

A THEILERIA ANNULATA SPOROZOITE SURFACE ANTIGEN
AS A POTENTIAL VACCINE FOR TROPICAL THEILERIOSIS

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This presentation is entirely the product of my own efforts and the work on which it is based was my own except where specifically stated in the text and in the acknowledgements section.

Susanna Williamson

November, 1988

DEDICATION

To my mother,
with love and thanks.

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ABBREVIATIONS

ACD	acid citrate dextrose solution
B-ME	B-mercaptoethanol
BoLA	bovine lymphocyte antigens
bp	base pairs
BPA	bovine plasma albumin
cdNA	complementary DNA
CFA	complete Freund's adjuvant
cpm	counts per minute
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
<u>E. coli</u>	<u>Esherichia coli</u>
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
GF	GUTS filtrate
GUTS	ground up tick supernatant
Gy	Gray
Hanks B.S.S.	Hanks balanced salt solution
HAT	hypoxanthine-aminopterin-thymidine
HT	hypoxanthine-thymidine
^{125}I	Iodine 125
IFA	incomplete Freund's adjuvant
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
IPTG	isopropyl B-D-thiogalactopyranoside

kb	kilobases
kdal	kilodaltons
λ gt11	lambda phage gt11
Mab	monoclonal antibody
MEM	minimum essential medium
MHC	major histocompatibility complex
NPS	no parasites seen
PBM	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCV	packed cell volume
PEG	polyethylene glycol
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
s.g.	specific gravity
SIP	stock isotonic Percoll
te	tick equivalent
wbc	white blood cell

ABSTRACT OF THESIS (Regulation 7.9)

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The aim of this study was to identify, and assess, one or more Theileria annulata sporozoite antigens as potential non-infective vaccine material for tropical theileriosis.

Theileria annulata was described with particular reference to literature concerning characteristics of the sporozoite stage, immunity and immunisation. The application of biotechnology to the production of recombinant DNA vaccines was reviewed and relevant examples were detailed.

Antisporozoite monoclonal antibodies (Mabs) were screened for their ability to inhibit sporozoite invasion of peripheral blood mononuclear cells in vitro. Two Mabs, 1A7 and 4B11, were shown to possess significant inhibitory activity. Strong sporozoite blocking activity was also detected in hyperimmune bovine sera, antisporozoite rabbit serum and sera from calves exposed to irradiated sporozoites. Mabs 1A7 and 4B11 both identified epitopes on the sporozoite surface. Mab 4B11 recognised a 17-20 kilodalton (kdal) antigen while the epitope detected by Mab 1A7 resided on several members of a group of proteins, of approximate molecular weights 35, 72, 63 and 54 kdal, believed to result from the processing of a high molecular weight precursor. Certain of the above polyclonal sera also recognised one or both of these epitopes.

In order to obtain material for immunisation trials, the gene sequence coding for the sporozoite epitope recognised by Mab 1A7 was cloned into the vector, λ gt11, and expressed in Escherichia coli (E. coli). Two recombinant phage clones, λ gt11-SR1 and λ gt11-SR2, containing this gene sequence were obtained. Analysis of the 330 base pair DNA sequence cloned in λ gt11-SR1 showed that three copies of the sequence were present in Eco R1 digested genomic DNA from uncloned parasite material while DNA from cloned parasite contained a single copy. Expression of the gene sequence was stage-specific, occurring only in sporoblast and sporozoite stages.

The recombinant DNA cloned in λ gt11-SR1 and λ gt11-SR2 was expressed in E. coli as B-galactosidase fusion proteins of 135 and 147 kdal respectively. These proteins reacted specifically with Mab 1A7 and also with antisporozoite rabbit serum and certain sera from calves exposed to live or irradiated sporozoites. Mice, rabbits and calves were immunised with the 135 kdal protein from λ gt11-SR1. Rabbits and calves developed a strong antibody response to this protein and the antibodies produced also recognised the native sporozoite epitopes, showing an identical pattern of reactivity to that seen with Mab 1A7. Significantly, the λ gt11-SR1 sera neutralised sporozoite infectivity very effectively in vitro, while control sera failed to do so. On challenge with live virulent sporozoites, the calves immunised with the λ gt11-SR1 protein became infected and underwent clinical reactions which were not significantly different from those observed in control B-galactosidase immunised, or unimmunised, calves. Discussion of these results concentrated on whether this failure to stimulate protective immunity reflected an inadequate method of antigen presentation, or whether antisporozoite immunity alone is insufficient to protect otherwise fully susceptible calves.

SUMMARY

The aim of this study was to identify potentially protective antigens of the infective, sporozoite, stage of Theileria annulata and to clone the relevant gene(s) to obtain one or more of these antigens as a recombinant protein with which to perform immunisation trials.

Theileria annulata and the disease it causes, tropical theileriosis, were described with particular reference to literature concerning characteristics of the sporozoite stage, immunity and immunisation. The application of biotechnology to the production of recombinant DNA vaccines was reviewed and relevant examples were detailed.

To this end, ant sporozoite monoclonal antibodies (Mabs) were raised and those giving positive fluorescence of formalin fixed sporozoites by the indirect fluorescent antibody test (IFAT) were selected. Nineteen such Mabs were screened for the ability to neutralise sporozoite infectivity for bovine peripheral blood mononuclear cells in vitro. Two ant sporozoite Mabs, 1A7 and 4B11, which demonstrated surface immunofluorescence of live sporozoites, exhibited a significant degree of sporozoite inhibition. These were chosen for further investigation. The in vitro assay was also used to detect sporozoite neutralising activity in hyperimmune bovine sera, sera from calves exposed to irradiated sporozoites and serum from rabbits on which infected ticks had fed.

Using SDS-PAGE Western blotting, Mabs 1A7 and 4B11 were shown to identify different epitopes on the sporozoite surface. Mab 1A7 specifically recognised an epitope present on several sporozoite

proteins of approximate molecular weights 85, 72, 63 and 54 kilodaltons (kdal) in Western blots. This was believed to reflect processing of a high molecular weight precursor. Mab 4B11 recognised a low molecular weight protein of 17-20 kdal, also located on the sporozoite surface. Some immune bovine sera and ant sporozoite rabbit serum also detected these two sporozoite epitopes.

Large amounts of one of the epitopes were made available as a recombinant protein by cloning and expressing the relevant theilerial gene fragment in Escherichia coli (E. coli). To achieve this a λ gt11 expression library, constructed using genomic DNA from T. annulata piroplasm DNA, was screened with Mabs 1A7 and 4B11. Two recombinant clones, λ gt11-SR1 and λ gt11-SR2, were obtained, both of which contained the gene sequence coding for the epitope recognised by Mab 1A7. The theilerial DNA insert of clone λ gt11-SR1 was 330 base pairs in size and hybridised to three DNA bands in EcoR1 digested genomic DNA from an uncloned parasite stock. These bands were segregated to single copies of the gene sequence in the DNA from cloned parasite material. Northern blot analysis using RNA from infected tick salivary glands showed expression of the λ gt11-SR1 insert to be stage specific, occurring only in sporoblast and sporozoite stages and not in macroschizonts or piroplasms, nor in uninfected tick salivary glands.

The recombinant DNA cloned in λ gt11-SR1 and λ gt11-SR2 was expressed in E. coli as B-galactosidase fusion proteins of 135 and 147 kdal respectively. These proteins reacted specifically with Mab 1A7 and also with ant sporozoite rabbit serum and certain sera from calves exposed to live or irradiated sporozoites. Immunisation trials

using the purified 135 kdal protein from λ gt11-SR1, were performed in mice, rabbits and calves. Inoculation of rabbits and calves with this fusion protein combined with Freund's adjuvant elicited a strong and specific antibody response. These antibodies also recognised the native sporozoite epitope when assessed by IFAT and Western blotting, the latter revealing exactly the same multiple reactivity of sporozoite proteins with anti λ gt11-SR1 sera as observed with Mab 1A7. Significantly, the same sera also neutralised sporozoite infectivity in vitro very effectively, while control sera failed to do so. On challenge with live virulent sporozoites, the calves immunised with the λ gt11-SR1 protein became infected and underwent clinical reactions which were not significantly different from those observed in control B-galactosidase immunised or unimmunised calves. Discussion of these results concentrated on whether this failure to stimulate protective immunity reflected an inadequate method of antigen presentation, or whether ant sporozoite immunity alone is insufficient to protect otherwise fully susceptible calves.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

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1.1. INTRODUCTION

Tropical theileriosis is a tick-borne haemoprotozoan disease threatening an estimated 250 million cattle in the Mediterranean littoral, North Africa, the Middle East, the Indian subcontinent and central Asia (Purnell, 1978; Robinson, 1982). The disease is caused by Theileria annulata, a protozoan parasite transmitted by ticks of the genus, Hyalomma (Dschunkowsky and Luhs, 1904), and acts as a major constraint on livestock production and improvement in many developing countries.

This constraint arises particularly where exotic cattle are being introduced in attempts to upgrade local beef or dairy stock. These cattle appear to be more susceptible to theileriosis than indigenous breeds, suffering higher mortality and more severe clinical signs (Yousif, 1969). In order to give these livestock improvement programmes a chance of success, the cattle need to be protected from theileriosis as well as from other tick-borne diseases such as babesiosis and anaplasmosis. This can, in a properly planned and managed system, be achieved using one of two principles; either the cattle can be protected from exposure to the disease agent through control of the tick vector and cattle movement regulations or they can be allowed to develop active immunity. In the latter case, if loss of animals and production is to be avoided, either the exposure to the parasite must be deliberate and controlled, through vaccination, or chemotherapy needs to be used to treat clinical cases as they arise. However, when considering the quality of surveillance that would be required to recognise and treat cases early enough, together with the high cost of theilericidal drugs, chemotherapy should ideally be reserved for use in the event of breakdown of other control methods.

Routine or strategic use of acaricides to control tick numbers is a well established method of limiting tick damage and the impact of tick-borne disease but suffers from concern about the development of tick resistance to acaricides (Young, Groocock and Kariuki, 1988). Also, a state of enzootic instability may result in which fully susceptible cattle exist in an area where tick-borne disease agents are endemic in the tick population (Lawrence and Norval, 1979). Therefore, any interruption to a tick dipping regime, whether through shortage of acaricide, political unrest, damage to equipment or simply inefficient implementation, can result in heavy losses in cattle suddenly exposed to infected ticks (Lawrence, Foggin and Norval, 1980).

Thus, the creation of cattle immune to tropical theileriosis in such a way that clinical disease is avoided and long term field challenge can be resisted is of paramount importance in limiting the effects of this disease in an otherwise susceptible cattle population.

Cattle recovering from tropical theileriosis either naturally or through drug treatment are immune to homologous challenge (Sergent, Donatien, Parrot and Lestoquard, 1945; Neitz, 1957). This immunity is believed to be mainly due to the action of genetically restricted cytotoxic T cells on schizont-infected lymphocytes (Preston, Brown and Spooner, 1983). However, with repeated exposure to sporozoites, an antibody mediated response develops which is directed against the sporozoite stage as shown by the fact that hyperimmune bovine serum blocks sporozoite infectivity for lymphocytes very effectively in vitro (Gray and Brown, 1981; Preston and Brown, 1985) and it is likely that this humoral mechanism plays a significant role in maintaining immunity.

Two methods of immunisation against theileriosis have been developed, both of which involve deliberately infecting cattle with live parasites. One, the infection and treatment method, involves inoculation of live sporozoites with simultaneous chemoprophylaxis to limit the clinical reaction and permit the subsequent development of immunity (Radley, 1981). The other method of immunisation, which has been widely applied in the Middle East, USSR, China and, more recently, India, Turkey and North Africa, is achieved by infecting cattle with attenuated Theileria annulata macroschizont-infected cells from in vitro cultures (Pipano and Tsur, 1966).

Both these methods of vaccination depend upon establishing active infection in the bovine host (Pipano, 1977) and thus incur the risks of causing clinical disease and carrier status and the problems of handling live parasite material under tropical conditions.

For these reasons, which have been emphasised by Doherty and Nussenzweig (1985) for Theileria parva, the cause of East Coast fever, efforts are being made to find alternative non-infective immunising material. To date, neither Theileria annulata schizont-infected cells killed by freeze drying and sonication (Pipano, Goldman, Samish and Friedhoff, 1977) nor plasma membranes prepared from Theileria parva parasitised lymphoblastoid cells (Emery, Morrison and Jack, 1986) have met with convincing success and more defined approaches have been adopted. For Theileria annulata, attention has so far been focussed particularly on the schizont-infected lymphocyte (Shiels, McDougall, Tait and Brown, 1986a; Preston, McDougall, Wilkie, Shiels, Tait and Brown, 1986) and, for Theileria parva, on the sporozoite stage (Musoke, Nantulya, Rurangirwa and Buscher, 1984; Dobbelaere, Spooner, Barry and Irvin, 1984).

With the exciting recent advances in biotechnology and their widespread implementation in research on all important disease agents of man and animals, new strategies for vaccine development have emerged. The aim of the work described in this thesis was to apply one of these strategies to the development of a subunit vaccine from the infective, sporozoite stage of Theileria annulata. The rationale adopted was similar to that behind much of the current work on the sporozoite stage of Plasmodium species, in particular of Plasmodium falciparum, the cause of perhaps the most important form of human malaria (Zavala, Tam, Cochrane, Quakyi, Nussenzweig and Nussenzweig, 1985).

First, the thesis describes how hybridoma technology was applied to the identification of individual sporozoite antigens and then how the resulting ant sporozoite monoclonal antibodies were screened for the ability to mimic hyperimmune bovine serum in blocking sporozoite invasion of lymphocytes in vitro. Monoclonal antibodies with a significant degree of blocking activity were selected and used to characterise the sporozoite antigens they recognise. One priority was then to obtain these proteins in sufficient quantities for immunisation experiments. To this end, a genomic DNA library of Theileria annulata was screened with the monoclonal antibodies resulting in the isolation of a recombinant expressing one of the relevant sporozoite epitopes. Following analysis of the recombinant DNA and the recombinant protein expressed, the protein was used in preliminary immunisation trials in mice, rabbits and calves. In this way, the potential of this particular sporozoite recombinant protein as vaccine material for tropical theileriosis was assessed.

1.2. THEILERIA ANNULATA AND TROPICAL THEILERIOSIS

1.2.1. The parasite

Two scientists taking part in a rinderpest control programme in, what was then, Southern Russia, noticed and investigated a fatal disease afflicting the Transcaucasian cattle of the region and first described the parasite now known as Theileria annulata (Dschunkowsky and Luhs, 1904). Observing the round or oval shape of this parasite and comparing it with that seen by Koch (1903a) in East Coast fever and described as Piroplasma parvum by Theiler (1904), they named it Piroplasma annulatum. Bettencourt, Franca and Borges (1907) proposed that Piroplasma annulatum and Piroplasma parvum be placed in a new genus, Theileria, on the basis of the distinctive shape of their erythrocytic stages. The existence of a schizont stage in several organs was revealed (Dschunkowsky and Luhs, 1909) and du Toit (1918) used this characteristic to define members of the Theileria genus. This definition stands today and all members of the Theileria genus have an exoerythrocytic stage which multiplies by schizogony in lymphoid mononuclear cells or cells from which they derive (Barnett, 1968). This distinguishes them from the closely related Babesia parasites which, if the controversially placed Babesia equi (Laveran, 1901) is overlooked, multiply exclusively during an erythrocytic stage.

Levine (1988) gives an up-to-date formal classification of Theileria which is shown below.

Phylum	AMPICOMPLEXA
Class	ACONOIDASIDA
Order	Piroplasmorida
Family	Theileriidae
Genus	<u>Theileria</u>

Theileria annulata (T. annulata) is synonymous with T. dispar and Gonderia annulata in the major reviews of this species by Sergent et al. (1945) and Neitz (1957) respectively.

The most important species of Theileria infecting cattle are listed in Table 1 together with their main features. Detailed accounts of these species are given by Barnett (1968), Uilenberg (1981a and b) and Uilenberg, Perié, Lawrence, de Vos, Paling and Spanjer (1982).

Hereon this review concentrates on T. annulata, the species studied in this thesis, with reference to T. parva, the cause of East Coast fever (ECF, Theiler, 1904), where research on this parasite has contributed to the understanding and knowledge of T. annulata and tropical theileriosis.

Until Sergent, Donatien, Parrot and Lestoquard (1931) effected biological transmission of T. annulata using the two host tick, Hyalomma detritum (syn. Hyalomma mauretanicum), its natural mode of transmission was unknown. By this time there were reports of bovine theileriosis distinct from ECF from numerous countries in Africa, Asia and Europe as summarised by Neitz (1957).

The demonstration of Hyalomma ticks as the natural vector and evidence from cross immunity trials (Adler and Ellenbogen, 1935, 1936; Sergent, Donatien, Parrot and Lestoquard, 1937; Sergent, Parrot, Lestoquard and Delpy, 1939a) indicating an immunological relationship between different isolates, led to the conclusion that the same parasite was involved in all three continents. The distribution of endemic theileriosis is therefore largely determined by that of its vector tick, Hyalomma, (Robinson, 1982) and is now known to exist in a wide geographic belt extending from Southern Europe and Northern

Table 1 Pathogenic Theileria species infecting cattle

<u>Theileria</u> species	Host	Geographical distribution	Disease	Severity of disease	Vector tick	Reference
<u>T. annulata</u>	Cattle Domestic buffalo (<u>Bubalus</u> <u>bubalis</u>)	Southern Europe and North Africa through Middle East to India and Southern USSR	Tropical theileriosis Mediterranean Coast fever	Moderate to severe	<u>Hyalomma</u> spp.	Dschunkowsky and Luhs (1904)
<u>T. parva</u> group:					<u>Rhipicephalus</u> spp.	Theiler (1904)
<u>T. parva parva</u>	Cattle Buffalo (<u>Syncerus</u> <u>caffer</u>)	East and Central Africa	Classical East Coast fever	Severe		
<u>T. parva</u> <u>lawrencei</u>	East and Central Africa	Corridor disease (cattle)	Severe			
<u>T. parva bovis</u>	Cattle ?Buffalo	Central Africa	January disease	Moderate to severe		
<u>T. mutans</u>	Cattle Buffalo	Africa Caribbean	Benign theileriosis Turning sickness	Usually none to mild but can be severe	<u>Amblyomma</u> spp.	Theiler (1906)
<u>T. orientalis</u> (<u>T. sergenti</u>)	Cattle Domestic buffalo	Worldwide Pathogenic strains in Russia, Japan, Far East	Theileriosis	Mild to moderate	<u>Haemaphysalis</u> spp.	Yakimoff and Dekhtereff (1930)
<u>T. taurotragi</u> (<u>Taurotragus</u> <u>oryx</u>) Cattle	Eland (<u>Taurotragus</u> <u>oryx</u>) Cattle	East and Central Africa	Theileriosis	Mild, occasionally severe	<u>Rhipicephalus</u> spp.	Martin and Brocklesby (1960)

Africa through the Middle East to South USSR and the Indian sub-continent (Purnell, 1978).

Domestic cattle both of Bos taurus and Bos indicus types are most commonly affected by T. annulata infection with domestic water buffalo (Bubalus bubalis) also being susceptible (Dhar, Bhattacharyulu and Gautam, 1973). There are isolated reports of other members of the family Bovidae becoming infected, namely the North American bison (Bison bison) and Tibetan yak (Bos grunniens) (Barnett, 1977), but no wild Bovidae are known to act as reservoirs of infection (Uilenberg, 1976). Although numerous attempts have been made to infect laboratory species with T. parva in particular (Dschunkowsky and Luhs, 1904; Brocklesby and Vidler, 1961, 1962; Guilbride, 1963) these have not met with success and there is no true laboratory animal model for Theileria species.

Transmission of theileriosis by the vector tick has been extensively reviewed (Sergent et al., 1945; Barnett, 1968; Robinson, 1982). Only the essential features of natural transmission of T. annulata are detailed below.

Ticks becoming infected by engorging on a patently infected animal will transmit infection in the following instar, but there is no persistence of infection from one tick generation to the next. Thus, transmission is transtadial but not transovarial. The Hyalomma species thought to be active vectors in the field may be classified according to the number of different hosts they feed on in one generation. Given that there are three instars per generation, there are one host (Hyalomma scupense, Markov, 1962), two host (Hyalomma detritum, Sergent et al., 1931), two or three host (Hyalomma

dromedarii, Tselishcheva, 1940) and three host (Hyalomma anatolicum, Kornienko and Shmyreva, 1944) ticks. The relative importance of these different species in transmitting theileriosis and the seasonality of disease outbreaks in part reflects this variation in the tick's biology. Transmission depends on an immature stage engorging on an infected bovine host, dropping off, moulting and reattaching to a fresh susceptible bovine host within a single generation (Robinson, 1982). In two host ticks, larvae or nymphs become infected on the first host before attaching to the second host and transmitting infection as the adult. Three host ticks can transmit infection as either nymphs or adults as they use a fresh host for each blood meal (Arthur, 1962; Robinson, 1982). The situation is complicated by the fact that any of these tick stages may feed on non-susceptible hosts. Sergent, Donatien, Parrot and Lestoquard (1936) were first to notice that three host ticks infected as larvae and then engorging as nymphs on a non-susceptible host were still able to transmit infection in the adult stage. This was confirmed by Bhattacharyulu, Chaudhuri and Gill (1975) who fed infective nymphs on a rabbit and found that the resultant adults were still infective. Adding to the complexity of transmission pattern is the observation that male adults may interrupt feeding to change hosts so making it possible for one tick to infect more than one host in the same season (Sergent et al., 1945). To explain how Hyalomma scupense, a one host tick, has been incriminated as a vector, Markov (1962) proposed that unfed adults migrated between animals.

Regardless of the above variables, two features of the transmission of T. annulata are clearly of particular interest with regard

to this study. Firstly, experiments by Sergent et al. (1936) showed the moulting process of the tick to be essential for the parasites ingested in the previous instar to develop and become infective. Secondly, until recently the belief that an initial feeding period of one to three days was required before ticks were able to transmit theileriosis was widely held (Sergent et al., 1936; Gautam, 1978; Samish and Pipano, 1978; Singh, Jagdish, Gautam and Dhar, 1979). Whilst this is an important feature and indicates that the parasite requires a period of maturation to its infective form, the stimulus of a blood meal is not an absolute requirement. Mazlum (1969) reported transmission of T. annulata by crushed infected Hyalomma dromedarii which were unfed. Work by Samish (1977 a and b) to investigate this observation indicates that high temperatures and humidity levels can substitute for this initial period of feeding.

1.2.2. Importance

Tropical theileriosis has long been recognised as a tick-borne disease of particular importance where the improvement of local dairy and beef breeds is being attempted in tropical and subtropical areas infested with the parasite (MacHattie, 1935; Sergent et al., 1945).

Exotic breeds have been and are being imported into such areas to improve the genetic potential of the indigenous stock and tropical theileriosis frequently takes its toll of these and their crossbred progeny. At best, theileriosis makes it essential for certain disease control measures to be instituted and diligently practised; at worst it can have devastating effects on a livestock improvement programme even leading to its failure. In the early 1920s for example, 16

Ayrshire bulls were imported into Iraq to obtain high yielding cross-bred milking cows (MacHattie, 1935). In the absence of any preventive measures against tick-borne diseases at dairies, the crossbred calves succumbed to theileriosis with 60% of the calf crop dying within three months of birth and those surviving having shown signs of infection and consequently retarded early growth. This was no isolated report; Rafyi and Maghani (1962) and Purnell (1978) describe the serious threat posed by T. annulata in Iran and Turkey respectively. Yousif (1969) recorded 44% of adult Friesians imported into Iraq becoming clinically infected where tick control was inadequate, with 29% of these dying.

The World Bank funded "Operation Flood" in India is in jeopardy because of the resistance being shown by farmers to the introduction of crossbred animals which are central to the aim to increase milk supplies. Part of this resistance is as a result of the greater susceptibility of crossbreds to disease amongst which theileriosis is one of the most important (Hashemi-Fesharki, 1988). Each mature cow is worth 3,000 Rupees, if she dies the average farmer and his family lose the equivalent of four months income, a vast sum compared to the loss of one of his local cows or buffalo (Mergos and Slade, 1987). Until effective tropical theileriosis immunisation reaches these areas one can understand the reluctance of these farmers to take on such a risk. That India is committed to a strategy of crossbreeding is clear from the fact that, by 1987, 48 frozen semen stations had been established allowing a total of 8.4 million inseminations with exotic semen annually and with plans to increase this number (Acharya and Chatterjee, 1987).

1.2.3. Life cycle

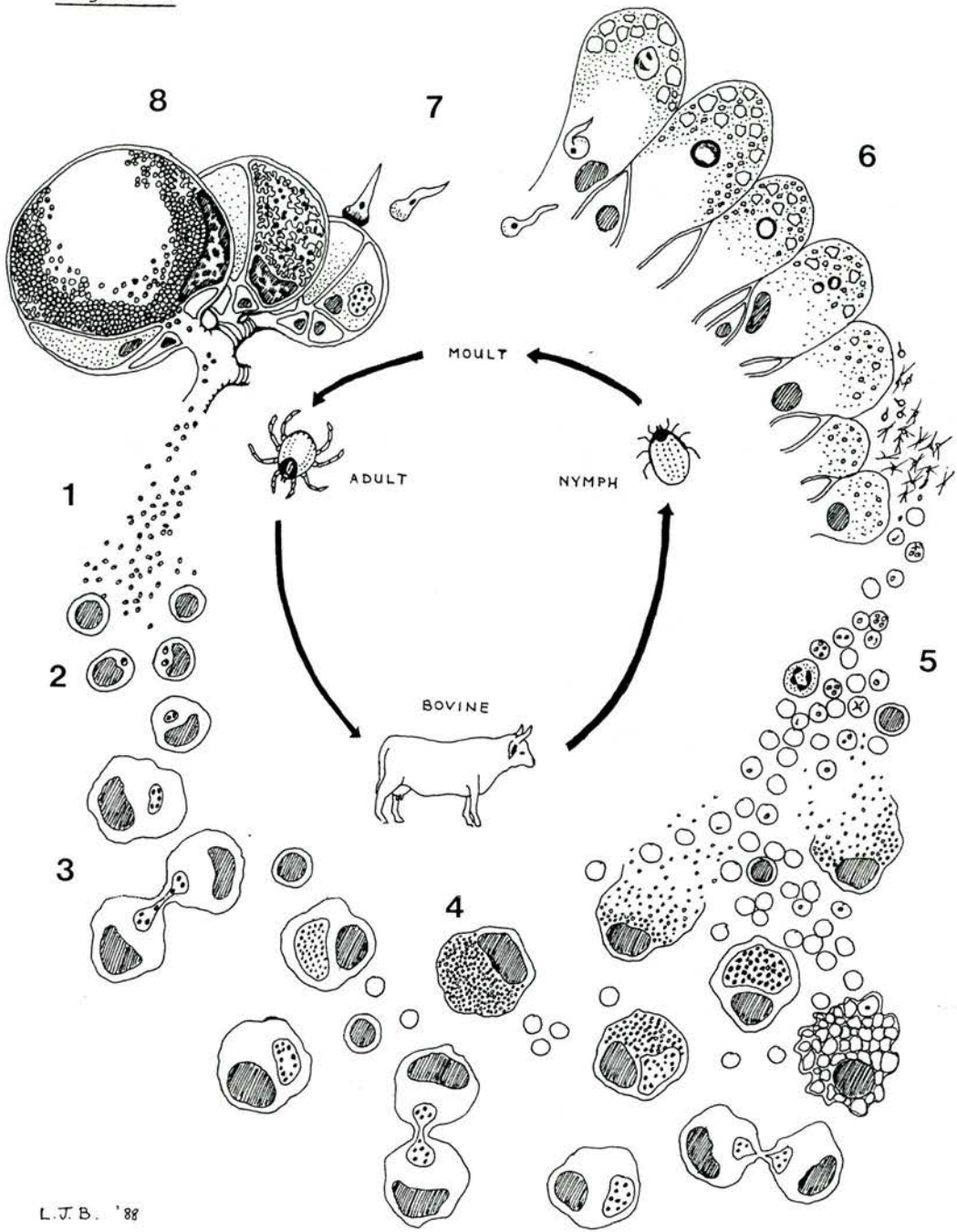
Detailed descriptions of bovine Theileria stages have been given by Cowdry and Danks (1933), Reichenow (1940), Sergeant et al. (1945) and Wilde (1967) while structural studies of tick stages of T. parva were made by Cowdry and Ham (1932) and Reichenow (1940), these were reviewed and updated for T. annulata by Schein, Buscher and Friedhoff (1975) and Schein and Friedhoff (1978). Together these authors have contributed to the present understanding of the life cycle which is illustrated in Figure 1 and summarised below.

Mature infective sporozoites are inoculated into the bovine host in the saliva of a feeding tick where they enter mononuclear cells. In vitro studies (Jura, Brown and Kelly, 1983) have shown this invasion to occur within minutes and the fact that the parasites cannot be detected at the site of a tick bite (Cowdry and Danks, 1933; Wilde, 1967) suggests that entry into host cells is equally rapid in vivo. Once interiorised, the sporozoite enlarges to form a transient trophozoite stage which develops into a macroschizont (Jura et al., 1983). At the same time, the infected cell becomes transformed and begins to divide (Hulliger, Wilde, Brown and Turner, 1964). The interaction between parasite and host which results in this disruption of normal cell growth control is poorly understood. Recent work has revealed DNA sequences homologous to oncogenes in the genome of T. annulata (Dyer and Tait, 1987); such oncogenes may be responsible for stimulating cell division. Whatever the mechanism operating the result is a fascinating example of subversion of the immune system to the benefit of the parasite (Bloom, 1979) since the macroschizont divides in synchrony with the mononuclear host cell

Figure 1 Life cycle of Theileria annulata

- 1 Sporozoites entering bovine host in tick saliva
- 2 Trophozoites in mononuclear cells
- 3 Macroschizonts in transformed mononuclear cells
- 4 Microschizont containing merozoites in transformed mononuclear cells
- 5 Piroplasms in erythrocytes
- 6 Gamogony cycle in tick gut cells
- 7 Kinetes in haemolymph
- 8 Sporogony cycle in tick salivary gland

Figure 1



resulting in an exponential increase in the number of lymphoblastoid cells, each of which is parasitised (Hulliger et al., 1964). This phenomenon was only described fully by observing T. parva infected cells in in vitro cultures (Hulliger et al., 1964). Similar observations had been made in vivo by Reichenow (1940) who had believed, correctly, that the macroschizont nuclei divide by binary fission and synchronous division of macroschizont and host cells had also been described by de Martini and Moulton (1973a) in histopathological sections of T. parva infected lymph nodes.

Macroschizont-infected cells disseminate around the body (de Martini and Moulton, 1973b) and a proportion differentiate into microschantons (Jarrett, Crighton and Pirie, 1969). The host cell is disrupted to release merozoites; the only bovine stage, other than the sporozoite, found extracellularly. These merozoites are infective for, and enter, erythrocytes and develop into piroplasms. Whilst intraerythrocytic schizogony of T. annulata is commonly observed (Conrad, Kelly and Brown, 1985), rupture of infected erythrocytes and subsequent reinvasion by the released piroplasms has not been shown to occur (Conrad, 1983). It is this piroplasm stage which is ingested by ticks as they take a blood meal.

The two main parasitic stages, macroschizont and piroplasm, occurring in the bovine phase of the life cycle are responsible for the clinical signs and pathology observed. In the early stages of infection when piroplasm parasitaemia is low and there is minor reduction in red blood cell count, calves die primarily of the effects of the proliferating macroschizont-infected cells which have disseminated around the body (de Martini and Moulton, 1973b; Eisler, 1988).

This uncontrolled mononuclear cell proliferation exerts a combination of effects. Apart from impaired function and destruction of lymphoid tissue, which is reflected clinically as a reduction in the number of circulating white blood cells, major organs are affected such as the lungs, liver, kidneys and adrenals which are infiltrated by parasitised lymphoblastoid cells as studied in T. parva infection by Steck (1928), Cowdry and Danks (1933) and de Kock (1957). Calves infected with T. annulata often survive the early stages of disease only to succumb to the pathogenic effects of the piroplasm stage (Barrett, 1977). A high piroplasm parasitaemia, sometimes in excess of 90%, may develop and is accompanied by a progressive reduction in red blood cell count (Hooshmand-Rad, 1976). The profound anaemia which can result is often fatal (ibid).

