 4-year period. Such stability of drug resistance is consistent with a number of laboratory studies (Gray and Roberts, 1971; Kaminsky and Zweygarth, 1989), and suggests that if there are any drug-sensitive trypanosome populations at Ghibe they do not have greater growth rates than drug-resistant populations in definitive hosts or vectors.

Earlier workers have indicated that resistance to diminazene is generally difficult to induce in trypanosomes (Fussgänger and Bauer, 1960; Bauer, 1962; Whiteside, 1963). However, in work with *Trypanosoma evansi* in mice, Osman et al. (1992) demonstrated that resistance to the compound will develop relatively readily if animals are immunosuppressed. Observations from a number of field situations in Africa in the 1950s and 1960s lead Whiteside (1960) to conclude that when resistance to diminazene develops as a result of using the compound, it is usually not associated with cross-resistance to either isometamidium or homidium. Thus, since diminazene aceturate was supposed to have been the only anti-trypanosomal compound used in cattle at Ghibe since the early 1980s, it was unclear how the very high levels and prevalence of multiple-drug resistance arose. Results from the aforementioned field studies of Whiteside (1960) caused this scientist to conclude that resistance to diminazene can develop as a result of cross-resistance associated with development of resistance to quinapyramine: a trypanocide currently recommended for use only in camels and horses, but which was often used as a

chemoprophylactic agent in cattle in the 1950s and 1960s (Fiennes, 1953; Rüchel, 1975). Since field studies are inherently uncontrolled, Section 2.5 describes work that was carried out to clarify this matter. In this work, resistance to quinapyramine was induced in a cloned, quinapyramine-sensitive, population of *T. congolense* under controlled laboratory conditions. When characterised in mice, the quinapyramine-resistant derivative was shown to be at least 40-times more resistant to quinapyramine than the parental population. The resistant derivative was also 8-fold more resistant to isometamidium, 28-fold more resistant to homidium, and 5.5-fold more resistant to diminazene than the parental population. Finally, unlike the parental population, the quinapyramine-resistant derivative was resistant to recommended therapeutic doses of homidium and diminazene in goats. Thus, induction of resistance to quinapyramine was associated with significant levels of cross-resistance to diminazene, homidium and isometamidium.

In conclusion, since resistance to quinapyramine is relatively easy to induce and is associated with cross-resistance to diminazene, isometamidium and homidium, in contrast to other anti-trypanosomal compounds (Section 2.5; Whiteside, 1960; Osman et al., 1992), the multiple-drug resistance observed at Ghibe appears most likely to be associated with use of quinapyramine at some time in the past. This could have occurred either within the valley or at some point within the land mass infested by the tsetse-fly population that is contiguous with that in the Ghibe valley. However, in light of the changing distribution of tsetse populations within Ethiopia over the past few decades (Slingenbergh, 1992; Leak and Mulatu, 1993), it is possible that the resistance originated elsewhere. In this context, it should be noted that Scott and Pegram (1974) described drug-resistant populations of *T. congolense* in Didessa and Angar valleys in Wollega province, Ethiopia. Furthermore, Dagnatchew et al. (1983) described the occurrence of drug-resistant *T. congolense* in Debeka and Miridicha, Sidamo province, and in Ghimbi and Ekoloko, Wollega province.

References:

Akol, G.W.O. and Murray, M. (1985) Induction of protective immunity in cattle by tsetse-transmitted cloned isolates of *Trypanosoma congolense*. *Annals of Tropical Medicine and Parasitology* 6, 617-627.

Bauer, B., Amsler-Delafosse, S., Clausen, P.-H., Kabore, I. and Petrich-Bauer, J. (1995) Successful application of deltamethrin pour on to cattle in a campaign against tsetse flies (Glossina spp.) in the pastoral zone of Samorogouan, Burkina Faso. *Tropical Medicine and Parasitology* 46, 183-189.

Bauer, F. (1962) The development of drug-resistance to Berenil in *Trypanosoma congolense*. *Veterinary Record* 74, 265-266.

Cunningham, M.P. (1966) Immunity in bovine trypanosomiasis. *East African Medical Journal* 43, 394-397.

Dagnatchew, Z., Lemecha, H. and Mulatu, W. (1983) Trypanocidal drug resistance problem in certain parts of Ethiopia. Trypanosomiasis Control Services, Ministry of Agriculture, Annual Report 1982-1983, pp. 52-57.

Dolan, R.B., Stevenson, P.G.W., Alushula, H. and Okech, G. (1992) Failure of chemoprophylaxis against bovine trypanosomiasis on Galana Ranch in Kenya. *Acta Tropica* 51, 113-121.

Doyle, J.J., de Gee, A.L.W. and Hirumi, H. (1980) Variable antigen-associated differences in the growth characteristics of *Trypanosoma brucei* and *Trypanosoma vivax*. *Insect Science and its Application* 1, 65-68.

Fiennes, R.N.T.W. (1953) The therapeutic and prophylactic properties of antrycide in trypanosomiasis of cattle. *British Veterinary Journal* 109, 280-295.

Fussgänger, R. and Bauer, F. (1960) Investigations on Berenil resistance of trypanosomes. *Veterinary Record* 72, 1118-1121.

Gray, A.R. and Roberts, C.J. (1971) The cyclical transmission of strains of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* 63, 67-89.

Hudson, J.R. (1944) Acute and subacute trypanosomiasis in cattle caused by *T. vivax*. *Journal of Comparative Pathology* 54, 108-119.

Kaminsky, R. and Zweygarth, E. (1989) Effect of *in vitro* cultivation on the stability of resistance of *Trypanosoma brucei brucei* to diminazene, isometamidium, quinapyramine, and Mel B. *Journal of Parasitology* 75, 42-45.

Leak, S.G.A. and Mulatu, W. (1993) Advance of *Glossina morsitans submorsitans* and *G. pallidipes* along the Ghibe-river system in southwest Ethiopia. *Acta Tropica* 55, 91-95.

Leak, S.G.A., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Peregrine, A.S., Rowlands, G.J. and Trail, J.C.M. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 1. Tsetse challenge and its relationship to trypanosome prevalence in cattle. *Acta Tropica* 53, 121-134.

Maxie, M.G., Losos, G.J. and Tabel, H. (1979) Experimental bovine trypanosomiasis (*Trypanosoma vivax* and *T. congolense*) I. Symptomatology and clinical pathology. *Tropenmedizin und Parasitologie* 30, 274-282.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R. and Moloo, S.K. (1984) Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe-passaged infections. *Infection and Immunity* 43, 735-738.

Osman, A.S., Jennings, F.W. and Holmes, P.H. (1992) The rapid development of drug-resistance by *Trypanosoma evansi* in immunosuppressed mice. *Acta Tropica* 50, 249-257.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M. (1994a) Effects of trypanosomiasis on growth and mortality of young East African Zebu cattle exposed to drug-resistant trypanosomes. *Preventive Veterinary Medicine* 21, 87-101.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M. (1994b) Effects of trypanosomiasis on reproduction of East African Zebu cows exposed to drug-resistant trypanosomes. *Preventive Veterinary Medicine* 21, 237-249.

Rüchel, W.-M. (1975) Chemoprophylaxis of bovine trypanosomiasis. Thesis, University of Göttingen, Göttingen, Germany, pp. 248.

Scott, J.M. and Pegram, R.G. (1974) A high incidence of *Trypanosoma congolense* strains resistant to homidium bromide in Ethiopia. *Tropical Animal Health and Production* 6, 215-221.

Slingenbergh, J. (1992) Tsetse control and agricultural development in Ethiopia. *World Animal Review* 70/71, 30-36.

Stevenson, P., Munga, L. and Dolan, R.B. (1993) The detrimental effects of frequent treatment of cattle with trypanocidal drugs. In *Proceedings of the International Scientific Council for Trypanosomiasis Research and Control, 22nd Meeting, Kampala, Uganda.* Colour Print Limited, Nairobi, OAU/STRC Publication No. 117, pp. 130-135.

Waitumbi, J.N., Murphy, N.B. and Peregrine, A.S. (1994) Genotype and drug-resistance phenotype of *Trypanosoma evansi* isolated from camels in northern Kenya. *Annals of Tropical Medicine and Parasitology* 88, 677-683.

Wellde, B.T., Hockmeyer, W.T., Kovatch, R.M., Bhogal, M.S. and Diggs, C.L. (1981) *Trypanosoma congolense*: natural and acquired resistance in the bovine. *Experimental Parasitology* 52, 219-232.

Whiteside, E.F. (1960) Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In *Proceedings of the International Scientific Committee for Trypanosomiasis Research, 8th meeting, Jos, Nigeria.* Commission for Technical Co-operation South of the Sahara, London, Publication No. 62, pp. 141-154.

Whiteside, E.F. (1963) A strain of *Trypanosoma congolense* directly resistant to Berenil. *Journal of Comparative Pathology* 73, 167-175.

Chapter 3

Role of invasion of the central nervous system in expression of resistance to diminazene

Section 3.0

- Introduction -

Probably the most significant component of the pathogenesis of human trypanosomiasis is invasion of the central nervous system (CNS) by either *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense*. Once the parasites have accessed the CNS in this "late" or "second" stage of the disease, the bioavailability of most anti-trypanosomal compounds appears to be greatly compromised due to their poor ability to cross the blood-brain barrier (Apted, 1980; Van Nieuwenhove et al., 1985). As a result, the chemotherapeutic repertoire for this stage of the disease is greatly reduced compared to the "early" or "first" stage of the disease (Van Nieuwenhove et al., 1985). Furthermore, because of the poor bioavailability, reappearance of trypanosomes in the bloodstream following treatment may be associated with recrudescence of parasites from within the CNS rather than intrinsic or acquired drug resistance (Hayes and Wolf, 1990; Hatz and Brun, 1992). Thus, in this situation, the trypanosome populations that reappear in the bloodstream have the same level of drug sensitivity as the trypanosome population present in the bloodstream prior to treatment (Brun et al., 1989).

In a similar manner to the pathogenesis of human trypanosomiasis, populations of *T. brucei brucei* and *T. vivax* have been shown to invade the CNS of goats (Whitelaw et al., 1985, 1988; Moulton, 1986), and *Trypanosoma simiae* has been shown to invade the CNS of pigs (Zweygarth and Röttcher, 1987). Thus, as described above, the occurrence of relapse infections with these trypanosome species following treatment may not necessarily be associated with decreased drug susceptibility of trypanosomes (Jennings et al., 1979; Whitelaw et al., 1985), but, instead, to invasion of the bloodstream by trypanosomes residing within the CNS after levels of anti-trypanosomal compounds have declined to sub-therapeutic concentrations (Morrison et al., 1983; Zweygarth and Röttcher, 1987). Masake et al. (1984) have demonstrated the presence of *T. congolense* within the CNS of cattle when this trypanosome species occurs as a mixed infection with *T. b. brucei.* Furthermore, Kalu (1985) has concluded that the brain is a source of relapse infections due to *T. congolense.* However, at the present time, the demonstration of CNS invasion in domestic livestock by *T. congolense* in the absence of other trypanosome species is not unequivocal.

Sections 2.1, 2.2, 2.3 and 2.4 describe a series of studies that were carried out on aspects of the epidemiology of trypanosomiasis in the Ghibe valley, Ethiopia, in the late 1980s and early 1990s. During this period, *T. congolense* was the most prevalent trypanosome species occurring in cattle at the site. Furthermore, a high proportion of infections with this species were resistant to treatment with diminazene aceturate. In light of the information given above, it is possible that the "resistance" was associated, in total or in part, with invasion of the CNS.

In order to determine whether "diminazene-resistant" *T. congolense* are able to resist treatment with diminazene because of invasion of the CNS, Section 3.1 describes the development of a cannulation methodology for repeated atraumatic collection of CSF from goats; a simple method for this purpose had not previously been described, but was required to ensure that multiple CSF samples could be collected from the same animal without blood contamination. Section 3.2 describes the application of this technique to determine whether the apparent diminazene resistance of a *T. congolense* population in goats is associated with invasion of the CNS. Finally, earlier workers have concluded that diminazene crosses the blood-brain barrier to a very limited extent (Williamson, 1970; Gutteridge and Coombs, 1977; Raseroka and Ormerod, 1986). However, since these conclusions were based on very little quantitative information, Section 3.3 describes the application of the cannulation technique to determine the pharmacokinetics of diminazene in CSF of goats.

References:

Apted, F.I.C. (1980) Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Eastern hemisphere. *Pharmacology and Therapeutics* 11, 391-413.

Brun, R., Baeriswyl, S. and Kunz, C. (1989) *In vitro* drug sensitivity of *Trypanosoma gambiense* isolates. *Acta Tropica* 46, 369-376.

Gutteridge, W.E. and Coombs, G.H. (1977) Biochemistry of parasitic protozoa. MacMillan, London, p. 20.

Hatz, C. and Brun, R. (1992) Zerebrale Trypanosomose: Aspekte zu Epidemiologie, Klinik, Therapie und Krankheitsverlauf. *Schweizerische Medizinische Wochenschrift* 122, 873-878.

Hayes, J.D. and Wolf, C.R. (1990) Molecular mechanisms of drug resistance. *Biochemical Journal* 272, 281-295.

Jennings, F.W., Whitelaw, D.D., Holmes, P.H., Chizyuka, H.G.B. and Urquhart, G.M. (1979) The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal for Parasitology* 9, 381-384.

Kalu, A.U. (1985) The effect of various doses of Berenil and Pentamidine isethionate in rats experimentally infected with *Trypanosoma congolense*. *Nigerian Veterinary Journal* 14, 52-55.

Masake, R.A., Nantulya, V.M., Akol, G.W.O. and Musoke, A.J. (1984) Cerebral trypanosomiasis in cattle with mixed *Trypanosoma congolense* and *T. brucei brucei* infections. *Acta Tropica* 41, 237-246.

Morrison, W.I., Murray, M., Whitelaw, D.D. and Sayer, P.D. (1983) Pathology of infection with *Trypanosoma brucei*: disease syndromes in dogs and cattle resulting from severe tissue damage. *Contributions to Microbiology and Immunology* 7, 103-119.

Moulton, J.E. (1986) Relapse infection after chemotherapy in goats experimentally infected with *Trypanosoma brucei*: pathological changes in central nervous system. *Veterinary Pathology* 23, 21-28.

Raseroka, B.H. and Ormerod, W.E. (1986) The trypanocidal effect of drugs in different parts of the brain. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 80, 634-641.

Van Nieuwenhove, S., Schechter, P.J., Declercq, J., Boné, G., Burke, J. and Sjoerdsma, A. (1985) Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-α-difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 79, 692-698.

Whitelaw, D.D., Gardiner, P.R. and Murray, M. (1988) Extravascular foci of *Trypanosoma vivax* in goats: the central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. *Parasitology* 97, 51-61.

Whitelaw, D.D., Moulton, J.E., Morrison, W.I. and Murray, M. (1985) Central nervous system involvement in goats undergoing primary infections with *Trypanosoma brucei* and relapse infections after chemotherapy. *Parasitology* 90, 255-268.

Williamson, J. (1970) Review of chemotherapeutic and chemoprophylactic agents. In *The African Trypanosomiasis*. Mulligan, H.W. (ed.). George Allen and Unwin, London, pp. 125-221.

Zweygarth, E. and Röttcher, D. (1987) The occurrence of *Trypanosoma (Nannomonas) simiae* in the cerebrospinal fluid of domestic pigs. *Parasitology Research* 73, 479-480.

Section 3.1

Peregrine, A.S. and Mamman, M. (1994) A simple method for repeated sampling of lumbar cerebrospinal fluid in goats. *Laboratory Animals* 28, 391-396.

A simple method for repeated sampling of lumbar cerebrospinal fluid in goats

Andrew S. Peregrine & Mohammed Mamman

International Laboratory for Research on Animal Diseases (ILRAD), PO Box 30709, Nairobi, Kenya

Summary

A technique has been developed for repeated sampling of cerebrospinal fluid from conscious goats by means of a catheter placed surgically in the subarachnoid space between the sixth and seventh lumbar vertebrae. Uncontaminated cerebrospinal fluid in excess of 1.0 ml could be obtained readily from the cannulated goats several times daily for up to 6 weeks. Cannulated goats did not appear stressed during, and in between, sampling periods.

Keywords Sampling method; cerebrospinal fluid; goats

The cerebrospinal pathway comprises the ventricles of the brain, the central canal of the spinal cord, the subarachnoid space and perivascular extensions of this space (Swenson 1977, Coles 1980). Contained within this pathway is cerebrospinal fluid (CSF), which has the function of protecting, nourishing and maintaining the homeostasis of the brain and spinal cord (Swenson 1977, Coles 1980).

Although CSF can be sampled with little difficulty, such procedures are not routine and are only indicated under special circumstances. In animals, single or repeated sampling of CSF can be achieved through a puncture made into the cerebral ventricles, or at one of two levels of the subarachnoid space; the cisterna magna, or in the lumbar region at a point beyond the termination of the spinal cord (Feldman 1989). Regardless of the method used, the process of obtaining CSF must be undertaken with great care and must be carried out with physical restraint, chemical sedation or local anaesthesia (Mayhew 1975, Coles 1980, Brewer 1987). General anaesthesia is usually essential, especially in procedures which require surgical placement of indwelling cannulae for long-term sampling of CSF from either the cerebral ventricles (Mayhew 1975, Beal & Bligh 1977, Hedlund *et al.* 1977, Allsop & Pauli 1985) or the cisterna magna (Falconer *et al.* 1985). In the absence of adequate restraint, or when a faulty technique is employed, the nervous system may be traumatized and may result in contamination of CSF (Mayhew 1975, Coles 1980, Allsop & Pauli 1985). In addition, infection may be introduced into the nervous system if the CSF is sampled under septic conditions. It is therefore contraindicated to sample CSF through any area of skin with an existing infection (Coles 1980). Because of some of these potential problems, experiments requiring repeated sampling of CSF for short or long-term durations are rarely carried out in animals.

With the hope of circumventing such problems, and to simplify the methodology for CSF cannulation, we have developed a safe, rapid and reliable surgical technique for cannulating the subarachnoid space between the sixth and seventh lumbar vertebrae in the goat. The technique

Correspondence to: AS Peregrine

Accepted 12 November 1993

permits repeated sampling of CSF in conscious animals and will therefore be useful for studies on infectious diseases of the nervous system in small ruminants.

Materials and methods

Animals

Twenty-two adult, male, castrated East African × Galla goats were used in the study. The animals weighed 18.3 ± 2.0 (15–22) kg and were fed a concentrate ration (Young Stock, Belfast Millers, Nairobi; 200 g/animal/day) throughout the experimental work. The animals were allowed free access to hay and water.

Cannulation apparatus

The apparatus used was the commercially available Epidural Minipack® (Portex, Kent, England; Lot No. 90G 18 AA01) which comprises a 10.5 cm long 18 G Tuohy needle (outer diameter 1.3 mm, inner diameter 1.0 mm × 80 mm) with graduations at the 3, 5 and 7 cm levels. The lumen of the needle was guarded by a plastic probe. A detachable plastic wing for ease of handling of the needle during operation was also supplied. The catheter was 90 cm long, nylon (outer diameter 0.9 mm), and open only at one of its two ends. The closed end, marked with 3 lateral 'eyes', was used for catheterization of the subarachnoid space between the sixth and seventh lumbar vertebrae. The pack also included a transparent tube for guiding the catheter into the needle, and a luer-lock connector consisting of upper and lower halves. The lumen of the lower half of the connector was guarded by an insert tube and a cover was included for closing the upper half of the connector (Fig. 1).

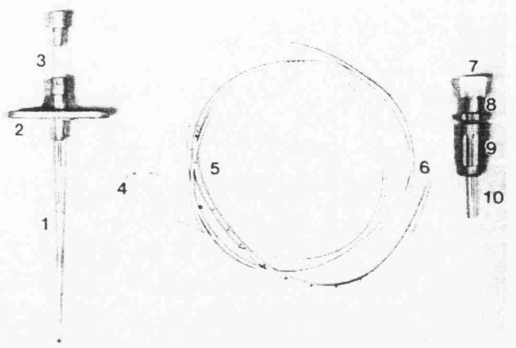


Fig 1 Epidural Minipack® (Portex, Kent, England). Components are shown here after removal from the sterile plastic pack. Key: 1, 18 G Tuohy needle; 2, detachable needle wing; 3, plastic probe in the lumen; 4, catheter guide; 5, graduated radio-opaque catheter; 6, closed end of the catheter; 7, cover; 8, 9, upper and lower halves, respectively, of the connector assembly; 10, insert tube

Presurgical care

The goats were starved for 24 h prior to beginning the surgical procedure. A rectangular area (40 cm × 15 cm) was then clipped and shaved in the lumbosacral region, with the animal in the standing position. The shaved area included the areas immediately cranial and caudal to the median sacral crest and the tubera coxae. The longitudinal axis along the lumbosacral spine region was then marked with a straight interrupted line, using a marker pen (Pentel Co., Japan). Subsequent to anaesthesia and placement of the animal in left lateral recumbency, the line aided in placement of the skin in its normal position in relation to the spinal longitudinal axis.

Routinely, each goat was administered with 0.2 ml of 2.0% xylazine (Chanazine®, Chanelle, Galway, Ireland) intravenously (i.v.) to ease induction of anaesthesia. Following premedication, the goats were placed in left lateral recumbency on the surgical table, then anaesthetized using a 5% solution of thiopentone sodium (Intraval®, May & Baker, Dagenham, England) administered dose-to-effect via the right jugular vein. Immediately after induction of anaesthesia the goats were intubated with a size 8 endotracheal tube (Rosch, Germany). After connection to an anaesthetic machine (Komesaroff®, Medical Developments Machine PTY Ltd, Sydney, Australia), goats were then maintained in surgical anaesthesia using an oxygen-methoxyfluorane-halothane mixture in a semi-closed system. The four legs of the animals were then tied together to ensure

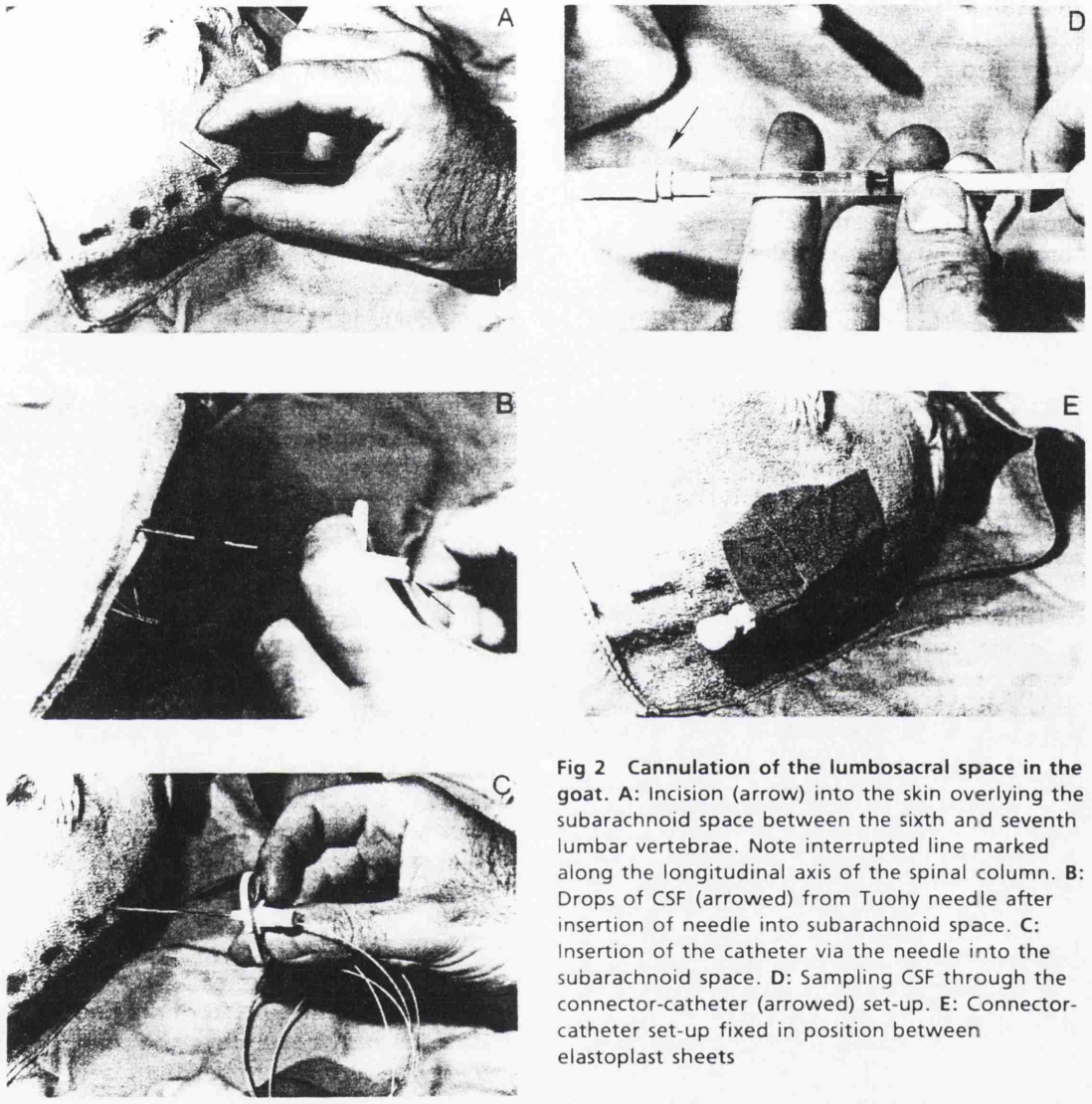


Fig 2 Cannulation of the lumbosacral space in the goat. A: Incision (arrow) into the skin overlying the subarachnoid space between the sixth and seventh lumbar vertebrae. Note interrupted line marked along the longitudinal axis of the spinal column. **B:** Drops of CSF (arrowed) from Tuohy needle after insertion of needle into subarachnoid space. **C:** Insertion of the catheter via the needle into the subarachnoid space. **D:** Sampling CSF through the connector-catheter (arrowed) set-up. **E:** Connector-catheter set-up fixed in position between elastoplast sheets

the spinal column was in a convex position. The shaved area of skin above the sixth and seventh lumbar vertebrae was then washed with a 1% solution of chlorhexidine gluconate (Savlon®, ICI Pharmaceuticals, Cheshire, England) and cleaned with a cotton swab. Sterile drapes were clipped to the trunk of the animal, leaving the surgical area exposed, and strict asepsis was maintained throughout the surgery.

Surgical procedure

Immediately prior to surgery, the epidural Minipack® was unpacked and the different parts identified and assembled. A 5 mm incision was then made into the skin overlying the subarachnoid space between the sixth and seventh lumbar vertebrae (Fig. 2A). The Tuohy needle, containing the lumen guard, was then inserted into the incision with the curved end orientated in a cranial direction. The winged outer end of the needle was then used to slowly advance the needle in a ventral direction through the tissues. When the 3 cm mark on the needle was at the level of the skin incision, the probe guarding the lumen of the needle was withdrawn. Gradually, the needle was advanced deeper until access

into the subarachnoid space was achieved. If anaesthesia was not sufficiently deep, entry into the space was accompanied by short-lasting opisthotonos. The appearance of clear normal CSF at the outer end of the needle indicated a successful cannulation (Fig. 2B). Immediately after appearance of the fluid, the catheter was inserted rapidly through the needle into the subarachnoid space (Fig. 2C). Care was taken to ensure that the correct end of the catheter was used for the catheterization. After approximately 7 cm of the catheter had been introduced into the space, the needle was carefully withdrawn, leaving the catheter in place. A pair of scissors was then used to cut off the excess portion of the catheter so that approximately 8 cm remained outside the incision site. The outer tip of the catheter was then positioned in the connector assembly, to a level just above the stop washer in the connector's lumen. The upper and lower halves of the connector were then twisted securely, but not too tightly, to fasten the catheter. At this stage, a sterile 1.0 ml syringe (Plastipak®, Becton Dickinson, Dublin, Ireland) was used to obtain a reference CSF sample (Fig. 2D). The white screw-cover was then used to close the outer end of the upper half of the connector.

In order that the catheter should not be accidentally pulled or damaged by the goat when conscious, a rectangular sheet of elastoplast (Leukoplast®, Beiersdorf AG, Hamburg, Germany), measuring approximately 8 × 6 cm, was placed on the shaved skin, immediately cranial to the cannula insertion site. The connector was then placed on the elastoplast in a cranial orientation, and another, but smaller (6 × 4 cm), piece of elastoplast used to affix the connector to the underlying elastoplast (Fig. 2E). The two sheets of elastoplast were then jointly sutured to the skin area using approximately six silk 2-0 (Supramid®, B. Braun, Melsungen AG, Germany) sutures. In this way, the connector was fixed semi-rigidly in position.

For each goat, the total time taken for the entire cannulation procedure varied between 30 and 60 min. At completion of the procedure, each goat was treated intramuscularly with oxytetracycline (Duphacycline LA®, Duphar, B.V., Amsterdam, Holland) at a dose of 20 mg/kg body weight. An oxytetracycline spray (Terramycin®, Pfizer Ltd, Sandwich, England) was also applied to the incision area. Generally, following some degree of ataxia, goats were able to stand and resume feeding within 1 h of the operation. Thereafter, the animals were housed communally. Goats did not interfere with each other's catheter assembly, presumably because the associated irritation caused animals to move away from each other.

Sampling procedure

Sampling was carried out with the aid of an assistant restraining the goat in a standing position. The operator's hands were cleaned with a solution of 70% alcohol, before removal of the connector cover. Using a sterile 1.0 ml syringe (Plastipak®, Becton Dickinson, Dublin, Ireland) tightly fitted to the mouth of the connector, the desired volume of CSF was then gradually aspirated. When the flow of CSF was compromised, infusion of 0.2 ml of 200 IU/ml of sterile heparin solution (Novo Nordisk, A/S Bagsvaerd, Denmark) in sterile distilled water at 27°C into the catheter was found to restore the flow. At completion of the sampling, the connector cover was returned into position, but without overtightening.

At the termination of an experiment, goats were administered 0.2 ml of 2.0% xylazine i.v. 3–5 min prior to the removal of the cannula. With the goat restrained in a standing position, the cannula was easily removed from the intervertebral space by a single rapid movement.

Results

Eighteen out of the 22 goats were cannulated successfully using the procedure described. One of the four other goats died immediately after induction of anaesthesia. In two others, clots of blood occluded the

lumen of the Tuohy needle and further attempts to gain access to the subarachnoid space using new needles were futile. In a fourth goat, the cannulation was of limited success because flow of the CSF ceased when the goat was still on the surgical table.

Cerebrospinal fluid samples in volumes of 0.5–1.0 ml (or more) were readily obtained for up to 42 days from goats when sampled once daily. Similar volumes could be obtained several times within a day when an interval of 5–10 min was allowed between sampling. Cannulated goats appeared and behaved normally and did not show signs of discomfort.

Although rarely observed, the main problem encountered in the study was cessation in the flow of CSF. Often, however, this was overcome by infusion of 0.2 ml of 200 IU/ml of heparin solution through the catheter, just prior to sampling. In some cases, the flow was intermittent with cessation of flow spanning a few days. When catheters from such animals were removed and examined, no visible damage or obstruction could be demonstrated. Restriction in flow may have been due to the presence of fibrous adhesions induced during the process of entry into the dural and subarachnoid spaces (Mayhew 1975, Falconer *et al.* 1985). Occasionally, cytological examination of CSF from freshly cannulated animals indicated the presence of erythrocytes. This, however, was usually confined to the first few days following surgery.

Discussion

The technique described in the present study permits collection of CSF from conscious goats by means of a catheter placed surgically in the subarachnoid space between the sixth and seventh lumbar vertebrae. Once in place, CSF in volumes of 0.5–1.0 ml (or more) could be collected once or several times daily, for 3–6 weeks. The catheterization procedure utilizes an inexpensive commercially available epidural cannulation pack and is completed within 1 h. It is therefore considerably easier than earlier-described techniques for collection of CSF which require expensive equipment, and take several hours to cannulate the cisterna magna (Pappenheimer *et al.* 1962, Falconer *et al.* 1985). Convulsions and leg paralysis are also problems that have been associated with previously described cisternal or lumbar cannulation methods (Allsop & Pauli 1985, Falconer *et al.* 1985). Furthermore, due to formation of extensive subarachnoid fibrous adhesions, such techniques have proved unsuitable for long-term studies (Pappenheimer *et al.* 1962). Such side effects were not associated with the present technique, and sampling of CSF could be carried out for up to 6 weeks without causing any apparent stress to the animal. This is almost twice as long as that reported by Falconer *et al.* (1985) for a cisterna magna cannulation technique in sheep. Finally, the technique described here has also circumvented the problem of CSF contamination with blood, which is frequently encountered with other methods (Strain *et al.* 1984, Allsop & Pauli 1985).

The described technique is particularly suitable for studying concentrations of endogenous substances, or therapeutic compounds in the lumbar CSF of healthy and diseased animals. To this end, it was used successfully to examine the CSF from 7 goats infected with *Trypanosoma congolense* for the presence of trypanosomal antigen; each animal was sampled once daily for 3–6 weeks (Mamman *et al.* 1994).

In view of the comparative advantages of the cannulation procedure described, the technique could have many applications for studies on the CSF in small ruminants. Since the catheterization apparatus is available in many sizes, the technique could also readily be applied to other animal species.

Acknowledgments We thank Messrs D. Kahoro and S. Kemei for their technical assistance during the surgical procedures. We are also grateful to Professor R. Ruberti for his advice concerning selection of the cannulation apparatus. Mr R. King is thanked for reviewing the manuscript. Mr D. Elsworth is thanked for providing the photographs. This is ILRAD Publication No. 1192.

References

Allsop TF, Pauli JV (1985) Magnesium concentrations in the ventricular and lumbar cerebrospinal fluid of hypomagnesaemic cows. *Research in Veterinary Science* **38**, 61–4

Beal AM, Bligh J (1977) Electrolyte concentrations in sheep cerebrospinal fluid. *Research in Veterinary Science* **22**, 382–3

Brewer BD (1987) Examination of the bovine nervous system. In *Veterinary Clinics of North America* (Baker JC, ed). Philadelphia: WB Saunders Co., pp 13–24

Coles EH (1980) *Cerebrospinal Fluid. Veterinary Clinical Pathology*, 3rd edn. Philadelphia: WB Saunders Co., pp 362–76

Falconer J, Owens PC, Smith R (1985) Cannulation of the cisterna magna in sheep: a method for chronic studies of cerebrospinal fluid. *Australian Journal of Experimental Biology and Medical Science* **63**, 157–62

Feldman BF (1989) Cerebrospinal fluid. In *Clinical Biochemistry of Domestic Animals* (Kaneko JJ, ed). New York: Academic Press, pp 835–65

Hedlund L, Lischko MM, Garner HE (1977) Ventricle cannulation in the calf. *Journal of Dairy Science* **60**, 997–1000

Mamman M, Moloo SK, Peregrine AS (1994) Relapse of *Trypanosoma congolense* infection in goats after diminazene aceturate is not a result of invasion of the central nervous system. *Annals of Tropical Medicine and Parasitology* **88**, 87–8

Mayhew IG (1975) Collection of cerebrospinal fluid from the horse. *Cornell Veterinarian* **65**, 500–11

Pappenheimer JR, Heisey SR, Jordan EF, Downer JD (1962) Perfusion of the cerebral ventricular system in unanaesthetized goats. *American Journal of Physiology* **203**, 763–74

Strain GM, Barta O, Olcott BM, Braun WF (1984) Serum and cerebrospinal fluid concentrations of immunoglobulin G in Suffolk sheep with scrapie. *American Journal of Veterinary Research* **45**, 1812–13

Swenson MJ (1977) *Duke's Physiology of Domestic Animals*. Ninth edn. Ithaca: Cornell University Press

Section 3.2

Mamman, M., Moloo, S.K. and Peregrine, A.S. (1994) Relapse of *Trypanosoma congolense* infection in goats after diminazene aceturate is not a result of invasion of the central nervous system. *Annals of Tropical Medicine and Parasitology* 88, 87-88.

Annals of Tropical Medicine and Parasitology, Vol. 88, No. 1, 87–88 (1994)

Relapse of *Trypanosoma congolense* infection in goats after diminazene aceturate is not a result of invasion of the central nervous system

The re-appearance of trypanosomes in infected animals following treatment, when re-infection and drug underdosage are excluded, is often attributed to drug resistance or the ability of parasites to survive in cryptic foci that are poorly accessible to trypanocidal drugs (Williamson, 1980). The latter phenomenon has been associated with some strains of *Trypanosoma brucei* in laboratory mice and rats (Jennings *et al.*, 1979; Abolarin *et al.*, 1982; Jennings and Gray, 1983), and *T. vivax* in goats (Whitelaw *et al.*, 1988), and is thought to be a result of invasion of sites such as the central nervous system (CNS).

We recently reported studies in which goats were infected by the feeding of *Glossina morsitans centralis* infected with a cloned population of *T. congolense* (IL 3274). Thereafter, animals were treated at various intervals following infection with diminazene aceturate at a single intramuscular (im) dose of 7·0 mg/kg body weight (bw) (Silayo *et al.*, 1992; Mamman *et al.*, 1993). It was observed that when treatment was administered on day 1 of infection, all the goats were cured. In contrast, infections were refractory to treatment when diminazene aceturate was administered on day 19 of infection, approximately 3 days after detection of trypanosomes in the peripheral blood. Since the parasitaemia profiles obtained from the latter group of animals mimic those observed with parasites which relapse from drug-inaccessible foci, the present experiment was carried out to determine whether invasion of the CNS contributed to the apparent resistance phenotype of *T. congolense* IL 3274 on day 19 of infection. The experimental protocols used by Silayo *et al.* (1992) and Mamman *et al.* (1993) were modified in the present study to include examination of the cerebrospinal fluid (CSF) for the presence of trypanosomes.

Seven adult, male, castrated East African × Galla goats were used. Sera were collected from these animals at the beginning of the experiment and were shown to be negative for *Trypanosoma* spp. antigen and anti-trypanosomal antibodies using ELISA (Nantulya and Lindqvist, 1989; Mamman *et al.*, 1993). Three days prior to initiation of infections, an epidural cannula (Minipack®, Portex, Kent, U.K.) was placed in the lumbar subarachnoid space of each goat to permit collection of CSF, in accordance with a previously developed technique (Peregrine and Mamman, in press). The goats were then infected with *T. congolense* IL 3274 by feeding five infected *G. m. centralis*, as described by Mamman *et al.* (1993). Infections in the goats were monitored by determination of the level of parasitaemia and the packed red blood cell volume per cent (Murray *et al.*, 1977; Paris *et al.*, 1982). Trypanosomes were first detected on days 12–16 post-infection. On day 19, a single im dose (7·0 mg/kg bw) of diminazene aceturate was administered to each goat. All goats showed temporary remission of parasitaemia within 48 h of the treatment. However, infections in all animals relapsed 6–8 days after drug treatment.

Lumbar CSF was collected daily from each goat from the day of cannulation until 3 days after trypanosomes had reappeared following treatment (days 25–27 post-infection). The cytology and presence of trypanosomes in each CSF sample were determined in three ways. First, within 10 min of collection, a 20 µl aliquot of each sample was dispensed onto a Neubauer haemocytometer and examined microscopically (400 ×). When CSF cell counts were very low, 500 µl of each sample was centrifuged (590 *g* for 3 min at 4°C) and then examined microscopically. Second, 200 µl aliquots were centrifuged at 42 *g* for 5 min and slides prepared from each sediment sample, dried at room temperature (27°C), stained with Diff Quick® (Baxter Health Care, Miami,

0003–4983/94/010087+02 $08.00/0

U.S.A.) and examined microscopically (1000 ×). Third, each sample was analysed for the presence of trypanosomal antigen using an ELISA (Nantulya and Lindqvist, 1989). In this assay, each sample was analysed undiluted, and at dilutions of 1:10 and 1:50.

None of the three techniques used to examine CSF revealed the presence of trypanosomes or trypanosomal antigen in any of the CSF samples collected from the goats, either prior to or after infection. The results therefore suggest that reappearance of *T. congolense* IL 3274 following treatment with diminazene aceturate on day 19 of infection is not associated with trypanosomes that have entered the CNS. However, the findings do not exclude the possibility that the parasites could survive treatment in such sites as the microcirculation, lymph nodes, spleen, heart or bone marrow (Maxie and Losos 1977; Banks 1978; WHO, 1986; Kalu *et al.*, 1988) where the bioavailability of diminazene may be compromised.

ACKNOWLEDGEMENTS. We are thankful to Dr. V. M. Nantulya and S. Minja, S. Kemei, D. Kahoro and B. Murithi for assistance during the study. This is ILRAD publication No. 1202.

M. MAMMAN
S. K. MOLOO
A. S. PEREGRINE*
International Laboratory for Research on Animal Diseases (ILRAD),
P.O. Box 30709,
Nairobi, Kenya

Received 27 July 1993, Accepted 28 September 1993

*Author to whom correspondence should be addressed.

REFERENCES

ABOLARIN, M. O., EVANS, D. A., TOVEY, D. G. & ORMEROD, W. E. (1982). *British Medical Journal*, **285**, 1380–1382.

BANKS, K. L. (1978). *Journal of Protozoology*, **25**, 241–245.

JENNINGS, F. W. & GRAY, G. D. (1983). In *Parasitic Infection to Parasitic Disease*, eds. Gigase, P. L. & Van Mark, E. A. E. pp. 147–154. Basel: Karger.

JENNINGS, F. W., WHITELAW, D. D., HOLMES, P. H., CHIZYUKA, H. G. B. & URQUHART, G. M. (1979). *International Journal for Parasitology*, **9**, 381–384.

KALU, A. U., LAWANI, F. A. & OMOTAINSE, S. O. (1988). *Bulletin of Animal Health and Production in Africa*, **36**, 77–79.

MAMMAN, M., KATENDE, J., MOLOO, S. K. & PEREGRINE, A. S. (1993). *Veterinary Parasitology*, **50**, 1–14.

MAXIE, M. G. & LOSOS, G. J. (1977). *Veterinary Parasitology*, **3**, 277–281.

MURRAY, M., MURRAY, P. K. & MCINTYRE, W. I. M. (1977). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **71**, 325–326.

NANTULYA, V. M. & LINDQVIST, K. J. (1989). *Tropical Medicine and Parasitology*, **40**, 267–272.

PARIS, J., MURRAY, M. & MCODIMBA, F. (1982). *Acta Tropica*, **39**, 307–316.

PEREGRINE, A. S. & MAMMAN, M. (in press). *Laboratory Animals*.

SILAYO, R. S., MAMMAN, M., MOLOO, S. K., ALIU, Y. O., GRAY, M. A. & PEREGRINE, A. S. (1992). *Research in Veterinary Science*, **53**, 98–105.

WHITELAW, D. D., GARDINER, P. R. & MURRAY, M. (1988). *Parasitology*, **97**, 1–11.

WILLIAMSON, J. (1980). Chemoresistance in trypanosomes. In *Report of the Expert Consultation on Research on Trypanosomiasis, Food and Agriculture Organization of the United Nations, Rome, 1–5 October 1979, pp. 84–89*. Rome: FAO.

WORLD HEALTH ORGANIZATION (1986). *Epidemiology and Control of African Trypanosomiasis*. Technical Report Series No. 739. Geneva: WHO.

Section 3.3

Mamman, M. and Peregrine, A.S. (1994) Pharmacokinetics of diminazene in plasma and cerebrospinal fluid of goats. *Research in Veterinary Science* 57, 253-255.

Research in Veterinary Science 1994, 57, 253-255

Pharmacokinetics of diminazene in plasma and cerebrospinal fluid of goats

M. MAMMAN, A. S. PEREGRINE, *International Laboratory for Research on Animal Diseases (ILRAD), PO Box 30709, Nairobi, Kenya*

The pharmacokinetics of diminazene in the cerebrospinal fluid (CSF) and plasma of five uninfected goats treated with single intramuscular doses of 3·5 mg diminazene base kg^{-1} bodyweight was investigated. The concentrations of the drug were determined by high performance liquid chromatography, and were three to four times lower in CSF than in plasma. The kinetics of the drug in CSF and plasma differed significantly with respect to C_{max}, t_{max}, AUC_{0-48h}, $AUMC_{0-48h}$, Cl and $V_{d(ss)}$.

AFRICAN trypanosomiasis is a vector-borne disease of man and animals caused by extracellular protozoan parasites of the genus *Trypanosoma*. In man, the disease is characterised in the early stages of infection by non-specific symptoms, but later a specific neurological syndrome occurs when the parasites invade the central nervous system (CNS); if left untreated the patients die (WHO 1986). The disease also poses a significant constraint on livestock production in sub-Saharan Africa (Murray and Gray 1984, Morrison et al 1985). In the absence of a vaccine, the control of the disease currently depends on the control of the vector and treatment with anti-trypanosomal drugs (Murray and Gray 1984).

Diminazene aceturate (Berenil, Veriben) is an aromatic diamidine that has been in use as a chemotherapeutic agent against animal trypanosomiasis since its introduction three decades ago (Jensch 1955, Williamson 1980). In general, the drug is effective when it is administered as a single intramuscular dose of 3·5 to 7·0 mg kg^{-1} bodyweight. However, treatment with diminazene aceturate is not effective against trypanosomes that have invaded the CNS (Jennings et al 1979, Abolarin et al 1982, Jennings and Gray 1983, Whitelaw et al 1988), and since the parasites which re-emerge from the CNS after treatment do not appear to be drug resistant (Jennings et al 1979, Whitelaw et al 1988), it is generally believed that the poor response results from the restricted entry of the drug into the nervous system, across the blood-brain-barrier (Williamson 1970). Evidence for this belief is provided by the fact that only small amounts of diminazene have been detected in the CNS of animals that have been treated with single doses of the drug (Kellner et al 1985, Onyeyili and Anika 1991). However, no rigorous investigations of the pharmacokinetics of the drug in the CNS have been carried out because a satisfactory technique to obtain samples of cerebrospinal fluid (CSF) in sufficient numbers and quantity was not available. Recently, a suitable technique has been developed (Peregrine and Mamman 1994) which has been used in the present study to compare the disposition of diminazene in the CSF and plasma of five uninfected East African/Galla crossbred goats.

The lumbar subarachnoid space between the sixth and seventh lumbar vertebrae of each goat was catheterised with an epidural apparatus (Minipack, Portex, Kent) while the goats were under general anaesthesia. Approximately 7 cm of the catheter was inserted in a cranial direction into the subarachnoid space. The outer tip of the catheter was then connected to a sampling assembly that was sutured to the skin immediately cranial to the site of insertion of the catheter. Twenty-four hours after insertion of the cannula, each goat received a single intramuscular dose of 3·5 mg diminazene base kg^{-1} bodyweight using a freshly prepared 7·0 per cent (w/v) solution of diminazene aceturate (Berenil, Hoechst) in sterile distilled water. Samples of CSF and jugular blood were obtained simultaneously from each animal at 0, 10, 15, 30 and 45 minutes and one-, one-and-a-half, two, three, four, six, eight, 10, 12, 14, 16, 18, 21, 24 and 48 hours after the administration of the drug, and samples were collected daily for another four weeks. The blood samples were collected into 5 ml evacuated tubes (Monoject, Sherwood Medical) containing potassium ethylenediamine tetra-acetate, and the plasma was separated after centrifugation at 400 *g* for 30 minutes. The plasma and CSF samples were then stored at –20°C until analysis. The diminazene in each sample was extracted by an ion-pair technique and measured by a high performance liquid chromatograhic technique with a sensitivity limit of 10 ng diminazene base ml^{-1} (Aliu and Ødegaard 1983, Aliu et al 1993). The concentration-time data were then analysed in terms of a non-compartmental model (Gibaldi and Perrier 1982).

Semi-logarithmic plots of the concentration of dimi-

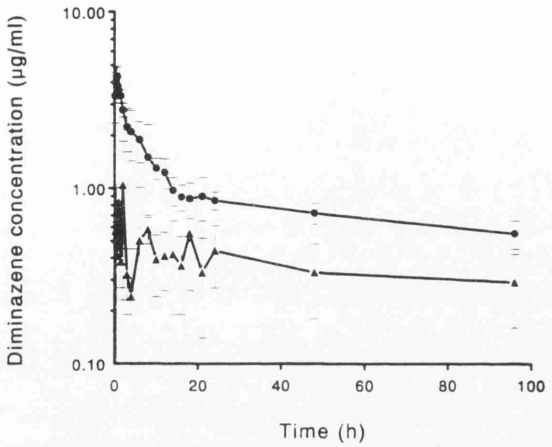


FIG 1: Mean (SD) concentrations of diminazene in plasma (●) and cerebrospinal fluid (▲) of five goats after the intramuscular administration of the drug at a dose of 3·5 mg diminazene base kg^{-1} bodyweight

nazene in the CSF and plasma of the goats during the first 96 hours after treatment are given in Fig 1. In general, the concentrations of the drug in the CSF were three to four times lower than those in the plasma at the same time. The values of C_{max}, t_{max}, AUC_{0-48h}, $AUMC_{0-48h}$, Cl and $V_{d(ss)}$ differed significantly between the CSF and the plasma (Table 1). The mean (SD) peak concentrations (C_{max}) were 1·04 (0·81) µg ml^{-1} in the CSF, compared with 4·31 (0·22) µg ml^{-1} in the plasma. The corresponding times (t_{max}) at which C_{max} occurred in the CSF and plasma were 2·00 (0·09) hours and 0·41 (0·29) hours, respectively. The differences in C_{max} and t_{max} between the CSF and plasma may be due to the marked ionisation of the drug at blood pH, resulting in its restricted and delayed passage into the CSF (Williamson 1970). Diminazene was detected in all the plasma samples collected from the five goats during the first four weeks after the treatment, but it could be detected in the CSF from only two of the five for the whole period.

The present findings, together with the results of studies with diminazene aceturate in cattle and dogs (Kellner et al 1985, Onyeyili and Anika 1991) and pentamidine, a structural analogue of diminazene, in human beings (Bronner et al 1991), indicate that aromatic diamidines pass the blood-brain barrier into the CNS, but in concentrations that are significantly lower than in the plasma. The anti-trypanosomal significance of the lower concentrations in the CSF remains to be established.

TABLE 1: Mean (SD) pharmacokinetic parameters of diminazene in plasma and cerebrospinal fluid (CSF) of five goats after the single intramuscular administration of the drug at a dose of 3·5 mg diminazene a base kg^{-1} bodyweight

Parameter	Units	Plasma	CSF
C_{max}	µg ml^{-1}	4·31 (0·22*)	1·04 (0·81*)
t_{max}	hours	0·41 (0·29*)	2·00 (0·09*)
AUC_{0-48h}	µg hours ml^{-1}	50·30 (1·07*)	19·8 (2·71*)
$AUMC_{0-48h}$	µg $hours^2$ ml^{-1}	830·5 (55·5*)	392·0 (76·4*)
MRT	hours	16·3 (1·4*)	20·6 (1·4*)
Cl	ml $minute^{-1}$ kg^{-1}	1·16 (0·02*)	3·07 (0·43*)
$V_{d(ss)}$	litres kg^{-1}	1·14 (0·11*)	3·74 (0·46*)

*P<0·05 for plasma and CSF parameters

Acknowledgement

The authors are grateful to Mr B. Murithi for technical assistance during the study. This is ILRAD publication number 1191.

References

ABOLARIN, M. O., EVANS, D. A., TOVEY, D. G. & ORMEROD, W. E. (1982) Cryptic stage of sleeping-sickness trypanosome developing in choroid plexus epithelial cells. *British Medical Journal* **285,** 1380-1382

ALIU, Y. O. & ØDEGAARD, S. (1983) Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in plasma. *Journal of Chromatography* **276,** 218-223

ALIU, Y. O. MAMMAN, M. & PEREGRINE, A. S. (1993) Pharmacokinetics of diminazene in female Boran (*Bos indicus*) cattle. *Journal of Veterinary Pharmacology and Therapeutics* **16,** 291-300

BRONNER, U., DOUA, F., ERICSSON, O., GUSTAFSSON, L. L., MIEZAN, T. W., RAIS, M. & ROMBO, L. (1991) Pentamidine concentrations in plasma, whole blood and cerebrospinal fluid during treatment of *Trypanosoma gambiense* infection in Cote d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85,** 608-611

GIBALDI, M. & PERRIER, D. (Eds) (1982) Pharmacokinetics. 2nd edn. New York, Marcel Dekker. pp 409-417

JENNINGS, F. W. & GRAY, G. D. (1983) Relapsed parasitaemia following chemotherapy of chronic *T brucei* infections in mice and its relation to cerebral trypanosomes. In From Parasitic Infection to Parasitic Disease. Vol 7. Eds P. L. Gigase and E. A. E. van Mark. Basel, Karger. pp 147-154

JENNINGS, F. W., WHITELAW, D. D., HOLMES, P. H., CHIZYUKA, H. G. B. & URQUHART, G. M. (1979) The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal for Parasitology* **9,** 381-384

JENSCH, H. (1955) 4,4'-Diamidino-diazoaminobenzeol, ein neues Mittel gegen Trypanosomen-und Babesien-Infektionen. *Arzneimittel Forschung* **5,** 634-635

KELLNER, H. M., ECKERT, H. G. & VOLZ, M. (1985) Studies in cattle on the disposition of the anti-trypanosomal drug diminazene aceturate (®Berenil). *Tropical Medicine and Parasitology* **36,** 199-204

MORRISON, W. I., MURRAY, M. & AKOL, G. W. O. (1985) Immune responses of cattle to African trypanosomes. In Immunology and Pathogenesis of Trypanosomiasis. Ed I. R. Tizard. Boca Raton, CRC Press. pp 103-131

MURRAY, M. & GRAY, A. R. (1984) The current situation on animal trypanosomiasis in Africa. *Preventive Veterinary Medicine* **2,** 23-30

ONYEYILI, P. A. & ANIKA, S. M. (1991) Diminazene aceturate residues in the tissues of healthy and *Trypanosoma congolense* and *Trypanosoma brucei brucei* infected dogs. *British Veterinary Journal* **147,** 155-161

PEREGRINE, A. S. & MAMMAN, M. (1994) A simple method for repeated sampling of lumbar cerebrospinal fluid in goats. *Laboratory Animals* (In press)

WHITELAW, D. D., GARDINER, P. R. & MURRAY, M. (1988) Extravascular foci of *Trypanosoma vivax* in goats: the central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. *Parasitology* **97,** 51-61

WHO (1986) Epidemiology and Control of African Trypanosomiasis. Report of a WHO Expert Committee. Technical Report Series 739. p 78

WILLIAMSON, J. (1970) Review of chemotherapeutic and chemoprophylactic agents. In The African Trypanosomiasis. Ed H. W. Mulligan. London, George Allen & Unwin. pp 125-221

WILLIAMSON, J. (1980) Chemoresistance in trypanosomes. In Report of the Expert Consultation on Research on Trypanosomiasis. Food and Agriculture Organization. October 1-5. Rome. FAO. pp 84-89

Received July 7, 1993
Accepted March 14, 1994

Section 3.4

- Discussion -

Various workers have demonstrated that treatment of domestic livestock with diminazene aceturate is not effective against populations of *T. brucei* subspecies and *T. vivax* that have invaded the CNS (Jennings et al., 1979; Abolarin et al., 1982; Jennings and Gray, 1983; Raseroka and Ormerod, 1986; Whitelaw et al., 1988). Thus, reappearance of these trypanosome species in the bloodstream following treatment with diminazene may be associated with recrudescence of parasites from the CNS rather than an ability of the parasites to withstand drug concentrations that eliminate drug-sensitive populations. In order to determine whether this phenomenon also applies to *T. congolense*, Section 3.1 describes the development of a cannulation methodology for repeated collection of CSF from conscious goats by means of a catheter placed surgically in the subarachnoid space between the sixth and seventh lumbar vertebrae. Uncontaminated CSF in excess of 1.0 ml could be obtained readily from the cannulated goats several times daily for up to 6 weeks, and cannulated goats did not appear stressed either during or between CSF sampling. Since this sampling frequency and duration of collection was a substantial improvement on previously described techniques (Pappenheimer et al., 1962; Falconer et al., 1985), it permitted a detailed investigation of the ability of *T. congolense* to invade the CNS.

Section 3.2 describes the use of the cannulation technique in goats to determine if reappearance of a population of *T. congolense* in the bloodstream following treatment with diminazene aceturate is associated with invasion of the CNS. Three days after cannulae were placed in seven goats the animals were infected with *T. congolense* IL 3274 via the bites of infected *Glossina morsitans centralis*. *Trypanosoma congolense* IL 3274 is a trypanosome clone originating from Burkina Faso and is resistant in goats to treatment with recommended doses of diminazene aceturate when administered after the onset of parasitaemia (Peregrine et al., 1991; Silayo et al., 1992; Mamman et al., 1993). Trypanosomes were first detected in the goats on days 12-16 following infection, and on day 19 the animals were treated with diminazene aceturate at a dose of 7.0 mg/kg b.w. Within 48 hours of treatment all the goats were non-parasitaemic. However, six to eight days later, trypanosomes were detected in the bloodstream of all the animals. Lumbar CSF was collected daily

from all the goats from before infection until 3 days after trypanosomes reappeared following treatment, and neither intact trypanosomes nor trypanosomal antigen were detected in any sample. Thus, reappearance of trypanosomes in the bloodstream following treatment did not appear to be associated with invasion of the CNS. However, it is possible that the trypanosomes could survive treatment in places such as lymph nodes, spleen, heart, bone marrow or the microcirculation where the bioavailability of diminazene might be compromised. Having said this, it appears most likely to have been associated with an ability of *T. congolense* IL 3274 to withstand concentrations of diminazene that are trypanocidal for diminazene-sensitive populations as this is consistent with data described by Silayo et al. (1992) and Gray and Peregrine (1993). These scientists characterised the drug sensitivity of *T. congolense* IL 3274 using an *in vitro* system in which metacyclic trypanosomes were incubated in the presence of various drug concentrations for 48 hours, and showed that the population was resistant to 10 μg diminazene aceturate/ml media, but sensitive to 50 μg/ml. In contrast, the diminazene-sensitive population *T. congolense* IL 1180 (Peregrine et al., 1991) was resistant to 1 μg/ml but sensitive to 5 μg/ml. In similar work, a high level of resistance to diminazene has also been demonstrated *in vitro* for an isolate of *T. congolense* that was collected from a cow in Ghibe in 1989 (Gray and Peregrine, 1993; Kaminsky et al., 1993). It would therefore appear that the resistance to diminazene observed amongst *T. congolense* populations at Ghibe was generally associated with an ability of the parasites to withstand trypanocidal drug concentrations, rather than an ability to invade the CNS.

In an attempt to determine whether diminazene would eliminate trypanosomes if they accessed the CNS, Section 3.3 describes an experiment in which the pharmacokinetics of diminazene were determined in CSF and plasma of five uninfected cannulated goats following treatment with diminazene aceturate at a dose of 3.5 mg/kg b.w. Unlike the routine "spinal tap" procedure which precludes frequent collection of CSF because of blood contamination (Burri et al., 1994), the cannulation technique described in Section 3.1 allowed large numbers of samples to be collected, e.g., 20 CSF samples over the first 48 hours following treatment. Diminazene concentrations were quantified in these samples using high-performance

liquid chromatography, and compared to drug concentrations in plasma samples collected from the same animals at the same time intervals following treatment. The data indicated that diminazene accesses the CNS, but that the kinetics of the drug in CSF and plasma differ significantly with respect to C_{max}, t_{max}, $AUC_{0\text{-}48h}$, $AUMC_{0\text{-}48h}$, Cl and $V_{d(ss)}$. Most importantly, the concentrations of diminazene in CSF were 3-4 times lower than those in plasma. Quite surprisingly, the difference between plasma and CSF drug concentrations was considerably less than that for either pentamidine in humans (Bronner et al., 1991), or melarsoprol in monkeys (Burri et al., 1994) and humans (Burri et al., 1993). For instance, even though melarsoprol is the most commonly used compound for treatment of second-stage trypanosomiasis in humans (Apted, 1980; Pépin and Milord, 1994), when trypanosomes have invaded the CNS, the concentrations of the drug in CSF of humans are 50-fold lower than those in serum, when quantified with a bioassay (Burri and Brun, 1992; Burri et al., 1993). Thus, the bioavailability of diminazene within the CNS would appear to be greater than that for either pentamidine or melarsoprol. As a result, the compound might eliminate CNS trypanosome infections if the parasites are highly sensitive to the drug. However, since CSF concentrations are usually insufficient to eliminate infections with *T. brucei* subspecies (Jennings et al., 1979; Abolarin et al., 1982; Jennings and Gray, 1983; Raseroka and Ormerod, 1986; Whitelaw et al., 1988), and *T. congolense* populations are generally less susceptible to diminazene than populations of *T. brucei* (Kaminsky et al., 1989, 1993), it would appear that CSF diminazene concentrations would be less likely to eliminate *T. congolense* CNS infections than *T. brucei* CNS infections.

In conclusion, the ability of *T. congolense* IL 3274 infections in goats to resist treatment with diminazene aceturate appears not to be associated with invasion of the CNS. Instead, while the work described in this Chapter has not ruled out the involvement of invasion of other "drug-inaccessible" sites in the drug-resistance phenotype, *in vitro* cultivation studies indicate that the resistance appears most likely to be associated with an ability of the parasites to withstand higher concentrations of diminazene than drug-sensitive populations. A similar property also appears to be

responsible for the diminazene resistance of *T. congolense* populations from Ghibe, Ethiopia (Chapter 2).

References:

Abolarin, M.O., Evans, D.A., Tovey, D.G. and Ormerod, W.E. (1982) Cryptic stage of sleeping-sickness trypanosome developing in choroid plexus epithelial cells. *British Medical Journal* 285, 1380-1382.

Apted, F.I.C. (1980) Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Eastern hemisphere. *Pharmacology and Therapeutics* 11, 391-413.

Bronner, U., Doua, F., Ericsson, Ö., Gustafsson, L.L., Miézan, T.W., Rais, M. and Rombo, L. (1991) Pentamidine concentrations in plasma, whole blood and cerebrospinal fluid during treatment of *Trypanosoma gambiense* infection in Côte d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85, 608-611.

Burri, C., Baltz, T., Giroud, C., Doua, F., Welker, H.A. and Brun, R. (1993) Pharmacokinetic properties of the trypanocidal drug melarsoprol. *Chemotherapy* 39, 225-234.

Burri, C. and Brun, R. (1992) An *in vitro* bioassay for quantification of melarsoprol in serum and cerebrospinal fluid. *Tropical Medicine and Parasitology* 43, 223-225.

Burri, C., Onyango, J.D., Auma, J.E., Burudi, E.M.E. and Brun, R. (1994) Pharmacokinetics of melarsoprol in uninfected vervet monkeys. *Acta Tropica* 58, 35-49.

Falconer, J., Owens, P.C. and Smith, R. (1985) Cannulation of the cisterna magna in sheep: a method for chronic studies of cerebrospinal fluid. *Australian Journal of Experimental Biology and Medical Science* 63, 157-162.

Gray, M.A. and Peregrine, A.S. (1993) An *in vitro* assay for drug sensitivity of *Trypanosoma congolense* using *in vitro*-derived metacyclic trypanosomes. *Acta Tropica* 54, 291-300.

Jennings, F.W. and Gray, G.D. (1983) Relapsed parasitaemia following chemotherapy of chronic *T. brucei* infections in mice and its relation to cerebral trypanosomes. In *From Parasitic Infection to Parasitic Disease. Vol. 7.* Gigase, P.L. and van Mark, E.A.E. (eds.). Karger, Basel, pp. 147-154.

Jennings, F.W., Whitelaw, D.D., Holmes, P.H., Chizyuka, H.G.B. and Urquhart, G.M. (1979) The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal for Parasitology* 9, 381-384.

Kaminsky, R., Chuma, F. and Zweygarth, E. (1989) *Trypanosoma brucei brucei*: expression of drug resistance *in vitro*. *Experimental Parasitology* 69, 281-289.

Kaminsky, R., Chuma, F. and Zweygarth, E. (1993) *Trypanosoma congolense*: *in vitro* susceptibility of bloodstream forms to diminazene and isometamidium. *Experimental Parasitology* 76, 213-215.

Mamman, M., Katende, J., Moloo, S.K. and Peregrine, A.S. (1993) Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology* 50, 1-14.

Pappenheimer, J.R., Heisey, S.R., Jordan, E.F. and Downer, J.D. (1962) Perfusion of the cerebral ventricular system in unanaesthetized goats. *American Journal of Physiology* 203, 763-774.

Pépin, J. and Milord, F. (1994) The treatment of human African trypanosomiasis. *Advances in Parasitology* 33, 1-47.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991). Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* 102, 93-100.

Raseroka, B.H. and Ormerod, W.E. (1986) The trypanocidal effect of drugs in different parts of the brain. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 80, 634-641.

Silayo, R.S., Mamman, M., Moloo, S.K., Aliu, Y.O., Gray, M.A. and Peregrine, A.S. (1992) Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate. *Research in Veterinary Science* 53, 98-105.

Whitelaw, D.D., Gardiner, P.R. and Murray, M. (1988) Extravascular foci of *Trypanosoma vivax* in goats: the central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. *Parasitology* 97, 51-61.

Chapter 4

Effect of duration of infection and size of trypanosome population on the sensitivity of trypanosomes to diminazene *in vivo*

Section 4.0

- Introduction -

Resistance to diminazene has been reported in populations of *Trypanosoma congolense* in Burkina Faso (Clausen et al., 1992; Bauer et al., 1995), Côte d'Ivoire (Küpper and Wolters, 1983), Ethiopia (Codjia et al., 1993), Kenya (Gitatha, 1979), Nigeria (Jones-Davies, 1968; Gray and Roberts, 1971a), Somalia (Ainanshe et al., 1992), Sudan (Mohamed-Ahmed et al., 1992), Tanzania (Mbwambo et al., 1988) and Zimbabwe (Joshua et al., 1995). Such resistance may be innate in origin (Williamson, 1960; Jones-Davies, 1967, 1968; Desquesnes et al., 1995). However, in general, it appears to be most commonly associated with short- or long-term use of quinapyramine, and, possibly, long-term use of diminazene (see Chapter 2; Bauer et al., 1995).

In areas where resistance to diminazene occurs as a "single"-resistance phenotype, such resistance can usually be controlled by use of a "sanative" drug pair, i.e., isometamidium or homidium (Whiteside, 1960; Aliu, 1981). However, when diminazene resistance occurs as a component of multiple-drug resistance (Gitatha, 1979; Schönefeld et al., 1987; Clausen et al., 1992; Mohamed-Ahmed et al., 1992; Codjia et al., 1993) sanative drug pairs will not be effective, particularly if the multiple resistance is associated with single infections (Codjia et al., 1993; Section 2.4). Research described in Chapter 2 has shown that such multiple-drug resistant infections can be effectively controlled if one integrates (i) administration of diminazene aceturate to infected animals to control pathogenic infections, with (ii) vector-control measures that produce a sizeable reduction in the tsetse population. Unfortunately, poor infrastructures in many countries preclude the establishment and maintenance of effective, long-term, tsetse control programs (Holmes and Scott, 1982). Thus, alternative measures for controlling drug-resistant infections are required. Baggot (1978) has indicated that if a pharmacologically active compound has a half-life in animals in excess of 9 hours, treatment with a single large dose is likely to be less efficacious than using a priming dose followed by a second dose after an appropriate interval. Since the mean half-life of diminazene in goats has been shown to be 21.4 hours (Aliu et al., 1984), Section 4.1 describes the evaluation of this hypothesis in goats infected with a diminazene-resistant clone of *T. congolense*.

In addition to the development of *de novo* therapeutic regimens for controlling diminazene-resistant infections using currently available anti-trypanosomal compounds, instability of resistance to diminazene in trypanosome populations would suggest that modifications to the current treatment regimens for anti-trypanosomal compounds may control such infections. In the past, resistance to diminazene in trypanosome populations has been shown to be relatively stable (Gray and Roberts, 1971a,b; Kaminsky and Zweygarth, 1989; Moloo and Kutuza, 1990; Zhang et al., 1993). As a result, the demonstration in Section 4.1 that the sensitivity of a trypanosome infection in goats to diminazene can change markedly during the early stages of infection was surprising. In Section 4.2 these observations were confirmed and the timing of the alteration in drug sensitivity was identified. However, the aetiology of the observation was still not clear. Since changes in the proportion of diminazene-resistant parasites in trypanosome populations could be responsible for the phenomenon, Section 4.3 describes a series of experiments that were carried out to determine the frequency with which drug-resistant trypanosomes occur in goats before and after treatment with diminazene. Because of the crude nature of the resultant estimates, due to limited animal numbers, Section 4.4 describes similar work that was carried out in mice to obtain more accurate estimates. In addition, the effect of trypanosome concentration on the level of resistance of trypanosomes to diminazene was also evaluated. Finally, since Section 4.3 demonstrated that a sizeable proportion of *T. congolense* parasites that arise in infected goats following treatment with diminazene aceturate are sensitive to the drug dosage used, Section 4.5 describes work that was carried out to determine if the apparent level of susceptibility of diminazene-sensitive trypanosomes to diminazene in goats is altered when they occur simultaneously with diminazene-resistant trypanosomes, i.e., in mixed infections.

References:

Ainanshe, O.A., Jennings, F.W. and Holmes, P.H. (1992) Isolation of drug-resistant strains of *Trypanosoma congolense* from the lower Shabelle region of Southern Somalia. *Tropical Animal Health and Production* 24, 65-73.

Aliu, Y.O. (1981) Approach to effective chemotherapy and chemoprophylaxis of animal trypanosomiasis in Nigeria. In *Proceedings, First National Conference on Tsetse and Trypanosomiasis Research in Nigeria, Kaduna, Nigeria, 10th-12th August, 1981.* Ilemobade, A.A. (ed.), Shereef Salam Press, Zaria, pp. 194-228.

Aliu, Y.O., Ødegaard, S. and Søgnen, E. (1984) Diminazene/Berenil: bioavailability and disposition in dairy goats. *Acta Veterinaria Scandinavica* 25, 593-596.

Baggot, J.D. (1978) Some aspects of clinical pharmacokinetics in veterinary medicine. II. *Journal of Veterinary Pharmacology and Therapeutics* 1, 111-118.

Bauer, B., Amsler-Delafosse, S., Clausen, P.-H., Kabore, I. and Petrich-Bauer, J. (1995) Successful application of deltamethrin pour on to cattle in a campaign against tsetse flies (Glossina spp.) in the pastoral zone of Samorogouan, Burkina Faso. *Tropical Medicine and Parasitology* 46, 183-189.

Clausen, P.-H., Sidibe, I., Kaboré, I. and Bauer, B. (1992) Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse fly challenge in the pastoral zone of Samorogouan, Burkina Faso. *Acta Tropica* 51, 229-236.

Codjia, V., Mulatu, W., Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authié, E., d'Ieteren, G.D.M. and Peregrine, A.S. (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. *Acta Tropica* 53, 151-163.

Desquesnes, M., La Rocque, S. (de) and Peregrine, A.S. (1995) French Guyanan stock of *Trypanosoma vivax* resistant to diminazene aceturate but sensitive to isometamidium chloride. *Acta Tropica* 60, 133-136.

Gitatha, S.K. (1979) *T. congolense* (Shimba Hills) resistant to various trypanocidal drugs. In *Proceedings of the 16th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yaoundé, Cameroon.* Eleza Services, Nairobi, OAU/STRC Publication No. 111, pp. 257-263.

Gray, A.R. and Roberts, C.J. (1971a) The cyclical transmission of strains of *Trypanosoma congolense* and *Trypanosoma vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* 63, 67-89.

Gray, A.R. and Roberts, C.J. (1971b) The stability of resistance to diminazene aceturate and quinapyramine sulphate in a strain of *Trypanosoma vivax* during cyclical transmission through antelope. *Parasitology* 63, 163-168.

Holmes, P.H. and Scott, J.M. (1982) Chemotherapy against animal trypanosomiasis. In *Perspectives in Trypanosomiasis Research.* Baker, J.R. (ed.), Research Studies Press, pp. 59-69.

Jones-Davies, W.J. (1967) The discovery of Berenil-resistant *Trypanosoma vivax* in Northern Nigeria. *Veterinary Record* 80, 531-532.

Jones-Davies, W.J. (1968) Berenil resistance in naturally occurring *Trypanosoma congolense*. *Bulletin of Epizootic Diseases of Africa* 16, 213-216.

Joshua, R.A., Obwolo, M.J., Bwangamoi, O. and Mandebvu, E. (1995) Resistance to diminazene aceturate by *Trypanosoma congolense* from cattle in the Zambezi Valley of Zimbabwe. *Veterinary Parasitology* 60, 1-6.

Kaminsky, R. and Zweygarth, E. (1989) Effect of *in vitro* cultivation on the stability of resistance of *Trypanosoma brucei brucei* to diminazene, isometamidium, quinapyramine, and Mel B. *Journal of Parasitology* 75, 42-45.

Küpper, W. and Wolters, M. (1983) Observation on drug resistance of *Trypanosoma (Nannomonas) congolense* and *Trypanosoma (Duttonella) vivax* in cattle at a feedlot in Northern Ivory Coast. *Tropenmedizin und Parasitologie* 34, 203-205.

Mbwambo, H.A., Mella, P.N.P. and Lekaki, K.A. (1988) Berenil (diminazene aceturate) resistant *Trypanosoma congolense* in cattle under natural tsetse challenge at Kibaha, Tanzania. *Acta Tropica* 45, 239-244.

Mohamed-Ahmed, M.M., Rahman, A.H. and Abdel Karim, E.I. (1992) Multiple drug-resistant bovine trypanosomes in South Darfur Province, Sudan. *Tropical Animal Health and Production* 24, 179-181.

Moloo, S.K. and Kutuza, S.B. (1990) Expression of resistance to isometamidium and diminazene in *Trypanosoma congolense* in Boran cattle infected by *Glossina morsitans centralis*. *Acta Tropica* 47, 79-89.

Schönefeld, A., Röttcher, D. and Moloo, S.K. (1987). The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. *Tropical Medicine and Parasitology* 38, 177-180.

Whiteside, E. F. (1960). Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In *Proceedings of the International Scientific Committee for Trypanosomiasis Research, 8th meeting, Jos, Nigeria.* Commission for Technical Co-operation South of the Sahara, London, Publication No. 62, pp. 141-153.

Williamson, J. (1960) Some problems in trypanocidal drug resistance. In *Proceedings of the International Scientific Committee for Trypanosomiasis Research, 8th meeting, Jos, Nigeria.* Commission for Technical Co-operation South of the Sahara, London, Publication No. 62, pp. 163-169.

Zhang, Z.Q., Giroud, C. and Baltz, T. (1993) *Trypanosoma evansi*: *in vivo* and *in vitro* determination of trypanocide resistance profiles. *Experimental Parasitology* 77, 387-394.

Section 4.1

Silayo, R.S., Mamman, M., Moloo, S.K., Aliu, Y.O., Gray, M.A. and Peregrine, A.S. (1992) Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate. *Research in Veterinary Science* 53, 98-105.

Research in Veterinary Science 1992, 53, 98-105

Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate

R. S. SILAYO, *Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture, PO Box 3019, Morogoro, Tanzania,* M. MAMMAN, S. K. MOLOO, *International Laboratory for Research on Animal Diseases, PO Box 30709, Nairobi, Kenya,* Y. O. ALIU, *Department of Physiology and Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria,* M. A. GRAY, *Kenya Trypanosomiasis Research Institute, PO Box 362, Kikuyu, Kenya,* A. S. PEREGRINE*, *International Laboratory for Research on Animal Diseases, PO Box 30709, Nairobi, Kenya*

Diminazene aceturate is one of a limited number of compounds currently marketed for treatment of trypanosomiasis in cattle, sheep and goats. The pharmacokinetics of the compound in goats suggest that double treatment with diminazene aceturate might enhance the compound's therapeutic activity. A study was therefore conducted in goats using two clones of *Trypanosoma congolense*, IL 3274 and IL 1180, which were previously shown to be resistant and sensitive, respectively, to single treatment with diminazene aceturate. The results indicated that, as compared to single treatment, double treatment with diminazene aceturate at a dose of 7·2 mg kg^{-1} bodyweight, at either eight or 24 hour intervals, did not greatly enhance the therapeutic activity of the drug. Furthermore, treatment with the same drug dose eliminated infections with *T congolense* IL 3274 when treatment was administered 24 hours after infected *Glossina morsitans centralis* had fed, but failed to do so if treatment was delayed until after goats were detected to be parasitaemic. This suggests that failure of *T congolense* IL 3274 to respond to treatment with diminazene may not be due to drug resistance per se.

CHEMOTHERAPY is an important means of control of trypanosomiasis in domestic livestock across Africa (Leach and Roberts 1981) and in cattle, sheep and goats relies upon the salts of three compounds: the phenanthridine, homidium; the phenanthridine aromatic amidine, isometamidium; and the aromatic diamidine, diminazene. Despite the proven efficacy of these compounds in the field over the past 30 years, chemotherapy of trypanosomiasis is beset with a number of problems: a limited range of compounds, the occurrence of drug resistance and drug toxicity (Williamson 1976, Leach and Roberts 1981).

Diminazene aceturate (Berenil, Hoechst; Veriben, Sanofi Animal Health) was introduced in 1955 as a therapeutic agent for the treatment of *Trypanosoma congolense, T vivax* and *T brucei* infections in cattle, sheep and goats. The manufacturers of the compound recommend that it should be administered to animals via the intramuscular route at doses of either 3·5 or 7 mg kg^{-1} bodyweight, and for some time after its introduction use of the compound was rarely associated with the development of drug resistance (Leach and Roberts 1981). However, subsequently, there have been reports of resistance to diminazene from parts of Africa (Jones-Davies 1967, Kupper and Wolters 1983, Mbwambo et al 1988), though whether the resistance described in these reports was true resistance to diminazene or cross resistance as a result of using other compounds is unclear (Leach and Roberts 1981).

Under ideal circumstances, when resistance to diminazene develops in the field, it is recommended that use of the compound should be terminated and that it be replaced with a compound that is chemically unrelated (that is, homidium or isometamidium [Whiteside 1958]). However, this is often not possible due to logistical and financial factors (Holmes and Scott 1982).

Studies in goats have shown that following intramuscular administration of diminazene aceturate at a dose of 3·5 mg kg^{-1} bodyweight,

*Reprint requests to Dr A. S. Peregrine

a maximal plasma concentration of 4·2 μg diminazene ml $^{-1}$ is attained 48 minutes after administration, and that the plasma fall-off is tri-exponential with an elimination half-life of 14 to 29 hours (Aliu et al 1984). Baggot (1978) has suggested that if a drug has a half-life in excess of nine hours, initiation of therapy with a priming dose and maintenance of blood levels by administering a second dose at an appropriate time interval is likely to be more successful than treatment with a single large dose. Studies on asinine and canine babesiosis have demonstrated that if diminazene aceturate is administered in two doses, 24 hours apart, it is safer and more efficacious than a single large dose (Singh et al 1980, Farwell et al 1982). Since the plasma concentration of diminazene in goats declines rapidly following administration, single dose therapy with diminazene aceturate, the recommended regime for treatment of African animal trypanosomiasis, may be responsible for the apparent lack of efficacy observed with diminazene in some areas. The aim of this study was therefore to determine whether double treatment with diminazene aceturate would enhance the therapeutic activity of the compound in goats as compared to single treatment.

Materials and methods

Animals

Adult castrated male East African cross Galla goats weighing 20 to 44 kg were obtained from a tsetse-free area of Kenya. The housing and management of these animals were as described by Whitelaw et al (1986).

Outbred Swiss white mice, weighing 25 to 35 g were obtained from the ILRAD breeding colony. This colony was derived from an OLAC (Oxfordshire Laboratory Animal Colony) 1976 strain.

Trypanosomes

Two populations of *T congolense* were used in this study: IL 3274 and IL 1180.

T congolense IL 3274 is a cloned population and is a derivative of field isolate Bankeledaga/83/CRTA/67, collected from a cow in Burkina Faso in 1983 (Pinder and Authie 1984). The derivation of clone IL 3274 from the field isolate was described by Peregrine et al (1991). In goats, IL 3274 was shown to be refractory to intramuscular treatment with diminazene aceturate at a dose of 7 mg kg^{-1} bodyweight when treatment was administered after trypanosomes were detected in jugular blood (A. S. Peregrine, unpublished data). In mice, IL 3274 has a diminazene aceturate 50 per cent curative dose (CD_{50}) value of 12·4 mg kg^{-1} bodyweight (Peregrine et al 1991).

T congolense IL 1180 is a double-cloned derivative of isolate Serengeti/71/STIB/212. The derivation of IL 1180 from this isolate has been described by Nantulya et al (1984). Previous studies have shown that this clone expresses a high level of sensitivity to diminazene in mice (CD_{50} = 2·3 mg kg^{-1} bodyweight [Peregrine et al 1991]) and in cattle (A. S. Peregrine, unpublished data).

Characterisation of T congolense *IL 3274 and* T congolense *IL 1180 in vitro*

The sensitivity of *T congolense* IL 3274 and *T congolense* IL 1180 to diminazene was determined in vitro (M. A. Gray, personal communication). Briefly, bloodstream forms collected from infected mice were adapted to in vitro cultivation and grown as epimastigote forms. Once established as epimastigote cultures they transformed to metacyclic trypanosomes (Gray et al 1987). Subsequently, samples of media containing 2×10^5 metacyclic trypanosomes ml^{-1} and various concentrations of diminazene aceturate (0·05 to 50 μg ml^{-1}), were incubated at 35°C in 5 per cent carbon dioxide in air for 48 hours. Samples (200 μl) of each drug dilution containing trypanosomes were then placed on a confluent monolayer of bovine aortic-endothelial cells. A complete medium change with medium free of drug was made two hours later and thereafter at 48 hour intervals. The sensitivity of the two *T congolense* clones was subsequently determined on the basis of whether or not, after five days incubation in medium free of drug, trypanosomes remained intact and viable. Previous work has indicated that viable trypanosomes present on day 5 are infective for mice (M. A. Gray, personal communication).

Tsetse flies

Teneral male *Glossina morsitans centralis*,

obtained from the ILRAD breeding colony, were infected with *T congolense* IL 3274 or *T congolense* IL 1180 by allowing them to feed on goats infected with either of the two populations. The techniques for producing infected flies, for ascertaining their infectivity and for using them to infect experimental cattle have been described by Whitelaw et al (1986). Goats were infected by allowing five infected tsetse flies to feed on the left flank of each animal.

Treatment with diminazene aceturate

Diminazene aceturate (Berenil; Hoechst, lot number 093D574) was freshly prepared by dissolving in sterile distilled water, at room temperature, to make a 7 per cent w/v solution. Doses of 7·2 mg kg^{-1} bodyweight were injected either singly or on two occasions, eight or 24 hours apart, deep into the right musculus semitendinosus.

Clinical observations

Skin thickness at the site where each tsetse was to be fed, and the breadth of the prefemoral lymph node draining the site of tsetse bites, were measured for three days before infection, and daily for 30 days following infection. Measurements were conducted with vernier callipers.

The rectal temperature of each animal was monitored daily for 30 days following infection and then three times weekly for a further 60 days.

The packed red blood cell volume percentage (PCV) was monitored daily until 90 days after infection, using a microhaematocrit centrifuge. Subsequently, the PCV was determined three times a week for a further 30 days.

Bodyweights were determined weekly throughout the experiment and when required for calculating drug doses. Observations for pain at the site of injection and signs of systemic toxicity were made immediately after treatment, then daily for seven days.

Monitoring of goat infections

Jugular blood samples were collected daily for 90 days following infection, into vacutainer tubes coated with potassium ethylenediamine tetraacetate (EDTA). Thereafter, for a further 30 days, blood samples were collected three times a week. Blood samples were examined for the presence of trypanosomes using the buffy-coat phase-contrast technique (Murray et al 1977) and the level of parasitaemia quantified.

In an attempt to detect levels of parasitaemia below the lower limit of detection of the buffy-coat phase-contrast technique, 0·5 ml samples of jugular blood were inoculated intraperitoneally into sublethally irradiated (600 rads) mice every two weeks following infection of the goats. Mice were then monitored three times a week, for 60 days, for the presence of parasites, by examining wet films of tail blood.

Experimental design

Twenty-three goats were weight-matched into four groups of five animals each (groups A, B, C and D) and one group of three animals (group E). Each goat was challenged with five infective tsetse flies and treated intramuscularly with 7·2 mg diminazene aceturate kg^{-1} bodyweight (Table 1). Animals in group A received a single dose of diminazene aceturate three days after all the animals had been detected parasitaemic to confirm that IL 3274 was resistant to this dosage regimen. Animals in groups B and C were also treated three days after all the animals in each group had been detected parasitaemic. However, they were then retreated with the same drug dosage eight or 24 hours later, respectively; intervals which were selected on the basis of previous pharmacokinetic studies (Aliu et al 1984) and which were deemed to be practical in the field. Since Jennings et al (1979) have demonstrated that, in mice, populations of *T brucei* may invade the central nervous system (CNS) within 14 days of infection, and that diminazene aceturate will not eliminate these infections, it was possible that the appearance of trypanosomes in animals in groups A, B and C after treatment was not due to drug resistance, but to invasion of a site, or sites, within the host which was inaccessible to diminazene. Thus, animals in group D were treated 24 hours after infection (at a time when trypanosomes still appear to be localised in the skin [Emery and Moloo 1981] and have not had access to sites within the body which are inaccessible to diminazene) to demonstrate whether IL 3274 was truly resistant to the drug dosage used. Animals in group E were infected with *T congolense* IL 1180 and treated after all the animals

TABLE 1: Experimental design, level of parasitaemia in experimental goats and response to treatment

	Group A	B	C	D	E
T congolense infection*	IL 3274	IL 3274	IL 3274	IL 3274	IL 1180
Prepatent periods (days)	13·4 ± 0·5	13·6 ± 1·1	14·0 ± 1·4	NT	12·0 ± 1·0
Day of treatment	19	19	19 + 20	1	19
Treatment with diminazene aceturate	7·2 mg kg^{-1} × 1	7·2 mg kg^{-1} × 2 8 h apart	7·2 mg kg^{-1} × 2 24 h apart	7·2 mg kg^{-1} × 1 24 h after infection	7·2 mg kg^{-1} × 1
Level of parasitaemia when treated	5·0 × 10^5 ml^{-1}	5·0 × 10^5 ml^{-1}	5·0 × 10^5 ml^{-1}	0·0 ml^{-1}	1·0 × 10^4 ml^{-1}
Number relapsed/ number treated	5/5	3/5	4/5	0/5	0/5
Relapse period (days)	9·4 ± 2·5	11·7 ± 2·1	14·3 ± 1·0	NT	NT

* Five infected *Glossina morsitans centralis* used to infect each animal
× 1 Treated once
× 2 Treated twice
h Hours
NT No trypanosomes detected

in the group had been detected parasitaemic to confirm the efficacy of a single dose of diminazene aceturate against a sensitive population.

High-performance liquid chromatography

At one, eight, nine, 24, 25 and 48 hours after administration of diminazene aceturate to the goats in groups B and C, 10 ml whole blood samples were drawn from the left jugular vein of each animal into EDTA-containing evacuated tubes. The samples were centrifuged at 1230 *g* and plasma subsequently separated and stored at −70°C until analysed. Intact diminazene in the plasma was extracted by an ion-pair technique and quantified by high-performance liquid chromatography (Aliu and Odegaard 1983).

Results

Development of parasitaemia

All animals except those in group D became parasitaemic following feeding of infective tsetse. The mean pre-patent periods were 13·6 ± 1·4 days for animals infected with *T congolense* IL 3274 and 12 ± 1 days for animals infected with *T congolense* IL 1180 (Table 1). In all animals, once trypanosomes were detected following infection they continued to be detected on every occasion until the day of treatment. At the time of treatment, animals in groups A, B and C, infected with *T congolense* IL 3274, had a mean level of parasitaemia of 5 × 10^5 trypanosomes ml^{-1} of jugular blood, while animals in group E, infected with *T congolense* IL 1180, had a mean level of parasitaemia of 1 × 10^4 ml^{-1}. All animals in groups A, B, C and E were treated on day 19 after infection. Animals in group D, treated 24 hours after infection, were aparasitaemic at the time of treatment.

Following treatment, animals infected with *T congolense* IL 3274 became aparasitaemic within an average interval of 48 hours. In contrast, animals infected with *T congolense* IL 1180 became aparasitaemic within an interval of 24 hours (data not given). In all animals in group A (which received treatment with a single dose of diminazene aceturate), trypanosomes reappeared following treatment within an interval of 9·4 ± 2·5 days. Similarly, in three of the five animals in group B and four of the five animals in group C, which received repeat treatment with diminazene aceturate, parasites reappeared following treatment within mean intervals of 11·7 ± 2·1 and 14·3 ± 1 days, respectively. In contrast, all five animals in group D, treated 24 hours after infection, were never subsequently detected parasitaemic. Similarly, all three animals in group E, infected with *T congolense* IL 1180 and treated with diminazene aceturate following detection of trypanosomes in jugular blood, were never detected parasitaemic following treatment.

All animals in which trypanosomes reappeared following treatment were detected parasitaemic on every occasion a blood sample was collected until day 47 after infection, when they were removed from the experiment because their PCVs had fallen below 18 per cent. Animals in which

trypanosomes could not be detected on day 47 (two in group B, one in group C, all five in group D and all three in group E) remained aparasitaemic for the entire observation period (120 days) following infection, both by phase-contrast examination of the buffy coat and by inoculation of blood into mice.

PCV

Following infection there was a progressive reduction in PCV in all animals, except those in group D, until treatment was administered on day 19 following infection. All animals in group D, which were treated 24 hours after infection with *T congolense* IL 3274, were never detected parasitaemic and maintained their PCV above 25 per cent.

Skin-fold thickness

Significant increases in skin-fold thickness occurred at the site of tsetse bites in all animals except those in group D (treated 24 hours following infection) (Table 2). The maximum skin-fold thickness was attained on days 12 to 13 in all animals infected with *T congolense* IL 3274 and on day 9 in animals infected with *T congolense* IL 1180. Infections with *T congolense* IL 1180 (group E) resulted in a significantly larger increase in skin-fold thickness than that which occurred in goats following infection with *T congolense* IL 3274 (groups A, B and C).

Breadth of draining prefemoral lymph node

Significant increases in the breadth of the prefemoral lymph node draining the site of tsetse bites occurred in all experimental animals (Table 2). This increase in breadth was detected as early as three days after infection (data not given) and was at a maximum on approximately day 13 following infection for animals in groups A, B, C and D, infected with *T congolense* IL 3274, and on day 12 for animals in group E, infected with *T congolense* IL 1180.

Characterisation of T congolense *IL 3274 and* T congolense *IL 1180 in vitro*

Using a culture system in which metacyclic trypanosomes were incubated in the presence of various drug concentrations for 48 hours, *T congolense* IL 3274 was shown to be resistant to 10 μg diminazene aceturate ml $^{-1}$ media, but sensitive to 50 μg ml $^{-1}$. In contrast *T congolense* IL 1180 was resistant to 1 μg ml $^{-1}$ but sensitive to 5 μg ml $^{-1}$.

High-performance liquid chromatography

Analysis of plasma samples from goats in groups B and C demonstrated that the mean plasma concentration of diminazene from one to 48 hours after treatment was in excess of 1·52 ± 0·11 μg ml $^{-1}$ (data not shown).

Discussion

This study has demonstrated that both the time of treatment in relation to the stage of infection with *T congolense*, and the number of treatments, influence the therapeutic activity of diminazene in goats.

In the work described here, infections with *T congolense* IL 3274 were not eliminated in any of

TABLE 2: Mean skin-fold thickness and prefemoral lymph node breadth in animals infected with *T congolense* IL 3274 or *T congolense* IL 1180

	Group A	B	C	D	E
T congolense infection	IL 3274	IL 3274	IL 3274	IL 3274	IL 1180
Skin-fold thickness					
Days to maximum size	11·8 ± 1·1	12·0 ± 1·0	13·4 ± 1·5	–	9·7 ± 0·6
% increase in size	160	210	160	–	260
Prefemoral lymph node breadth					
Days to maximum size	12·6 ± 1·1	12·4 ± 0·9	12·6 ± 0·5	16·8 ± 4·7	11·0 ± 1·0
% increase in size	200	310	270	220	400

– No increase in thickness

the animals in group A. In contrast, infections were eliminated in two animals in group B and in one animal in group C. Thus, repeat treatment with diminazene aceturate at a dose of 7·2 mg kg^{-1} bodyweight eliminated the infection in three out of 10 animals. Furthermore, in the other seven animals reappearance of trypanosomes following treatment was significantly delayed compared to animals in group A, treated with diminazene aceturate on only one occasion. Since the mean plasma concentration of diminazene over the first 48 hours following treatment was $1{\cdot}52 \pm 0{\cdot}11$ μg ml^{-1} for animals in groups B and C, and metacyclic trypanosomes of *T congolense* IL 3274 remained viable after 48 hours incubation in vitro in media containing diminazene aceturate at a concentration of 10 μg ml^{-1}, the inability of double treatment with diminazene aceturate to eliminate infections with IL 3274 in seven out of 10 animals was not surprising. It would therefore appear that when animals are infected with trypanosomes that express a high level of resistance to diminazene, double treatment with diminazene aceturate, at either eight or 24-hour intervals, does not greatly enhance the therapeutic activity of the drug.

Whether *T congolense* IL 3274 is truly resistant to diminazene in goats is open to question as a result of findings with animals in group D. These animals were infected with IL 3274 via the bites of infected tsetse and treated 24 hours later with diminazene aceturate at a dose of 7·2 mg kg^{-1} bodyweight. However, in contrast to animals in group A, trypanosomes were not detected in any of the five animals following treatment. Thus, *T congolense* IL 3274 appeared to be sensitive to diminazene when goats were treated 24 hours after feeding of infected tsetse (group D), but resistant when treated on day 19 (group A). There is, therefore, an apparent difference in sensitivity of IL 3274 to diminazene between day 1 and day 19 after tsetse fly-transmitted infection. The basis of this phenomenon is unclear.

The development of local skin reactions, or chancres, at the site of inoculation of metacyclic trypanosomes by tsetse is a well-recognised phenomenon in man (Fairbairn and Godfrey 1957). A similar lesion has also been observed in goats following tsetse transmission of *T congolense* (Emery and Moloo 1981) and was produced in the study described here by both *T congolense* IL 3274 and *T congolense* IL 1180. Fluid expressed from these lesions has been shown to contain trypanosomes several days before they can be detected in the peripheral blood (Roberts et al 1969). Furthermore, microscopic examination of such lesions seven days after tsetse infected with *T congolense* have fed on rabbits, has demonstrated the presence of trypanosomes in the dermal connective tissue (Luckins and Gray 1978) which have been termed 'local-reaction' or 'lesion' forms (Roberts et al 1969). It is, therefore, possible that trypanosomes would be present in this extravascular site 24 hours after tsetse have fed and that the apparent sensitivity of IL 3274 to diminazene on day 1 is because the life-cycle stage of IL 3274 present in the goat on day 1 differs in its sensitivity to diminazene from that present on day 19 (that is, bloodstream forms). Trypanosomes which are morphologically similar to lesion forms of *T congolense* have been observed in vitro (Gray et al 1979). However, in vitro, this life-cycle stage does not appear to differ from metacyclic forms in its sensitivity to diminazene (M. A. Gray, unpublished data).

A second factor that might be responsible for the apparent difference in drug sensitivity of *T congolense* IL 3274 between day 1 and day 19 could be a difference in the pharmacokinetics of diminazene between the blood and the dermis. Aliu et al (1984) have described the pharmacokinetics of diminazene in the blood of goats but no data exist for the dermis. It is possible that significantly higher concentrations of diminazene are attained in the dermis than in blood and this possibility is currently under investigation.

Invasion of a site, or sites, within the goat which is, or are, inaccessible to diminazene is a third factor that could be responsible for the apparent drug resistance expressed by IL 3274 on day 19. Such a phenomenon has been described in mice infected with *T brucei* (Jennings et al 1977, 1979). Treatment with diminazene aceturate eliminated infections if the drug was administered seven days after infection. However, trypanosomes reappeared following administration of the drug if treatment was delayed until 14 days after infection. The appearance of trypanosomes was subsequently shown to be associated with trypanosomes that had invaded the CNS. While *T brucei* has been demonstrated to occur within the CNS of cattle (Masake et al 1984) and *T vivax* has been detected in the aqueous humour of goats (Whitelaw et al 1988), *T congolense* has only been demonstrated

within the CNS of ruminants when animals were infected simultaneously with both *T congolense* and *T brucei* (Masake et al 1984).

One further possible explanation for the apparent difference in sensitivity of IL 3274 between day 1 and day 19 of infection is that within this period the level of drug resistance expressed by the trypanosomes had altered. Peregrine et al (1991) have conducted experiments in mice which demonstrate that resistance to isometamidium is not a stable phenotype in *T congolense*. It is possible that the same holds true for diminazene.

Finally, there were clear differences between *T congolense* IL 3274 and *T congolense* IL 1180 in terms of the chancre development elicited and the reaction that occurred in the prefemoral lymph node draining the site of tsetse bites. Infection with *T congolense* IL 1180 resulted in an increase in skin-fold thickness that was larger, and occurred earlier, than that elicited by *T congolense* IL 3274. The same was true for the reaction elicited in the draining prefemoral node. For both IL 1180 and IL 3274, the maximum increase in skin-fold thickness occurred one to two days before the detection of trypanosomes in the blood. Furthermore, enlargement of the draining lymph node accompanied the chancre reaction in all animals in groups A, B, C and E. This is consistent with the results of previous studies in goats (Emery and Moloo 1981). However, unlike those studies, in the study described here, enlargement of the draining lymph node was not necessarily indicative of ensuing parasitaemia: goats in group D, treated 24 hours after infection, exhibited significant increases in the breadth of the lymph node draining the site of tsetse bites but were not subsequently detected parasitaemic. In a similar manner, studies in rabbits have demonstrated that development of a chancre is not always indicative of subsequent systemic infection (Silayo 1984).

Acknowledgements

The authors thank Mr F. McOdimba, Mr A. Adema, Mr S. Kemei and the staff of ILRAD's Tsetse Vector Laboratory for technical assistance. Thanks are also due to Mr J. Scott for his help with the analysis of the data, Ms P. Otieno and Ms D. Lewa for secretarial assistance and Dr A. Teale for comments on the manuscript. M.A.G. received financial support for participation in this work from the Overseas Development Administration of the United Kingdom. This is ILRAD publication number 986.

References

ALIU, Y. O. & ODEGAARD, S. (1983) Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in plasma. *Journal of Chromatography* **276**, 218-223

ALIU, Y. O., ODEGAARD, S. & SOGNEN, E. (1984) Diminazene/Berenil: Bioavailability and disposition in dairy goats. *Acta Veterinaria Scandinavica* **25**, 593-596

BAGGOT, J. D. (1978) Some aspects of clinical pharmacokinetics in veterinary medicine. II. *Journal of Veterinary Pharmacology and Therapeutics* **1**, 111-118

EMERY, D. L. & MOLOO, S. K. (1981) The dynamics of the cellular reactions elicited in the skin of goats by *Glossina morsitans morsitans* infected with *Trypanosoma (Nannomonas) congolense* or *T (Duttonella) vivax*. *Acta Tropica* **38**, 15-28

FAIRBAIRN, H. & GODFREY, D. G. (1957) The local reaction in man at the site of infection with *Trypanosoma rhodesiense*. *Annals of Tropical Medicine and Parasitology* **51**, 464-470

FARWELL, G. E., LeGRAND, E. K. & COBB, C. C. (1982) Clinical observations on *Babesia gibsoni* and *Babesia canis* infections in dogs. *Journal of the American Veterinary Medical Association* **180**, 507-511

GRAY, M. A., BROWN, C. G. D., LUCKINS, A. G. & GRAY, A. R. (1979) Maintenance of infectivity of *Trypanosoma congolense in vitro* with explants of infected skin at 37°C. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **73**, 406-408

GRAY, M. A., HIRUMI, H. & GARDINER, P. R. (1987) Salivarian trypanosomes: insect forms. In *In vitro* Methods for Parasite Cultivation. Eds A. E. R. Taylor and J. R. Baker. London, Academic Press. pp118-152

HOLMES, P. H. & SCOTT, J. M. (1982) Chemotherapy against animal trypanosomiasis. In Perspectives in Trypanosomiasis Research. Ed J. R. Baker. Chichester, Research Studio Press. pp59-69

JENNINGS, F. W., WHITELAW, D. D. & URQUHART, G. M. (1977) The relationship between duration of infection with *Trypanosoma brucei* in mice and the efficiency of chemotherapy. *Parasitology* **75**, 143-153

JENNINGS, F. W., WHITELAW, D. D., HOLMES, P. H., CHIZYUKA, H. G. B. & URQUHART, G. M. (1979) The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal for Parasitology* **9**, 381-384

JONES-DAVIES, W. J. (1967) The discovery of Berenil-resistant *Trypanosoma vivax* in northern Nigeria. *Veterinary Record* **80**, 531-532

KUPPER, W. & WOLTERS, M. (1983) Observations on drug resistance of *Trypanosoma (Nannomonas) congolense* and *Trypanosoma (Duttonella) vivax* in cattle in a feedlot in the northern Ivory Coast. *Tropenmedizin und Parasitologie* **34**, 203-205

LEACH, T. M. & ROBERTS, C. J. (1981) The present status of chemotherapy and chemoprophylaxis of animal trypanosomiasis in the Eastern Hemisphere. *Pharmacology and Therapeutics* **13**, 91-147

LUCKINS, A. G. & GRAY, A. R. (1978) An extravascular site of development of *Trypanosoma congolense*. *Nature* **272**, 613-614

MASAKE, R. A., NANTULYA, V. M., AKOL, G. W. O. & MUSOKE, A. J. (1984) Cerebral trypanosomiasis in cattle with mixed *Trypanosoma congolense* and *T brucei brucei* infections. *Acta Tropica* **41**, 237-246

MBWAMBO, H. A., MELLA, P. N. P. & LEKAKI, K. A. (1988) Berenil (diminazene aceturate)-resistant *Trypanosoma congolense* in cattle under natural tsetse challenge at Kibaha, Tanzania. *Acta Tropica* **45**, 239-244

MURRAY, M., MURRAY, P. K. & McINTYRE, W. I. M. (1977) An improved parasitological technique for diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **65**, 254-255

NANTULYA, V. M., MUSOKE, A. J., RURANGIRWA, F. R. & MOLOO, S. K. (1984) Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe-passaged infections. *Infection and Immunity* **43**, 735-738

PEREGRINE, A. S., KNOWLES, G., IBITAYO, A. I., SCOTT, J. R., MOLOO, S. K. & MURPHY, N. B. (1991) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* **102**, 93-100

PINDER, M. & AUTHIE, E. (1984) The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica* **41**, 247-252

ROBERTS, C. J., GRAY, M. A. & GRAY, A. R. (1969) Local skin reactions in cattle at the site of infection with *Trypanosoma congolense* by *Glossina morsitans* and *G tachinoides*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **63**, 620-624

SILAYO, R. S. (1984) *Trypanosoma congolense*: chemotherapy and immunity to cyclically transmitted infections in rabbits. PhD thesis, Centre for Tropical Veterinary Medicine, University of Edinburgh

SINGH, B., BANERJEE, D. P. & GAUTAM, O. P. (1980) Comparative efficacy of diminazene diaceturate and imidocarb dipropionate against *Babesia equi* infection in donkeys. *Veterinary Parasitology* **7**, 173-179

WHITELAW, D. D., BELL, I. R., HOLMES, P. H., MOLOO, S. K., HIRUMI, H., URQUHART, G. M. & MURRAY, M. (1986) Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. *Veterinary Record* **118**, 722-726

WHITELAW, D. D., GARDINER, P. R. & MURRAY, M. (1988) Extravascular foci of *Trypanosoma vivax* in goats: the central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. *Parasitology* **97**, 1-11

WHITESIDE, E. F. (1958) The maintenance of cattle in tsetse-infested country. A summary of four years' experience in Kenya. Report of International Scientific Council for Trypanosomiasis Research, 7th meeting, Brussels, Belgium, 1958. CCTA Publication 41, pp83-90

WILLIAMSON, J. (1976) Chemotherapy of African trypanosomiasis. *Tropical Diseases Bulletin* **73**, 531-542

Received June 10, 1991
Accepted January 13, 1992

Section 4.2

Mamman, M., Katende, J., Moloo, S.K. and Peregrine, A.S. (1993) Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology* 50, 1-14.

Veterinary Parasitology, 50 (1993) 1–14
0304-4017/93/$06.00

Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats

M. Mamman, J. Katende, S.K. Moloo, A.S. Peregrine*
International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya

(Accepted 11 February 1993)

Abstract

Twenty-five goats were randomly allocated to five groups of five animals each and infected with *Trypanosoma congolense* IL 3274 via the bites of infected *Glossina morsitans centralis*. At intervals of 1, 4, 8, 12 or 19 days following infection, each group of five animals was treated intramuscularly with diminazene aceturate at a dose of 7.0 mg kg^{-1} body weight (b.w.). While treatment on Day 1 eliminated infections in all five goats, treatment on Day 19 did not cure any of the animals; in groups treated 4, 8 or 12 days following infection, two of five goats in each group were cured. Since the alteration in apparent resistance of *T. congolense* IL 3274 between Day 1 and Day 19 could have been due to alteration in expression of drug resistance by trypanosomes as the population expanded, the experiment was repeated using trypanosomes that reappeared in the animals that had been treated with diminazene aceturate on Day 19. On Day 36, when all five animals were parasitaemic, five groups of teneral *G. m. centralis*, each containing 160 flies, were fed on one occasion on each of the five goats (one group of testse flies per goat). Thereafter, each group of tsetse flies was maintained on clean rabbits. When infective, five flies from each group were allowed to feed on two naive goats each (i.e. two goats per group of tsetse flies). One animal in each pair was treated 24 h after infection with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w., the other was treated on Day 19, when parasitaemic, with the same drug dosage. As before, treatment 24 h following infection eliminated infections in all animals, but when treatment was delayed until Day 19, trypanosomes in all animals were refractory to treatment. Thus, although tsetse flies were infected with trypanosomes that had arisen in infected goats following treatment with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w., when the same flies were allowed to feed on clean goats, the resultant infections were sensitive to treatment with the same drug dosage when administered 24 h following infection. These data therefore indicate that there is a significant alteration in diminazene sensitivity of IL 3274 between Day 1 and Day 19 and that this is associated with an alteration in the resistance phenotype of the trypanosomes.

Introduction

Diminazene aceturate (Bauer, 1955) is the most commonly used agent for treating trypanosomosis in domestic livestock and is marketed as a commercial preparation (Berenil®, Veriben®) consisting of 44.5% diminazene ace-

*Corresponding author.

turate and 55.5% of the chemical stabilizer antipyrine. While reports of resistance to diminazene in the field continue to be made (Jones-Davies, 1967; MacLennan and Na'Isa, 1970; Kupper and Wolters, 1983; Mbwambo et al., 1988), the mechanisms of resistance in trypanosomes are poorly understood. Studies in vitro have demonstrated pronounced differences among trypanosome populations concerning their sensitivity to diminazene (Brun and Kunz, 1989; Kaminsky et al., 1989; Silayo et al., 1992), thus showing that innate differences in drug sensitivity may play a role. However, since the central nervous system is believed to be inaccessible to diminazene (Gutteridge and Coombs, 1977) and may be a source of relapse infections following treatment of mice infected with *Trypanosoma brucei* (Jennings et al., 1979) and goats infected with *T. vivax* (Whitelaw et al., 1988), the ability of a trypanosome population to be refractory to treatment may also be dependent upon the ability of trypanosomes to gain entry to such sites.

In preliminary work, Silayo et al. (1992) reported a study that was carried out in goats infected with a clone of *T. congolense*: IL 3274. Infections were initiated in goats via the bites of infected *Glossina morsitans centralis* and animals were cured if treated 24 h after challenge with a single intramuscular (i.m.) dose of 7.0 mg diminazene aceturate kg^{-1} body weight (b.w.). However, if treatment with the same drug dosage was delayed until 19 days after infection, trypanosomes reappeared in all goats within 7–12 days following treatment. The work described here was carried out to elucidate the mechanism(s) behind these observations.

Materials and methods

Experiment animals

Adult castrated male East African Galla cross-bred goats were obtained from an area of Kenya known to be free of tsetse flies. The goats weighed 15–28 kg and were maintained as described previously (Whitelaw et al., 1986). Prior to the experiment, sera were collected from all goats and examined for the presence of trypanosome antigens (Nantulya and Lindqvist, 1989) and anti-trypanosome antibodies (see below), using enzyme-linked immunosorbent assays (ELISAs).

Outbred C_3H.He mice weighing 20–35 g were obtained from the ILRAD breeding colony (originally derived from Ola:MF1 and hairy progeny MF1-hr/Ola). Mice were allowed access to water and a pelleted ration (Unga Feeds Ltd., Nairobi) on an ad libitum basis.

Trypanosomes

Trypanosoma congolense clone IL 3274 (Peregrine et al., 1991) is a derivative of stock Banankeledaga/83/CRTA/67, which was isolated from a bull

in Burkina Faso (Pinder and Authie, 1984). Earlier work has shown that this stock expresses high levels of resistance to both diminazene aceturate and isometamidium chloride (Authie, 1984; Moloo and Kutuza, 1990). In goats, *T. congolense* IL 3274 has previously been shown to be resistant to single i.m. treatment with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w. when treatment was administered approximately 3 days after first detection of parasitaemia (Silayo et al., 1992). In mice, the diminazene aceturate CD_{50} value (dose of drug required to cure 50% of infected animals) for *T. congolense* IL 3274 is 12.4 mg kg^{-1} b.w. (Peregrine et al., 1991).

Trypanosoma congolense IL 1180 is a doubly cloned derivative of stock Serengeti/77/STIB/212 which was isolated from a lion in Tanzania (Geigy and Kauffmann, 1973). The derivation of *T. congolense* IL 1180 from this isolate has been described previously (Nantulya et al., 1984). In mice, the diminazene aceturate CD_{50} value of *T. congolense* IL 1180 is 2.3 mg kg^{-1} b.w. (Peregrine et al., 1991).

Tsetse flies

Teneral male *G. m. centralis* flies were infected with either *T. congolense* IL 3274 or *T. congolense* IL 1180 by allowing them to feed on infected goats. The methods used to infect flies, to identify those with mature infections and to infect animals, have been described previously (Whitelaw et al., 1986). Goats were infected by allowing five infected tsetse flies to engorge on the left flank of each animal.

Treatment of goats with diminazene aceturate

At different time intervals following tsetse infection (see Table 1), goats in each experimental group were treated with diminazene aceturate (Berenil®, Hoechst AG, Germany) at a dose of 7.0 mg kg^{-1} b.w., administered deep into the right vastus medialis muscle. A fresh 7.0% (w/v) solution of the drug was prepared in sterile distilled water on the day of administration. Berenil® batch Number 093D574 was used throughout this work.

Monitoring goat infections

For 7 days prior to infection and for 30 days following the infection, blood samples were collected daily from the left jugular vein of each goat into 10 ml evacuated tubes containing dipotassium ethylenediamine tetra-acetate. Blood samples were collected in a similar manner, three times a week, for a further 152 days. The packed red blood cell volume per cent (PCV) of each sample was determined prior to examination of the buffy coat for the presence of trypanosomes (Murray et al., 1977). The level of parasitaemia was scored in

accordance with the method of Paris et al. (1982). Throughout the experiment, sera were collected from each goat once a week and stored at −70°C until analysed for the presence of trypanosome antigens or anti-trypanosome antibodies.

The body weight of each goat was determined weekly throughout the study and whenever required for calculation of the drug dose for treatment.

Detection of low levels of parasitaemia

At 3 day intervals, from Day 7 to Day 22 following infection, 0.2 ml aliquots of blood (collected from each goat as described above) were inoculated intraperitoneally (i.p.) into sublethally irradiated (600 rads) mice (three mice per goat per day). Subsequently, the mice were monitored three times a week for 70 days for the presence of trypanosomes by microscopic examination (×250) of wet films of tail blood.

Detection of anti-trypanosome antibodies and trypanosome antigens

Sera obtained from goats were examined for the presence of *T. congolense*-specific antibodies using a modified version of the technique described by Vos et al. (1988) to detect antibodies to common antigens of *T. vivax*. Ten sublethally irradiated (600 rads) rats were infected with *T. congolense* ILNat 2.1 (Majiwa et al., 1985). At the first peak of parasitaemia, the rats were exsanguinated and the blood collected into sodium citrate (final concentration 0.8% w/v). The blood was centrifuged at 400×*g* for 15 min and trypanosomes in the buffy coat separated from blood cellular components using a diethylaminoethyl-cellulose (DE 52, Beckman, USA) column, in accordance with the method of Lanham and Godfrey (1970). A lysate was then prepared by freezing and thawing the trypanosomes on one occasion, followed by sonication on ice for 5 min. The lysate was then centrifuged at 18 000×*g* for 1 h and the supernatant concentrated by ultrafiltration overnight at 4°C through a PM 10 membrane (Amicon, Massachussets, USA). The resultant concentrate was then centrifuged at 1230×*g* for 30 min and the supernatant fractionated on a DE 52 column (Beckman, USA). The absorbance of each fraction was determined at 260 nm and 280 nm on a DU-50 spectrophotometer (Beckman, USA) to determine the protein concentration. Each fraction was then stored at −70°C until screened for its ability to detect serum anti-trypanosome antibodies. A selected fraction with optimal activity was prepared in Dulbecco's phosphate buffered saline (DPBS), pH 7.4, and used to coat 96-well flat-bottomed microtitre plates (Nunc-Immuno Plate, Polysorp, Denmark); 20 μg of protein, in 100 μl, was added to each well and incubated overnight at 4°C. The method of McGuire et al. (1979) was used to prepare rabbit anti-goat IgG/IgM. This reagent was subsequently conjugated to hor-

seradish peroxidase (Wilson and Nakane, 1978). In experiments to screen sera for anti-trypanosome antibodies, sera collected from goats (as described above) were screened at an initial dilution of 1:250 (v/v) in bovine lymphocyte lysate (BLL) (Katende et al., 1987) containing 0.01% (v/v) Tween 20 (Sigma Chemical Co., USA). Further dilutions of sera were made in BLL diluted 1:2 (v/v) with the buffer (DPBS+0.01% (v/v) Tween 20) that was used for washing the microtitre plates; diluted sera were added to ELISA plates and incubated at room temperature for 30 min. The horseradish peroxidase-conjugate was then added and used at a dilution of 1:2000 (v/v) in the same buffer that was used for dilutions of sera and incubated for 1 h at 37°C. After washing the plates, a solution containing sodium citrate buffer (pH 4.0), 1.0% (v/v) hydrogen peroxide and the chromogen 2, 2′-azinobis (3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS, Serva, USA) was added to each plate. The ELISA plates were then kept in the dark at room temperature for 1 h to allow colour development (Katende et al., 1990). Thereafter, absorbance was measured at 414 nm in a Titertek Multiskan MCC/340 (Flow Laboratories, Finland).

Sera from goats were also examined for the presence of *T. congolense* antigen using an antigen-capture ELISA described by Nantulya and Lindqvist (1989).

Experiment designs

Experiment 1

The design of Experiment 1 is shown in Table 1. Twenty-five goats were allocated to five groups of five animals each (Groups A, B, C, D and E) and

Table 1
Experiment design and development of infection in goats treated with a single intramuscular dose of 7.0 mg diminazene aceturate kg^{-1} body weight on Days 1, 4, 8, 12 or 19 after challenge with tsetse flies infected with *Trypanosoma congolense* IL 3274 or IL 1180

Group	No. of goats infected	*T. congolense* challenge[a]	Time of treatment (days following infection)	Treatment with diminazene aceturate (mg kg^{-1} i.m.)	No. cured/ No. treated	First day detected parasitaemic following infection Mean ± SD (Range)
A	5	IL 3274	1	7.0	5/5	–[b]
B	5	IL 3274	4	7.0	2/5	47 ± 25 (30–76)
C	5	IL 3274	8	7.0	2/5	25 ± 3 (22–28)
D	5	IL 3274	12	7.0	2/5	26 ± 4 (23–31)
E	5	IL 3274	19	7.0	0/5	17 ± 2 (15–19)
F	5	IL 1180	19	7.0	5/5	12 ± 1 (11–13)

[a]Five infective *Glossina morsitans centralis* per goat.
[b]Parasitaemia was never detected in any of the five goats in this group.

infected with *T. congolense* IL 3274 via the bites of infective *G. m. centralis* (five tsetse flies per goat). A further five goats, constituting drug-sensitivity controls (Group F), were similarly infected but with flies infected with *T. congolense* IL 1180. Subsequent to the infection, a single dose of 7.0 mg diminazene aceturate kg^{-1} b.w. was administered i.m. to each goat in Groups A, B, C and D, on Days 1, 4, 8 and 12, respectively. Goats in Groups E and F were also treated i.m. with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w., however, treatment was administered on Day 19, approximately 3 days after all goats in these two groups were first detected parasitaemic.