The piroplasms ingested by the tick however, ensure the survival of Theileria and the completion of the life cycle. Inside the tick gut, the ingested erythrocytes lyse and the liberated piroplasms develop into male and female gametes which fuse (Schein et al., 1975). The resulting zygote invades cells in the gut wall (Schein, Warnecke and Kirmse, 1977). Concurrently with the tick moulting, motile kinetes are released into the haemolymph in which they migrate to the salivary glands (Schein and Friedhoff, 1978). The parasite enters and resides in acinar cells of the salivary gland until sporogony occurs when mature sporozoites develop and pass into the tick saliva and so to the bovine host, thus completing the life cycle (ibid).

1.2.4. Sporogony

Studies of the development of T. parva in the tick salivary glands (Cowdry and Ham, 1932; Reichenow, 1940; Purnell and Joyner, 1968) have been complemented by similar work on T. annulata (Hadani, Pipano and Dinür, 1969; Schein and Friedhoff, 1978) and show the two parasites to behave in a very similar fashion. Kinetes invade alveolar cells of the salivary gland acini immediately before the tick moults when the alveolar cells are still immature. The kinete rounds up and becomes multinucleate, steadily increasing in size and number. Once this initial differentiation is complete and the sporonts are formed, parasite development is suspended until sporogony is stimulated. Survival of Theileria in the salivary glands is impressive; adult Rhipicephalus appendiculatus infected with T. parva can still be infective after 18 months under natural conditions (Young, Leitch, Dolan, Newson, Ngumi and Omwoyo, 1983). This obviously increases the chance of successful transmission of Theileria.

Maturation and differentiation of the parasite within the salivary glands to generate vast numbers of infective sporozoites is achieved by the process of sporogony and salivary gland activation at the start of tick feeding is coincident with a rapid renewal of parasite multiplication (Schein and Friedhoff, 1978). The infected alveolar cells enlarge massively as the parasites within divide at a tremendous rate and differentiate from sporoblasts to mature sporozoites, one sporont giving rise to thousands of sporozoites (Martin, Barnett and Vidler, 1964).

Studies on T. parva-infected Rhipicephalus appendiculatus have shown that it is between the second and third days of tick feeding

that maximal parasite proliferation occurs as monitored by incorporation of tritiated nucleic acid precursors into parasite DNA and RNA (Dobbelaere, Irvin, Spooner and Ocama, 1983). Active protein synthesis has been demonstrated to occur simultaneously by immunogold labelling of a sporozoite surface antigen (Dobbelaere, Webster, Leitch, Voigt and Irvin, 1985a). The same phenomenon undoubtedly occurs in T. annulata-infected Hyalomma, probably a little earlier after tick feeding has begun (Bhattacharyulu et al., 1975).

Sporogony is essential for the maturation of the infective sporozoites and is the time when the Theileria parasite differentiates and acquires the ability to recognise and invade bovine host cells. This is thought to be achieved by the synthesis of functionally important sporozoite surface proteins (Dobbelaere et al., 1985).

The stimulus for sporogony is not tick feeding per se since high temperatures alone can stimulate the development of infective sporozoites in both T. annulata (Samish, 1977a) and T. parva (Young, Leitch and Omwoyo, 1979). Backless tick explants cultured in vitro also showed maturation of T. parva within the salivary gland when cultured at 37°C although their infectivity was not confirmed (Bell, 1980). The common factor in those experiments which result in infective ticks is that the temperature is raised to and maintained at around 37°C. Unpublished preliminary work by Drs. Roger Hall and Philip Mason has detected the presence of "heat shock" genes in the Theileria genome. The temperature rise may activate these genes and result in the expression of proteins which stimulate sporogony. Friedhoff (1986) speculated that sporogony was the result of transformation of alveolar host cells at a molecular level in a manner

similar to that proposed for transformation of host lymphocytes by the macroschizont (Dyer and Tait, 1987). There is no evidence that this is the case and, anyway, it is normal for acinar cells of tick salivary glands to undergo major activation and transformation during feeding (Binnington, 1978).

An idea of the relative importance of high environmental temperature and humidity and tick feeding in stimulating sporogony in the field was given by Young, Leitch and Mutugi (1984). They found that although sporogony was effectively stimulated by high temperature, parasites sometimes failed to complete sporogony in unfed ticks, maybe because tick feeding supplies nutrients or salivary gland changes essential to the process of sporogony. Nevertheless sporogony occurring in unfed ticks has important epidemiological implications since it may allow a tick to be infective as soon as it attaches and before acaricides, which are the primary defence against tick-borne diseases, can take effect.

1.2.5. Sporozoite entry into mononuclear cells

The precise interaction between T. annulata sporozoites and bovine mononuclear cells resulting in sporozoite entry has been studied in vitro by Jura et al. (1983). Whilst the mechanism would be expected to be similar for different Theileria species, these authors found some notable differences from those working on T. parva (Fawcett, Doxsey, Stagg and Young, 1982). Both groups agreed that the entry process was rapid, occurring within the first five minutes of contact between sporozoites and cells. The T. annulata sporozoites, averaging 0.9 μm in length, attached to target cells by their basal

end while T. parva sporozoites made contact in any orientation. Following initial contact, sporozoite and bovine cell membranes became progressively more closely apposed and entry of both parasites occurred by endocytosis involving an invagination of the host cell membrane which remained intact. Jura (1984) believed this to be an active, temperature dependent, energy requiring process in direct contrast to Fawcett et al. (1982) who detected no reduction in invasion when the temperature was lowered to 1-2°C, suggesting that the process was a passive one. In neither case did there appear to be an obvious role in invasion for the pleiomorphic rhoptries located at the apical end of the sporozoite. Immunoelectron studies of sporozoite entry were performed using protein A-colloidal gold conjugate to visualise a monoclonal antibody which binds to the T. parva sporozoite surface (Webster, Dobbelaere and Fawcett, 1985). The results were very revealing; free sporozoites were labelled but those inside cells were not and local areas of label were visible on the surface of infected cells. Immunofluorescence using similar material lent supporting evidence that the sporozoite surface coat is shed during entry into the host cell (Dobbelaere et al., 1985a).

Having gained entry to the host cell the Theileria parasite has to avoid destruction by lysosomes which could fuse with the invaginated portion of host cell membrane (Hirsch, 1972). It appears that its survival is achieved through the action of micronemes within the sporozoite which causes dispersal of the host cell membrane or parasitophorus vacuole, this suggestion is by inference from the observation that the micronemes and host cell membrane disappear at the same time (Fawcett et al., 1982).

Although no specific receptor sites have been identified, certain mononuclear cell subsets are markedly more susceptible to infection with T. annulata than others; class II bearing cells, monocytes and B cells being highly susceptible (Spooner, Innes, Glass and Brown, 1988). If this is due to selective sporozoite infectivity, specific recognition would have to occur, presumably through receptor interaction.

1.2.6. Immunity

a) General

Dschunkowsky and Luhs (1904) tried and failed to transmit T. annulata artificially using infected blood. They repeated the attempt using recipient cattle obtained from a theileriosis free area and were successful (Neitz, 1957). This was the first indication that cattle could resist infection with T. annulata.

Subsequently, cross-immunity trials were widely used to determine the relationship of Theileria parasites involved in different outbreaks of the disease (Sergent et al., 1937; Adler and Ellenbogen, 1935, 1936). While there is no cross-immunity between T. annulata and other Theileria species (Sergent et al., 1945; Neitz, 1957) but strong immunity to homologous challenge in recovered cattle, the cross-immunity between different T. annulata stocks is very variable. Parasites isolated from within a geographic area can differ as much from each other as from parasites originating from geographically distinct regions (Sergent et al., 1937; Sergent et al., 1939a; Gill, Bansal, Bhattacharyulu, Kaur and Singh, 1980). T. annulata stocks have been shown to differ on the basis of glucose phosphate isomerase

isoenzyme patterns (Melrose and Brown, 1979; Melrose, Brown and Sharma, 1980) and schizont staining monoclonal antibody profiles (Shiels, McDougall, Tait and Brown, 1986b). Similar monoclonal antibody reagents detect antigenic diversity in T. parva isolates (Pinder and Hewett, 1980; Minami, Spooner, Irvin, Ocamo et al., 1983) and DNA probes have also been produced which reveal diversity at the level of the genome (Conrad, Iams, Brown, Sohanpal and ole-Moi Yoi, 1987; Allsopp and Allsopp, 1988). However, none of these three approaches have yet been able to predict cross-immunity and so replace in vivo cattle experiments.

Some features of the immunity engendered in recovered cattle were revealed in a series of experiments by Sargent et al. (1945) using large numbers of cattle. The resistance to homologous challenge acquired after a single episode of infection did not break down in the face of repeated challenge. However, in the absence of challenge immunity began to decline after the first six months. They also made the observation that immunity conferred by tick bite, in other words by sporozoites, was stronger than that resulting from the inoculation of blood, implying that life cycle stages before the schizont are responsible for stimulating a component of the protective immune response (Sargent, Donatien, Parrot, Lestoquard and Delpy, 1939b). Pipano (1978) found the same to be true; adult dairy cows immunised with schizonts and then challenged with sporozoites frequently suffered heavy clinical symptoms compared to cows immunised with sporozoites before exposure to the same challenge. Perhaps eliciting the same mechanism, Musoke et al. (1984) were able to obtain cross-immunity against two T. parva stocks which normally

broke through the immunity stimulated by just a single dose of a third stock. They achieved this by giving repeated inoculations of sporozoites of the immunising stock before challenging with the others. Their conclusion was that multiple exposure to sporozoites may broaden the spectrum of immunity.

It is possible to transmit T. annulata by the inoculation of blood taken from an animal in the early stages of a patent primary infection (Sergent et al., 1945; Tsur, 1965) and animals recovering from infection established in this manner are immune to homologous challenge. The sporozoite stage is therefore not essential to the development of immunity although it may significantly modify the immunity stimulated as mentioned above.

b) Humoral immune responses

An offshoot of attempts to find an effective treatment for ECF led to field veterinarians in East Africa using immune serum in passive transfer experiments. Robson, Yeoman and Ross (1961) set up an experiment to try and confirm reports from Tanganyika that the inoculation of serum from cattle in endemic areas into cattle with ECF was curative (Garden, Tanganyika Territory Report; cited by Robson et al., 1961). They immunised cattle by exposing them to field challenge under tetracycline cover over a 28 day period.

Immune serum was transferred to 15 cattle and resulted in all surviving exposure to the parasite. Other workers were not able to repeat this (Wilde, 1967; Muhammed, Lauermann and Johnson, 1975) and the possibility that effective serum levels of tetracycline had persisted could account for the results. To produce immune serum

comparable to that used therapeutically in Tanganyika one would have to feed vast numbers of infected ticks on experimental cattle since animals in endemic areas are being challenged by several hundred infected ticks each week. It is interesting to speculate that this constant and long term sporozoite challenge may enhance the relative importance of the humoral component of immunity. Earlier, Koch (1903b) had claimed some degree of success in a trial treatment of clinical ECF using serum from cattle immunised by blood inoculation on four occasions but Theiler (1907) was unable to repeat this.

In response to the controversy surrounding the role of antibodies in immunity to Theileria infections, Muhammed et al. (1975) took advantage of the recently developed indirect fluorescent antibody test (IFAT) used to diagnose exposure to Theileria infection (Lohr and Ross, 1969; Pipano and Cahana, 1969; Burr ridge, 1971; Burr ridge and Kimber, 1972). They measured IFAT titres to T. parva macroschizont and piroplasm antigens to ascertain that donor sera had high antibody levels to these stages and that recipients had no serological evidence of previous exposure to ECF, both being possible reasons for the conflicting results obtained previously. They failed to alter the course of T. parva infection in the recipients which received serum before and on the day of infection with sporozoites. No information was available on the ant sporozoite antibody titres.

Uncertainty also surrounds the role played by maternal antibody in colostrum digested by calves born to immune dams and early reports conflict (Wilde, 1967). Burr ridge and Kimber (1973) demonstrated that the majority of a group of immune cows produced colostrum containing significant levels of antibody but only two of the eight calves

feeding on this colostrum reflected this in a significant rise in serum antibody levels. All eight calves were fully susceptible to challenge (Cunningham, Urquhart, Brown, Burr ridge and Morzaria, 1988). Again only antischizont and antipiroplasm IFAT titres were measured. There is still much interest in the possible role of colostral antibody, particularly ant sporozoite antibody, in protecting young calves born to dams in areas where they are under constant tick challenge and a research project in Muguga, Kenya is currently investigating this particular question (A.S. Young, personal communication).

Antibodies reacting with the macroschizont and piroplasm stages consistently develop in cattle exposed to Theileria infection (Burr ridge and Kimber, 1972) and are useful in making the IFAT a reliable diagnostic test. However, there is now enough evidence to conclude that they do not play a role in protective immunity. Some of this evidence comes from in vivo work in which cattle immunised with extracted schizont or piroplasm antigens develop antimacroschizont or antipiroplasm antibody levels comparable to those of Theileria-immunised cattle but remain susceptible to challenge (Wagner, Duffus and Burr ridge, 1974). Complementary in vitro studies summarised by Wilde (1967) showed that the infectivity and multiplication of T. parva schizont-infected cells in culture were unaffected by exposure to immune serum even when complement was added (Hulliger, Brown and Wilde, 1965). Sera from cattle immunised with sporozoites do not recognise the surface of either schizont-infected mononuclear cells (Duffus, Wagner and Preston, 1978; Creemers, 1982) or piroplasm-infected red blood cells (Hall, 1988) which may explain why antibody does not appear to affect these stages (Wilde, 1967; J. Glascodine, personal communication).

All the experiments mentioned above once again failed to assess levels of antisporozone antibody in any form. This may have been a significant omission; the role of antisporozone antibody has been under particularly close scrutiny since Gray and Brown (1981) reported the ability of immune bovine serum to significantly neutralise T. annulata infectivity for bovine mononuclear cells in vitro. The same activity was found in serum from T. parva immune cattle and became more marked when cattle were challenged several times with large numbers of infected ticks (Musoke, Nantulya, Buscher, Masake and Otim, 1982). Preston and Brown (1985) also found the sporozoite neutralising activity of serum to increase after sequential T. annulata sporozoite challenge and showed that two independent inhibitory mechanisms were operating; one reducing sporozoite invasion of cells and the other retarding the transformation of trophozoite infected mononuclear cells into proliferating macroschizont infected cells.

The sporozoite neutralising action of immune serum was effective against homologous and heterologous T. annulata sporozoites but T. parva immune serum had little effect on the infectivity of T. annulata sporozoites (Gray and Brown, 1981).

More recently, two groups working on T. parva have each produced an antisporozone monoclonal antibody with similar ability to neutralise sporozoite infectivity (Dobbelaere et al., 1984; Musoke et al., 1984). Significantly, both monoclonals neutralised sporozoites from a range of T. parva stocks, some of which do not cross protect in in vivo cross-immunity trials.

c) Cell mediated responses

Evidence that cattle which recover from infection with material containing schizont-infected cells are immune (Sergent et al., 1945; Neitz, 1957; Tsur, 1965) and that antibody against the macroschizont stage is not protective (Hulliger et al., 1965; Wilde et al., 1974) led those working on immunity to Theileria to consider it likely that cell mediated mechanisms, acting on the macroschizont or the macroschizont infected cell, were responsible for protective immunity.

Emery (1981) strengthened this belief when he succeeded in transferring immunity to ECF between calves by inoculating susceptible calves around the time of infection with syngeneic thoracic duct lymphocytes harvested from their T. parva immune chimaeric twins. The protection afforded by this cell transfer, while an exciting result, gave little indication as to the nature of the effector mechanisms. This aspect was being investigated for T. parva in vitro using peripheral blood mononuclear (PBM) cells isolated from cattle. Pearson, Lundin, Dolan and Stagg (1979) had demonstrated mixed lymphocyte reactions in PBM from immune and non-immune cattle when exposed to T. parva-infected cell lines in vitro. Functional activity was assayed in these responding PBM populations and only PBM from ECF immune cattle were found to possess cytotoxicity directed against T. parva transformed cell lines (ibid). Emery, Eugui, Nelson and Tenywa (1981) and Eugui and Emery (1981) showed this cytotoxicity to change from non-specific to specific with the development of immunity such that immune cattle possess genetically restricted cytotoxic cells which lyse autologous but not allogeneic infected target cells.

Morrison, Goddeeris, Teale, Baldwin et al. (1986a) give a comprehensive account of the current knowledge of cell mediated immune mechanisms operating on T. parva macroschizont-infected cells on which much of the work on immunity to T. annulata was based, as reviewed by Hall (1988).

Immunity to the macroschizont stage of T. annulata appears to be the result of the combined effects of three immune cell types, two with cytotoxic and one with cytostatic activity. Cells from cattle destined to recover from primary infection show two waves of cytotoxic activity. The first exhibits BoLA restriction, where BoLA defines the Class I bovine major histocompatibility complex (MHC) profile. Thus, these cytotoxic cells will kill infected cell lines which are BoLA matched (i.e. have the same BoLA type as themselves) and not those which are mismatched (Preston et al., 1983). The second wave of cytotoxic cells do not show BoLA restricted killing (ibid). Over a more prolonged period, which includes the time when cytotoxic cells are active, adherent cells exhibit cytostatic effects on macroschizont-infected cells (Preston and Brown, 1988). This cytostasis is not BoLA restricted and is mediated via a soluble factor. Preston and Brown (1988) speculate that tumour necrosis factor (Cerami and Beutler, 1988) may be responsible and may also account for the inhibition of transformation of trophozoite-infected cells by immune serum (Preston and Brown, 1985; Hall, 1988). The adherent cells secreting this factor are presumed to be macrophages. The identify of the cytotoxic cells has been inferred primarily from their properties and also partly by analysis using monoclonal antibody markers for specific cell types (E.A. Innes, personal communication). Those showing BoLA restriction

appear to be genetically restricted cytotoxic T cells, while the non-restricted cytotoxic cells appearing later are probably natural killer cells (Preston et al., 1983). On challenge, similar effector mechanisms are activated. Cytostatic cells appear and show the same kinetics as during primary infection (Preston and Brown, 1988). The cytotoxic cells appear a little earlier and again show two peaks of activity. However, both peaks tend to consist predominantly of BoLA restricted cytotoxicity (Preston et al., 1983). This reflects the activation of memory cytotoxic T cells rather than natural killer cells which do not become established in the immunological memory (Roitt, Brostoff and Male, 1985).

These cytotoxic and cytostatic cells are assumed to recognise either a parasite antigen or an altered host antigen on the surface of the macroschizont-infected cell. Shiels et al. (1986a) have raised monoclonals to T. annulata-infected cell lines. One of these not only reacts specifically with the surface of infected cells but also mediates complement lysis of such cells (Preston et al., 1986). It is not yet known if this antigen is recognised by bovine T cells.

The cell mediated immune responses to sporozoite, merozoite and piroplasm stages have not been investigated. Eisler (1988) remarked upon the appearance of large numbers of macrophages in the lymph node draining the site of infection. These macrophages were present in the lymph nodes of calves surviving the acute stages of infection, whether or not the calves were destined to recover, and exhibited marked erythrophagocytosis - both uninfected and infected erythrocytes being ingested. Whether this represents a significant cell mediated response with relevance to protective immunity, akin to the role of

macrophages in immunity to babesiosis (Losos, 1986) has yet to be determined. The failure of immune serum to recognise the surface of the piroplasm-infected cell suggests that opsonisation by antibody is not a feature of antitheilerial immunity (Glascodine, personal communication).

1.2.7. Immunisation

Present methods of immunisation against Theileria depend on establishing active infection in the bovine host using either sporozoites or schizont-infected cells (Pipano, 1977; Brown, Crawford, Kanhai, Njuguna and Stagg, 1978; Radley, 1978).

Infected blood containing schizonts of a mild T. annulata strain was being used very early on as a crude form of vaccination (Sergent, Donatien, Parrot and Lestoquard, 1932). This meant that there was little impetus for developing an alternative type of immunising material for tropical theileriosis (Brown, 1981). In contrast, transmission of T. parva using infected blood proved highly inconsistent and was abandoned as a means of immunisation (Theiler, 1912; Theiler and du Toit, 1929). Largely for this reason, most work on immunisation methods involving sporozoites has been performed using T. parva and, following the encouraging results using a blood vaccine for T. annulata (Sergent et al., 1932), methods using schizont material have been developed in laboratories working on tropical theileriosis (Tsur and Adler, 1965; Pipano and Tsur, 1966). In both instances, immunisation is believed to be effected solely as a result of infection of the animal.

a) Immunisation with sporozoites

The principles of the infection and treatment method of immunisation developed for ECF (Radley, 1981) can be applied to T. annulata (Samish and Pipano, 1981). In this, cattle are deliberately infected with virulent sporozoites and simultaneously treated chemoprophylactically such that mild or inapparent infections result but immunity is still able to develop (Radley, 1981).

The achievement of a practical infection and treatment method followed on from three important developments: infection of cattle with ground up ticks (Theiler and du Toit, 1929), the cryopreservation of ground up infected tick suspensions allowing stocks of sporozoites of proven and titrated infectivity to be stored (Cunningham, Brown, Burridge and Purnell, 1973), and the observation that administration of tetracyclines during the prepatent period of ECF limits the clinical reaction (Neitz, 1953). The considerable effort involved in refining this method to its present state has been reviewed and summarised by Brocklesby and Bailey (1965), Cunningham (1977), Purnell (1977) and Radley (1981). The chemoprophylactic component still relies primarily on the use of tetracyclines usually given as a long acting formulation of oxytetracycline (Terra LA, Pfizer) on day 0 or as a short acting preparation on days 0 and 3 or 4 after infection (Gill, Bhattacharyulu, Kaur and Singh, 1978; Young et al., 1988). More recently the discovery of a new series of theilericidal quinones; menoctone (McHardy, Haigh and Dolan, 1976), parvaquone (Clexon, Wellcome; McHardy and Morgan, 1981) and buparvaquone (McHardy, Wekesa, Hudson and Randall, 1985), has made another group of compounds available for chemoprophylaxis and chemotherapy of theileriosis (Dolan, 1981).

There are good indications that T. annulata is also susceptible to their effects (Brown and Masion, 1981; Taylor, 1985), buparvaquone being particularly effective. The observation that the early use of menoctone could block infection so effectively that no immunity developed (McHardy et al., 1976) served as a warning that the timing of the chemoprophylactic use of parvaquone and buparvaquone may have to be delayed until several days after the inoculation of sporozoite stabilate to permit effective immunisation (Dolan, 1981; Young et al., 1988).

As part of a series of experiments with the tissues of T. parva-infected ticks, inactivated sporozoites were administered with adjuvants. These caused no reaction to inoculation and left the recipient cattle susceptible to ECF (Wilde, Brown, Hulliger, Gall and MacLeod, 1968). Subsequent to this and in the light of the success of those working on malarial sporozoites, the effects of gamma irradiation on the infectivity and immunogenicity of Theileria sporozoites were studied (Cunningham, Brown, Burridge, Musoke, Purnell and Dargie, 1973; Purnell, Burridge, Cunningham, Emu, Irvin, Ledger, Njuguna, Payne and Radley, 1974; Srivastava and Sharma, 1977; Singh, Jagdish and Gautam, 1979). Irradiated malarial sporozoites have been used in similar experiments with some success from an early date. Mulligan, Russell and Mohan (1941) used ultraviolet light to irradiate avian malarial sporozoites and obtained a substantial degree of protection against lethal challenge. More recently, others have successfully immunised animals with the irradiated sporozoites of Plasmodium berghei (Nussenzweig, Vanderberg and Most, 1969a) and Plasmodium knowlesi (Gwadz, Cochrane, Nussenzweig and Nussenzweig, 1970).

None of the in vivo experiments using either T. parva (Cunningham et al., 1973; Purnell et al., 1974) or T. annulata (Srivastava and Sharma, 1977; Singh et al., 1979) irradiated sporozoites have shown effects which cannot be explained simply as an effective reduction in the number of infective sporozoites. There was no evidence of true attenuation; thus cattle which were immune to challenge had all shown evidence of infection establishing during immunisation while those which were susceptible had not. However, studying the effects of irradiation in vitro, Brown (1981) reported that T. annulata sporozoites irradiated at 640 Gy did lose the ability to transform to macroschizonts although they still entered host cells. Those sporozoites exposed to lower doses of irradiation still infected and transformed bovine lymphoid cells but their ability to do so was impaired, their infectivity being inversely proportional to the irradiation dose used as suggested by the in vivo results.

b) Immunisation with macroschizonts or macroschizont-infected cells

While immunisation against ECF, based on the use of the T. parva sporozoite infection and treatment method, is still in its infancy, T. annulata immunisation is being put into practice in many of the countries where tropical theileriosis is endemic. This now involves the inoculation of macroschizont-infected mononuclear cells taken from in vitro cultured cell lines (Pipano, 1977). Long term passage of such cell lines causes them to become attenuated such that their pathogenicity is markedly reduced but their infectivity is left unimpaired (Pipano and Tsur, 1966; Hooshmand-Rad, 1973). When tissue culture cells are inoculated, infection transfers from the donor cells to those of the recipient (Wilde, Hulliger and Brown, 1966; Brown et al., 1978).

This transfer to the cells of the animal being inoculated is believed to be essential for the stimulation of protective immunity which correlates with the knowledge that the cytotoxic T cells involved are genetically restricted (Emery et al., 1981; Eugui and Emery, 1981; Preston et al., 1983). The mechanism of transfer of the macroschizont from the cells of the inoculum to those of the new host is not yet understood but it is seen that T. annulata transfers much more readily than T. parva (Brown, 1981). This inability of T. parva macroschizonts to transfer efficiently is thought to be responsible for the poor results observed when cattle are immunised against ECF with allogeneic T. parva-infected lymphoblastoid cell lines, successful immunisation only being obtained when large numbers of cells are inoculated (Brown et al., 1978; Brown, 1981). T. parva-infected cell lines which are autologous (Buscher, Morrison and Nelson, 1984) or BoLA matched with the recipient cattle (Dolan, Teale, Stagg, Kemp et al., 1984) establish infection and subsequent immunity in a majority of animals when only small numbers of cells are inoculated, comparable to the number of T. annulata-infected cells required when the BoLA relationship between the inoculated cells and the recipients is unknown (Pipano, 1981). Apart from the excessive number of cultured cells which would be required to reliably infect most cattle with T. parva macroschizont infected cells, this method of immunisation is not practical in the field because inevitably a proportion of the immunised animals would have a similar BoLA type to that of the donor cells resulting in lethal infections establishing (Morrison and Buscher, 1983). Spooner et al. (1988) suggests that this difference between T. parva and T. annulata is because they infect different cell types; T. parva transforming T

cells, B cells and null cells (Baldwin, Black, Brown, Conrad et al., 1988) and T. annulata infecting Class II positive cells, monocytes and B cells (Spooner et al., 1988).

Immunity to tropical theileriosis stimulated by immunising with attenuated allogeneic macroschizont-infected cells has two components; one being antiparasite as described earlier, the other being antiBoLA and consisting of cytotoxic T cells which recognise the allogeneic donor cells as foreign (Innes, Millar, Brown and Spooner, 1988). In an experiment involving challenge with the same material as used for immunisation, cattle showed marked antiBoLA and minor antiparasite responses (Innes et al., 1988). Evidence from this experiment and another performed by Pipano et al. (1977) suggests that a graft rejection phenomenon occurs when allogeneic cell lines are used to immunise which can significantly (Innes et al., 1988) or even totally (Pipano et al., 1977) block parasite transfer to the recipient. This has important implications, especially as this form of vaccination is being widely adopted, since if revaccination becomes necessary it may prove ineffective in boosting immunity to the parasite.

Another problem which may afflict vaccination campaigns dependent on macroschizont immunisation is that the immunity may be less broad spectrum and less complete when subjected to tick challenge than that afforded by an immunisation method based on the sporozoite stage (Sergent et al., 1939b; Pipano, 1978 and Musoke et al., 1984).

As was the case for sporozoites, inactivated schizont material has not stimulated protective immunity, whether crude (Wilde, 1967) or purified to a greater or lesser degree (Wagner et al., 1874; Pipano et al., 1977; Emery et al., 1986).

c) Immunisation with piroplasms

The possibility of immunising cattle with piroplasms remains relatively unexplored. Irvin and Gill (1981) point out that the pathogenic effects of the macroschizont stage would not be prevented and that such a vaccine would be similar to a malaria gamete vaccine (Carter and Chen, 1976) since it would benefit the host population more than it would benefit the individual. A vaccine based on the piroplasm stage might prove more beneficial in reducing the severity of T. annulata infection than T. parva because of the more severe anaemia which occurs in the former (Barnett, 1968).

Wagner et al. (1974) were unable to stimulate protective immunity in cattle immunised with extracted piroplasm antigen in spite of the high antipiroplasm antibody titres they developed. Attempts to immunise cattle using separated piroplasm-infected red blood cells have only succeeded due to contamination of the immunising material with schizont-infected mononuclear cells (Pipano, 1972).

There is no reported work on immunisation with the merozoite stage of either T. annulata or T. parva, principally because of the difficulty in obtaining reasonable quantities of this stage of the parasite (Morrison, Lalor, Goddeeris and Teale, 1986b).

1.3. RECOMBINANT DNA VACCINES

The production of recombinant DNA vaccines involves strategies designed to prepare, select, clone and express individual genes or gene fragments from pathogens of man and animals. The biotechnology, developed within the last 15 years, has given rise to, perhaps, the most dynamic and potentially rewarding of areas in the search for novel methods of disease control. It involves the splicing of a segment of foreign DNA into the DNA of a vector such as a bacterial plasmid or double stranded DNA bacteriophage. The vector is then transferred into a host cell where replication and regulated expression of the foreign DNA occurs along with the DNA of the vector. This provides a source of defined antigenic material and the basis for a genetically engineered subunit vaccine.

1.3.1. Advantages

Whether it is worth attempting to develop a recombinant vaccine depends on the characteristics of a particular pathogenic organism and whether existing methods of immunisation are satisfactory. Thus a vaccine like that for yellow fever virus which is safe, effective and long lasting (WHO, 1957) is unlikely to be superceded by a subunit vaccine. Even for those diseases where in the future a subunit vaccine could supplement or replace an existing one with advantage the changeover is likely to be slow; whole organism vaccines have a time proven effectiveness.

The most powerful drive for developing a recombinant vaccine is for diseases where there is no readily available method of immunisation. Hepatitis B is one such example. Since the virus cannot

readily be grown in culture, the surface antigen of the virus used as the vaccine had to be obtained from the plasma of chronic carriers making it both expensive and inaccessible (Arnon, Shapira and Jacob, 1983).

A closer analogy to the disease being examined in this thesis is malaria. There is a pressing need for an effective vaccine for malaria and intensive research is in the process of producing and testing recombinant proteins which represent antigens from different stages of the life cycle (Howard, 1987).

Development of a recombinant vaccine may allow a novel application in the control of a disease for which a more conventional vaccine is also used. The recombinant vaccinia virus vaccine for rabies, in which the immunogenic surface glycoprotein of the rabies virus has been inserted into the genome of the vaccinia virus, is effective by oral administration. The vaccinia virus replicates in tonsillar tissue and the amplified expression of the rabies virus glycoprotein is sufficient to stimulate high neutralising antibody titres in the mammalian host which are protective on challenge with street rabies virus (Blancou, Kieny, Lathe, Lecocq et al., 1986). Effective immunity provided by oral dosing is unique to this form of rabies vaccine and means that it could be used to immunise wildlife and so help prevent the spread of rabies.

The use of whole and particularly, live organisms as vaccines can sometimes cause individual cases, or even outbreaks, of clinical disease. This can occur through mutations of the genotype as in the reversion to virulence of attenuated live oral poliovirus vaccines (Kew, Nottay, Hatch, Nakano and Obijeski, 1981). Much too often

foot and mouth disease outbreaks have followed the use of batches of incompletely inactivated virus to vaccinate cattle (FAO, 1981). This is a risk for many vaccines and, since the starting material for their manufacture is a live, fully virulent, organism, any laboratory involved in their production is a potential source of infection. Escapes do occur, foot and mouth disease virus again being an example where this has occurred (FAO, 1981).