Experiment 2

In Experiment 1, tsetse flies were infected by allowing them to feed on a goat that had been inoculated with a stabilate of *T. congolense* IL 3274. In order to determine whether the different diminazene sensitivity results observed in goats in Groups A and E (treated on Day 1 and Day 19, respectively, following infection with *T. congolense* IL 3274) were due to an alteration in the sensitivity of trypanosome populations to diminazene between Day 1 and Day 19, Experiment 1 was repeated with tsetse flies that were fed on goats in Group E after trypanosomes reappeared following treatment with diminazene aceturate. Briefly, groups of clean *G. m. centralis* were fed on each of the goats in Group E (160 flies per goat) on Day 36 following infection (17 days following treatment). Thereafter, each batch of flies was maintained on separate rabbits and allowed to feed every day, except at weekends, for 30 days. Those with mature infections were then identified as previously described (Dwinger et al., 1988), and infective flies from each group were fed on two normal goats each (five tsetse flies per goat). Twenty-four hours after the challenge, one goat in each pair received a single i.m. dose of 7.0 mg diminazene aceturate kg^{-1} b.w.; the other goat served as an infectivity control and was not treated until Day 19 following challenge. The five goats that were treated on Day 1 comprised Group Et(1). The five goats that were not treated until Day 19 are referred to as Group Et(19).

Results

Experiment 1

Development of infection

Following infection of animals with *T. congolense* IL 1180 (Group F), trypanosomes were first detected by the buffy-coat phase-contrast technique on Days 11–13 following infection. In contrast, the interval between infection and first detection of trypanosomes was significantly longer (15–19 days) in animals infected with *T. congolense* IL 3274 (Group E; Table 1). Unlike Groups A–D, animals in Groups E and F were not treated until after detec-

tion of trypanosomes. The responses of the animals in each experiment group to treatment are given in Table 1. While infections were eliminated in all animals in Groups A and F, treatment eliminated infections in only two of five animals in each of Groups B, C and D. Furthermore, infections in all five animals in Group E were refractory to treatment. The mean (± SD) and range of parasitaemia pre-patent periods observed in those animals that were detected parasitaemic following challenge in Groups B, C, D and E are given in Table 1.

Detection of low level parasitaemia by mouse inoculation

Trypanosomes were not detected in any of the mice that were inoculated with blood samples collected from goats in Groups A, B and D (Table 2). This was also true for four of the five goats in Group C, in which case trypanosomes were detected in mice inoculated with blood collected from one animal on Day 22 following infection (14 days following treatment). With animals in Group E, one of the five goats was shown to be parasitaemic by mouse inoculation on Day 10 following infection; on Days 16 and 19 all five goats were shown to be parasitaemic by this method. Although treatment on Day 19 with diminazene aceturate failed to eliminate infections in the five animals (see Table 1) only one of the five animals was demonstrated to be parasitaemic by mouse inoculation on Day 22 (Table 2). In Group F, one of the five animals was shown to be parasitaemic by mouse inoculation on Day 7. This increased to five of five animals on Days 13, 16 and 19 following infection. Treatment of animals in this group on Day 19 eliminated infections in all five animals (Table 1); none of the animals were demonstrated parasitaemic by mouse inoculation on Day 22 (Table 2). In general, inoculation of

Table 2
Detection of trypanosomes in goats by inoculation of blood into mice

Group	*T. congolense* challenge[a]	Day of treatment[b] after challenge	Day of blood collection following challenge[c]					
			7	10	13	16	19	22
A	IL 3274	1	0/5[d]	0/5	0/5	0/5	0/5	0/5
B	IL 3274	4	0/5	0/5	0/5	0/5	0/5	0/5
C	IL 3274	8	0/5	0/5	0/5	0/5	0/5	1/5
D	IL 3274	12	0/5	0/5	0/5	0/5	0/5	0/5
E	IL 3274	19	0/5	1/5	2/5	5/5	5/5	1/5
F	IL 1180	19	1/5	4/5	5/5	5/5	5/5	0/5

[a]Five infective *Glossina morsitans centralis* per goat.
[b]Intramuscular treatment with 7.0 mg diminazene aceturate kg^{-1} body weight.
[c]0.2 ml of each blood sample inoculated into three mice each.
[d]Value indicates number of goats in group shown to be parasitaemic by inoculation of blood into mice.

goat blood into mice resulted in earlier detection of trypanosomes than was achieved using the buffy-coat phase-contrast technique.

Antigen and antibody ELISAs

Table 3 shows the parasitaemia, serum trypanosome antigen and serum anti-trypanosome antibody profiles in all Experiment 1 animals for the first 35 days following infection. Of the five groups of goats infected with *T. congolense* IL 3274, the length of time between infection and treatment with diminazene aceturate was longest in Group E: 19 days. On Days 7 and 14 following feeding of tsetse flies, sera from goats in Group E were negative for anti-trypanosome antibody; trypanosome antigens were only detected in one ani-

Table 3
Levels of parasitaemia, serum trypanosomal antigen and serum anti-trypanosomal antibody in goats within Experiment 1: Days 0–35

Group	*T. congolense* challenge[a]	Day of treatment[b] after challenge		Days after challenge with infected tsetse					
				0	7	14	21	28	35
A	IL 3274	1	Parasitaemia[c]	0/5	0/5	0/5	0/5	0/5	0/5
			Serum antigen[d]	0/5	0/5	0/5	0/5	0/5	0/5
			Serum antibody[d]	0/5	0/5	0/5	0/5	0/5	0/5
B	IL 3274	4	Parasitaemia	0/5	0/5	0/5	0/5	0/5	1/5
			Serum antigen	0/5	0/5	0/5	1/5	0/5	0/5
			Serum antibody	0/5	0/5	0/5	0/5	0/5	1/5
C	IL 3274	8	Parasitaemia	0/5	0/5	0/5	0/5	2/5	3/5
			Serum antigen	0/5	0/5	1/5	1/5	2/5	2/5
			Serum antibody	0/5	0/5	2/5	2/5	2/5	2/5
D	IL 3274	12	Parasitaemia	0/5	0/5	0/5	0/5	1/5	3/5
			Serum antigen	0/5	0/5	1/5	1/5	0/5	1/5
			Serum antibody	0/5	0/5	1/5	1/5	0/5	2/5
E	IL 3274	19	Parasitaemia	0/5	0/5	0/5	1/5	1/5	5/5
			Serum antigen	0/5	0/5	1/5	3/5	1/5	0/5
			Serum antibody	0/5	0/5	0/5	4/5	4/5	4/5
F	IL 1180	19	Parasitaemia	0/5	0/5	5/5	0/5	0/5	0/5
			Serum antigen	0/5	0/5	4/5	2/5	4/5	1/5
			Serum antibody	0/5	0/5	2/5	2/5	3/5	2/5

[a]Five infective *Glossina morsitans centralis* per goat.
[b]Intramuscular treatment with 7.0 mg diminazene aceturate kg^{-1} body weight.
[c]No. of goats detected parasitaemic/No. goats treated, using the buffy-coat phase-contrast technique (Murray et al., 1977).
[d]No. of sera positive/No. of goat sera tested, using ELISAs.

mal on Day 14 (see Table 3). However, on Day 21, antibody and antigen were detected in four of five, and three of five goats, respectively. In contrast, neither antigen nor antibody were detected in sera from the five goats in Group A, and in two animals in each of Groups B, C and D throughout the entire 182-day sampling period (data not given); the same animals were never detected parasitaemic. Treatment therefore appeared to eliminate infections in all these animals. By contrast, in animals in Groups B, C and D that were detected parasitaemic following treatment, low anti-trypanosome antibody titres were detected from Day 14 following the challenge; detection of anti-trypanosome antibody was generally coincident with first detection of trypanosomes and/or antigenaemia in a given animal (Table 3). In goats infected with IL 1180 (Group F), in which treatment eliminated infections, both trypanosome antigen and anti-trypanosome antibody were detectable from Day 14 following the infection (Table 3). In some animals, anti-trypanosome antibody and/or trypanosome antigen were detected until Day 126 following the infection (data not shown).

Experiment 2

The five goats in Group Et(1), treated with diminazene aceturate 24 h after infection, were never detected parasitaemic by the buffy-coat phase-contrast technique during the entire 152-day observation period following treatment. In contrast, all five goats in Group Et(19) were detected parasitaemic 15–22 days following infection; trypanosomes were detected in four goats by Day 19; these goats received diminazene aceturate in a single dose of 7.0 mg kg^{-1} b.w. i.m. on Day 19. The same treatment was given to the other goat in this group on Day 22, the same day it was first detected parasitaemic. Infections in all five goats relapsed 7–12 days after the treatment.

Discussion

Trypanosoma congolense clone IL 3274 was transmitted to goats via the bites of infective *G. m. centralis* and animals were treated with diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w. at various intervals between Day 1 and Day 19 following infection. While infections were eliminated in all goats that received treatment on Day 1, infections relapsed in all goats that were treated on Day 19 (after detection of trypanosomes). There was, therefore, a significant alteration in the sensitivity of *T. congolense* IL 3274 to diminazene between Day 1 and Day 19 following tsetse-transmitted infections, confirming an earlier study (Silayo et al., 1992). In order to ascertain when the alteration in sensitivity between Day 1 and Day 19 occurred, groups of five goats were each infected in a similar manner and treated on Days 4, 8 or 12 following infection; infections in three of five goats in each group were refrac-

tory to treatment. Thus, although the trypanosome population was fully sensitive to diminazene on Day 1, by Day 4 the population expressed significant resistance to diminazene.

In the above experiment, tsetse flies were infected by allowing them to feed on a goat that had been infected with a stabilate of *T. congolense* IL 3274. One possible explanation for the apparent alteration in resistance to diminazene between Day 1 and Day 19 following infection is that there was a spontaneous increase in the level of drug resistance of the trypanosomes between these 2 days. This was investigated by repeating the aforementioned experiment, but using tsetse flies that were infected with a subpopulation of *T. congolense* IL 3274 that reappeared in goats following treatment with diminazene aceturate at a dose 7.0 mg kg^{-1} b.w.; batches of tsetse flies were infected by allowing them to feed on each of the five animals in the first experiment in which trypanosomes had reappeared following treatment with diminazene aceturate on Day 19. Infective flies were then used to infect ten naive goats. Five of the goats received diminazene aceturate at a dose of 7.0 mg kg^{-1} b.w. on Day 1 following challenge; infections in all five animals were eliminated. The other five goats were not treated until Day 19; infections in all five animals were refractory to treatment. These results therefore provide further evidence that the sensitivity of *T. congolense* IL 3274 to diminazene is dependent on the duration of infection at the time treatment is instituted.

In work carried out with a stock of *T. congolense* in mice, Sones and Holmes (1992) demonstrated that the sensitivity of the population to isometamidium was dependent on the size of the population used to characterise the sensitivity; as the population size was increased, the minimum curative dose for the population also increased. Such a population size-dependent effect may have been responsible for the observations in the experiments described here. However, the biological basis for the apparent alteration in resistance phenotype with population size is unclear, particularly since a cloned population was used.

Results from the first experiment demonstrated that when *T. congolense* IL 3274 was transmitted to goats via infective tsetse flies, the trypanosomes were fully sensitive to treatment with diminazene on Day 1, but expressed significant resistance to diminazene in some animals by Day 4. Several studies have indicated that when tsetse flies infected with *T. congolense* are allowed to feed on ruminants, the flies infect the animals by depositing metacyclic trypanosomes within the dermis of the skin (Luckins and Gray, 1978; Emery and Moloo, 1981; Akol and Murray, 1982). Four days later, the trypanosomes access the afferent lymph draining the dermis (Dwinger et al., 1990). Furthermore, it is not until 2–3 weeks following infection that trypanosomes are first detected in the blood (Luckins and Gray, 1978; Emery and Moloo, 1981; Akol and Murray, 1982; Dwinger et al., 1988, 1990). Thus, while the trypanosomes would appear to be localised in the dermis at the time an infection is

initiated, by Day 4 they have begun to leave this site. When located in the skin the trypanosomes exist in a distinct morphological form. As a result of this location and/or various ultrastructural features they have been referred to as 'extravascular forms' (Gray et al., 1985), 'intracutaneous forms' (Dwinger et al., 1988) or 'intermediate forms' (Gray et al., 1985; Dwinger et al., 1988). Intermediate forms have ultrastructural features such as a small mitochondrion, a well differentiated secretory reticulum and a lack of large lipid inclusions (Dwinger et al., 1988), which distinguish them from both metacyclic and bloodstream-form trypanosomes. One possible explanation for the apparent alteration in sensitivity to diminazene which occurs between Day 1 and Day 19 is that this life-cycle stage differs in sensitivity to diminazene compared with life-cycle stages which occur in the host once the trypanosomes migrate away from the dermis. In recent work, it has been shown that compounds closely related to diminazene, such as pentamidine, exert growth-retarding effects on human peripheral lymphocytes, particularly in the G_0 phase of the cell-division cycle (Stauffert et al., 1990). It remains to be established whether the susceptibility of trypanosomes to diminazene is greatest when cells are in G_0, and whether on Day 1 following tsetse challenge *T. congolense* in the restricted environment of the dermis exist in a quiescent, non-proliferating state characteristic of the G_0 phase of the cell-division cycle (Pardee, 1989).

An alternative explanation for the apparent alteration in sensitivity to diminazene during the early stages of infection is that during this period the trypanosomes access sites within the host which are inaccessible to diminazene; when the systemic drug levels decline they are then able to re-emerge from such sites. Such a phenomenon occurs in mice when infected with *T. brucei* (Jennings et al., 1979). In order to determine whether the same phenomenon played a role in the observations described here, we have in other work collected cerebrospinal fluid from seven goats on a daily basis from Day 1 to Day 19 following tsetse-transmitted infection with *T. congolense* IL 3274; all cerebrospinal fluid samples were negative for trypanosome antigen (Nantulya and Lindqvist, 1989; M. Mamman, unpublished data, 1992). The results indicate that invasion of the central nervous system between Days 1 and 19 is unlikely and therefore probably does not explain the alteration in sensitivity of *T. congolense* IL 3274 between Day 1 and Day 19 following infection.

In view of the potential threat that drug resistance poses to the control of trypanosomosis in domestic livestock it is important that the biological basis for the results described herein is elucidated. Such information may lead to the design of intervention strategies capable of overcoming apparent drug resistance, thereby maximising the useful life of current trypanocidal compounds.

Acknowledgements

The technical assistance provided by the staff of ILRAD's Tsetse, Large and Small Animal Units is greatly acknowledged. This is ILRAD publication No. 1075.

References

Akol, G.W.O. and Murray, M., 1982. Early events following challenge of cattle with tsetse infected with *Trypanosoma congolense*: development of the local skin reaction. Vet. Rec., 110: 295–302.

Authie, E., 1984. Mis en evidence d'une resistance aux trypanocides parmi des souches de *Trypanosoma congolense* recemment isolees au Burkina. Rev. Elevage Med. Vet. Pays Trop., 37: 219–235.

Bauer, F., 1955. Ergbenisse der klinischen prufüng von Berenil. Vet. Med. Nachr., 3: 152–154.

Brun, R. and Kunz, C., 1989. In vitro drug sensitivity test for *Trypanosoma brucei* subgroup bloodstream trypomastigotes. Acta Trop., 46: 361–368.

Dwinger, R.H., Rudin, W., Moloo, S.K. and Murray, M., 1988. Development of *Trypanosoma congolense, T. vivax* and *T. brucei* in the skin reaction induced by infected *Glossina morsitans centralis*: a light and electron microscopical study. Res. Vet. Sci., 44: 154–163.

Dwinger, R.H., Murray, M. and Moloo, S.K., 1990. Parasite kinetics and cellular responses in goats infected and superinfected with *Trypanosoma congolense* transmitted by *Glossina morsitans centralis*. Acta Trop., 47: 23–33.

Emery, D.L. and Moloo, S.K., 1981. The dynamics of the cellular reactions elicited in the skin reactions produced in goats by *Glossina morsitans morsitans* infected with *Trypanosoma (Nannomonas) congolense* or *T. (Duttonella) vivax*. Acta Trop., 38: 15–28.

Geigy, R. and Kauffmann, M., 1973. Sleeping sickness survey in the Serengeti area (Tanzania), 1971. I. Examination of large mammals for trypanosomes. Acta Trop., 3: 12–23.

Gray, M.A., Ross, C.A., Taylor, A.M., Tetley, L. and Luckins, A.G., 1985. In vitro cultivation of *Trypanosoma congolense*: the production of infective metacyclic trypanosomes cultured on bovine endothelial cell monolayers. Acta Trop., 42: 99–111.

Gutteridge, W.E. and Coombs, G.H., 1977. Biochemistry of Parasitic Protozoa. Macmillan Press, London, 20 pp.

Jennings, F.W., Whitelaw, D.D., Holmes, P.H., Chizyuka, H.G.B. and Urquhart, G.M., 1979. The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. Int. J. Parasitol., 17: 361–384.

Jones-Davies, W.J., 1967. The discovery of Berenil-resistant *Trypanosoma vivax* in Northern Nigeria. Vet. Rec., 80: 531–532.

Kaminsky, R., Chuma, F. and Zweygarth, E., 1989. *Trypanosoma brucei brucei*: expression of drug resistance in vitro. Exp. Parasitol., 69: 281–289.

Katende, J.M., Musoke, A.J., Nantulya, V.M. and Goddeeris, B.M., 1987. A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for the diagnosis of bovine trypanosomiasis. Tropenmed. Parasitol., 38: 41–44.

Katende, J.M., Goddeeris, B.M., Morzaria, S.P., Nkonge, C.G. and Musoke, A.J., 1990. Identification of *Theileria mutans*-specific antigen for use in an antibody and antigen detection ELISA. Parasite Immunol., 12: 419–433.

Kupper, W. and Wolters, M., 1983. Observations on drug resistance of *Trypanosoma (Nanno-*

monas) congolense and *Trypanosoma (Duttonella) vivax* in cattle at a feedlot in northern Ivory Coast. Tropenmed. Parasitol., 34: 203–205.

Lanham, S.M. and Godfrey, D.G., 1970. Isolation of salivarian trypanosomes of man and other mammals using DEAE-cellulose. Exp. Parasitol., 28: 521–534.

Luckins, A.G. and Gray, A.R., 1978. An extravascular site of development of *Trypanosoma congolense*. Nature, 272: 613–634.

MacLennan, K.J.R. and Na'Isa, B.K., 1970. Relapsing *Trypanosoma vivax* infections in Nigerian Zebu Cattle treated with diminazene aceturate. Trop. Anim. Health Prod., 2: 189–195.

Majiwa, P.A.O., Masake, R.A., Nantulya, V.M., Hamers, R. and Matthysens. G., 1985. *Trypanosoma (Nannomonas) congolense*: identification of two karyotypic groups. EMBO J., 4: 3307–3313.

Mbwambo, H.A., Mella, P.N.P. and Lekaki, K.A., 1988. Berenil (diminazene aceturate)-resistant *Trypanosoma congolense* in cattle under natural tsetse challenge at Kibaha, Tanzania. Acta Trop., 45: 239–244.

McGuire, T.C., Musoke, A.J. and Kurtti, T., 1979. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages and skin. Immunology, 38: 249–256.

Moloo, S.K. and Kutuza, S.B., 1990. Expression of resistance to isometamidium and diminazene in *Trypanosoma congolense* in Boran cattle infected by *Glossina morsitans centralis*. Acta Trop., 47: 79–89.

Murray, M., Murray, P.K. and McIntyre, W.I.M., 1977. An improved parasitological technique for diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg., 65: 254–255.

Nantulya, V.M. and Lindqvist, K.J., 1989. Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T. congolense* and *T. Brucei* infections in cattle. Trop. Med. Parasit., 40: 267–272.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R. and Moloo, S.K., 1984. Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe-passaged infections. Infect. Immun., 43: 735–738.

Pardee, A.B., 1989. G_1 events and regulation of cell proliferation. Science, 246: 603–608.

Paris, J., Murray, M. and McOdimba, F., 1982. A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis. Acta Trop., 39: 307–316.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B., 1991. Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. Parasitology, 102: 93–100.

Pinder, M. and Authie, E., 1984. The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. Acta Trop., 41: 247–252.

Silayo, R.S., Mamman, M., Moloo, S.K., Aliu, Y.O., Gray, M.A. and Peregrine, A.S., 1992. Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate. Res. Vet. Sci., 53: 98–105.

Sones, K.R. and Holmes, P.H., 1992. The influence of the size of the initial inoculum on the efficacy of isometamidium (samorin) on a stock of *Trypanosoma congolense*. Acta Trop., 51: 213–216.

Stauffert, I., Paulini, H., Steinmann, U., Sippel, H. and Estler, C.J., 1990. Investigations on mutagenicity and genotoxicity of pentamidine and some related trypanocidal diamidines. Mutat. Res., 245: 93–98.

Vos, G.J., Moloo, S.K., Nelson, R.T and Gardiner, P.R., 1988. Attempts to protect goats against challenge with *Trypanosoma vivax* by initiation of primary infections with large numbers of metacyclic trypanosomes. Parasitology, 97: 383–392.

Whitelaw, D.D., Bell, I.R., Holmes, P.H., Moloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M., 1986. Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. Vet. Rec., 118: 722–726.

Whitelaw, D.D., Gardiner, P.R. and Murray, M., 1988. Extravascular foci of *Trypanosoma vivax* in goats: central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. Parasitology, 97: 1–11.

Wilson, M.B. and Nakane, P.K., 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: W. Knapp, K., Holubar and G. Wick (Editors), Immunofluorescence and Related Staining Techniques. Elsevier, Biomedical Press, North Holland, pp. 215–224.

Section 4.3

Mamman, M., Williams, D.J.L., Murphy, N.B. and Peregrine, A.S. (1995) Apparent rarity of diminazene-resistant trypanosomes in goats infected with a diminazene-resistant population of *Trypanosoma congolense*. *Research in Veterinary Science* 58, 113-118.

Research in Veterinary Science 1995, 58, 113-118

Apparent rarity of diminazene-resistant trypanosomes in goats infected with a diminazene-resistant population of *Trypanosoma congolense*

M. MAMMAN, D. J. L. WILLIAMS, N. B. MURPHY, A. S. PEREGRINE, *International Laboratory for Research on Animal Diseases (ILRAD), PO Box 30709, Nairobi, Kenya*

SUMMARY

Experiments were carried out in goats to determine the frequency with which diminazene-resistant trypanosomes occur in parasite populations before and after the intramuscular treatment of the goats with diminazene aceturate. *Trypanosoma congolense* IL 3274, a diminazene-resistant clone, was used to initiate infections in three groups of five goats. The goats in the first group were treated with diminazene aceturate at a dose of 7·0 mg kg^{-1} bodyweight within 10 seconds of infection; one of the goats was cured. All of the second group, which received no treatment, became parasitaemic. The third group of goats received the same dose of drug as the first group but three days after all of them were first detected parasitaemic; trypanosomes reappeared in all the five goats. When this third group was treated, the frequency of trypanosomes resistant to the drug dosage was estimated to be less than 1 in 10^3. The parasites which reappeared after the treatment of these animals were used to infect two additional groups of five goats intravenously. The goats in one group were treated with the same dose of drug as before, within 10 seconds of infection and were all cured. In contrast, the five goats in the second, untreated, group became parasitaemic. Finally, when the goats in which the infections had relapsed were retreated with diminazene aceturate at the same dose rate, the level of parasitaemia temporarily decreased by at least 10^3 trypanosomes ml^{-1}. These findings suggest that diminazene-resistant *T congolense* occur at low levels in trypanosome populations despite attempts to select for a population resistant to the dose of drug used.

DIMINAZENE aceturate (Berenil Hoechst, Ganaseg, Veriben) is an aromatic diamidine that was introduced as a chemotherapeutic agent for animal trypanosomiasis more than three decades ago (Bauer 1955, Fussgänger and Bauer 1958). Unlike other trypanocidal drugs, resistance to diminazene was initially rarely reported and slow to develop (Hawking 1963, Williamson 1976). However, in later years, there have been several reports of resistance to diminazene in parts of the African continent (Jones-Davies 1967, 1968, MacLennan and Na'Isa 1970, Küpper and Wolters 1983, Mbwambo et al 1988, Clausen et al 1992, Mohamed-Ahmed et al 1992). Although resistance to diminazene can be demonstrated in many field isolates both in vivo and in vitro, the underlying mechanisms which lead to the emergence of drug resistance are poorly understood (Hawking 1963, Williamson 1980, Leach and Roberts 1981). Furthermore, the in vivo biodynamics of drug-resistant trypanosomes, and the frequency with which drug-resistant trypanosomes occur in populations before drug treatment, and in relapse populations after treatment, are poorly understood.

In this study, the authors have investigated the diminazene-resistance phenotype of trypanosomes in goats and attempted to estimate the proportions of trypanosome populations that are refractory to treatment, both before and after treatment.

MATERIALS AND METHODS

Experimental animals

Thirty-five adult, male, castrated East African cross Galla goats were obtained from a tsetse-free area of Kenya. At the beginning of the experiment, samples of serum were collected from all the goats and found to be negative for trypanosomal antigen and anti-trypanosomal antibodies when examined by ELISA techniques (Nantulya and Lindqvist 1989, Mamman et al 1993). Throughout the study the animals were kept in fly-proof housing, fed 200 g concentrates daily and allowed free access to hay and water.

C3H/He mice were obtained from the ILRAD breeding colony, weighed 20 to 35 g and were fed water and a pelleted ration (Unga Feeds, Nairobi) ad libitum.

Trypanosomes

Peregrine et al (1991) have descibed the derivation of *Trypanosoma congolense* clone IL 3274 from a stock which was originally isolated from a bull in Burkina Faso (Pinder and Authie 1984). In goats, *T congolense* IL 3274 is resistant to intramuscular treatment with diminazene aceturate at a dose of 7·0 mg kg^{-1} bodyweight when the drug is administered after the detection of trypanosomes (Silayo et al 1992, Mamman et al 1993).

Infection and treatment of animals

A freshly prepared 7·0 per cent (w/v) solution of diminazene aceturate (Berenil, Hoechst) in sterile distilled water was used to treat the goats at a dose of 7·0 mg kg^{-1} bodyweight. The drug was administered intramuscularly in the right vastus medialis muscle.

Monitoring of goat infections

Three millilitre samples of jugular blood were taken daily from each goat into evacuated tubes containing dipotassium ethylenediamine tetra-acetic acid (EDTA)

TABLE 1: Experimental design and the response to treatment with diminazene aceturate of groups of goats infected with *Trypanosoma congolense*

Group	Number of goats infected	*Trypanosoma congolense* clone*	Treatment with diminazene aceturate Dose (mg kg^{-1})	Route	Time after infection	Number cured/ number infected	Mean (SD) and range of prepatent period (days)	
A	5	IL 3274†	–	–	–	0/5	19·4 (3·1)	(16-23)
B	5	IL 3274‡	7·0	im	<10 seconds	1/5	20·0 (4·7)	(13-23)
C	5	IL 3274‡	–	–	–	0/5	5·2 (1·8)	(4-8)
D	5	IL 3274‡	7·0	im	10 days	0/5	5·6 (1·5)	(3-7)
D	5	IL 3274§	7·0	im	29 days	0/5	6·2 (1·8)	(3-7)
E	5	IL 3274**	7·0	im	<10 seconds	5/5	No trypanosomes detected	
F	5	IL 3274**	–	–	–	0/5	6·6 (1·5)	(5-8)

* 1·0 X 10^5 trypanosomes per goat inoculated via the left jugular vein, † Infection in this group was initiated from a stabilate of the parasite in phosphate-saline-glucose, – treatment not given, ‡ Infections were initiated with trypanosomes occurring in group A on day 28 of infection, im Intramuscular treatment via the right vastus medialis muscle, § Infections in these goats had relapsed after the first treatment. ** Infection in this group was initiated with trypanosomes that had reappeared in group D 14 days after the first treatment

beginning seven days before infection and continuing for 30 days after infection. Thereafter, blood samples were collected weekly for a further five months. At each sampling, the packed red blood cell volume (PCV) was measured by the haematocrit centrifugation technique, followed by an examination of the buffy coat for trypanosomes (Murray et al 1977). The method of Paris et al (1982) was used to score the level of parasitaemia; the minimum number of parasites detected by the method was 10^2 ml^{-1} blood.

Experimental design

Thirty goats were randomly divided into six groups (A, B, C, D, E and F) of five animals (Table 1) and used as indicated in Fig 1. The goats in group A were infected intravenously with *T congolense* IL 3274, prepared in phosphate-saline-glucose (PSG). Twenty-eight days later, after the detection of trypanosomes, samples of blood from each of the five goats were used to infect three goats, one in each of groups B, C and D, intravenously with 1·0 x 10^5 trypanosomes per goat. Within 10 seconds of infection, diminazene aceturate was administered at a single intramuscular dose of 7·0 mg kg^{-1} bodyweight to each animal in group B. The goats in group C were not treated and served as infectivity controls for group B.

During the rising phase of the first peak of parasitaemia, each goat in group D was treated intramuscularly with diminazene aceturate at a dose of 7·0 mg kg^{-1} bodyweight. After the treatment, all five goats became aparasitaemic but relapsed within nine days of the treatment. On day 15 after the treatment, trypanosomes from each of the five goats were inoculated intravenously (1·0 x 10^5 trypanosomes per goat) into two goats, one in each of groups E and F. Within 10 seconds of infection, each goat in group E was treated intramuscularly with diminazene aceturate at a dose of 7·0 mg kg^{-1} bodyweight. The animals in group F were not treated and functioned as infectivity controls for the animals in group E. Finally, 19 days after the first treatment, the five goats in group D received a further single dose of 7·0 mg kg^{-1} bodyweight of diminazene aceturate; the 19-day interval was used because it maximised the elimination of the drug after the first treatment, but resulted in the goats having PCV values above 15 per cent at the time of the second treatment.

Determination of variable surface glycoprotein (VSG) antibody responses by fluorescence activated cell sorter (FACS) analysis

In the animals in group D, experiments were carried out

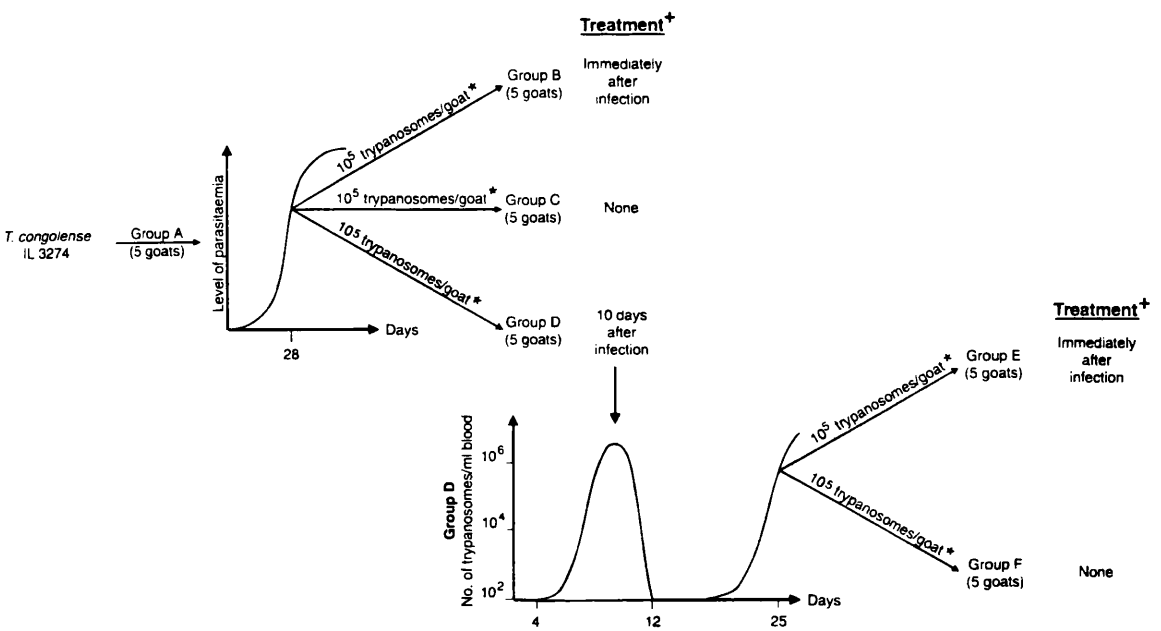


FIG 1: Methodology used for infection and treatment of experimental groups. *Intravenous infection, + 7·0 mg diminazene aceturate kg^{-1} bodyweight by the intramuscular route

to determine the contribution of the host's immune response to the disappearance of trypanosomes after each of the two treatments. Sera were collected from the goats weekly throughout the experiment and stored at −70°C until analysis. Stabilates of trypanosomes were prepared from each goat on the two occasions that the goats were treated with diminazene aceturate, shortly before the drug was administered, and cryopreserved in liquid nitrogen (Dar et al 1972). The stabilites were later used to infect groups of sublethally irradiated (650 rads) mice and the resultant trypanosome populations separated from other cellular elements on a diethylaminoethyl-cellulose (DE 52, Beckman) column (Lanham and Godfrey 1970). The reactivity of the parasites to antibodies in the goat sera was analysed by using a combination of immunolgical and FACS methods (Naessens et al 1988, Williams et al 1990, 1991).

Comparative pharmacokinetics of diminazene in plasma

The goats in group D were treated with diminazene aceturate (7·0 mg kg^{-1} bodyweight) intramuscularly on days 10 and 29 of infection. The same treatment was administered once intravenously to each of five additional uninfected goats; the intravenous data were used to calculate the values of some pharmacokinetic parameters in the goats that were treated intramuscularly. Over the first 48 hours after each of the drug treatments a series of 5·0 ml blood samples was collected from each animal into EDTA-containing Monoject tubes (Sherwood Medical), as described by Aliu et al (1993). Paired-ion extraction and high performance liquid chromatography of diminazene in the plasma of these samples were as previously described (Aliu and Ødegaard 1983, Aliu et al 1993). The resulting plasma concentration-time data for diminazene were rigorously analysed by using standard compartmental and non-compartmental methods (Gibaldi and Perrier 1982, Aliu et al 1993). The derivation, and the symbols used to describe the pharmacokinetic parameters are as previously described (Allen et al 1982, Gibaldi and Perrier 1982, Riviere 1988, Kinabo and McKellar 1989). The values of the pharmacokinetic parameters were compared by using a one-way analysis of variance at the 5 per cent significance level. All the values are expressed as arithmetic means (SD), except for $t_{1/2}\lambda_z$ (terminal elimination half-life) which is given as a geometric mean.

RESULTS

The parasitological results obtained in the six groups of goats are summarised in Table 1. Of the five goats in group B, each of which received diminazene aceturate within 10 seconds after infection, four were detected to be parasitaemic 13 to 23 days after the treatment; the other goat appeared to be cured because it was not detected as parasitaemic during an observation period of 180 days. In comparison, the five untreated goats of group C became parasitaemic four to eight days after being infected. Similarly, the five goats in group D became parasitaemic within three to seven days after being infected. However, after treatment with diminazene aceturate 10 days after infection, a period of aparasitaemia was observed in all five, but trypanosomes reappeared in all the goats within five to nine days of the treatment. When the goats were treated again on day 29, a temporary aparasitaemia occurred in all the animals, but all

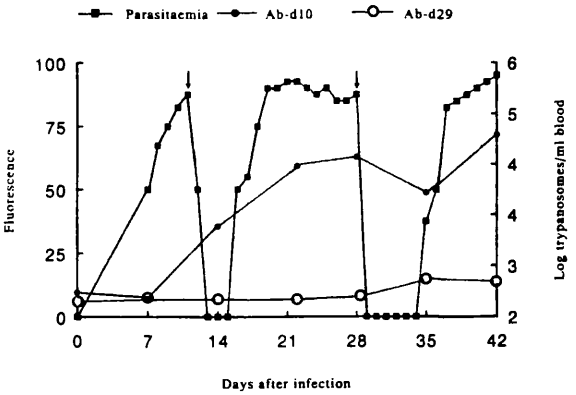


FIG 2: Mean level of parasitaemia and antibody before and after the intramuscular administration of single doses (7·0 mg kg^{-1}) of diminazene aceturate to the five goats in group D. Arrows indicate times of drug treatment. The goats were infected via the jugular vein with bloodstream forms of *T congolense* IL 3274 (10^5 trypanosomes per goat) obtained from the goats in group A 28 days after infection. Trypanosome stabilates were prepared from the goats in group D 10 days (day of the first treatment) and 29 days (day of the second treatment) after infection. The parasites were multiplied in sublethally irradiated mice. Serum samples which were collected weekly from the goats from day 0 to day 42 of infection were analysed, at a dilution of 1:100, using FACS to determine the relative amount of fluorescence bound to each population of parasites present on day 10 (Ab-d10) and day 29 (Ab-d29)

five animals again became parasitaemic within seven days of the treatment. After the second treatment, the level of parasitaemia decreased from at least 10^5 trypanosomes ml^{-1} blood in all the animals, to less than 10^2 trypanosomes ml^{-1}.

The five goats in group E were infected with parasites that reappeared in the group D animals after their first treatment with diminazene aceturate, but they were all cured by treatment with a single dose of diminazene aceturate administered within 10 seconds after the infection; the goats were never detected as parasitaemic during a 150-day period of observation after the treatment. In contrast, all the untreated control goats of group F which were similarly infected with trypanosomes from group D goats, were detected as parasitaemic within five to eight days after they were infected.

Determination of variable surface glycoprotein antibody responses by FACS analysis

The results of the FACS analysis showed that the sera collected from group D goats seven days after infection (three days before the first treatment) contained very low titres of specific antibodies against the variable antigen types that were present on day 10 after infection (Fig 2). However, there was an increase in antibody titre four days after treatment, and the titres continued to rise, reaching a peak 28 days after infection. Up to 28 days after infection, there were relatively low levels of specific antibodies to the variable antigen types that were present in the goats on the day of the second treatment (29 days after infection).

Comparative pharmacokinetics of diminazene in plasma

After the administration of diminazene aceturate to the animals in group D, a series of blood samples was collected to determine whether the disappearance of trypanosomes after the second treatment was associated with higher plasma concentrations of diminazene as a result of residual amounts remaining after the first treatment.

TABLE 2: Pharmacokinetic parameters of diminazene in uninfected and *Trypanosoma congolense*-infected goats after the administration of a single dose of 7·0 mg kg^{-1} by the intravenous or intramuscular routes

Parameter	Units	Route of administration		
		[1]Intravenous	[2]Intramuscular	[3]Intramuscular
K_a	hours	NA	21·3 (13·1)	8·67 (8·07)
C_{max}	μg ml^{-1}	86·9 (111·5)	7·48 (2·81)	9·58 (6·34)
T_{max}	hours	NA	0·21 (0·04)	0·36 (0·18)
$AUC_{0-\infty}$	μg hour^{-1} ml^{-1}	33·6 (3·2)*	29·7 (3·4)‡	136·1 (56·4)*‡
MRT	hours	21·1 (2·6)*	20·5 (6·8)‡	210·0 (134·4)*‡
$t_{1/2}\lambda_z$	hours	11·7 (9·8-13·7)*	16·7 (10·7-30·0)‡	137·4 (74·3-332)*‡
Cl	ml min^{-1} kg^{-1}	1·74 (0·16)*	1·97 (0·22)‡	0·48 (0·17)*‡
$V_{d(ss)}$	litre kg^{-1}	2·20 (0·31)*	2·39 (0·66)‡	5·10 (1·04)*‡
V_c	litre kg^{-1}	0·12 (0·11)*‡	0·73 (0·15)*	075 (0·31)‡

1 Uninfected goats (n=5), 2 First treatment administered to group D animals (n=5) on day 10 of infection (during the rising phase of the first peak of parasitaemia), 3 Second treatment administered to group D animals 19 days after the first treatment (during the rising phase of relapse parasitaemia), K_a absorption constant, NA Not applicable. C_{max} Peak plasma concentration, T_{max} Time at which C_{max} occured, $AUC_{0-\infty}$ Area under the plasma concentration-time curve determined from time 0 to infinity, MRT Mean residence time, $t_{1/2}\lambda_z$ Terminal elimination half-life, Cl Apparent total body clearance, $V_{d(ss)}$ Apparent volume of distribution at steady-state, V_c Volume of the central compartment. All data indicate means (SD) except for $t_{1/2}\lambda_z$ (given as geometric means with ranges in parenthesis); * and ‡ Values with the same symbol are significantly different (P<0·05)

The values of the pharmacokinetic parameters determined after the intravenous treatment of uninfected goats, and after the first and second intramuscular doses in the infected goats of group D, are presented in Table 2.

DISCUSSION

This study has provided preliminary information on the susceptibility to diminazene of *T congolense* IL 3274 in goats and of subpopulations of the clone which reappeared after treatment with diminazene aceturate. *T congolense* IL 3274 was initially allowed to proliferate in five goats. Before the first peak of parasitaemia, parasites in the jugular blood were collected and used to initiate infections in three groups of five goats by the intravenous inoculation of 10^5 trypanosomes per goat. The animals in the first group were each treated with a single intramuscular dose of 7·0 mg kg^{-1} bodyweight of diminazene aceturate within 10 seconds of infection. The early treatment was carried out to minimise the chances of the parasites invading sites that might be poorly accessible to the drug (Jennings et al 1979, Whitelaw et al 1988). Although the treatment was administered at this early stage of infection, four of the five goats subsequently became parasitaemic. The five goats in the second, untreated, group also became parasitaemic. Thus, resistance to diminazene aceturate at a dose of 7·0 mg kg^{-1} bodyweight was expressed at an inoculum of 10^5 trypanosomes per goat. This result is in contrast with earlier observations in which five out of five goats infected with the same parasite clone, via the bites of five tsetse flies, were cured by the same dose of drug, provided that the treatment was administered 24 hours after the initiation of the infection (Silayo et al 1992, Mamman et al 1993). However, the numbers of trypanosomes present in these animals when they were treated would have been considerably fewer than 10^5 because tsetse flies infected with *T congolense* extrude approximately 100 metacyclic trypanosomes at each feed (Whitelaw et al 1986). As a result, the infections would have been expected to be more sensitive to treatment (Sones and Holmes 1992).

The animals in the third group were used in studies to determine the frequency of parasites in a trypanosome population that are able to withstand a particular drug dosage in vivo. Three days after each of the goats in this group were first detected as parasitaemic each animal was treated with diminazene aceturate; after a temporary remission of the parasitaemia, trypanosomes were detected in each of the five goats. The level of parasitaemia decline, from more than 10^5 trypanosomes ml^{-1} to less than 10^2 trypanosomes ml^{-1} (the lower limit of detection of the diagnostic technique used [Paris et al 1982]) after the treatment, indicated that at the time of the treatment less than 0·1 per cent of the trypanosome population was resistant to the dose of drug used. In the second part of the study, the trypanosomes which reappeared in these animals after the first treatment were used to infect two other groups of five goats, each of which was inoculated intravenously with 10^5 trypanosomes. The goats in one of the groups received diminazene aceturate within 10 seconds of infection, to determine the diminazene susceptibility of the parasites when they were present only in the cardiovascular system and before they could invade sites less accessible to the drug. Surprisingly, all the five goats were cured. In contrast, trypanosomes were detected in all the five goats in the second, untreated, control group. Assuming a high level of infectivity of the trypanosomes (M. Mamman, unpublished observations), these results suggest that less than 1 in 10^5 of the parasites used to infect the two groups of goats survived this dose of diminazene aceturate. It therefore appears that the majority of the parasites in the relapse population were sensitive to the dose of diminazene aceturate used to treat the goats. To confirm this conclusion, the group of goats in which the parasites had relapsed after treatment on day 10 of infection was treated again with the same dose of diminazene aceturate on day 29. If all the trypanosomes that reappeared in these goats after the first treatment had been resistant to the dose of drug, the second treatment would have been expected to have had no effect on the level of parasitaemia. However, the second treatment resulted in the parasitaemia decreasing from more than 10^5 trypanosomes m^{-1}, to less than 10^2 trypanosomes ml^{-1}; the trypanosomes reappeared in all the goats within seven days of treatment.

The decrease in the parasitaemia after the second treatment may have been due to some event other than the administration of the drug. For instance, antibody responses to variable surface glycoproteins are known to clear parasites from the bloodstream of infected hosts (Morrison et al 1982). Thus, the titres of antibodies against variable antigen types were measured before the first and second drug treatments. The results indicated that antibodies specific for variable antigen types did not appear to account for the

decline in parasitaemia observed after each treatment. It would appear, therefore, that the remission in parasitaemia after the second treatment resulted from the exposure of the parasites to the drug. High-performance liquid chromatographic analyses indicated that the area under the concentration-time curve ($AUC_{0-\infty}$), the elimination half life, and the steady-state volume of distribution of the drug were significantly greater after the second treatment (Table 2). Thus, a greater concentration of diminazene was maintained in the plasma after the second dose, than after the first. However, despite the differences in the kinetics of the drug between the two treatments caution is necessary in associating the pharmacokinetic parameters with the decline in parasitaemia after the second treatment, because it is not clear which of these parameters reliably indicates the ability of the drug to eliminate trypanosomes.

In the first part of the study, a stabilate of *T congolense* IL 3274 was inoculated into five goats. Before the first peak of parasitaemia, trypanosomes were collected from each of these animals and inoculated intravenously into five additional goats (10^5 per animal) which were treated within 10 seconds of infection with diminazene aceturate; the infections in four of the five animals were refractory to treatment. However, when the same experimental protocol was used with a subpopulation of *T congolense* IL 3274 that had reappeared in goats following treatment with the same dose of diminazene aceturate the infections were eliminated from all the goats. Thus, the first drug treatment did not increase the frequency of 'diminazene-resistant' trypano-somes within the parasite population, but rather resulted in an apparent decrease in the frequency of resistant trypanosomes. It is possible that the parasites accumulated diminazene during their first exposure to the drug and that this sensitised them to further treatments. However, the fact that a repeat treatment with diminazene did not eliminate an infection with the same trypanosome clone from the animals of group D provides evidence against this hypothesis. Alternatively, the FACS analyses suggest that *T congolense* IL 3274 underwent antigenic variation during the course of the infection (data not shown). This would suggest that different variable antigen types were present in the two groups of experimental animals at the time of treatment. Thus, the possibility that different degrees of drug sensitivity are associated with different variable antigen types could account for the difference in drug sensitivity observed between the two experimental groups.

In summary, the sensitivity of *T congolense* clone IL 3274 to diminazene has been determined in infected goats both before and after treatment with the drug. The findings suggest that diminazene-resistant trypanosomes occur at low frequencies in such populations. However, some of the findings are based on assumptions concerning the infectivity and diminazene-sensitivity of the parasite populations. More rigorous determinations of the frequency with which diminazene-resistant trypanosomes occur in vivo require experiments with larger numbers of experimental animals.

ACKNOWLEDGEMENTS

The authors acknowledge the skilled technical assistance provided by Mr S. Kemei in the parasitological and pharmacokinetic experiments, and Messrs M. Opollo, P. Mucheru and J. Magondu in the FACS analysis. Mr D. Elsworth is thanked for assistance with the graphics. This is ILRAD publication number 1207.

REFERENCES

ALIU, Y. O., MAMMAN, M. & PEREGRINE, A. S. (1993) Pharmacokinetics of diminazene in female Boran (*Bos indicus*) cattle. *Journal of Veterinary Pharmacology and Therapeutics* **16**, 291-300

ALIU, Y. O. & ØDEGAARD, S. (1983) Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in plasma. *Journal of Chromatography* **276**, 218-223

ALLEN, L., KIMURA, K., MACKICHAN, J. & RITSCHEL, W. A. (1982) Manual of symbols, equations and definitions in pharmacokinetics. *Journal of Clinical Pharmacology* **22** (Supplement), S1-S23

BAUER, F. (1955) Ergebnisse der klinischen prüfung von Berenil. *Veterinärmedezinische Nachrichten* **3**, 152-154

CLAUSEN, P.-H., SIDIBE, I., KABORE, I. & BAUER, B. (1992) Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse fly challenge in the pastoral zone of Samorogouan, Burkina Faso. *Acta Tropica* **51**, 229-236

DAR, F. K., LIGTHART, G. S. & WILSON, A. J. (1972) Cryopreservation of pathogenic African trypanosomes *in situ*. *Journal of Protozoology* **19**, 494-497

FUSSGÄNGER, R. & BAUER, F. (1958) Berenil ein neues chemotherapeuticum in der veterinarmedizin. *Medizin und Chemie* **6**, 504-531

GIBALDI, M. & PERRIER, D. (1982) Pharmacokinetics. New York, Marcel Dekker

HAWKING, F. (1963) Chemotherapy of trypanosomiasis. In Experimental Chemotherapy. Vol. 1. Eds R. J. Schnitzer and F. Hawking. New York, Academic Press. pp 129-256

JENNINGS, F. W., WHITELAW, D. D., HOLMES, P. H., CHIZYUKA, H. G. B. & URQUHART, G. M. (1979) The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal for Parasitology* **9**, 381-384

JONES-DAVIES, W. J. (1967) The discovery of Berenil-resistant *Trypanosoma vivax* in Northern Nigeria. *Veterinary Record* **80**, 531-532

JONES-DAVIES, W. J. (1968) Berenil resistance in naturally occurring *Trypanosoma congolense*. *Bulletin of Epizootic Diseases of Africa* **16**, 213-216

KINABO, L. D. B. & McKELLAR, Q. A. (1989) Current models in pharmacokinetics: applications in veterinary pharmacology. *Veterinary Research Communications* **13**, 141-157

KÜPPER, W. & WOLTERS, M. (1983) Observations on drug resistance of *Trypanosoma (Nannomonas) congolense* and *T (Duttonella) vivax* in cattle at a feedlot in the northern Ivory Coast. *Tropenmedizin und Parasitologie* **34**, 203-205

LANHAM, S. M. & GODFREY, D. G. (1970) Isolation of salivarian trypanosomes of man and other mammals using DEAE-cellulose. *Experimental Parasitology* **28**, 521-534

LEACH, T. M. & ROBERTS, C. J. (1981) Present status of chemotherapy and chemoprophylaxis of animal trypanosomiasis in the Eastern Hemisphere. *Pharmacology and Therapeutics* **13**, 91-147

MACLENNAN, K. J. R. & NA'ISA, B. K. (1970) Relapsing *Trypanosoma vivax* infections in Nigerian Zebu cattle treated with diminazene aceturate. *Tropical Animal Health and Production* **2**, 189-195

MAMMAN, M., KATENDE, J., MOLOO, S. K. & PEREGRINE, A. S. (1993) Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology* **50**, 1-14

MBWAMBO, H. A., MELLA, P. N. P. & LEKAKI, K. A. (1988) Berenil (diminazene aceturate)-resistant *Trypanosoma congolense* in cattle under natural tsetse challenge at Kibaha, Tanzania. *Acta Tropica* **45**, 239-244

MOHAMED-AHMED, M. M., RAHMAN, A. H. & ABDEL-KARIM, E. I. (1992) Multiple drug-resistant bovine trypanosomes in South Darfur Province, Sudan. *Tropical Animal Health and Production* **24**, 179-181

MORRISON, W. I., BLACK, S. J., PARIS, J., HINSON, C. A. & WELLS, P. W. (1982) Protective immunity and specificity of antibody responses elicited in cattle by irradiated *Trypanosoma brucei*. *Parasite Immunology* **4**, 395-407

MURRAY, M., MURRAY, P. K. & McINTYRE, W. I. M. (1977) An improved parasitological technique for diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **71**, 325-326

NAESSENS, J., NEWSON, J., WILLIAMS, D. J. L. & LUTJE, V. (1988) Identification of isotypes and allotypes of bovine immunoglobulin M with monoclonal antibodies. *Immunology* **63**, 569-574

NANTULYA, V. M. & LINDQVIST, K. J. (1989) Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax*, *T congolense* and *T brucei* infections in cattle. *Tropical Medicine and Parasitology* **40**, 267-272

PARIS, J., MURRAY, M. & McODIMBA, F. (1982) A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis. *Acta Tropica* **39**, 307-316

PEREGRINE, A. S., KNOWLES, G., IBITAYO, A. I., MOLOO, S. K. & MURPHY, N. B. (1991) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* **102**, 93-100

PINDER, M. & AUTHIE, E. (1984) The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica* **41**, 247-252

RIVIERE, J. E. (1988) Veterinary clinical pharmacokinetics. Part II: Modelling. *Compendium of Continuing Education in Small Animal Practice* **10**, 314-327

SILAYO, R. S., MAMMAN, M., MOLOO, S. K., ALIU, Y. O., GRAY, M. A. & PEREGRINE, A. S. (1992) Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate. *Research in Veterinary Science* **53**, 98-105

SONES, K. R. & HOLMES, P. H. (1992) The influence of the size of the initial inoculum on the efficacy of isometamidium (samorin) on a stock of *Trypanosoma congolense*. *Acta Tropica* **51**, 213-216

WHITELAW, D. D., BELL, I. R., HOLMES, P. H., MOLOO, S. K., HIRUMI, H., URQUHART, G. M. & MURRAY, M. (1986) Isometamidium chloride prophy-

laxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. *Veterinary Record* **118,** 722-726

WHITELAW, D. D., GARDINER, P. R. & MURRAY, M. (1988) Extravascular foci of *Trypanosoma vivax* in goats: central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. *Parasitology* **97,** 1-11

WILLIAMS, D. J. L., NAESSENS, J., SCOTT, J. R. & McODIMBA, F. A. (1991) Analysis of peripheral leucocyte populations in N'Dama and Boran cattle following a rechallenge infection with *Trypanosoma congolense*. *Parasite Immunology* **13,** 171-185

WILLIAMS, D. J. L., NEWSON, J. & NAESSENS, J. (1990) Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Veterinary Immunology and Immunopathology* **24,** 267-283

WILLIAMSON, J. (1976) Chemotherapy of African trypanosomiasis. *Tropical Diseases Bulletin* **73,** 531-542

WILLIAMSON, J. (1980) Report of the Expert Consultation on Research on Trypanosomiasis. Food and Agriculture Organization, Rome. pp 84-89

Received July 26, 1993
Accepted July 7, 1994

Section 4.4

Mamman, M., Gettinby, G., Murphy, N.B., Kemei, S. and Peregrine, A.S. (1995) Frequency of diminazene-resistant trypanosomes in populations of *Trypanosoma congolense* arising in infected animals following treatment with diminazene aceturate. *Antimicrobial Agents and Chemotherapy* 39, 1107-1113.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1995, p. 1107–1113
0066-4804/95/$04.00+0

Vol. 39, No. 5

Frequency of Diminazene-Resistant Trypanosomes in Populations of *Trypanosoma congolense* Arising in Infected Animals Following Treatment with Diminazene Aceturate†

MOHAMMED MAMMAN,[1] GEORGE GETTINBY,[2] NOEL B. MURPHY,[1] SAMMY KEMEI,[1] AND ANDREW S. PEREGRINE[1]*

International Laboratory for Research on Animal Diseases, Nairobi, Kenya,[1] and Department of Statistics and Modelling Science, University of Strathclyde, Glasgow, Scotland[2]

Received 25 July 1994/Returned for modification 13 December 1994/Accepted 4 March 1995

The frequency of trypanosomes resistant to diminazene aceturate at a dose of 25 mg/kg of body weight was investigated for populations of *Trypanosoma congolense* IL 3274 which reappeared in infected mice after intraperitoneal treatment with diminazene aceturate at the same dosage. At inoculum sizes of 10^2, 10^3, 10^4, 10^5, and 10^6 trypanosomes per mouse, the relapse populations were used to initiate infections in five groups of 100 mice each by the intravenous route. Immediately after infection, 50 mice in each group were treated intraperitoneally with diminazene aceturate at the aforementioned dosage; the other 50 mice functioned as untreated controls. Thereafter, all animals were monitored for 100 days for the presence of trypanosomes. In each group, trypanosomes were detected in 50 of 50 control mice, indicating 100% infectivity for all five inoculum sizes. In contrast, in the groups of 50 mice infected with 10^2, 10^3, 10^4, 10^5, and 10^6 trypanosomes and treated with diminazene aceturate, trypanosomes were detected in 4, 11, 13, 28, and 39 of 50 mice, respectively. By logistic regression, a good fit was found between the number of mice identified as parasitemic and the inoculum sizes. Maximum likelihood estimates for the proportions of trypanosomes resistant to diminazene aceturate at 25 mg/kg of body weight for the inoculum of 10^2, 10^3, 10^4, 10^5, and 10^6 organisms were 8.335×10^{-4}, 2.485×10^{-4}, 3.02×10^{-5}, 8.3×10^{-6}, and 1.6×10^{-6}, respectively. These findings indicate that the majority of the relapse trypanosomes were susceptible to the drug dosage used for selecting the population and that, surprisingly, the calculated proportion of organisms which survived drug exposure varied inversely with the inoculum size. Further experiments with mice indicated that the inverse relationship did not result from alterations in the pharmacokinetics of the drug with different inoculum sizes. The data therefore suggest that parasite inoculum size and drug dosage are important factors in estimating the apparent frequency of diminazene-resistant trypanosomes in populations of *T. congolense* occurring in vivo.

African trypanosomiasis is a disease complex caused by unicellular protozoan parasites belonging to the genus *Trypanosoma*. Pathogenic trypanosomes cause clinical disease in humans and are an important factor limiting development of domestic livestock in Africa (33).

Diminazene aceturate (Berenil [Hoechst AG, Frankfurt, Germany] or Veriben) is an aromatic diamidine that was introduced in 1955 as a therapeutic agent for treatment of animal trypanosomiasis. In trypanosomatids, the drug appears to selectively block replication of kinetoplast DNA (27, 35). However, the primary target(s) for diminazene's trypanocidal activity remain(s) unknown.

Experimental studies of rodents indicate that the number of trypanosomes used to initiate an infection has a significant influence on the subsequent growth of trypanosomes in vivo (9, 16–18, 31); an increase of the inoculum size shortened the parasitemia prepatent period and the time to reach the first peak of parasitemia. However, it also resulted in an increased density of trypanosomes at the first peak of parasitemia (10, 18). Such differences in density appear to inhibit trypanosome proliferation in vivo in a density-dependent fashion, characteristic of the parasite strain (9, 10). For example, Diffley et al. (17) have shown that at levels of parasitemia exceeding 10^8 trypanosomes per ml of blood, a strain of *Trypanosoma brucei gambiense* exhibited a significantly prolonged generation time compared with that of the same strain at lower levels of parasitemia.