The motivation behind the production of a recombinant vaccine for foot and mouth disease has, therefore primarily been the need for a safe vaccine since the existing vaccine is efficient and cheap (Pereira, 1981). In fact, researchers have moved on from recombinant foot and mouth disease proteins to synthetic peptides representing portions of the immunogenic viral protein 1. One such peptide, when coupled to keyhole limpet haemocyanin and mixed with adjuvant, given on a single occasion to guinea pigs, stimulated neutralising antibody and protected them from challenge with live foot and mouth disease virus (Bittle, Houghton, Alexander, Shinnick *et al.*, 1982).

As mentioned for poliovirus, mutations can occur during replication of the attenuated vaccine organism. This is believed to be a possible origin of canine parvovirus which may have resulted from a batch of feline enteritis vaccine in which a mutation had occurred to change the host specificity of the virus (McCandlish, 1979).

These points are of direct relevance to vaccination against theileriosis. Even when properly attenuated, inoculation of schizont infected cells and the establishment of infection in the recipient can cause clinical reactions in some individuals (Pipano, 1977). In the infection and treatment method, clinical reactions are more common,

their frequency being related to the parasite stock and the chemoprophylactic regime adopted (Radley, 1981). Both methods of immunisation can result in the carrier state where immunised animals harbour low numbers of piroplasms (Sergent et al., 1945; Barnett, 1977; Radley, 1981). Carrier animals would not result from the use of a recombinant vaccine assuming the immunity it stimulated was strong enough to prevent infection reaching the piroplasm stage on challenge. The carrier state is of concern because it makes parasite available for the infection of ticks (Barnett, 1968). Situations where this matters would arise if immunised animals were moved to theileriosis free areas which have the tick vector, or if use of vaccine introduced new parasite stocks into an area and immunity to local stocks was not cross-protective. This could put unimmunised cattle at risk (Radley, 1981). Thus an effective recombinant vaccine would be safer for both immunised and non-immunised stock.

Other deleterious effects of using live vaccines which would be avoided if a recombinant one was available, include allergic reactions, immunosuppression and possible contamination with other infectious agents. The inoculation of material derived from another animal can cause reactions, particularly if repeated. Schizont infected cells constitute such material and Ouhelli, Innes, Walker, Brown et al. (1988) show that cattle immunised with 10^8 T. annulata infected allogeneic cells underwent an early temperature rise which was not seen in cattle receiving BoLA matched infected cell line. The possibility of a graft rejection type of response to repeated schizont immunisation has been discussed.

Rinderpest vaccine has been suspected of causing immunosuppression at the time of immunisation. While this was certainly a problem with the goat-adapted virus vaccine, the tissue culture derived vaccine employed now is much safer (Rossiter and Kariuki, 1987) although some still suspect the rinderpest vaccine virus could interfere with the development of immunity to other vaccines given at the same time (Jeggo, Wardley and Corteyn, 1987). In view of the fact that the Theileria parasite infects and destroys cells of the immune system, the use of vaccines which establish active infection in the host may bring Theileria under suspicion as a potential immunosuppressive agent.

The presence of contaminating infectious agents in the cells or medium of a vaccine such as that used for T. annulata must be checked for rigorously.

The use of recombinant DNA technology makes the task of producing and testing individual antigens easier in that large quantities of recombinant protein are available for purification. An antigen produced as a recombinant protein can be defined at the level of both the DNA and the amino acid sequence which can yield valuable information about both the protein and the immune response it elicits (Bachrach, 1982).

Lastly, the stability of a recombinant vaccine would make the practicalities of storage, delivery and administration significantly easier than the strict requirements for present Theileria vaccine material and its limited life once resuscitated from storage in liquid nitrogen (Brown, 1983).

1.3.2. Hybridoma technology

Hybridoma technology and monoclonal antibody production are subjects for review in their own right. With the advent of monoclonal antibody technology, a powerful tool for the identification, isolation and assessment of antigens, particularly parasite antigens, was made available. The progress towards the production of recombinant proteins as potential vaccines has been greatly facilitated by the use of monoclonal antibodies. For this reason, a brief review of the techniques involved in their production is included here.

The development of cell fusion techniques (Harris and Watkins, 1965) enabled Köhler and Milstein (1975) to produce hybridoma cell lines by fusing spleen cells from immunised mice with murine myeloma cells using inactivated Sendai virus. The standard technique for cell fusion in hybridoma technology follows the same principles but uses polyethylene glycol as the fusing agent (Goding, 1980). To ensure that only hybrids grow following cell fusion a strategy devised by Littlefield (1964) is followed in which the myeloma cell line used is a mutant lacking the enzyme, hypoxanthine guanine phosphoribosyl transferase (HGPRT). After fusion, the cells are grown in hypoxanthine-aminopterin-thymidine (HAT) medium (Szybalska and Szybalski, 1962) which contains the folic acid antagonist, aminopterin. In the presence of aminopterin, the mutant myeloma cells are unable to use salvage pathways to synthesis purines and they die. However, if fused with spleen cells, which possess HGPRT, the resulting hybrids can survive in HAT medium. Unfused spleen cells die naturally after several days in culture (Goding, 1980).

Since 1979, myeloma cell lines such as X63-Ag-8 clone 653 (Kearney, Radbruch, Liesegang and Rajewsky, 1979) have been available. These are unable to synthesise or secrete their own immunoglobulin chains. Thus, antibodies secreted by hybridomas established using these myeloma cells, derive only from stimulated spleen cells. Once hybridomas secreting the desired antibodies have been identified they are cloned (Goding, 1980; Oi and Herzenberg, 1980) to obtain cell lines, each of which secretes unlimited quantities of monoclonal antibody which, by definition, is specific for a single antigenic determinant.

Pearson, Pinder, Roelants, Kar, Lundin, Mayor-Withey and Hewett (1980) describe the application of the above technology to the derivation and detection of antiparasite monoclonal antibodies, in particular to Trypanosoma and Theileria species. Monoclonal antibodies have been raised against most parasitic protozoa and tend to fall into two categories; those useful in diagnosis and detection of antigenic diversity and those used to attempt to identify protective antigens. Monoclonals of the former type have proved useful in characterising different isolates or stocks of Plasmodium falciparum (McBride, Walliker and Morgan, 1982; Knowles, Davidson, McBride and Jolley, 1984), Leishmania braziliensis (McMahon, Pratt, Bennett and David, 1982), Theileria parva (Pinder and Hewett, 1980; Minami, Spooner, Irvin, Ocama, Dobbelaere and Fujinaga, 1983), Theileria annulata (Shiels, McDougall, Tait and Brown, 1986b) and others.

There are numerous examples of monoclonal antibodies raised against parasite antigens which may play a role in stimulating protective immunity (Gamble, 1987). The beauty of this approach is that

the functional antiparasite activity of antibody, which by definition is known to interact with a single parasite molecule to exert its effects, can be assessed using in vivo or in vitro techniques. In this manner, some prediction can be made of the likelihood of a particular parasite antigen being a useful candidate as a vaccine.

To give a few examples; monoclonal antibodies have been selected which identify stage specific antigens of the malaria parasite. Amongst those recognising the sporozoite stage are some of particular interest which neutralise sporozoite infectivity (Yoshida, Nussenzweig, Potocnjak, Nussenzweig and Aikawa, 1980). Antigens on the blood stages of Plasmodium falciparum have been identified by monoclonals which inhibit parasite multiplication (Saul, Myler, Schofield and Kidson, 1984). Affinity purification of a major surface antigen (p 190) of late blood stages of this parasite was made possible using a monoclonal antibody (Hall, Hyde, Goman, Simmons et al., 1984). Subsequent immunisation trials using the purified protein suggested that p 190 can protect monkeys from falciparum malaria (ibid). Antigamete monoclonal antibodies raised against Plasmodium gallinaceum are able to block transmission of malaria by suppressing the infectivity of the parasites to Aedes aegypti mosquitoes (Kaushal, Carter, Renner, Grotendorst et al., 1983).

Similar work has yielded useful monoclonals against other protozoan parasites which show the ability to inhibit infective stages from entering their target cells, as described for the sporozoites of Eimeria tenella (Danforth, 1983), Eimeria bovis (Whitmire, Kyle, Speer and Burgess, 1988) and Theileria parva (Musoke et al., 1984; Dobbelaere et al., 1984) or the Babesia divergens merozoite (Winger, Canning and Culverhouse, 1987).

1.3.3. Development of recombinant vaccines for malarial sporozoites and hepatitis B virus

Ideally, the choice of vector and expression system results in the production of a recombinant protein possessing the same biochemical characteristics, and therefore potentially the same immunogenic and protective properties, as the native molecule. The likelihood of this being achieved, using any one strategy, is hard to predict and is best described with reference to two examples. These detail the development of potential malaria sporozoite and hepatitis B vaccines.

a) Malarial sporozoites

Monoclonal antibodies which recognise the circumsporozoite (CS) protein of the infective, sporozoite, stage of malaria may block sporozoite infectivity in vitro (Hollingdale, Zavala, Nussenzweig and Nussenzweig, 1982) and in vivo (Potocnjak, Yoshida, Nussenzweig and Nussenzweig, 1980). This circumsporozoite protein has been shown to possess a single immunodominant region in which different monoclonal antibodies raised against the sporozoite surface react with identical epitopes. These monoclonals also inhibit the subsequent binding of polyclonal antisera from mice and man immunised with irradiated sporozoites, indicating that they also recognise this region (Zavala, Cochrane, Nardin, Nussenzweig and Nussenzweig, 1983). The CS proteins from different Plasmodium species are structurally similar and are not glycosylated (Santoro, Cochrane, Nussenzweig, Nardin et al., 1983).

Analysis of the CS protein after its synthesis as several subunit peptides revealed that a sequence of eight amino acids form the

epitope (Schlesinger, Cochrane, Gwadz, Godson et al., 1984). Since short peptide regions were found to contain the relevant epitope, complex folding of the molecule was unlikely to be a requirement for epitope formation. This feature, together with the absence of glycosylation, made expression in E. coli likely to be successful. These indications were subsequently found to be correct and the gene coding for the circumsporozoite protein from several Plasmodium species has been cloned and expressed in E. coli (Ellis, Ozaki, Gwadz, Cochrane et al., 1983; Enea, Arnot, Schmidt, Cochrane et al., 1984a; Young, Hockmeyer, Gross, Ballou et al., 1985). The whole CS gene from Plasmodium knowlesi and Plasmodium falciparum has been cloned and sequenced (Ozaki, Svec, Nussenzweig, Nussenzweig and Gordon, 1983; Dame, Williams, McCutchan, Weber et al., 1984; Enea, Ellis, Zavala, Arnot et al., 1984b). These groups have shown the CS protein to consist of multiple tandem repeats in the amino acid sequence such that, when expressed, about half of each CS molecule consists of epitope repeats. It is therefore not surprising that this epitope shows such strong immunogenicity (Nussenzweig and Nussenzweig, 1984).

The expressed recombinant peptides of the Plasmodium falciparum CS protein are of particular importance because of their potential use as a vaccine for human malaria. When it was revealed that, if the whole CS protein was expressed in E. coli, the expressed product was unstable (Dame et al., 1984; Young et al., 1985), gene fragments were introduced into plasmid vectors which, on expression in E. coli, yielded recombinant proteins which corresponded to peptide repeats containing the CS epitope. These were found to be immunogenic in

mice and the polyclonal non-specific sera raised were shown to block sporozoite invasion of human hepatoma cells in vitro (Young et al., 1985; Mazier, Mellouk, Beaudoin, Texier et al., 1986). Subsequent immunisation trials in human volunteers have only achieved low levels of antibody and partial protection has only been engendered in individuals repeatedly inoculated with high doses of the recombinant protein (Ballou, Sherwood, Neva, Gordon et al., 1987). Whether this reflects the fact that antibody to the CS protein is not alone sufficient to protect humans from malaria or whether the current developments in antigen presentation (Que, Cryz, Ballou, Fürer et al., 1988) will overcome these difficulties remains to be seen.

b) Hepatitis B virus

In order to obtain the immunogenic surface antigen of hepatitis B virus (HBsAg) it was necessary to extract it from the plasma of hepatitis B virus carrier donors which was both expensive and laborious, requiring careful monitoring of vaccine material to ensure that no live virus was present (Tiollais, Charnay and Vyas, 1981). There was, therefore, considerable drive for the production of an alternative means of producing HBsAg. The native HBsAg from plasma occurs as spherical or tubular 22 nm particles formed through complex folding of the molecule (Tiollais et al., 1981). This, and the presence of glycoprotein, made it appear that complex post-translational modification would be required to obtain anything resembling the native HBsAg and retaining its properties. In spite of the fact that no such post-translation modification will occur in E. coli, several

groups used this expression system to clone and express genes from the hepatitis B virus. As predicted, E. coli has not been selected as the expression system for production of recombinant HBsAg for vaccine purposes. Yields were found to be low (Pumpen, Kozlovskaya, Borisova, Bichko et al., 1984) and the conventional determinants recognised on native HBsAg which are related to protective immunity, were not recognised on the HBsAg expressed from E. coli, presumably because of the absence of structural changes and glycosylation (Fujisawa, Ito, Ikeyama and Kikuchi, 1985). However E. coli was not abandoned as an expression system for hepatitis B virus. The core antigen of the virus has been expressed fused to B-galactosidase in E. coli (Cohen and Richmond, 1982). The expressed product forms particles equivalent to 27 nm core particles isolated from the human liver (*ibid*). These recombinant core particles are immunogenic in laboratory animals and may well be of diagnostic value (Stahl, MacKay, Magazin, Bruce and Murray, 1982). Of interest regarding HBsAg expressed in E. coli is the disclosure that, although the recombinant protein does not contain the protective epitopes of the native HBsAg, it does bear epitopes on another region of the protein which enables the HBsAg to bind human serum albumin (Fujisawa et al., 1985). This, so called, human serum albumin receptor has been implicated as the mechanism by which the hepatitis B virus exhibits hepatotropism (Tiollais et al., 1981).

In further attempts to obtain recombinant HBsAg bearing intact and immunogenic protective epitopes, the relevant gene was cloned into plasmid vectors and expressed in the yeast, Saccharomyces cerevisiae (Valenzuela, Medina, Rutter, Ammere and Hall, 1982).

The multimeric HBsAg complexes expressed in yeast cells are similar to the 22 nm particles and possess the complete antigenic activity of native HBsAg (Valenzuela et al., 1982). The ability of eukaryotic yeast cells to yield a recombinant protein bearing the protective epitopes of HBsAg, in spite of their inability to glycosylate proteins, may reflect the closer similarity in gene expression and protein synthesis between yeast cells and eukaryotic mammalian cells compared to that between the prokaryote E. coli and mammalian cells (Old and Primrose, 1985). Chimpanzees immunised with yeast derived recombinant HBsAg were protected from challenge with high doses of infective hepatitis B virus (Murray, Bruce, Hinner, Wingfield et al., 1984) after which immunisation trials were performed in humans.

In these, the antibody responses of human recipients of this yeast derived recombinant HBsAg have been compared with the responses of those given HBsAg extracted from plasma. No significant differences were observed between the two in terms of quantity, quality or specificity of antibody response (Brown, Stanley, Howard, Zuckerman and Steward, 1986). The development of this recombinant immunogen has been so successful that it has become available as commercial vaccine (Engerix B, Smith, Kline and French).

A third host cell expression system has been used to express cloned hepatitis B virus genes in mammalian cells.

The HBsAg gene has been expressed in cultured rat and mouse cell lines (Siddiqui, 1983) as have other genes of the hepatitis B virus (Gough and Murray, 1982). Early work by Hirschman, Price, Garfinkel, Christman and Acs (1980) succeeded in expressing the whole genome of the hepatitis B virus in HeLa cells. The cells showed

marked cytopathic changes and hepatitis B core and surface antigens were produced in culture. Under the electron microscope these could be seen to form the 42 nm particles with central cores typical of the intact virus (ibid). This effectively meant that a method for studying hepatitis B virus and its effects on cells in vitro had been developed and also, by showing HeLa cells to be susceptible to the cytopathic effects of the virus, provided an in vitro system for testing sera and vaccines for the presence of live hepatitis B virus.

Thus, while the requirements for expression of an immunologically effective recombinant protein may narrow the choice of expression systems available, each system has its merits and it may be only by using each system that these are revealed.

1.3.4. Bacteriophage lambda gt11 expression system

a) Molecular biology

Bacteriophage lambda gt11 (λ gt11) is an expression vector constructed from other λ gt phage vectors and described by Young and Davis (1983a and b). λ gt11 contains the E. coli lac Z gene and within the B-galactosidase coding region is a unique EcoR1 digestion site, upstream of the B-galactosidase translation termination codon.

Insertion of foreign DNA into this EcoR1 site inactivates the B-galactosidase and cells bearing recombinant phage are therefore unable to utilise the chromogenic substrate, 5-bromo-4 chloro-3-indolyl-B-D-galactosidase (X-gal). Growth in the presence of X-gal yields blue colonies for non-recombinant and white colonies for recombinant phage (Miller, 1972) allowing identification and selection of recombinants. Up to 8.3 kb foreign DNA can be inserted into λ gt11 and leave it functional (Old and Primrose, 1985).

E. coli cells infected with recombinant λ gt11 express the foreign DNA inserts as B-galactosidase fusion proteins. This expression is under the control of prokaryotic gene signals of E. coli and is induced in the presence of isopropyl B-D-thiogalactopyranoside (IPTG). If the DNA insert is in the correct orientation and reading frame, the induced fusion protein contains a sequence of amino acids identical to that of the native protein. Antibodies can then be used in a screening immunoassay to select recombinants containing the DNA sequence coding for a particular protein. Young and Davis (1983a) first described model λ gt11 recombinants containing mouse α -amylase and chicken ovalbumin cDNAs as inserts. Then Kemp, Coppel, Cowman, Saint, Brown and Anders (1983) applied the technique to isolating cDNA clones coding for malarial blood stage antigens using human serum to screen the recombinant phage. Since then, λ gt11 expression libraries have been constructed using genomic or circular DNA from a wide spectrum of parasitic organisms such as protozoa (Gill, Timms and Kemp, 1987), cestoda (Bowtell, Saint, Rickard and Mitchell, 1984) and trematoda (Lanar, Pearce and Sher, 1985). In certain E. coli strains, λ gt11 phage undergoes replication by the lytic cycle cells lysing as the phage propagates, thus forming a visible phage plaque (Herskowitz, 1973). In addition, the foreign DNA expressed by recombinant λ gt11 may result in a foreign protein which is toxic to the host cell and might kill it before sufficient amounts of the antigen are produced. These problems do not prevent such strains being used as hosts for λ gt11 during library screening; large amounts of lac operon repressor (product of the lac 1 gene) prevent lac Z directed expression of the fusion protein during the initial hours of plaque



formation (Huynh, Young and Davis, 1985). Only when the number of infected cells around the plaque is reasonably large is lac Z-directed expression induced by the addition of IPTG (ibid). In this way detectable amounts of the recombinant protein are produced and bound by the nitrocellulose paper before being degraded where they are available for screening with antibody (Young, Bloom, Grossikinsky, Ivanyi et al., 1985). Since the objective of cloning genes in λ gt11 is usually to obtain substantial amounts of recombinant protein, these E. coli strains are only suitable for library screening and alternative strains have been developed for expression of larger amounts of recombinant protein (Young and Davis, 1983a). These contain a mutation (hfl A 150) which enhances the frequency of phage lysogeny. This means that cells infected with phage do not lyse because the phage undergoes replication by the lysogenic cycle where phage DNA is integrated with host cell DNA and divides in synchrony with it (Herskowitz, 1973). Thus, high cell densities of lysogens can be cultured before inducing lac Z-directed expression of the recombinant protein.

b) Advantages

When recombinant DNA is expressed in E. coli, the proteins produced are potentially unstable due to the inherent tendency of bacteria to rapidly degrade foreign polypeptides (Charnay, Gervais, Louise, Galibert and Tiollais, 1980; Edman, Hallewell, Valenzuela, Goodman and Rutter, 1981). By inserting DNA into the E. coli lac Z gene present in λ gt11 the recombinant protein is produced fused to B-galactosidase. This close association with a normal bacterial

protein has been shown to enhance the stability of the fusion protein (Itakura, Hirose, Crea, Riggs et al., 1977). In addition E. coli strains which host λ gt11 are mutants deficient in protein degradation pathways (Young and Davis, 1983a). Together these features increase the yield of recombinant protein expressed by increasing the lifetime of the fusion protein.

Another advantage of using the E. coli lac Z gene is that the expression is under the control of E. coli promoters, making it more likely that the sequence of foreign DNA will be efficiently transcribed and translated.

In a recombinant DNA library these two characteristics of λ gt11, efficient expression and increased stability of recombinant proteins, increase the chances of detecting any one antigen when a large number of recombinants are screened.

More recently, since immunisation trials have been used to test recombinant proteins as vaccines (Murray et al., 1984; Winther, Allen, Bomford and Brown, 1986; Ballou et al., 1987), the importance of using carrier proteins (Que, Cryz, Ballou, Fürer et al., 1988) and adjuvants (Zanetti, Sercarz and Salk, 1987) has been realised. In this respect, the B-galactosidase portion of recombinant fusion proteins can act as a carrier protein (Shuman, Silhavy and Beckwith, 1980). It has already been established that antibodies are formed against amino acid sequences located at the amino terminus end of B-galactosidase (*ibid*). The principle behind the role of a carrier protein is based on the realisation that small antigenic determinants with specificity for, and capacity to react with, an Ig receptor on a B cell were not able to stimulate an antibody response on their own.

If these small peptides were coupled to an immunogenic molecule recognised by T cells (the carrier) an antibody response to the peptide (the hapten) could be obtained in what is known as an anti-hapten response (Mitchison, 1971, cited by Lachmann, Strangeways, Vyakarnam and Evan, 1986). Thus, T cells would recognise determinants on the carrier part of the fusion protein and deliver help to B cells which recognise the hapten (Roitt et al., 1985).

The relevant features of T. annulata and tropical theileriosis, particularly in relation to the development of the sporozoite stage, immunity and immunisation, have been reviewed together with the current application of recombinant DNA technology to the development and production of new non-infective subunit vaccines.

The role of immunity to the Theileria sporozoite in protection remains unclear although in vitro evidence that an effector mechanism exists for ant sporozoite antibodies to block sporozoite infectivity has been described.

Given the progress of malariologists along a similar line of investigation, a research project was initiated to identify and assess T. annulata sporozoite antigens of potential importance in stimulating protective immunity. Regardless of whether successful vaccine material was obtained, it was hoped that the study would help determine what contribution ant sporozoite immunity makes to protective immunity in vivo. If successful vaccine material was produced this would not only provide a non-infective vaccine, or a component of such a vaccine, with greater ease of storage and handling in tropical conditions, but might also, based on the sporozoite stage, induce immunity giving broader and more complete protection than that elicited using schizont infected cells.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

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This chapter describes the work involved in the establishment and maintenance of the Theileria life cycle in vivo and the Theileria parasite in vitro. The provision of parasite material, the generation of reagents such as peripheral blood lymphocytes and in vivo cattle experiments were essential requirements for work described in later chapters and the basic methods behind each of these are detailed below.

2.1. MATERIALS

Reagents which are not fully detailed in the text are described in Appendix I.1. and Appendix III gives the full names and addresses of supplier companies.

2.1.1. Culture media

a) Purified deionised water

Unless otherwise specified all water used was highly purified as follows. The water was first deionised in a MILLI RO 60 water purification system (Millipore) and at the same time purified by reverse osmosis. The water was then given a final polishing with a MILLI Q water purification system (Millipore).

The final product had a resistivity of 18 megaohms, indicating a high degree of purity.

b) Sporozoite separation media

i) Surface sterilisation of ticks: Eagles minimum essential medium (MEM Gibco, BRL) with Hanks' salts but without L-glutamine and sodium bicarbonate was supplemented with double strength antibiotics (200 units/ml penicillin, 200 µg/ml streptomycin) and

nystatin (100 units/ml). Sterile 7.5% sodium bicarbonate solution (Gibco, BRL) was added until the phenol red indicator in the medium had turned orange (pH 7.0-7.2). This medium was used to wash ticks during surface sterilisation.

ii) Tick grinding media: When no further purification of sporozoites was to be carried out, ticks were ground in Eagles MEM supplemented as above and containing 3.5% bovine plasma albumin (BPA, Armour Fraction V, Sigma). This medium, known as MEM/3.5% BPA, was filter sterilised (0.22 μm , Millipore) before the addition of nystatin and the pH was adjusted as above.

When sporozoites were to be purified further, ticks were ground in Hanks balanced salt solution free of calcium and magnesium ions and without sodium bicarbonate (Gibco, BRL) containing 1% BPA (Hanks B.S.S./1% BPA). Double strength antibiotics were included and the pH adjusted using sodium bicarbonate solution as above. The medium was filter sterilised (0.22 μm) after the BPA had been added.

In later experiments when sporozoites were purified prior to protein analysis the BPA was omitted.

iii) Stock isotonic Percoll: Sporozoites were purified by separation on a discontinuous density gradient prepared using media of different densities. These media were prepared using stock isotonic Percoll (SIP). SIP had the same tonicity as the medium used to grind ticks and was made by mixing Percoll (Pharmacia), a colloid of silica particles, with concentrated Hanks B.S.S. with or without 1% BPA as follows:

SIP

<u>with BPA</u>		<u>without BPA</u>	
Hanks B.S.S. x 10	5 ml	Hanks B.S.S. x 10	2 ml
BPA	0.5 g	Percoll	20 ml
Sodium bicarbonate (Analar, BDH)	1 mg	The pH was adjusted using sterile 1M hydrochloric acid until the SIP was just turning orange from red.	
After filtering (0.22 μ m) 2 ml of the above was added to 20 ml Percoll.			

iv) Percoll gradient: The media of different densities used to form the Percoll gradient were prepared from SIP and Hanks B.S.S. mixed in different proportions. When BPA was being included it was present in SIP and Hanks B.S.S., likewise when excluded it was absent from both SIP and Hanks B.S.S. The volumes of SIP and Hanks B.S.S. required to achieve the stated step densities were different, depending on whether BPA was included as shown below.

<u>Step density</u> (specific gravity) s.g.	<u>% SIP</u>	<u>ml SIP</u>	<u>ml Hanks B.S.S./</u> <u>1% BPA</u>	<u>ml required</u> <u>for gradient</u>
With BPA:				
1.095	76.0	7.60	2.40	5
1.070	54.0	5.40	4.60	5
1.050	36.0	1.80	3.20	2
1.030	18.0	0.90	4.10	2
Without BPA:				
1.095	76.5	7.65	2.35	5
1.070	55.5	5.55	4.45	5
1.050	39.0	1.95	3.05	2
1.030	22.0	1.10	3.90	2

In order to form the Percoll gradient, the above media were layered on top of each other in order of decreasing density into a

thick walled 40 ml glass centrifuge tube (2.5 cm diameter, MSE 40) held at 45° to the horizontal.

c) Cell culture media

i) Foetal calf serum: Foetal calf serum (FCS, Flow Labs.) was used to supplement the media for all Theileria and hybridoma cell lines. As some FCS batches are unsatisfactory for Theileria culture, particularly T. parva culture, samples of prospective FCS batches were tested for their suitability before purchase. The growth of a fastidious T. parva cell line in medium containing the test FCS was compared with its growth in the medium containing the current FCS in use over at least three passages of the culture.

Early hybridoma cultures were supplemented with FCS specially selected for their growth (Section 3.2.4, Myoclone, Gibco, BRL). Once well established, the hybridoma cell lines were cultured in the same medium as used for Theileria cell lines. FCS was heat inactivated at 56°C for 30 minutes and stored at -20°C.

ii) Complete medium - RPMI 1640/16.7% FCS: Liquid RPMI 1640 with 25 mM Hepes (Gibco, BRL) was originally described for use in human leucocyte culture by Moore, Gerner and Franklin (1967). Sterile supplements were added to give complete medium containing 16.7% FCS, 2 mM L-glutamine (Gibco) and 100 units/ μ g per ml of penicillin/streptomycin. The medium was stored at 4°C. Fresh glutamine was added to unused medium after two weeks. This medium was used to culture all Theileria-infected lymphoblastoid cell lines and established hybridoma cell lines.

2.1.2. General equipment

a) Filters

All filters were obtained from Millipore.

i) 0.22 μm sterile Millex-GV filter units were used for filter sterilisation.

ii) A 22 mm diameter glass microfibre AP prefilter and a 25 mm diameter 8 μm high porosity cellulose ester MF filter were held together in an O ring of a Swinnex filter holder and used to obtain an 8 μm filtrate of ground up tick supernatants. They were sterilised before use by autoclaving at 15 p.s.i. for 15 minutes.

b) Culture equipment

All glassware, such as bottles, flasks and centrifuge tubes; some reusable plasticware and other autoclavable materials were soaked in a non residue-forming detergent (0.5-1.0% Decon 75, Decon Labs.), rinsed three times in tap water and three times in purified deionised water and then sterilised by autoclaving at 15 p.s.i. for 15 minutes.

Glass pipettes were soaked in 0.5-1.0% Decon 75 and then flushed 12 times with tap water, followed by three flushes with purified water before being sterilised by dry heat in a hot air oven at 170-180°C for 90 minutes.

Cultures were grown in disposable plastic vessels, either in tissue culture wells of 2 cm^2 , 1 cm^2 (Costar) or 0.38 cm^2 (Nunc, Gibco, BRL) or in tissue culture flasks of 25, 75 or 125 cm^2 (Nunc, Gibco, BRL).

2.1.3. Theileria stocks

a) Origins

Three uncloned stocks of Theileria annulata were used, each isolated from a different geographic area and named accordingly as Ankara from Turkey (Schein et al., 1975), Hissar from India (Gill, Bhattacharyulu and Kaur, 1976) and Gharb from Morocco (Ouhelli, 1985). All three stocks were brought to the Centre for Tropical Veterinary Medicine (CTVM), Edinburgh, in infected ticks; H. a. anatolicum infected with Hissar or Gharb stocks and H. a. excavatum infected with the Ankara stock. Since then they have all been passaged and maintained in H. a. anatolicum of Indian origin (Bhattacharyulu et al., 1975).

One stock of T. parva was used. This was the Muguga stock described by Brocklesby, Barnett and Scott (1961). T. parva Muguga was obtained in the tick, Rhipicephalus appendiculatus, from the Muguga tick colony (Bailey, 1960) and was also used uncloned.

The species and stocks used in any particular experiment are detailed in the relevant sections.

b) Storage

The Theileria parasites were stored as cryopreserved ground up tick stabilates and macroschizont-infected cell lines in liquid nitrogen. The methods of cryopreservation and resuscitation are described in Sections 2.2.5. and 2.2.6.

c) Maintenance

In vivo maintenance of T. annulata in H. a. anatolicum and T. parva in Rhipicephalus appendiculatus was achieved by periodic

passage of parasite through calves as described by Walker, Fletcher, McKellar, Bell and Brown (1985) for T. annulata and Bailey (1960) for T. parva. In vitro maintenance was by culture of established Theileria-infected cell lines which is described fully in Section 2.2.5.

2.1.4. Cattle

Purebred and crossbred Bos taurus calves from dairy breeds and of 2-6 months old were used. These were obtained from local farms and kept in isolation for three weeks during which time their health was monitored and screening for Salmonella infection carried out. The calves were weaned onto a home produced pellet diet with ad libitum hay and water. They were kept on straw litter in tick free buildings and each calf was identified by an ear tag number.

2.2. METHODS

2.2.1. Ticks

The practical aspects of maintaining T. parva in Rhipicephalus appendiculatus colonies have been described by Bailey (1960) and Branagan (1969) while others reported methods of obtaining high infection rates in ticks (Young and Leitch, 1981). Many of the general principles can be applied to H. a. anatolicum as a vector for T. annulata as detailed by Walker et al. (1985).