Growth and differentiation of trypanosomes appear to be regulated by both host (7, 8, 38) and parasite (4, 9, 37, 39) factors. There is also evidence that cyclic AMP (cAMP) is directly involved in such regulation, since high levels of parasitemia have been shown to be associated with an elevation in intracellular levels of cAMP and prolongation of the doubling time in *Trypanosoma brucei brucei* (29). The dynamics of growth of mixed populations of trypanosomes within the same host have received considerable interest, and it appears that rapidly growing strains competitively outgrow, and dominate, slowly growing subpopulations (46). There is also evidence to suggest that some drug-susceptible trypanosomes grow at relatively faster rates than drug-resistant strains, which may explain why, in the absence of chemotherapy, drug-susceptible populations appear to outgrow drug-resistant populations in vivo (12, 21, 36, 49). While the frequencies with which drug-susceptible and drug-resistant trypanosomes occur in vivo are unknown, we have shown in goats that the majority of trypanosomes which repopulate the blood following treatment with diminazene aceturate appear to remain susceptible to the drug dosage that was used (28). However, in that study, the proportions of the populations which survived drug treatment were not determined. In this report, we present estimates of these

* Corresponding author. Mailing address: ILRAD, P.O. Box 30709, Nairobi, Kenya. Phone: 254-2-630743. Fax: 254-2-631499. Electronic mail address: a.peregrine@cgnet.com.

† International Laboratory for Research on Animal Diseases publication 1206.

TABLE 1. Proportions of mice with diminazene-resistant trypanosomes after infection with relapse[a] populations of *T. congolense* IL 3274 and estimated proportions of diminazene-resistant trypanosomes of each inoculum size

Inoculum size (no. of trypanosomes/mouse)	No. of mice		Estimated proportion of resistant trypanosomes
	Observed parasitemic/treated	Predicted parasitemic[b]/treated	
10^2	4/50	3.9/50	8.335×10^{-4}
10^3	11/50	8.6/50	2.485×10^{-4}
10^4	13/50	16.9/50	3.020×10^{-5}
10^5	28/50	27.8/50	8.300×10^{-6}
10^6	39/50	37.8/50	1.600×10^{-6}

[a] The population had relapsed in infected mice following i.p. treatment with 25 mg of diminazene aceturate/kg of b.w.
[b] Estimated by using a logistic model (see the text).

TABLE 2. Diminazene susceptibility of *T. congolense* IL 3274 that reappeared in infected mice after i.p. treatment with 25 mg of diminazene aceturate per kg of b.w.

Dose of diminazene aceturate (mg/kg of b.w.)	Proportion of parasitemic mice[a] by inoculum size (no. of trypanosomes/mouse)	
	10^5	10^6
0.0	25/25	25/25
10.0	25/25	25/25
20.0	15/25	25/25
30.0	2/25	13/25
40.0	4/25	4/25
50.0	2/25	2/25

[a] Number of mice detected as parasitemic/number of mice treated (25 mg of diminazene aceturate per kg of b.w. administered i.p. immediately after infection by the i.v. route).

proportions based on studies carried out with mice that simulate the dose-response relationship previously observed for goats.

MATERIALS AND METHODS

Experimental animals. Swiss white mice (25 to 38 g each) obtained from the International Laboratory for Research on Animal Diseases breeding colony were used throughout the study. They were allowed free access to both water and a pelleted ration (Unga Feeds Ltd., Nairobi, Kenya).

Trypanosomes. *Trypanosoma congolense* IL 3274 (40) is a cloned population which was derived from a stock, Banankeledaga/83/CRTA/67, that was isolated from a bull in Burkina Faso (41). In goats, infections with this parasite are refractory to treatment with diminazene aceturate at an intramuscular dose of 7.0 mg/kg of body weight (b.w.) when treatment is administered on day 19 of infection (47).

Treatment. Diminazene aceturate (Berenil) freshly prepared in sterile distilled water was used for intraperitoneal (i.p.) treatment of mice. Calculations for the different doses of diminazene aceturate used in the study were corrected such that the required amounts of drug were contained in a volume of 0.2 ml of sterile distilled water. The same volume of sterile distilled water was administered i.p. to infected, but untreated, control mice.

Study design. *T. congolense* IL 3274 was initially expanded in sublethally (650-rad) irradiated mice. On day 9 of infection, the resultant population was used to infect nonirradiated mice i.p. at a dose of 10^7 trypanosomes per mouse. After detection of trypanosomes, the mice were treated i.p. with diminazene aceturate at a dose of 25 mg/kg of b.w. This dosage was used since it resulted in a parasitemia profile that was similar to that seen for goats treated intramuscularly with diminazene aceturate at a dose of 7.0 mg/kg of b.w.; the treatment caused temporary remission of parasitemia, which was subsequently followed by the reappearance of trypanosomes. At this stage, the mice were anaesthetized with diethyl ether, and exsanguinated by cardiac puncture and the blood was collected into sodium citrate (final concentration, 1.0% [wt/vol]). The number of trypanosomes per milliliter in the pooled blood was quantified with a Neubauer hemocytometer. Thereafter, the blood was diluted in phosphate saline glucose to prepare trypanosome inocula of five different sizes. With an inoculum volume of 0.2 ml, mice were infected intravenously (i.v.), via the tail vein, with 10^2, 10^3, 10^4, 10^5, or 10^6 trypanosomes. Inocula of each size were used to infect a group of 100 nonirradiated mice. Within a minute of infection, mice were either treated i.p. with diminazene aceturate at a dose of 25 mg/kg of b.w. or administered 0.2 ml of distilled water i.p. Administration of diminazene aceturate and distilled water was carried out in an alternating manner: i.e., mice 1, 3, 5, and 7, etc., were administered diminazene aceturate; mice 2, 4, 6, and 8, etc., were administered only distilled water. Immediate treatment following infection was carried out in order to minimize the chances of parasites invading drug-inaccessible sites. In this way, 50 mice in each group were administered diminazene aceturate; the other 50 mice were administered only sterile distilled water and functioned as infectivity controls. Following infection, development of parasitemia in all mice was monitored for 100 days by examining wet films of tail blood at a magnification of ×250; any mouse detected as parasitemic was removed from the experiment.

Estimation of the proportion of diminazene-resistant trypanosomes in inocula. The relationship between the fraction of the mice detected as parasitemic following treatment with diminazene aceturate and the trypanosome inoculum sizes was investigated by using logistic regression. Parameter estimation using maximum likelihood methods was undertaken with GLIM version 3.77 (44).

Characterization of the diminazene susceptibility of *T. congolense* IL 3274 that reappeared in mice after treatment. In a similar manner to that described above in the paragraph on study design, the subpopulation of *T. congolense* IL 3274 which reappeared in mice after treatment with diminazene aceturate at a dose of 25 mg/kg of b.w. was diluted in phosphate saline glucose to prepare inocula of 10^5 and 10^6 trypanosomes in 0.2 ml. Inocula of each size were used to i.v. infect six groups of 25 mice each. Thereafter, the mice in each group were treated immediately with diminazene aceturate i.p. at doses of 0, 10, 20, 30, 40, or 50 mg/kg of b.w. For 100 days following treatment, all mice were monitored for development of parasitemia by examining wet films of tail blood at a magnification of ×250; mice detected as parasitemic were removed from the experiment.

Values of the doses of diminazene aceturate required to cure 50% of the infected mice (CD_{50}) were determined by logit analysis and the minimum chi-square method (22).

Characterization of the diminazene susceptibility of clones derived from *T. congolense* IL 3274. Experiments to determine the diminazene sensitivity of clones of *T. congolense* IL 3274 that arose in mice prior to, or following, treatment with diminazene aceturate were carried out. Bloodstream forms of the parasite were used to infect a group of nonirradiated mice i.p. (10^7 trypanosomes per mouse). Following detection of trypanosomes, on day 3 of infection, each mouse was treated i.p. with diminazene aceturate at a dose of 25 mg/kg of b.w. Subsequently, all mice exhibited temporary remission of parasitemia followed by reappearance of trypanosomes. Trypanosome populations occurring in these animals were cloned on days 2 and 3 of infection (prior to treatment) by the method of Barry and Gathuo (6). They were also cloned on days 18 to 20 of infection, when trypanosomes had reappeared following treatment. Each clone was thereafter expanded in sublethally (650-rad) irradiated mice. The resultant populations were then used to infect groups of nonirradiated mice at an inoculum size of 10^7 trypanosomes per mouse. On day 3 of infection, the mice were treated i.p. with diminazene aceturate at a dose of 25 mg/kg of b.w. All mice were then monitored for 100 days for the development of parasitemia, as described above.

Pharmacokinetics of diminazene in mice. In the first mouse study, all five groups of mice were treated with diminazene aceturate at a dose of 25 mg/kg of b.w. In order to determine whether differences in the disposition of diminazene occurred as the trypanosome inoculum size was increased, the pharmacokinetics of the drug for three groups of mice were determined. Group A consisted of noninfected mice which were treated i.p. with diminazene aceturate at a dose of 25 mg/kg of b.w. Mice in group B were infected i.v. (10^6 trypanosomes per mouse) with a population of *T. congolense* IL 3274 that had reappeared in infected mice after treatment with diminazene aceturate at a dose of 25 mg/kg of b.w. Immediately after infection, the mice were treated i.p. with diminazene aceturate at a dose of 25 mg/kg of b.w. Mice in group C were not infected but were treated i.v. with diminazene aceturate at a dose of 15 mg/kg of b.w. This last group was included to provide data required to determine values of a number of pharmacokinetic parameters.

Following treatment, blood samples were obtained from mice in each of groups A, B, and C after 5, 10, 15, 30, 40, and 45 min and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 21, 24, and 48 h. Since ion pair extraction of diminazene from blood (2) requires a volume of 1 ml, samples from two treated mice were pooled in a 3-ml potassium-EDTA-containing Vacutainer (Becton Dickinson) tube for each time. Furthermore, at each sampling time, 10 mice were used to obtain five replicate samples. The methods used for plasma separation, storage, and analysis of diminazene by high-performance liquid chromatography were as previously described (1, 2). Plasma concentration-time data sets were modelled by using NONLIN version 3.0 (Statistical Consultants Inc.) and STATIS version 3.0 (ClydeSoft), with JANA (Statistical Consultants Inc.) to determine initial parameter estimates. The Akaike and Schwartz information criteria (45, 53) were used for selection of the compartmental model that best fitted each concentration-time data set. Symbols of compartmental parameters are used in this paper as commonly defined (3, 20, 25, 43). Noncompartmental parameters, such as the

TABLE 3. Coefficients and exponents describing disposition of diminazene following its administration at single doses of 15.0 mg/kg of b.w. i.v. or 25.0 mg/kg of b.w. i.p. to noninfected and *T. congolense*-infected mice

Route of drug administration (group)	C_1 (μg/ml)	C_2 (μg/ml)	C_3 (μg/ml)	λ_1 (h^{-1})	λ_2 (h^{-1})	λ_3 (h^{-1})
i.p. (noninfected)	14.24 ± 10.37*#	2.79 ± 0.43*	0.46 ± 0.45	23.30 ± 17.10*	1.31 ± 0.64*	0.07 ± 0.05
i.p. (infected)	13.21 ± 8.23‡	1.42 ± 1.77	0.28 ± 0.17	15.17 ± 16.45	0.83 ± 0.96	0.03 ± 0.03
i.v. (noninfected)	2.01 ± 1.44*‡	0.93 ± 0.40*	0.24 ± 0.08	5.36 ± 1.67*	0.45 ± 0.10*	0.04 ± 0.01

[a] Arithmetic mean values (± standard deviations) with the same symbols (* and ‡) in the same column are significantly different ($P < 0.05$).

area under the concentration-time curve ($AUC_{0-\infty}$) and the area under the (first) moment curve ($AUMC_{0-\infty}$) determined by the trapezoidal rule from time zero to infinity, mean residence time (MRT), apparent total body clearance (CL) and apparent volume of distribution at steady state [$V_{d(ss)}$], were calculated in accordance with standard equations (20). Bioavailability (F) represents the percentage ratio of $AUC_{0-\infty}$ (i.p.) to $AUC_{0-\infty}$ (i.v.). Other parameters used in this paper are as follows: maximum concentration in plasma (C_{max}), volume of the central compartment (V_c), zero time intercepts of the rapid (C_1) and slow (C_2) distribution phases, zero time intercept of the terminal elimination phase (C_3), rapid (λ_1) and slow (λ_2) exponents of the distribution phases, hybrid rate constant for the terminal elimination phase (λ_3), and rapid ($t_{1/2\lambda_1}$) and slow ($t_{1/2\lambda_2}$) distribution half-life ($t_{1/2}$), terminal elimination $t_{1/2}$ ($t_{1/2\lambda_3}$), elimination rate constants from the central compartment (k_{el}), $t_{1/2}$ for elimination from central compartment ($t_{1/2k_{el}}$), transfer rate constant from central-to-shallow (k_{12}) and central-to-deep (k_{13}) peripheral compartments, and transfer rate constants from deep (k_{31}) and shallow (k_{21}) peripheral compartments to the central compartment. All statistical analyses were carried out with Minitab version 8.2 (Minitab Inc.). The geometric means (range) are given for $t_{1/2}$ parameters. The values of other parameters are expressed as arithmetic means ± standard deviations. A single classification analysis of variance was used to test differences between values of pharmacokinetic parameters determined in the three groups at the 5% significance level.

RESULTS

Estimation of the proportions of resistant trypanosomes in inocula. Five groups of 100 mice each were infected i.v. with a population of *T. congolense* IL 3274 that relapsed in infected mice following i.p. treatment with diminazene aceturate at a dose of 25 mg/kg of b.w. The results obtained for each of the five groups are presented in Table 1. In each group, 50 of 50 control mice were detected as parasitemic, thus indicating 100% infectivity for all five inoculum sizes. In contrast, in the groups of mice inoculated with 10^2, 10^3, 10^4, 10^5, or 10^6 trypanosomes and treated with diminazene aceturate at a dose of 25 mg/kg of b.w., trypanosomes were detected in 4, 11, 13, 28, or 39 of 50 mice, respectively. Blood from the mice that remained aparasitemic was not infective to irradiated mice (data not shown). In addition, sera from the same parasite-negative mice were negative for *T. congolense* antigen when examined by enzyme-linked immunosorbent assay (34) (data not shown).

The data presented in Table 1 show that not all mice infected with *T. congolense* IL 3274 and treated with diminazene aceturate became parasitemic. Furthermore, mice receiving smaller inocula appeared less likely to become parasitemic than mice receiving larger inocula. Logistic regression was used to investigate the relationship between the probability of a mouse being parasitemic and the size of the inoculum. A relationship of the following form was found to be a good fit: $\ln(p/[1-p]) = -4.27 + 0.90 \log_{10}(\text{IS})$, where p is the proportion of mice detected as parasitemic following treatment with diminazene aceturate and IS is the trypanosome inoculum size. The 95% confidence intervals for the intercept and slope were (−5.38, −3.16) and (0.66, 1.14), respectively. Table 1 shows the closeness of fit between the observed number of mice that were detected as parasitemic and the number that were predicted to be parasitemic by the logistic equation.

The possibility that each inoculum contained a mixture of susceptible and resistant trypanosomes, and that sampling variation resulted in some inocula not containing any resistant trypanosomes, was investigated as a possible explanation for the relationship between the inoculum size and the proportion of mice that became parasitemic. For an inoculum of size n trypanosomes containing a proportion Θ of resistant trypanosomes, the probability of a mouse receiving at least one resistant trypanosome is calculated as follows: $1 - (1 - \Theta)^n$. By using the observed proportion of animals detected as parasitemic (Table 1) as an estimate of this proportion, it was possible to obtain an estimate of Θ for each inoculum size. For inocula of 10^2, 10^3, 10^4, 10^5, and 10^6 trypanosomes, the maximum likelihood estimates for Θ were 8.335×10^{-4}, 2.485×10^{-4}, 3.02×10^{-5}, 8.3×10^{-6}, and 1.6×10^{-6}, respectively. Likelihood ratio testing confirmed that the estimates were significantly different.

Diminazene susceptibility of relapse populations of *T. congolense* IL 3274. Table 2 summarizes the diminazene aceturate susceptibility data for *T. congolense* IL 3274 that appeared in mice after treatment with diminazene aceturate at a dose of 25 mg/kg of b.w. Regardless of the inoculum size, trypanosomes were detected in all mice that were treated with diminazene aceturate at doses of either 0 or 10 mg/kg of b.w. Similarly, trypanosomes were detected in 25 of 25 mice which were treated with 20 mg/kg of b.w. after infection with 10^6 trypanosomes. In the groups of mice that were infected with 10^3 trypanosomes and treated with diminazene aceturate at doses of 20, 30, 40, or 50 mg/kg of b.w., 15, 2, 4, or 2 of 25 mice, respectively, were detected as parasitemic. In the groups of mice that were infected with 10^6 trypanosomes and treated with diminazene aceturate at doses of 30, 40, or 50 mg/kg of b.w., 13, 4, or 2 of 25 mice, respectively, were detected as parasitemic. Based on these data, the diminazene aceturate CD_{50} value determined for mice infected with 10^6 trypanosomes was 29.88 mg/kg of b.w. and was significantly ($P < 0.001$) greater than that determined for mice infected with 10^3 trypanosomes (CD_{50}, 22.28 mg/kg of b.w.).

Characterization of the diminazene susceptibility of clones derived from *T. congolense* IL 3274. A total of 15 clones were examined: 5 were prepared on day 2 of infection, 6 were prepared on day 3, 1 was prepared on day 18, 1 was prepared on day 19, and 2 were prepared on day 20. All clones were refractory to treatment with a single dose of 25 mg of diminazene aceturate per kg of b.w.

Pharmacokinetics of diminazene in mice. Values of the pharmacokinetic parameters determined from the noninfected and *T. congolense*-infected groups of mice after treatment with diminazene are shown in Tables 3 to 5. The C_{max} of diminazene for both the noninfected (17.49 ± 10.25 μg/ml) and *T. congolense*-infected (14.89 ± 10.05 μg/ml) groups were observed at 5 min following treatment. For the noninfected group treated i.p., F, $AUC_{0-\infty}$, and $t_{1/2\lambda_3}$ were 66.16% ± 20.97%, 9.70 ± 1.83 μg · h/ml, and 10.71 h, respectively. Values of these parameters for the infected group were 87.00% ± 15.71%, 13.33 ± 3.83 μg · h/ml, and 25.70 h, respectively. Although the

TABLE 4. Pharmacokinetic parameters[a] of diminazene in noninfected and *T. congolense*-infected mice after administration of the drug at doses of 15.0 mg/kg of b.w. i.v. and 25.0 mg/kg of b.w. i.p.

Route of drug administration (group)	$t_{1/2}\lambda_1$ (h)	$t_{1/2}\lambda_2$ (h)	$t_{1/2}\lambda_3$ (h)	$t_{1/2}\beta_{el}$ (h)	k_{21} (h^{-1})	k_{12} (h^{-1})	k_{13} (h^{-1})	k_{31} (h^{-1})	k_{el} (h^{-1})	V_c (liters/kg)
i.p. (noninfected)	0.036 (0.013–0.086)*	0.57 (0.29–0.84)[b]	10.71 (4.11–24.54)	0.38 (0.17–0.68)*	2.00 ± 1.18*	14.59 ± 14.78	2.88 ± 1.77*	0.25 ± 0.29	4.96 ± 0.46*	1.92 ± 1.25[b]
i.p. (infected)	0.07 (0.02–0.028)	1.86 (0.28–19.23)	25.70 (8.07–105.92)	0.45 (0.15–0.79)‡	1.88 ± 1.46‡	8.64 ± 9.86	3.60 ± 2.36‡	0.14 ± 0.13	2.64 ± 3.40	2.17 ± 1.04‡
i.v. (noninfected)	0.13 (0.10–0.25)*	1.51 (1.23–2.27)*	17.37 (12.59–31.80)	1.94 (1.26–3.20)*‡	0.37 ± 0.12*‡	2.45 ± 1.73	0.53 ± 0.26*‡	0.12 ± 0.04	2.36 ± 1.27*	5.30 ± 2.12*‡

[a] Values with the same symbols (* and ‡) in the same column are significantly different ($P < 0.05$). Geometric mean (range) values are given for $t_{1/2}\lambda_1$, $t_{1/2}\lambda_2$, $t_{1/2}\lambda_3$, and $t_{1/2}\beta_{el}$; all other parameters are arithmetic means ± standard deviations.

values of these parameters were higher for the infected group than for the noninfected group treated i.p., they did not differ significantly. For the noninfected group treated i.p., CL (28.25 ± 5.73 ml/min/kg) was very similar to that for the infected group (28.62 ± 5.73 ml/min/kg). Similarly, the MRT for the infected group (50.04 ± 46.24 h) was not significantly longer than that for the same noninfected group (17.89 ± 5.39 h). A similar trend was observed with $V_{d(ss)}$, which was large for the infected group (84.29 ± 54.68 liters/kg) but not significantly different from that for the noninfected group (47.32 ± 15.24 liters/kg). There was also no significant difference between the V_c of the noninfected (1.92 ± 1.25 liters/kg) and infected (2.17 ± 1.04 liters/kg) groups. However, the V_c of the noninfected group treated i.v. (5.30 ± 2.12 liters/kg) was significantly greater than those of both the noninfected and infected i.p. treatment groups. Similarly, the geometric mean $t_{1/2}k_{el}$ from the central compartment of the i.v. group was significantly higher than those of the i.p. treatment groups. In contrast, the C_{max} for the i.v. group (3.19 ± 1.17 μg/ml) was significantly lower than those for both i.p. treatment groups. There were no significant differences between the i.v. and i.p. treatment groups with respect to $AUC_{(0-\infty)}$, $AUMC_{(0-\infty)}$, MRT, CL, and $V_{d(ss)}$.

DISCUSSION

In the work presented here, experiments were carried out to investigate the dynamics of expression of diminazene resistance amongst trypanosomes which reappear in infected mice after treatment with diminazene aceturate. *T. congolense* IL 3274 was used for these studies. In initial work, experiments were carried out to examine the trypanosome populations which reappeared in infected mice after treatment with diminazene aceturate at a dose of 25 mg/kg of b.w. Inocula of 10^2, 10^3, 10^4, 10^5, and 10^6 trypanosomes were injected i.v. into large numbers of mice. Immediately thereafter, their susceptibility to i.p. treatment with diminazene aceturate at a dose of 25 mg/kg of b.w. was determined. In the absence of treatment, trypanosomes were detected in the blood of all mice that were infected. In additional work, infectivity was demonstrated at the level of individual trypanosomes (data not shown). In mice which were infected and treated, however, different proportions of animals were cured, depending on the inoculum size. The number of mice that became parasitemic, despite treatment, progressively increased from 4 in 50 to 39 in 50 mice from the smallest (10^2-trypanosome) to the largest (10^6-trypanosome) inoculum sizes used. A close fit to these data was obtained when the data were modelled by logistic regression. The observed data were also analyzed by using a mathematical model to determine the proportions of diminazene-resistant and -susceptible trypanosomes that were present in the inoculum populations. The model assumed that these proportions were fixed constants, regardless of the inoculum size. The resultant data indicated that with all five inoculum sizes, less than 0.1% of the trypanosomes were resistant to the drug dosage used. Thus, the majority of the populations appeared to be susceptible to diminazene aceturate at this dosage. Surprisingly, however, the proportion of the population that contained resistant trypanosomes did not remain constant but varied inversely with the inoculum size.

The above data indicate that the subpopulation of *T. congolense* IL 3274 which reappeared in infected animals after treatment with diminazene aceturate at a dose of 25 mg/kg of b.w. contained trypanosomes that were both resistant and susceptible to the drug dosage used. There could be several ex-

TABLE 5. Values of noncompartmental pharmacokinetic parameters[a] of diminazene for noninfected and *T. congolense*-infected mice after administration of the drug at single doses of 15.0 mg/kg of b.w. i.v. and 25 mg/kg of b.w. i.p.

Route of drug administration (group)	C_{max} (μg/ml)	$AUC_{0-\infty}$ (μg · h/ml)	$AUMC_{0-\infty}$ (μg · h²/ml)	MRT (h)	CL (ml/min/kg)	$V_{d(ss)}$ (liters/kg)	F (%)
i.p. (noninfected)	17.49 ± 10.25*	9.70 ± 1.83	174.76 ± 67.23	17.89 ± 5.39	28.25 ± 5.73	47.32 ± 15.24	66.16 ± 20.97
i.p. (infected)	14.89 ± 10.05‡	13.33 ± 3.83	785.41 ± 914.91	50.04 ± 46.24	28.62 ± 5.73	84.29 ± 54.68	87.00 ± 15.71
i.v. (noninfected)	3.19 ± 1.17*‡	9.14 ± 1.92	218.40 ± 111.29	22.97 ± 8.65	28.28 ± 5.73	36.99 ± 10.52	NA[b]

[a] Values with the same symbols (* and ‡) in the same column are significantly different ($P < 0.05$). The values are arithmetic means ± standard deviations.
[b] NA, not applicable.

planations for this observation. First, it has been shown that populations of *T. brucei* in mice (23) and *Trypanosoma vivax* in goats (52) can evade treatment and establish relapse parasitemias by invading the central nervous system. It is therefore possible that a similar phenomenon accounted for the presence of the drug-susceptible trypanosomes that were observed in the relapse populations described herein, although immediate treatment should have circumvented this possibility. Secondly, sites other than the central nervous system may serve as cryptic foci (51). Thus, since *T. congolense* can localize in the microvasculature (5, 24, 30), such sites may serve as cryptic foci for the parasite if they are poorly perfused with blood. A third possibility is that the diminazene resistance of *T. congolense* IL 3274 is unstable, as has previously been reported for isometamidium in *T. congolense* (40). It is therefore possible that during expansion of the trypanosome population, a drug-susceptible variant with a growth rate more rapid than that of a drug-resistant population dominates the relapse population (49). However, since there appeared to be little variation in diminazene susceptibility among clones derived from *T. congolense* IL 3274, this appears to be unlikely.

The present study also demonstrated that the level of diminazene resistance of *T. congolense* IL 3274 varies directly with the inoculum size. However, the calculated drug-resistant proportion of each population varied in an inverse linear manner with the inoculum size (Fig. 1). The reasons underlying this last observation were investigated in two experiments. In the first, we examined whether the resistance of the trypanosome population decreased as the inoculum size was increased. This question was investigated by characterizing the susceptibilities of relapse populations of two inoculum sizes (10^3 and 10^6 trypanosomes) to different doses of diminazene aceturate in mice. The results indicated that the diminazene aceturate CD_{50} value for mice infected with 10^6 trypanosomes was 29.88 mg/kg of b.w. and was significantly greater than that for mice infected with 10^3 trypanosomes, 22.28 mg/kg of b.w. These observations therefore indicate that there is a direct relationship between the inoculum size and the level of resistance of a population, as described by Sones and Holmes (48). The object of the second experiment was to determine whether the inverse relationship between trypanosome inoculum size and the proportion of surviving trypanosomes occurred because the parasites were exposed to decreasing concentrations of the drug as the inoculum size was increased as a result of the effects of parasite numbers on drug pharmacokinetics. The resultant data indicated that the disposition of diminazene did not vary significantly with the trypanosome inoculum size. Lastly, it is possible that the observed apparent decrease in the proportion of drug-resistant trypanosomes with increasing inoculum sizes was associated with improvements in the anti-variant-specific surface glycoprotein antibody responses as the inoculum size was increased. However, in light of the inverse linear relationship between $\log_{10}$(trypanosome inoculum) and $\log_{10}$(proportion of trypanosome population resistant to diminazene), we do not believe that this is a logical explanation for a number of reasons. Firstly, the relationship between these two parameters, as shown in Fig. 1, indicates that for every 10-fold increase in the size of the trypanosome inoculum there was a decrease in $\log_{10}$ (proportion of diminazene-resistant trypanosomes) by 0.692 ± 0.031. Thus, if antibody responses were responsible for these observations the functional activity of the antibody response would have had to increase by the same proportion with each 10-fold increase in inoculum size. Such a linear relationship between the antibody response and the quantity of trypanosome antigen is inconsistent with experimental findings (8). Furthermore, by extrapolation from experiments carried out with cattle, it would appear that the smallest inoculum sizes used in our experiment would be insufficient to elicit an anti-variant-specific surface glycoprotein response (32).

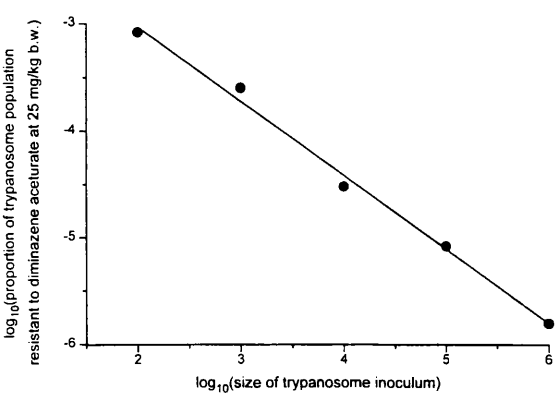


FIG. 1. Relationship between *T. congolense* IL 3274 inoculum size and the proportion of the population resistant to diminazene aceturate at a dose of 25 mg/kg of b.w. (using data on observed parasitemia from Table 1).

The maximum concentration that a trypanosome population attains in rodents is regulated by both host factors (7, 8) and host-independent mechanisms associated with the parasite population (9). The results described here have demonstrated that diminazene susceptibility is dependent upon the concentration of a trypanosome population in vivo. Other studies have shown that the concentration of a population may influence the rate of proliferation of individual trypanosomes (9, 10), thus potentially altering the proportions of the population in the different stages of the cell division cycle. While little is known about the mechanism of action of diminazene on trypanosomes, evidence from other systems indicates that diminazene acts on organisms at specific stages of the cell division cycle (11, 26, 42, 50). A full explanation of the results observed

in the present study requires an extensive study of the factors which influence trypanosomal cell division and proliferation and of whether there is developmental and/or cell division cycle control of expression of drug resistance in vivo.

Finally, work with pentamidine, an aromatic diamidine closely related to diminazene, has indicated that the molecule is transported into bloodstream forms of *T. b. brucei* via a carrier-mediated process that is concentrative and substrate specific (15). Additional work with pentamidine and diminazene has indicated that their uptake in *T. b. brucei* is mediated by adenosine transporters (13, 19) and that differences in transport activity are associated with expression of resistance to these compounds (13, 14, 19). The role of these processes in the work described herein awaits investigation.

ACKNOWLEDGMENTS

We thank C. Robertson of the University of Strathclyde, Glasgow, United Kingdom, and J. Rowlands, International Livestock Centre for Africa, for discussion concerning statistical analyses. B. Williams of the London School of Tropical Medicine and Hygiene, London, United Kingdom, is also thanked for helpful discussions. M. Mulindi is thanked for secretarial assistance.

REFERENCES

1. **Aliu, Y. O., M. Mamman, and A. S. Peregrine.** 1993. Pharmacokinetics of diminazene in female Boran (*Bos indicus*) cattle. J. Vet. Pharmacol. Ther. **16**:291–300.
2. **Aliu, Y. O., and S. Ødegaard.** 1983. Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in plasma. J. Chromatogr. **276**:218–223.
3. **Allen, L., K. Kimura, J. Mackichan, and W. A. Ritschel.** 1982. Manual of symbols, equations and definitions in pharmacokinetics. J. Clin. Pharmacol. **22**(Suppl.):S1–S23.
4. **Bakhiet, M., T. Olsson, C. Edlund, B. Höjeberg, K. Holmberg, J. Lorentzen, and K. Kristensson.** 1993. A *Trypanosoma brucei brucei*-derived factor that triggers CD8$^+$ lymphocytes to interferon-γ secretion: purification, characterization and protective effects *in vivo* by treatment with a monoclonal antibody against the factor. Scand. J. Immunol. **37**:165–178.
5. **Banks, K. L.** 1978. Binding of *Trypanosoma congolense* to the walls of blood vessels. J. Protozool. **25**:241–245.
6. **Barry, J. D., and H. Gathuo.** 1984. Antigenic variation in *Trypanosoma vivax*: isolation of a serodeme. Parasitology **89**:49–58.
7. **Black, S. J., R. M. Jack, and W. I. Morrison.** 1983. Host-parasite interactions which influence the virulence of *Trypanosoma* (*Trypanozoon*) *brucei brucei* organisms. Acta Trop. **40**:11–18.
8. **Black, S. J., C. N. Sendashonga, P. A. Lalor, D. D. Whitelaw, R. M. Jack, W. I. Morrison, and M. Murray.** 1983. Regulation of growth and differentiation of *Trypanosoma* (*Trypanozoon*) *brucei brucei* in resistant (C57Bl/6) and susceptible (C3H/He) mice. Parasite Immunol. **5**:465–478.
9. **Bungener, W.** 1982. Behaviour of a *Trypanosoma brucei brucei* stock (STIB 348C) in mice. 2. Course of infections with basic type and several variant types. Trop. Med. Parasitol. **33**:243–248.
10. **Bungener, W.** 1984. Behaviour of a *Trypanosoma brucei brucei* stock (STIB 348C) in mice. 4. Different course of primary parasitemia in trypanosome variants of different virulence. Trop. Med. Parasitol. **35**:115–118.
11. **Byres, T. J., B. G. Kim, L. E. King, and E. R. Hugo.** 1991. Molecular aspects of the cell cycle and encystment of *Acanthamoeba*. Rev. Infect. Dis. **13**(Suppl. 5):S3378–S3384.
12. **Cantrell, W.** 1956. Behaviour of mixtures of oxophenarsin-resistant and unmodified strains of *Trypanosoma equiperdum*. Exp. Parasitol. **5**:178–190.
13. **Carter, N. S., and A. H. Fairlamb.** 1993. Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature (London) **361**:173–176.
14. **Damper, D., and C. L. Patton.** 1976. Pentamidine transport and sensitivity in *brucei*-group trypanosomes. J. Protozool. **23**:349–356.
15. **Damper, D., and C. L. Patton.** 1976. Pentamidine transport in *Trypanosoma brucei*—kinetics and specificity. Biochem. Pharmacol. **25**:271–276.
16. **Diffley, P., and J. O. Scott.** 1984. Immunological control of chronic *Trypanosoma brucei gambiense* in outbred rodents. Acta Trop. **43**:335–342.
17. **Diffley, P., J. O. Scott, K. Mama, and T. N. Tsen.** 1987. The rate of proliferation among African trypanosomes is a stable trait that is directly related to virulence. Am. J. Trop. Med. Hyg. **36**:535–540.
18. **Diffley, P., J. E. Strickler, C. L. Patton, and B. H. Waksman.** 1980. Detection and quantification of variant-specific antigen in the plasma of rats and mice infected with *Trypanosoma brucei brucei*. J. Parasitol. **66**:185–191.
19. **Fairlamb, A. H., N. S. Carter, M. Cunningham, and K. Smith.** 1992. Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. Mol. Biochem. Parasitol. **53**:213–222.
20. **Gibaldi, M., and D. Perrier.** 1982. Pharmacokinetics. p. 409–417. Marcel Dekker, New York.
21. **Hawking, F.** 1963. Drug-resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, berenil and other compounds. Ann. Trop. Med. Parasitol. **57**:262–282.
22. **Hewlett, P. S., and R. L. Plackett.** 1979. The interpretation of quantal responses in biology. p. 22–37. Edward Arnold, London.
23. **Jennings, F. W., D. D. Whitelaw, P. H. Holmes, H. G. B. Chizyuka, and G. M. Urquhart.** 1979. The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. Int. J. Parasitol. **9**:381–384.
24. **Kalu, A. U., F. A. Lawani, and S. O. Omotainse.** 1988. Localization of *Trypanosoma congolense* following diminazene aceturate therapy in ruminants. Bull. Anim. Health Prod. Afr. **36**:77–79.
25. **Kinabo, L. D. B., and Q. A. McKellar.** 1989. Current models in pharmacokinetics: applications in veterinary pharmacology. Vet. Res. Commun. **13**: 141–157.
26. **Kishore, P., S. Gupta, D. K. Srivastava, and O. P. Shukla.** 1990. Action of methylglyoxal bis (guanyl hydrazone) and related antiprotozoals on *Acanthamoeba culbertsoni*. Indian J. Exp. Biol. **28**:1174–1179.
27. **Macadam, R. F., and J. Williamson.** 1972. Drug effects on the fine structure of *Trypanosoma rhodesiense*: diamidines. Trans. R. Soc. Trop. Med. Hyg. **66**:897–904.
28. **Mamman, M., D. J. L. Williams, N. B. Murphy, and A. S. Peregrine.** 1995. Apparent rarity of diminazene-resistant trypanosomes in goats infected with a diminazene-resistant population of *Trypanosoma congolense*. Res. Vet. Sci. **58**:113–118.
29. **Mancini, P. E., and C. L. Patton.** 1981. Cyclic 3′,5′-adenosine monophosphate levels during the developmental cycle of *Trypanosoma brucei brucei* in the rat. Mol. Biochem. Parasitol. **3**:19–31.
30. **Maxie, M. G., and G. J. Losos.** 1977. Release of *Trypanosoma congolense* from the microvasculature of cattle by Berenil. Vet. Parasitol. **3**:277–281.
31. **Miller, E. N., and M. J. Turner.** 1981. Analysis of antigenic types appearing in first relapse populations of clones of *Trypanosoma brucei*. Parasitology **82**:63–80.
32. **Morrison, W. I., S. J. Black, J. Paris, C. A. Hinson, and P. W. Wells.** 1982. Protective immunity and specificity of antibody responses elicited in cattle by irradiated *Trypanosoma brucei*. Parasite Immunol. **4**:395–407.
33. **Murray, M., and A. R. Gray.** 1984. The current situation on animal trypanosomiasis in Africa. Prev. Vet. Med. **2**:23–30.
34. **Nantulya, V. M., and K. J. Lindqvist.** 1989. Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax*, *T. congolense* and *T. brucei* infections in cattle. Trop. Med. Parasitol. **40**:267–272.
35. **Newton, B. A., and E. W. F. Le Page.** 1967. Preferential inhibition of extranuclear deoxyribonucleic acid synthesis by the trypanocide Berenil. Biochem. J. **105**:50P.
36. **Oehler, R.** 1914. Untersuchungen uber den Dimorphismus von *Trypanosoma brucei*. Z. Hyg. Infektionskr. **77**:356–370.
37. **Olsson, T., M. Bakhiet, C. Edlund, B. Höjeberg, P. H. Van Der Meide, and K. Kristensson.** 1991. Bidirectional activating signals between *Trypanosoma brucei* and CD8$^+$ T cells: a trypanosome-released factor triggers interferon-γ production that stimulates parasite growth. Eur. J. Immunol. **21**:2447–2454.
38. **Olsson, T., M. Bakhiet, B. Höjeberg, Å. Ljungdahl, C. Edlund, G. Andersson, H.-P. Ekre, W.-P. Fung-Leung, T. Mak, H. Wigzell, U. Fiszer, and K. Kristensson.** 1993. CD8 is critically involved in lymphocyte activation by a *T. brucei brucei*-released molecule. Cell **72**:715–727.
39. **Olsson, T., M. Bakhiet, and K. Kristensson.** 1992. Interactions between *Trypanosoma brucei* and CD8$^+$ T cells. Parasitol. Today **8**:237–239.
40. **Peregrine, A. S., G. Knowles, A. I. Ibitayo, S. K. Moloo, and N. B. Murphy.** 1991. Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. Parasitology **102**:93–100.
41. **Pinder, M., and E. Authie.** 1984. The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. Acta Trop. **41**:247–252.
42. **Poot, M., K. Kausch, J. Kohler, T. Haaf, and H. Hoehn.** 1990. The minor-groove binding ligands netropsin A and berenil cause polyploidisation via impairment of the G_2 phase of the cell cycle. Cell Struct. Funct. **15**:151–157.
43. **Riviere, J. E.** 1988. Veterinary clinical pharmacokinetics. Part II. Modelling. Compend. Cont. Educ. Small Anim. Pract. **10**:314–327.
44. **Royal Statistical Society.** 1985. GLIM statistical package, version 3.77.
45. **Schwartz, G.** 1978. Estimating the dimension of a model. Ann. Stat. **6**:461–464.
46. **Seed, J. R.** 1978. Competition among serologically different clones of *Trypanosoma brucei gambiense in vivo*. J. Protozool. **25**:526–529.
47. **Silayo, R. S., M. Mamman, S. K. Moloo, Y. O. Aliu, M. A. Gray, and A. S. Peregrine.** 1992. Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate. Res. Vet. Sci. **53**:98–105.
48. **Sones, K. R., and P. H. Holmes.** 1992. The influence of the size of the initial

inoculum on the efficacy of isometamidium (samorin) on a stock of *Trypanosoma congolense*. Acta Trop. **51**:213–216.
49. **Sones, K. R., P. H. Holmes, and G. M. Urquhart.** 1989. Interference between drug-resistant and drug-sensitive stocks of *Trypanosoma congolense* in goats. Res. Vet. Sci. **47**:75–77.
50. **Stauffert, I., H. Paulini, U. Steinmann, H. Sippel, and C. J. Estler.** 1990. Investigations on mutagenicity and genotoxicity of pentamidine and some related trypanocidal diamidines. Mutat. Res. **245**:93–98.
51. **Waitumbi, J. N., H. C. Brown, F. W. Jennings, and P. H. Holmes.** 1988. The relapse of *Trypanosoma brucei brucei* infections after chemotherapy in rabbits. Acta Trop. **45**:45–54.
52. **Whitelaw, D. D., P. R. Gardiner, and M. Murray.** 1988. Extravascular foci of *Trypanosoma vivax* in goats: central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. Parasitology **97**:1–11.
53. **Yamaoka, K., T. Nakagawa, and T. Uno.** 1978. Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. J. Pharmacokinet. Biopharm. **6**:165–175.

Section 4.5

Burudi, E.M.E., Peregrine, A.S., Majiwa, P.A.O., Mbiuki, S.M. and Murphy, N.B. (1994) Response of diminazene-resistant and diminazene-susceptible *Trypanosoma congolense* to treatment with diminazene when occurring as a mixed infection in goats. *Annals of Tropical Medicine and Parasitology* 88, 595-606.

Annals of Tropical Medicine and Parasitology, Vol. 88, No. 6, 595–606 (1994)

Response of diminazene-resistant and diminazene-susceptible *Trypanosoma congolense* to treatment with diminazene when occurring as a mixed infection in goats

By E. M. E. BURUDI, A. S. PEREGRINE*, P. A. O. MAJIWA
International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya

S. M. MBIUKI
Department of Clinical Studies, Faculty of Veterinary Medicine, University of Nairobi, P.O. Box 29053, Nairobi, Kenya

AND N. B. MURPHY
International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya

Received 19 July 1994, Revised 22 August 1994, Accepted 24 August 1994

A study was carried out to determine whether a drug-resistant trypanosome population could influence the survival of a drug-sensitive population in mixed infections in goats. To identify both populations during the course of a mixed infection, a system for distinguishing them was developed; using a nucleotide sequence of a cDNA that was derived from *Trypanosoma congolense* ILNat 3.3 (IL 1616), a pair of 20-mer primers was designed which, in a PCR, amplified a 900-bp sequence from the diminazene-sensitive trypanosome, *T. congolense* IL 1180, but not the diminazene-resistant trypanosome, *T. congolense* IL 3247. The PCR technique detected 100 pg of IL 1180 DNA when mixed with 25 ng of total genomic DNA of IL 3274, as determined by gel electrophoresis and ethidium bromide-staining of the PCR products. Using the 900-bp PCR product as a ^{32}P-labelled probe on Southern blots, the sensitivity was increased 100-fold. Three groups of five goats each were infected with IL 1180 (group A), IL 3274 (group B) or both clones simultaneously (group C), and treated with diminazene aceturate at a dose of 7·0 mg/kg body weight following detection of trypanosomes. Three other groups of three goats each were similarly infected and kept as untreated controls. All group A animals were cured, while all in group B and four animals in group C relapsed. Trypanosomes were harvested from all animals at regular intervals up to 60 days post treatment. Using the PCR techniques, IL 1180 DNA could not be detected in any post-treatment trypanosome DNA sample. It therefore appeared, on the basis of the sensitivity of the DNA detection systems used, that IL 1180 is unable to survive treatment with diminazene aceturate when mixed with IL 3274 in goats.

Chemotherapy is the most commonly used method of controlling African trypanosomiasis in domestic livestock (Ilemobade, 1987). However, the development of resistance to the drugs that are used is a major constraint to their efficacy. While resistance to each of the commonly used trypanocides has been described (Leach and Roberts, 1981), the development of resistance to diminazene is particularly intriguing since it is a drug that is very rapidly eliminated (Aliu *et al.*, 1984). Therefore, resistance would not be expected to

*Author to whom correspondence should be addressed.

0003–4983/94/060595+12 $08.00/0

develop as rapidly as for prophylactic drugs, which have longer half-lives (Whiteside, 1960). However, the incidence of resistance to diminazene (Jones-Davies, 1967; Gray and Roberts, 1971; Ilemobade, 1979) appears to be similar to that to other trypanocides, such as isometamidium and homidium. While this is somewhat surprising, it should be noted that trypanosome field isolates that are resistant to diminazene are often also resistant to quinapyramine (Whiteside, 1960). Thus, such isolates could have been directly selected by quinapyramine usage (Ndoutamia *et al.*, 1993) and developed cross-resistance to diminazene (Whiteside, 1960; Gray and Roberts, 1971).

Studies on the resistance of trypanosome populations to diminazene aceturate are often carried out in definitive hosts by characterizing the sensitivity of the populations to treatment with the standard recommended doses of 3·5 and 7·0 mg/kg body weight (b.w.) (Leach and Roberts, 1981). However, the sensitivity of *Trypanosoma congolense* to diminazene depends on the stage of infection when treatment is administered (Mamman *et al.*, 1993). Furthermore, for diminazene-resistant populations, the majority of *T. congolense* trypanosomes which re-appear in goats following treatment with diminazene aceturate appear to be sensitive to the dosage that was used (Mamman *et al.*, 1995). This latter finding is surprising, and could be due to the ability of diminazene-sensitive trypanosomes to survive treatment when mixed with trypanosomes that are resistant to diminazene. The drug-resistant population could have caused this phenomenon by altering the pharmacodynamics of diminazene. Alternatively, the drug-resistant population could have altered the growth rate of the drug-sensitive population (Sones *et al.*, 1989); this effect would alter the sensitivity of the 'drug-sensitive' population to diminazene since the drug appears to act on cells at specific stages of the cell-division cycle (Poot *et al.*, 1990; Stauffert *et al.*, 1990).

The present study was carried out to establish whether a diminazene-sensitive clone of *T. congolense* could survive treatment with diminazene aceturate when mixed with a diminazene-resistant clone in goats. Since the study used two savannah-type clones of *T. congolense*, it was necessary to develop a highly sensitive technique that could differentiate the two clones. At present, only some variable surface glycoprotein (VSG) gene sequences (Majiwa *et al.*, 1986), type-specific DNA probes (Majiwa *et al.*, 1985) and the arbitrary primer PCR (AP-PCR; Waitumbi and Murphy, 1993) are available for use in distinguishing different types of *T. congolense*. A PCR technique for this purpose was developed and its sensitivity and specificity evaluated in the present study. The technique was used to screen for the diminazene-sensitive clone in trypanosome populations collected from infected goats, both before and after treatment with diminazene aceturate.

MATERIALS AND METHODS

Experimental Animals

Twenty-four East African × Galla goats, aged 1–2 years, were obtained from an area of Kenya that is devoid of trypanosomiases and tsetse flies. Thereafter, the animals were fed hay and concentrate supplements as described by Whitelaw *et al.* (1986), and allowed water *ad libitum*. Sera were collected from these animals on the day of infection and were shown to be negative for anti-trypanosomal antibodies using an IFAT (Katende *et al.*, 1987). Only animals with a packed red cell volume (PCV) within the normal range (22%–38%) (Jain, 1986) were used. Outbred male Swiss mice, each weighing 20–30 g, were sublethally irradiated (650 rad) and used for expansion of trypanosome numbers for infection of goats, and isolation of trypanosomal DNA.

Parasites

Two clones of *T. congolense* were used: IL 1180 and IL 3274. *Trypanosoma congolense* IL 1180 (ILNat 3.1) was derived after one passage in mice from clone IL 968, a derivative of STIB 212 which was isolated from a lion in the Serengeti region of Tanzania (Geigy and Kauffmann, 1973). Previous work has shown this population to be sensitive to diminazene aceturate in goats at a dose of 3·5 mg/kg b.w.

TABLE
Experimental design

Group	*No. of goats*	*Strain(s) of* T. congolense *used to infect goats (i.v.)*	*Treatment with diminazene aceturate (i.m.)*
A	5	IL 1180	7 mg/kg b.w. ADP
B	5	IL 3274	7 mg/kg b.w. ADP
C	5	IL 1180 & IL 3274	7 mg/kg b.w. ABP
D	3	IL 1180	No treatment
E	3	IL 3274	No treatment
F	3	IL 1180 & IL 3274	No treatment

ADP, after detection of trypanosomes; ABP, after animals in both groups A and B became parasitaemic and the animals in group C were all parasitaemic; b.w., body weight; i.v., intravenously; i.m., intramuscularly.

(Peregrine *et al.*, 1991). *Trypanosoma congolense* IL 3274 was derived from isolate Banankeledaga/83/CRTA/67, collected from a cow in Burkina Faso (Pinder and Authie, 1984), and shown to be resistant to diminazene aceturate in goats at a dose of 7·0 mg/kg b.w. (Peregrine *et al.*, 1991).

Reagents

The recombinant plasmid p1616/5 was isolated from a cDNA library made from mRNA of *T. congolense* IL 1616 (ILNat 3.3; Majiwa *et al.*, 1993). The cDNA encodes the IL 1616 VSG (unpublished obs.). Diminazene aceturate (Berenil®; Hoechst, Germany) was obtained commercially and reconstituted as a 7% (w/v) solution in sterile distilled water. Oligonucleotides ILO 1044 (5′ TGCCGATGGTGGAGGTTTCC 3′) and ILO 1045 (5′ TGCTCAGCATGGTGATGCTG 3′) were designed on the basis of partial nucleotide sequence information from the ends of the insert of the recombinant plasmid p1616/5 (unpubl. obs.). ILO 1044, ILO 1045 and the arbitrary primer ILO 525 (5′ CGGACGTCGC 3′) were synthesized using phosphoramidite chemistry on a model 381A DNA synthesizer (Applied Biosystems, U.S.A.). PCR buffer and *Taq* DNA polymerase were obtained from Promega Corp., U.S.A. Ultra-pure agarose was obtained from BRL Life Technologies, Inc., U.S.A., and (α-^{32}P)-dCTP was purchased from Amersham International, U.K.

Experimental Design

Twenty-four goats were divided into three groups of five animals each (groups A, B and C), and three groups of three animals each (groups D, E and F) (see Table), such that mean body weights in each group were approximately similar. The goats were then infected using trypanosomes obtained from blood of infected mice which was diluted in sterile phosphate–saline–glucose (PSG), pH 8; 1×10^6 *T. congolense* IL 1180 were inoculated into the left jugular vein or 1×10^6 *T. congolense* IL 3274 were inoculated into the right jugular vein or both inoculations were given to each goat. Thereafter, the goats were examined on a daily basis for the presence of trypanosomes using the buffy-coat, phase-contrast technique (Murray *et al.*, 1977). Treatment of the goats with diminazene aceturate was then carried out as shown in the Table. Goats in groups A and B were treated with 7 mg diminazene aceturate/kg b.w. following development of parasitaemia. Goats in group C were treated when all the goats in groups A, B and C had detectable parasitaemias. Goats in groups D, E and F served as non-treatment controls for the different infections. Animals were removed from the experiment when their PCV became <12%.

Monitoring of Infections and Collection of Samples

Blood samples were collected into potassium ethylenediamine tetra-acetate (EDTA)-containing vacutainer tubes prior to infection, to ascertain the pre-infection PCV values. Following infection, goats were monitored daily until treatment, and three times a week following treatment, for PCV and parasitaemia. During the entire experimental period of 120 days (from the day of infection to the day of termination of the experiment), two sets of trypanosome blood stabilates were prepared from each animal twice weekly and stored in liquid nitrogen (Dar *et al.*, 1972). Thereafter, sublethally irradiated mice were inoculated with blood collected 3 days before infection, and on days 18, 32, 46 and 60 of infection. The resultant parasites were cryopreserved in blood samples in liquid nitrogen. Finally, blood buffy-coats were also prepared from each goat twice a week and preserved at − 80°C.

DNA Extraction

Trypanosomes isolated on a diethyl amino-ethyl (DEAE)–cellulose column (Lanham and Godfrey, 1970) were resuspended in 500 µl TNE [25 mM Tris–HCl (pH 8·0), 100 mM NaCl, 5 mM EDTA] and DNA was prepared, and its concentration estimated, according to standard procedures (Sambrook *et al.*, 1989).

Buffy-coat samples that were used in the PCR were processed by suspending the cells in phosphate-buffered saline (pH 7·4), and boiling with Chelex-100 (BioRad, U.S.A.) as described by Walsh *et al.* (1991). Non-infected goat blood buffy-coat preparations were processed similarly to serve as negative controls.

PCR Reactions

The cycling parameters for AP-PCR were 94°C for 1 min, 40°C for 1·5 min, 72°C for 2 min, for 30 cycles, and finally an extension at 72°C for 5 min. For all sequence-specific primer PCR reactions with ILO 1044 and ILO 1045 the parameters were as follows: 94°C for 45 s, 60°C for 1 min, 72°C for 1·5 min, for 40 cycles, followed by an extension at 72°C for 5 min. The PCR products were resolved by electrophoresis in 2% (w/v) agarose gels in 1 × TAE [0·04 M Tris–acetate, 0·001 M EDTA (pH 8·3)] buffer, stained with ethidium bromide, and then visualized with an ultraviolet (U.V.) transilluminator (Sambrook *et al.*, 1989).

Purification of the 900-bp Diagnostic Probe for IL 1180 from p1616/5 PCR Product

For Southern blotting analysis, a 900-bp PCR product was generated from p1616/5 with ILO 1044 and ILO 1045 as primers. This product was purified after electrophoresis in a 1·5% (w/v) agarose gel run in 0·5 × TAE buffer and visualized on the transilluminator after staining with ethidium bromide. The gel segment containing the 900-bp PCR product was excised, and the DNA purified, as described by He *et al.* (1992). Phenol-chloroform extraction and ethanol precipitation were carried out on the eluate to further purify the DNA (Sambrook *et al.*, 1989).

Labelling of the 900-bp PCR Product

^{32}P-Labelling of the 900-bp PCR product was carried out using a Multiprime kit (Amersham, U.K.) according to the manufacturer's instructions, in the presence of 50 µCi of (α-^{32}P)-dCTP (3000 Ci/mmol; Amersham, U.K.). The labelled DNA was then separated from the unincorporated (α-^{32}P)-dCTP on a Sephadex G-50 column by the spun-column method, as described by Ausubel (1988). Estimation of the specific activity of the probe was performed by scintillation in a Beckman LS 6800 scintillation counter (Beckman Instruments Inc., U.S.A.).

Southern Blotting and Hybridization

Southern blotting of DNA from agarose gels to nylon filters (Schleicher and Schuell, Germany) was carried out essentially as described by Southern (1975). The DNA was then fixed by U.V. irradiation in a U.V. Stratalinker (Stratagene, U.S.A.) for 90 s. Prehybridization of filters was for 2 h and hybridization was carried out, with 10^6 counts/min/ml of ^{32}P-labelled probe, overnight at 65°C. Filters were then washed twice

for 30 min in 0·1 × SSC (where 20 × SSC is 3 M sodium chloride and 0·3 M sodium citrate in distilled water, pH 7·0), 0·1% SDS (w/v) (Sambrook *et al.*, 1989) at 65°C. Thereafter, the filters were exposed to X-ray films (Fuji, Japan) with an intensifying screen (Du Pont de Nemours, U.S.A.) at − 70°C for between 30 min and 2 weeks. After this period of exposure the films were developed using an automatic developer (Fuji, Japan).

PCR on Samples From Goats

Blood stabilates collected from goats were expanded in sublethally irradiated mice and the trypanosome DNA isolated. The DNA was dissolved in TE [10 mM Tris–HCl (pH 7·5), 0·1 mM EDTA] and stored at 4°C until required. Buffy-coat preparations taken on the day of treatment and non-infected goat blood buffy-coat preparations were processed by the method of Walsh *et al.* (1991) and used in the PCR as template DNA.

RESULTS

Development of Parasitaemia

On the basis of detection of trypanosomes by the buffy-coat, phase-contrast technique, animals only infected with *T. congolense* IL 1180 (groups A and D) had an average prepatent period following infection of 3 days. Animals in group A were treated with diminazene aceturate on day 18 post-infection and all five animals became aparasitaemic within 24 h. Thereafter, no relapse infections were detected in any of the group A goats during the entire period of 102 days following treatment. In contrast, the untreated control animals (group D) remained parasitaemic for the entire experimental period; none of the animals had to be removed from the experiment because of low PCV.

Animals that were only infected with *T. congolense* IL 3274 (groups B and E) had an average prepatent period following infection of 14 days. Treatment of animals in group B was carried out on day 18 post-infection. In all group B goats, blood trypanosomes apparently disappeared within 48 h of treatment but reappeared within 14 days of treatment and thereafter persisted for the entire experimental period. All the group B animals maintained their PCV above 12%. In contrast, all the untreated control animals (group E) were persistently parasitaemic throughout the experiment and were removed from the experiment (one on day 25 and the remainder on day 57) due to low PCV.

Animals infected with both IL 1180 and IL 3274 (groups C and F) had an average prepatent period following infection of 3.3 days. However, since animals in group B (single infections with IL 3274) were not found parasitaemic until 14 days post-infection, animals in group C were not treated until day 18 post-infection. Within 24 h of treatment, all group C animals became aparasitaemic, but trypanosomes reappeared in four of the five treated animals within 17 days of treatment; all four animals then remained parasitaemic for the remainder of the experimental period and maintained their PCV above 12%. Of the three non-treatment control animals (group F), one was removed from the experiment on day 32 post-infection due to low PCV. The other two remained parasitaemic until the termination of the experiment since they were able to maintain their PCV above 12%.

Arbitrary Primer-PCR

PCR reactions were carried out on DNA from the two trypanosome clones individually, and when mixed to mimic mixed infections. The results showed differences in 'fingerprint' patterns generated from the two DNA alone [Fig. 1(A); lanes 1 and 2]. When the two trypanosome DNA were mixed, a combination of the two polymorphisms was generated (data not shown). Fingerprint patterns generated in the AP-PCR with ILO 525 as a primer distinguished the two clones of trypanosomes, 0·8- and 0·9-kb PCR products only being present in IL 1180 and a 0·4-kb product only being present in IL 3274 [Fig. 1(A)]. A further PCR product of 0·1 kb was amplified intermittently and could not be used to distinguish IL 1180 from IL 3274. This technique was used to demonstrate the presence of IL 3274 in all

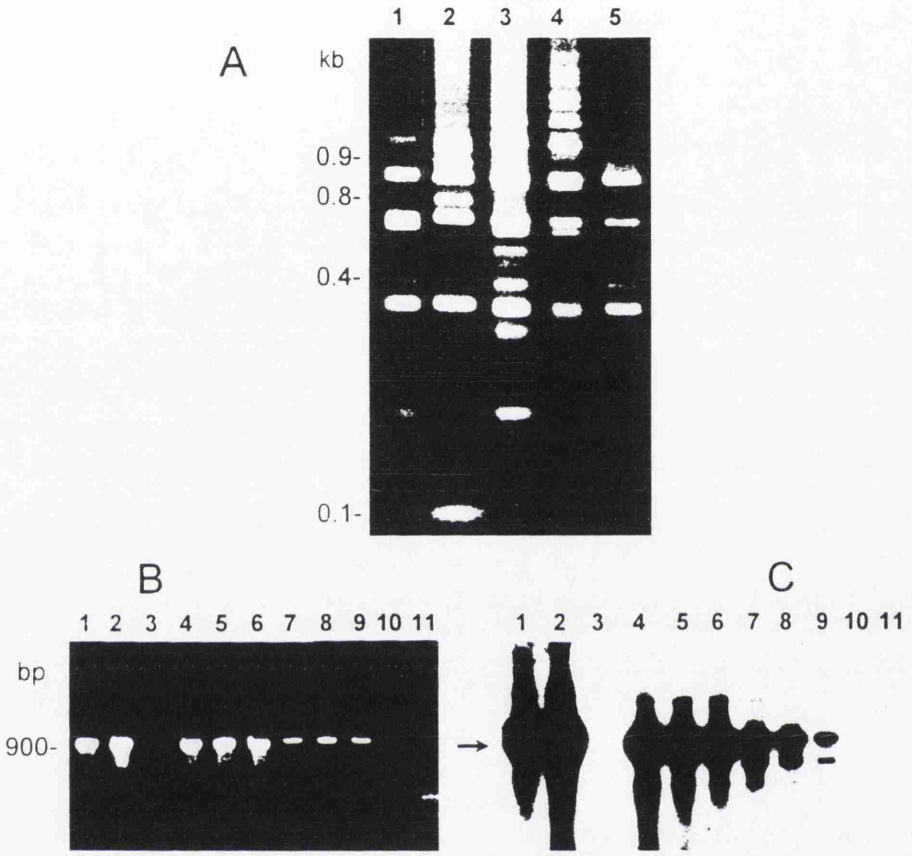


Fig. 1. (A) Arbitrary primer-PCR 'fingerprints' using primer ILO 525 on the total DNA of trypanosome populations collected 18 days post-treatment. Lane 1, IL 3274 DNA control; 2, IL 1180 DNA control; 3, 4 and 5, DNA of trypanosome populations collected from goats CJ 517, CJ 522 and CJ 532 (all infected with IL 3274 and IL 1180, and treated), respectively. (B) *Trypanosoma congolense* IL 1180 DNA titration in a PCR using primers ILO 1044 and ILO 1045. A photograph of an ethidium bromide-stained 2% agarose gel is shown. Lanes 1, 2, and 3, IL 1180 DNA, plasmid p1616/5 and IL 3274 DNA, respectively, as templates; 4–11, 25 ng each of IL 3274 DNA and 25 ng, 10 ng, 5 ng, 1 ng, 500 pg, 100 pg, 10 pg and 1 pg, respectively, of IL 1180 DNA. (C) DNA from the gel shown in (B) transferred to a nylon filter and hybridized with the ^{32}P-labelled, 900-bp probe. An autoradiograph of the hybridization is shown.

relapse trypanosome populations of mixed infections following treatment of the infected animals. However, because the technique lacked sensitivity in distinguishing mixtures of the two parasite clones, and because the technique was not applicable to blood buffy coats (due to interference by host lymphocyte DNA), the AP-PCR was considered inappropriate for analysing relapse infections in goats.

Sensitivity and Specificity of the Population-Specific PCR Method

Since the AP-PCR technique was not sufficiently sensitive to distinguish DNA from one parasite when mixed with DNA from a different parasite, and since it could not be applied to unpurified DNA, an alternative strategy was adopted. Two primers, ILO 1044 and ILO 1045, were designed on the basis of sequence information from a cDNA clone,

p1616/5, which, from Southern blot hybridization analysis, appeared to hybridize under highly stringent conditions only to DNA from clones of STIB 212, including IL 1180. PCR reactions were carried out with the DNA from the two trypanosome clones individually and mixed to mimic mixed infections *in vivo*. A fixed concentration of IL 3274 DNA was used and the IL 1180 DNA concentration was varied to determine the sensitivity of the technique for detecting IL 1180 DNA when the trypanosomes occur in mixed infections. The sensitivity of the PCR, by standard agarose gel electrophoresis, staining of the DNA in the gel with ethidium bromide, and visualizing the products under U.V. light, was 100 pg of IL 1180 DNA when mixed with 25 ng of IL 3274 DNA [Fig. 1(B)]. The sensitivity could be improved at least 100-fold by hybridizing blots of the PCR products with the ^{32}P-labelled, 900-bp PCR product of p1616/5 [Fig. 1(C)].

It was also found that there was increased amplification of non-specific sequences as the proportion of IL 1180 to IL 3274 DNA decreased. However, since the PCR products amplified by the primers in the two genomes were of different sizes [Fig. 1(B); non-specific products from IL 3274 are evident in lanes 9–11] and there was no cross-hybridization between the 900-bp PCR product amplified in p1616/5 and IL 3274 [Fig. 1(C)], it was demonstrated that the PCR could distinguish the two trypanosome populations.

Analysis of Trypanosome Samples from Goat Blood

Trypanosome isolates were prepared from the blood of four goats in group C (CJ 517, CJ 522, CJ 532 and CJ 537), one goat in group D (CJ 530) and one goat in group F (CJ 565), on days 0, 18, 32, 46 and 60 post-treatment.

Parasites in a mouse-blood stabilate (obtained by infecting sublethally irradiated mice with goat blood collected on the day of treatment) were first expanded in numbers in irradiated mice. Subsequently, the trypanosomes were isolated and DNA purified. The DNA was then subjected to PCR amplification with primers ILO 1044 and ILO 1045. IL 1180 was present in animals with mixed infections (data not shown). Also, using buffy-coat preparations from goats as template DNA in the PCR with primers ILO 1044 and ILO 1045, it was shown that prior to treatment all trypanosome populations from group C animals contained IL 1180 DNA [Fig. 2(A), lanes 4, 5, 7, 8].

As previously mentioned, of the five goats in group C that were infected with both IL 1180 and IL 3274, four relapsed following treatment; these were all parasitaemic 18 days after treatment. Relapse populations occurring in these four animals on days 18, 32, 46 and 60 post-treatment were examined. Genomic DNA from these samples was used as template in the PCR reactions. Alongside these samples, PCR reactions were also carried out on purified IL 1180 DNA as a positive control and IL 3274 DNA as a negative control. The results demonstrated the apparent absence of IL 1180 in relapse populations of mixed infections on days 18, 32, 46 and 60 post-treatment. However, they did demonstrate the presence of IL 1180 in both single IL 1180 infections (group D) and non-treated mixed infections (group F), again in samples taken on days 18, 32, 46 and 60 post-treatment; a 900-bp sequence was amplified in DNA samples containing IL 1180 genomic DNA [Fig. 2(B); lanes 8–10 and 18–21]. It should be noted that in Fig. 2(B), lane 14 [containing relapse parasites from animal CJ 537 (Group C)], a PCR product with a size of approximately 600 bp was observed. This occurred as a result of non-specific amplification of an IL 3274 DNA sequence, but this product did not hybridize to the 900-bp probe [Fig. 2(C); lane 14]. Hybridization of the 900-bp, ^{32}P-labelled probe to a Southern blot of the gel confirmed the observations made by visual examination of the ethidium bromide-stained products in the gel [Fig. 2(C)]. Furthermore, the ^{32}P-labelled probe only hybridized to the 900-bp PCR products. Despite 14 days' exposure of the film to the hybridized filter, no hybridization signal was observed in lanes loaded with samples from group C goats with relapse infections (data not shown). This therefore indicated that, at the level of detection of

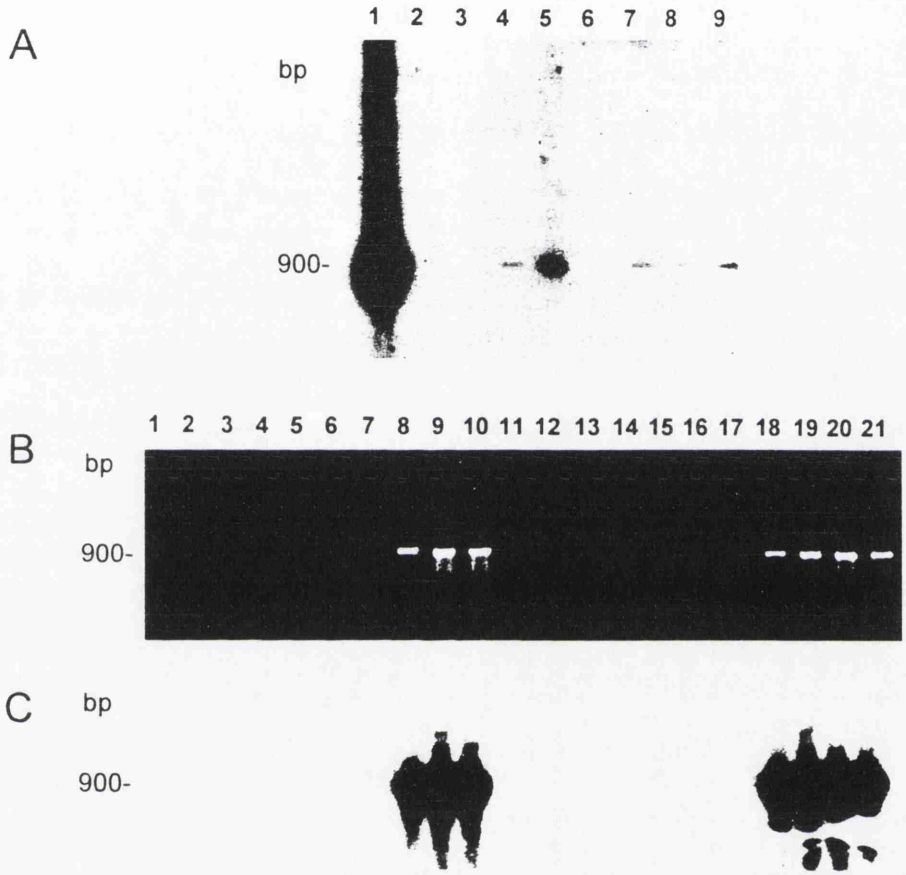


Fig. 2. PCR amplification using primers ILO 1044 and ILO 1045 on DNA from trypanosome populations collected from parasitaemic goats. (A) Buffy-coat preparations collected on the day of treatment. Lane 1, IL 1180 DNA control; 2, IL 3274 DNA control; 3, blank, 4, 5, 7, 8 and 9, samples from goats CJ 517, CJ 522, CJ 532, CJ 537 and CJ 565 (all infected with IL 3274 and IL 1180), respectively; 6, sample from goat CJ 530 (infected with IL 1180). DNA in the gel was blotted onto a nylon filter and hybridized with the ^{32}P-labelled, 900-bp probe. An autoradiograph is shown. (B) Buffy-coat preparations collected on various days post-treatment. Lanes 1–4, samples from goat CJ 517 (mixed infection and treated) on days 18, 32, 46 and 60, respectively; 5–7, samples from goat CJ 522 (mixed infection and treated) on days 32, 46 and 60, respectively; 8–10, samples from goat CJ 530 (infected with IL 1180 and not treated) on days 32, 46 and 60, respectively; 11–13, samples from goat CJ 532 (mixed infection and treated) on days 18, 46 and 60, respectively; 14–17, samples from goat CJ 537 (mixed infection and treated) on days 18, 32, 46 and 60, respectively; 18–20, samples from goat CJ 565 (mixed infection and not treated) on days 18, 32 and 46, respectively; 21, IL 1180 DNA control. An ethidium bromide-stained 2% agarose gel is shown. (C) Autoradiograph of the gel shown in (B) when transferred to a nylon filter and hybridized with the ^{32}P-labelled, 900-bp probe.

the radiolabelled probe, IL 1180 was not detectable in relapse trypanosome populations occurring in animals that had had mixed infections.

DISCUSSION

The present study was carried out to determine whether the diminazene-resistant pheno-

type of trypanosome populations is altered in mixed infections. *Trypanosoma congolense* IL 1180 and IL 3274 were used for this work since earlier studies in goats (Mamman *et al.*, 1993) had shown them to be sensitive and resistant, respectively, to treatment with diminazene aceturate at a dose of 7·0 mg/kg b.w. Because the two clones of *T. congolense* were both savannah-type *T. congolense*, it was necessary to find a system that could differentiate them at the molecular level. A population-specific PCR amplification technique was therefore developed which utilized a pair of oligonucleotide primers which were specific for IL 1180, and did not amplify IL 3274 or goat DNA. The transcript, a segment of which is contained in the plasmid p1616/5, is expressed in bloodstream forms of IL 1616 but not insect stages of the parasite life-cycle (unpub. obs.). This sequence encodes a bloodstream-form VSG-gene sequence and its use is comparable with studies by Gibson *et al.* (1988) in which VSG-gene sequences were used to distinguish *T. brucei gambiense* from *T. b. rhodesiense* and *T. b. brucei*.

To test the possibility that IL 3274 might allow the survival of small numbers of IL 1180 following treatment with diminazene aceturate, there was a requirement for a PCR technique with a high level of sensitivity for detecting IL 1180 DNA when mixed with other DNA. To improve the sensitivity of the PCR detection method, the 900-bp PCR product from p1616/5 was purified and ^{32}P-labelled to act as a probe on Southern blots of the PCR products.

In initial studies on the sensitivity of the PCR, based on ethidium bromide-staining of gels in which the PCR products had been separated, as little as 100 pg of IL 1180 DNA could be detected in a 25-μl reaction containing a total of 25·1 ng of total genomic DNA. Since one genome equivalent of *T. congolense* consists of approximately 7×10^7 base pairs (Borst *et al.*, 1982), and each base pair has a molecular mass of approximately 660, 100 pg of DNA is equivalent to 1300 trypanosomes. Using the radiolabelled probe, the sensitivity was improved by 100-fold, i.e., an amount of DNA equivalent to 13 trypanosomes. The probe demonstrated specificity for the 900-bp PCR product from IL 1180, since no such product was detected in either IL 3274 DNA or goat DNA. Using probes developed from repetitive DNA sequences of trypanosomes, Gibson *et al.* (1988) were able to detect as few as 100 trypanosomes by the direct dot-blot technique. The relatively low sensitivity of the PCR technique described in this study, using a pair of VSG gene-specific primers, could be due to the small copy number of the sequence in the genome. It could also be due to inhibition by IL 3274 DNA in the PCR, as previously observed by Diaz *et al.* (1992).

To determine whether the diminazene-susceptible clone, IL 1180, could survive treatment with diminazene aceturate in goats when mixed with a diminazene-resistant clone, IL 3274, goats were simultaneously and intravenously infected with both clones in order to evade the effects of the interference phenomenon described previously (Dwinger *et al.*, 1986, 1989; Sones *et al.*, 1989). Since the two clones had different growth rates, treatment of animals with mixed infections was carried out at a time when it was extrapolated, from animals with single infections, that both clones would have developed to a detectable level of parasitaemia. Using the PCR techniques, it was demonstrated that IL 1180 was present in all animals examined with mixed infections prior to treatment [groups C and F; Fig. 2(A)]. Since trypanosomes reappeared in four of the goats in group C within 17 days post-treatment, trypanosomes were examined that were collected from these animals on days 18, 32, 46 and 60 post-treatment. Parasitaemic blood from these goats was inoculated into mice to make trypanosome stabilates, and to expand their numbers for DNA extraction. This adaptation may have had some influence on the relative proportions of IL 1180 and IL 3274, since we have found that the former clone grows much faster than the latter in mice, although the latter clone appears to be more pathogenic than the former. IL 1180 could not be detected in any of the relapse populations using either of the PCR techniques employed. Thus, on the basis of the sensitivity of these techniques [demonstrated

in Fig. 1(B) and (C)], it was concluded that, although IL 1180 was present in mixed infections pre-treatment [Fig. 2(A)], the population was cleared when the animals were treated with diminazene aceturate at a dose of 7·0 mg/kg b.w. [Fig. 2(B) and (C)]. Thus, IL 1180 appears not to be refractory to treatment with diminazene at this dosage when mixed with a resistant population.

Using IL 3274, Mamman *et al.* (1995) showed that, when infected goats are treated with diminazene, the majority of the parasites which relapse following treatment appears to be sensitive to the dosage used. Using the two PCR techniques in the study described here, as little as 1 pg of IL 1180 total genomic DNA could be detected in 25 ng of relapse trypanosome DNA. It is therefore possible that IL 1180 may have been present in relapse populations at levels lower than this limit of detection; suppression of growth to below the limit of detection could have been mediated by the other trypanosome population, as described previously (Sones *et al.*, 1989). However, this hypothesis would appear unlikely since IL 1180 was always detected in animals with mixed infections that were not treated (group F).

In conclusion, the described PCR technique allows easy monitoring of different populations of trypanosomes of the same type in mixed infections. It therefore facilitates studies on the influence of different populations on each other during the course of infections *in vivo*. Such studies will provide information that will lead to a better understanding of the dynamics of trypanosome populations in the field.

ACKNOWLEDGEMENTS. The technical assistance of S. Kemei, S. Wasike and F. Shikhubari is greatly appreciated. E. M. E. B. was supported by an ILRAD fellowship and a Deutscher Akademischer Austauschdienst (DAAD) scholarship. M. Mulindi is thanked for typing the manuscript. This is ILRAD publication number 1305.

REFERENCES

ALIU, Y. O., ØDEGAARD, S. & SOGNEN, E. (1984). Diminazene/Berenil: bioavailability and disposition in dairy goats. *Acta Veterinaria Scandinavica*, **25**, 593–596.

AUSUBEL, F. M. (1988). Preparation and analysis of DNA. In *Current Protocols in Molecular Biology*, Vol. 1, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. pp. 2.0.1–2.10.3. New York: Greene Publishing and Wiley-Interscience.

BORST, P., VAN DER PLOEG, M., VAN HOEK, J. F. M., TAS, J. & JAMES, J. (1982). On the DNA content and ploidy of trypanosomes. *Molecular and Biochemical Parasitology*, **6**, 13–23.

DAR, F. K., LIGTHART, G. S. & WILSON, A. J. (1972). Cryopreservation of pathogenic African trypanosomes *in situ*: metacyclic and bloodstream forms. *Journal of Protozoology*, **19**, 494–497.

DIAZ, C., NUSSENZWEIG, V. & GONZALEZ, A. (1992). An improved polymerase chain reaction assay to detect *Trypanosoma cruzi* in blood. *American Journal of Tropical Medicine and Hygiene*, **46**, 616–623.

DWINGER, R. H., MURRAY, M., LUCKINS, A. G., RAE, P. F. & MOLOO, S. K. (1986). Interference between different serodemes of *T. congolense* in the establishment of superinfections in goats following transmission by tsetse. *Parasite Immunology*, **8**, 293–305.

DWINGER, R. H., MURRAY, M., LUCKINS, A. G., RAE, P. F. & MOLOO, S. K. (1989). Interference in the establishment of tsetse-transmitted *Trypanosoma congolense*, *T. brucei* or *T. vivax* superinfections in goats already infected with *T. congolense* or *T. vivax*. *Veterinary Parasitology*, **30**, 177–189.

GEIGY, R. & KAUFFMANN, N. M. (1973). Sleeping sickness survey in the Serengeti area (Tanzania), 1971. Part I. Examination of large mammals for trypanosomes. *Acta Tropica*, **30**, 12–23.

GIBSON, W. C., DUKES, P. & GASHUMBA, J. K. (1988). Species-specific DNA probes for the identification of African trypanosomes in tsetse flies. *Parasitology*, **97**, 63–73.

GRAY, A. R. & ROBERTS, C. J. (1971). The cyclical transmission of strains of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology*, **63**, 67–89.

HE, M., LIU, H., WANG, Y. & AUSTEN, B. (1992). Optimized centrifugation for rapid elution of DNA from agarose gels. *Genetic Analysis Techniques and Applications*, **9**, 31–33.

ILEMOBADE, A. A. (1979). Drug sensitivity of mouse-infective *Trypanosoma vivax* isolates in cattle and sheep. In *Proceedings of the 16th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yaounde, Cameroon*, pp. 251–253. Nairobi: Organization of African Unity/ Scientific Council for Trypanosomiasis Research.

ILEMOBADE, A. A. (1987). Chemotherapy against African animal trypanosomiasis: its strengths and limitations. In *African Trypanotolerant Livestock Network: Livestock Production in Tsetse Affected Areas of Africa*, pp. 265–273. Nairobi: International Livestock Centre for Africa/International Laboratory for Research on Animal Diseases.

JAIN, C. N. (1986). The goat: normal haematology with comments on response to disease. In *Schalms Veterinary Hematology*, 4th Edn., pp. 225–239. Philadelphia: Lea and Febiger.

JONES-DAVIES, W. J. (1967). The discovery of Berenil-resistant *Trypanosoma vivax* in Northern Nigeria. *Veterinary Record*, **80**, 531–532.

KATENDE, J. M., MUSOKE, A. J., NANTULYA, V. M. & GODDEERIS, B. M. (1987). A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for the diagnosis of bovine trypanosomiasis. *Tropenmedizin und Parasitologie*, **38**, 41–44.

LANHAM, S. M. & GODFREY, D. G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Experimental Parasitology*, **28**, 521–534.

LEACH, T. M. & ROBERTS, C. J. (1981). Present status of chemotherapy and chemoprophylaxis of animal trypanosomiasis in the eastern hemisphere. *Pharmacology and Therapeutics*, **13**, 91–147.

MAJIWA, P. A. O., MAINA, M., WAITUMBI, J. N., MIHOK, S. & ZWEYGARTH, E. (1993). *Trypanosoma (Nannomonas) congolense*: molecular characterization of a new genotype from Tsavo, Kenya. *Parasitology*, **106**, 151–162.

MAJIWA, P. A. O., MASAKE, R. A., NANTULYA, V. M., HAMMERS, R. & MATTHYSSENS, G. (1985). *Trypanosoma (Nannomonas) congolense*: identification of two karyotypic groups. *EMBO Journal*, **4**, 3307–3313.

MAJIWA, P. A. O., YOUNG, J. R., HAMMERS, R. & MATTHYSSENS, G. (1986). Minichromosomal variable surface glycoprotein genes and molecular karyotypes of *Trypanosoma (Nannomonas) congolense*. *Gene*, **41**, 183–192.

MAMMAN, M., KATENDE, J., MOLOO, S. K. & PEREGRINE, A. S. (1993). Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology*, **50**, 1–14.

MAMMAN, M., WILLIAMS, D. J. L., GETTINBY, G., MURPHY, N. B. & PEREGRINE, A. S. (1995). Frequency of diminazene-resistant trypanosomes and pharmacokinetics of diminazene in *Trypanosoma congolense*-infected goats. *Research in Veterinary Science*, in press.

MURRAY, M., MURRAY, P. K. & MCINTYRE, W. I. M. (1977). An improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **71**, 325–326.

NDOUTAMIA, G., MOLOO, S. K., MURPHY, N. B. & PEREGRINE, A. S. (1993). Derivation and characterization of a quinapyramine-resistant clone of *Trypanosoma congolense*. *Antimicrobial Agents and Chemotherapy*, **37**, 1163–1166.

PEREGRINE, A. S., KNOWLES, G., IBITAYO, A. I., SCOTT, J. R., MOLOO, S. K. & MURPHY, N. B. (1991). Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology*, **102**, 93–100.

PINDER, M. & AUTHIE, E. (1984). The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica*, **41**, 247–252.

POOT, M., KAUSCH, K., KOHLER, J., HAAF, T. & HOEHN, H. (1990). The minor-groove binding DNA-ligands netropsin, distamycin A and berenil cause polyploidisation via impairment of the G_2 phase of the cell cycle. *Cell Structure and Function*, **15**, 151–157.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

SONES, K. R., HOLMES, P. H. & URQUHART, G. M. (1989). Interference between drug-resistant and drug-sensitive stocks of *Trypanosoma congolense* in goats. *Research in Veterinary Science*, **47**, 75–77.

SOUTHERN, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*, **98**, 503–517.

STAUFFERT, I., PAULINI, H., STEINMANN, U., SIPPEL, H. & ESTLER, C. J. (1990). Investigations on mutagenicity and genotoxicity of pentamidine and some related trypanocidal diamidines. *Mutation Research*, **245**, 93–98.

WAITUMBI, J. N. & MURPHY, N. B. (1993). Inter- and intra-species differentiation of trypanosomes by genomic fingerprinting with arbitrary primers. *Molecular and Biochemical Parasitology*, 58, 181–186.

WALSH, P. S., METZGER, D. A. & HIGUCHI, R. (1991). Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, **10**, 506–513.

WHITELAW, D. D., BELL, I. R., HOLMES, P. H., MOLOO, S. K., HIRUMI, H., URQUHART, G. M. & MURRAY, M. (1986). Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. *Veterinary Record*, **118**, 722–726.

WHITESIDE, E. F. (1960). Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In *Proceedings of the Eighth Meeting of the International Scientific Committee for Trypanosomiasis Research, Jos, Nigeria*, pp. 141–154. Lagos: Commission de Cooperation Technique en Afrique au Sud du Sahara.

Section 4.6

- Discussion -

The work described in Section 4.1 was carried out to determine whether multiple, as compared to single, treatment with diminazene aceturate would increase the drug's therapeutic efficacy against a *T. congolense* population in goats that is resistant to the maximum recommended dose regimen for the drug. The study used a cloned population, *T. congolense* IL 3274 (Pinder and Authie, 1984; Peregrine et al., 1991), which when inoculated into goats via the bites of infective *Glossina morsitans centralis*, produced infections that were resistant in five out of five animals to single intramuscular treatment with diminazene aceturate at a dose of 7.2 mg/kg b.w. on day 19 of infection. Section 3.2 indicates that this resistance was not associated with invasion of the CNS. In contrast, *in vitro* characterisation of metacyclic trypanosomes of *T. congolense* IL 3274 has shown that this population is approximately ten times more resistant to diminazene than a reference diminazene-sensitive population (Section 4.1; Gray and Peregrine, 1993). Thus, the resistance of *T. congolense* IL 3274 to diminazene appears to be associated with an ability of the trypanosomes to withstand higher concentrations of diminazene than diminazene-sensitive populations.

When goats infected with *T. congolense* IL 3274 were treated on day 19 following tsetse transmission with diminazene aceturate at a dose of 7.2 mg/kg b.w. and retreated 8 or 24 hours later with the same drug dose, infections were eliminated in two out of five and one out of five animals, respectively. Thus, retreatment at these intervals slightly improved the therapeutic efficacy of diminazene.

In similar work that was carried out in mice infected with a "diminazene-resistant" *T. congolense*, Silayo (1991) concluded that two-dose treatment with diminazene aceturate at an 8-hour interval did not significantly improve the therapeutic activity of the compound compared to single treatment. In contrast, two-dose treatment at 24 to 96-hour intervals resulted in a significant improvement. Differences in the diminazene sensitivity of *T. congolense* IL 3274 and the *T. congolense* population used by Silayo (1991) would not appear to be responsible for the different results since *T. congolense* IL 3274 is significantly less sensitive to diminazene than the other population (Peregrine et al., 1991; Silayo, 1991). Instead, the much higher

doses of diminazene aceturate used by Silayo (1991), as compared to those used in Section 4.1, facilitated by diminazene's higher maximum tolerated dose in mice compared to goats, and differences in the drug's pharmacokinetics in the two animal species (Aliu et al., 1984; Mamman et al., 1995), are most likely to be responsible for the difference.

In addition to evaluating the efficacy of diminazene when given to goats on two occasions, Section 4.1 also demonstrated that while single treatment with diminazene aceturate at a dose of 7.2 mg/kg b.w. failed to eliminate *T. congolense* IL 3274 in any goat when administered on day 19 of infection (when all animals were parasitaemic), the same dosage eliminated *T. congolense* IL 3274 if administered 24 hours following the feeding of infective flies on the goats. It is clear that the parasite burdens in the animals at these two stages of infection were very different. Thus, with heterogeneous trypanosome populations the level of drug resistance of the population would be expected to increase with increasing population size (Sones and Holmes, 1992). However, since trypanosomes are classically assumed to be either resistant or sensitive to a given dosage of diminazene, because resistance to the compound has been shown to be stable in trypanosome populations (Gray and Roberts, 1971a,b; Kaminsky and Zweygarth, 1989; Zhang et al., 1993), the size of the trypanosome population should not be a relevant variable in the work described in Section 4.1 since *T. congolense* IL 3274 is a cloned population. Either way, the data suggest that resistance to diminazene is not a stable phenotype in *T. congolense* IL 3274.

Section 4.2 describes work that was carried out in goats to determine when the alteration in diminazene sensitivity of *T. congolense* IL 3274 occurs between day 1 and day 19 following tsetse-transmitted infection. The results indicated that by day 4 the trypanosome population expressed resistance to diminazene. At this stage of infection the parasites have begun to migrate away from the skin into the afferent lymphatics draining the skin (Dwinger et al., 1990), but have not entered the bloodstream (Luckins and Gray, 1978, Emery and Moloo, 1981). Since trypanosomes in the dermis of the skin differ structurally from metacyclic and

bloodstream-form trypanosomes (Gray et al., 1985; Dwinger et al., 1988) it is possible that the diminazene sensitivity of the dermal life-cycle stage differs from that of trypanosomes in the lymphatics and bloodstream. Alternatively, differences in the pharmacokinetics of diminazene in the dermis compared to other sites in the host (Mamman et al., in press) may result in the apparent difference in drug sensitivity. In similar work reported by Silayo (1987), tsetse-transmitted *T. congolense* infections in rabbits were shown to be susceptible to treatment with diminazene when administered less than 8 days following infection, resistant to treatment with the drug when given on days 8-10, and susceptible to treatment if given more than 10 days following infection. The author associated the drug resistance observed on days 8-10 with inaccessibility of diminazene to trypanosomes within the chancre, which is at its maximum size at this time. However, a similar phenomenon was not observed in the goats described in this Chapter, in which maximum chancre reactions would have occurred on approximately day 10 following challenge (Emery and Moloo, 1981). Instead, there was a progressive increase in the level of drug resistance of the infections over time, suggesting that the basis of the drug resistance observed in the goats was different from that described in rabbits.

In order to evaluate the stability of resistance to diminazene in *T. congolense* IL 3274, Section 4.2 describes an experiment that was carried out with five goats infected with *T. congolense* IL 3274 and treated with 7.0 mg diminazene aceturate/kg b.w. on day 19 of infection. Seventeen days after this treatment, when trypanosomes had reappeared in all the animals, groups of teneral *G. m. centralis* were fed on one occasion on each animal (by definition, the trypanosomes present in the goats at this time should all have been resistant to the drug dosage used). Thereafter, when infective, ten flies from each group were fed on 2 non-infected goats per group (i.e., five flies per goat). One of the two goats in each pair was then treated 24 hours after infection with diminazene aceturate at a dose of 7.0 mg/kg b.w. The second goat was treated on day 19 with the same drug dosage. As before, treatment on day 1 eliminated all infections while treatment on day 19 failed to eliminate infections in any animal. Thus, after transmission through tsetse flies, the

diminazene sensitivity of the trypanosome populations ingested by the flies appeared to revert back to that of the parental trypanosome population. Such an observation would be consistent with the hypothesis that intra-dermal and bloodstream-form trypanosomes differ in their sensitivity to diminazene. However, it is unclear why the diminazene sensitivity of tsetse-transmitted infections in goats on day 1 was apparently the same for both *T. congolense* IL 3274 and a subpopulation of *T. congolense* IL 3274 that reappeared in goats following treatment with diminazene. The data would suggest that despite application of drug pressure the two populations had the same level of resistance to diminazene.

In Section 4.2, observations on the change in level of parasitaemia in goats following treatment with diminazene aceturate indicated that even with a cloned "diminazene-resistant" population of *T. congolense* the majority of trypanosomes in the bloodstream are not resistant to treatment with diminazene. Section 4.3 describes work that was carried out in goats to determine the frequency with which diminazene-resistant trypanosomes occur in populations of *T. congolense* IL 3274, before and after treatment with diminazene. On the basis of the reduction in parasitaemia that occurred following treatment of the original infection with diminazene aceturate, the frequency of diminazene-resistant trypanosomes was estimated to be less that $1:10^3$. Quite surprisingly, however, the ratio did not appear to be any less in trypanosome populations that reappeared in animals following treatment with diminazene. Thus, despite using a cloned population of *T. congolense*, and attempts to decrease the diminazene sensitivity of the trypanosome population by application of drug pressure, the proportion of diminazene-resistant trypanosomes remained less that $1:10^3$. This is consistent with the aforementioned results in Section 4.2 and indicates that the majority of trypanosomes which reappear in the bloodstream following treatment are sensitive to the dose of diminazene that was used. Such a phenomenon may be associated with growth advantages of drug-sensitive trypanosomes compared to drug-resistant trypanosomes (Cantrell, 1956; Hawking, 1963; Sones et al., 1989; Mutugi et al., 1995). Alternatively, the occurrence of diminazene-sensitive trypanosomes in parasite populations that arise in the bloodstream of goats following treatment with diminazene could also be due

to an ability of diminazene-sensitive trypanosomes to survive treatment when mixed with trypanosomes that are drug resistant. In order to investigate this hypothesis, Section 4.5 describes the response of a mixed infection in goats, comprising a diminazene-sensitive clone (*T. congolense* IL 1180) and a diminazene-resistant clone (*T. congolense* IL 3274), to treatment with diminazene aceturate. Using a nucleotide sequence of a cDNA that was derived from *T. congolense* IL 1180, a pair of 20-mer primers was designed which, in a PCR, amplified a 900-bp sequence from *T. congolense* IL 1180 but not *T. congolense* IL 3274. Groups of 5 goats each were then infected with *T. congolense* IL 1180 (group A), *T. congolense* IL 3274 (group B), or both clones simultaneously (group C), and treated with diminazene aceturate at a dose of 7.0 mg/kg b.w. following detection of trypanosomes. Infections in all goats in group A were eliminated. In contrast, all infections in group B goats and in four of the five goats in group C were resistant to treatment. Using the PCR methodology, *T. congolense* IL 1180 DNA could not be detected in any DNA sample collected from group C goats up to 60 days following treatment. Thus, on the basis of the sensitivity of this detection technique, *T. congolense* IL 1180 appeared unable to survive treatment with diminazene in goats when mixed with *T. congolense* IL 3274. This therefore suggests that the high proportion of diminazene-sensitive trypanosomes that appear in goats following treatment with diminazene are not present in the animals at the time of treatment.

In order to obtain a more accurate quantitative estimate of the proportion of diminazene-resistant trypanosomes that occur *in vivo* following treatment with diminazene aceturate, a mouse model was developed in which parasitaemia profiles following treatment with diminazene aceturate resembled those observed in the aforementioned goats. In this model (Section 4.4) mice were infected with *T. congolense* IL 3274 and thereafter treated with diminazene aceturate at a dose of 25 mg/kg b.w. since this dose produced a parasitaemia profile similar to that observed in goats infected with *T. congolense* IL 3274 following treatment with the drug at a dose of 7.0 mg/kg b.w. (Sections 4.2 and 4.3). In Section 4.4 the frequency of trypanosomes resistant to diminazene aceturate at a dose of 25 mg/kg b.w. was investigated in a subpopulation of *T. congolense* IL 3274 which reappeared in mice

after treatment with diminazene aceturate at this dosage. At population sizes of 10^2, 10^3, 10^4, 10^5 and 10^6 organisms, maximum likelihood estimates for the proportions of trypanosomes resistant to this drug dosage in mice were 8.34×10^{-4}, 2.49×10^{-4}, 3.02×10^{-5}, 8.3×10^{-6} and 1.6×10^{-6}, respectively. These data indicate that the majority of parasites in the trypanosome population that reappeared in the bloodstream following treatment were susceptible to the drug dosage used for selecting the population. Furthermore, and quite surprisingly, the calculated proportion of organisms which survived drug exposure varied inversely with the inoculum size. To the best of this writer's knowledge, this is the first time that this has been described and suggests that the level of diminazene resistance of a trypanosome population is, at least in part, dependent on the concentration of the trypanosomes. Additional experiments described in Section 4.4 indicated that this inverse relationship did not result from alterations in the pharmacokinetics of diminazene with different trypanosome population sizes. Bungener (1982, 1984) has shown that increasing the concentration of trypanosomes *in vivo* results in inhibition of trypanosome proliferation in a concentration-dependent manner, characteristic of the trypanosome strain. Thus, since diminazene appears to act on cells at specific stages of the cell-division cycle (Kishore et al., 1990; Poot et al., 1990; Stauffert et al., 1990), the alteration in the proportion of *T. congolense* IL 3274 that is resistant to diminazene with different population sizes could be due to changes in the proportion of the trypanosome population in different stages of the cell-division cycle at different parasite concentrations, due to different growth rates (Turner et al., 1996).

References:

Aliu, Y.O., Ødegaard, S. and Søgnen, E. (1984) Diminazene/Berenil: bioavailability and disposition in dairy goats. *Acta Veterinaria Scandinavica* 25, 593-596.

Bungener, W. (1982) Behaviour of a *Trypanosoma brucei brucei* stock (STIB 348C) in mice. 2. Course of infections with basic type and several variant types. *Tropical Medicine and Parasitology* 33, 243-248.

Bungener, W. (1984) Behaviour of a *Trypanosoma brucei brucei* stock (STIB 348C) in mice. 4. Different course of primary parasitaemia in trypanosome variants of different virulence. *Tropical Medicine and Parasitology* 35, 115-118.

Cantrell, W. (1956) Behaviour of mixtures of oxophenarsin-resistant and unmodified strains of *Trypanosoma equiperdum*. *Experimental Parasitology* 5, 178-190.

Dwinger, R.H., Murray, M. and Moloo, S.K. (1990) Parasite kinetics and cellular responses in goats infected and superinfected with *Trypanosoma congolense* transmitted by *Glossina morsitans centralis*. *Acta Tropica* 47, 23-33.

Dwinger, R.H., Rudin, W., Moloo, S.K. and Murray, M. (1988) Development of *Trypanosoma congolense*, *T. vivax* and *T. brucei* in the skin reaction induced by infected *Glossina morsitans centralis*: a light and electron microscopical study. *Research in Veterinary Science* 44, 154-163.

Emery, D.L. and Moloo, S.K. (1981) The dynamics of the cellular reactions elicited in the skin of goats by *Glossina morsitans morsitans* infected with *Trypanosoma (Nannomonas) congolense* or *T. (Duttonella) vivax*. *Acta Tropica* 38, 15-28.

Gray, A.R. and Roberts, C.J. (1971a) The cyclical transmission of strains of *Trypanosoma congolense* and *Trypanosoma vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* 63, 67-89.

Gray, A.R. and Roberts, C.J. (1971b) The stability of resistance to diminazene aceturate and quinapyramine sulphate in a strain of *Trypanosoma vivax* during cyclical transmission through antelope. *Parasitology* 63, 163-168.

Gray, M.A. and Peregrine, A.S. (1993) An *in vitro* assay for drug sensitivity of *Trypanosoma congolense* using *in vitro*-derived metacyclic trypanosomes. *Acta Tropica* 54, 291-300.

Gray, M.A., Ross, C.A., Taylor, A.M., Tetley, L. and Luckins, A.G. (1985) *In vitro* cultivation of *Trypanosoma congolense*: the production of infective metacyclic trypanosomes cultured on bovine endothelial cell monolayers. *Acta Tropica* 42, 99-111.

Hawking, F. (1963) Drug-resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, berenil and other compounds. *Annals of Tropical Medicine and Parasitology* 57, 262-282.

Kaminsky, R. and Zweygarth, E. (1989) Effect of *in vitro* cultivation on the stability of resistance of *Trypanosoma brucei brucei* to diminazene, isometamidium, quinapyramine, and Mel B. *Journal of Parasitology* 75, 42-45.

Kishore, P., Gupta, S., Srivastava, D.K. and Shukla, O.P. (1990) Action of methylglyoxal bis (guanyl hydrazone) and related antiprotozoals on *Acanthamoeba culbertsoni*. *Indian Journal of Experimental Biology* 28, 1174-1179.

Luckins, A.G. and Gray, A.R. (1978) An extravascular site of development of *Trypanosoma congolense*. *Nature* 272, 613-614.

Mamman, M., Gettinby, G., Murphy, N.B., Kemei, S. and Peregrine, A.S. (1995) Frequency of diminazene-resistant trypanosomes in populations of *Trypanosoma congolense* arising in infected animals following treatment with diminazene aceturate. *Antimicrobial Agents and Chemotherapy* 39, 1107-1113.

Mamman, M., McKeever, D.J., Aliu, Y.O. and Peregrine, A.S. (in press) Pharmacokinetics of diminazene in plasma and lymph of goats. *American Journal of Veterinary Research.*

Mutugi, M.W., Boid, R. and Luckins, A.G. (1995) Differences in cloning and sub-cloning success rates in four stocks of *Trypanosoma evansi* and variation in suramin resistance of the clones. *Veterinary Parasitology* 60, 213-220.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* 102, 93-100.

Pinder, M. and Authie, E. (1984) The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica* 41, 247-252.

Poot, M., Kausch, K., Kohler, J., Haaf, T. and Hoehn, H. (1990) The minor-groove binding DNA-ligands netropsin, distamycin A and berenil cause polyploidisation via impairment of the G_2 phase of the cell cycle. *Cell Structure and Function* 15, 151-157.

Silayo, R.S. (1987) On curative and prophylactic effects of diminazene aceturate *Trypanosoma congolense* and *T. brucei*. In *Proceedings, 19th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Lomé, Togo*. Modern Lithographic (K) Ltd., Nairobi, OAU/STRC Publication No. 114, pp. 265-274.

Silayo, R.S. (1991) Studies on efficacy of repeat trypanocidal drug treatment against drug-resistant *Trypanosoma congolense* infections. In *Proceedings, 21st Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yamoussoukro, Côte d'Ivoire*. Modern Lithographic (K) Ltd., Nairobi, OAU/STRC Publication No. 116, p. 186.

Sones, K.R. and Holmes, P.H. (1992) The influence of the size of the initial inoculum on the efficacy of isometamidium (samorin) on a stock of *Trypanosoma congolense*. *Acta Tropica* 51, 213-216.

Sones, K.R., Holmes, P.H. and Urquhart, G.M. (1989) Interference between drug-resistant and drug-sensitive stocks of *Trypanosoma congolense* in goats. *Research in Veterinary Science* 47, 75-77.

Stauffert, I., Paulini, H., Steinmann, U., Sippel, H. and Estler, C.J. (1990) Investigations on mutagenicity and genotoxicity of pentamidine and some related trypanocidal diamidines. *Mutation Research* 245, 93-98.

Turner, C.M.R., Aslam, N. and Angus, S.D. (1996) Inhibition of growth of *Trypanosoma brucei* parasites in chronic infections. *Parasitology Research* 82, 61-66.

Zhang, Z.Q., Giroud, C. and Baltz, T. (1993) *Trypanosoma evansi*: *in vivo* and *in vitro* determination of trypanocide resistance profiles. *Experimental Parasitology* 77, 387-394.

Chapter 5

Molecular basis of isometamidium's activity

Section 5.0

- Introduction -

At the present time the most commonly used compound for chemoprophylaxis of trypanosomiasis in cattle, sheep and goats is isometamidium chloride (Kinabo and Bogan, 1988). Widespread use of the compound since its first description by Berg (1960) has clearly demonstrated the drug's ability to reduce mortality and increase productivity of domestic livestock under both medium and high levels of trypanosomiasis risk (Logan et al., 1984; Trail et al., 1985; M[a]loo et al., 1987). However, the duration of the compound's prophylactic action at standard recommended doses varies widely, ranging from less than one month to at least six months (Robson, 1962; Fairclough, 1963; Wiesenhutter et al., 1968; Lewis and Thomson, 1974; Pinder and Authie, 1984; Whitelaw et al., 1986). Variation in the duration of prophylaxis appears largely to be associated with differing levels of sensitivity of trypanosomes to the compound (Peregrine et al., 1988). In this context, not surprisingly for a compound that has been in use for 35 years, the incidence of resistance to isometamidium appears to have been increasing in recent years (Section 1.1).

Since it is unlikely that new anti-trypanosomal compounds will be marketed during the next decade, because of prohibitive development costs, the derivation of techniques that will rapidly quantify the drug-resistance phenotype of trypanosomes will provide a means to more accurately determine the prevalence of different resistance phenotypes than is currently possible. As a result, recommendations could be given more quickly than at present on the compounds that are most likely to abrogate the development of drug resistance at the site, thereby maximising the long-term efficacy of the currently available compounds. However, at present, such assays are not available. Trypanosome isolates can be characterised for their drug sensitivity using definitive hosts (Gray and Roberts, 1971), laboratory rodents (Hawking, 1963) and *in vitro* cultivation techniques (Kaminsky and Brun, 1993). Unfortunately, each of these methods is associated with various problems (Section 1.1). Furthermore, none can be used to rapidly quantify the drug-resistance phenotype of large numbers of trypanosome populations. As a result, it is currently not possible to obtain precise, up-to-date, estimates for the prevalence of drug-resistant infections in the field. In an attempt to address these problems, Eisler et al.

(1993) have described an enzyme-linked immunosorbent assay that will quantify the isometamidium concentration in cattle sera. When used in combination with a trypanosome diagnostic test, the assay can be used as a rapid method for indirectly determining the level of isometamidium resistance of trypanosome infections. However, since it does not determine the maximum level of resistance of trypanosomes it is limited in application. A direct technique for rapidly quantifying the level of resistance to isometamidium is therefore still required, but requires precise information on the molecular basis of the drug's mode of action, and resistance to the compound, before it can be developed.

Although isometamidium has been used in the field for 35 years, very little is known about it's mode of action. In earlier work, the molecule was shown to bind to DNA (Wagner, 1971; Kinabo and Bogan, 1987) and to inhibit DNA polymerases (Marcus et al., 1982), RNA polymerases (Lantz and Van Dyke, 1972), purine nucleotide synthesis (Henderson et al., 1977), polyamine metabolism (Bacchi, 1981) and mitochondrial type II topoisomerase (Shapiro and Englund, 1990). As a result, the primary mode of action of isometamidium would appear to be blockade of nucleic acid synthesis (Kinabo and Bogan, 1988). However, since all except the last of these studies were carried out with purified subcellular fractions, the contribution of the aforementioned stated activities to the trypanocidal activity of the compound *in vivo* has not been clearly defined. The work described in this Chapter was carried out to determine the biochemical basis of isometamidium's mode of action in intact trypanosomes, and the genetic basis of resistance to the compound.

Data presented in Section 5.1 describe a series of parasitological experiments that were carried out to determine whether there was significant variation in resistance to isometamidium amongst clones derived from a drug-resistant stock of *T. congolense*. Such information was required to determine the most suitable material for further studies. In additional work, information on the stability of resistance to isometamidium in *T. congolense* was obtained by determining whether there was significant variation in resistance to isometamidium amongst a set of clones derived from one of the aforementioned clones.

Earlier work has demonstrated the autofluorescent property of isometamidium and has used this property to characterise the compound's intracellular localisation in mammalian cells (Philips et al., 1967). In Section 5.2, the autofluorescent property of the molecule was used in fluorimetry to characterise the interaction of the molecule with a drug-sensitive clone of *T. congolense*. The resultant data indicate the mechanism by which isometamidium enters these trypanosomes.

Finally, in work described by Sutherland et al. (1991), fluorescence microscopy was used to characterise the interaction of isometamidium with the drug-sensitive clone of *T. congolense* mentioned above; after 20 minutes incubation of bloodstream forms in 0.5 μg isometamidium chloride/ml at 27°C, diffuse surface fluorescence was observed, along with a focal area of fluorescence in the posterior region of the trypanosomes. However, because of the relatively low level of resolution of the technique, it was not possible to identify the precise intracellular localisation of the drug. In an attempt to circumvent this problem Section 5.3 describes the derivation, and characterisation, of anti-isometamidium monoclonal antibodies that were subsequently used in electron-microscopy immunocytochemistry (Section 5.4) to characterise isometamidium's intracellular localisation in the same clone of *T. congolense*. Section 5.4 also describes the use of electron-microscopy autoradiography for the same purpose.

References:

Bacchi, C.J. (1981) Content, synthesis, and function of polyamines in trypanosomatids: relationship to chemotherapy. *Journal of Protozoology* 28, 20-27.

Berg, S.S. (1960) Structure of isometamidium (M&B 4180A), 7-m-amidinophenyldiazoamino-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride, the red isomer present in metamidium. *Nature* 188, 1106-1107.

Eisler, M.C., Gault, E.A., Smith, H.V., Peregrine, A.S. and Holmes, P.H. (1993) Evaluation and improvement of an enzyme-linked immunosorbent assay (ELISA) for the detection of isometamidium in bovine serum. *Therapeutic Drug Monitoring* 15, 236-242.

Fairclough, R. (1963) A comparison of Metamidium, Samorin, Berenil and Ethidium bromide under field conditions in Kenya. *Veterinary Record* 75, 855-858.

Gray, A.R. and Roberts, C.J. (1971) The cyclical transmission of strains of *Trypanosoma congolense* and *Trypanosoma vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* 63, 67-89.

Hawking, F. (1963) Drug-resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, Berenil, and other compounds in mice. *Annals of Tropical Medicine and Parasitology* 57, 262-282.

Henderson, J.F., Battell, M.L., Zombor, G. and Khoo, M.K.Y. (1977) Effects of ethidium and isometamidium on adenosine triphosphate catabolism and purine nucleotide synthesis in Ehrlich ascites tumor cells *in vitro*. *Cancer Research* 37, 3434-3441.

Kaminsky, R. and Brun, R. (1993) *In vitro* assays to determine drug sensitivities of African trypanosomes: a review. *Acta Tropica* 54, 279-289.

Kinabo, L.D.B. and Bogan, J.A. (1987) Binding of isometamidium to calf thymus DNA and lipids: pharmacological implications. *Journal of Veterinary Pharmacology and Therapeutics* 10, 357-362.

Kinabo, L.D.B. and Bogan, J.A. (1988) The pharmacology of isometamidium. *Journal of Veterinary Pharmacology and Therapeutics* 11, 233-245.

Lantz, C. and Van Dyke, K. (1972) *Plasmodium berghei*: inhibited incorporation of AMP-8-^{3}H into nucleic acids of erythrocyte free material parasites by acridines, phenanthridines, and 8-aminoquinolines. *Experimental Parasitology* 31, 255-262.

Lewis, A.R. and Thomson, J.W. (1974) Observations on an isometamidium resistant strain of *Trypanosoma congolense* in Rhodesia. *Rhodesian Veterinary Journal* 4, 62-67.

Logan, L.L., Goodwin, J.T., Tembely, S. and Craig, T.M. (1984) Maintaining zebu Maure cattle in a tsetse infested area of Mali. *Tropical Animal Health and Production* 16, 1-12.

M[a]loo, S., Chema, S., Connor, R., Durkin, J., Kimotho, P., Maehl, Mukendi, F., Murray, M., Rarieya, M. and Trail, J. (1987) Efficacy of chemoprophylaxis for East African Zebu cattle exposed to trypanosomiasis in village herds in Kenya. In *Proceedings, 19th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Lomé, Togo*. Modern Lithographic (K) Ltd., Nairobi, OAU/STRC Publication No. 114, pp. 282-287.

Marcus, S.L., Kopelman, R., Koll, B. and Bacchi, C.J. (1982) Effects of exogenous polyamine and trypanocides on the DNA polymerase activities from *Trypanosoma brucei brucei*, mouse thymus and murine leukemia virus. *Molecular and Biochemical Parasitology* 5, 231-243.

Peregrine, A.S., Ogunyemi, O., Whitelaw, D.D., Holmes, P.H., Moloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M. (1988) Factors influencing the duration of isometamidium chloride (Samorin) prophylaxis against experimental challenge with metacyclic forms of *Trypanosoma congolense*. *Veterinary Parasitology* 28, 53-64.

Philips, F.S., Sternberg, S.S., Cronin, A.P., Sodergren, J.E. and Vidal, P.M. (1967) Physiologic disposition and intracellular localization of isometamidium. *Cancer Research* 27, 333-349.

Pinder, M. and Authie, E. (1984) The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica* 41, 247-252.

Robson, J. (1962) Prophylaxis against trypanosomiasis in Zebu cattle. IV. A field trial of metamidium and isometamidium. *Veterinary Record* 74, 913-917.

Shapiro, T.A. and Englund, P.T. (1990) Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. *Proceedings of the National Academy of Sciences of the USA* 87, 950-954.

Sutherland, I.A., Peregrine, A.S., Lonsdale-Eccles, J.D. and Holmes, P.H. (1991) Reduced accumulation of isometamidium by drug-resistant *Trypanosoma congolense*. *Parasitology* 103, 245-251.

Trail, J.C.M., Murray, M., Sones, K., Jibbo, J.M.C., Durkin, J. and Light, D. (1985) Boran cattle maintained by chemoprophylaxis under trypanosomiasis risk. *Journal of Agricultural Science* 105, 147-166.

Wagner, T.E. (1971) Physical studies on the interaction of lysergic acid diethylamide and trypanocidal dyes with DNA and DNA-containing genetic material. *Progress in Molecular and Subcellular Biology* 2, 152-162.

Whitelaw, D.D., Bell, I.R., Holmes, P.H., Moloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M. (1986) Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in cattle. *Veterinary Record* 118, 722-726.

Wiesenhutter, E., Turner, D.B. and Kristensen, K.A. (1968) Aspects of current bovine trypanosomiasis control in Tanzania - a comparative field trial of available chemoprophylactics under ranching conditions. *Bulletin of Epizootic Diseases of Africa* 16, 419-424.

Section 5.1

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* 102, 93-100.

Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*

A. S. PEREGRINE, G. KNOWLES, A. I. IBITAYO, J. R. SCOTT, S. K. MOLOO *and* N. B. MURPHY

International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya

(*Received 3 July 1990; revised 28 August 1990; accepted 29 August 1990*)

SUMMARY

Nine clones were derived from a drug-resistant *Trypanosoma congolense* stock (IL 2856) and characterized in mice for their sensitivity to isometamidium chloride and diminazene aceturate. All clones were derived from the stock without drug selection and expressed high levels of resistance to isometamidium chloride (50% curative dose [CD_{50}] values ranging from 1·5 to 5·1 mg/kg) and intermediate to high levels of resistance to diminazene aceturate (CD_{50} values ranging from 5·1 to 21·0 mg/kg). By contrast, the isometamidium chloride and diminazene aceturate CD_{50} values for a drug-sensitive clone, *T. congolense* IL 1180, were 0·018 mg/kg and 2·3 mg/kg, respectively. For both drugs, there appeared to be significantly different levels in expression of drug resistance amongst the 9 clones derived from IL 2856. Isoenzyme analysis of 7 enzymes showed that all 9 clones expressed the same electrophoretic variants. Thus, all 9 clones were identical for these phenotypic markers. The clone which expressed the highest level of resistance to isometamidium in mice (IL 3270) was transmitted to Boran cattle via the bite of infected *Glossina morsitans centralis*. IL 3270 produced an infection rate in tsetse of 5·0%. The resulting infections in cattle were shown to be resistant to intramuscular treatment with 2·0 mg/kg isometamidium chloride and 14·0 mg/kg diminazene aceturate. This contrasts with doses of 0·25 mg/kg isometamidium chloride or 3·5 mg/kg diminazene aceturate which are deemed sufficient to cure fully sensitive infections. Finally, 9 clones (subclones) were derived from IL 3270 and characterized in mice for their sensitivity to isometamidium chloride. Seven of the subclones expressed a significantly lower level of resistance to isometamidium than the parental clone and amongst the subclones there was significant variation in resistance. Thus, expression of a high level of resistance to isometamidium appears to be unstable in the rodent host and at least a component of the genetic determinant(s) for this drug-resistant phenotype is (are) likely to be unstable.

Key words: *Trypanosoma congolense*, clone, isometamidium chloride, diminazene aceturate, drug resistance, *Glossina morsitans centralis*.

INTRODUCTION

Chemotherapy and chemoprophylaxis of trypanosomiasis in cattle, sheep and goats currently relies upon the administration of either homidium chloride (Novidium®, RMB Animal Health Ltd, England), homidium bromide (Ethidium®, CAMCO, England), diminazene aceturate (Berenil®, Hoechst AG., W. Germany; Veriben®, Sanofi Animal Health Ltd, England) or isometamidium chloride (Samorin®, RMB Animal Health Ltd, England). Trypanosomes are usually not resistant to both diminazene and isometamidium (Whiteside, 1960). Therefore, these 2 compounds have been termed a 'sanative pair' (Whiteside, 1958), and in instances of resistance to one drug the application of the other drug of the sanative pair would usually control the disease. Thus, a protocol in which the application of diminazene aceturate is alternated with isometamidium chloride has successfully controlled trypanosomiasis in ranching conditions (Trail *et al.* 1985) and at the village level (Maloo *et al.* 1987). Nevertheless, experimental and field-based studies have demonstrated the occurrence of resistance to both diminazene aceturate (Jones-Davies, 1967; MacLennan & Na'Isa, 1970; Gray & Roberts, 1971; Gitatha, 1979; Schonefeld, Rottcher & Moloo, 1987; Mbwambo, Mella & Lekaki, 1988) and isometamidium chloride (Scott & Pegram, 1974; Kupper & Wolters, 1983; Pinder & Authie, 1984; Schonefeld *et al.* 1987). However, the available data would suggest that, at present, resistance to these trypanocides is not widespread.

Pinder & Authie (1984) and Authie (1984) isolated 6 stocks of *T. congolense* which expressed a high level of resistance to both isometamidium and diminazene when examined in mice. It was not known whether the double-resistance phenotype of these stocks was because they contained at least 2 distinct populations, one of which expressed resistance to diminazene and the other which expressed resistance to isometamidium, or because the stocks contained parasites which expressed resistance to both drugs. If the latter was the case, then the use of the sanative pair

would be ineffective to treat such infections. In order to answer these questions we have derived 9 clones from one of the aforementioned stocks and characterized them in mice for their sensitivity to both isometamidium and diminazene and also for electrophoretic variants of 7 enzymes. Subsequently, a series of subclones was derived from one of these clones and characterized in mice for their sensitivity to isometamidium to determine the stability of resistance to isometamidium in *T. congolense*.