The management and manipulation of ticks to enable the provision of large numbers of sporozoites of the required stock when necessary was mainly carried out by Dr. Alan Walker and Mrs. June Fletcher. Those aspects relating to this study are outlined below.

a) Infection of nymphal ticks

One thousand to 2,000 unfed nymphal ticks were applied to, and confined on, a calf on day 8 of a primary infection. Engorged ticks were collected daily from day 11 (day 3 post application) with peak numbers dropping around day 13 to coincide with rising piroplasm parasitaemia. Unless the calf died, nymphs continued to drop to about day 17. Figure 6 (a-d) shows the timing of nymph application and nymph drop in relation to the parasitaemia. Nymphs were moulted and stored at 28°C and 18°C respectively. The infection rates of the resultant adults was determined using methyl green and pyronin stains (Walker, McKellar, Bell and Brown, 1979) to stain the Theileria parasite within the activated acini of the tick salivary glands.

Infection rates were generally very high for T. annulata with 100% of ticks and more than 300 acini per adult tick infected.

b) Challenge of calves

Infected adult ticks were allowed to feed on calves to provide a challenge for several calves as detailed in Section 3.2.2. Equal numbers of female and male ticks were confined to the calves' ears in cotton bags.

c) Provision of infective sporozoites

In order to obtain infective sporozoites, infecting adult H. a. anatolicum were partially fed for three days on the ears of rabbits before being removed to harvest sporozoites (Walker et al., 1979). This partial tick feed stimulates sporogony and allows maturation of sporoblasts to sporozoites without significant loss of parasite into the rabbit in the tick saliva.

2.2.2. Isolation of sporozoites

Since this thesis centres on a study of the infective sporozoite, the provision of ample quantities of this stage was of prime concern. Mature sporozoites of high infectivity in crude tick homogenates or homogenate filtrates were suitable for infection or challenge of calves, stabilate preparation and in vitro infection of peripheral blood mononuclear cells (PBM).

For IFAT antigen or sporozoite protein analysis, clean sporozoite preparations free of contaminating tick proteins were required. The basic methods of sporozoite separation described here were developed in response to these demands by previous workers (Brown, Stagg, Purnell, Kanhai and Payne, 1973; Brown, 1979; Walker and McKellar, 1983).

a) Ground up tick supernatant (GUTS) and GUTS filtrate

Three day fed adult T. annulata infected H. a. anatolicum were washed with water, counted and dried. To remove debris and surface sterilise they were washed once in 1% benzalkonium chloride (Roccal, Winthrop), three times in 70% ethanol and four times in warmed (37°C) MEM containing double strength antibiotics and nystatin. Ticks were left in the fourth medium wash for at least 10 minutes.

Medium for grinding ticks for GUTS or GF was MEM/3.5% BPA with double strength antibiotics and nystatin. Ticks were usually ground at a concentration of four tick equivalents (te) per ml and the total volume of grinding medium to be used was divided into aliquots so that there were several changes of medium during the grinding. Thus, ice cold MEM/3.5% BPA was added to the ticks and they were transferred to a mortar to be thoroughly ground using a pestle while removing and

adding grinding medium until no intact ticks remained. The resulting tick suspension was centrifuged at 100 G for five minutes (MSE Chil-spin) at 4°C and the ground up tick supernatant (GUTS) recovered. If GUTS filtrate (GF) was required, the supernatant was filtered using an AP prefilter and 8 μ MF filter (see Section 2.1.2.). Dilutions of GUTS or GF were made in grinding medium and the concentration expressed as the number of tick equivalents (te) per ml.

To assess the quality of GUTS and GF preparations, Giemsa-stained cytocentrifuge smears (cytospins) were prepared from GUTS or GF diluted to one te per ml and centrifuged at 110 G for five minutes (Cytospin 2, Shandon Instruments). Figure 2 shows an example of a cytospin of infected GF.

b) Percoll purified sporozoites

This method of separating mature sporozoites from contaminating tick material and sporoblasts using density gradient centrifugation was developed from that described by Walker and McKellar (1983).

When sporozoites were being purified for IFAT antigen or immunisation of mice for monoclonal antibody production, BPA was included in the grinding medium, SIP and step densities. When purified for SDS-PAGE, Western blotting and surface labelling, BPA was excluded. Section 2.1.1. describes the preparation of Percoll gradients.

Preparation of GF to load onto the Percoll gradient followed the same protocol as described above except that the grinding medium was Hanks B.S.S. Eight to 10 ml GF at four te per ml (32-40 te in

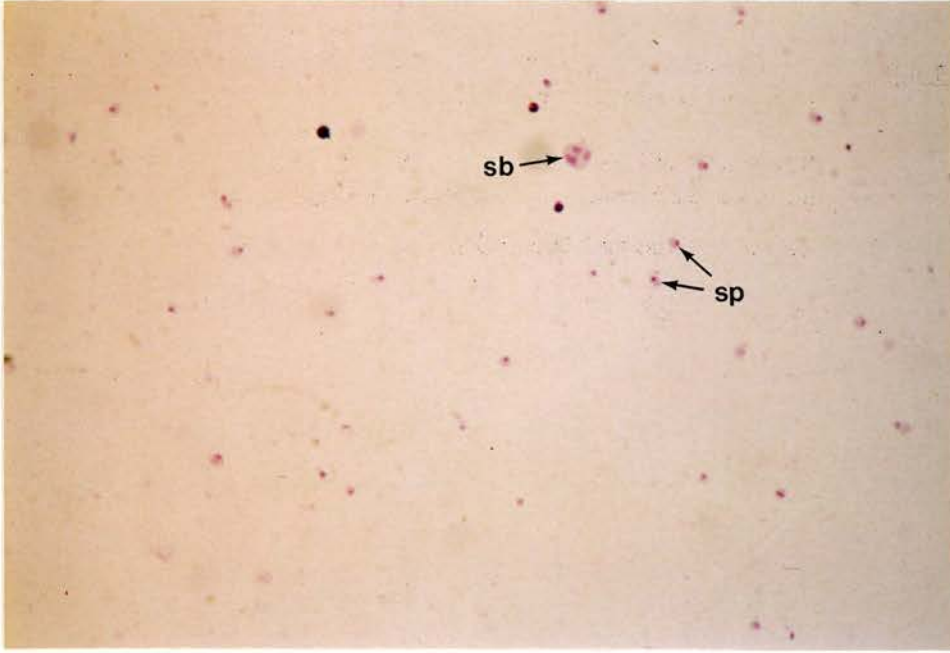
Figure 2 Ground up tick supernatant filtrate from T. annulata
Ankara infected ticks.

Giemsa-stained cytospin of GF at 1 te per ml
Arrows indicate sporozoites (sp) and sporoblast (sb)
(x 1,100 magnification)

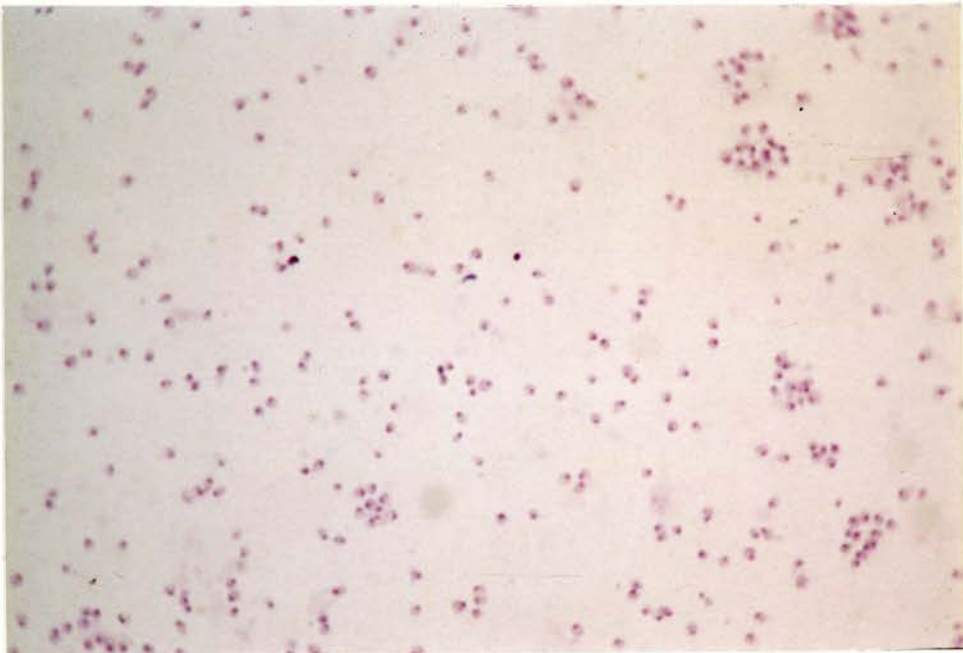
Figure 3 Percoll-purified T. annulata Ankara sporozoites

Giemsa-stained cytospin
(x 1,100 magnification)

2



3



total) was carefully layered onto the gradient and the positions of interfaces marked on the tube. The tube was then centrifuged using a swing out rotor at 2,500 G (4,000 rpm) at 4°C. This causes sporozoites to band at the 1.070/1.095 s.g. interface leaving tick debris above and below. Using Pasteur pipettes, GF and gradient steps above the sporozoite band were removed. The sporozoites were harvested into 20 ml phosphate buffered saline (PBS) and, to remove the Percoll, the mixture was centrifuged at 1500 G (3,300 rpm) for 30 minutes at 4°C.

The sporozoite pellet was difficult to visualise and therefore the supernatant fluid was removed by pipetting down to within 100 µl of the bottom of the tube. The pellet was resuspended in PBS or Hanks/1% BPA by repeated pipetting. A cytopsin was prepared in order to assess purity and numbers of sporozoites as shown in Figure 3.

2.2.3. Isolation of peripheral blood mononuclear cells (PBM)

Bovine PBM were required to provide target cells for sporozoite infectivity and inhibition assays or to enable infected cell lines to be established in vitro using PBM from an experimental animal.

The technique used for separation of PBM was based on that described by Brown (1987). Blood was collected into heparin (20-40 units/ml) or by defibrination using glass beads (BDH). For small volumes of blood, each 10 ml collected into sterile lithium heparin (Vacutainer, Becton Dickinson) was diluted with 10 ml PBS and layered directly onto 8 ml of a Ficoll Paque gradient (Pharmacia) before

centrifugation at 800 G at the interface (2,600 rpm) for 30 minutes at 15°C (MSE Chilspin). If larger volumes of blood were being handled, the buffy coat was separated first by centrifuging 50 ml volumes at 2,800 G for 20 minutes at 4°C. The buffy coat was diluted in 9 ml PBS and layered onto 8 ml Ficoll Paque for centrifugation as above. PBM were harvested from and below the interface and washed twice in PBS for 10 minutes, and then five minutes, at 350 G, 15°C. The cell pellet was resuspended in cold complete medium (RPMI 1640, 16.7% FCS) and the cell concentration determined (Coulter Electronics) and adjusted as necessary. A Giemsa-stained cytopsin was prepared to check the quality of the separation and the cell type present. Figure 4 shows PBM separated from defibrinated bovine blood.

2.2.4. In vitro infection of bovine PBM

Brown, Stagg, Purnell, Kanhai and Payne (1973) first infected lymphoid cells from cattle in vitro with T. parva-infected tick material. This advance made the assessment of the bovine humoral response to the Theileria parasite, particularly to the sporozoite stage (Gray and Brown, 1981; Preston and Brown, 1985) possible.

In vitro infections were carried out in 0.38 cm² or 1 cm² tissue culture wells, in 96 and 48 well plates respectively, according to the amount of material available and the purpose of the experiment.

PBM concentration was 2-4 x 10⁶ cells per ml and GF was used at between 0.25 and 2 te per ml. Wells were seeded with GF first, then an equal or double the volume of PBM was added to each well. Outer wells of plates were filled with sterile PBS as these wells

Figure 4 Bovine peripheral blood mononuclear cells isolated from defibrinated blood.

Giemsa-stained cytospin of PBM at 2×10^6 cells per ml.

(x 550 magnification)

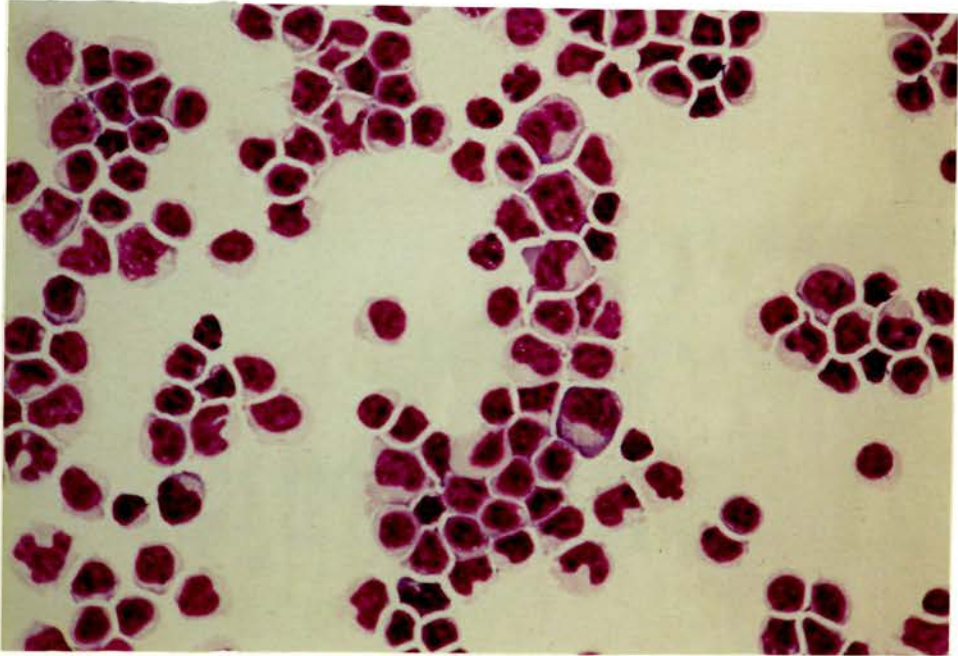
Figure 5 T. annulata Hissar infected lymphoblastoid cell line.

Giemsa-stained cytospin

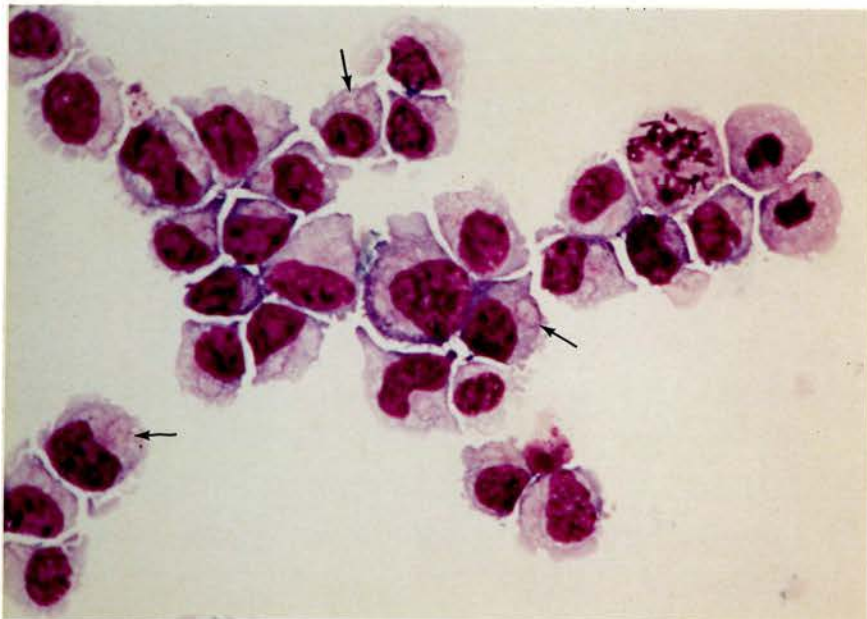
Arrows indicate macroschizonts in the cytoplasm of infected cells.

(x 550 magnification)

4



5



were susceptible to evaporation during incubation. The cultures were then placed in a humidified incubator gassed with 5% CO₂ (LEEC) and incubated at 37°C.

Medium was changed and cytopins taken at three day intervals until 30% or more cells were infected when the cultures were transferred to larger wells or 25 cm² flasks. The rate of cell growth was assessed using the inverted microscope (Diavert, Leitz, Wetzlar). Subculture was carried out every 2-3 days at increasing dilutions until, with established cell lines, cells were diluted 1:10 with medium, cell concentration being maintained at levels ranging between 1×10^5 to 2×10^6 per ml. Figure 5 illustrates an established T. annulata lymphoblastoid cell line.

2.2.5. Cryopreservation

a) Theileria-infected cell lines

Dimethyl sulphoxide (DMSO) has been used as a cryoprotectant for living cells for many years (Lovelock and Bishop, 1959) and has proved to be satisfactory for use with Theileria-infected cell lines (Wathanga, Jones and Brown, 1986). Cells from cultures growing at a density of $1-2 \times 10^6$ cells per ml were cryopreserved in complete medium containing 10% DMSO (Analar, BDH) at a cell concentration of approximately 10^7 cells per ml in 1 ml vials (Nunc, Gibco). After 24 hours at -80°C the vials were transferred to a liquid nitrogen bank (LR40, BOC) for long term storage. The precise method for cryopreservation is given by Brown (1983).

b) Sporozoites

Ground up tick supernatant (GUTS) from infected ticks at a concentration ranging from 1-4 te per ml was cryopreserved in the presence of 7.5% glycerol (Analar, BDH) in MEM/3.5% BPA. Aliquots were placed in 1 ml vials (Nunc, Gibco) and after 24 hours at -80°C were stored in liquid nitrogen. Brown (1983) details the method involved.

2.2.6. Resuscitation

Brown (1987) gives an account of the resuscitation of Theileria-infected cell lines and sporozoites from cryopreservation in liquid nitrogen. The different protocols for each reflect differences in the properties of the cryoprotectants; DMSO and glycerol. Thus, cell lines were thawed to 37°C and rapidly removed from the presence of DMSO by centrifugation (200 G, 10 minutes) and resuspension in fresh complete medium in which they were then incubated. By this method, the estimated viability of the resuscitated cells was greater than 90% (Wathanga et al., 1986).

Sporozoite stabilates were also thawed rapidly in a 37°C water bath but were then left at room temperature (20°C) to allow equilibration over 15-45 minutes. When being used to infect cattle in vivo, several vials of a given stock were resuscitated and their contents pooled. Aliquots were taken from this pool to inoculate calves to ensure consistency in the infective dose. The infectivity of a resuscitated stabilate was estimated to be about 10% of that of the original material (Brown, 1987).

2.2.7. Cloning Theileria-infected cell lines

The provision of cloned parasite was not a prerequisite for the study of sporozoite antigens. However, results were interpreted in the knowledge that all three T. annulata stocks were likely to contain a mixed population of parasites (Shiels et al., 1986b). Clones were only used in work on characterisation of the DNA of a sporozoite recombinant (Section 6.3.3.). These clones were the result of previous work by Wathanga (1984) and Wilkie (personal communication) at the CTVM.

The clones were derived in several ways; Wathanga (1984) took established cell lines and cloned them by limit dilution or by the isolation of single cells using similar methods to those applied to T. parva lymphoblastoid cell lines by Munderloh and Kurtti (1982) and Nelson and Hirumi (1981). Although these clones derived from the growth of a single cell they are not necessarily true parasite clones since at the time of infection more than one sporozoite may have entered a single lymphocyte. The other method used was to clone at the time of in vitro infection of bovine PBM with sporozoites such that increasing dilutions of sporozoites were added to a constant number of cells in tissue culture wells until an end point was reached after which no infection established. Here end point cultures were presumed to be clones deriving from the infection of a single cell with a single sporozoite.

Cloned cell lines which were used were:

i) Those derived from PBM of calf 46 - T. annulata Hissar 46 clones 2, 3 and 4; T. annulata Ankara 46 clones A, 2 and 3: These were cloned by limit dilution or isolation of single infected cells from an infected cell line as described by Wathanga (1984).

ii) Those derived from PBM of calf 139 - T. annulata Ankara 139 clones D4, D6, E5: These were cloned by end point titration of sporozoites (G. Wilkie, personal communication).

When glucose phosphate isomerase (GPI) isoenzyme patterns of the clones were compared with that of the parent stock pattern, they showed segregation of the band pattern in line with that expected when clones are established (Melrose et al., 1980; T.R. Melrose, personal communication).

2.2.8. Cattle

a) Infection

Primary infection of calves was by subcutaneous inoculation of sporozoites harvested from ticks (Section 2.2.2.) or cryopreserved stocks of sporozoite stabilate in front of the shoulder. The draining prescapular lymph node was one which could be readily palpated and biopsied. The area in front of the shoulder was shaved and a sterile syringe and 19 gauge, one inch needle used to inoculate the stabilate. Parasite stocks, doses and sites of inoculation are given with the appropriate experiment.

b) Challenge

Challenge was effected either by subcutaneous inoculation of sporozoite stabilate as described above or by allowing infected adult ticks to feed on the ears of the calf. In the latter case the draining lymph node was the left or right parotid lymph node.

c) Monitoring of reaction

In order to follow the calves' reaction to infection or challenge regular observations of clinical conditions and haematological and parasitological changes were made.

i) Clinical condition: From day 0 rectal temperatures were taken daily and recorded on a day sheet. Calves were visited twice by stockmen and 3-7 times weekly for routine experimental procedures. The clinical features of particular note during the course of the disease included pyrexia; inappetence; weakness; enlargement of lymph nodes, particularly the node draining the site of inoculation; colour of mucous membranes indicating anaemia or icterus; superficial haemorrhages in the form of petechiae; subcutaneous oedema; hyperpnoea and coughing indicating pulmonary involvement; the colour and state of faeces and urine indicating internal haemorrhage, intravascular haemolysis or impaired liver function and the degree of dehydration as assessed by skin elasticity. Monthly liveweights were recorded in kilogrammes. Detailed recording of clinical condition was not made but was an important part of the monitoring process and served as an initial indicator of the progress of the infection and the course the disease was taking. It was also used to aid prognosis and help decide whether a calf should be destroyed on humane grounds.

ii) Haematology: From day 0 haematological parameters were measured at least three times a week and at certain times, for example, at the onset or approaching the peak of piroplasm parasitaemia, were measured daily. Jugular venous blood samples were taken into tubes (Vacutainer, Becton Dickinson) containing the disodium salt of

ethylenediamine tetraacetic acid (EDTA) for haematological examination and the preparation of thin blood smears. Smears were air dried, fixed in absolute methanol (BDH) and stained for 40 minutes in 5% Giemsa stain (Shute, 1966) in phosphate buffer pH 7.2 (BDH). Packed cell volume (PCV) was determined using haematocrit tubes (Harshaw Chemicals) centrifuged at 12,000 rpm for six minutes (Biofuge A, Heraeus Sepatech) and measured in a microhaematocrit reader (Hawksley).

Total white and red blood cell counts were measured using an electronic particle counter (Coulter Electronics) and expressed as the number of cells $\times 10^3$ per μl and $\times 10^6$ per μl respectively.

iii) Parasitology: The regional lymph node draining the site of inoculation was palpated regularly from day 4 and, at the first sign of enlargement, was biopsied. Lymph node biopsy smears were made, fixed and stained as described for blood. Examination of blood and lymph node smears was carried out under oil immersion ($\times 50$, $\times 100$ Fluotar lenses) using an Ortholux II microscope (Leitz, Wetzlar, Germany).

Lymph node smears were examined for the presence of hyperplastic cells (H), macroschizonts (Ma) and microschizonts (Mi) and the presence of any one of these was rated as:

+	less than 1% of cells	} under	
++	1-5% of cells		x 100
+++	greater than 5% of cells		lens

Other features such as macrophage invasion, erythrophagocytosis and schizont degeneration were noted. Lymph node biopsy smears were made daily until macroschizonts had been observed and confirmed the following day. Thereafter, biopsy smears were examined at two day intervals.

Blood smears were examined primarily for the presence and number of intraerythrocytic piroplasms. Other features such as thrombocytopaenia or immature red blood cells indicative of regenerative anaemia were noted. Piroplasm parasitaemia was expressed as the percentage of erythrocytes infected regardless of how many piroplasms were present within individual erythrocytes. Percentage parasitaemias were counted to as low as 0.1%, below this parasitaemia was recorded as + if a piroplasm was observed in approximately 200 high power fields representing about 100,000 red blood cells, and, if none were seen, as NPS (no parasites seen).

iv) Serology: Blood for serum was collected monthly from day 0, serum separated and stored at -20°C. The serological test employed was the indirect fluorescent antibody test (IFAT) described by Burrige and Kimber (1972). IFAT was used to monitor calf antibody levels to various stages of the parasite during experiments. The details of the test and preparation of the antigens are described in Sections 3.2.5. and 3.2.6.

2.2.9. Lethal infections in cattle

The purpose of establishing potentially lethal theilerial infections in calves by inoculation of live sporozoites was to obtain a high piroplasm parasitaemia and supply material for piroplasm IFAT antigen, piroplasm protein analysis, glucose phosphate isomerase isoenzyme studies and extraction of parasite DNA. The other important objective was to transmit infection to ticks to replenish stocks of H. a. anatolicum infected with T. annulata and Rhipicephalus appendiculatus infected with T. parva.

The calves used for tick feeding and blood collection were often acting as controls in experiments involving other calves undergoing infection or challenge. An example of the clinical reaction, haematological and parasitological changes and how these relate to the timing of tick feeding and blood collection is given for each Theileria species and stock used during this research (Figure 6). Table 2 gives details of the sporozoite stabilates used and calves infected with them.

Table 2 Sporozoite stabilate inoculations for lethal infections in calves

Calf number	Breed	Sex	Parasite stock	Stabilate dose*		Local lymph node for biopsy
				volume ml	te	
P70	Ayrshire	Male	<u>T. a.</u> Ankara	2.5	3.3	RPG
143 N	Friesian	Male	<u>T. a.</u> Hissar	1.0	0.1	RPG
930	Hereford	Female	<u>T. a.</u> Gharb	2.0	4.3	LPG
931	Hereford	Female	<u>T. p.</u> Muguga	1.2	9.2	RPG
929	Hereford	Male	<u>T. p.</u> Muguga	1.2	9.2	RPG

*Stabilate inoculated subcutaneously

PG = prescapular gland

L = left

R = right

Figure 7 shows the appearance of the lymph node draining the site of sporozoite inoculation on day 13 of a lethal infection when piroplasm parasitaemia was reaching a peak as illustrated in Figure 8.

Figure 6 Lethal T. annulata and T. parva infections in calves: graphs to show changes in body temperature, haematology and parasitosis during the course of infection.

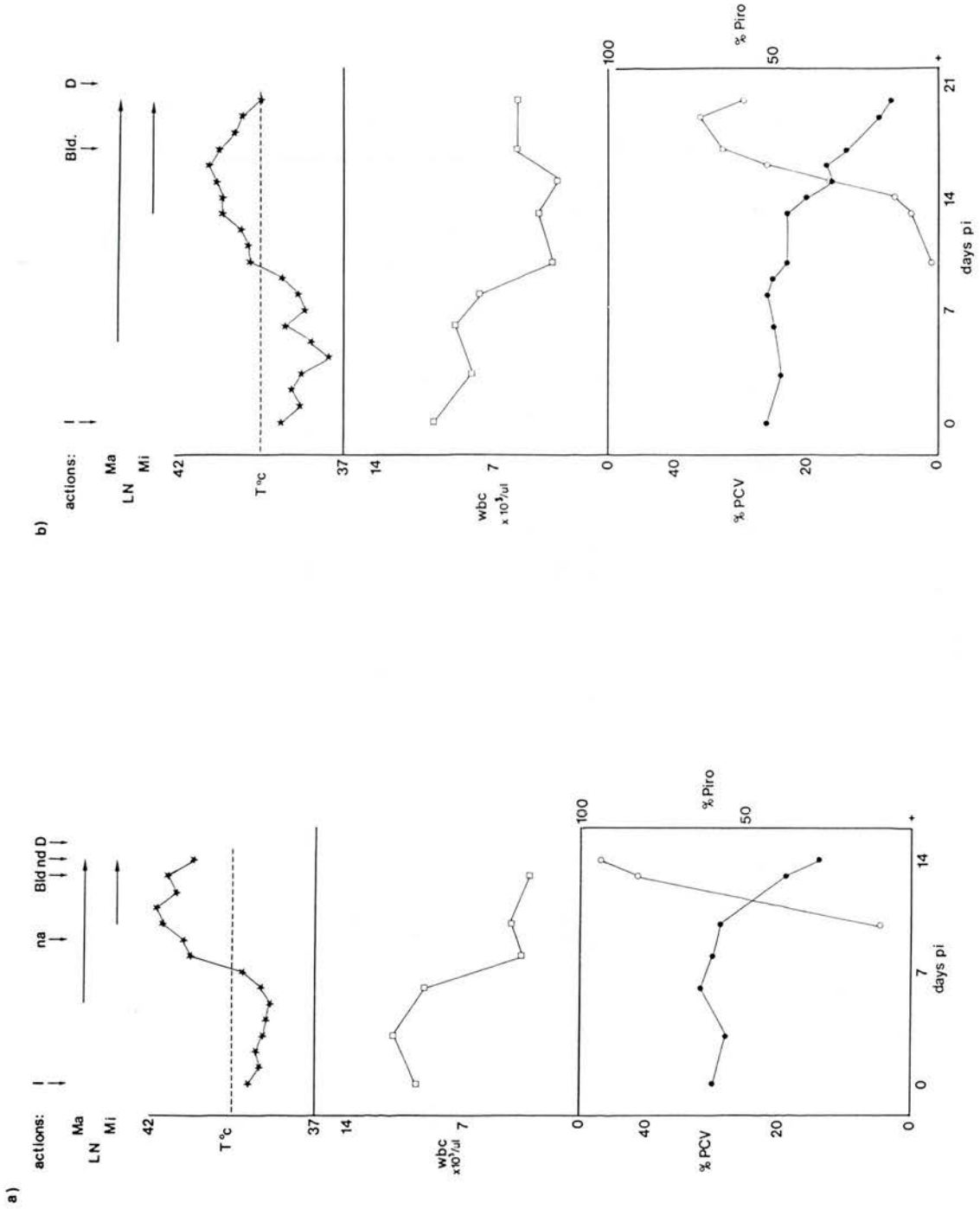
- a Calf P70 infected with T. annulata Ankara
- b Calf 143N infected with T. annulata Hissar
- c Calf 930 infected with T. annulata Gharb
- d Calf 931 infected with T. parva Muguga
- e Calf 929 infected with T. parva Muguga

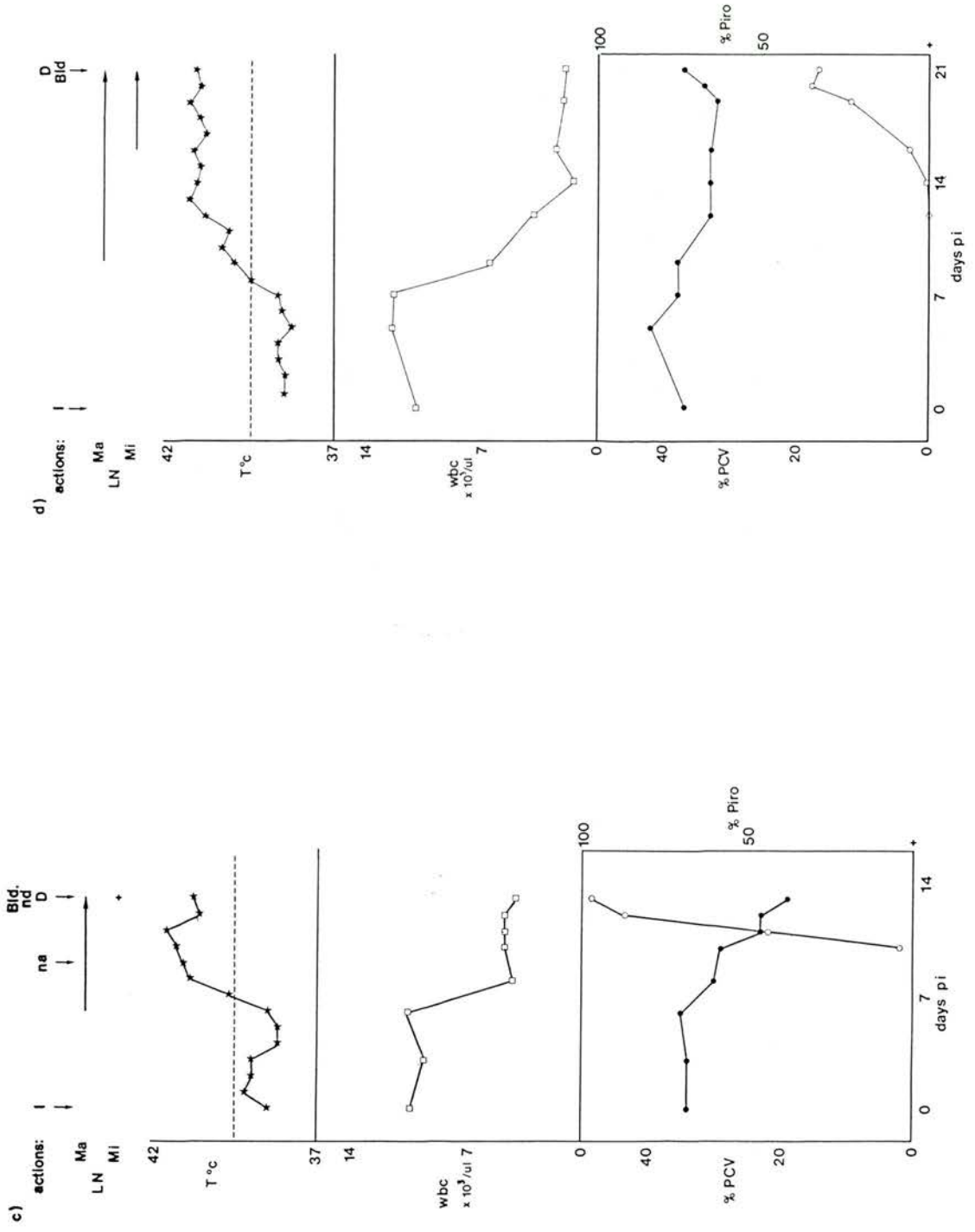
The times when tick nymphs were applied to (na) or dropped from (nd) infected calves and blood was collected (bld) are indicated.