MATERIALS AND METHODS

Experimental animals

Boran (*Bos indicus*) calves were obtained from a ranch on Kapiti Plain, Machakos District, Kenya; an area not inhabited by tsetse. All calves were screened for the presence of antibodies to *T. congolense*, *T. vivax* and *T. brucei* using an immunofluorescent antibody test (Katende *et al.* 1987). Antibodies to these trypanosome species were not detected in any of the animals. Outbred Swiss white mice were obtained from the ILRAD breeding colony, which was originally derived from an O.L.A.C. (Oxfordshire Laboratory Animal Colonies) 1976 strain. Mice weighed between 25 and 35 g at the beginning of each experiment and a mean mouse weight of 30 g was used for drug computations.

Trypanosomes

T. congolense isolate Banankeledaga/83/CRTA/67 was made from a cow in Banankeledaga, Burkina Faso, in 1983 (Pinder & Authie, 1984). 'Stabilate 2', derived from the parental isolate, was a gift to ILRAD by the Centre de Recherches sur les Trypanosomoses Animales, Bobo-Dioulasso, Burkina Faso, and was subsequently redesignated *T. congolense* IL 2462. The derivation of the working stock *T. congolense* IL 2856 from *T. congolense* IL 2462 and the parental isolate has been briefly described by Moloo & Kutuza (1990). A full description of this derivation is shown in Fig. 1. Also given in Fig. 1 is the derivation of the clones and subclones used in the present study.

T. congolense IL 2856 was inoculated intraperitoneally (i.p.) into irradiated (650 rad.) mice. Seven days later, blood from these mice was inoculated into another group of irradiated (650 rad.) mice and 5 days later trypanosome clones 1–7 were derived as previously described (Barry & Gathuo, 1984). Clones 8 and 10 were derived following one further passage in irradiated mice (see Fig. 1). Nine clones (subclones) were derived from IL 2856 clone 1 by expansion of the clone in irradiated mice and cloning according to the method of Barry & Gathuo (1984). *T. congolense* IL 1180 is a doubly-cloned derivative of an isolate from the Serengeti, Tanzania (Natulya *et al.* 1984). *T. congolense* IL 2642 is a doubly-cloned population derived from an isolate from Busogo, Uganda (Peregrine *et al.* 1988). Earlier studies have demonstrated that both IL 1180 and IL 2642 are highly sensitive to isometamidium chloride (Peregrine *et al.* 1988; Sones, Njogu & Holmes, 1988) and diminazene aceturate (A.S. Peregrine, unpublished data).

Characterization of drug sensitivity

In order to characterize the drug sensitivities of the various populations, stabilates of each clone and subclone were expanded in irradiated mice. Prior to the first peak of parasitaemia, blood was collected in heparin and the parasitaemia estimated using a Neubauer haemocytometer. Groups of non-irradiated mice (6/group) were inoculated i.p. with $1{\cdot}0 \times 6^6$ bloodstream forms and treated 6 h later with varying doses of isometamidium chloride (0·125–16·0 mg/kg body weight) or diminazene aceturate (0·25–64·0 mg/kg body weight). Trypanocides were administered i.p. in 0·2 ml of sterile water. Following treatment, mice were monitored twice weekly for 60 days, for the presence of parasites, by examination of tail blood. Using standard logic analyses, the sensitivities of each clone to isometamidium chloride and diminazene aceturate were expressed as 50% curative dose (CD_{50}) values, i.e., the dose of drug required to cure 50% of infected mice. Kendall's rank correlation test (Sprent, 1989) was used to determine whether there was any correlation between the isometamidium chloride and diminazene aceturate CD_{50} values of each clone.

Drug sensitivity tests in cattle were conducted employing deep intramuscular administration of drugs, 3 days following first detection of parasitaemia. Thereafter, cattle were monitored three times weekly for 150 days following treatment.

Isometamidium chloride (Samorin®) Lot Number DX0105 and diminazene aceturate (Berenil®) Lot Number 093D574 were used throughout these studies.

Tsetse

Teneral male *Glossina morsitans centralis*, obtained from the ILRAD-bred colony, were infected with either *T. congolense* stock IL 2856 or *T. congolense* IL 2856 clone 1 (also designated IL 3270) by allowing them to feed on infected Boran calves. Flies were first fed on infected calves 7 days after parasites were first detected in the blood buffy-coat (Murray, Murray & McIntyre, 1977). Subsequently, flies were maintained by daily feeding, except at weekends, on the infected calves for 25 days. Groups of 180 flies, fed in such a manner, were dissected using standard methods, to determine the infection rate produced by the 2 *T. congolense* populations.

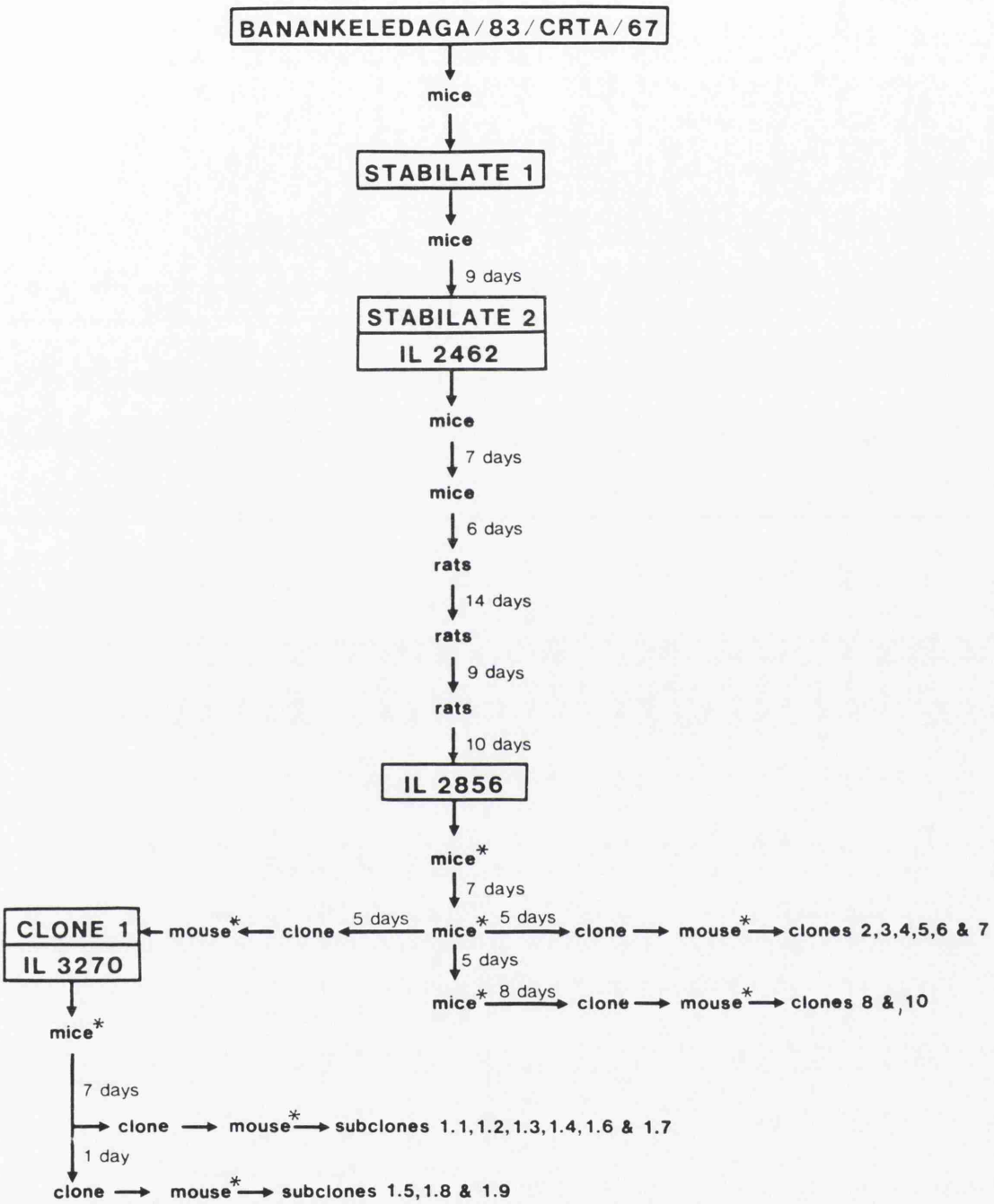


Fig. 1. Pedigree of *Trypanosoma congolense* stock IL 2856 and the clones derived from this stock.
* Sublethally irradiated (650 rad.).

Monitoring of cattle infections

Jugular blood samples were collected 3 times weekly into vacutainer tubes coated with ethylenediamine tetra-acetic acid and the levels of parasitaemia estimated using phase-contrast microscopic examination of the blood buffy-coat (Murray *et al.* 1977; Paris, Murray & McOdimba, 1982).

Isoenzyme analysis

Electrophoresis of trypanosome enzymes was carried out on thin-layer starch gels. Parasite lysates were adsorbed onto cotton threads which were then inserted into the gels according to the method of Wraxall & Culliford (1968). The enzymes studied were EC 5.3.1.9, glucose phosphate isomerase (GPI); EC 2.7.5.1, phosphoglucomutase (PGM);

EC 1.1.1.37, malate dehydrogenase (MDH); EC 2.6.1.1, aspartate amino transferase (ASAT); EC 2.6.1.2, alanine amino transferase (ALAT); EC 1.2.1.12, glyceraldehyde phosphate dehydrogenase (GAPDH); and the peptidase activity which hydrolyses the substrate L-leucylglycylglycine (PEP 1). The electrophoretic conditions and staining procedures to reveal enzymic activity were those given by Young & Godfrey (1983).

RESULTS

T. congolense stock IL 2856 and 9 clones derived from this population were characterized in mice for their sensitivity to isometamidium chloride (see Table 1). Whilst the isometamidium chloride CD_{50} for IL 2856 was 3·6 mg/kg, the CD_{50} values for the clones derived from this stock ranged from 1·5 to 5·1 mg/kg. Eight of the clones expressed a significantly ($P < 0·05$) lower level of resistance to isometamidium chloride than the stock from which they were derived (Table 1). Only 1 clone (clone 1; IL 3270) expressed a level of resistance which was not significantly different from the parental stock. In addition, amongst the 9 clones there was significant variation in expression of resistance to isometamidium. In contrast to the 9 IL 2856 clones, the CD_{50} values for 2 isometamidium-sensitive clones, IL 1180 and IL 2642, were 0·018 and 0·007 mg/kg, respectively. Thus, all 9 clones derived from IL 2856 expressed a 500- to 700-fold higher level of resistance to isometamidium than did *T. congolense* IL 1180 and IL 2642.

In further studies, IL 2856 and the same 9 derivative clones were characterized in mice for their sensitivity to diminazene aceturate (see Table 2). The diminazene aceturate CD_{50} values for the 9 clones ranged from 6·4 to 21·0 mg/kg and 5 of the 9 clones expressed a significantly lower level of resistance to diminazene than the parental stock. Furthermore, amongst the clones, there was significant variation in resistance to diminazene aceturate. When compared to *T. congolense* IL 1180, a clone which is sensitive to the normal clinical dose of diminazene aceturate (3·5 mg/kg) in goats (A. S. Peregrine, unpublished data), all the clones were more resistant to diminazene. Using Kendall's rank correlation test, there was no correlation between the clones' expression of resistance to isometamidium and diminazene, at the 5 % level.

Because drug sensitivity data obtained in mice cannot be directly extrapolated to the situation in cattle (Hawking, 1963; Sones *et al.* 1988), *T. congolense* IL 3270 was screened for its sensitivity to isometamidium and diminazene in cattle. The clone produced an infection rate in tsetse of 5·0%. Infective tsetse were fed on Boran calves and the resulting infections screened for their sensitivity to isometamidium chloride. Intramuscular treatment with doses up to 2·0 mg/kg failed to cure the infections in cattle (Table 3). Furthermore, populations which relapsed after treatment with 0·5, 1·0 and 2·0 mg/kg isometamidium chloride were not cured by treatment with 3·5, 7·0 and 10·5 mg/kg diminazene aceturate, respectively. Infections which relapsed after treatment with diminazene aceturate at the aforementioned doses also failed to be eliminated when the same cattle were treated with 17·5 mg/kg diminazene aceturate.

All 9 of the clones derived from *T. congolense* IL 2856 were characterized for their electrophoretic variants of the enzymes GPI, PGM, MDH, ASAT, ALAT, GAPDH and PEP 1. For each enzyme all clones expressed the same variants.

Finally, 9 clones (subclones 1·1–1·9) were derived from *T. congolense* IL 3270 and characterized in mice for their sensitivity to isometamidium (see Table 4). Whilst the isometamidium chloride CD_{50} for the

Table 1. Sensitivity of *Trypanosoma congolense* populations to isometamidium chloride in mice

Population	Designation	CD_{50} (mg/kg)*	95 % C.I. (mg/kg)†
T. congolense IL 2856 stock	IL 2856	3·6	3·0–4·3
T. congolense IL 2856 clone 1	IL 3270	5·1	3·2–8·0
T. congolense IL 2856 clone 2	IL 3271	1·5	1·3–1·8
T. congolense IL 2856 clone 3	IL 3272	2·2	1·9–2·6
T. congolense IL 2856 clone 4	IL 3273	2·5	2·0–2·9
T. congolense IL 2856 clone 5	IL 3274	1·5	1·3–1·7
T. congolense IL 2856 clone 6	IL 3275	1·9	1·6–2·2
T. congolense IL 2856 clone 7	IL 3276	2·0	1·7–2·4
T. congolense IL 2856 clone 8	IL 3277	1·6	1·4–1·9
T. congolense IL 2856 clone 10	IL 3279	2·2	1·9–2·5
T. congolense IL 1180‡		0·018	0·013–0·025
T. congolense IL 2642‡		0·007	0·006–0·009

* CD_{50}, Curative dose for 50 % of the infected population.
† C.I., Confidence interval.
‡ Reference sensitive clone.

Table 2. Sensitivity of *Trypanosoma congolense* populations to diminazene aceturate in mice

Population	CD_{50} (mg/kg)*	95 % C.I. (mg/kg)†
T. congolense IL 2856 stock	35·7	12·1–105
T. congolense IL 2856 clone 1	9·6	8·1–11·4
T. congolense IL 2856 clone 2	18·2	12·4–26·6
T. congolense IL 2856 clone 3	7·0	5·6–8·8
T. congolense IL 2856 clone 4	9·2	7·6–11·3
T. congolense IL 2856 clone 5	12·4	9·5–16·1
T. congolense IL 2856 clone 6	21·0	12·1–36·7
T. congolense IL 2856 clone 7	13·5	10·3–17·8
T. congolense IL 2856 clone 8	6·4	5·1–7·9
T. congolense IL 2856 clone 10	9·4	7·9–11·4
T. congolense IL 1180‡	2·3	2·0–2·6

* CD_{50}, Curative dose for 50 % of the population.
† C.I., Confidence interval.
‡ Reference sensitive clone.

Table 3. Studies in cattle on the sensitivity of *Trypanosoma congolense* IL 2856 clone 1 (NL 3270) to isometamidium chloride and diminazene aceturate (all treatments given intramuscularly)

Drug	Dose (mg/kg)	Number treated	Number cured
Isometamidium chloride	0·5	2	0
	1·0	7	0
	2·0	7	2
Diminazene aceturate*	3·5	2	0
	7·0	2	0
	10·5	2	0

* Relapses after treatment with 0·5, 1·0 and 2·0 mg/kg isometamidium chloride were treated with 3·5, 7·0 or 10·5 mg/kg diminazene aceturate, respectively.

Table 4. Sensitivity of *Trypanosoma congolense* IL 3270 subclones to isometamidium chloride in mice

Population	Designation	CD_{50} (mg/kg)*	95 % C.I. (mg/kg)†
IL 3270 (IL 2856 clone 1)		5·1	3·2–8·0
IL 3270 subclone 1.1	IL 3486	1·8	1·5–2·1
IL 3270 subclone 1.2	IL 3487	1·4	1·2–1·6
IL 3270 subclone 1.3	IL 3488	1·4	1·2–1·5
IL 3270 subclone 1.4	IL 3489	0·9	0·8–1·0
IL 3270 subclone 1.5	IL 3490	1·9	1·7–2·2
IL 3270 subclone 1.6	IL 3491	3·0	2·5–3·7
IL 3270 subclone 1.7	IL 3492	1·3	1·1–1·4
IL 3270 subclone 1.8	IL 3493	2·7	2·2–3·3
IL 3270 subclone 1.9	IL 3494	1·7	1·5–2·0

* CD_{50}, Curative dose for 50 % of the population.
† C.I., Confidence interval.

parental clone (IL 3270) was 5·1 mg/kg, the CD_{50} values for the 9 subclones ranged from 0·9 to 3·0 mg/kg: 7 of the 9 subclones expressed a significantly lower ($P < 0·05$) level of resistance than IL 3270. In addition, amongst the subclones, there was significant variation in expression of resistance to isometamidium.

DISCUSSION

These studies have demonstrated that clones of *T. congolense* can express high levels of resistance to both isometamidium and diminazene, and that resistance to isometamidium is not a stable phenotype.

The studies have confirmed that *T. congolense* IL 2856, a stock derived from an isolate described by Pinder & Authie (1984) and Authie (1984), expresses a high level of resistance to both isometamidium and diminazene in mice. Similarly, Moloo & Kutuza (1990) have previously shown that this stock expresses a high level of resistance to isometamidium and diminazene in cattle. Similar multidrug-resistant phenotypes, with trypanosomes resistant to diminazene and a phenanthridine (i.e. isometamidium or homidium) have also been described for isolates of *T. congolense* or *T. vivax* from The Central African Republic (Finelle & Yvore, 1962), Nigeria (Gray & Roberts, 1971), Kenya (Gitatha, 1979; Schonefeld *et al.* 1987), Ivory Coast (Kupper & Volters, 1983) and Ethiopia (V. Codjia, unpublished data). Since diminazene and isometamidium, or diminazene and homidium, are usually sanative combinations (Whiteside, 1958) it is possible that the trypanosome populations in these reports expressed resistance to both drugs because they consisted of 2 phenotypically distinct subpopulations; one resistant to diminazene and the other resistant to a phenanthridine. Whether this was the case was not clear from the above studies.

Nine clones were therefore derived, without drug selection, from *T. congolense* IL 2856 and characterized in mice for their sensitivities to both isometamidium and diminazene. The studies demonstrated that all 9 clones expressed high levels of resistance to isometamidium and moderate to high levels of resistance to diminazene. Thus, clones of *T. congolense* can express resistance to both compounds. Furthermore, amongst the clones, there was significant variation in expression of resistance to both isometamidium and diminazene. Since most of the clones expressed significantly lower levels of resistance to isometamidium and diminazene than the parental stock, this indicates that drug sensitivity studies using clones to characterize field isolates should be interpreted with caution since lower levels of resistance may be obtained.

In addition to drug resistance phenotypes, electrophoretic patterns for 7 enzymes were determined for the 9 clones and their parental stock: all 10 populations expressed the same isoenzyme variants. Therefore, all 9 clones appeared to be identical for these phenotypic markers. Thus, although clones derived from stock IL 2856 expressed significantly different levels of resistance to isometamidium and diminazene, this difference is probably not due to the stock containing a mixed population of trypanosomes. In a similar manner, clones derived from isolates of *Plasmodium falciparum* have been shown to express significantly different levels of resistance to chloroquine (Thaithong, 1983; Webster *et al.* 1985), pyrimethamine (Thaithong, 1983) and mefloquine (Webster *et al.* 1985). However, in contrast to the isoenzyme homogeneity of the trypanosome clones in the present study, the sets of clones described in these studies on *P. falciparum* appeared to be heterogeneous on the basis of isoenzyme electrophoretic variants and other phenotypic markers. Since clones of *T. brucei* may differ in their growth rates *in vivo* (Seed, 1978; Barry, Le Ray & Herbert, 1979), these studies may have important implications for scientists using mice or *in vitro* cultivation to characterize the level of drug resistance expressed by field isolates of trypanosomes: adaptation to, or long-term maintenance in mice or *in vitro* systems may select for or against certain clones. However, preliminary data reported by Brown *et al.* (1987) have indicated that adaptation of *T. congolense* populations to cultivation *in vitro*, and cyclical transformation *in vitro*, appears to have little effect on the expression of resistance against isometamidium in mice. In contrast, with *P. falciparum*, Le Bras *et al.* (1983) have suggested that there appears to be a direct relationship between resistance to chloroquine and a population's adaptability to cultivation *in vitro*.

In addition to expressing high levels of resistance to isometamidium and diminazene in mice, *T. congolense* clones IL 3270 has also been shown to express a high level of resistance to both compounds in cattle. Although mice are often used to screen trypanosome populations for their sensitivity to isometamidium (Scott & Pegram, 1974; Pinder & Authie, 1984) there appears to be an inconsistent correlation between the level of resistance expressed in mice and that expressed in cattle (Hawking, 1963; Sones *et al.* 1988). Since the two screening systems may select for or against certain subpopulations, and the work described herein has demonstrated heterogeneity of drug resistance amongst trypanosomes belonging to a stock, the apparent lack of correlation between the two systems may be because stocks were used in the aforementioned studies.

Finally, 9 clones (subclones) were derived from *T. congolense* clone IL 3270 and characterized for their sensitivity to isometamidium in mice. Amongst the subclones there was significant variation in expression of resistance. In addition, 7 of the 9 subclones were significantly more sensitive than IL 3270. These results suggest that trypanosomes of a similar genetic background may differ significantly in expression of resistance to isometamidium and that at least a component of the genetic control of this phenotype is likely to be unstable. Thus, resistance to isometamidium should not be a stable phenotype. Nyeko *et al.* (1989) have provided data which support this latter conclusion: multiple passages of a *T. congolense* stock in mice resulted in a significant reduction in the population's resistance to isometamidium. In contrast, other workers have reported that resistance to isometamidium is stable when trypanosomes are maintained *in vivo* (Gray &

Roberts, 1971) or *in vitro* (Kaminsky & Zweygarth, 1989). Further work is required to determine whether the methods used to quantify resistance in these studies were sufficiently sensitive to detect small alterations in expression of resistance.

It is conceivable that host components play a role in the apparent levels of drug resistance expressed by the clones and subclones in this study. However, since every population was characterized in the same strain of mouse and repeat experiments produced consistent results (data not given), it is more likely that the observed phenotypic resistance levels are due to unstable genetic component(s). This appears to be true for both resistance phenotypes. However, since the levels of resistance to both drugs appeared to vary independently of each other, this would suggest that the unstable elements differ for each drug. Thus, from the present results, it is possible to envisage that gene amplification (Brown, Tlsty & Schimke, 1983) or the production of unstable extrachromosomal elements (Beverley *et al.* 1988) may be responsible for expression of resistance to both isometamidium and diminazene. It should be possible to identify such sequences in drug-resistant trypanosomes and this possibility is under investigation.

RMB Animal Health Ltd kindly donated the isometamidium chloride used in these studies. The technical assistance of Mr S. Kemei is gratefully acknowledged and Ms D. Lewa is thanked for typing the manuscript.

This is ILRAD publication number 905.

REFERENCES

AUTHIE, E. (1984). Mise en évidence d'une résistance aux trypanocides parmi des souches de *Trypanosoma congolense* récemment isolées au Burkina. *Revue d'Elevage et de Medécine Vétérinaire des Pays Tropicaux* **37** (No. spécial), 219–35.

BARRY, J. D. & GATHUO, H. (1984). Antigenic variation in *Trypanosoma vivax*: isolation of a serodeme. *Parasitology* **89**, 49–58.

BARRY, J. D., LE RAY, D. & HERBERT, W. J. (1979). Infectivity and virulence of *Trypanosoma* (*Trypanozoon*) *brucei* for mice. IV. Dissociation of virulence and variable antigen type in relation to pleomorphism. *Journal of Comparative Pathology* **89**, 465–70.

BEVERLEY, S. M., ELLENBERGER, T. E., IOVANNISCI, D. M., KAPLER, G. M., PETRILLO-PEIXOTO, M. & SINA, B. J. (1988). Gene amplification in *Leishmania*. In *The Biology of Parasitism: A Molecular and Immunological Approach* (ed. England, P. T. & Sher, A.) pp. 314–48. New York: A. R. Liss, Inc.

BROWN, H. C., ROSS, C. A., HOLMES, P. H., LUCKINS, A. G. & TAYLOR, A. M. (1987). Adaptation of *Trypanosoma congolense* stocks to *in vitro* culture does not change their sensitivity to isometamidium. *Acta Tropica* **44**, 373–4.

BROWN, P. C., TLSTY, T. D. & SCHIMKE, R. T. (1983). Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea. *Molecular and Cellular Biology* **3**, 1097–107.

FINELLE, P. & YVORE, P. (1962). Quelques observations sur la chimioresistance. In *International Scientific Council for Trypanosomiasis Research, 9th meeting, Conakry, Guinea*, CCTA publication No. 88, pp. 107–10.

GITATHA, S. K. (1979). *T. congolense* (Shimba hills) resistant to various trypanocidal drugs. In *International Scientific Council for Trypanosomiasis Research and Control, 16th meeting, Yaounde, Cameroon*, OAU/STRC publication No. 111, pp. 257–63.

GRAY, A. R. & ROBERTS, C. J. (1971). The cyclical transmission of strains of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* **63**, 67–89.

HAWKING, F. (1963). Drug-resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, Berenil and other compounds in mice. *Annals of Tropical Medicine and Parasitology* **57**, 262–82.

JONES-DAVIES, W. J. (1967). The discovery of Berenil-resistant *Trypanosoma vivax* in Northern Nigeria. *The Veterinary Record* **80**, 531–2.

KAMINSKY, R. & ZWEYGARTH, E. (1989). Effect of *in vitro* cultivation on the stability of resistance of *Trypanosoma brucei brucei* to diminazene, isometamidium, quinapyramine and Mel B. *Journal of Parasitology* **75**, 42–5.

KATENDE, J. M., MUSOKE, A. J., NANTULYA, V. M. & GODDEERIS, B. M. (1987). A new method of fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescent antibody test for diagnosis of bovine trypanosomiasis. *Tropical Medicine and Parasitology* **38**, 41–4.

KUPPER, W. & WOLTERS, M. (1983). Observations on drug resistance of *Trypanosoma* (*Nannomonas*) *congolense* and *Trypanosoma* (*Duttonella*) *vivax* in cattle at a feedlot in the northern Ivory Coast. *Tropenmedizin und Parasitologie* **34**, 203–5.

LE BRAS, J., DELORON, P., RICOUR, A., ANDRIEU, B., SAVEL, J. & COULAUD, J. P. (1983). *Plasmodium falciparum*: drug sensitivity *in vitro* of isolates before and after adaptation to continuous culture. *Experimental Parasitology* **56**, 9–14.

MacLENNAN, K. J. R. & NA'ISA, B. K. (1970). Relapsing *Trypanosoma vivax* infections in Nigerian Zebu cattle treated with diminazene aceturate. *Tropical Animal Health and Production* **2**, 189–95.

MALOO, S., CHEMA, S., CONNOR, R., DURKIN, J., KIMOTHO, P., MAEHL, MUKENDI, F., MURRAY, M., RARIEYA, M. & TRAIL, J. (1987) Efficacy of chemoprophylaxis for East African Zebu cattle exposed to trypanosomiasis in village herds in Kenya. In *International Scientific Council for Trypanosomiasis Research and Control, 19th meeting, Lome, Togo*, OAU/STRC publication No. 114, pp. 282–7.

MBWAMBO, H. A., MELLA, P. N. P. & LEKAKI, K. A. (1988). Berenil (diminazene aceturate)-resistant *Trypanosoma congolense* in cattle under natural tsetse challenge at Kibaha, Tanzania. *Acta Tropica* **45**, 239–44.

MOLOO, S. K. & KUTUZA, S. B. (1990). Expression of resistance to isometamidium and diminazene in *Trypanosoma congolense* in Boran cattle infected by *Glossina morsitans centralis*. *Acta Tropica* **47**, 79–89.

MURRAY, M., MURRAY, P. K. & MCINTYRE, W. I. M. (1977). An improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **71**, 325–6.

NANTULYA, V. M., MUSOKE, A. J., RURANGIRWA, F. R. & MOLOO, S. K. (1984). Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe passaged infections. *Infection and Immunity* **43**, 735–8.

NYEKO, J. H. P., GOLDER, T. K., OTIENO, L. H. & SSENYONGA, G. S. Z. (1989). *Trypanosoma congolense*: drug resistance during cyclical transmissions in tsetse flies and syringe passage in mice. *Experimental Parasitology* **69**, 357–62.

PARIS, J., MURRAY, M. & MCODIMBA, F. (1982). A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. *Acta Tropica* **39**, 307–16.

PEREGRINE, A. S., OGUNYEMI, O., WHITELAW, D. D., HOLMES, P. H., MOLOO, S. K., HIRUMI, H., URQUHART, G. M. & MURRAY, M. (1988). Factors influencing the duration of isometamidium chloride (Samorin) prophylaxis against experimental challenge with metacyclic forms of *Trypanosoma congolense*. *Veterinary Parasitology* **28**, 53–64.

PINDER, M. & AUTHIE, E. (1984). The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica* **41**, 247–52.

SCHONEFELD, A. R., ROTTCHER, D. & MOLOO, S. K. (1987). The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. *Tropical Medicine and Parasitology* **38**, 177–80.

SCOTT, J. M. & PEGRAM, R. G. (1974). A high incidence of *Trypanosoma congolense* strains resistant to homidium bromide in Ethiopia. *Tropical Animal Health and Production* **6**, 215–21.

SEED, J. R. (1978). Competition among serologically different clones of *Trypanosoma brucei gambiense in vivo*. *Journal of Protozoology* **25**, 526–9.

SONES, K. R., NJOGU, A. R. & HOLMES, P. H. (1988). Assessment of sensitivity of *Trypanosoma congolense* to isometamidium chloride: a comparison of tests using cattle and mice. *Acta Tropica* **45**, 153–64.

SPRENT, P. (1989). *Applied Nonparametric Statistical Methods*. London: Chapman & Hall Ltd.

THAITHONG, S. (1983). Clones of different sensitivities in drug-resistant isolates of *Plasmodium falciparum*. *Bulletin of the World Health Organization* **61**, 709–12.

TRAIL, J. C. M., MURRAY, M., SONES, K., JIBBO, J. M. C., DURKIN, J. & LIGHT, D. (1985). Boran cattle maintained by chemoprophylaxis under trypanosomiasis risk. *Journal of Agricultural Science* **105**, 147–66.

WEBSTER, H. K., THAITHONG, S., PAVANAND, K., YONGVANITCHIT, K., PINSWASDI, C. & BOUDREAU, E. F. (1985). Cloning and characterization of mefloquine-resistant *Plasmodium falciparum* from Thailand. *American Journal of Tropical Medicine and Hygiene* **34**, 1022–7.

WHITESIDE, E. F. (1958). The maintenance of cattle in tsetse-infested country. A summary of four years' experience in Kenya. In *International Scientific Council for Trypanosomiasis Research, 7th meeting, Bruxelles, Belgium*, CCTA publication No. 41, pp. 83–90.

WHITESIDE, E. F. (1960). Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In *International Scientific Council for Trypanosomiasis Research, 8th meeting, Jos, Nigeria*, CCTA publication No. 62, pp. 141–53.

WRAXALL, B. G. D. & CULLIFORD, B. J. (1968). A thin layer starch gel method for enzyme typing blood strains. *Journal of the Forensic Science Society* **8**, 81.

YOUNG, C. J. & GODFREY, D. G. (1983). Enzyme polymorphism and the distribution of *Trypanosoma congolense* isolates. *Annals of Tropical Medicine and Parasitology* **77**, 467–81.

Section 5.2

Zilberstein, D., Wilkes, J., Hirumi, H. and Peregrine, A.S. (1993) Fluorescence analysis of the interaction of isometamidium with *Trypanosoma congolense*. *Biochemical Journal* 292, 31-35.

Biochem J (1993) 292, 31–35 (Printed in Great Britain)

Fluorescence analysis of the interaction of isometamidium with *Trypanosoma congolense*

Dan ZILBERSTEIN,* Jonathan WILKES,† Hiroyuki HIRUMI† and Andrew S. PEREGRINE†‡

*Department of Biology, Technion, Israel Institute of Technology, Technion City, Haifa 32000, Israel, and †International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya

Isometamidium chloride (Samorin) is the only compound recommended for prophylaxis against bovine trypanosomiasis in sub-Saharan Africa. The fluorescence property of this compound was used to investigate the interaction of the molecule with *in vitro*-derived bloodstream forms of *Trypanosoma congolense* IL 1180. Incubation of isometamidium with trypanosomes at 37 °C for 180 min resulted in a gradual alteration of the $\lambda_{max.}$ with time (from 600 to 584 nm) and an increase in the intensity of trypanosome-associated fluorescence of approx. 2-fold. The alteration in fluorescence was temperature-dependent and inhibited by the addition of *N*-ethylmaleimide. In contrast, with intact cells addition of digitonin caused a rapid increase in fluorescence intensity to approximately four times that observed with intact cells. Uptake of isometamidium was also determined using radiolabelled drug; the results indicated that the time course of the uptake process resembled the fluorescence profile and was temperature-dependent. The results therefore indicate that the alteration of fluorescence is due to interaction of isometamidium with an intracellular component(s) and that isometamidium is transported across the plasma membrane via a protein carrier. The data also indicate that the described fluorescence technique can be used to investigate the role of membrane transport in resistance to isometamidium.

INTRODUCTION

In sub-Saharan Africa, chemotherapy of trypanosomiasis in cattle, goats and sheep is dependent upon the salts of three compounds: the aromatic diamidine, diminazene (Jensch, 1958), the phenanthridine, homidium (Watkins and Woolfe, 1952), and the phenanthridine aromatic amidine, isometamidium (Berg, 1960). While the salts of diminazene (Berenil, Veriben) and homidium (Novidium, Ethidium) are used as therapeutic agents, only isometamidium chloride (Samorin, Trypamidium) is marketed as both a prophylactic and a therapeutic agent (Leach and Roberts, 1981). Thus chemoprophylaxis of bovine trypanosomiasis is restricted to the use of only one compound, namely isometamidium chloride.

Isometamidium chloride is 8-[(*m*-amidinophenylazo)amino]-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (see Figure 1). The compound has been shown to be a highly effective prophylactic agent in the field (Wiesenhutter et al., 1968; Trail et al., 1985) and is probably the most widely used of the available trypanocides for bovine trypanosomiasis. However, resistance to the compound has been described at a number of sites across Africa (Lewis and Thomson, 1974; Kupper and Wolters, 1983; Pinder and Authie, 1984; Schönefeld et al., 1987).

Although isometamidium has been used in the field for over 30 years, very little is known about its mechanism of action. Various studies have indicated that the compound interacts with a number of intracellular molecules (Henderson et al., 1977; Bacchi et al., 1980; Bacchi, 1981; Shapiro and Englund, 1990) and, therefore, that there may be several modes of action.

In earlier work, the fluorescence property of isometamidium was used to study localization of the compound in mammalian cells (Philips et al., 1967). Similar analyses were recently conducted to examine the interaction of isometamidium with bloodstream forms of *Trypanosoma* (*Nannomonas*) *congolense* clones *in vitro*; an inverse relationship was observed between the intensity of trypanosome-associated fluorescence and the level of resistance to isometamidium expressed by the clones *in vivo* (Sutherland et al., 1991). It is unclear, however, whether these changes in fluorescence characteristics are associated with the trypanocidal action of isometamidium. In the present work, the interaction of isometamidium with an isometamidium-sensitive clone of *T. congolense* was analysed by quantitative fluorescence measurements and with radiolabelled isometamidium. The results indicate that isometamidium is transported into the trypanosome and that this process is mediated by a protein in the plasma membrane.

MATERIALS AND METHODS

Materials

Isometamidium chloride (Samorin) was a gift from RMB Animal Health Ltd.; digitonin and *N*-ethylmaleimide (NEM) were purchased from Sigma.

Trypanosome

T. congolense IL 1180 is a doubly cloned derivative (Nantulya et al., 1984) of an isolate collected from a lion in the Serengeti National Park, Tanzania (Geigy and Kauffmann, 1973). On the basis of molecular karyotype (Majiwa et al., 1986) and hybridization to a repetitive DNA sequence (Majiwa et al., 1985), this clone is a savannah-type *T. congolense* (Young and Godfrey, 1983). The clone expresses a high level of sensitivity to isometamidium chloride both in mice [50% curative dose (CD_{50}) = 18 μg·kg body weight^{-1} (Peregrine et al., 1991)] and in

Abbreviations used: PBSG, phosphate-buffered saline + 5 mM glucose; NEM, *N*-ethylmaleimide.

‡ To whom correspondence should be sent.

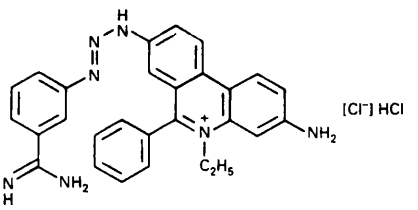


Figure 1 Molecular structure of isometamidium

cattle [sensitive to intramuscular treatment with 1.0 μg·kg body weight^{-1} (Sones et al., 1988)].

Preparation of trypanosomes

Bloodstream forms of *T. congolense* IL 1180 were propagated in exponential-phase growth in an axenic culture system *in vitro* (Hirumi and Hirumi, 1991). Briefly, primary cultures were initiated by pipetting tail blood from infected mice into wells of a 24-well culture plate (10 μl blood/well) containing 2.0 ml of medium. Trypanosomes were then subcultivated in T-25 flasks and maintained at 34 °C in HMI-93 medium, which consists of Iscove's modified Dulbecco's minimal essential medium (Flow Laboratories) supplemented with 0.05 mM bathocuproine sulphate, 1.5 mM L-cysteine, 0.5 mM hypoxanthine, 0.12 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.16 mM thymidine, 20% (v/v) heat-inactivated young-goat serum and 5% (v/v) Serum Plus (Hazleton Biologics, Lenaxa, KS, U.S.A.). Trypanosomes propagated in this system were morphologically similar to those seen in blood from infected mice, were covered with a surface coat as determined by electron microscopy and were infective for mice (Hirumi and Hirumi, 1991). Trypanosomes in culture medium were prepared for experiments by centrifuging at 1000 *g* for 10 min. They were then resuspended in Dulbecco's phosphate-buffered saline containing 5 mM glucose (PBSG), pH 7.4, centrifuged at 1000 *g* for 10 min and resuspended in PBSG to a final concentration of 1.0×10^8 trypanosomes·ml^{-1}. Parasites were kept at room temperature prior to experiments. In this state they were stable for at least 5 h, i.e., fluorescence activity remained the same over this period.

Fluorescence measurements

The reaction mixture (final volume 3.0 ml), consisting of PBSG and isometamidium chloride at various concentrations (50 or 100 ng·ml^{-1}), was placed in a 3.5 ml glass cuvette. Parasites were added to a final density of $(0.67–1.0) \times 10^7$ cells·ml^{-1}. Fluorescence was recorded on an SLM Aminco 8000 fluorimeter with excitation and emission wavelengths of 374 and 584 nm respectively (slit width of 10 nm for both excitation and emission). The cuvette mounting was temperature-controlled throughout the measurements. Data analyses were conducted using an SLM version 2.2 spectrum processor (SLM Instruments Inc., Urbana, IL, U.S.A.).

Uptake of radiolabelled isometamidium

The reaction mixture (0.6 ml) consisted of PBSG, 100 μM hypoxanthine and 1 μg·ml^{-1} [^{14}C]isometamidium (22.7 Ci·mol^{-1}). Assays were started by adding 0.1 ml of a trypanosome suspension containing 2×10^8 cells·ml^{-1} to the reaction mixture and incubating it at 37 °C. The mixture was subsequently placed in an Eppendorf microcentrifuge tube over a layer of silicone oil (1.02 g·ml^{-1}) which itself was placed over a 50 μl aliquot of 12% $HClO_4$ (1.1 g·ml^{-1}). Uptake was terminated by centrifugation at 15800 *g* for 1–2 min. The level of [^{14}C]radioactivity in 40 μl aliquots of $HClO_4$ lysates were subsequently determined by liquid-scintillation counting.

RESULTS AND DISCUSSION

The development of a sensitive quantitative assay for isometamidium is essential for investigations on the mechanism of action of this compound in trypanosomes. Such an experimental system does not currently exist. In the work described here, the fluorescence properties of isometamidium were analysed.

In initial studies, the excitation spectrum of isometamidium at a constant emission wavelength of 600 nm was determined when the compound was dissolved in PBS; the maximum emission wavelength was achieved at 374 nm (results not shown). When using 374 nm as the excitation wavelength the emission spectra displayed two peaks: at 600 nm and at 645 nm (Figure 2a). It should be noted that the intensity of both peaks are similar. When lysates of *T. congolense* IL 1180 were added to isometamidium, there was an immediate shift in the $\lambda_{max.}$ at 600 nm to 584 nm; there was also an increase in fluorescence intensity of more than 4-fold (Figure 2b). In contrast, neither an alteration in $\lambda_{max.}$ nor a significant increase in fluorescence intensity were observed at 645 nm. We therefore examined the interaction of isometamidium with intact bloodstream forms of *T. congolense* IL 1180. When parasites were added to a solution containing isometamidium at a concentration of 100 ng·ml^{-1}, there was a gradual time-dependent alteration in both the $\lambda_{max.}$ from that of the free compound (600 nm) to that of bound isometamidium, and an increase in fluorescence intensity at 600 nm over the experimental period of 180 min. However, neither an alteration in fluorescence intensity nor $\lambda_{max.}$ was observed at 645 nm (Figure 3). The shift in $\lambda_{max.}$ from 600 nm to 584 nm and the increase in fluorescence intensity resemble similar observations with a number of fluorescence indicators, in which their interaction with a specific ligand caused changes in their emission and/or excitation spectra. Examples are the calcium fluorescence probes quin 2, fura 2 and indo 2 (Tsien et al., 1982; Grynkiewicz et al., 1985; Philosoph and Zilberstein, 1989), and pH indicators such as 2,7-bis(carboxyethyl)-5,6-carboxyfluorescein (Rink et al., 1982; Zilberstein et al., 1989). The change in fluorescence properties of these compounds are determined by the concentration of the specific ligand. They have therefore been exploited for the determination of ligand concentrations inside various cells. By analogy to these various indicators, we quantified the alteration in fluorescence intensity of isometamidium by calculating $\Delta F_{(584-645)}$ as a function of time (Figure 4). The rate of increase in fluorescence with time gradually decreased with time and therefore demonstrated a saturation process with first-order steady-state kinetics. The gradual increase observed using intact bloodstream forms of *T. congolense* IL 1180 was in contrast with the immediate shift in fluorescence obtained when cell lysates were added to isometamidium. The results therefore suggested that the parasites' plasma membrane forms a barrier between the drug and its ligand. This hypothesis was therefore investigated by determining changes in fluorescence with time using constant excitation and emission wavelengths of 374 nm and 584 nm respectively.

The effect of temperature on the rate of increase in fluorescence with time is illustrated in Figure 5. When cells were incubated at

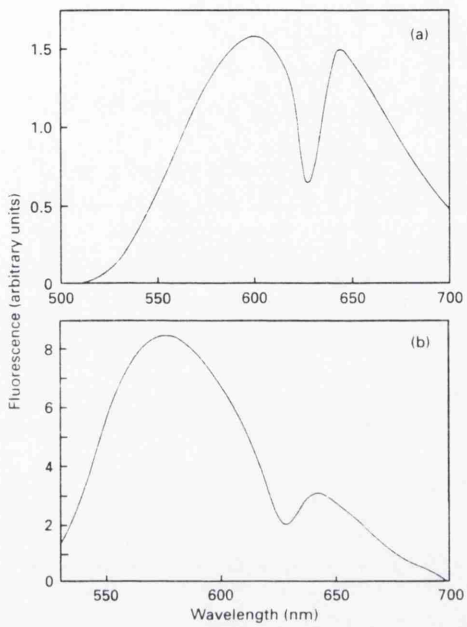


Figure 2 Emission spectra of isometamidium

(**a**) Spectrum analysis of isometamidium chloride at 100 ng·ml^{-1} was carried out at 37 °C using an excitation wavelength of 374 nm. (**b**) Emission spectrum of isometamidium when incubated with digitonin-treated bloodstream forms of *T. congolense*. Spectrum analysis was carried out, as described in (**a**), on the digitonin-treated trypanosomes at the final time point given in Figure 6(a).

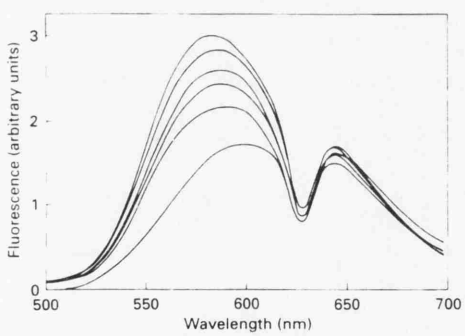


Figure 3 Alteration in the emission spectrum of isometamidium at various time intervals after incubation with *T. congolense* bloodstream forms

Parasites in PBSG (1×10^7·ml^{-1}) containing 100 ng·ml^{-1} isometamidium chloride were incubated at 37 °C. Aliquots (3 ml each) were placed in a glass cuvette at 0, 30, 45, 75, 105 and 180 min (lower plot to upper plot respectively), and the emission spectrum of isometamidium analysed as described in Figure 2(a).

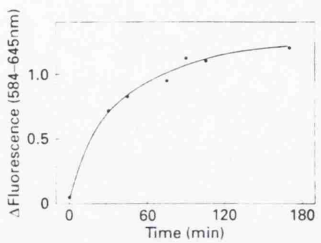


Figure 4 Time course of the increase in the fluorescence of isometamidium when incubated with bloodstream forms of *T. congolense*

Each data point was derived from the fluorescence spectra given in Figure 3 by subtracting the fluorescence signal at 645 nm from that at 584 nm.

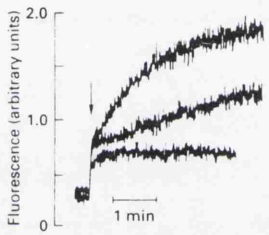


Figure 5 Effect of temperature on the fluorescence of isometamidium when incubated with bloodstream forms of *T. congolense*

Trypanosomes (final density 1×10^7·ml^{-1}, arrow) were added to PBSG containing 100 ng·ml^{-1} isometamidium chloride at 4, 15 or 37 °C (lower, middle or upper plot respectively). Assays were carried out using excitation and emission wavelengths of 374 and 584 nm respectively.

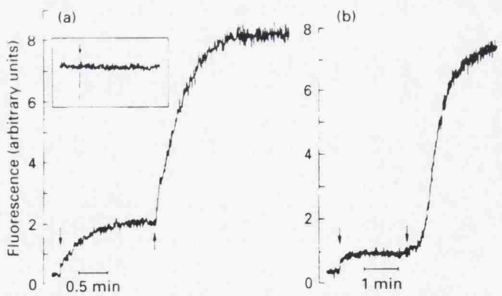


Figure 6 Effect of digitonin on the interaction of isometamidium with bloodstream forms of *T. congolense*

Fluorescence assays were carried out at 37 °C (**a**) or at 4 °C (**b**), as described in Figure 5. The first arrow indicates the addition of cells (6.7×10^6 cells·ml^{-1}). Digitonin was added to a final concentration of 10 μM, indicated by the second arrow. The inset illustrates the effect of addition of 10 μM digitonin to isometamidium in PBSG.

37 °C there was an initial rapid increase in fluorescence followed by a relatively slower phase. When the temperature was reduced to 15 °C the rate of increase in fluorescence was reduced. Furthermore, at 4 °C no change in fluorescence was observed. This further indicated that the gradual increase in fluorescence with intact parasites, and the rate of change, was temperature-dependent. To assess further the role of the plasma membrane in this process, parasites were partially permeabilized with digitonin (Figure 6). Digitonin has previously been used to partially permeabilize the plasma membrane of various kinetoplastids (Docampo and Vercesi, 1989; Philosoph and Zilberstein, 1989), thereby facilitating the study of cellular enzymic activities in a semi-intact system. Thus cells treated with digitonin should be freely permeable to small molecules such as isometamidium.

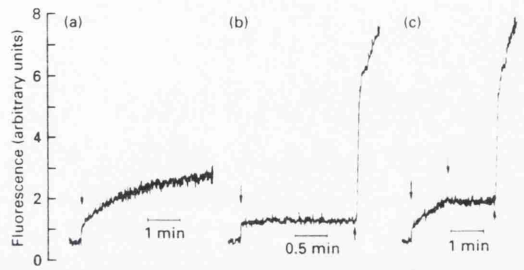


Figure 7 Effect of NEM on the fluorescence of isometamidium when incubated with bloodstream forms of *T. congolense*

Assays were carried out at 37 °C and fluorescence analyses conducted as described in Figure 5, using a trypanosome density of 1×10^7 cells·ml^{-1}. (**a**) Control, without addition of NEM or digitonin; (**b**) cells treated with 1 mM NEM 10 min prior to addition to isometamidium in PBSG (first arrow); 10 μM digitonin added at second arrow; (**c**) cells added at first arrow to isometamidium in PBSG; 1 mM NEM added at second arrow; 10 μM digitonin added at third arrow.

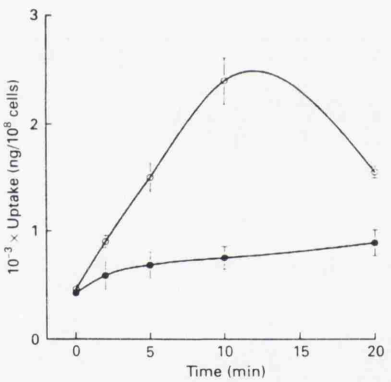


Figure 8 Time course of uptake of isometamidium by bloodstream forms of *T. congolense* IL 1180

Parasites (3×10^7 cells·ml^{-1}) were incubated in PBSG + 100 μM hypoxanthine containing [^{14}C]isometamidium (1 μg·ml^{-1}, 22.7 Ci·mol^{-1}) at 37 °C (○) and at 4 °C (●). At various intervals of time, uptake was terminated by centrifugation of 0.7 ml aliquots as described in the Materials and methods section.

Addition of 10 μM digitonin to cells that had been preincubated with isometamidium chloride at 37 °C for 4 min resulted in an approx. 4-fold increase in the fluorescence intensity (Figure 6a). Furthermore, digitonin had a similar effect when added to cells that were preincubated at 4 °C (Figure 6b), where no increase in fluorescence was observed in intact cells. In a control experiment, digitonin had no effect on the fluorescence characteristics of isometamidium chloride (see the inset to Figure 6a).

NEM is a non-permeable thiol-group-reactive reagent and is therefore used to inhibit the activity of enzymes and membrane transporters exposed to the extracellular side of plasma membranes. Preincubation of parasites with NEM inhibited the increase in fluorescence (Figures 7a and 7b). When trypanosomes were first incubated with isometamidium, the addition of NEM resulted in immediate cessation of the increase in fluorescence (Figure 7c). In both experiments the addition of digitonin resulted in a rapid increase in the fluorescence, similar to that illustrated in Figure 6.

The foregoing observations indicated that, in order for isometamidium to interact with the ligand causing alterations in its fluorescence, it has to cross the parasite's plasma membrane. Moreover, the inhibition of the increase in fluorescence by NEM, suggested that isometamidium is transported across the parasite's plasma membrane via a protein carrier.

In order to assess the role of carrier-mediated transport in a more direct manner, an experiment was set up to measure uptake of ^{14}C-labelled isometamidium. Figure 8 illustrates the time course of the uptake of radiolabelled isometamidium by bloodstream forms of *T. congolense* IL 1180. When evaluated at 37 °C, a rapid initial rate of uptake was observed, which was followed by a gradual decrease in uptake, eventually resulting in a decline in the level of the label. When transport assays were carried out at 4 °C, very little uptake with time was observed (Figure 8). Thus, in a similar manner to the fluorescence data, uptake of [^{14}C]isometamidium demonstrated steady-state kinetics and temperature-dependency. The decline in cellular drug accumulation observed between 10 and 20 min may be a consequence of the 10-fold higher concentration of drug utilized compared with the fluorescence studies, and may be indicative of a toxic effect of the drug, or induction of an efflux process. Finally, addition of excess non-radioactive isometamidium to cells at steady-state transport resulted in rapid efflux of the radioactive compound from the cells (results not shown). In conclusion, these results with radiolabelled isometamidium are consistent with recent work by Sutherland et al. (1992) in which it was concluded that uptake of [^{14}C]isometamidium in populations of *T. congolense* is carrier-mediated.

Collectively our observations indicate that, in order for isometamidium to interact with the ligand causing an alteration in its fluorescence, it has to cross the parasite's plasma membrane. Moreover, the inhibition of the increase in fluorescence by NEM and the temperature-dependent uptake of radiolabelled isometamidium strongly suggests that isometamidium is transported across the parasite's plasma membrane by a protein carrier.

The results from this work constitute the first quantitative fluorescence measurements of the interaction of isometamidium with *T. congolense*. This technique can be exploited to investigate transport of isometamidium and its specific interaction internally. Furthermore, such interactions could be used to study the role of such parameters in drug-resistant populations of *T. congolense* and in other pathogenic African trypanosomes.

RMB Animal Health Ltd. are thanked for providing the isometamidium chloride (Samorin) used in this work. Mrs. K. Hirumi is thanked for technical assistance in the cultivation of trypanosomes. This is ILRAD publication no. 1027.

REFERENCES

Bacchi, C. J. (1981) J. Protozool. **28**, 20–27
Bacchi, C. J., Nathan, H. C., Hutner, S. H., Duch, D. S. and Nichol, C. A. (1980) in Current Chemotherapy and Infectious Disease (Nelson, J. D. and Grassi, C., eds.), vol. 2, pp. 1119–1121, American Society for Microbiology, Washington
Berg, S. S. (1960) Nature (London) **188**, 1106–1107
Docampo, R. and Vercesi, A. E. (1989) J. Biol. Chem. **264**, 108–111
Geigy, R. and Kauffmann, M. (1973) Acta Trop. **3**, 12–23
Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
Henderson, J. F., Batteil, M. L., Zombor, G. and Khoo, M. K. Y. (1977) Cancer Res. **37**, 3434–3441
Hirumi, H. and Hirumi, K. (1991) Parasitology **102**, 225–236
Jensch, H. (1958) Med. Chem. **6**, 134–169
Kupper, W. and Wolters, M. (1983) Tropenmed. Parasitol. **34**, 203–205
Leach, T. M. and Roberts, C. J. (1981) Pharmacol. Ther. **13**, 91–147
Lewis, A. R. and Thomson, J. W. (1974) Rhod. Vet. J. **4**, 62–67

Majiwa, P. A. O., Masake, R. A., Nantulya, V. M., Hamers, R. and Matthyssens, G. (1985) EMBO J. **4**, 3307–3313
Majiwa, P. A. O., Young, J. R., Hamers, R. and Matthyssens, G. (1986) Gene **41**, 183–192
Nantulya, V. M., Musoke, A. J., Rurangirwa, F. R. and Moloo, S. K. (1984) Infect. Immun. **43**, 735–738
Peregrine, A. S., Knowles, G., Ibitayo, A. I., Scott, J. R., Moloo, S. K. and Murphy, N. B. (1991) Parasitology **102**, 93–100
Philips, F. S., Sternberg, S. S., Cronin, A. P., Sodergren, J. E. and Vidal, P. M. (1967) Cancer Res. **27**, 333–349
Philosoph, H. and Zilberstein, D. (1989) J. Biol. Chem. **264**, 10420–10424
Pinder, M. and Authie, E. (1984) Acta Trop. **41**, 247–252
Rink, T. J., Tsein, R. Y. and Pozzan, T. (1982) J. Cell Biol. **95**, 189–196
Schonefeld, A. R., Rottcher, D. and Moloo, S. K. (1987) Trop. Med. Parasitol. **38**, 177–180
Shapiro, T. and Englund, P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 950–954
Sones, K. R., Njogu, A. R. and Holmes, P. H. (1988) Acta Trop. **45**, 153–164
Sutherland, I. A., Peregrine, A. S., Lonsdale-Eccles, J. D. and Holmes, P. H. (1991) Parasitology **103**, 245–251
Sutherland, I. A., Mounsey, A. and Holmes, P. H. (1992) Parasitology **104**, 461–467
Trail, J. C. M., Murray, M., Sones, K., Jibbo, J. M. C., Durkin, J. and Light, D. (1985) J. Agric. Sci. **105**, 147–166
Tsien, R. Y., Pozzan, T. and Rink, T. J. (1982) J. Cell Biol. **94**, 325–334
Watkins, T. I. and Woolfe, G. (1952) Nature (London) **169**, 506
Wiesennutter, E., Turner, D. B. and Kristensen, K. A. (1968) Bull. Epizoot. Dis. Afr. **16**, 419–424
Young, C. J. and Godfrey, D. G. (1983) Ann. Trop. Med. Parasitol. **77**, 467–481
Zilberstein, D., Philosoph, H. and Gepstein, A. (1989) Mol. Biochem. Parasitol. **36**, 109–118

Received 11 September 1992/30 November 1992; accepted 4 December 1992

Section 5.3

Peregrine, A.S., Eisler, M.C., Katende, J., Flynn, J.N., Gault, E.A., Kinabo, L.D.B. and Holmes, P.H. (1994) Generation of monoclonal antibodies to the anti-trypanosomal drug isometamidium. *Hybridoma* 13, 289-294.

HYBRIDOMA
Volume 13, Number 4, 1994
Mary Ann Liebert, Inc., Publishers

Generation of Monoclonal Antibodies to the Anti-Trypanosomal Drug Isometamidium

ANDREW S. PEREGRINE,[1] MARK C. EISLER,[2] JOSEPH KATENDE,[1] J. NORMAN FLYNN,[3] ELIZABETH A. GAULT,[2] LUDOVIC D.B. KINABO,[4,5] and PETER H. HOLMES[2]

ABSTRACT

Mice were immunized with either an isometamidium–human serum albumin (HSA) conjugate or an isometamidium-porcine thyroglobulin conjugate (PTG). Thereafter, monoclonal antibodies (MAbs) IL-A 1001, IL-A 1002, IL-A 1003, 5F7.B7, and 5F7.C9 were generated and selected on the basis that they recognized conjugated and unconjugated isometamidium, but lacked cross-reactivity with the carrier molecules. All five MAbs were of the IgG_1 isotype. Each of the five MAbs was assessed in a competitive ELISA for isometamidium; in each case, the minimum level of detection was approximately 10 ng/ml. Each MAb exhibited approximately 0.1% cross-reactivity with the anti-trypanosomal compound diminazene. However, based on their cross-reactivity with the anti-trypanosomal compound homidium, the MAbs could be divided into two groups; IL-A 1001, IL-A 1002, and IL-A 1003, produced using an isometamidium–HSA conjugate as an immunogen, exhibited low levels of cross-reactivity (approximately 0.1%). In contrast, 5F7.B7 and 5F7.C9, produced using an isometamidium-PTG conjugate as an immunogen, exhibited high levels of cross-reactivity.