Key:

- I day 0, stabilate inoculated
- D died or destroyed
- LN regional lymph node
- Ma macroschizonts observed
- Mi microschizonts observed
- T°C rectal temperature ★—★
- wbc white blood cell count □—□
- % PCV % packed cell volume ●—●
- % Piro % piroplasm parasitaemia ○—○
- days pi days post infection

Section 2.2.9. details the stabilates used to infect these calves.





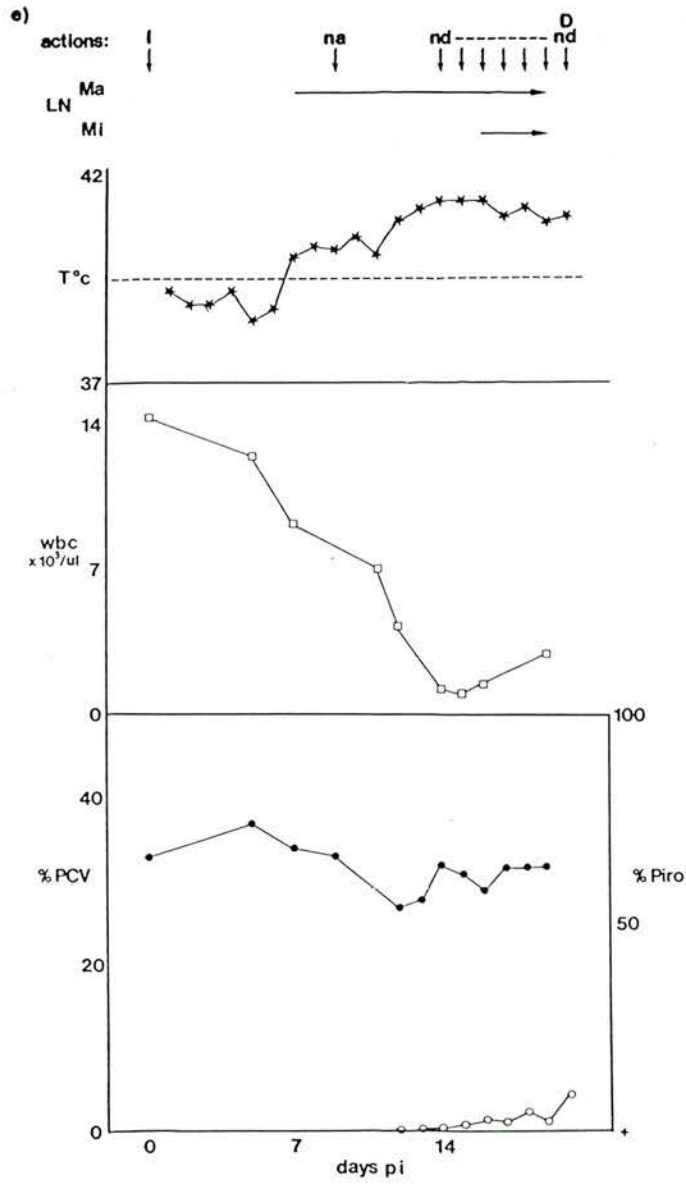


Figure 7 Lymph node biopsy smear on day 13 of lethal T. annulata Hissar infection from the regional prescapular lymph node.

Giemsa-stained cytospin showing:

mφ macrophage exhibiting erythrophagocytosis

ma macroschizont

mi microschizont

me merozoites

(x 550 magnification)

Figure 8 Blood smear on day 14 of lethal T. annulata Gharb infection.

Giemsa-stained cytospin with arrows showing piroplasms in red blood cells at a parasitaemia of 89%.

(x 1,100 magnification)

Figure 9 T. annulata Hissar piroplasms purified from piroplasm infected blood.

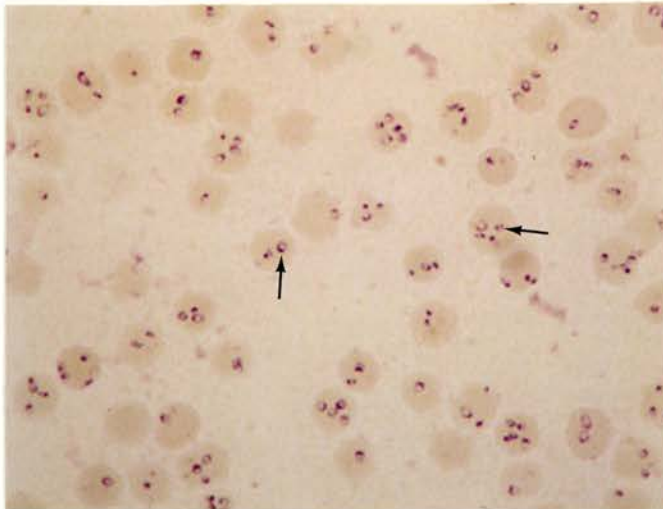
Giemsa-stained cytospin showing clumped piroplasms.

(x 1,100 magnification)

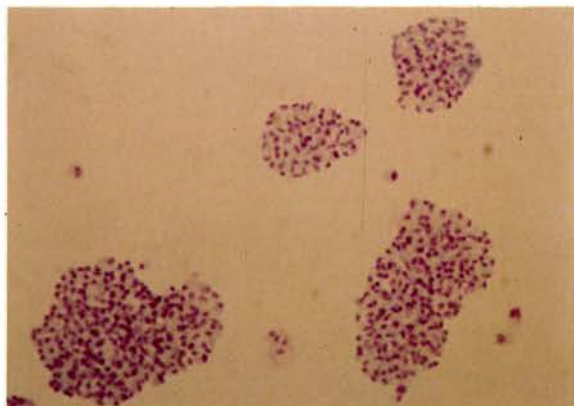
7



8



9



2.2.10. Isolation of piroplasms

The purification of theilerial piroplasms from bovine blood by removal of white blood cells (Fulton and Grant, 1956) and lysis of red blood cells (Shortman, Williams and Adams, 1972) was necessary to provide material for SDS-PAGE and Western blotting and isolation of parasite DNA with minimal host DNA contamination.

Up to one litre of blood was collected from a Theileria-infected calf at the time of peak parasitaemia. Anticoagulant in the form of 20% ACD or defibrination prevented the blood from clotting, the latter requiring the blood to be filtered through sterile gauze. Piroplasm parasitaemia and packed cell volume were measured so that an estimate of the expected piroplasm yield could be made.

After centrifugation at 2,400 G for 20 minutes at 4°C the plasma and, where possible, buffy coat were removed and PBS added to resuspend the packed erythrocyte layer. After three such washes in PBS (centrifugation for 20 minutes, 4°C, 2,400 G) an estimate of the pellet volume was made and four pellet volumes of PBS added. Meanwhile cellulose CF11 powder (Whatman) columns were prepared. Thirty-five ml dry CF11 powder was packed to about 30 ml using PBS and the column was left to run dry. Fifty ml diluted blood was then applied to each CF11 column and the white blood cell depleted blood coming through was kept on ice. Thirty ml of PBS washed the column through and a sample of the pooled blood was removed for a white blood cell haemocytometer count. Having ascertained that the count was less than 2×10^4 cells per ml the purification was continued. Blood which had run through the CF11 columns was washed twice in PBS (15 minutes, 2,000 G, 4°C) and resuspended in an equal volume of PBS before mixing

with preheated (37°C) red blood cell lysis buffer (0.83% NH₄ Cl, 0.17M Tris pH 7) at a ratio of one volume blood to nine volumes buffer. Incubation of the mixture for 3-5 minutes at 37°C changed the colour to a clear deep red indicating complete red blood cell lysis. The released piroplasms were collected by centrifugation at 2,000 G, 10 minutes, 4°C and then washed three times in PBS. The volume of the final pellet was estimated and, if the piroplasms were not being used immediately, they were rapidly frozen in liquid nitrogen and stored at -80°C. Cytocentrifuge smears were made from blood after passing through the CF11 columns and post lysis to assess the purity and quality of the piroplasms and Figure 9 shows an example of purified piroplasms.

Table 3 gives an example for each Theileria stock of the quantity of piroplasms harvested in the above manner together with details of factors influencing the piroplasm content of the blood.

The day of infection on which peak parasitaemia occurred depended on both the sporozoite dose (te) and the parasite stock. The piroplasm yield was influenced by a combination of factors including the volume of blood collected, the parasitaemia and the packed cell volume.

Table 3 Blood collection and piroplasm yields from calves undergoing lethal infections

Calf number	Parasite stock	Day of infection on which blood collected	Volume of blood collected ml	Method of collection	% parasitaemia	% PCV	Piroplasm pellet volume ml
P70	<u>T. a.</u> Ankara	13	400	into 100 ml ACD	83	19	1.75
143 N	<u>T. a.</u> Hissar	17	450	defibrinated	65	14	1.5
930	<u>T. a.</u> Gharb	13	800	into 200 ml ACD	97	19	2.0
931	<u>T. p.</u> Muguga	21	800	into 200 ml ACD	33	37	1.0

CHAPTER THREE
IMMUNISATION WITH SPOROZOITES
AND ASSESSMENT OF DERIVED
ANTIBODIES USING IFAT

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3.1. INTRODUCTION

During infection with T. annulata the bovine host is exposed to successive life cycle stages, each presenting many different antigens resulting in a complex assay of responses. In this study of the potential of sporozoite antigens as immunising material, initial work was orientated towards distinguishing the response which is stimulated by the sporozoite from that elicited by other stages. To this end, antibodies recognising sporozoite antigens were raised as reagents to identify, in particular those antigens with a role in stimulating protective immunity.

Sporozoites were first studied antigenically when Kimber, Purnell and Sellwood (1973) carried out immunofluorescent assays with serum from recovered cattle using cryostat sections of T. parva infected ticks and obtained positive reactions. Interest in the humoral response to sporozoites developed rapidly when the sporozoite neutralising ability of immune bovine serum was revealed (Gray and Brown, 1981) and confirmed (Musoke et al., 1982; Preston and Brown, 1985) as reviewed in Section 1.

Mitchell (1984) discussed methods of identifying potential targets for a protective immune response on the parasite surface. In order to avoid random selection, purification and testing of parasite molecules, defined and relevant antibody reagents are vital.

Hyperimmune bovine serum was an obvious source of antibody to generate to identify sporozoite antigens and was known to be likely to possess strong neutralising activity (Musoke et al., 1982; Preston and Brown, 1985).

However, the presence of antibodies to macroschizont and piroplasm stages in such serum (Burridge and Kimber, 1972) makes it less satisfactory than sera from animals which have been exposed solely to the sporozoite stage.

Two methods were used to raise ant sporozoite antisera which were intended to be restricted to the recognition of sporozoite antigens. One method was to immunise a rabbit with live T. annulata sporozoites since in this species the parasite does not establish infection (Neitz, 1957). This in vivo observation has been tested by attempting to infect rabbit PBM with sporozoites in vitro without success (C.G.D. Brown, personal communication). Although there has been one report of macroschizont development in a New Zealand White rabbit (Dipeolu and Ogunji, 1977) this has never been convincingly reproduced and the rabbit immunised with live Theileria sporozoites was considered to have been exposed to one stage only. The other method used was to inoculate calves with irradiated sporozoites in experiments to study the effect of different levels of irradiation on sporozoites and their subsequent effectiveness as immunising material. The intention was to be able to choose postimmunisation but prechallenge antisera from calves in which patent infection with schizonts or piroplasms was not detected during immunisation with irradiated sporozoites, but which showed a significant degree of immunity to challenge.

A limitation to the use of rabbit or bovine polyclonal sera alone to identify sporozoite antigens is that, by using whole organisms to immunise, the serum obtained inevitably recognises a number of antigens, many of which will not be relevant to protective immunity. A problem therefore exists in distinguishing those antigens which

stimulate antibody with sporozoite neutralising activity from others of no functional significance.

To circumvent this problem the same strategy as used by those studying the sporozoite stage of T. parva was adopted (Musoke et al., 1984; Dobbelaere et al., 1984). Mice were immunised with T. annulata sporozoites and hybridoma technology was applied to produce monoclonal antibodies which recognised single antigenic determinants on these sporozoites, enabling them to be studied individually.

This chapter describes how this battery of polyclonal and monoclonal antibodies were generated in order to identify sporozoite antigens.

3.2. MATERIALS AND METHODS

3.2.1. Experimental animals

Calves as described in Section 2.1.4.; 2-6 month old New Zealand White female rabbits; inbred Balb/c female mice.

3.2.2. Immunisation protocols

a) Immunisation of calves with live virulent sporozoites

Calves 54X, 134X, N53 and N61 all underwent repeated exposure to sporozoites. Following recovery from sporozoite stabilate immunisation (Section 2.2.8.) either due to a non lethal infection or through drug treatment with buparvaquone (Clexon, Coopers Animal Health) at 5 mg/kg on day 14, the calves were challenged on three occasions with live sporozoites (Sections 2.2.2. and 2.2.8.) or by adult tick feeds (Sections 2.2.1. and 2.2.8.) as indicated in Table 4. Serum from these calves following third challenge was considered to be hyperimmune.

Table 4 Immunisation and challenge of calves with live virulent sporozoites

a) Calves N53 and N61

Day	Action	<u>T. annulata</u> stock	Dose te	Inoculation s/c
0	Immunisation	Gharb STAB	0.2	RPG
14	Drug treated buparvaquone 5 mg/kg		-	-
30	Challenge 1	Gharb STAB	0.2	LPG
62	Challenge 2	Hissar STAB	4.0	LPG
		Ankara STAB	3.0	RPG
93	Challenge 3	Gharb GUTS	20.0	RPG

b) Calves 54X and 134X

Day	Action	<u>T. annulata</u> stock	Dose te	Inoculation s/c
0	Immunisation	Hissar STAB (54X)	4.0	RPG
		Gharb GUTS (134x)	5.0	RPG
35/41 (54X)/ (134X)	Challenge 1	Ankara STAB	3.0	LPG
		GUTS	6.0	
56/62	Challenge 2	Gharb GUTS (54X)	2.0	RPG
		Hissar GUTS (134X)	2.0	RPG
83/89	Challenge 3	Hissar	20 x <u>H.a.a.</u>	R ear
		Gharb	20 x <u>H.a.a.</u>	L ear
			(10 female, 10 male in each case)	

STAB = cryopreserved stabilate; GUTS = fresh GUTS

H.a.a. = infected adult H. a. anatolicum;

RPG = right prescapular gland; LPG = left prescapular gland

s/c = subcutaneous

b) Immunisation of calves with irradiated sporozoites

Antisporozoite sera were obtained from calves taking part in a series of experiments designed to test the effect of different irradiation levels on live sporozoites and their subsequent effectiveness as immunising material. Serum was taken from four calves, each of which received sporozoites irradiated by a 137 Caesium source at a different dose level. The details of their immunisation and subsequent challenge with virulent unirradiated sporozoites are shown in Table 5.

c) Immunisation of rabbits by infected and uninfected tick feeding

Antisporozoite serum was raised in rabbit 28 by allowing T. annulata-infected H. a. anatolicum adult ticks to feed on its ears on five occasions over a six month period according to the protocol in Table 6. In order to provide rabbit serum with antibodies to tick components to act as a control for the serum raised in rabbit 28, two rabbits (8 and 9) were fed on by uninfected adult H. a. anatolicum as shown in Table 6.

Blood was collected for serum on days 0, 73, 168 and 189 for rabbit 28 and days 0, 71 and 78 for rabbits 8 and 9.

d) Immunisation of mice with live sporozoites

Two Balb/c mice received repeated immunising doses of Percoll-purified T. annulata Ankara sporozoites (Section 2.2.2.) over several months as shown in Table 7.

The mouse which had received the final immunisation intravenously was killed three days after its final dose and its spleen removed for use in the production of monoclonal antibodies (see Section 3.2.4.).

Table 5 Immunisation of calves with irradiated sporozoites and their subsequent challenge

Action	Calf number: Irradiation dose of sporozoites (Gray)	L21		N44		L37		102	
		Day	Parasite	Day	Parasite	Day	Parasite	Day	Parasite
Immunisation 1	Stock	0	Ankara	0	Gharb	0	Ankara	0	Ankara
	Dose te		2 GUTS		3 GUTS		5 GF		4 GUTS
Immunisation 2	Stock	14	Ankara	35	Gharb	28	Ankara	28	Ankara
	Dose te		2 GUTS		4 GUTS		4 GF		4 GUTS
Immunisation 3	Stock	-	-	49	Gharb	77	Ankara	-	-
	Dose te		-		4 GUTS		3 GF		-
Immunisation 4	Stock	-	-	56	Gharb	-	-	-	-
	Dose te		-		4 GUTS		-		-
Challenge 1	Stock	49	Ankara	63	Gharb	91	Ankara	42	Ankara
	Dose te		2 GUTS		16 x <u>H.a.a.</u> (8 female, 8 male)		0.9 STAB		0.9 STAB
Challenge 2	Stock	90	Ankara	-	-	119	Ankara	-	-
	Dose te		4 GUTS		-		6 GUTS		-

STAB = cryopreserved stabilate; GUTS = fresh GUTS; H.a.a. = adult H. a. anatolicum; GF = GUTS filtrate

Table 6 Infected and uninfected tick feeds on rabbits

a) Rabbit 28: Infected H. a. anatolicum

Day	Tick numbers	<u>T. annulata</u> stock	Duration of tick feed (days)
0	60	Gharb	most 3
	80	Ankara	20 ticks 5
14	120	Hissar	most 3 20 ticks 5
35	60	Ankara	3
63	160	Hissar	most 3 20 ticks 5
179	60	Ankara	most 3
	60	Hissar	20 ticks 7

b) Rabbits 8 and 9: Uninfected H. a. anatolicum

Day	Tick numbers	Duration of tick feed
0	120	To engorgement (7 days)
28	40	To engorgement (7 days)
56	120	To engorgement (7 days)

Table 7 Immunisation of mice with live sporozoites for monoclonal antibody production

Day	Dose te	Route of administration
0	6.4	Intraperitoneal
14	16.0	Intraperitoneal
42	16.0	Intraperitoneal
204	4.0	1 mouse intravenous 1 mouse intraperitoneal

Blood was collected at the same time to obtain serum for IFAT. The other mouse which had a final intraperitoneal dose was kept alive and bled for serum on day 5 postimmunisation.

3.2.3. Hybridoma culture media

Once media had been made up they were stored at 4°C and used within 14 days. Stock solutions of media constituents are detailed in Appendix I.2.2.

a) Complete medium

RPMI 1640 medium (Gibco) was supplemented with 16.7% FCS, 2 mM L-glutamine, 100 units/ μ g per ml of penicillin/streptomycin, 2 μ g per ml amphotericin B (fungizone) and 5×10^{-5} M B-mercaptoethanol (B-ME).

Myoclone (Gibco), a FCS specially selected for its suitability in supporting the growth of myeloma and hybridoma cells, was used to supplement RPMI 1640 medium until hybridoma cell lines were cloned and established when the RPMI 1640 complete medium used to culture Theileria-infected cell lines was found to be satisfactory. B-ME was included in the medium as a reducing agent to minimise the damaging effects of oxidising agents released by dying cells and so promote growth of healthy hybridoma cells. Penicillin, streptomycin and fungizone were included to control bacterial, yeast or fungal contamination in hybridoma cultures, all being of particular threat to slowly establishing cultures or cloning work.

b) Incomplete medium

Incomplete RPMI 1640 medium was made up in the same way as complete medium but excluding FCS.

c) HAT or HT medium

HAT or HT medium was prepared by the addition of 4 ml 50 x HAT or HT to 96 ml complete RPMI 1640 medium to give 2 x HAT or HT. If 1 x HAT or HT medium was required, 2 ml 50 x HAT or HT was added to 98 ml complete medium.

3.2.4. Monoclonal antibody productiona) Preparation of myeloma cells

Mouse myeloma cell line P3-X63-Ag 8 clone 653 which does not synthesise or secrete immunoglobulin chains (Kearney et al., 1979) was resuscitated ten days prior to the day of fusion from storage in liquid nitrogen.

These myeloma cells were maintained in complete medium in 25 and 75 cm² tissue culture flasks (Flow) in the logarithmic phase of growth by subculturing every 48 hours, seeding rates being between 1 and 2×10^5 cells per ml. The myeloma cells were passaged as above and 24 hours later were harvested for the fusion. After two washes in incomplete medium (200 G, 7 minutes, 15°C) the cells were resuspended in 10 ml fresh incomplete medium and counted using 0.2% trypan blue. The total number of myeloma cells was 1.4×10^7 and all were used for the fusion with mouse spleen cells. A Giemsa-stained cyto-spin of the cells at this stage showed them to be healthy blastoid cells, many exhibiting mitotic figures.

b) Preparation of mouse spleen cells

The Balb/C mouse given sporozoites intravenously three days previously was killed by cervical dislocation and blood for serum was taken by cardiac puncture. The spleen was removed in an aseptic

manner, cut into small fragments and transferred into 3 ml incomplete medium. The barrel of a sterile 20 ml syringe was used to crush the spleen thoroughly, after which more medium was added and the suspension was frequently pipetted to disrupt the clumps of spleen cells. Having been allowed to stand for several minutes the cell suspension was centrifuged at 200 G, 15°C for seven minutes. The pellet was washed twice in incomplete medium before suspending in 10 ml fresh incomplete medium and counting an aliquot diluted in white blood cell counting fluid. 1.2×10^8 spleen cells were obtained and used for the fusion. A cytopspin showed the spleen lymphocytes to be healthy but with only a small percentage (less than 1%) blastoid.

c) Fusion of myeloma and spleen cells

The medium and PEG solutions (Appendix I.2.3.) were warmed to 37°C. Myeloma and spleen cells were mixed together in a conical glass 50 ml tube and centrifuged for seven minutes, 200 G at 15°C. The supernatant was poured off and a Pasteur pipette used to remove the last few drops. Over the period of a minute, 0.5 ml PEG solution 1 was added to the combined cell pellet using a 1 ml pipette while mixing with the pipette end as each drop was added.

In the same manner, 0.5 ml PEG solution 2 was added over one minute. In the following 2-3 minutes the clumps of cells were teased apart using the pipette tip to form a cell suspension in the PEG mixture. To this 9 ml complete medium was added dropwise over 3-5 minutes while mixing continuously. Finally 40 ml complete medium was mixed with the cell suspension to bring the total volume to 50 ml. The cells were then pipetted out into 5 x 96 well flat bottomed

microtitre plates labelled 1-5, each well receiving 100 μ l volume, and the plates were incubated overnight in a humidified 37°C incubator in 5% CO₂ in air.

d) Harvesting peritoneal macrophages

Balb/c mice from which peritoneal macrophages were to be obtained were killed by cervical dislocation. The abdominal wall was exposed by dissection in an aseptic manner. Four to five ml ice cold incomplete medium was injected into the peritoneal cavity and then withdrawn after several minutes, taking care to avoid penetrating the mouse's intestine. The cells were pelleted by centrifugation at 200 G for seven minutes at 4°C before being resuspended in 10 ml of the appropriate medium at 37°C and counted. Generally peritoneal macrophages were included in the medium at a concentration of about 2×10^5 cells per ml whenever cultures were established in new vessels and when cloning.

e) Post fusion protocol and initial IFAT screening

Twenty four hours after the fusion, 100 μ l of double strength HAT medium containing 2.4×10^5 peritoneal macrophages per ml was dispensed into each well of plates 1-5. The plates were incubated again at 37°C, 5% CO₂.

On day 7 after the fusion, microtitre plates were examined for any turbidity or untoward medium colour change; red indicating excessive cell death or yellow and turbid indicating contamination. Wells showing contamination were emptied and rinsed four times with 70% ethanol. A partial medium change was made by carefully removing 100 μ l supernatant medium and replacing with 100 μ l fresh warmed 1 x HAT medium.

On day 9 after the fusion, plates 1-5 were screened for cell growth under the inverted microscope. Wells in which definite focal colonies were growing were marked. On day 11 after the fusion, hybridoma culture supernatants were harvested from the wells marked as showing good cell growth on day 9. One hundred and ten μl was removed from each well and replaced with 110 μl fresh 1 x HAT medium. Indications that the time was appropriate for screening included cell growth covering at least 70% of the microtitre well bottom - an estimated 3×10^5 cells are needed to cover the bottom of a microtitre well - and medium colour change towards yellow. Four days had passed since the medium change, ample time for secreted antibody to accumulate.

The harvested supernatants were screened by IFAT against formalin fixed T. annulata Ankara sporozoites.

f) Expansion of hybridoma cultures

Cultures from which the supernatants gave strong sporozoite fluorescence in the initial IFAT screen were selected for expansion into 1 cm^2 wells. The microtitre well contents were resuspended and 100 μl was transferred to a 1 cm^2 well containing 400 μl 1 x HAT medium with peritoneal macrophages. The original microtitre well culture was maintained by regular addition of fresh medium until the cell cultures derived from it were established in larger vessels and cryopreserved. Once hybridoma cells were growing well in these 1 cm^2 wells, the culture supernatant was retested by IFAT for antisporezoite activity and, if still giving a positive reaction, cloning and further expansion were carried out. Further expansion involved the transfer

of 1 cm² well contents to 2 cm² wells and thence to 25 cm² (10 ml) flasks. At all stages, cultures in original wells were maintained, culture supernatants were retested for antiparasite activity and peritoneal macrophages were included in the medium on the initial transfer of a culture. One cm² wells and 25 cm² flasks were set up with medium containing macrophages 24 hours prior to culture transfer to allow time for medium to become conditioned. All cultures (parents and clones) were maintained in 1 x HAT medium until transferred to 2 cm² cultures when 1 x HT medium was employed. Medium changes and culture passage were made in much the same way as described for Theileria-infected cell lines in Section 2.2.4. Once parent and cloned hybridoma cultures had been growing for some time (12 weeks) they were gradually changed to the same complete medium as used for Theileria cultures and no longer required peritoneal macrophage feeder cells.

g) Cloning hybridoma cultures

Cloning was achieved by limiting dilution (Oi and Herzenberg, 1980) in 96 well microtitre plates (0.38 cm²). The 1 cm² culture selected for cloning was thoroughly resuspended and the cell concentration and viability determined using trypan blue and counting cells using a haemocytometer. Then dilutions were made down to five cells per ml in 1 x HAT medium containing peritoneal macrophages. These dilutions were plated out as follows (100 µl per well):

24 wells @ 100 cells/ml	i.e.	10 cells/well
24 wells @ 50 cells/ml	i.e.	5 cells/well
24 wells @ 10 cells/ml	i.e.	1 cells/well
24 wells @ 5 cells/ml	i.e.	0.5 cells/well

The 24 wells which contained a representative 0.5 cell each had the highest probability that a colony establishing originated from a single cell and was a true clone. Therefore it was from these wells that cloned cultures were selected. From day 7 after cloning, microtitre plates were screened under the inverted microscope and those wells appearing to contain early clone colonies deriving from a single cell were marked. Once nearing confluence, the culture supernatants from at least 10 clones from each parent cell line were screened by IFAT and positive clone cultures were expanded in a similar manner to parent cultures.

h) Cryopreservation and resuscitation of hybridoma cultures

The methods for freezing and thawing the hybridoma cells were identical to those followed for Theileria-infected cell lines (see Sections 2.2.5. and 2.2.6.).

i) Harvesting monoclonal antibody

Monoclonal antibody was obtained in hybridoma culture supernatant or in ascites fluid from hybridomas grown intraperitoneally in syngeneic Balb/c mice, the latter being approximately 100-fold more concentrated in antibody. Culture supernatants were collected after 48 or 72 hours cell growth when the culture was being passaged. The discarded medium was centrifuged at 1,400 G for 10 minutes at 4°C and the supernatant stored at -20°C.

To produce ascites, recipient mice were given intraperitoneal injections of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Sigma) one week before intraperitoneal inoculation of 5×10^6 live hybridoma cells in complete medium. These cells were taken from

cultures in the exponential phase of growth. Hybridoma growth and ascites production followed 1-4 weeks later and ascites tapped from the mice was diluted in complete medium, filtered (0.22 μ) and stored in liquid nitrogen. Repeated freeze thawing of supernatants and ascites was avoided.

j) Immunoglobulin class of monoclonal antibodies

Immunoglobulin class was determined using two methods; IFAT in which class-specific fluorescein conjugates were employed as second antibody (goat antimouse IgG-FITC or goat antimouse IgM-FITC, Nordic Immunologicals) at 1:40 dilutions; and an Ouchterlony agar gel radial diffusion test (Serotec) where 75 μ l undiluted culture supernatant placed in the central well acted as antigen and antibodies against the different mouse immunoglobulin classes and subclasses (IgA, IgM, IgG1, IgG2a, IgG2b, IgG3) were located in surrounding wells. After overnight incubation in a humidified atmosphere, the presence of a white precipitation line between the central well and an antibody well identified the monoclonal antibody class (Ouchterlony and Nilsson, 1973).

k) Nomenclature of hybridomas

Parent cell lines were named after the original fusion plate and well from which they were selected, for example, plate 1 well A7 gave rise to 1A7. Clones were named firstly after their parent and secondly after the well of the limit dilution plate from which the clone was taken, such as 1A7/G8.

3.2.5. IFAT antigens

All IFAT antigens were prepared on glass slides. Fifteen well slides (Flow Lab) were used for sporozoite antigens while other antigens were prepared on these or plain 0.8-1 mm glass slides (BDH). Plain glass slides were cleaned in a 50:50 mixture of ether and ethanol before use.

Antigen was applied to multiwell slides by expressing and then removing 20 μ l from each well to leave a thin film which was rapidly air dried. On plain slides, antigen was applied as a thin even smear covering the whole surface.

Antigen slides were wrapped and stored at -20°C in the presence of silica gel. One slide from each batch prepared was methanol fixed and Giemsa-stained to assess antigen quality.