INTRODUCTION

In much of sub-Saharan Africa, trypanosomiasis is a major constraint to the development of the livestock industry.[1] Because a vaccine is currently not available for the disease, control of trypanosomiasis is dependent upon the use of chemotherapeutic and chemoprophylactic agents in domestic livestock, and also on control of the vector (*Glossina* spp.). In some parts of the African continent, the rearing of trypanotolerant breeds of livestock also constitutes a control strategy.[2]

Control of trypanosomiasis in cattle, sheep, and goats with anti-trypanosomal compounds is currently dependent upon the salts of three compounds: isometamidium (Samorin®, Trypamidium®), homidium (Novidium®, Ethidium®), and diminazene (Berenil®, Veriben®). Of these compounds, only isometamidium is used routinely as a chemoprophylactic agent.[3]

Isometamidium is a phenanthridine-aromatic amidine, chemically known as 7-(*m*-amidinophenyl-diazoamino)-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride; $C_{28}H_{25}ClN_7HCl$, molecular weight 531.5 (Fig. 1).[4] Although the compound has been used for over 30 years, little is known about its mode of action. Various workers have demonstrated that the molecule interferes with DNA polymerases,[5] purine nucleotide synthesis,[6] polyamine metabolism,[7] and mitochondrial type II topoisomerase.[8] However, the contribution of such activities to anti-trypanosomal activity *in vivo* is not known.

In this report, we describe the production and characterization of a panel of monoclonal antibodies (MAbs) raised against isometamidium. Such reagents represent a potential tool for determining the site of action of isometamidium within trypanosomes. Furthermore, the substitution of monoclonal reagents

[1]International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya.
[2]Department of Veterinary Physiology, University of Glasgow Veterinary School, Bearsden, Glasgow G61 1QH, Scotland.
[3]Department of Veterinary Pathology, University of Glasgow Veterinary School, Bearsden, Glasgow G61 1QH, Scotland.
[4]Department of Veterinary Pharmacology, University of Glasgow Veterinary School, Bearsden, Glasgow G61 1QH, Scotland.
[5]Present address: P.O. Box 3017, Department of Veterinary Physiology, Biochemistry, Pharmacology and Toxicology, Sokoine University of Agriculture, Morogoro, Tanzania.

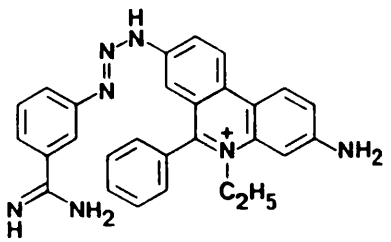


FIG. 1. Molecular structure of isometamidium.

into a recently described assay for the detection of isometamidium, based on a polyclonal antiserum,[9] might facilitate standardization of the anti-isometamidium immunological reagent used in this procedure.

MATERIALS AND METHODS

Anti-trypanosomal drugs

Isometamidium chloride (Samorin®) and homidium chloride (Novidium®) were obtained from RMB Animal Health Ltd., U.K. Homidium bromide (Ethidium®) was obtained from Camco Animal Health, U.K., and Diminazene aceturate (Berenil®) was obtained from Hoechst AG, Germany.

Isometamidium conjugates

An isometamidium-human serum albumin (HSA) conjugate was prepared as described previously.[10] Briefly, 100 mg of HSA (Sigma, Poole, Dorset, UK) was added to 2 ml of distilled water into which 4 mg of isometamidium chloride had previously been dissolved. The mixture was then gently mixed until the HSA had dissolved. One milliliter of 0.25% aqueous glutaraldehyde (vol/vol) (BDH Chemicals Ltd., Poole, Dorset, UK) was then added dropwise with constant mixing, and the solution allowed to stand for 2 hr at room temperature. The reaction was stopped by adding 100 μl of 1 M lysine (BDH Chemicals Ltd., UK). Unbound isometamidium was removed by dialysis in saline overnight at room temperature. The conjugate was then lyophilized.

Isometamidium-porcine thyroglobulin (PTG) and isometamidium-chicken egg albumin (CEA) conjugates were prepared as described previously[11]; 50 mg of isometamidium chloride was dissolved in 2 ml of a solution containing equal volumes of HCl (3.3 M) and $NaNO_2$ (128 mM) at 4°C. After 10 min, this solution was added to 4 mg PTG (Sigma, UK) or CEA (Sigma, UK) to give a final ratio (wt/wt) of 1:5 (isometamidium/PTG), or 1:80 (isometamidium/CEA). The reaction was allowed to proceed for 2 hr before dialysis overnight against 5% acetic acid. The conjugates were then stored at 4°C until use.

Preparation of MAbs

BALB/c mice, bred at the International Laboratory for Research on Animal Diseases, were primed intraperitoneally (i.p.) with 1.25 μg of isometamidium–HSA conjugate emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI). On days 15, 28, and 57, the animals were immunized i.p. with 30 μg of isometamidium–HSA conjugate emulsified in incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, MI). Two further i.p. immunizations with 15 μg of isometamidium–HSA conjugate, in IFA, were given on days 91 and 175, before a final intravenous boost with 15 μg of isometamidium–HSA in 100 μl of Dulbecco's phosphate-buffered saline (PBS).

Spleen cells were removed from the mice 3 days after the final boost and fused with the mouse myeloma mutant clone X63.Ag8.653[12] using established methods.[13] Resultant hybridomas were cultured and cloned as previously described.[14] After the first cloning the specificity of the antibodies for isometamidium was determined in an ELISA ("ELISA 1"). Hybridomas secreting isometamidium-specific antibodies were recloned. The resultant supernatants were then screened for the ability to bind free isometamidium, and for cross-reactivity with homidium and diminazene, using a second ELISA ("ELISA 2"). Hybridomas secreting antibody that bound to free isometamidium and did not cross-react with either homidium or diminazene at 10^{-5} M in this system were subsequently recloned and called IL-A 1001, IL-A 1002, and IL-A 1003. The isotype of each MAb was determined by immunodiffusion in 1% (wt/vol) agarose (Sigma, UK), and by ELISA, using class-specific antibodies from Litton Bionetics (Kensington, USA) and Hyclone (Logan, UT), respectively. Selected hybridomas were injected i.p. into mice primed with 0.5 ml of pristane (Aldrich, Gillingham, Dorset, UK) 10 days earlier to produce ascitic fluid. The IgG fraction was then purified as described previously.[15] The specificity and cross-reactivity of the IgG fractions were determined in ELISA 3.

BALB/c mice, bred at the University of Glasgow Veterinary School, were initially immunized i.p. with 50 μg of isometamidium-PTG emulsified in CFA, and then boosted i.p. with 5 μg of the same immunogen emulsified in IFA, on days 25, 55, 83, 133, and 192. The mice were screened by testing sera using ELISA 4a, and those showing the highest indirect ELISA titers, and highest displacement by liquid-phase isometamidium, were selected for generation of MAbs. On day 203, each animal received 5 μg of isometamidium-PTG administered intravenously in 100 μl sterile normal saline. On the fourth day following this i.v. immunization, the mice were killed and the spleen cells fused with myeloma cells as described above. Screening of hybridomas was performed using ELISA 4a and selected hybridomas (5F7.B7 and 5F7.C9) were cloned by limiting dilution. The isotype of MAbs in supernatants was determined using the INNO-line immunoassay system (Innogenetics N.V., Belgium). Cross-reactivity was determined using ELISA 4b.

Enzyme-linked immunosorbent assays

ELISA 1—Binding to HSA and Isometamidium–HSA: Ninety-six-well plates (PolySorp, Nunc, Denmark) were incubated overnight at 4°C with either 30 μg of HSA/ml or 30 μg of isometamidium–HSA conjugate/ml (100 μl per well), dissolved in PBS, pH 7.4. The plates were then washed with PBS supplemented with 0.1% Tween 20 (Sigma, UK) (PBS-T) and incubated with hybridoma supernatants for 1 hr at 37°C followed by washing with PBS-T. The anti-HSA and anti-isometamidium antibodies were then detected by using a goat anti-mouse

horseradish peroxidase (HRP) conjugate[16] at a dilution of 1/3,000 (vol/vol) in goat-lymphocyte lysate[17]; 100 µl was added to each well and incubated at 37°C for 1 hr. After washing in PBS-T, the substrate, 1% (vol/vol) hydrogen peroxide, and the chromogen, 40 mM 2,2'-azinobis(3-ethyl-benzthiazoline 6-sulphonic acid) ammonium salt (ABTS, Serva, Germany) were added and the color was allowed to develop in the dark for 1 hr. The optical density was then determined in a Titertec reader (Flow Laboratories, UK) at 414 nm.

ELISA 2—A Competition Assay to Determine the Specificity of Antibodies for Drugs: Each hybridoma supernatant or ascites IgG fraction (at 1.0 mg protein/ml) was diluted 50 times in saline + 0.1% (vol/vol) Tween 20 (Sigma, UK) (ST). Thereafter aliquots (100 µl) of each supernatant/ascites dilution were added to equal volumes of ST containing isometamidium chloride, homidium chloride or diminazene aceturate at 2×10^{-5} M, 2×10^{-6} M, 2×10^{-7} M, 2×10^{-8} M, 2×10^{-9} M, or 2×10^{-10} M. Appropriate controls were also included. Each mixture was then incubated overnight at 4°C. Ninety-six-well plates (PolySorp, Nunc, Denmark) were coated with isometamidium–HSA conjugate as described for ELISA 1. After coating, plates were washed with ST, 100 µl of supernatant/ascites mixtures was added to wells, and the plates were incubated at 37°C for 1 hr. Subsequently, after washing with ST and PBS-T, binding of antibody to isometamidium–HSA conjugate was detected as described for ELISA 1.

ELISA 3—Competition Assay to Determine the Specificity of Antibodies for Drugs (More Sensitive Than ELISA 2): Ninety-six-well microtiter plates (Immulon 4, Dynatech Laboratories, UK) were coated overnight at 4°C with isometamidium–HSA conjugate diluted optimally (1/8,000) in carbonate/bicarbonate buffer, pH 9.2, and washed five times in a 1/5 dilution of PBS in distilled water containing 0.05% (vol/vol) Tween 20 (PBS-Ti), and blotted dry.

Isometamidium chloride, homidium bromide, and diminazene aceturate standards in normal bovine serum (Gibco, UK) were prepared by serial dilution. Standards, diluted 1/20 in optimal dilutions (1/16,000–1/32,000) of ascitic fluids in PBS-Ti, were added to ELISA plates which were incubated and shaken at 37°C for 1 hr, and washed five times as described previously.

Murine immunoglobulins were detected using a goat anti-mouse immunoglobulin–HRP conjugate[16] diluted 1/3,000 in PBS-Ti containing 5% normal goat serum. The conjugate was incubated at 37°C for 1 hr, and plates were washed five times as described previously.

ELISAs were developed using tetramethylbenzidine (TMB; Sigma, UK) in sodium acetate/citric acid buffer, pH 6.0, containing 1.3 mM hydrogen peroxide. The color reaction was stopped after 10 min using 2 M sulfuric acid, and absorbances read at 450 nm.

All well volumes were 100 µl.

ELISA 4a—Competition Assay to Determine the Specificity of Antibodies for Isometamidium: Microtiter plates (Immulon 4, Dynatech Laboratories, UK) were coated overnight at 4°C with isometamidium–CEA conjugate optimally diluted (1/2,000) in carbonate/bicarbonate buffer, pH 9.2, and washed five times as described above (ELISA 3).

Mouse sera or culture supernatants were diluted (sera, 1/50; supernatants, 1/2) in either PBS-Ti alone, or PBS-Ti containing 10^{-5} M isometamidium chloride, and added to ELISA plates, which were incubated and shaken at 37°C for 1 hr, and thereafter washed five times as described previously.

Immunoglobulins in murine sera were detected using rabbit anti-mouse immunoglobulin alkaline–phosphatase (AP) conjugate (Sigma, UK) diluted 1/800 in PBS-Ti. Immunoglobulins in culture supernatants were detected using rabbit anti-mouse immunoglobulin–HRP conjugate (Sigma, UK) diluted 1/1,600. Conjugates were incubated at 37°C for 1 hr, and washed five times as described previously.

Alkaline-phosphatase ELISAs were developed using *para*-nitrophenyl phosphate (Sigma, UK) in diethanolamine buffer, pH 9.8; color reactions were stopped after 30 min using 3 M NaOH, and absorbances were read at 405 nm. Horseradish peroxidase ELISAs were developed using TMB as described above (ELISA 3).

All well volumes were 100 µl.

ELISA 4b—Competition Assay to Determine the Specificity of Antibodies for Different Drugs: Microtiter plates were coated and washed as in ELISA 4a (see above). Culture supernatants were diluted optimally (B7, 1/2,000; C9, 1/1,000) in PBS-Ti containing a range of concentrations of isometamidium chloride, homidium bromide or diminazene aceturate, and added to microtiter plates that were incubated at 37°C for 1 hr, and washed five times as described previously.

Immunoglobulins retained on the solid-phase were detected using HRP and TMB as in ELISA 4a.

All well volumes were 100 µl.

Cross-reactivity

Cross-reactivity of anti-isometamidium MAbs with diminazene and homidium were calculated by comparing the concentration of each drug that resulted in 50% inhibition of the optical density obtained in the absence of competing drug. Cross-reactivity was expressed as the concentration of cross-reacting drug divided by the concentration of isometamidium chloride × 100%.

RESULTS

Production of MAbs to isometamidium

Spleen cells were collected from BALB/c mice that had been immunized with an isometamidium–HSA conjugate and used to generate hybridomas. After cloning of the hybridomas, the resultant MAbs were tested for their specificity by ELISA, using plates coated with isometamidium–HSA or HSA. Hybridomas secreting MAbs that recognized HSA were discarded. Isometamidium-specific hybridomas were recloned. Cross-reactivity of the resulting MAbs with homidium and diminazene, and their ability to bind free isometamidium, were then ascertained in a competition ELISA (see Materials and Methods, ELISA 2). MAbs that failed to bind to isometamidium over the range 10^{-5}–10^{-10} M were discarded. Similarly, MAbs exhibiting cross-reactivity with either homidium or diminazene at any concentration over this range were also discarded. Only three MAbs remained after this selection procedure; all were derived from the same original clone. After a further cloning, they were named IL-A 1001, IL-A 1002, and IL-A 1003. All three MAbs

were of the IgG_1 isotype. For each hybridoma, ascites was produced in mice. Using ELISA 1, the maximum dilution to give positive binding for each IgG fraction was shown to be 1/78,125. Using ELISA 2, none of the three MAbs demonstrated cross-reactivity with either homidium or diminazene over the concentration range of 10^{-5}–10^{-10} M. However, binding to free isometamidium was demonstrated at concentrations down to 10^{-7} M for IL-A 1001, and 10^{-8} M for IL-A 1002 and IL-A 1003.

Spleen cells collected from BALB/c mice immunized with isometamidium–PTG were successfully used to generate hybridomas. After cloning and testing of hybridomas using ELISA 4a, two clones were obtained that secreted anti-isometamidium MAbs. Both MAbs were IgG_1 and recognized both conjugated and unconjugated drug. These MAbs were named 5F7.B7 and 5F7.C9.

Specificity of MAbs

MAbs IL-A 1001, IL-A 1002, and IL-A 1003 were examined in ELISA 3, in which competition with the three anti-trypanosomal drugs, isometamidium, homidium, and diminazene, was determined using microtiter plates coated with the isometamidium–HSA conjugate. With each of the three MAbs, similar results were obtained (Fig. 2). On the basis of comparisons of the drug concentrations required to produce 50% competition, the cross-reactivity of the MAbs for diminazene was approximately 0.1% in all cases. The cross-reactivity for homidium was slightly less.

Using ELISA 3, MAb IL-A 1001 resulted in the most sensitive ELISA for isometamidium, with 50% competition occurring just below 10 ng/ml; because the inhibition curve was very steep, this was approximately the minimum detectable concentration. Finally, for all three MAbs, the gradients of the inhibition curves for homidium and diminazene were less than the respective inhibition profiles for isometamidium (see Fig. 2). This would indicate that the affinity of the MAbs for isometamidium was greater than that for either homidium or diminazene.

The cross-reactivities of MAbs 5F7.B7 and 5F7.C9 for homidium and diminazene were determined using ELISA 4b (Fig. 3). MAb 5F7.B7 exhibited approximately 100% cross-reactivity to homidium. Furthermore, MAb 5F7.C9 exhibited greater reactivity with homidium than with isometamidium; the amount of drug resulting in 50% reduction in optical density in ELISA 4b was 10 times less (wt/wt) for homidium than for isometamidium. Finally, cross-reactivity with diminazene was 0.1% or less for both MAbs.

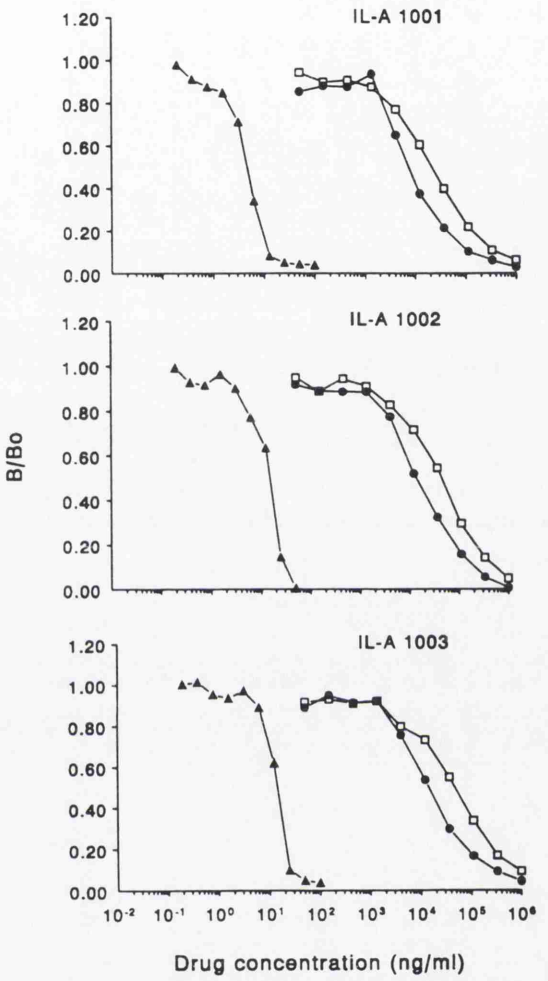


FIG. 2. Inhibition profiles for MAbs IL-A 1001, IL-A 1002, and IL-A 1003 in ELISA 3. (▲) Isometamidium chloride; (●) diminazene aceturate; (□) homidium chloride. B, Optical density of test sample; Bo, optical density of zero-drug control.

DISCUSSION

Two different immunization methods and hybridoma screening strategies were used to generate a panel of five MAbs that recognized the chemoprophylactic compound isometamidium. Because isometamidium is a hapten, mice were immunized with either an isometamidium–HSA conjugate or an isometamidium–PTG conjugate. MAbs were then selected that did not recognize the hapten carriers. The five MAbs were similar in their ability to detect isometamidium in competition ELISAs, and all exhibited slight (approximately 0.1%) cross-reactivity with diminazene. However, there were differences in their cross-reactivities with homidium. The MAbs produced using the isometamidium–HSA conjugate as both immunogen and as solid-phase antigen in hybridoma screening assays (IL-A 1001, IL-A 1002, and IL-A 1003) exhibited a relatively low level of cross-reactivity with homidium, similar to that obtained with diminazene (Fig. 2). (Because these three MAbs were derived from the same hybridoma clone, and have similar inhibition profiles, it is likely that they are the same antibodies.) In contrast, the MAbs obtained using an isometamidium–PTG conjugate as immunogen, and isometamidium–CEA in hybridoma screening assays (5F7.B7 and 5F7.C9) showed a higher level of cross-reactivity with homidium. In fact, one of the two MAbs (5F7.C9) exhibited greater reactivity with homidium than with isometamidium.

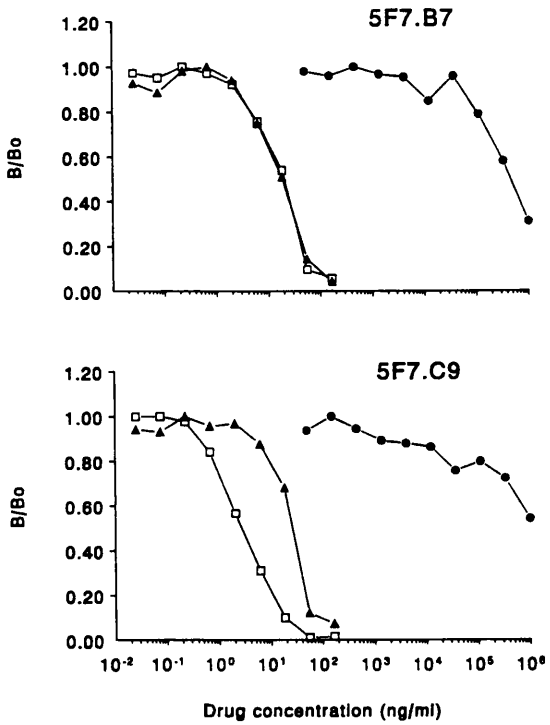


FIG. 3. Inhibition profiles of MAbs 5F7.B7 and 5F7.C9 in ELISA 4b. (▲) Isometamidium chloride; (●) diminazene aceturate; (□) homidium chloride. B, Optical density of test sample; Bo, optical density of zero-drug control.

Recent work has demonstrated that isometamidium is transported into *Trypanosoma congolense via* a protein carrier in the plasma membrane.[18] However, the mode of action, and therefore final localization of the molecule, currently remain unknown. The described MAbs may constitute a valuable reagent to determine the ultrastructural localization of isometamidium in trypanosomes, for example, by co-localization of the MAbs with colloidal gold.[19] Such information may thereby facilitate a definitive description of the compound's mode of action.

Expression of resistance to isometamidium by trypanosomes has been described in many parts of Africa.[20–27] However, the molecular mechanism(s) underlying this phenomenon are unknown. Because it appears unlikely that new trypanocides will be introduced in the near future, it is important that the efficacy of the currently used anti-trypanosomal drug is maintained for as long as possible. To this end, an ELISA has been developed for the detection and quantification of isometamidium in bovine sera.[9] Such an assay, when used in conjunction with a diagnostic test for the presence of trypanosome infection,[28] can be used as an indirect assay for drug resistance. The described isometamidium–ELISA[9] uses biotinylated anti-isometamidium IgG from sheep, for which isometamidium in a biological sample competes with a solid-phase immobilized isometamidium–protein conjugate. Quantification of drug concentrations is over a 100-fold range (minimum level of detection approximately 0.1 ng/ml), and isometamidium can be detected in cattle sera for at least 100 days following treatment.[9] This represents a significant increase in sensitivity compared to previously described spectrophotometric,[29] high-performance liquid chromatography[30,31] and radioimmunoassay[10] techniques that have minimum limits of detection in serum of 0.7 μg/ml, 20 ng/ml, 10 ng/ml, and 29 ng/ml, respectively.

Because monoclonal anti-isometamidium reagents might simplify standardization of the isometamidium-ELISA, the five MAbs described here were evaluated for their possible inclusion in such an assay; for four of the MAbs, 50% inhibition occurred at approximately 10 ng/ml and the quantitative range of the assay was reduced to a maximum of 10-fold. Thus, compared to the polyclonal sheep anti-isometamidium reagent, the affinity of the four MAbs for isometamidium was greatly reduced. In contrast, the fifth MAb, 5F7.C9, resulted in a displacement curve that approached the level of sensitivity required for use on samples from cattle; the concentration resulting in 50% competition in ELISA 4a was approximately 2.5 ng/ml. However, this MAb exhibited greater reactivity for homidium than isometamidium.

Finally, the cross-reactivity of MAbs for homidium and diminazene is an important criterion for selection of MAbs for an isometamidium–ELISA because both homidium and diminazene are used routinely in cattle as anti-trypanosomal compounds. Cross-reactivity for homidium at the level of 0.1% should not pose a problem in the interpretation of results from unknown field samples, because homidium concentrations in cattle sera are similar to those of isometamidium.[32] However, such cross-reactivity may constitute a potential problem within the first few days following treatment with diminazene since, at this time, the concentrations of diminazene in cattle sera are approximately 1,000 times those of isometamidium.[33,34]

ACKNOWLEDGMENTS

M.C.E., E.A.G., and P.H.H. gratefully acknowledge the financial support of O.D.A. (UK) under Holdback Project No. R4653H. RMB Animal Health Ltd., UK are thanked for the gift of Samorin® and Novidium® used in this study. Dr. J. Naessens is thanked for reviewing the manuscript. This is ILRAD publication No. 1244.

REFERENCES

1. Murray M and Gray AR: The current situation on animal trypanosomiasis in Africa. Prev Vet Med 1984;2:23–30.
2. Doyle JJ, Moloo SK, and Borowy NK: Development of improved control methods of animal trypanosomiasis: a review. Prev Vet Med 1984;2:43–52.
3. Kinabo LDB and Bogan JA: The pharmacology of isometamidium. J Vet Pharmacol Therapeut 1988;11:233–245.
4. Berg SS: Structure of isometamidium (M & B 4180a), 7-*m*-amidinophenyl-diazoamino-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride, the red isomer present in metamidium. Nature 1960;188:1106–1107.
5. Marcus SL, Kopelman R, Koll B, and Bacchi CJ: Effects of exogenous polyamine and trypanocides on the DNA polymerase activities from *Trypanosoma brucei brucei*, mouse thymus and murine leukemia virus. Mol Biochem Parasitol 1982;5:231–243.

6. Henderson JF, Battell ML, Zombor G, and Khoo MKY: Effects of ethidium and isometamidium on adenosine triphosphate catabolism and purine nucleotide synthesis in Ehrlich ascites tumor cells *in vitro*. Cancer Res 1977;37:3434–3441.
7. Bacchi CJ: Content, synthesis, and function of polyamines in trypanosomatids: relationship to chemotherapy. J Protozool 1981;28:20–27.
8. Shapiro TA and Englund PT: Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. Proc Natl Acad Sci USA 1990;87:950–954.
9. Eisler MC, Gault EA, Smith HV, Peregrine AS, and Holmes PH: Evaluation and improvement of an enzyme-linked immunosorbent assay (ELISA) for the detection of isometamidium in bovine serum. Therapeut Drug Monitor 1993;15:236–242.
10. Kinabo LDB and Bogan JA: Development of a radioimmunoassay for isometamidium. Vet Res Commun 1988;12:375–382.
11. Whitelaw DD, Gault EA, Holmes PH, Sutherland IA, Rowell FJ, and Phillips A: Development of an enzyme-linked immunosorbent assay for the detection and measurement of the trypanocidal drug isometamidium chloride in cattle. Res Vet Sci 1991;50:185–189.
12. Kearney JS, Radbruch A, Liesegang B, and Rajewsky K: A new mouse myeloma cell line which has lost immunoglobulin expression that permits the construction of antibody-secreting hybridomas. J Immunol 1979;123:1548–1550.
13. Oi VT and Herzenberg LA: Immunoglobulin-producing hybrid cell lines. In *Selected Methods in Cellular Immunology*. Mishell BB and Shiigi SM (Eds.), W.H. Freeman & Co., San Francisco, 1980, pp. 351–372.
14. Naessens J, Newson J, Bensaid A, Teale AJ, Magondu JG, and Black SJ: De novo expression of T cell markers on *Theileria parva*-transformed lymphoblasts in cattle. J Immunol 1985;135: 4183–4188.
15. Sileghem M, Saya R, Ellis JA, Flynn JN, Peel JE, and Williams DJL: Detection and neutralization of bovine tumor necrosis factor by a monoclonal antibody. Hybridoma 1992;11:617–627.
16. Katende JM, Goddeeris BM, Morzaria SP, Nkonge CG, and Musoke AJ: Identification of a *Theileria mutans*-specific antigen for use in an antibody and antigen detection ELISA. Parasite Immunol 1990;12:419–433.
17. Katende JM, Musoke AJ, Nantulya VM, and Goddeeris BM: A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for the diagnosis of bovine trypanosomiasis. Trop Med Parasitol 1987;38:41–44.
18. Zilberstein D, Wilkes J, Hirumi H, and Peregrine AS: Fluorescence analysis of the interaction of isometamidium with *Trypanosoma congolense*. Biochem J 1993;292:31–35.
19. Webster P and Grab DJ: Intracellular colocalization of variant surface glycoprotein and transferrin-gold in *Trypanosoma brucei*. J Cell Biol 1988;106:279–288.
20. Gray AR and Roberts CJ: The cyclical transmission of strains of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs. Parasitology 1971;63:67–89.
21. Lewis AR and Thomson JW: Observations on an isometamidium resistant strain of *Trypanosoma congolense* in Rhodesia. Rhodesian Vet J 1974;4:62–67.
22. Gitatha SK: *T. congolense* (Shimba hills) resistant to various trypanocidal drugs. In *International Scientific Council for Trypanosomiasis Research and Control, 16th meeting, Yaounde, Cameroon*. OAU/STRC publication No. 111, Nairobi, 1979, pp. 257–263.
23. Kupper W and Wolters M: Observations on drug resistance of *Trypanosoma (Nannomonas) congolense* and *Trypanosoma (Duttonella) vivax* in cattle at a feedlot in the northern Ivory Coast. Tropenmed Parasitol 1983;34:203–205.
24. Pinder M and Authie E: The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. Acta Trop 1984;41: 247–252.
25. Schonefeld AR, Rottcher D, and Moloo SK: The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. Trop Med Parasitol 1987;38:177–180.
26. Mohamed-Ahmed MM, Rahman AH, and Abdel Karim EI: Multiple drug-resistant bovine trypanosomes in South Darfur Province, Sudan. Trop Anim Health Prod 1992;24:179–181.
27. Codjia V, Mulatu W, Majiwa PAO, Leak SGA, Rowlands GJ, Authie E, d'Ieteren GDM, and Peregrine AS: Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. Acta Trop 1993; 53:151–163.
28. Luckins AG: Methods for diagnosis of trypanosomiasis in livestock. World Anim Rev 1992;70/71:15–20.
29. Philips FS, Sternberg SS, Cronin AP, Sodergren JE, and Vidal PM: Physiologic disposition and intracellular localization of isometamidium. Cancer Res 1967;27:333–349.
30. Perschke H and Vollner L: Determination of the trypanocidal drugs homidium, isometamidium and quinapyramine in bovine serum or plasma using HPLC. Acta Trop 1985;42:209–216.
31. Kinabo LDB and Bogan JA: Solid-phase extraction and ion-pair reversed-phase HPLC of isometamidium in bovine serum and tissues. Acta Trop 1988;45:165–170.
32. Gilbert RJ and Newton BA: Ethidium bromide: Pharmacokinetics and efficacy against trypanosome infections in rabbits and calves. Parasitology 1982;85:127–148.
33. Kellner H-M, Eckert GH, and Voltz MH: Studies in cattle on the disposition of the antitrypanosomal drug diminazene diaceturate (®Berenil). Trop Med Parasitol 1985;36:199–204.
34. Aliu YO, Mamman M, and Peregrine AS: Pharmacokinetics of diminazene in female Boran (*Bos indicus*) cattle. J Vet Pharmacol Therapeut 1993;16:291–300.

Address reprint requests to:
Dr. Andrew S. Peregrine
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

Received for publication: 1/17/94
Accepted: 2/22/94

Section 5.4

Wells, C., Wilkes, J. and Peregrine, A.S. (1995) Use of fluorescence, immunoelectron-microscopy and autoradiography techniques to localise Samorin® in *Trypanosoma congolense*. In *Proceedings of the 23rd Meeting of the International Scientific Council for Trypanosomiasis Research and Control*, 11th-15th September, 1995, Banjul, The Gambia. OAU/STRC Publication, Abstract P527.

Introduction

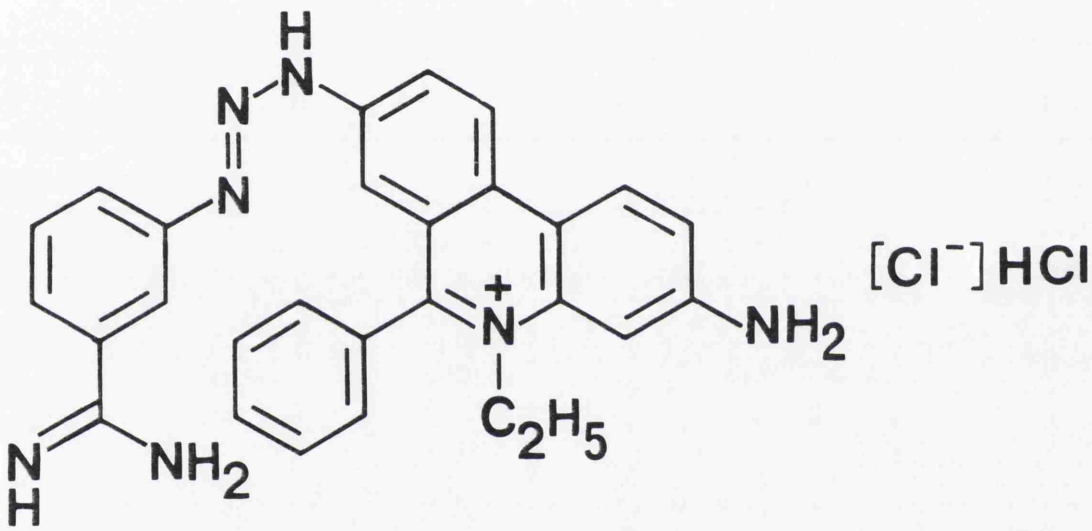
Samorin®/Trypamidium® is a phenanthridine-aromatic amidine (see Fig. 1) that is commonly used in cattle, sheep and goats as a prophylactic agent against trypanosomiasis. Although the compound has been used for over 30 years, very little is known about it's mode of action. Various studies have demonstrated that the molecule interferes with DNA polymerases (Marcus *et al.*, 1982), purine nucleotide synthesis (Henderson *et al.*, 1977), polyamine metabolism (Bacchi, 1981), and mitochondrial type II topoisomerase (Shapiro and Englund, 1990). However, the contribution of such activities to the compound's anti-trypanosomal activity *in vivo* is not known. Recent work has demonstrated that the major active component of Samorin, isometamidium, is transported into *Trypanosoma congolense* via a protein carrier in the plasma membrane (Zilberstein *et al.*, 1993). However, the subsequent target(s) within the cell with which the drug interacts have not been fully characterised.

In this work we demonstrate targeting of Samorin to organelles within *T. congolense* during early stages of incubation with the drug, using three different techniques. Such information will lead to a better understanding of the compound's mode of trypanocidal action *in vitro* and *in vivo*.

Materials

Trypanosoma congolense IL 1180 is a doubly cloned derivative of an isolate collected from a lion in the Serengeti National Park, Tanzania (Nantulya *et al.*, 1984). The clone is highly sensitive to Samorin, having a 50% curative dose value (dose of drug required to cure 50% of infected animals) in mice of 18 μg/kg body weight (Peregrine *et al.*, 1991). Furthermore, in cattle the trypanosome population is sensitive to intramuscular treatment with the drug at a dose of 1.0 μg/kg body weight (Sones *et al.*, 1988). In the work described here, all experiments were carried out with bloodstream-form parasites.

Fig. 1 Molecular structure of isometamidium chloride


HN
N
N
NH2
N
+
C2H5
NH2
N
H
[Cl⁻]HCl

Monoclonal antibody (Mab) SA3/141.8.6 is a mouse IgG_1 isotype specific for isometamidium (Peregrine *et al.*, 1994).

Methods

Fluorescence microscopy. The auto-fluorescence property of isometamidium when taken up by eukaryotic cells (Philips *et al.*, 1967) was utilised in this study. Trypanosomes were incubated at 37°C in culture medium containing 1 μg/ml Samorin for 2, 5, 15 and 30 minutes. Cells were then pelleted rapidly, washed in phosphate-buffered saline (PBS) and cytospun onto glass microscope slides for examination by U.V. and differential interference contrast microscopy (DIC).

Immunoelectron microscopy (immuno EM). Trypanosomes were incubated with Samorin, as above, for 30 minutes. The cells were then washed thoroughly with three changes of PBS, fixed in 0.5% glutaraldehyde, 4% paraformaldehyde, 0.2% picric acid, 0.5 mM calcium chloride in 0.1 M phosphate buffer, pH 7.2, for 1 hour, and processed into Lowicryl K4M following the method of Burleigh *et al.* (1993). Sections (60 nm) were then mounted on formvar-coated grids, incubated with Mab SA3/141.8.6, then labelled with a goat anti-mouse gold probe.

Autoradiography. Trypanosomes were incubated at 37°C in culture medium containing 1.3 μg/ml ^{3}H-labelled Samorin (a gift from RMB Animal Health Limited, U.K.) for 15 seconds, 1, 2, 5 and 15 minutes. The uptake was stopped by centrifuging the cells through a layer of silicone oil into fixative containing 2.5% glutaraldehyde, 0.5 mM calcium chloride, 0.25 M sucrose in 0.1 M cacodylate buffer, pH 7.4; free Samorin remained in the supernatant layer above the oil. Cells were then processed into epoxy resin. Sections (60 nm) were cut, mounted on formvar-coated nickel grids and coated with Ilford L4 emulsion. After exposure for 6 weeks in a light-free dessicator the autoradiographs were developed and viewed.

Results

After 2 minutes incubation, fluorescence could be detected throughout the trypanosomes with an area of brighter fluorescence in the region of the flagellar pocket (Fig. 2A). At 5, 15 and 30 minutes the fluorescence in this region increased in intensity, with an area of fluorescence brighter than the background extending through the posterior part of the trypanosome (Figs. 2B, C & D).

Immuno EM revealed labelling throughout the trypanosomes with an area of more intense labelling in the kinetoplast and the mitochondrion. The endocytic organelles were not labelled (Fig. 3).

Autoradiography gave a signal in the kinetoplast after 15 seconds (Fig. 4). At 2 and 5 minutes the signal was strong in the kinetoplast (Figs. 5 and 6, respectively). At 15 minutes (the longest period one could maintain viable trypanosomes in the toxic environment) the signal appeared diffusely distributed throughout the mitochondrion, cytosol and nucleus.

Discussion

Biochemical data obtained by Zilberstein *et al.* (1993) indicate that uptake of Samorin by *T. congolense* bloodstream forms *in vitro* is a rapid, temperature-dependent, saturable event. Using the auto-fluorescent property of the drug we have observed a rapid, focal accumulation of fluorescence in an area adjacent to the flagellar pocket. Immuno EM indicated that the drug was concentrated in the kinetoplast. This latter method, however, proved unsuitable for studies of incubation times shorter than 30 minutes since incubations of at least 30 minutes were required to allow sufficient amounts of drug to be taken up to obtain good signals. Unfortunately, by this time the cells were probably being overloaded with drug. As a result, we may have been inducing toxic damage to the cellular organelles, resulting in passage of the drug across membranes that are normally impermeable. Furthermore, efflux mechanisms may have been activated, which may not be the

Fig. 2 Fluorescence within cells incubated for 2 (A), 5 (B), 15 (C) and 30 (D) minutes. Differential interference contrast images of the same trypanosomes are below with arrow indicating the flagellar pocket.

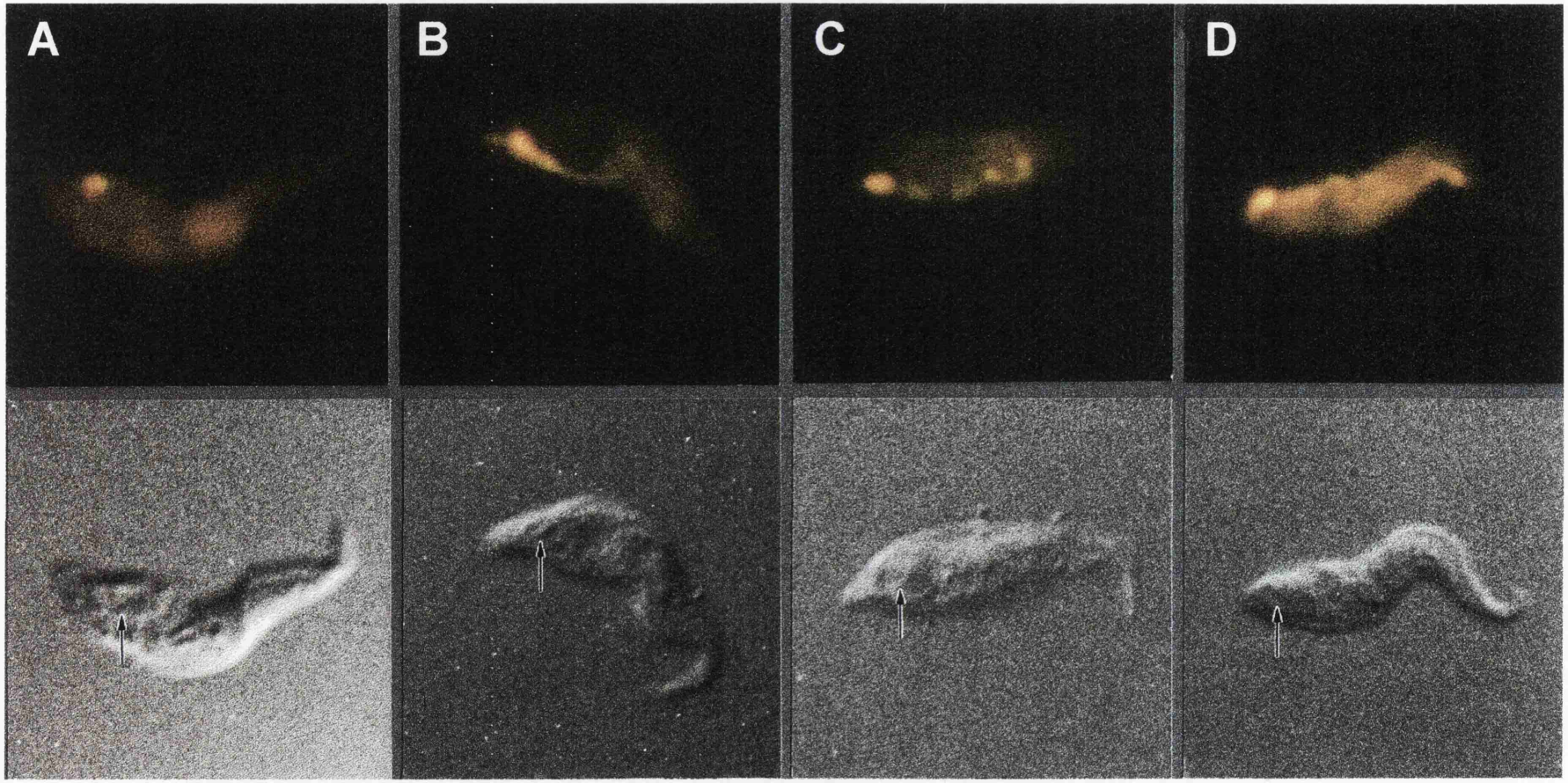

A
B
C
D

Fig. 3 Immuno EM using Mab SA3/141.8.6 shows Samorin diffusely distributed within the cytosol and concentrated in the kinetoplast (K). Note the flagellar pocket (FP) and endocytic organelles (E) are almost free of label. Bar = 0.5 μm.

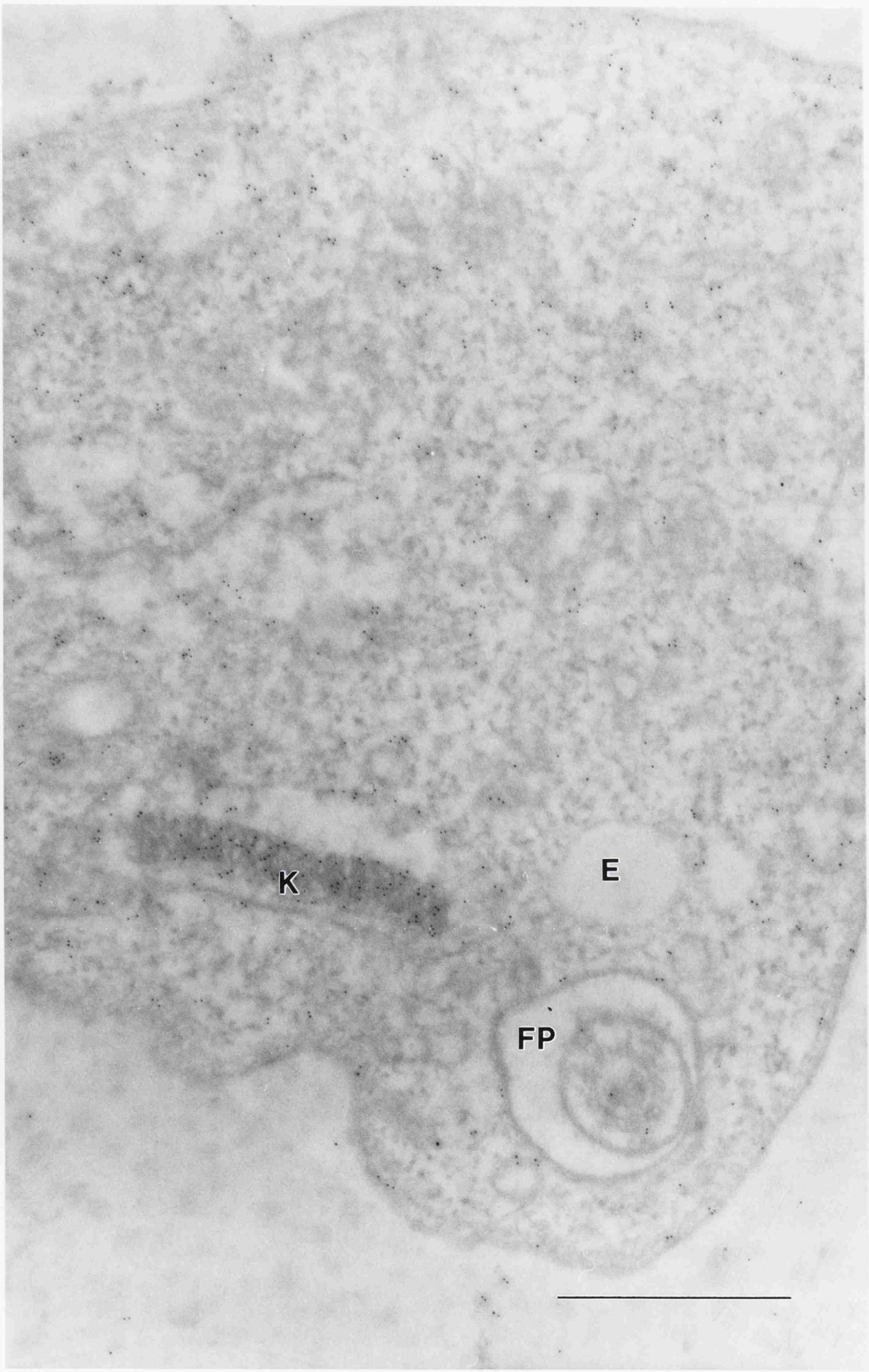

K
E
FP

Fig. 4 Autoradiograph of a trypanosome incubated for 15 seconds with tritiated Samorin. Signal is detected only in the kinetoplast. Bar = 0.5 μm.

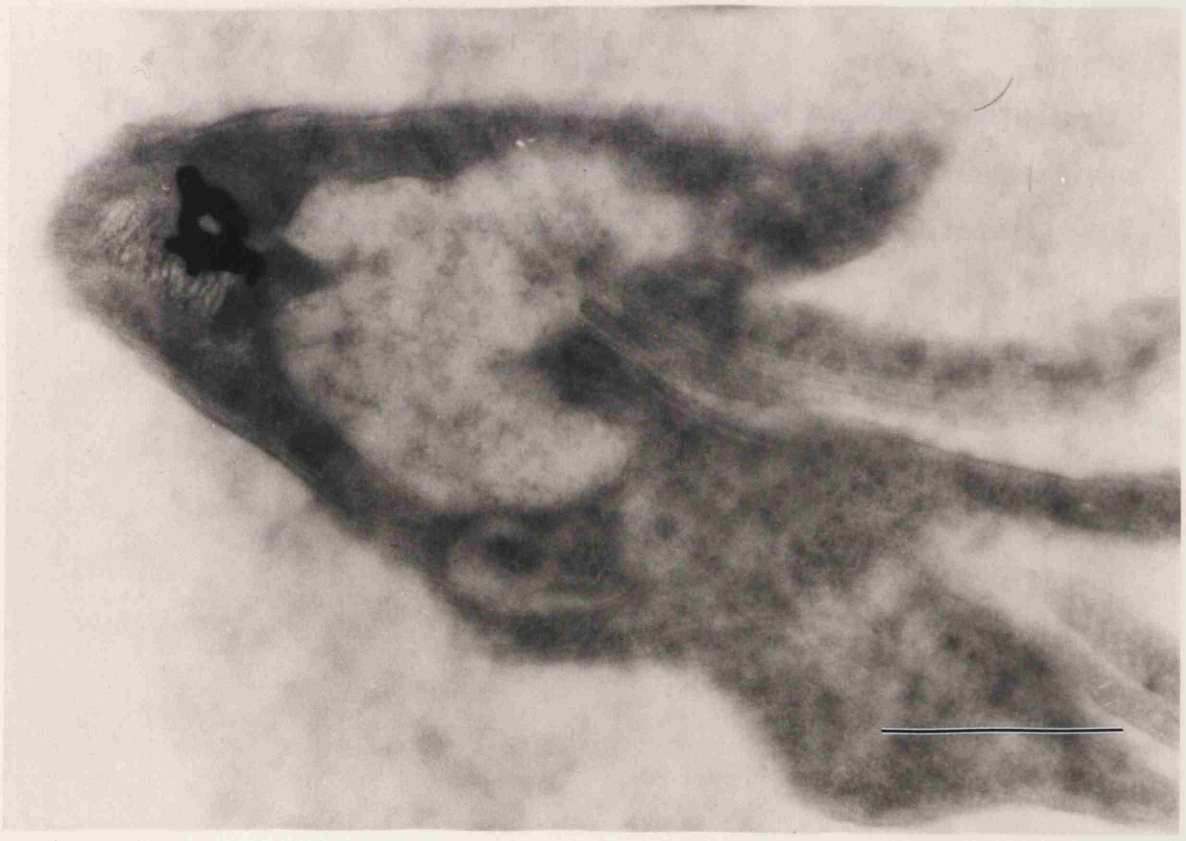


Fig. 5 Autoradiograph of trypanosomes incubated for 2 minutes with tritiated Samorin. Signal is in the kinetoplast. The extracellular signal may originate from a small piece of damaged trypanosome to which the drug binds strongly.
Bar = 2.0 μm.

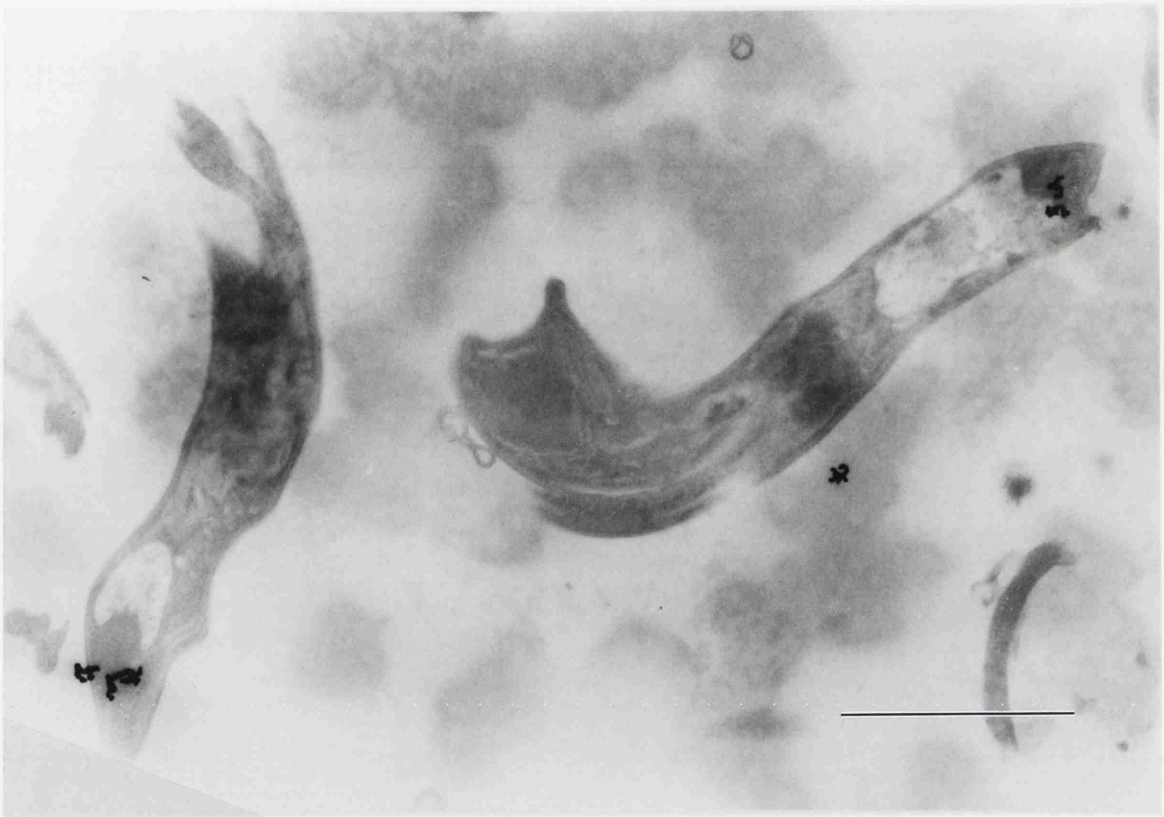
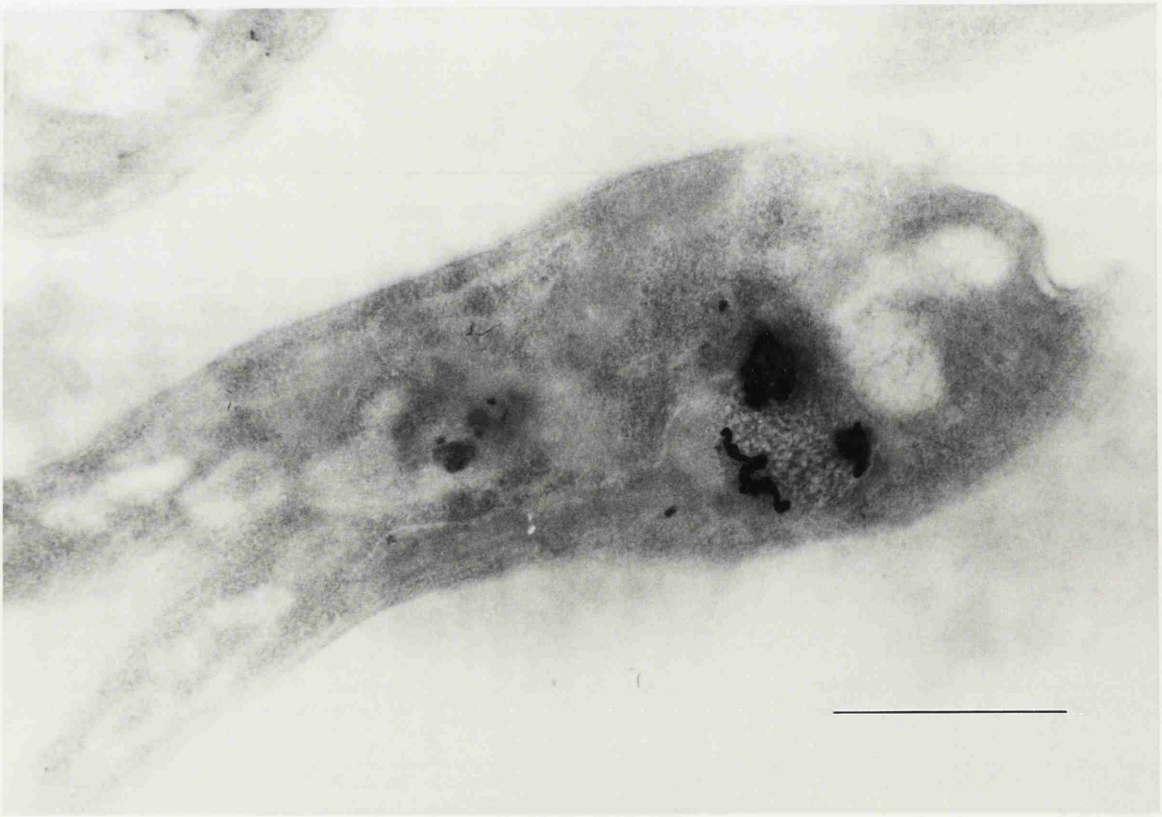


Fig. 6 Autoradiograph of trypanosome incubated for 5 minutes with tritiated Samorin. The signal is still confined to the kinetoplast and is stronger than the earlier time points. Bar = 0.5 μm.



case with physiological concentrations of the drug. Autoradiography proved the solution to this problem; by carefully adjusting development times and conditions we were able to show uptake and localisation of Samorin as early as 15 seconds after addition of the drug to the incubation mixture. An increase in the signal within the kinetoplast occurred over the next few minutes, after which the drug appeared to also accumulate within the mitochondrion. In conclusion, these data indicate that the primary site of Samorin's accumulation is the kinetoplast. This is consistent with Samorin's mode of action being associated with inhibition of mitochondrial type II topoisomerase (Shapiro and Englund, 1990).

In the future the techniques described here will be used to fully characterise uptake and possible efflux of Samorin in *T. congolense*. The methods will also be used to compare Samorin-resistant and Samorin-sensitive clones of *T. congolense* to investigate possible differences in drug uptake and localisation.

References

Bacchi, C.J. (1981) Content, synthesis, and function of polyamines in trypanosomatids: relationship to chemotherapy. *Journal of Protozoology* 28, 20-27.

Burleigh, B.A., Wells, C.W., Clarke, M.W. and Gardiner, P.R. (1993) An integral membrane glycoprotein associated with an endocytic compartment of *Trypanosoma vivax*: identification and partial characterization. *Journal of Cell Biology* 120, 339-352.

Henderson, J.F., Battell, M.L., Zombor, G. and Khoo, M.K.Y. (1977) Effects of ethidium and isometamidium on adenosine triphosphate catabolism and purine nucleotide synthesis in Ehrlich ascites tumor cells *in vitro*. *Cancer Research* 37, 3434-3441.

Marcus, S.L., Kopelman, R., Koll, B. and Bacchi, C.J. (1982) Effects of exogenous polyamine and trypanocides on the DNA polymerase activities from *Trypanosoma brucei brucei*, mouse thymus and murine leukemia virus. *Molecular and Biochemical Parasitology* 5, 231-243.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R. and Moloo, S.K. (1984) Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe passaged infections. *Infection and Immunity* 43, 735-738.