Anhydrous acetone (BDH) used in antigen fixation was stored in the presence of a 5 \AA molecular sieve (Sigma)

a) Sporozoite antigen

i) Acetone fixation: Percoll-purified sporozoites (Section 2.2.2.) washed once in PBS to remove the Percoll were resuspended in PBS containing 1% BPA at 64 te/ml, i.e. 0.5 ml PBS per 32 te Percoll preparation. Approximately 80 multiwell slides could be prepared from 0.5 ml sporozoite suspension as described above. Slides were fixed in anhydrous acetone for 15 minutes and allowed to dry.

ii) Formalin fixation: To 32 te of Percoll-purified sporozoites in 1.0 ml PBS an equal volume of ice-cold 1:10 formalin (3.7% formaldehyde in PBS) was added dropwise. After 10 minutes fixation, 20 ml ice-cold PBS was added and the fixed sporozoites were pelleted by

centrifugation at 1,500 G for 15 minutes at 4°C. The sporozoites were washed in PBS three times centrifuging each time as described above. The final sporozoite pellet was resuspended in 0.5 ml PBS/1% BPA and spotted onto multiwell slides.

b) Macroschizont antigen

Preparation of macroschizont antigen was based on the technique of Burridge and Kimber (1972).

i) Acetone fixation: Macroschizont-infected lymphoblastoid cells in logarithmic phase of growth at approximately 10^6 cells per ml were resuspended in medium and pelleted by centrifugation at 200 G for 10 minutes at 4°C. The cells were washed three times, centrifuging as above each time and the volume of the final cell pellet was determined. Three volumes of PBS with 1% BPA were used to resuspend the cells which were held at 4°C while slides were prepared. Once the antigen had dried, slides were fixed in dry acetone for 15 minutes and then dried.

ii) Formalin fixation: Macroschizont-infected cells during the exponential phase of growth were washed twice in PBS as described above. After the second wash the cells were resuspended to a concentration of 5×10^7 per ml and an equal volume of ice-cold 1:10 formalin added dropwise. The cells were allowed to fix for 10 minutes at 4°C before washing three times in PBS and finally resuspending to 0.5×10^7 cells per ml. Slides were prepared from this suspension and air dried.

c) Piroplasm antigen

Approximately 5 ml blood was collected from a Theileria-infected calf with a patent piroplasm parasitaemia, preferably over 20%, into 150 ml ice-cold PBS (Burridge, 1971). The blood suspension was centrifuged at 400 G for 10 minutes at 4°C and the supernatant together with the buffy coat was discarded. After resuspending the packed erythrocytes and washing them three times in PBS, the volume of packed erythrocytes was estimated. Two volumes of PBS with 1% BPA were added and the suspension used to prepare IFAT antigen, generally on plain glass slides. These slides were acetone fixed for 15 minutes and dried.

3.2.6. IFAT method

a) Fixed antigen

The indirect fluorescent antibody test was used to assess antibody response to the different parasite stages in immunised calves, to assess antisporezoite antibody response in rabbits and to screen hybridoma cultures for antisporezoite antibody production. The method employed was based on that described by Burridge (1971) and Burridge and Kimber (1972).

Antigen slides were thawed from -20 to 4°C over 30 minutes and then to room temperature over a further 30 minutes. Acetone fixed slides were washed three times in PBS prior to use; piroplasm slides underwent four rinses to ensure all the haemoglobin was washed out. The slides were drained and then left to dry. Serum or monoclonal antibody dilutions were made in PBS. Antigen slides were placed in a humidified chamber and 10 µl of the antibody dilution

was pipetted into the appropriate well and recorded. At least one well on each antigen slide acted as a second antibody control and received PBS instead of first antibody. After a 30 minute incubation at room temperature the slides were washed in three changes of PBS and dried. Fluorescein labelled second antibody (Appendix I.2.1.) was applied at a 1:80 dilution (antibovine and antirabbit conjugates) or 1:40 (antimouse conjugate) dilution made in PBS. Twenty μ l of diluted conjugate was pipetted onto each well and the slides were incubated for another 30 minutes. After three PBS washes the slides were dried and mounted using a 66% glycerol mountant before being examined for fluorescence using a x 25 or x 40 oil immersion objective (Phaco 2 or 3) on an ultraviolet epi-illuminated fluorescence microscope (Leitz Orthoplan, Wetzlar).

Fluorescence was graded according to intensity as shown below:

- +++ very intense fluorescence
- ++ bright fluorescence
- + positive fluorescence
- ± weak fluorescence
- no fluorescence, negative

The highest serum dilution still producing positive (+) fluorescence was taken as the end point dilution.

b) Viable sporozoites

Surface immunofluorescence with live sporozoites was performed to determine whether antibody was reacting with sporozoite surface epitopes.

The method used Percoll-purified sporozoites which, after one wash, were resuspended thoroughly in 0.5 ml PBS and dispensed in

40-50 μ l aliquots into 1.5 ml plastic centrifuge tubes, hereafter called Eppendorf tubes (Alpha Labs). An equal volume of monoclonal antibody as 1:100 ascites or undiluted culture supernatant or anti-serum (diluted to 1:10, 1:80, 1:100) was added and the mixture incubated at room temperature for 30-45 minutes. Then 1.0 ml PBS was added to each tube and the sporozoites were pelleted by centrifugation at 1,500 G on a microcentrifuge (Biofug A, Heraeus Sepatech) for 15 minutes. The supernatant was carefully removed and each pellet was resuspended in 50 μ l of ice-cold 1:10 formalin solution and left to fix for five minutes. 1.0 ml PBS was added and the resuspended sporozoites centrifuged at 1,500 G for five minutes. Sporozoites were washed twice with PBS in this manner. After the last wash the supernatant was removed and the pellet resuspended in 30-50 μ l of the appropriate diluted fluorescein conjugate (Appendix I.2.1.). After a 60 minute incubation at room temperature the sporozoites were washed three times in PBS as above. The final pellet was resuspended in 25 μ l PBS, spotted onto the wells of a multiwell slide, dried, mounted and examined for fluorescence.

3.3. RESULTS

3.3.1. Clinical reactions of calves

The clinical reactions to immunisation(s) and challenge(s) are shown graphically in Figure 10 (a-f). Where calves were paired, the reaction of just one of the calves has been given.

Calf N53 (Figure 10a)

During primary infection this calf suffered a sharp rise in body temperature (peak 41.3°C) and piroplasm parasitaemia (peak 12.5%) which were both halted following drug treatment. PCV, which fell to its lowest level of 12% on day 17, rapidly recovered to normal levels as the parasitaemia declined. Following all three challenges, body temperature and PCV remained unchanged while Giemsa-stained biopsy smears of the lymph node draining the site of challenge inoculum showed transient hyperplasia but no parasitosis. A low level of piroplasm parasitaemia remained in the blood which increased marginally on challenge, particularly following challenges 2 and 3.

Calf 54X (Figure 10b)

The general picture was similar to that of calf N53. This calf recovered spontaneously from primary infection and not through drug treatment and a more prolonged clinical reaction was observed with piroplasm parasitaemia reaching a peak of 45% on day 22. On this day PCV had fallen to 13%. Again, like calf N53, PCV and body temperature did not rise following any of the three challenges although, unlike N53, macroschizonts were observed as well as hyperplasia in the lymph node biopsy following the first challenge. Piroplasms were continuously present in the blood at low levels during challenge and showed slight increases following each of the three challenges.

Calf L21 (Figure 10c)

The first immunising dose of sporozoites irradiated at 320 Gy established active infection in the calf as exhibited by the appearance of macroschizonts in the local lymph node on day 12 and piroplasms

on day 15. This reaction overlapped the time of the second immunising dose of 320 Gy irradiated sporozoites. PCV and body temperature values had returned to preimmunisation levels by the time the calf was challenged and showed no changes in response to either challenge 1 or 2. Piroplasms remained at a low level.

Calves N44 and L37 (Figure 10d and e)

These calves demonstrated similar responses to immunisation and challenge. Immunisation with irradiated sporozoites (500 or 640 Gy) caused body temperature to rise up to 40°C. This was of short duration as were the hyperplastic reactions in the local lymph nodes. There was no evidence of infection establishing and blood and lymph node remained negative for parasite. When calf N44 was challenged it became infected and exhibited a moderate degree of clinical reaction, piroplasm parasitaemia reaching 19% as did PCV. The calf went on to recover from this. Calf L37 was challenged twice and underwent a relatively mild reaction to challenge 1 with piroplasms at a level of 7.5%, PCV only falling to 20% and the most significant changes being detected in the local lymph node. The second challenge evoked no recrudescence in parasites over the time the calf was monitored.

Calf 102 (Figure 10f)

This calf showed even less response to immunisation with 840 Gy irradiated sporozoites. Body temperature remained steady and only mild hyperplasia was observed in the lymph node following the initial immunising dose. However on challenge a severe clinical reaction developed resulting in death 24 days after challenge.

Figure 10 Clinical reactions of calves during immunisation and challenge with live virulent or irradiated sporozoites.

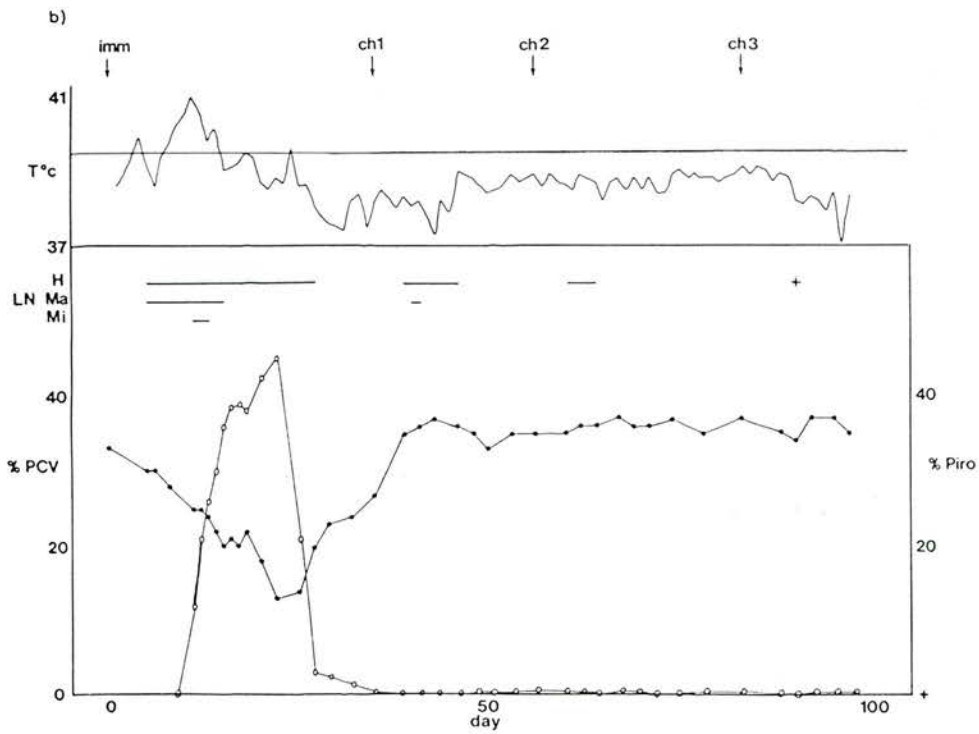
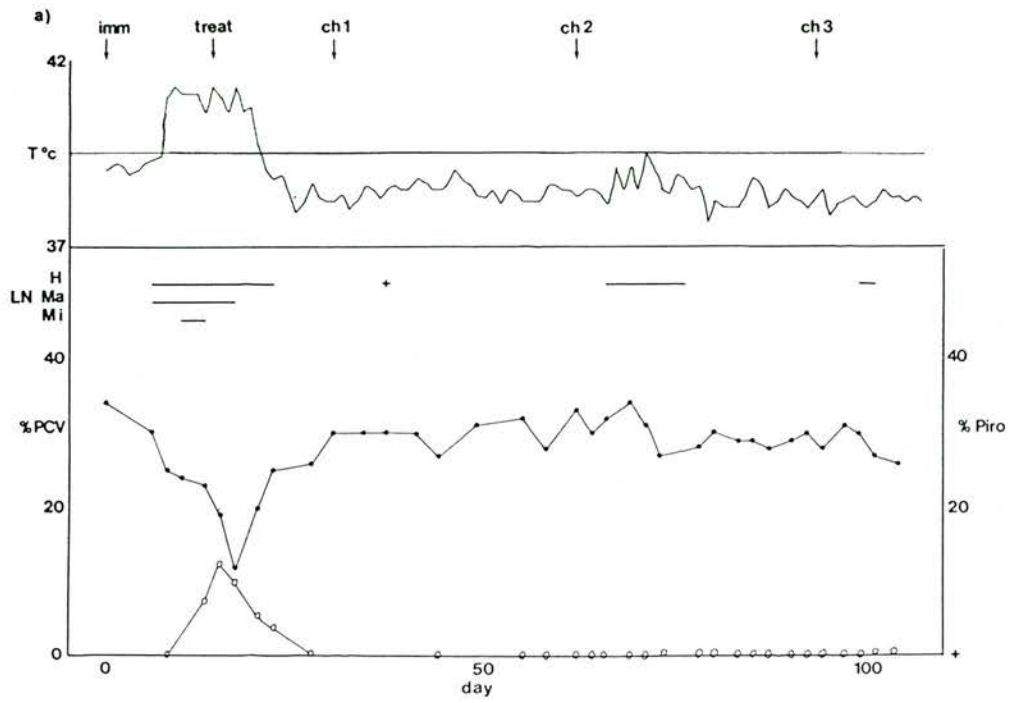
- a Calf N53 immunised with live virulent sporozoites and drug treated.
- b Calf 54X immunised with live virulent sporozoites and drug treated.
- c Calf L21 immunised with sporozoites irradiated at 320 Gy.
- d Calf N44 immunised with sporozoites irradiated at 500 Gy.
- e Calf L37 immunised with sporozoites irradiated at 640 Gy.
- f Calf 102 immunised with sporozoites irradiated at 840 Gy.

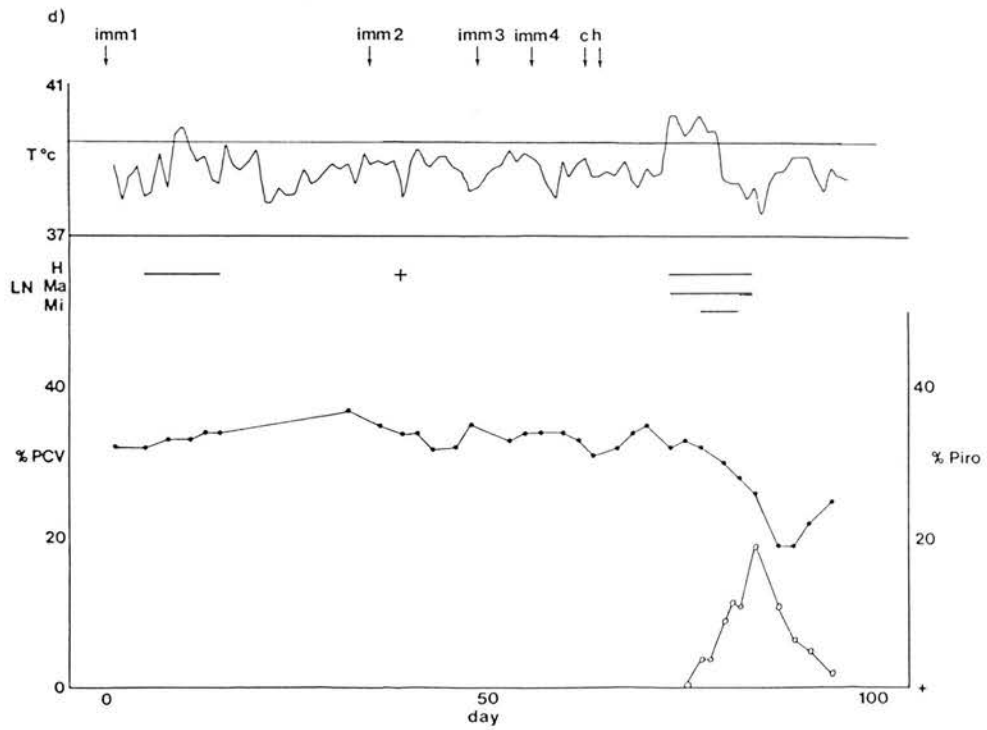
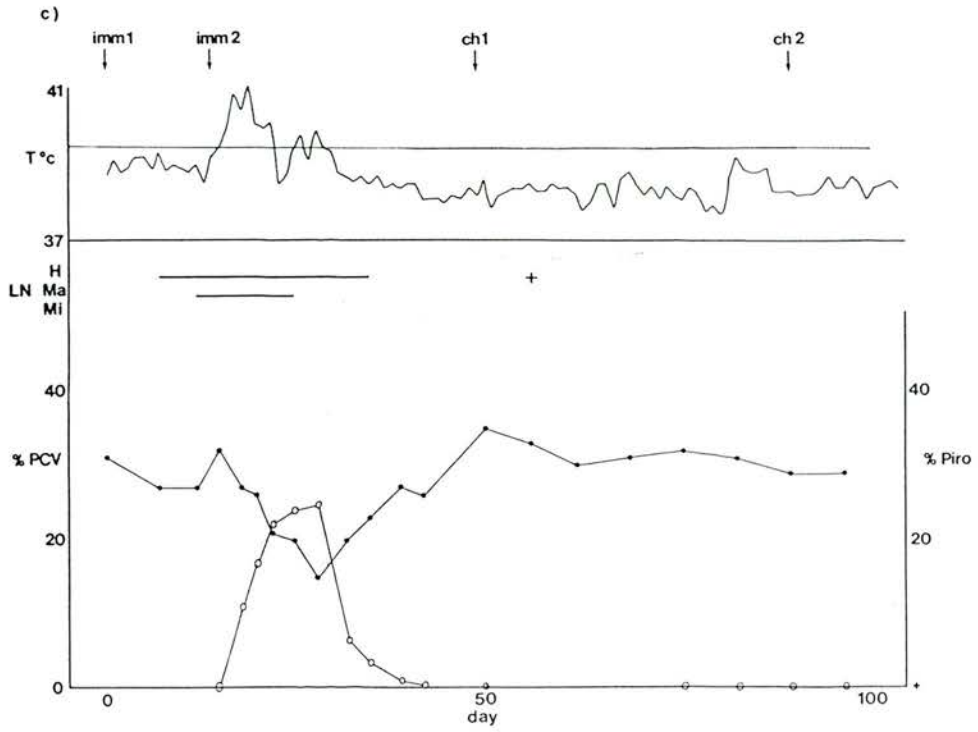
All calves were challenged with live virulent sporozoites.

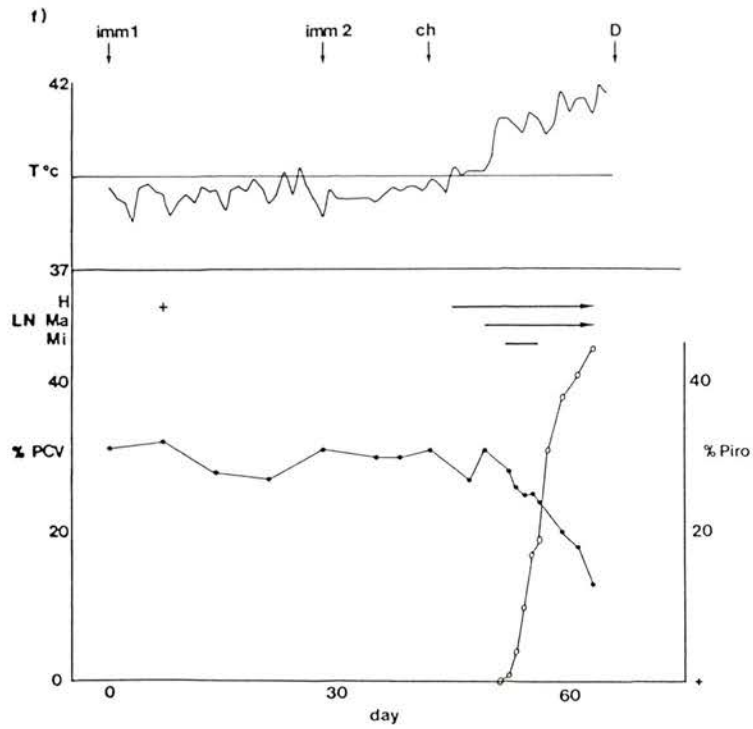
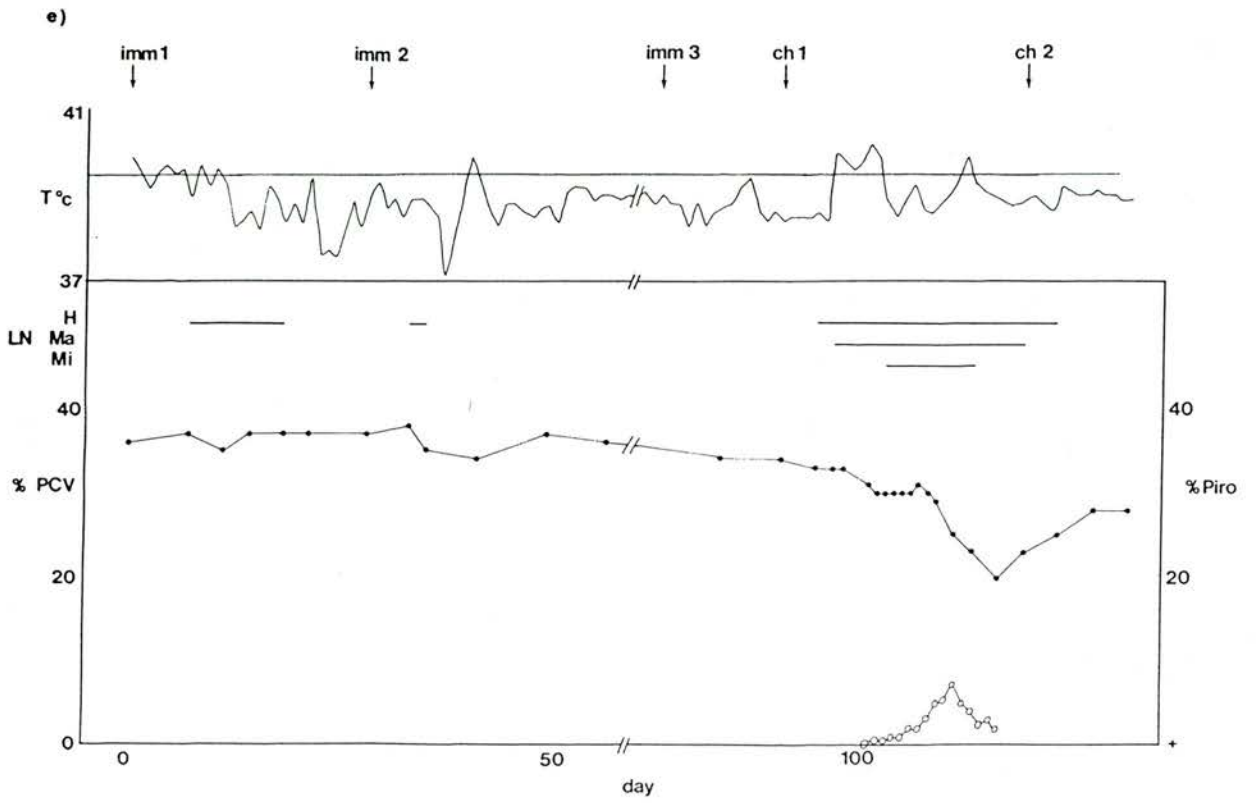
Key:

- imm immunisation
- ch challenge
- s serum taken
- D died or destroyed
- LN regional lymph node
- H hyperplasia observed
- Ma macroschizonts observed
- Mi microschizonts observed
- T°C rectal temperature
- % PCV % packed cell volume ●—●
- % Piro % piroplasm parasitaemia ○—○

Section 3.2.2. details the immunisation and challenge protocols used for these calves.







3.3.2. IFAT results: calf sera

Table 8 indicates the end point titres by IFAT for the calves' sera tested against different stock and stage antigens during the course of immunisation and challenge.

Calves N53, N61, 54X and 134X immunised with live virulent sporozoites all developed titres of $1/160$ to $1/640$ to all three parasite antigens. These titres were very similar between stocks when the IFAT reactions with different parasite stocks were tested.

Calf L21 which became infected during immunisation with 320 Gy irradiated sporozoites but recovered, developed a very high anti-sporozoite titre of $1/2560$ postchallenge. Titres against other parasite stages were also high.

In response to immunisation with 500 and 640 Gy irradiated sporozoites, calves L37 and N44 developed ant sporozoite titres of $1/40$ and $1/160$ respectively. In calf L37 an antimacroschizont titre developed more slowly but reached the same value ($1/40$) as that against sporozoites after immunisation 3. Calf N44 failed to develop a titre against Ankara macroschizont antigen even after four immunising doses. Neither calf showed antiproplasm activity on IFAT until challenge when titres developed to all three stages of parasite antigen.

For calf N44 the prechallenge ant sporozoite titre cross-reacted well with T. annulata Hissar sporozoites ($1/160$) and to a degree with Gharb sporozoites ($1/40$). There was also some cross-reaction producing a low titre to Hissar macroschizont antigen ($1/40$).

Calf 102 which received 840 Gy irradiated sporozoites, developed a low level sporozoite titre of $1/40$ during immunisation with very little other IFAT reaction and showed no dramatic increase in titres before dying from the challenge reaction.

Table 8 Reciprocal IFAT end point titres for calves immunised with live virulent or irradiated sporozoites

Calf + immunising material	Day	Serum	Reciprocal end point titre								
			sp	A schiz	piro	sp	H schiz	piro	sp	G schiz	piro
N53 live sporozoites	-1	Preimmunisation	10	-	-	10	10	-	10	10	-
	58	Postchallenge 1	160	160	160	40	160	160	160	160	160
	90	Postchallenge 2	160	640	640	160	640	640	640	640	640
N61 live sporozoites	-1	Preimmunisation	40	-	-	40	10	-	40	-	-
	58	Postchallenge 1	160	160	160	40	160	-	160	160	160
	90	Postchallenge 2	160	640	640	160	640	640	160	640	640
54X live sporozoites	0	Preimmunisation	-	10	-	-	10	-	-	10	-
	35	Postimmunisation	160	40	160	40	40	640	-	40	160
	56	Postchallenge 1	160	160	160	160	160	640	-	160	160
	83	Postchallenge 2	640	640	640	640	640	2560	-	640	640
	90	Postchallenge 3	640	640	640	640	640	2560	-	640	640
	95	Postchallenge 3	640	640	640	640	640	640	-	640	640
134X live sporozoites	0	Preimmunisation	-	10	-	10	10	-	-	-	-
	41	Postimmunisation	40	40	160	40	40	160	-	40	640
	62	Postchallenge 1	160	160	640	160	160	640	-	160	640
	89	Postchallenge 2	160	160	640	160	160	640	-	160	640
	101	Postchallenge 3	160	160	640	160	160	640	-	160	640
L21 320 Gy irradiated sporozoites	0	Preimmunisation	-	-	-	-	-	-	-	-	-
	28	Postimmunisation 2	-	640	640	-	-	-	-	-	-
	56	Postchallenge 1	640	640	640	-	-	-	-	-	-
	76	Postchallenge 1	2560	2560	2560	640	-	-	2560	2560	-
	104	Postchallenge 2	2560	640	10240	-	-	-	-	-	-
N44	0	Preimmunisation	10	10	-	10	10	-	10	40	-
	62	Postimmunisation (4 d 50 Kr)	160	-	-	160	40	-	40	40	-
	92	Postchallenge	160	160	640	160	160	640	160	160	640
L37 640 Gy irradiated sporozoites	0	Preimmunisation	-	-	-	-	-	-	-	-	-
	28	Postimmunisation 1	10	-	-	-	-	-	-	-	-
	77	Postimmunisation 2	40	10	-	-	-	-	-	-	-
	91	Postimmunisation 3	40	40	-	-	-	-	-	-	-
	119	Postchallenge 1	40	40	40	-	-	-	-	-	-
	127	Postchallenge 2	640	640	160	-	-	-	-	-	-
	152	Postchallenge 2	160	640	160	-	-	-	-	-	-
102	0	Preimmunisation	-	-	-	-	-	-	-	-	-
	28	Postimmunisation (1 x 84 Kr)	10	10	-	-	-	-	-	-	-
	42	Postimmunisation (2 x 84 Kr)	40	10	-	-	-	-	-	-	-
	63	Postchallenge	40	10	40	-	-	-	-	-	-

A = T. annulata Ankara; H = T. annulata Hissar; G = T. annulata Gharb;
 sp = sporozoite antigen; schiz = macroschizont antigen; piro = piroplasm;
 f = formalin fixed; a = acetone fixed

3.3.3. IFAT results: rabbit sera

Sera raised in rabbits 28 and 8 by feeding Theileria-infected or uninfected adult H. a. anatolicum ticks respectively were tested by IFAT for antisporozone activity. Titres against formalin-fixed T. annulata Ankara sporozoite antigen are shown in Table 9 for rabbit 28. Rabbit 8 day 0 and day 78 serum gave no fluorescence even at the lowest dilution ($1/10$).

3.3.4. IFAT results: mouse sera

Serum was taken from the Balb/c mice three (intravenous recipient) or five (intraperitoneal recipient) days after their final immunising dose and was tested by IFAT against various sporozoite antigens and against acetone fixed T. annulata Ankara schizont and piroplasm antigen. Normal mouse serum was included as a negative control while an antisporozone monoclonal antibody, 4E5 (Shiels, personal communication) was used as a positive control.

Table 10 shows the IFAT results for the above sera. Using formalin fixed sporozoite antigen prepared from the T. annulata Ankara with which the mice were immunised, an IFAT titre of $1/160$ was observed for the intraperitoneal recipient. This was greater than the titre of $1/40$ obtained for the mouse immunised intravenously, while normal mouse serum gave no fluorescent reaction. A low titre to Ankara macroschizont antigen was detected in both immunised mice. Neither showed any antipiroplasm reaction by IFAT (data not shown).

Table 9 IFAT results: sera from rabbit fed on by T. annulata infected Hyalomma ticks

Day of serum	R e c i p r o c a l I F A T t i t r e s	
	Formalin fixed sporozoite	Formalin fixed macroschizont Acetone fixed piroplasm
0	0	-
73	640	10
168	160	10
189	640	40

All antigens: T. annulata Ankara Sera from rabbit 28

- No detectable titre (less than $1/10$)

Table 10 IFAT results: sera from mice immunised with purified sporozoites

Antibody	Reciprocal dilution	Antigen				
		A sp a f	H sp a	G sp a f	A schiz a	
4E5 culture supernatant	1	+ ±	+	+ +	-	
	4	± -	+	± ±	-	
Normal mouse serum	10	± ±	+	+ +	±	
	40	- -	±	- ±	-	
i/p immunised mouse serum	10	+ +	+	+ +	+	
	40	± +	+	+ +	+	
	160	- +	+	+ +	±	
	640	- ±	-	± ±	±	
	2560	- -	-	- -	-	
i/v immunised mouse serum	10	+ +	+	+ +	+	
	40	± +	+	+ +	±	
	160	- ±	±	± ±	-	
	640	- -	-	- -	-	
	2560	- -	-	- -	-	

A = T. annulata Ankara

H = T. annulata Hissar

G = T. annulata Gharb

sp = sporozoite antigen

schiz = macroschizont antigen

f = formalin fixed

a = acetone fixed

3.3.5. IFAT results: monoclonal antibodies

a) Primary screen

On day 9 after the fusion, 26% of the microtitre wells contained cultures with distinct colonies growing. The culture supernatants of 74 of these were taken for testing for antisporozone activity using formalin fixed T. annulata Ankara sporozoite antigen.

Twenty of the 74 supernatants gave strong fluorescence of sporozoites and the cultures corresponding to these 20 were expanded, cloned and cryopreserved.

b) Stable clones

Following cloning and loss of some secreting cultures, 19 stable cloned hybridoma cell lines were established secreting antisporozone monoclonal antibodies. One non-secretor, 4C7/G7, was retained for use as a control hybridoma cell line.

Table 11 shows the IFAT reactions of these 19 monoclonal antibodies when tested against sporozoite, macroschizont and piroplasm stages of T. annulata Ankara and sporozoites of T. annulata Gharb. Figure 11 shows examples of the fluorescence obtained with monoclonals recognising formalin fixed T. annulata Ankara sporozoites.

Antisporozone activity appeared to cross-react well with sporozoites of the Gharb stock from Morocco; all 19 of the monoclonals reacting positively with this antigen.