Peregrine, A.S., Eisler, M.C., Katende, J., Flynn, J.N., Gault, E.A., Kinabo, L.D.B. and Holmes, P.H. (1994) Generation of monoclonal antibodies to the anti-trypanosomal drug isometamidium. *Hybridoma* 13, 289-294.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991) Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* 102, 93-100.

Philips, F.S., Sternberg, S.S., Cronin, A.P., Sodergren, J.E. and Vidal, P.M. (1967) Physiologic disposition and intracellular localization of isometamidium. *Cancer Research* 27, 333-349.

Shapiro, T.A. and Englund, P.T. (1990) Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. *Proceedings of the National Academy of Sciences of the USA* 87, 950-954.

Sones, K.R., Njogu, A.R. and Holmes, P.H. (1988) Assessment of sensitivity of *Trypanosoma congolense* to isometamidium chloride: a comparison of tests using cattle and mice. *Acta Tropica* 45, 153-164.

Zilberstein, D., Wilkes, J., Hirumi, H. and Peregrine, A.S. (1993) Fluorescence analysis of the interaction of isometamidium with *Trypanosoma congolense*. *Biochemical Journal* 292, 31-35.

Section 5.5

- Discussion -

Section 5.1 describes a series of experiments that were carried out to characterise the genetic basis of resistance to isometamidium. In initial work, nine clones were derived from an isometamidium-resistant stock of *T. congolense* that originated from Burkina Faso (Pinder and Authie, 1984) and characterised in mice for their sensitivity to isometamidium. Despite all nine clones expressing the same electrophoretic variants of seven enzymes, and therefore belonging to the same zymodeme, significant variation in resistance to isometamidium was observed amongst the clones. This is consistent with the work of others (Mutugi et al., 1995) and indicated the importance of using cloned populations in subsequent studies. In further work, nine clones were derived, without drug selection, from the clone amongst the aforementioned nine clones that had the highest level of resistance to isometamidium. When the isometamidium sensitivities of the subclones were characterised in mice, the data indicated that seven of the subclones expressed significantly lower levels of resistance to isometamidium than the parental clone. Furthermore, there was significant variation in resistance to isometamidium amongst the subclones. Thus, expression of resistance to isometamidium appears to be unstable in mice, and at least part of the genetic component(s) for this resistance phenotype appears likely to be unstable. This conclusion is consistent with the work of Nyeko et al. (1989), but inconsistent with the work of others (Gray and Roberts, 1971; Brown et al., 1987; Kaminsky and Zweygarth, 1989). Differences in results among these workers may have been associated with the qualitative manner in which some assessed the level of isometamidium resistance. Similarly, use of uncloned trypanosome populations by some workers could have compromised conclusions about the stability of drug resistance.

At the present time the genetic basis of resistance to isometamidium is unclear. If the trait is monogenic in nature it would indicate that the observed instability of resistance to isometamidium is most likely associated with some form of gene amplification. Alternatively, if it is polygenic, any combination of gene amplification, gene transfer, gene deletion, point mutations, loss of *cis*-acting regulatory elements, loss or dysfunction of *trans*-acting factors, transcriptional activation, or hypo- or hyper-methylation, could play a role (Hayes and Wolf, 1990).

Work is currently underway at the International Livestock Research Institute, Kenya, to determine the genetic basis of resistance to isometamidium in *T. congolense* using representation difference analysis.

In Section 5.2 the autofluorescent property of isometamidium was used to characterise the interaction of the compound with bloodstream forms of a drug-sensitive *T. congolense*. Earlier workers have used this property to evaluate the interaction of isometamidium with trypanosomes and mammalian cells in a qualitative manner (Philips et al., 1967; Sutherland et al., 1991). In Section 5.2 quantitative fluorescence measurements were used for the same purpose and indicated that the observed alteration in isometamidium's fluorescence, that occurs upon incubation with bloodstream forms of *T. congolense*, is due to interaction of the compound with an intracellular component(s). Furthermore, the drug was shown to be transported across the plasma membrane via a protein carrier, consistent with the conclusions of Sutherland et al. (1992a) and Wilkes et al. (1995) from studies on uptake of ^{14}C-isometamidium in *T. congolense*. In further work, Wilkes et al. (1995) have shown that the aforementioned changes in fluorescence are associated with constraints on isometamidium's free rotation in solution. In addition, interaction of the molecule with purified DNA produced fluorescence changes that are similar to those observed with intact trypanosomes, and addition of DNase 1 to trypanosomal lysates resulted in greater than 85% disruption of the binding sites. As a result, the changes in isometamidium's fluorescence that are observed upon incubation with trypanosomes appear to be primarily associated with interaction of the compound with trypanosomal DNA (Wilkes et al., 1995).

Sutherland et al. (1991) used fluorescence microscopy to characterise the intracellular localisation of isometamidium in bloodstream forms of *T. congolense.* However, because of the low level of resolution of the technique it was not possible to identify the precise organellar localisation of the molecule. In an attempt to develop a more sensitive drug-localisation technique, Section 5.3 describes the production of monoclonal antibodies (Mabs) to isometamidium that could be used in electron-microscopy immunocytochemistry, in combination with colloidal gold

(Section 5.4), to identify isometamidium's organellar localisation. In brief, the Mabs were produced by immunising mice with either an isometamidium-human serum albumin conjugate or an isometamidium-porcine thyroglobulin conjugate. The resultant Mabs were then selected on the basis that they recognised conjugated and unconjugated isometamidium, but lacked cross-reactivity with the carrier molecule. When one of the selected Mabs was used in immunoelectron microscopy to examine drug-sensitive bloodstream forms of *T. congolense* that had been incubated at 37°C in isometamidium for 30 minutes, when intact and viable, diffuse labelling was observed throughout the cytosol and nucleus of the cells (Section 5.4). In contrast, intense labelling was observed in the kinetoplast and mitochondrion, while the endocytic organelles appeared unlabelled. Unfortunately, however, drug localisation could not be identified with incubation periods shorter than 30 minutes. Thus, while the data suggested that isometamidium's primary site of localisation was the kinetoplast, the fact that drug could not be detected in trypanosomes at incubation periods less than 30 minutes, in contrast to fluorescence and radiolabelled drug-uptake studies (Sutherland et al., 1991, 1992a; Wilkes et al., 1995; Section 5.2), indicated that a more sensitive technique was required to characterise drug uptake at earlier time points. Furthermore, since there was diffuse labelling of the cytosol and nucleus at 30 minutes incubation, this suggested that the cells were overloaded with drug at this time point. As a result, the observed drug localisation could have been an artefact.

In order to address these concerns, an electron-microscopy autoradiography methodology was developed in which isometamidium could be detected in bloodstream-forms of *T. congolense* after as little as 15 seconds incubation at 37°C in medium containing tritiated isometamidium (Section 5.4). After 15 seconds a signal could only be detected within the kinetoplast. Similarly, after 2 and 5 minutes incubation the signal was still clearly localised to the kinetoplast; longer incubations gave more diffuse labelling throughout the mitochondrion, cytosol and nucleus. Thus, in a consistent manner to recent biochemical data by other authors, in which cleavage of kDNA-topoisomerase complexes was suggested to be the main action of isometamidium (Shapiro and Englund, 1990; Shapiro, 1993), the drug appears to

exert it's activity primarily at the level of the kinetoplast. Finally, since no drug was found associated with endocytic vesicles, endocytosis via the flagellar pocket does not appear to play a significant role in uptake of isometamidium by bloodstream forms of *T. congolense.*

In work carried out to determine the molecular basis of resistance to isometamidium in bloodstream-form trypanosomes, decreased levels of drug accumulation have been observed in drug-resistant populations of *T. congolense* (Sutherland et al., 1992a; Section 2.4). Furthermore, but not confirmed with biochemical data, mathematical modelling led Sutherland et al. (1992b) to conclude that reduced drug accumulation in drug-resistant *T. congolense* is not a result of reduced uptake of isometamidium. Thus, a drug-efflux mechanism associated with a P-glycoprotein plasma-membrane transporter, as reported in neoplastic cells (Gottesman and Pastan, 1988a), malaria parasites (Krogstad et al., 1987; Martin et al., 1987), *Trypanosoma cruzi* (Neal et al., 1989) and *Leishmania* spp. (Neal et al., 1989; Dey et al., 1994), could be responsible for the resistance. However, while blockers of this transporter have been shown to increase the accumulation of isometamidium in drug-resistant *T. congolense* (Sutherland et al., 1992a), the same compounds have been shown to have no effect on the level of isometamidium resistance of *T. b. brucei* populations *in vitro* (Kaminsky and Zweygarth, 1991). Furthermore, Fairlamb et al. (1992) have inferred that the strongly ionic nature of isometamidium is not consistent with the hydrophobic substrate-specificity of multidrug (P-glycoprotein) transporters (Gottesman and Pastan, 1988b; Juranka et al., 1989). Detailed biochemical analyses are therefore required to determine the role, if any, that P-glycoprotein transporters play in resistance to isometamidium in pathogenic African trypanosomes. Furthermore, definitive biochemical evidence for an active isometamidium efflux process is still lacking. Finally, in light of recent work that has implicated the role of nucleoside transporters in resistance to melaminophenyl arsenical drugs (Carter and Fairlamb, 1993), diminazene and pentamidine (Carter et al., 1995), and melarsomine (Ross and Barns, 1996), the role of these transporters in resistance to isometamidium also requires clarification.

References:

Brown, H.C., Ross, C.A., Holmes, P.H., Luckins, A.G. and Taylor, A.M. (1987) Adaptation of *Trypanosoma congolense* stocks to *in vitro* culture does not change their sensitivity to isometamidium. *Acta Tropica* 44, 373-374.

Carter, N.S., Berger, B.J. and Fairlamb, A.H. (1995) Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. *Journal of Biological Chemistry* 270, 28153-28157.

Carter, N.S. and Fairlamb, A.H. (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature* 361, 173-176.

Dey, S., Papadopoulou, B., Haimeur, A., Roy, G., Grondin, K., Dou, D., Rosen, B.P. and Ouellette, M. (1994) High level arsenite resistance in *Leishmania tarentolae* is mediated by an active extrusion system. *Molecular and Biochemical Parasitology* 67, 49-57.

Fairlamb, A.H., Carter, N.S., Cunningham, M. and Smith, K. (1992) Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Molecular and Biochemical Parasitology* 53, 213-222.

Gottesman, M.M. and Pastan, I. (1988a) Resistance to multiple chemotherapeutic agents in human cancer cells. *Trends in Pharmacological Science* 9, 54-58.

Gottesman, M.M. and Pastan, I. (1988b) The multidrug transporter, a double-edged sword. *Journal of Biological Chemistry* 263, 12163-12166.

Gray, A.R. and Roberts, C.J. (1971) The cyclical transmission of strains of *Trypanosoma congolense* and *Trypanosoma vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* 63, 67-89.

Hayes, J.D. and Wolf, C.R. (1990) Molecular mechanisms of drug resistance. *Biochemical Journal* 272, 281-295.

Juranka, P.F., Zastawny, R.L. and Ling, V. (1989) P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. *FASEB Journal* 3, 2583-2592.

Kaminsky, R. and Zweygarth, E. (1989) Effect of *in vitro* cultivation on the stability of resistance of *Trypanosoma brucei brucei* to diminazene, isometamidium, quinapyramine, and Mel B. *Journal of Parasitology* 75, 42-45.

Kaminsky, R. and Zweygarth, E. (1991) The effect of verapamil alone and in combination with trypanocides on multidrug-resistant *Trypanosoma brucei brucei*. *Acta Tropica* 49, 215-225.

Krogstad, D.J., Gluzman, I.Y., Kyle, D.E., Oduola, A.M.J., Martin, S.K., Milhous, W.K. and Schlesinger, P.H. (1987) Efflux of chloroquine from *Plasmodium falciparum*: mechanism of action of chloroquine resistance. *Science* 238, 1283-1285.

Martin, S.K., Oduola, A.M.J. and Milhous, W.K. (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science* 235, 899-901.

Mutugi, M.W., Boid, R. and Luckins, A.G. (1995) Differences in cloning and sub-cloning success rates in four stocks of *Trypanosoma evansi* and variation in suramin resistance of the clones. *Veterinary Parasitology* 60, 213-220.

Neal, R.A., van Bueren, J., McCoy, N.G. and Iwobi, M. (1989) Reversal of drug resistance in *Trypanosoma cruzi* and *Leishmania donovani* by verapamil. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 83, 197-198.

Nyeko, J.H.P., Golder, T.K., Otieno, L.H. and Ssenyonga, G.S.Z. (1989) *Trypanosoma congolense*: drug resistance during cyclical transmissions in tsetse flies and syringe passage in mice. *Experimental Parasitology* 69, 357-362.

Philips, F.S., Sternberg, S.S., Cronin, A.P., Sodergren, J.E. and Vidal, P.M. (1967) Physiologic disposition and intracellular localization of isometamidium. *Cancer Research* 27, 333-349.

Pinder, M. and Authie, E. (1984) The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta Tropica* 41, 247-252.

Ross, C.A. and Barns, A.M. (1996) Alteration to one of three adenosine transporters is associated with resistance to Cymelarsan in *Trypanosoma evansi*. *Parasitology Research* 82, 183-188.

Shapiro, T.A. (1993) Inhibition of topoisomerases in African trypanosomes. *Acta Tropica* 54, 251-260.

Shapiro, T.A. and Englund, P.T. (1990) Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. *Proceedings of the National Academy of Sciences of the USA* 87, 950-954.

Sutherland, I.A., Mounsey, A. and Holmes, P.H. (1992a) Transport of isometamidium (Samorin) by drug-resistant and drug-sensitive *Trypanosoma congolense*. *Parasitology* 104, 461-467.

Sutherland, I.A., Mounsey, A., Eisler, M. and Holmes, P.H. (1992b) Kinetic modelling of isometamidium chloride (Samorin) uptake by *Trypanosoma congolense*. *Parasitology* 105, 91-95.

Sutherland, I.A., Peregrine, A.S., Lonsdale-Eccles, J.D. and Holmes, P.H. (1991) Reduced accumulation of isometamidium by drug-resistant *Trypanosoma congolense*. *Parasitology* 103, 245-251.

Wilkes, J.M., Peregrine, A.S. and Zilberstein, D. (1995) The accumulation and compartmentalization of isometamidium chloride in *Trypanosoma congolense*, monitored by its intrinsic fluorescence. *Biochemical Journal* 312, 319-329.

Chapter 6

General discussion and conclusions

Trypanosomiasis and the tick-borne diseases theileriosis, babesiosis, anaplasmosis and cowdriosis are the major disease constraints to livestock production in much of sub-Saharan Africa (Winrock, 1992). Furthermore, they are also significant constraints to livestock production in many other areas of the developing world. Comparison of the compounds currently available for control of tsetse-transmitted trypanosomiases and tick-borne diseases indicates that there are two major differences in the efficacy of the compounds available for these two groups of diseases. Firstly, drug resistance is a major problem associated with the compounds available for trypanosomiasis. In contrast, drug resistance has rarely, if ever, been described in definitive hosts for the compounds available for treatment or prophylaxis of theileriosis, babesiosis, anaplasmosis and cowdriosis (Section 1.1). Secondly, problems with drug resistance appear to be exactly the opposite for the compounds currently available for control of the vectors of these diseases; while resistance to all chemical classes of acaricides has been described in all important tick species, singly or combined, in many field situations (Mathewson, 1984; Nolan, 1990), no resistance to any insecticide has been reported in *Glossina* spp. (Jordan, 1986).

Drug resistance has been reported for all three anti-trypanosomal compounds currently recommended for use in cattle, sheep and goats (i.e., isometamidium, homidium and diminazene) at many sites within Africa, and to a lesser extent in other parts of the world (Section 1.1). This resistance would largely appear to be associated with underdosing and the lengthy period that all three compounds have been used (greater than 35 years) (Stephen, 1986). However, while the lack of resistance to compounds such as parvaquone, buparvaquone and halofuginone may be associated with the relatively short period that these compounds have been in use, it would appear that duration of drug use and underdosing are not the only factors responsible for failure of development of drug resistance in *Theileria* spp., *Babesia* spp., *Anaplasma* sp. or *Cowdria* sp., since imidocarb and the tetracyclines have been in use for almost as long as the anti-trypanosomal compounds yet resistance to these drugs has not been described in these pathogens. In general, it would appear that differences in drug pressure and disease pathogenesis are the major factors

responsible for differences in the incidence of resistance to anti-trypanosomal compounds and resistance to the drugs used to treat tick-borne diseases. However, the mutagenic activity of some anti-trypanosomal compounds (Goldring et al., 1970; Hixon et al., 1975; MacGregor and Johnson, 1977) may also be responsible.

Over the last 20 years there appears to have been an increase in the incidence and prevalence of drug-resistant trypanosomes (Section 1.1). However, the true incidence and prevalence of such infections is unclear because of the lack of direct techniques to rapidly quantify the level of resistance of large numbers of trypanosome populations (Holmes, 1991; Joshua et al., 1995). Furthermore, the factors that determine the epidemiology of drug-resistant infections have not been clearly defined. Because of this, the research described in Chapters 2, 3, 4 and 5 of this thesis was carried out to characterise aspects of drug action and drug resistance in *T. congolense*.

In initial work, a model was developed to analyse longitudinal parasitological data from cattle in the field for evidence of drug-resistant infections. When applied to cattle in the Ghibe valley, Ethiopia, the model indicated that the prevalence of diminazene-resistant *T. congolense* infections increased from 6% to 14% between 1986 and 1989 (Section 2.1). This would suggest that diminazene resistance was a significant and increasing problem at the site.

The presence of diminazene-resistant infections in cattle at Ghibe was confirmed by characterising the drug sensitivity of trypanosome isolates collected from cattle at Ghibe in 1989 in naïve cattle (Section 2.2). The results demonstrated that greater than 90% of *T. congolense* infections were resistant to the maximum recommended dose of diminazene aceturate, and that a very high proportion of the isolates were also resistant to the maximum recommended doses of both isometamidium chloride and homidium chloride in cattle. This was not the first description of multiple-drug resistant trypanosome infections in domestic livestock since such infections have previously been described in Burkina Faso, Kenya, Nigeria and Sudan (Gitatha, 1979; Ilemobade, 1979; Schönefeld et al., 1987; Clausen et al., 1992; Mohamed-

Ahmed et al., 1992). However, to the best of this writer's knowledge, this was the first description of multiple-drug resistance in *T. congolense* that was expressed at the level of individual trypanosomes. In similar work, Kaminsky and Zweygarth (1991) have described clones of *T. b. brucei,* derived from isolates collected in Somalia, that express high levels of resistance to both isometamidium and diminazene.

The origin of the multiple-drug resistance in *T. congolense* populations at Ghibe is unclear. Firstly, it is possible that it is intrinsic in origin (Hayes and Wolf, 1990). Secondly, in light of the reciprocal cross-resistance that is observed between isometamidium and homidium (Whiteside, 1960; Peregrine et al., submitted), it is possible that the multiple-drug resistance arose as a result of independent acquired resistance to both diminazene and a phenanthridine. However, in light of the difficulty that various researchers have encountered in producing resistance to diminazene in trypanosomes under controlled laboratory conditions (Fussgänger and Bauer, 1960; Bauer, 1962; Whiteside, 1963), it appears from work by Whiteside (1960) and that described in Section 2.5 that quinapyramine cross-resistance is the most likely aetiology of the multiple-drug resistance. Furthermore, since the multiple-resistance phenotype was associated with many different populations of *T. congolense*, both in 1989 and 1993 (Sections 2.2 and 2.4, respectively), and the prevalence and level of drug resistance was approximately similar at these two time points, this would suggest that drug resistance developed independently in different animals infected with different trypanosome populations, and that this selection process occurred prior to 1989. It should be noted, moreover, that in light of the changes in tsetse distribution that have occurred within south-western Ethiopia over the past three decades (Slingenbergh, 1992; Leak and Mulatu, 1993), it is unclear whether the drug resistance at Ghibe arose within or outside the valley. For example, drug-resistant populations of *T. congolense* have been described within domestic livestock in Didessa and Angar valleys, Wollega Province (Scott and Pegram, 1974), and within Debeka and Miridicha, Sidamo Province, and Ghimbi and Ekoloko, Wollega Province (Dagnatchew et al., 1983), Ethiopia. As a result, it would appear

that drug-resistant infections may have been widespread in the south-western part of Ethiopia for some time.

Demonstration of a high prevalence of multiple-drug resistant *T. congolense* infections in cattle at Ghibe in both 1989 and 1993 (Sections 2.2 and 2.4) suggests stability of this phenotype over a period of four years. Since trypanosomiasis has been shown to compromise the productivity of cattle at Ghibe (Rowlands et al., 1994a, b), and cattle are required to work the heavy clay soils in the valley, this disease, compounded by drug resistance, limits the production of meat and milk, as well as food crops (Swallow, 1995). Thus, because the health of people, land, crops and livestock cannot be separated from each other in integrated farming systems (DeVries et al., 1994), as at Ghibe, the detrimental effects of trypanosomiasis on human nutrition would be expected to increase the prevalence and impact of various human diseases (USDA, 1992; Sharma et al., 1996). In this context, farmers at Ghibe have recently been interviewed to determine the factors responsible for changes in land use at the site over the past 40 years, and the impact of these factors on people's livelihoods. The resultant information indicated that trypanosomiasis only became a serious problem in livestock at Ghibe after 1979/1980. In association with this change in impact of the disease there appears to have subsequently been a significant negative effect of trypanosomiasis on the quantity and variety of crops and livestock products available at Ghibe, and thus the diet of people living within the valley (Reid et al., in preparation).

Section 2.1 indicates that from 1986 to 1989 the prevalence of *T. vivax* infections in cattle at Ghibe was considerably lower than *T. congolense*. Recent analyses of longitudinal data for *T. vivax* infections in cattle at the site from 1986 to 1994, as described for *T. congolense* (Section 2.1), suggest that the *T. vivax* populations are generally not resistant to diminazene (G.J. Rowlands, personal communication). This finding requires confirmation by laboratory characterisation of field isolates, and would explain the lower prevalence of *T. vivax*, as compared to *T. congolense*, infections in the cattle.

In light of the apparent increase in prevalence of drug-resistant infections in livestock across Africa (Section 1.1), development of strategies for controlling drug resistance in trypanosomes, especially multiple-drug resistance, should contribute towards improving livestock health in sub-Saharan Africa. Section 2.3 describes an experiment that was carried out at Ghibe to evaluate the impact of tsetse control on the apparent prevalence of multiple-drug resistant infections in cattle. In this study, an integrated control programme was implemented in which administration of diminazene aceturate to cattle, when required, was combined with deltamethrin-impregnated stationary "targets" for control of the tsetse-fly populations. Subsequent to initiation of the control programme the relative density of the main vector at Ghibe, *Glossina pallidipes*, declined from a mean of 1.9 flies/trap/day in the 12-month period preceding initiation of tsetse control to a mean of 0.4 flies/trap/day in the 12-month period following initiation. In addition, the apparent prevalence of *T. congolense* infections in cattle fell from approximately 30% to 5% over the same period, despite a high prevalence of multiple-drug resistant infections. Both a reduction in the trypanosome challenge and expression of acquired immunity by cattle (Akol and Murray, 1985) would appear to be factors that contributed to the decline in apparent prevalence of infections. Thus, multiple-drug resistant infections in livestock can be controlled if one integrates chemotherapy with effective vector control.

Since populations of *T. brucei brucei*, *T. vivax* and *T. simiae* have been shown to invade the CNS of domestic livestock (Whitelaw et al., 1985, 1988; Moulton, 1986; Zweygarth and Röttcher, 1987), the persistence of *T. congolense* infections in cattle at Ghibe after treatment could have been associated with invasion of this organ (Jennings et al., 1979; Whitelaw et al., 1985). However, while a number of authors have concluded that *T. congolense* will invade the CNS (Haase et al., 1981; Masake et al., 1984; Kalu, 1985), the demonstration of such invasion in the absence of other trypanosome species is at present equivocal because the techniques used to evaluate CNS invasion could have introduced trypanosomes into CNS tissue or CSF. In order to avoid any ambiguity in such studies a cannulation technique was developed for repeated collection of CSF from conscious goats, using a catheter placed surgically

in the subarachnoid space between the sixth and seventh lumbar vertebrae (Section 3.1). Using this technique, volumes of uncontaminated CSF in excess of 1.0 ml could be obtained readily from cannulated goats several times daily for up to 6 weeks.

In further work the cannulation technique was used to determine whether the diminazene resistance of a *T. congolense* population in goats was associated with invasion of the CNS (Section 3.2). In this study, seven cannulated goats were infected with *T. congolense* IL 3274; a population that is resistant to treatment with recommended doses of diminazene aceturate when administered to goats after the onset of parasitaemia (Silayo et al., 1992; Mamman et al., 1993). Lumbar CSF was collected daily from all the goats until 3 days after the trypanosomes reappeared in the bloodstream following treatment, and neither intact trypanosomes nor trypanosomal antigen were detected in any sample. Thus, reappearance of parasites following treatment did not appear to be associated with invasion of the CNS. Instead, *in vitro* studies have demonstrated that *T. congolense* IL 3274 is able to withstand concentrations of diminazene that are trypanocidal for diminazene-sensitive trypanosomes (Gray and Peregrine, 1993). Since the same also appears to be the case for *T. congolense* populations from Ghibe (Gray and Peregrine, 1993; Kaminsky et al., 1993), these data collectively suggest that the diminazene resistance observed amongst *T. congolense* populations at Ghibe was, in general, not associated with an ability of the parasites to invade the CNS of cattle.

Finally, the subarachnoid cannulation technique was also used to determine the pharmacokinetics of diminazene in CSF of goats (Section 3.3). The data indicated that the concentrations of the drug in CSF were 3-4 times lower than those in plasma, and that the kinetics of the drug in CSF and plasma differed significantly with respect to all the pharmacokinetic parameters that were obtained. Thus, if trypanosomes accessed CSF they would be less likely to be eliminated in this fluid than parasites present in plasma. Quite surprisingly, the difference between plasma and CSF diminazene concentrations was considerably less than that described for either pentamidine (an aromatic diamidine closely related to diminazene that is used

to treat "early stage" sleeping sickness) in humans (Bronner et al., 1991), or melarsoprol (an organic arsenical used to treat "late-stage" sleeping sickness) in both monkeys (Burri et al., 1994) and humans (Burri et al., 1993). The bioavailability of diminazene within CSF would therefore appear to be greater than that for either pentamidine or melarsoprol.

Depending on the pharmacokinetics and pharmacodynamics of a compound, multiple, rather than single, administration may enhance the compound's therapeutic efficacy. Since the dosage regimens for the currently used anti-trypanosomal compounds were generally derived in an empirical manner, it is possible that multiple-treatment regimens may enhance their activity against "drug resistant" infections (Silayo, 1991). Thus, because it is unlikely that new anti-trypanosomal compounds will be introduced in the near future for use in cattle, sheep or goats, an experiment was carried out to evaluate this hypothesis with diminazene in goats (Section 4.1). However, when goats infected with *T. congolense* IL 3274 were treated at the first peak of parasitaemia with 7.2 mg diminazene aceturate/kg b.w. on two occasions, either 8 or 24 hours apart, this only slightly improved the therapeutic efficacy of the compound. These double treatment regimens can therefore not be recommended for trypanosome infections that have the same level of drug resistance as *T. congolense* IL 3274. However, since *T. congolense* IL 3274 expresses a relatively high level of diminazene resistance (Section 4.1; Gray and Peregrine, 1993) it is possible that such treatment regimens might be more efficacious against trypanosome populations with lower levels of resistance. This requires investigation.

At present, very little information is available on the epidemiology of drug-resistant trypanosomes. In particular, the factors responsible for development, maintenance and abrogation of drug-resistant infections have not been clearly defined. Work described in Section 2.5 and by Peregrine et al. (submitted) has clearly shown that subcurative treatment with quinapyramine and isometamidium will relatively rapidly lead to the development of resistance to these compounds, particularly the former, in trypanosome populations. However, the stability of such resistance, especially in mixed infections, is not clear. Various scientists have concluded that the

diminazene-resistance phenotype is stable (Gray and Roberts, 1971a,b; Kaminsky and Zweygarth, 1989; Zhang et al., 1993). In contrast, the data presented in Sections 4.1 and 4.2 indicate that the diminazene sensitivity of *T. congolense* IL 3274 in goats decreases significantly during the first 19 days following tsetse transmission, with the major decrease in drug sensitivity occurring between day 1 and day 4 of infection (Section 4.2). Differences in the drug sensitivity of the parasite life-cycle stages present on these two days (Gray et al., 1985; Dwinger et al., 1988), or differences in the pharmacokinetics of diminazene at the different sites in which the trypanosomes reside on these two days (Mamman et al., in press), may account for the observations.

In further studies on the stability of resistance to diminazene, goats were infected with *T. congolense* IL 3274, by tsetse transmission, and treated on day 19 of infection with diminazene aceturate at a dose of 7.0 mg/kg b.w. (Section 4.2). After trypanosomes had subsequently reappeared in the bloodstream of all animals, teneral *G. m. centralis* were fed on one occasion on each animal. Thereafter, when infective, the flies were fed on uninfected goats. As before, treatment with diminazene aceturate on day 1 eliminated all infections, while treatment on day 19 failed to eliminate any infection. Thus, despite the flies being infected with trypanosomes that were "resistant" to treatment with diminazene in goats, the diminazene sensitivity of the trypanosome population ingested by the tsetse flies appeared to revert back to that of the parental trypanosome population after cyclical development and transmission. The diminazene sensitivity of day-1 tsetse-transmitted infections was therefore the same for both the parental *T. congolense* population and a subpopulation that reappeared in goats following treatment with diminazene. Thus, drug pressure did not appear to have altered the diminazene sensitivity of *T. congolense* IL 3274. In light of these findings, additional work was carried out to determine the frequency with which diminazene-resistant trypanosomes occur in populations of *T. congolense* IL 3274 in goats before and after treatment with diminazene (Section 4.3). In parasitaemias occurring in animals prior to treatment the frequency of diminazene-resistant trypanosomes was shown to be less than $1:10^3$. Furthermore, the ratio did not appear to be any less in trypanosome

populations that reappeared in the blood of animals following treatment with diminazene. This is consistent with the aforementioned results in Section 4.2, and indicates that the majority of trypanosomes which reappear in the bloodstream following treatment are not resistant to the dosage of diminazene aceturate that was administered. Such a phenomenon could be associated with a compromised viability of trypanosomes that express increased levels of drug resistance (Cantrell, 1956; Hawking, 1963; Sones et al., 1989). Alternatively, it could be associated with a high level of instability of resistance to diminazene. Lastly, it could be associated with an ability of diminazene-sensitive trypanosomes to survive treatment when occurring simultaneously with diminazene-resistant trypanosomes. This last hypothesis was investigated in goats that were infected simultaneously with both a diminazene-sensitive clone of *T. congolense*, IL 1180, and the diminazene-resistant clone of *T. congolense*, IL 3274, that was used in earlier work (Section 4.5). Subsequent to treatment with diminazene, the trypanosome populations that reappeared in the bloodstream were analysed using a PCR technique that was specific for the drug-sensitive population. On the basis of the sensitivity of the PCR technique, DNA from the drug-sensitive trypanosome population could not be detected in any post-treatment trypanosome DNA sample. As a result, this suggests that the drug-sensitive trypanosomes present in trypanosome populations that reappear in the bloodstream following treatment are not present in animals at the time of treatment but arise subsequent to treatment.

Estimates of the proportion of diminazene-sensitive trypanosomes in goat parasitaemias (Section 4.3) were inherently crude because of the small number of animals that could be used. In order to obtain estimates with a higher level of accuracy, a mouse model system was developed in which treatment of *T. congolense* IL 3274 infections with diminazene aceturate at a dose of 25 mg/kg b.w. produced parasitaemia profiles that were similar to those observed in goats following treatment with the drug at a dose of 7.0 mg/kg b.w. (Section 4.4). When trypanosomes that reappeared in mice after treatment with 25 mg diminazene aceturate/kg b.w. were characterised for their sensitivity to this drug dosage, maximum likelihood estimates for the proportions of drug-resistant trypanosomes in

population sizes of 10^2, 10^3, 10^4, 10^5 and 10^6 organisms were 8.34 x10^{-4}, 2.49 x 10^{-4}, 3.02 x 10^{-5}, 8.3 x 10^{-6} and 1.6 x 10^{-6}, respectively. In addition to these data indicating that diminazene-resistant trypanosomes constitute a very small proportion of the trypanosome population that reappears in the bloodstream of mice after treatment, they also indicate that the apparent proportion of trypanosomes which survives drug exposure varies inversely with the population size. Thus, since trypanosome growth rates *in vivo* appear to be dependent on the density of the trypanosome population (Bungener, 1982, 1984), and diminazene acts on cells at a specific stage of the cell-division cycle (Kishore et al., 1990; Poot et al., 1990; Stauffert et al., 1990), these results could be associated with different proportions of trypanosomes in different population sizes being in different stages of the cell-division cycle. Definitive elucidation of the aetiology of this phenomenon requires the development of markers for each specific stage of the trypanosome's cell-division cycle.

In light of the current problems associated with rapidly quantifying the drug-resistance phenotype of trypanosome field isolates (Section 1.1), a final series of experiments were undertaken to elucidate the molecular basis of isometamidium's action, and resistance to the compound (Chapter 5). Such information should lead to the development of a rapid, biochemical-based, method for quantifying the level of isometamidium resistance in trypanosomes. The research focused on isometamidium since it is the most commonly used chemoprophylactic compound in cattle (Kinabo and Bogan, 1988). Furthermore, because of the reciprocal cross-resistance that is observed between isometamidium and homidium (Whiteside, 1960; Peregrine et al., submitted) it was probable that much of the resultant data would be directly applicable to homidium.

In initial work significant variation in resistance to isometamidium was demonstrated in mice amongst clones derived from a stock of *T. congolense* (Section 5.1). This is consistent with resistance to suramin in populations of *T. evansi* (Mutugi et al., 1995) and clearly indicates that studies on the molecular basis of resistance to isometamidium are greatly simplified when using cloned material. In

further work, significant variation in resistance to isometamidium was also demonstrated in mice amongst nine clones derived from one of the aforementioned clones of *T. congolense* (Section 5.1). Thus, expression of resistance to isometamidium would appear to be unstable. Furthermore, in light of the approximate three-fold variation in resistance to isometamidium amongst the subclones (CD_{50} values ranged from 0.9 to 3.0 mg/kg b.w.), such variation in the field, if associated with differing growth rates amongst clones, could result in relatively rapid temporal changes in the level of isometamidium resistance of trypanosome populations. Definitive research on the genetic basis of this resistance is currently being carried out by applying representation difference analysis to isogenic clones of *T. congolense* with different levels of sensitivity to isometamidium (Peregrine et al., submitted; P.A.O. Majiwa, personal communication).

In the first of a series of cell biology experiments that were carried out to define isometamidium's mode of action, the drug's autofluorescence property (Philips et al., 1967) was used to characterise the interaction of the molecule with trypanosomes. Quantitative assessment of the changes in fluorescence that occur upon incubation with bloodstream forms of a drug-sensitive clone of *T. congolense*, IL 1180, indicated that the alterations occur as a result of interaction with an intracellular component(s) (Section 5.2). In subsequent work these changes have been shown to be associated with constraints to isometamidium's free rotation, as a result of interacting with trypanosomal DNA (Wilkes et al., 1995).

In additional work, incubation of *T. congolense* IL 1180 bloodstream forms with digitonin (a partial permeabiliser of the plasma membrane of kinetoplastids) and N-ethylmaleimide (a non-permeable thiol-group-reactive reagent) demonstrated that isometamidium is transported into the parasites via a plasma membrane-associated protein carrier (Section 5.2). This is consistent with the work of Sutherland et al. (1992a) and Wilkes et al. (1995), and suggests that isometamidium enters *T. congolense* via a carrier-mediated transport process and subsequently, relatively

rapidly, associates with trypanosomal DNA. Either the nucleus or the kinetoplast would therefore appear to be the major site of action of the compound.

After incubation of drug-sensitive *T. congolense* bloodstream forms with isometamidium, fluorescence microscopy indicates punctate, rather than diffuse, localisation of the molecule (Sutherland et al., 1991). However, definitive identification of the organellar localisation of the molecule using this technique is not possible because of its low level of resolution. As part of an approach to overcome this problem, anti-isometamidium Mabs were produced by immunising mice with either an isometamidium-human serum albumin conjugate or an isometamidium-porcine thyroglobulin conjugate (Section 5.3). Mabs for use in further studies were then selected on the basis that they (a) lacked cross-reactivity with the carrier molecule, and (b) recognised both conjugated and unconjugated isometamidium. One of these Mabs, with a high level of functional affinity, was then used in immunoelectron microscopy to examine sections of *T. congolense* IL 1180 bloodstream forms after incubation with isometamidium (Section 5.4). The resultant electron micrographs demonstrated relatively high levels of labelling in the kinetoplast and mitochondrion, in contrast to the nucleus. Furthermore, relatively low levels of labelling were observed in endocytic organelles. As a result, the data suggest that endocytosis via the flagellar pocket does not play a major role in uptake of isometamidium. In addition, the major site of action of isometamidium would appear to be the kinetoplast/mitochondrion. However, since the technique could only detect the drug in trypanosomes after 30 minutes incubation at 37°C, and at this time point high background labelling indicated that the parasites were overloaded with drug, definitive conclusions about the drug's localisation could not be made. As a result, an electron-microscopy autoradiography technique was developed that enabled one to characterise uptake of ^{3}H-labelled Samorin in *T. congolense* IL 1180 bloodstream forms (Section 5.4). After 15 seconds, 2 minutes and 5 minutes incubation at 37°C a signal was only detected within the kinetoplast. This would therefore suggest that isometamidium's primary mode of action occurs within the kinetoplast, probably by inhibition of mitochondrial type II topoisomerase (Shapiro and Englund, 1990; Shapiro, 1993). Furthermore, since no drug was observed

associated with endocytic vesicles, as with the aforementioned immunoelectron-microscopy technique, this confirms that endocytosis via the flagellar pocket does not play a major role in uptake of isometamidium by *T. congolense* bloodstream forms.

The above findings have demonstrated the primary mechanism by which isometamidium enters drug-sensitive *T. congolense* bloodstream forms, and the apparent primary site of action. However, the role of these processes in resistance to the compound is yet to be defined. Decreased levels of isometamidium accumulation have been observed in drug-resistant populations of *T. congolense* (Sutherland et al., 1992a; Section 2.4) and could be associated with reduced uptake, increased efflux or increased metabolism of isometamidium by the parasites. In this context, mathematical modelling of radiolabelled-drug uptake data led Sutherland et al. (1992b) to conclude that reduced uptake of isometamidium is not responsible for reduced drug accumulation in resistant parasites, thereby inferring the possible involvement of an efflux process. However, definitive biological data to confirm this conclusion is yet to be obtained (Kaminsky and Zweygarth, 1991; Sutherland et al., 1992a). Recent work has indicated that reduced uptake of organic-arsenical and aromatic-diamidine anti-trypanosomal compounds via nucleoside transporters plays a major role in resistance to these compounds in trypanosomes (Carter and Fairlamb, 1993; Carter et al., 1995; Ross and Barns, 1996). In contrast, however, impaired activity of nucleoside transporter(s) does not appear to play a significant role in resistance to isometamidium (J. Wilkes, personal communication). Lastly, a consistent relationship has very recently been observed between the potential difference across the mitochondrial membranes and the level of resistance of wild-type *T. congolense* bloodstream forms to isometamidium (Wilkes et al., submitted). Further work is required to characterise the molecular basis of this finding and the spatial extent to which this mechanism is associated with resistance to isometamidium. If the same mechanism mediates resistance to isometamidium in geographically disparate isolates, then the information may be used to develop a biochemical diagnostic for rapidly quantifying the level of isometamidium resistance of large numbers of trypanosome populations.

In conclusion, drug resistance in trypanosomes appears to be a worsening problem which, in the absence of new compounds, will increasingly pose a threat to the productivity of domestic livestock maintained in trypanosomiasis-endemic areas. It is therefore important that the epidemiology of drug-resistant trypanosomes is fully defined so that optimal methods for controlling drug resistance can be identified and implemented, thereby maintaining the efficacy of the current anti-trypanosomal compounds for as long as possible. To this end, the work described in this thesis was carried out, firstly, to develop tools for more accurately determining the prevalence and level of drug resistance in the field than was possible when the work was initiated. Secondly, a series of experiments were carried out to determine the contribution of various factors to the development, maintenance and abrogation of drug-resistant trypanosome populations in the field. Finally, the efficacy of various potential methods for controlling drug-resistant infections in domestic livestock was evaluated; control of pathogenic multiple-drug resistant infections in cattle in the field was shown to be achieved by integrating anti-trypanosomal chemotherapy with vector control.

Development of drug resistance in trypanosomes is a phenomenon that is not unique to these parasites. Similar, if not greater, problems with drug resistance are increasingly being associated with acaricides and anthelmintics throughout the world (Nolan, 1990; Waller, 1994). Thus, since trypanosomiasis rarely occurs in the field as an isolated disease entity, control methods for trypanosomiasis must not be considered in isolation, but rather as a component of a comprehensive health-control package. In this respect, the potential detrimental effects of trypanosomiasis control strategies on the efficacy of control methods for other diseases require consideration when rationalising control strategies for trypanosomiasis. For example, while the use of synthetic pyrethroids in "pour-on" formulations appears to be a particularly promising method for control of tsetse populations (Bauer et al., 1995), the long-term effect of such formulations on the rate of development of resistance to synthetic pyrethroids in tick populations is unclear. This particular concern needs addressing in the near future. Finally, since information on the optimal control of drug

resistance in trypanosomes requires definitive data on the epidemiology of the various phenotypes, future research on drug resistance in trypanosomes will need to focus on the derivation of biochemical markers for the different drug-resistance phenotypes and their application in the field.

References:

Akol, G.W.O. and Murray, M. (1985) Induction of protective immunity in cattle by tsetse-transmitted cloned isolates of *Trypanosoma congolense*. *Annals of Tropical Medicine and Parasitology* 6, 617-627.

Bauer, B., Amsler-Delafosse, S., Clausen, P.-H., Kabore, I. and Petrich-Bauer, J. (1995) Successful application of deltamethrin pour on to cattle in a campaign against tsetse flies (Glossina spp.) in the pastoral zone of Samorogouan, Burkina Faso. *Tropical Medicine and Parasitology* 46, 183-189.

Bauer, F. (1962) The development of drug-resistance to Berenil in *Trypanosoma congolense*. *Veterinary Record* 74, 265-266.

Bronner, U., Doua, F., Ericsson, Ö., Gustafsson, L.L., Miézan, T.W., Rais, M. and Rombo, L. (1991) Pentamidine concentrations in plasma, whole blood and cerebrospinal fluid during treatment of *Trypanosoma gambiense* infection in Côte d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85, 608-611.

Bungener, W. (1982) Behaviour of a *Trypanosoma brucei brucei* stock (STIB 348C) in mice. 2. Course of infections with basic type and several variant types. *Tropical Medicine and Parasitology* 33, 243-248.

Bungener, W. (1984) Behaviour of a *Trypanosoma brucei brucei* stock (STIB 348C) in mice. 4. Different course of primary parasitaemia in trypanosome variants of different virulence. *Tropical Medicine and Parasitology* 35, 115-118.

Burri, C., Baltz, T., Giroud, C., Doua, F., Welker, H.A. and Brun, R. (1993) Pharmacokinetic properties of the trypanocidal drug melarsoprol. *Chemotherapy* 39, 225-234.

Burri, C., Onyango, J.D., Auma, J.E., Burudi, E.M.E. and Brun, R. (1994) Pharmacokinetics of melarsoprol in uninfected vervet monkeys. *Acta Tropica* 58, 35-49.

Cantrell, W. (1956) Behaviour of mixtures of oxophenarsin-resistant and unmodified strains of *Trypanosoma equiperdum*. *Experimental Parasitology* 5, 178-190.

Carter, N.S., Berger, B.J. and Fairlamb, A.H. (1995) Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. *Journal of Biological Chemistry* 270, 28153-28157.

Carter, N.S. and Fairlamb, A.H. (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature* 361, 173-176.

Clausen, P.-H., Sidibe, I., Kaboré, I. and Bauer, B. (1992) Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse fly challenge in the pastoral zone of Samorogouan, Burkina Faso. *Acta Tropica* 51, 229-236.

Dagnatchew, Z., Lemecha, H. and Mulatu, W. (1983) Trypanocidal drug resistance problem in certain parts of Ethiopia. Trypanosomiasis Control Services, Ministry of Agriculture. Annual Report 1982-1983, pp. 52-57.

DeVries, J., Gudahl, D., Hoey, J., Pelant, R.K. and Shumaker, J. (1994) Livestock for a small Earth. The role of animals in a just and sustainable world. Aaker, J. (ed.). Seven Locks Press, Washington, D.C., pp. 111.

Dwinger, R.H., Rudin, W., Moloo, S.K. and Murray, M. (1988) Development of *Trypanosoma congolense*, *T. vivax* and *T. brucei* in the skin reaction induced by infected *Glossina morsitans centralis*: a light and electron microscopical study. *Research in Veterinary Science* 44, 154-163.

Fussgänger, R. and Bauer, F. (1960) Investigations on Berenil resistance of trypanosomes. *Veterinary Record* 72, 1118-1121.

Gitatha, S.K. (1979) *T. congolense* (Shimba Hills) resistant to various trypanocidal drugs. In *Proceedings, 16th meeting, International Scientific Council for Trypanosomiasis Research and Control, Yaoundé, Cameroon.* OAU/STRC Publication No. 111, pp. 257-263.

Goldring, E.S., Grossman, L.I., Krupnick, D., Cryer, D.R. and Marmur, J. (1970) The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with Ethidium bromide. *Journal of Molecular Biology* 52, 323-335.

Gray, A.R. and Roberts, C.J. (1971a) The cyclical transmission of strains of *Trypanosoma congolense* and *Trypanosoma vivax* resistant to normal therapeutic doses of trypanocidal drugs. *Parasitology* 63, 67-89.

Gray, A.R. and Roberts, C.J. (1971b) The stability of resistance to diminazene aceturate and quinapyramine sulphate in a strain of *Trypanosoma vivax* during cyclical transmission through antelope. *Parasitology* 63, 163-168.

Gray, M.A. and Peregrine, A.S. (1993) An *in vitro* assay for drug sensitivity of *Trypanosoma congolense* using *in vitro*-derived metacyclic trypanosomes. *Acta Tropica* 54, 291-300.

Gray, M.A., Ross, C.A., Taylor, A.M., Tetley, L. and Luckins, A.G. (1985) *In vitro* cultivation of *Trypanosoma congolense*: the production of infective metacyclic trypanosomes cultured on bovine endothelial cell monolayers. *Acta Tropica* 42, 99-111.

Haase, M., Bernard, S. and Guidot, G. (1981) Trypanosomiasis in Zebu cattle. Reappearance of *Trypanosoma congolense* in brain tissue after treatment with Berenil. *Revue d'Elevage Médecine Vétérinaire des Pays Tropicaux* 34, 149-154.

Hawking, F. (1963) Drug-resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, berenil and other compounds. *Annals of Tropical Medicine and Parasitology* 57, 262-282.

Hayes, J.D. and Wolf, C.R. (1990) Molecular mechanisms of drug resistance. *Biochemical Journal* 272, 281-295.

Hixon, S.C., White, W.E and Yielding, K.L. (1975) Bypass by photoaffinity labeling of blocked metabolic activation of Ethidium: confirmation of the role for covalent Ethidium attachment in mitochondrial mutagenesis. *Biochemical and Biophysical Research Communications* 66, 31-35.

Holmes, P.H. (1991) New opportunities for the diagnosis and control of animal diseases in the tropics. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85, 165-167.

Ilemobade, A.A. (1979) Drug sensitivity of mouse infective *Trypanosoma vivax* isolates in cattle and sheep. In *Proceedings, 16th meeting, International Scientific Council for Trypanosomiasis Research and Control, Yaoundé, Cameroon.* OAU/STRC Publication No. 111, pp. 251-253.

Jennings, F.W., Whitelaw, D.D., Holmes, P.H., Chizyuka, H.G.B. and Urquhart, G.M. (1979) The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal for Parasitology* 9, 381-384.

Jordan, A.M. (1986) Trypanosomiasis control and African rural development. Longman Singapore Publishers (Pte) Ltd., p. 140.

Joshua, R.A., Obwolo, M.J., Bwangamoi, O. and Mandebvu, E. (1995) Resistance to diminaz[e]ne aceturate by *Trypanosoma congolense* from cattle in the Zambezi Valley of Zimbabwe. *Veterinary Parasitology* 60, 1-6.

Kalu, A.U. (1985) The effect of various doses of Berenil and Pentamidine isethionate in rats experimentally infected with *Trypanosoma congolense*. *Nigerian Veterinary Journal* 14, 52-55.

Kaminsky, R., Chuma, F. and Zweygarth, E. (1993) *Trypanosoma congolense*: *in vitro* susceptibility of bloodstream forms to diminazene and isometamidium. *Experimental Parasitology* 76, 213-215.

Kaminsky, R. and Zweygarth, E. (1989) Effect of *in vitro* cultivation on the stability of resistance of *Trypanosoma brucei brucei* to diminazene, isometamidium, quinapyramine, and Mel B. *Journal of Parasitology* 75, 42-45.

Kaminsky, R. and Zweygarth, E. (1991) The effect of verapamil alone and in combination with trypanocides on multidrug-resistant *Trypanosoma brucei brucei*. *Acta Tropica* 49, 215-225.

Kinabo, L.D.B. and Bogan, J.A. (1988) The pharmacology of isometamidium. *Journal of Veterinary Pharmacology and Therapeutics* 11, 233-245.

Kishore, P., Gupta, S., Srivastava, D.K. and Shukla, O.P. (1990) Action of methylglyoxal bis (guanyl hydrazone) and related antiprotozoals on *Acanthamoeba culbertsoni*. *Indian Journal of Experimental Biology* 28, 1174-1179.

Leak, S.G.A. and Mulatu, W. (1993) Advance of *Glossina morsitans submorsitans* and *G. pallidipes* along the Ghibe-river system in southwest Ethiopia. *Acta Tropica* 55, 91-95.

MacGregor, J.T. and Johnson, I.J. (1977) In vitro metabolic activation of Ethidium bromide and other phenanthridinium compounds: mutagenic activity in *Salmonella typhimurium*. *Mutation Research* 48, 103-108.

Mamman, M., Katende, J., Moloo, S.K. and Peregrine, A.S. (1993) Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology* 50, 1-14.

Mamman, M., McKeever, D.J., Aliu, Y.O. and Peregrine, A.S. (in press) Pharmacokinetics of diminazene in plasma and lymph of goats. *American Journal of Veterinary Research.*

Masake, R.A., Nantulya, V.M., Akol, G.W.O. and Musoke, A.J. (1984) Cerebral trypanosomiasis in cattle with mixed *Trypanosoma congolense* and *T. brucei brucei* infections. *Acta Tropica* 41, 237-246.

Mathewson, M.D. (1984) The future of tick control: a review of the chemical and non-chemical options. In *Impact of Disease on Livestock Production in the Tropics*. Riemann, H.P. and Burridge, M.P. (eds.). Elsevier, Amsterdam, pp. 559-568.

Mohamed-Ahmed, M.M., Rahman, A.H. and Abdel Karim, E.I. (1992) Multiple drug-resistant bovine trypanosomes in South Darfur Province, Sudan. *Tropical Animal Health and Production* 24, 179-181.

Moulton, J.E. (1986) Relapse infection after chemotherapy in goats experimentally infected with *Trypanosoma brucei*: pathological changes in central nervous system. *Veterinary Pathology* 23, 21-28.

Mutugi, M.W., Boid, R. and Luckins, A.G. (1995) Differences in cloning and sub-cloning success rates in four stocks of *Trypanosoma evansi* and variation in suramin resistance of the clones. *Veterinary Parasitology* 60, 213-220.

Nolan, J. (1990) Acaricide resistance in single and multi-host ticks and strategies for its control. *Parasitologia* 32, 145-153.

Peregrine, A.S., Gray, M.A. and Moloo, S.K. (submitted) Cross-resistance phenotype associated with induction of resistance to isometamidium chloride in a clone of *Trypanosoma congolense*. *The Journal of Antimicrobial Chemotherapy*.

Philips, F.S., Sternberg, S.S., Cronin, A.P., Sodergren, J.E. and Vidal, P.M. (1967) Physiologic disposition and intracellular localization of isometamidium. *Cancer Research* 27, 333-349.

Poot, M., Kausch, K., Kohler, J., Haaf, T. and Hoehn, H. (1990) The minor-groove binding DNA-ligands netropsin, distamycin A and berenil cause polyploidisation via impairment of the G_2 phase of the cell cycle. *Cell Structure and Function* 15, 151-157.

Reid, R.S., Nyawira, V., Wootton, S., Kruska, R.L., Wilson, C.J. and Mulatu, W. (in preparation) Land-use change in response to changes in livestock disease severity and population pressure in southwestern Ethiopia.

Ross, C.A. and Barns, A.M. (1996) Alteration to one of three adenosine transporters is associated with resistance to Cymelarsan in *Trypanosoma evansi*. *Parasitology Research* 82, 183-188.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M. (1994a) Effects of trypanosomiasis on growth and mortality of young East African Zebu cattle exposed to drug-resistant trypanosomes. *Preventive Veterinary Medicine* 21, 87-101.

Rowlands, G.J., Mulatu, W., Authié, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M. (1994b) Effects of trypanosomiasis on reproduction of East African Zebu cows exposed to drug-resistant trypanosomes. *Preventive Veterinary Medicine* 21, 237-249.

Schönefeld, A., Röttcher, D. and Moloo, S.K. (1987) The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. *Tropical Medicine and Parasitology* 38, 177-180.

Scott, J.M. and Pegram, R.G. (1974) A high incidence of *Trypanosoma congolense* strains resistant to homidium bromide in Ethiopia. *Tropical Animal Health and Production* 6, 215-221.

Shapiro, T.A. (1993) Inhibition of topoisomerases in African trypanosomes. *Acta Tropica* 54, 251-260.

Shapiro, T.A. and Englund, P.T. (1990) Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. *Proceedings of the National Academy of Sciences of the USA* 87, 950-954.

Sharma, M., Garcia, M., Qureshi, A. and Brown, L. (1996) Overcoming nutrition: is there an ecoregional dimension? Food, Agriculture, and the Environment Discussion Paper 10. International Food Policy Research Institute, Washington, U.S.A., pp. 19.

Silayo, R.S. (1991) Studies on efficacy of repeat trypanocidal drug treatment against drug-resistant *Trypanosoma congolense* infections. In *Proceedings, 21st Meeting of the International Scientific Council for Trypanosomiasis Research and Control, Yamoussoukro, Côte d'Ivoire.* Modern Lithographic (K) Ltd., Nairobi, OAU/STRC Publication No. 116, p. 186.

Silayo, R.S., Mamman, M., Moloo, S.K., Aliu, Y.O., Gray, M.A. and Peregrine, A.S. (1992) Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate. *Research in Veterinary Science* 53, 98-105.

Slingenbergh, J. (1992) Tsetse control and agricultural development in Ethiopia. *World Animal Review* 70/71, 30-36.

Sones, K.R., Holmes, P.H. and Urquhart, G.M. (1989) Interference between drug-resistant and drug-sensitive stocks of *Trypanosoma congolense* in goats. *Research in Veterinary Science* 47, 75-77.

Stauffert, I., Paulini, H., Steinmann, U., Sippel, H. and Estler, C.J. (1990) Investigations on mutagenicity and genotoxicity of pentamidine and some related trypanocidal diamidines. *Mutation Research* 245, 93-98.

Stephen, L.E. (1986) Trypanosomiasis: a veterinary perspective. Pergamon Press, pp. 454-479.

Sutherland, I.A., Mounsey, A., Eisler, M. and Holmes, P.H. (1992b) Kinetic modelling of isometamidium chloride (Samorin) uptake by *Trypanosoma congolense*. *Parasitology* 105, 91-95.

Sutherland, I.A., Mounsey, A. and Holmes, P.H. (1992a) Transport of isometamidium (Samorin) by drug-resistant and drug-sensitive *Trypanosoma congolense*. *Parasitology* 104, 461-467.

Sutherland, I.A., Peregrine, A.S., Lonsdale-Eccles, J.D. and Holmes, P.H. (1991) Reduced accumulation of isometamidium by drug-resistant *Trypanosoma congolense*. *Parasitology* 103, 245-251.

Swallow, B. (1995) Tsetse: Ethiopian success. African Farming July/August, p.21.

USDA (1992) Nutrition: Eating for good health. United States Department of Agriculture. Agriculture Information Bulletin 685, pp. 208.

Waller, P.J. (1994) The development of anthelmintic resistance in ruminant livestock. *Acta Tropica* 56, 233-243.

Whitelaw, D.D., Gardiner, P.R. and Murray, M. (1988) Extravascular foci of *Trypanosoma vivax* in goats: the central nervous system and aqueous humor of the eye as potential sources of relapse infections after chemotherapy. *Parasitology* 97, 51-61.

Whitelaw, D.D., Moulton, J.E., Morrison, W.I. and Murray, M. (1985) Central nervous system involvement in goats undergoing primary infections with *Trypanosoma brucei* and relapse infections after chemotherapy. *Parasitology* 90, 255-268.

Whiteside, E.F. (1960) Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. In *Proceedings, 8th meeting, International Scientific Committee for Trypanosomiasis Research, Jos, Nigeria.* CCTA Publication 62, pp. 141-154.

Whiteside, E.F. (1963) A strain of *Trypanosoma congolense* directly resistant to Berenil. *Journal of Comparative Pathology* 73, 167-175.

Wilkes, J.M., Mulugeta, W., Wells, C. and Peregrine, A.S. (submitted) Mitochondrial energetics and drug resistance in African trypanosomes. *Biochemical Journal.*

Wilkes, J.M., Peregrine, A.S. and Zilberstein, D. (1995) The accumulation and compartmentalization of isometamidium chloride in *Trypanosoma congolense*, monitored by its intrinsic fluorescence. *Biochemical Journal* 312, 319-329.

Winrock (1992) Assessment of animal agriculture in sub-Saharan Africa. Winrock International, Arkansas, U.S.A., pp. 125.

Zhang, Z.Q., Giroud, C. and Baltz, T. (1993) *Trypanosoma evansi*: *in vivo* and *in vitro* determination of trypanocide resistance profiles. *Experimental Parasitology* 77, 387-394.

Zweygarth, E. and Röttcher, D. (1987) The occurrence of *Trypanosoma (Nannomonas) simiae* in the cerebrospinal fluid of domestic pigs. *Parasitology Research* 73, 479-480.