No fluorescence of piroplasms was observed with any of the antisporozone monoclonals; several did cross-react with the macroschizont-infected lymphocyte antigen of T. annulata Ankara giving a variety of fluorescence patterns.

Table 11 IFAT results: monoclonal antibodies tested against different stages and stocks of *T. annulata*

Monoclonal antibody (Mab)	IFAT antigen				
	Ankara sp f	Gharb sp f	Ankara sp a	Ankara sch a	Ankara piro a
Supernatants:					
4B11/C12	++	++	+	± speckly cytoplasm	-
4A7/A8	+	+	-		-
5D1/D11	+	+	+	± schizont	-
5F1/E11	+	+	±		-
1B4/B12	++	+	±	± speckly cytoplasm	-
1A7/G8	++	+	+	-	-
4A7/E8	+	+	±		-
4A7/B12	+	+	±		-
4D3/E8	+	+	±		-
5F1/H11	+	+	+		-
4B11/E9	++	+	+	+ speckly cytoplasm	-
4A7/D10	+	+	+		-
4A7/E12	+	+	+	-	-
4B11/B12	+	+	+	± speckly cytoplasm	-
4B11/G11	+	+	+	+ speckly cytoplasm	-
4G5/C10	+	+	±	+ speckly cytoplasm	-
5F1/G12	+	+	+		-
4B11/H12	+	+	+	? speckly cytoplasm	-
5D1/D12	+	+	±	-	-
*4C7/G7	-	-	-	-	-
*4E5	±	±	±		-
*1C7	-	-	-	+	-
Ascites:					
4B11/C12	++	++		± speckly cytoplasm	-
4A7/A8	+	+		+ schizont	-
5D1/D11	+	±			-
5F1/E11	+	+		-	-
4G5/C10	+			± speckly cytoplasm	-
1A7/G8	+			-	-
4D3/E8	±			± schizont	-
*7B10	±	-		+ surface	-

***Controls:**

4C7/G7 supernatant from non-secreting hybridoma cell line

1C7 antimacroschizont Mab (Shiels *et al.*, 1986a)

7B10 anti-infected lymphocyte surface Mab (Shiels *et al.*, 1986b)

4E5 antiprotozoite Mab (B. Shiels, personal communication)

sp = sporozoite antigen; schiz = macroschizont antigen;
piro = piroplasm antigen; f = formalin fixed; a = acetone fixed

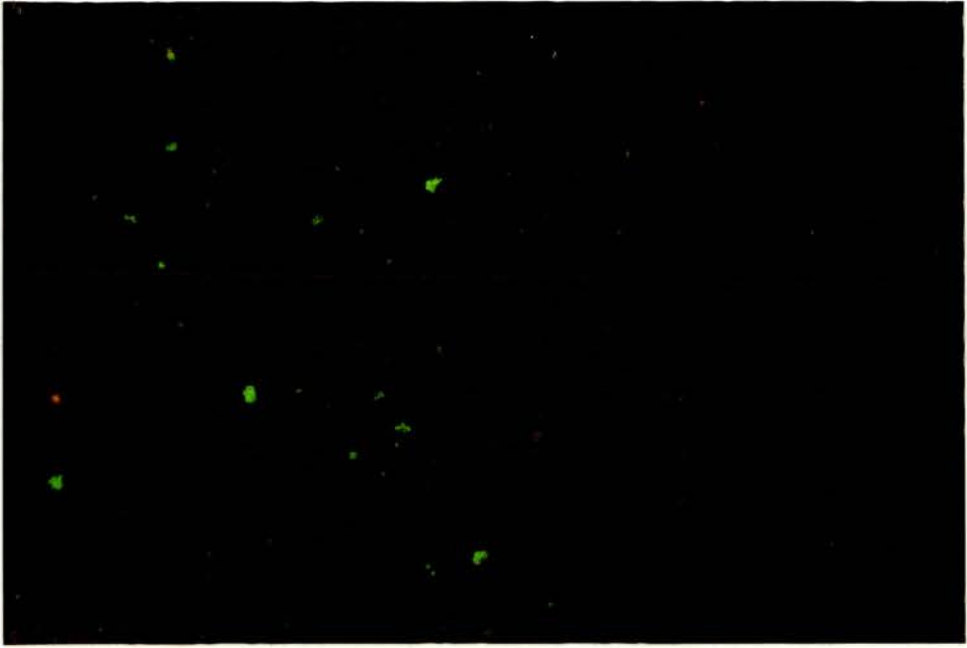
Figure 11 Immunofluorescence of formalin fixed T. annulata Ankara sporozoites in the IFAT using monoclonal antibodies:

a) 4B11 (x 440 magnification)

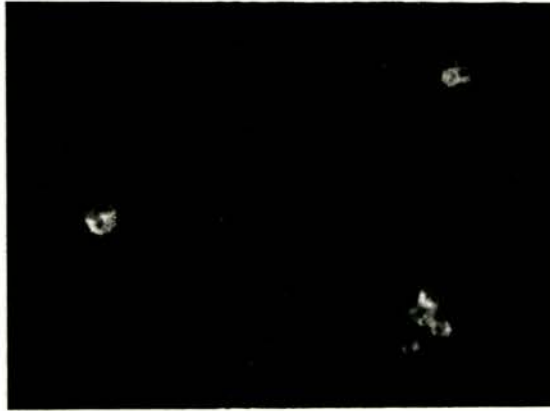
b) 1A7 (x 1,500 magnification)

These clearly show that the use of monoclonals 4B11 and 1A7 in the IFAT results in fluorescence located at or near the sporozoite surface.

a)



b)



3.3.6. Immunoglobulin class of monoclonal antibodies

Two antisporezoite monoclonal antibodies, 1A7/G8 and 4B11/C12, were subjected to IFAT and the Ouchterlony test to determine their immunoglobulin class as described. By both methods the two monoclonals were found to be of immunoglobulin class M (IgM). Figure 12 shows the appearance of the Ouchterlony test result.

3.3.7. Surface immunofluorescence of sporozoites

Live Percoll-purified *T. annulata* Ankara sporozoites were used in surface IFAT against several monoclonal antibodies and antisera and the reactions obtained are detailed in Table 12.

Table 12 Surface immunofluorescence of live sporozoites with different antibodies

Antibody	Dilution	Fluorescence
Monoclonal antibodies:		
4B11/C12	A 10^{-1}	++
	A 10^{-2}	+
	S	+
		} - good definition
1A7/G8	A 10^{-1}	+
	A 10^{-2}	±
	S	+
		} - weaker than 4B11/C12
7B10	A 10^{-1}	-
	A 10^{-2}	-
	S	-
Bovine antiserum:		
134X day 0	1/1	+
	1/80	-
day 101	1/1	++
	1/80	+
Rabbit antiserum:		
28	day 0 1/80	-
	day 73 1/80	++

A = ascites; S = supernatant

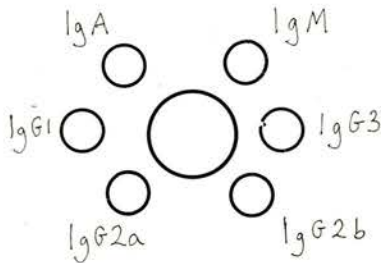
7B10 = control monoclonal antibody (Shiels et al., 1986a)

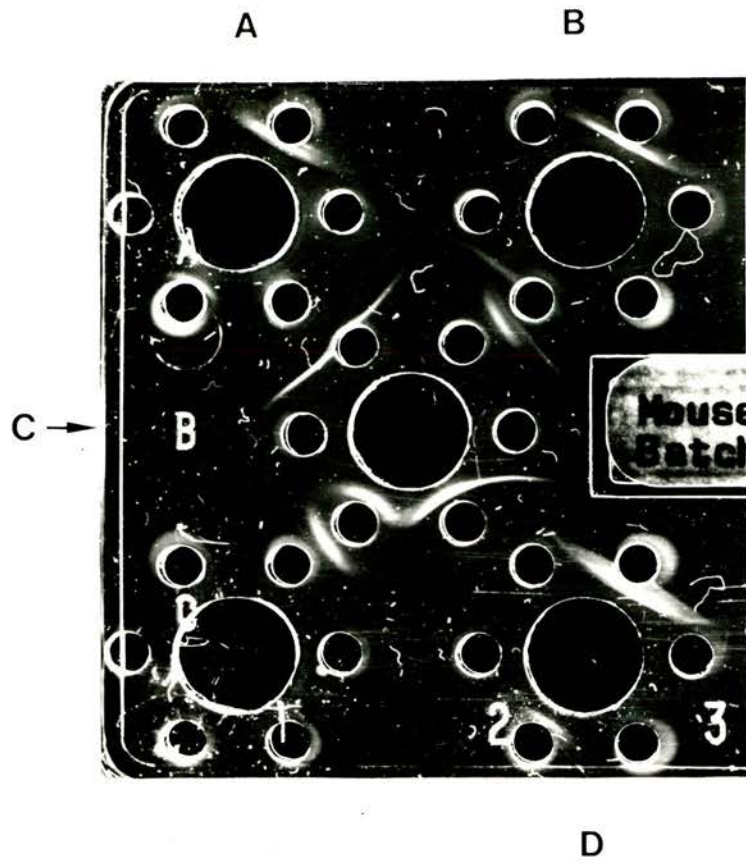
Figure 12 Precipitation lines in the Ouchterlony test used to determine the immunoglobulin class of anti-sporozoite monoclonal antibodies.

Immunoglobulin class:

- A 4B11 IgM
- B 1A7 IgM
- C 4H5 IgG2 control IgG2 monoclonal
(Shiels et al., 1986a)
- D 3D6D3 IgM control IgM monoclonal
(see Section 4.2.5.)

Test format:





3.4. DISCUSSION

The calves in which hyperimmune serum was raised (N53, N61, 54X, 134X) all became immune during their primary infection as shown by the subsequent lack of reaction to challenge on three occasions and the development of IFAT titres to all three parasite stages; sporozoite, schizont and piroplasm. Antisporozoite titres were established after infection but either remained static or showed only a four-fold rise after three challenges. This suggests that the re-exposure to sporozoites was not sufficient to boost the calves' humoral response to sporozoites dramatically. It is possible that more long term and repeated sporozoite inoculation is required to obtain a convincing anamnestic response. For example, workers studying Plasmodium falciparum, and wishing to use antibody with restricted specificity to sporozoite and liver stages, obtained serum from an individual constantly exposed to malaria over 20 years while under chloroquine prophylaxis. This subject exhibited extremely high antibody titres to sporozoites as assessed by IFAT (Guerin-Marchand, Druilhe, Galey, Londono et al., 1987).

During challenge of the calves it is likely that the proportions of each antibody class stimulated were changing. This may not have been reflected in IFAT titres but may be of enormous functional significance. Musoke et al. (1982) found that antisporozoite antibody class following a primary infection was almost exclusively IgM while serum from cattle boosted two to three times showed antisporozoite activity to be predominantly IgG. They used solid phase radio-immunoassay to detect antibody so titres are hard to compare directly with those detected by IFAT. However their results show that there was as

much variation in antibody titre between calves within a group as between titres exhibited by individual calves after primary infection and after repeated exposure to sporozoite challenge. Thus some calves had antibody titres after primary infection equivalent to titres exhibited by hyperimmune calves. It seems that IFAT titres alone may not be a good indication of hyperimmunity to sporozoites.

The calves involved in the irradiated sporozoite experiments present a complicated spectrum of reactions and IFAT titres. In simple terms, the results can be interpreted as showing that the lowest level of sporozoite irradiation studied here (320 Gy, calf L21) may have reduced the infective dose but nevertheless did not prevent the calf from becoming infected and then recovering to behave in a fashion much like one of the hyperimmunised calves above during challenge.

Srivastava and Sharma (1977) reported prolonged incubation periods and less severe disease in two groups of calves receiving T. annulata sporozoites irradiated with 50 or 100 Gy. However two out of three calves still died in each group, all of which became infected and their observations could be attributed more to reduction in the infective dose as observed in calf L21 than to true attenuation of the parasite.

Similarly, Purnell et al. (1974) studied the effect of inoculating cattle with suspensions derived from T. parva infected Rhipicephalus appendiculatus irradiated over a wider range of 0-600 Gy. At lower irradiation doses (200 Gy or less), calves became infected by the inoculated parasites and were immune to challenge. Of two calves which showed no reaction to the first inoculum, one died on challenge and

the other survived challenge undergoing only a mild reaction. Above 250 Gy irradiation, parasites failed to infect cattle in all but one case which died. These cattle remained susceptible to challenge. Thus, only one animal could be considered to have become immune without showing clinical reaction, and therefore perhaps without infection establishing. There was no good evidence for attenuation of sporozoites and the observations made by Purnell, Ledger and Obatre (1972) that increasing doses of irradiation produced increasing numbers of morphologically abnormal parasites in acini may account for the reactions by indicating a progressive reduction in parasite numbers inoculated.

In this study a high level of irradiation (840 Gy, calf 102) reduced sporozoite infectivity and effective immunogenicity leaving the calf susceptible to challenge with live sporozoites.

Between these two extremes, calves L37 and N44 received sporozoites irradiated at intermediate dose levels and while infection did not appear to establish in either calf during immunisation on the basis of absence of detectable schizonts in repeated biopsies of the local lymph node and low or undetectable levels of antibody to schizont and piroplasm stages, neither calf died on challenge. L37 underwent a mild clinical reaction and N44 a moderate one before recovery. This is compared to the reaction of two control calves challenged with the same stabilate inoculum which either died or demonstrated severe clinical signs (data not shown). If one accepts that infection was not established in calves L37 and N44 the implication is that the irradiated sporozoites alone were able to stimulate a protective immune response. In vitro studies on irradiated Plasmodium berghei sporozoites

have revealed that some retain the ability to infect cultured hepatoma cells and transform to the trophozoite stage but are unable to develop further into the schizont (Sigler, Leland and Hollingdale, 1984). Preliminary in vitro observations on the invasion of bovine lymphocytes by irradiated T. annulata sporozoites in this laboratory have indicated that a similar phenomenon may be occurring (Brown, 1981) in which case both humoral responses to sporozoites and cell mediated responses to the trophozoite-infected cell may have been elicited in calves N44 and L37. The relative importance of each has yet to be determined but if sporozoite surface proteins on irradiated sporozoites remain functional as regards infectivity, those involved in stimulation of neutralising antibody, which may be the same proteins, are also likely to be intact. Serum taken after immunisation with irradiated sporozoites but before challenge in calf N44 cross-reacted well between the immunising T. annulata Ankara stock and T. annulata Hissar and, to a lesser extent with T. annulata Gharb, while there was minor antimacroschizont and no antipiroplasm response detectable. The observation that cross-reaction between sporozoites of different parasite stocks is present where there is virtually no cross-reaction between life cycle stages within the same stock suggests that the protective immunity generated may be restricted to sporozoite challenge and leave animals susceptible to challenge with macroschizont infected cells though this would not be a risk during natural challenge. Whether this sporozoite stock cross-reactivity correlates with cross-immunity remains to be determined. The specificity of protective immunity produced by irradiated Plasmodium berghei sporozoites has been investigated by Nussenzweig, Vanderberg, Most and Orton (1969) and they

showed that the immunological differences between sporozoites and blood forms of one Plasmodium species may be greater than those between sporozoites of different species. Although their observation refers to species rather than stocks of the same species as in this study, the principle applies to both. Also, cross-immunity, where it occurred, between sporozoite species was unrelated to the cross-immunity between the respective blood stages. Immunity was stage specific; challenge of sporozoite immunised mice with homologous blood stage parasite resulted in no significant degree of protection.

Therefore, the sera from calves N53, N61, 54X, 134X and L21 could be classified as hyperimmune sera reflecting exposure to all parasite stages. Sera from calves N44 and L37 provided what could tentatively be described as stage specific immune sera, apparently only exposed to sporozoites. Calf 102, inoculated with sporozoites receiving the highest doses of irradiation, was susceptible to challenge and failed to develop significant IFAT titres to any stage; its serum could, therefore, provide a useful comparison for sera from the other animals.

The rabbits immunised by tick feeding predictably gave strong or negligible ant sporozoite IFAT reactions according to whether infected or uninfected ticks had fed. The serum from rabbit 28 represents sporozoite specific antiserum with a high titre without the complication of possible trophozoite development since sporozoites do not infect rabbits (Neitz, 1957; C.G.D. Brown, personal communication). It follows that sporozoites remain extracellular and exposed to the immune system for longer than in cattle and, in consequence, high levels of ant sporozoite antibody were likely to

develop. At the same time the slow release of sporozoites into the dermis during tick feeding probably serves as a very effective method of antigen delivery to an area rich in cells of the immune system which have infiltrated the site of tick attachment (Gill and Walker, 1985; Walker and Fletcher, 1986).

The immunisation regime followed for the mice and for monoclonal antibody production appeared to elicit disappointingly low levels of ant sporozoite antibody in the mouse used for the fusion which was inoculated intravenously. Since this mouse's serum was taken only three days post-inoculation, the low IFAT titre may be due to the fact that the anamnestic response was still in its early stages. A higher ant sporozoite IFAT titre was exhibited by serum from the mouse receiving its sporozoite dose intraperitoneally. Serum was taken later from this mouse and antibody levels may have had time to rise in response to the final inoculation. However, low antibody levels at the time of spleen removal need not imply that poor numbers of ant sporozoite secreting hybridomas will be obtained. Indeed the opposite may be true. There is evidence that recently activated B cells or plasma cell precursors, as yet non-secreting, fuse preferentially (Goding, 1980) and for this reason fusion is recommended on day 3 after the final immunisation (Oi, Jones, Goding, Herzenberg and Herzenberg, 1978). The mouse had "rested" for several months after the initial immunisation and was then boosted with an intravenous sporozoite dose as recommended by Goding (1980). Other workers (Pearson et al., 1980) have found that the intravenous route for the final immunisation results in high numbers of specific hybrids because memory lymphocytes are selectively stimulated in the spleen.

The fusion itself involved a spleen:myeloma cell ratio of 10:1 which has achieved good results previously, although ratios anywhere between 1:1 and 10:1 seem to be satisfactory (Oi et al., 1978; Galfre, Howe, Milstein, Butcher and Howard, 1977).

Nineteen cloned antisporezoite monoclonal antibodies were established from this fusion which may appear rather few considering that 15 parent cultures were cloned. However, during expansion, cloning and culture of these hybridomas, many cultures previously identified as antisporezoite being antibody secretors suddenly stopped secreting. Prior to cloning, this can easily result by overgrowth of secretors by non-secreting hybridomas especially as antibody production, by its use of cell resources, makes a secreting hybridoma cell line less able to compete for growth with non-secretors (Goding, 1980). Early cloning is helpful in this respect but cessation of secretion is still a frequent occurrence, particularly if hybrids are stressed by unfavourable culture conditions. Reasons for loss of antibody secreting ability in a cloned cell line may be loss of chromosomal material (Goding, 1980) or instability in gene expression (Pearson et al., 1980; Galfre and Milstein, 1981). To minimise the risk of losing positive hybridoma cell lines, conditions were optimised; medium changes were partial and frequent, all overgrowth avoided, peritoneal macrophages were used to condition the medium and act as feeder cells and, once HAT medium was no longer required, cells were grown in HT medium for at least a week until the aminopterin and its effects had gone.

The IFA test was chosen to screen for antisporezoite antibody activity because, like enzyme linked immunosorbent assays, it is quick

to perform, simple and sensitive although it is a subjective test. These are important criteria to consider when large numbers of hybrid supernatants are to be tested and make IFAT and ELISA preferable to more laborious techniques such as coprecipitation of radio-labelled parasite components. IFAT has the additional advantage of allowing visualisation of the site of antibody recognition.

Antisporozoite activity of all 19 established monoclonal antibodies was observed more easily when formalin fixed sporozoite antigen was used rather than acetone fixed antigen. Since formalin fixation is reputed to stabilise surface membrane antigens while preserving antigenicity (Nantulya and Doyle, 1977) this may indicate that most of the monoclonals were reacting with epitopes on the sporozoite surface. Acetone tends to have a much more destructive effect on cell surface membranes and allows them to become partially permeable to immunoglobulin (Biberfeld, Biberfeld, Molnar and Fagraeus, 1974). This makes it a less suitable fixative when testing for antibodies directed against surface epitopes.

The monoclonal antibodies were raised against the Ankara stock of T. annulata and all showed cross-reaction with T. annulata Gharb sporozoites by IFAT. Musoke et al. (1984) also found monoclonal antibody cross-reaction between sporozoites of several T. parva stocks and suggested that a common protective antigenic determinant may be present in all T. parva stocks.

Cross-reaction with other life cycle stages was observed to a lesser extent. None of the monoclonals staining piroplasms and only three giving schizont fluorescence, two of which were very weak. Several others showed a weak and less localised fluorescence of the

infected cell cytoplasm. This may have been a non-specific reaction although there are precedents for antigenic cross-reaction between parasite life cycle stages (Hope, Hall, Simmons, Hyde and Scaife, 1984; Shiels et al., 1986b; Szarfman, Lyon, Walliker, Quakyi et al., 1988).

The immunoglobulin class for monoclonal antibodies (Mabs), 1A7/A8 and 4B11/C12 was determined as IgM. To obtain IgM and not IgG monoclonal antibodies after a long immunisation schedule may be reconciled with the fact that a high early IgM peak is stimulated in an anamnestic response (Roitt, 1980). Much shorter protocols for raising T. parva sporozoite recognising antibodies have produced an IgM (Musoke et al., 1984) and an IgG3 (Dobbelaere et al., 1984) monoclonal.

Finally, surface immunofluorescence of sporozoites was convincingly obtained when the same two Mabs (1A7/G8 and 4B11/C12) and hyperimmune rabbit and bovine sera were reacted with purified live sporozoites. This indicated that the monoclonals and at least some antibodies in the polyclonal sera recognise epitopes exposed on the surface of sporozoites. Thus, sporozoite coat proteins of T. annulata are immunogenic.

The IgG3 monoclonal antibody against T. parva sporozoites has also been shown to recognise a surface antigen (Dobbelaere, Shapiro and Webster, 1985) although the monoclonal derived from the use of crude freeze thawed T. parva sporozoites as immunising material rather than intact live sporozoites as in this study.

With the antibodies raised as described above, it was possible to continue to determine if the antibodies could be shown to be protective and then to extend the study to identify the antigens they recognise.

CHAPTER FOUR
SCREENING OF ANTISPOROZOITE ANTIBODIES
FOR SPOROZOITE NEUTRALISING ACTIVITY

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4.1. INTRODUCTION

The main aim of this study was to identify sporozoite antigens capable of eliciting a protective response. Since the IFAT used in the initial assessment of ant sporozoite activity by antisera or monoclonal antibodies gives no indication of the functional ability of an antibody as regards its role in protection, another method of assessment was necessary.

The choice of method was influenced by the absence of a convenient laboratory animal model for the Theileria parasite (Irvin, Brown and Crawford, 1972; Irvin, Purnell, Brown and Cunningham, 1975). If such a model were available, in vivo passive transfer experiments would be an option as employed by Potocnjak et al. (1980) investigating the protective activity of monoclonals against a malarial sporozoite surface antigen.

The mouse has also been used for passive transfer experiments to assess monoclonals recognising the surface of the invasive tachyzoite stage of Toxoplasma gondii (Johnson, McDonald and Neoh, 1983; Kasper, Currie and Bradley, 1985).

The protective potential of immune serum has classically been judged by similar passive transfer experiments and also by evidence of passive immunity in the offspring of immune mothers as observed in the young of mice immunised with irradiated malarial sporozoites (Orjih, Cochrane and Nussenzweig, 1981). Passive transfer of anti-sporozoite antibodies to infants occurs in areas where malaria is endemic (Nardin, Nussenzweig, Bryan and McGregor, 1981). Passive transfer experiments using serum from animals immune to theilerial infection have been described (Koch, 1903; Theiler, 1907; Robson et

al., 1961; Muhammed et al., 1975) with conflicting results being reported. The possibility of transfer of protective antibodies to calves in colostrum has also been studied (Burridge and Kimber, 1973; Cunningham et al., 1988) without yielding convincing results. However, antisporezoite antibody levels were not specifically measured in any of these studies and their implications as regards antisporezoite immunity are unclear.

Assay methods involving a combination of in vivo and in vitro components in which antibody is incubated with the infective parasite prior to inoculation into susceptible animals have been developed in several parasite-host systems. Nussenzweig, Vanderberg and Most (1969a) prevented infection of susceptible mice with malarial sporozoites by incubating them in serum from mice immunised with irradiated sporozoites. Using a similar protocol, those working on the T. parva sporozoite have detected neutralising activity by sera containing antisporezoite antibodies from cattle and rabbits (Musoke et al., 1982) and by an antisporezoite monoclonal antibody (Dobbelaere, et al., 1984).

To assess a large number of sera or monoclonals against sporozoites of T. annulata or T. parva in this way would require the use of many cattle making the exercise excessively expensive, laborious and, perhaps, unethical. Therefore an entirely in vitro screening assay was chosen.

Since 1973 there has been a system for reproducibly infecting bovine PBM with sporozoites in vitro (Brown et al., 1973). Into this system Gray and Brown (1981) and later others (Musoke et al., 1984; Dobbelaere et al., 1984; Preston and Brown, 1985) were able to

introduce antibodies to create a sporozoite inhibition assay. Inhibition of invasion assays have been used to test antibodies in vitro against parasites such as Toxoplasma gondii for some time (Jones, Len and Hirsch, 1975) while malarial sporozoite inhibition assays (Hollingdale et al., 1982; Hollingdale, Nardin, Tharavanij, Schwartz and Nussenzweig, 1984) only became possible at the same time as the in vitro cell culture systems were developed (Hollingdale, McCullough, Leef and Beaudoin, 1981).

All assays of this type work on the same principle, whereby the infective stage is incubated with antiserum or monoclonal antibody and then allowed access to the appropriate target cells under conditions in which infection would normally occur. This is followed by a quantitative assessment of the infection rate. By including suitable controls the degree of inhibition can be determined for any one antibody.

This chapter describes the further development and application of a sporozoite inhibition assay which was used to demonstrate the sporozoite neutralising activity of immune bovine serum (Preston and Brown, 1985) to assess the antibodies described in Section 3. The aim was to use the assay to discriminate and select ant sporozoite antibodies with potential protective capacity from others by screening for the functional ability to block sporozoite invasion of PBM in vitro.

4.2. MATERIALS AND METHODS

4.2.1. Sporozoites

Theileria annulata sporozoites from Ankara, Gharb or Hissar stocks were obtained as GUTS filtrate (GF) as described in Section 2.2.2. GF was used at a concentration of 0.25-2.0 te/ml in MEM/3.5% BPA.

4.2.2. Peripheral blood mononuclear cells (PBM)

PBM from a susceptible cow or calf were separated using the Ficoll-Paque method (Section 2.2.3). The cells were used at a concentration of $2-4 \times 10^6$ /ml in complete medium (RPMI 1640/16.7% FCS).

4.2.3. Antibodies

Antisera were thawed from storage at -20°C and used undiluted and diluted $1/4$, $1/8$, $1/16$.

Monoclonal antibodies (Mabs) were used either as undiluted fresh culture supernatant taken from hybridoma culture after 2-4 days' growth, or as diluted ascites from hybridoma inoculated mice.

Ascites was diluted to $1/10$, $1/100$, $1/1000$ for inclusion in the assays.

Dilutions were made in complete tissue culture medium and, where necessary, antibody solutions were filter sterilised using a 0.22μ filter (Millex G-V, low protein binding, Millipore).

4.2.4. Sporozoite inhibition assay

Inhibition assays were carried out in one cm^2 wells of 48 well tissue culture plates (Costar).

For each sporozoite:antibody combination, 0.25 ml GF was mixed with an equal volume of antibody in four replicate wells except in initial Mab screening assays when two or three replicate wells were set up. These were allowed to incubate at 37°C, 5% CO₂ for one hour. In a few assays involving monoclonal antibodies, complement was added in the form of rabbit serum (Gibco) after 30 minutes incubation following which plates were incubated a further 30 minutes. Then 1-2 x 10⁶ PBM in 0.5 ml complete medium were added to each well and incubation continued. It should be noted that since sporozoites and antibody were mixed 1:1, the effective concentration of antibody was half the stated concentration.

4.2.5. Controls

Controls were included in each assay by incubating sporozoites either with medium alone, with medium containing no antibody (e.g. supernatant from 4C7/G7 a non-secreting hybridoma) or with antibody lacking ant sporozoite activity.

Preimmunisation sera acted as controls for ant sporozoite sera while the controls for ant sporozoite monoclonal antibodies were monoclonals raised against something other than sporozoites. These were:

7B10: which reacts with the surface of the infected lymphocyte (Shiels et al., 1986a)

1E11 which react with the macroschizont (Shiels et al., 1986b)
1C7: (1E11 cross-reacts with sporozoites)

3D6D3: an anti-Taenia saginata IgM monoclonal donated by Dr. L.J. Harrison

4.2.6. Assessment of inhibition

To assess the blocking action of the antibodies, 50 μ l samples were taken from the resuspended contents of each of the wells in order to prepare Giemsa-stained cytopins.

Cytopins were taken from each well any time from day 2, usually on day 4 and later in some cases. Two hundred cells were counted from each cytopin to obtain the percentage of trophozoite or macro-schizont infected cells. Percent inhibition was calculated by comparing the median percentage infection for the control monoclonal or antiserum (x) with that for the test monoclonal or antiserum (y) in the equation:

$$\% \text{ inhibition} = \frac{x - y}{x} \times 100$$

Where sample numbers permitted ($n \geq 4$), results were analysed using the Mann-Whitney U Test for non-parametric data (Siegel, 1956). Results for a test antibody described as significantly different, differ at the $P < 0.05\%$ level from the results of the appropriate control antibody.

4.3. RESULTS

Median percentage infection rates and range values from which the figures were constructed and the statistical analyses were made are tabulated in Appendix II.

4.3.1. Sporozoite neutralising activity: bovine antisera

a) Hyperimmune sera from calves immunised with live virulent sporozoites

i) N53 and N61: These two calves were hyperimmunised with repeated doses of live virulent infective sporozoites. During primary

infection they were treated with buparvaquone and recovered. Figure 13a shows the sporozoite neutralising activity of their serum during the course of their hyperimmunisation. A substantial degree of inhibition was observed in the serum from both calves after being exposed to two doses of sporozoites (day 58). This increased after a third sporozoite inoculation (day 90) but the increase was only significant for calf N61. Although serum from day 107, two weeks after a fourth dose of live sporozoites, showed a slightly reduced neutralising action this was not found to be significantly different from day 90's serum for either calf.

ii) 54X and 134X: These two calves were also hyperimmunised by repeated inoculation of live virulent sporozoites. They recovered naturally from the primary infection. Figure 13b shows the strong (100%) neutralising effect of their hyperimmune sera. Cultures containing 134X sera were maintained for 18 days at which time there was still no sign of infection observed in any well containing day 101 serum.

b) Sera from calves immunised with irradiated sporozoites

Selected sera from calves L21, N44, L37 and 102, which had been immunised with sporozoites irradiated at different dose levels, were tested for sporozoite neutralising activity. The assay included sera taken before immunisation, after immunisation but before challenge and, where available, after challenge with virulent sporozoites. Pre- and post-immunisation sera from N53 were included as a positive control. Figure 13c shows the different levels of infection and inhibition obtained. Undiluted sera from all calves inhibited 100% of sporozoite

Figure 13 Sporozoite inhibition assays to test bovine sera.

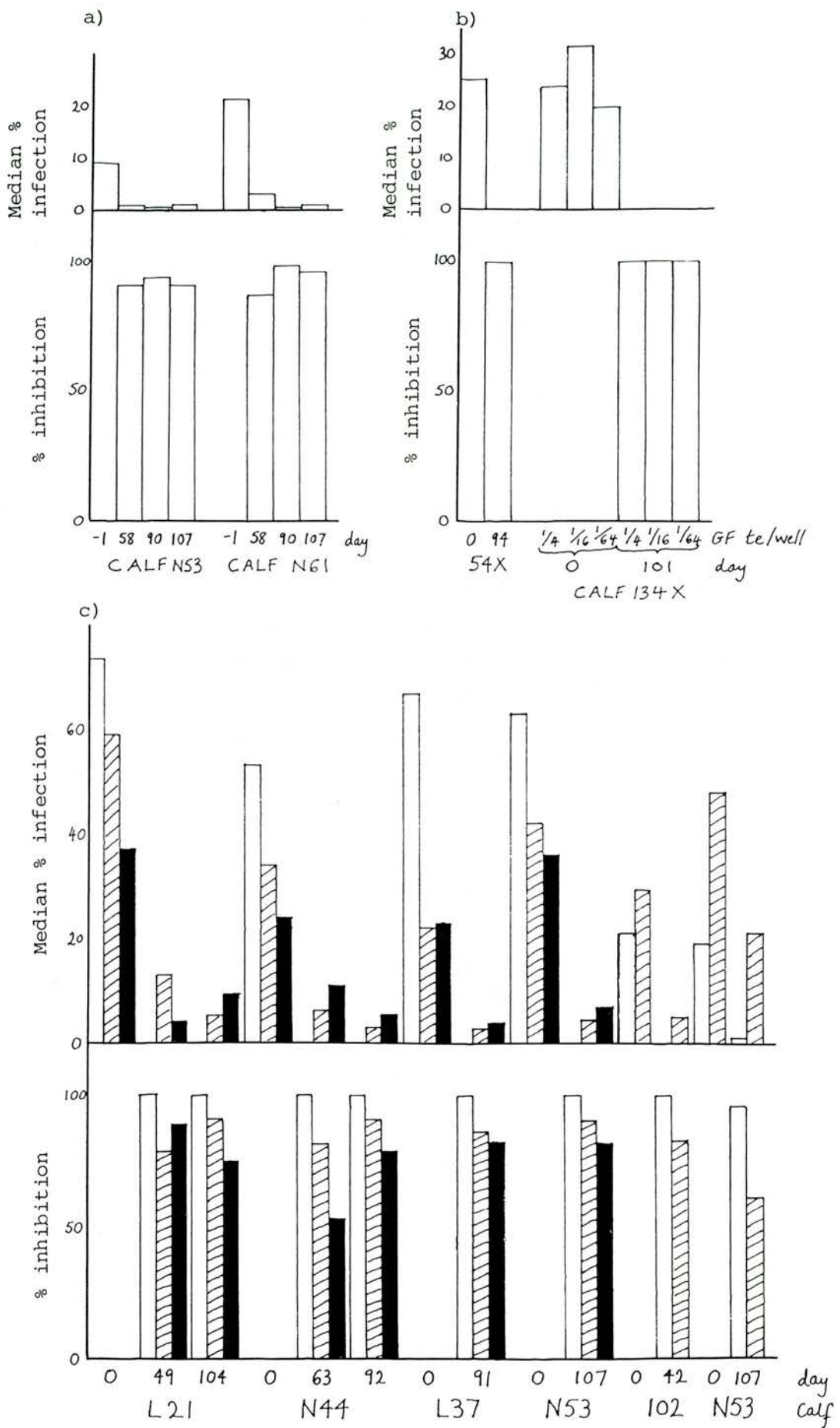
Bar charts showing median % infection and inhibition levels in assays to test sera from:

- a) Calves N53 and N61 hyperimmunised with live virulent sporozoites after recovery following drug treatment.
- b) Calves 54X and 134X hyperimmunised with live virulent sporozoites after recovering naturally.
- c) Calves L21, N44, L37 and 102 (and calf N53 as a control) immunised with irradiated sporozoites.

Open bars represent assays using undiluted sera.

Shaded bars represent assays using serum diluted $\frac{1}{4}$.

Closed bars represent assays using serum diluted $\frac{1}{16}$.



infectivity irrespective of the irradiation dose of the sporozoites they received. In those calves which survived, this inhibition remained at a high level after challenge. The level of inhibition was reduced by dilution of the sera, the degree of reduction not being significantly different between calves.

4.3.2. Sporozoite neutralising activity: rabbit antisera

a) Antisporozoite serum from rabbit 28 against two *T. annulata* stocks

Rabbit 28 was immunised by repeatedly feeding *T. annulata*-infected *H. a. anatolicum* ticks on its ears. Preimmune and immune sera were included in a neutralisation assay using sporozoites from Ankara and Gharb stocks. Figure 14a indicates that immune serum from rabbit 28 possessed strong blocking activity against both parasite stocks, such that even a $1/16$ serum dilution inhibited 100% of sporozoite infectivity and prevented infection from establishing. Both preimmune and immune sera, when undiluted, showed a potent non-specific action on sporozoite invasion. Therefore, rabbit sera were diluted to at least $1/4$ for inclusion in subsequent inhibition assays.

b) Antisporozoite serum from rabbit 28 compared with serum from rabbit 8

Rabbit 8 was also fed on repeatedly by adult *H. a. anatolicum* but, unlike rabbit 28, the ticks were uninfected. Thus, sera from rabbit 8 were supplementary controls for the possibility of a non-specific neutralising effect resulting from anti-tick responses. In this way (Figure 14b), serum diluted $1/4$ showed significant inhibition for both rabbits although the inhibition by rabbit 28 serum (100%) was much stronger than that by rabbit 8 serum (41%) after they

had been fed on by ticks. When the same sera were diluted to $1/8$ the neutralising activity of rabbit 8 serum (20.8%) was no longer significant while that of rabbit 28 serum remained at a high level (98.9%).

c) Effect of heat inactivation on sporozoite neutralising activity

Sporozoites of a third T. annulata stock, Hissar, were used in an assay to assess the effect of heat inactivation on day 0 and immune sera from rabbit 28. The sera were heat inactivated for 30 minutes at 56°C and used at a $1/4$ dilution. Figure 14c was derived from results obtained after just 48 hours culture. At this time after infection normal infected cells may still contain parasites at the trophozoite stage. Therefore, infection levels were taken to include both trophozoite and schizont-infected cells.

A high level of infection had established by day 2 in the cultures containing day 0 sera. Heat inactivated day 0 serum allowed a significantly higher level of infection to develop (41%) than untreated day 0 serum (19%). However, heat inactivation of immune day 73 serum had no detectable effect on its sporozoite neutralising activity; both untreated and heat inactivated day 73 serum abolished sporozoite infectivity.

d) Assessment of sporozoite blocking versus inhibition of lymphocyte transformation activity

In this experiment a sporozoite inhibition assay was set up in duplicate. One tissue culture plate was set up in the normal manner; day 0 and day 73 sera from rabbit 28 were added to sporozoites one hour before PBM were added. The other plate was set up with sporozoites

Figure 14 Sporozoite inhibition assays to test rabbit sera.

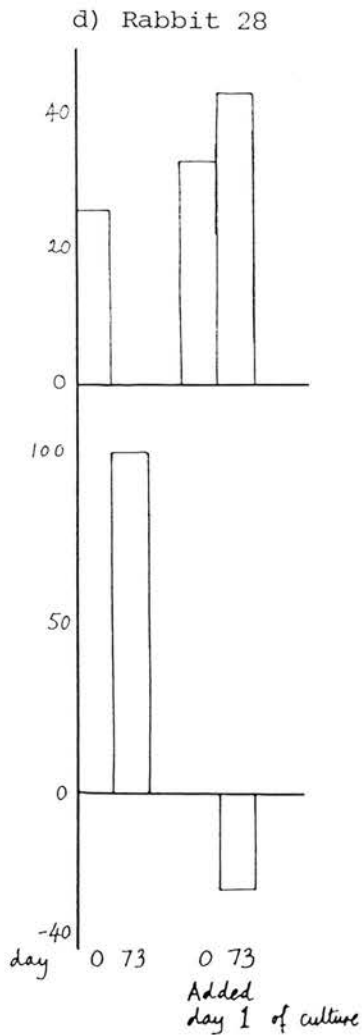
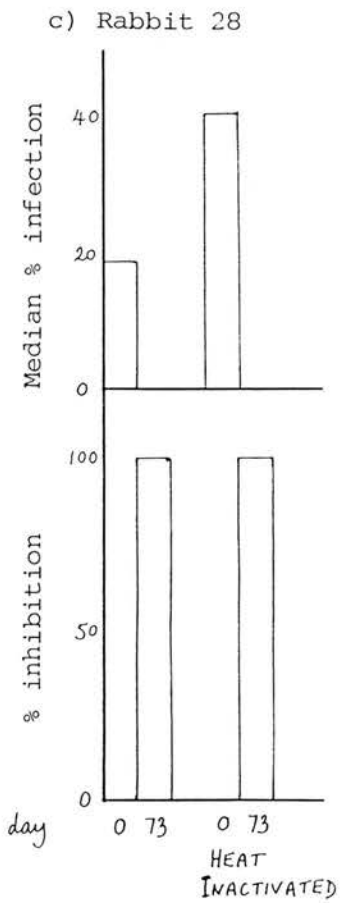
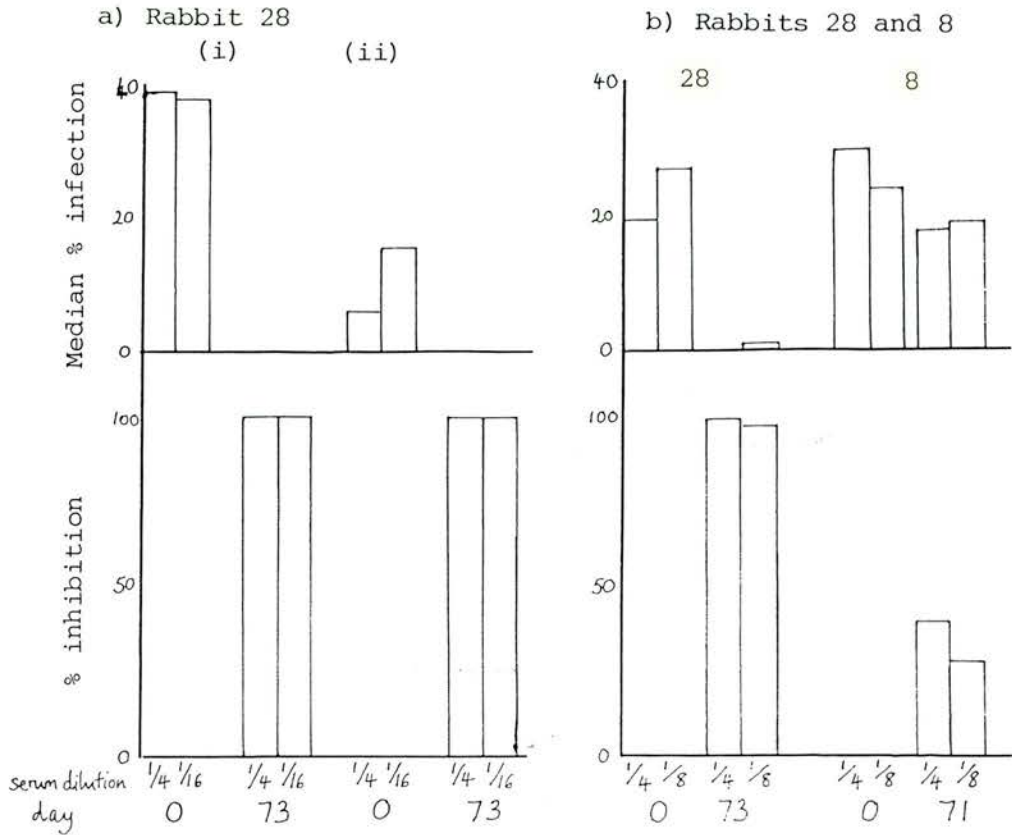
Bar charts showing median % infection and inhibition levels in assays to test sera from:

- a) Rabbit 28 fed on by infected ticks
Tested vs. T. annulata Ankara (i) and Gharb (ii) sporozoites.

- b) Rabbit 28 and rabbit 8 (fed on by uninfected ticks)
Tested vs. T. annulata Ankara sporozoites

- c) Rabbit 28
Tested vs. T. annulata Hissar sporozoites
Sera used: normal and heat inactivated

- d) Rabbit 28
Tested vs. T. annulata Hissar sporozoites
Sera added: as normal on day 0 of culture
 on day 1 of culture



and PBM but sera were not added until 24 hours later. After a further 48 hours (total 3 days) incubation, cytopins were made from all wells and Figure 14d shows the results obtained. As before, immune (day 73) serum from rabbit 28 added on day 0 of the assay, completely blocked sporozoite infectivity. When added on day 1 of the assay this serum had no significant effect on the level of infection establishing compared to cultures containing day 0 serum added on day 1. Cultures to which serum was added on day 1 developed a higher level of infection than those containing serum from day 0 but the difference was not found to be significant.

4.3.3. Sporozoite neutralising activity: ant sporozoite monoclonal antibodies

a) Primary screen of 19 ant sporozoite monoclonals

All 19 secreting hybridoma cell lines selected on the basis of giving positive fluorescence of formalin fixed sporozoites in the IFAT were screened for functional ability to neutralise sporozoite infectivity. Four wells were set up for each sporozoite:Mab combination. Complement (rabbit serum, Gibco) was added to two of these as described in Section 4.2.4. to a concentration of 1:10 in the sporozoite:Mab mixture. This proved toxic to all cultures, resulting in cell death. Consequently, results are only presented of the evaluation of sporozoite invasion inhibition without complement.

Table 13 shows the results obtained.

b) Secondary screen of selected ant sporozoite monoclonals

After the preliminary screen, 12 Mabs were selected for retesting on the basis of having blocked 45% or more of the sporozoite infectivity.

Table 13 Primary screen of antsporozoite monoclonal antibodies for sporozoite neutralising activity

Monoclonal antibody		Median % infection ⁺ n = 2	% inhibition ⁺⁺	
4B11/C12	S	1.0	85*	
	A	2.5	62*	
4A7/A8	S	12.5	-92	
	A	2.0	69*	
5D1/D11	S	2.0	69*	
	A	5.5	15	
5F1/E11	S	6.5	0	
	A	1.0	85*	
1A7/G8	S	3.5	46*	
4A7/E8	S	5.5	15	
4A7/B12	S	5.5	15	
4D3/E8	s	9.0	-38	
5F1/H11	S	5.5	15	
4B11/E9	S	0.5	92*	
4A7/D10	S	6.5	0	
4A7/E12	S	4.5	31	
4B11/B12	S	0.5	92*	
4B11/G11	S	2.0	69*	
4G5/C10	S	2.0	69*	
5F1/G12	S	6.5	0	
4B11/H12	S	1.0	85*	
5D1/D12	S	2.5	62*	
1B4/B12	S	1.0	85*	
7B10	S	5.0	23	
	A	6.5	0	
Controls	1E11	S	3.0	54*
	4C7/G7	S	5.0	23
	Medium only	6.5	0	

Sporozoites: T. annulata Ankara, 0.25 te/well

S = undiluted supernatant from 4 day hybridoma cultures

A = ascites diluted 1:100

+ = on day 4 of assay

++ = calculated against medium only

* = Mab neutralising over 45% sporozoite infectivity

Various changes were made in the experimental detail to try and ensure that observed effects were not artefacts:

i) Rabbit complement at a 1:10 working dilution had proved too toxic to be of use and was included at 1:20 in this experiment.

ii) Supernatants were taken from 2-day rather than 4-day hybridoma cultures since the latter might contain a higher proportion of dying cells releasing factors into the medium. These could affect sporozoite infectivity in a non-specific manner.

iii) To try and obtain a higher level of infection in control cultures by day 4, more concentrated GF was used.

Table 14 shows the results. Again no data is shown for the effect of complement since even a 1:20 dilution proved too toxic with very few cells becoming infected.

c) Tertiary screen of selected ant sporozoite monoclonals

Of the six Mabs appearing in the first two screening experiments to possess some ability to block sporozoite invasion into PBM (at least 55% inhibition compared with control Mab), three were selected to re-examine this inhibitory activity.

Again, changes were made to experimental detail to try and improve the inhibition assay:

i) PBM were isolated from the blood of a calf since the PBM from the mature cow used previously appeared to be less susceptible to infection with sporozoites than cells from less mature animals.

ii) GF was used at two dilutions.

iii) Mab ascites was used at three dilutions.

Table 14 Secondary screen of selected ant sporozoite monoclonal antibodies for sporozoite neutralising activity

Monoclonal antibody	Median % infection ⁺ n = 2	% inhibition vs. control Mab Medium		
<u>Ascites</u> (1:100)				
4B11/C12	0.0	100*	100*	
4A7/A8	5.0	-43	23	
5F1/E11	6.5	-86	13	
4G5/C10	11.5	-220	-53	
4D3/E8	7.0	-100	7	
1A7/G8	1.5	57*	80*	
control: 7B10	3.5	0	53	
<u>Supernatants</u> (undiluted)				
4B11/C12	1.5	75*	80*	
5D1/D11	5.0	17	33	
1A7/G8	2.5	58*	67*	
4B11/E9	0.5	92*	93*	
4A7/E12	4.5	25	40	
4B11/B12	0.5	92*	93*	
4B11/G11	1.5	75*	80*	
4G5/C10	6.5	-8	13	
4B11/H12	1.5	75*	80*	
5D1/D12	4.0	33	47	
1B4/B12	3.0	50	60*	
controls	{ 1E11	6.0	0	20
	{ 4C7/G7	5.5	8	27
	{ medium only	7.5	-25	0

Sporozoites: T. annulata Ankara, 0.3 te/ml

+ = on day 4 of assay

* = Mab neutralising over 55% of sporozoite infectivity

iv) As well as control Mabs, 7B10 and 1E11, Mabs 5D1/D11 (ascites) and 5D1/D12 (supernatant) were included as controls. These two Mabs were produced during the same fusion as the test Mabs and gave positive results with sporozoites on IFAT but had not possessed significant blocking action in the previous two experiments.

Complement was not added to any wells.

Table 15 shows the results obtained.

Inhibition rates were low in this experiment with supernatants being inactive. For day 4 cultures, the infection rate was high in the cultures containing 0.25 te GF. Thus, in these cultures the blocking activity of all but the strongest ascites (1:10) may have been masked.

d) Sporozoite neutralising activity of two ant sporozoite monoclonals, 4B11/C12 and 1A7/G8

Two ant sporozoite Mabs, 4B11/C12 and 1A7/G8, were selected for further testing. They had most consistently exhibited a substantial degree of sporozoite neutralising activity.

Assay i): The Mabs were used individually and in combination to see whether an additive inhibitory effect was obtained. The calf sera were included to provide a positive control. Figure 15 illustrates the results.

Ascites at 1:10 and 1:100 dilutions demonstrated strong blocking activity for both individual monoclonals 4B11/C12 (80-93%) and 1A7/G8 (45-87%). When used together, the inhibition rates did not increase substantially (61-93%). The culture supernatant of control Mab 7B10 allowed such low levels of infection (Appendix II) to establish that it was not possible to detect significant inhibition in

Table 15 Tertiary screen of selected antisporezoite monoclonal antibodies for sporezoite neutralising activity

Monoclonal antibody	Median % infection ⁺ n = 2	% inhibition vs. 7B10	% inhibition vs. 5D1
<u>GF 0.25 te/well</u>			
4B11/C12 A 1:10	26.0	52*	61*
A 1:100	57.5	-20	10
A 1:1000	52.0	3	8
S	38.0	22	7
1A7/G8 A 1:10	33.5	38	50*
A 1:100	40.0	17	37
A 1:1000	42.5	21	25
S	42.5	13	-4
4B11/E9 S	50.5	-3	-23
<u>GF 0.05 te/well</u>			
4B11/C12 A 1:100	9.5	58*	69*
S	16.0	-45	0
1A7/G8 A 1:100	11.0	51*	64*
S	13.5	-23	16
4B11/E9 S	23.5	-114	-47

Sporozoites: T. annulata Ankara

A = ascites

S = undiluted culture supernatant

+ = on day 4 of assay

* = Mab neutralising over 50% of sporezoite infectivity

cultures containing supernatants from 4B11/C12 and 1A7/G8. It was realised, at this point, that the use of 7B10 as the control monoclonal might have been making the calculated inhibition values lower than they really were. Preston et al. (1986) actually report that the inclusion of Mab 7B10 in the medium of schizont-infected cultures severely limits the growth of these cultures. Although not actually inhibiting sporozoite invasion of lymphocytes, 7B10 may have reduced the subsequent growth of cells which had become infected.

Assay ii): Culture supernatants from Mabs 4B11/C12 and 1A7/G8 were retested for neutralising activity in an assay using different controls; Mab 1C7 which recognises a macroschizont antigen (Shiels et al., 1986b) and an IgM class Mab raised against Taenia saginata. This assay was performed in microtitre wells as it was part of a larger inhibition assay (see Section 7.3.2.). Fifty μl GF (0.05 te) and 50 μl undiluted Mab supernatant were mixed in each well before adding 100 μl PBM (2×10^5 cells) 60 minutes later. The bar chart in Figure 15b indicates the infection and inhibition rates which resulted.

4B11/C12 and 1A7/G8 supernatants both showed convincing and significant degrees of sporozoite inhibition; 97% and 77% respectively. While the anti-schizont Mab, 1C7, inhibited 35% of infectivity it was not found to be significantly different from the control Mab.

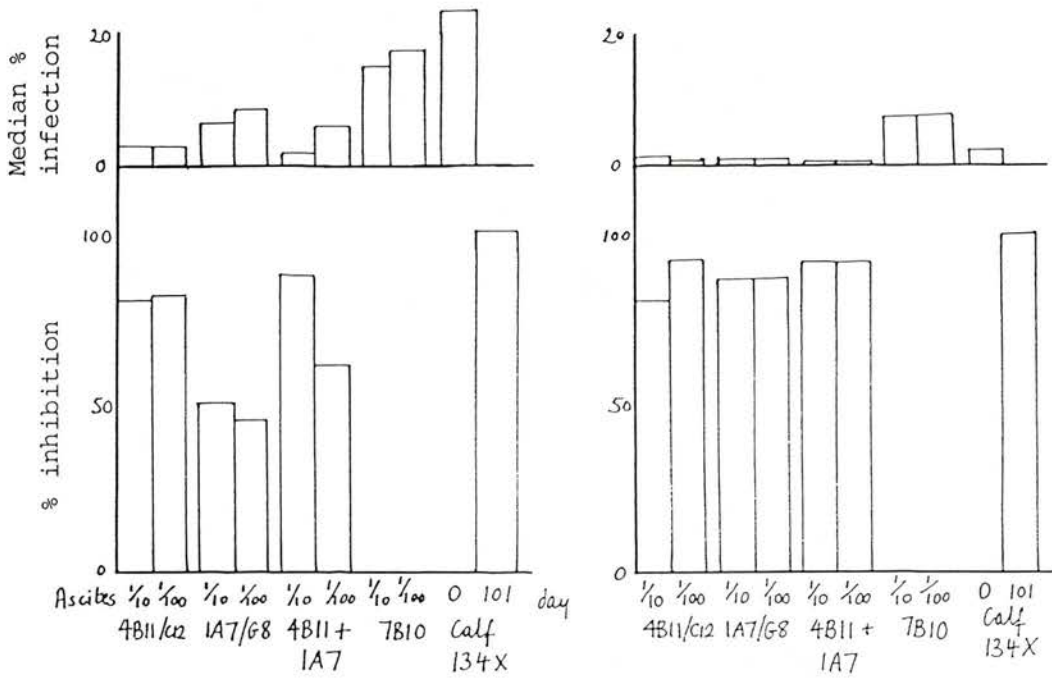
To illustrate the appearance of cultures from which cell counts were made in a sporozoite inhibition assay, Figure 16 shows a Giemsa-stained cytopsin of a culture in which sporozoite invasion a) was and b) was not blocked.

Figure 15 Sporozoite inhibition assays to test antisporezoite monoclonal antibodies.
 Bar charts showing median % infection and % inhibition levels in assays to test monoclonals 1A7/G8 and 4B11/C12.

a) individually and in combination:

i) GF 1/4 te/well

ii) GF 1/16 te/well



b) individually with different control monoclonals

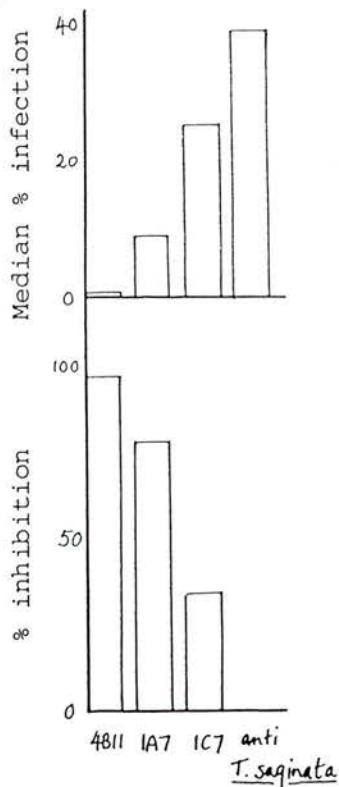


Figure 16 Cultures of bovine peripheral blood mononuclear cells resulting from incubation of sporozoites in neutralising antibody (a) or antibody with no neutralising effect (b).

Giemsa-stained cytopins taken on day 3 of a sporozoite inhibition assay:

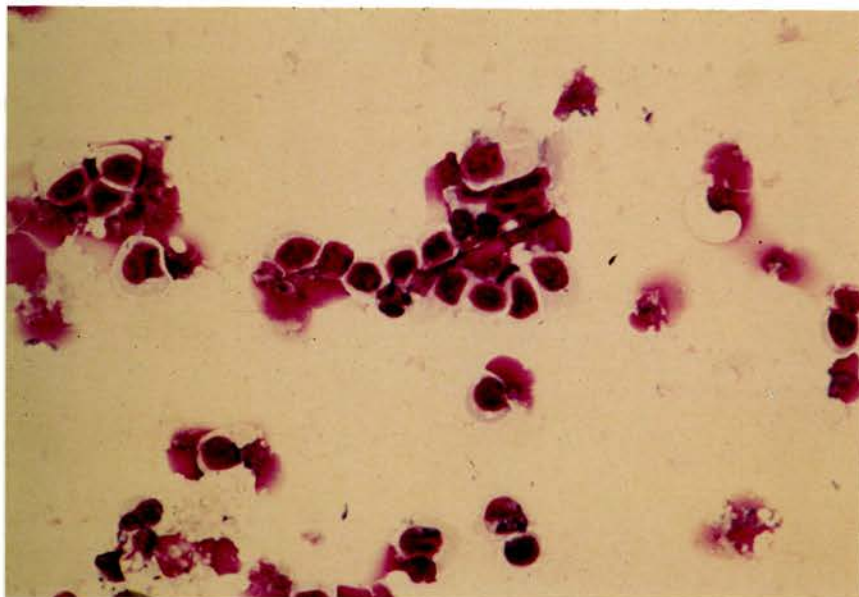
- a) uninfected mononuclear cells; 100% inhibition of sporozoites (x 550 magnification)
- b) macroschizont infected cells; no inhibition of sporozoites (x 1,100 magnification)

Arrows indicate macroschizonts in infected cells.

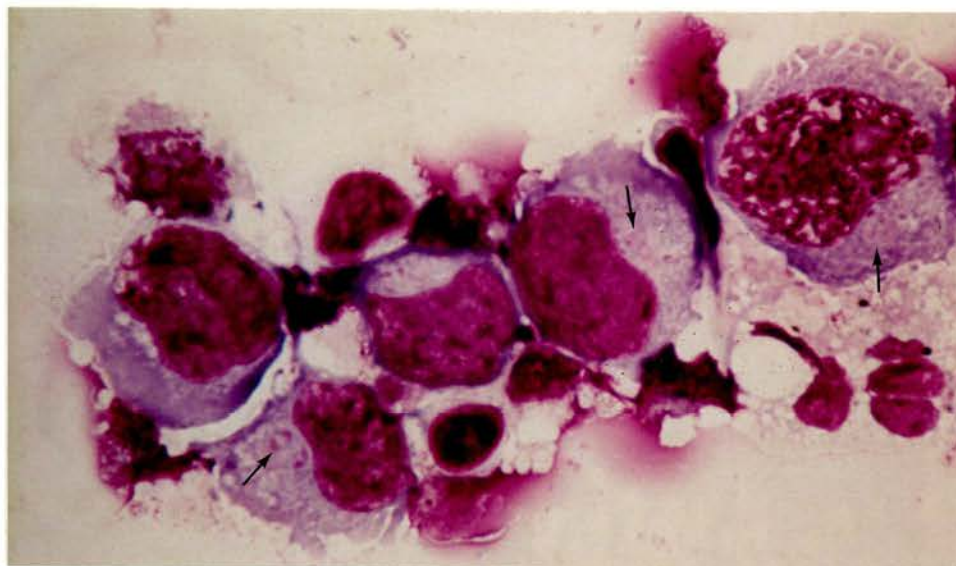
Figure 17 Trophozoite infected bovine peripheral blood mononuclear cells 48 hours after in vitro infection.

Giemsa-stained cytopins showing two examples of intracellular trophozoites (arrowed).
(x 1,100 magnification)

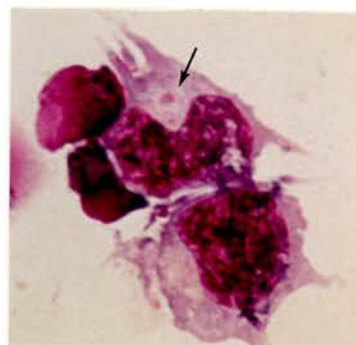
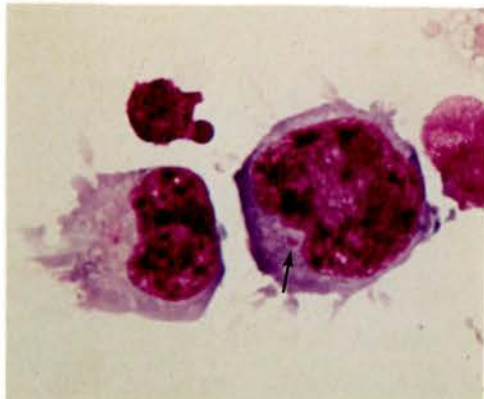
16 a



b



17



4.4. DISCUSSION

In using an in vitro assay to assess inhibition of sporozoite invasion into target cells by antibody, such an assay must satisfy certain demands. These can be considered in terms of predictability and reliability.

a) Predictability

The assay should be standardised so that known positive and negative controls yield consistent levels of inhibition and infection respectively. Into such a standardised system, test antibodies could be introduced and compared with others as interassay variation would be minimal. This predictability, or lack of it, was one of the areas where difficulties were encountered in these sporozoite inhibition assays. Three sources of variation were present.

i) Sporozoites: The quantum of infection per well was expressed as the number of tick equivalents. This is an accepted way of indicating the concentration of a filtrate (GF) of ground up tick supernatant (Brown, 1983). Since sporozoites were not counted, the number placed in each well could, and did, vary between different GF preparations, differences in the infection rate of ticks being one cause of this variation. It might therefore seem logical to institute sporozoite or infected salivary gland acini counts (Irvin, Boarer, Dobbelaere, Mahan, Masake and Ocama, 1981) and adjust GF to contain a standard number of sporozoites per unit volume in a manner similar to that carried out for sporozoites of Plasmodium falciparum and Plasmodium vivax by Hollingdale et al. (1984). However, this would in no way assure standard sporozoite infectivity, other factors play as important a role in determining sporozoite infectivity. One of

these, sporozoite maturity, is affected by the duration of the tick feed (Purnell and Joyner, 1968; Purnell, Brown, Cunningham, Burridge et al., 1973); the age of the tick and the tick's efficiency at feeding (Young and Leitch, 1981; Walker et al., 1985). This last effect itself is governed by a host of other factors. These include the degree of inbreeding in the tick population; the resistance of ticks shown by the rabbit on which the ticks are feeding, be it innate or immunological as may occur if the rabbit has been used for a tick feed on a previous occasion (Brossard, 1977; Chiera, Newson and Cunningham, 1985); and the level of T. annulata infection in the salivary glands. Very high infection rates can adversely affect the tick's survival and ability to feed (Schein and Friedhoff, 1978; Walker et al., 1985). Sporoblasts are presumed to be non-infective and immature sporozoites less infective than those which have reached full maturity in the salivary gland. Variation in any of the above can alter the range of maturity within the sporozoite population and thus influence the infectivity.

Finally, parasite stocks and, within stocks, different batches of infection appear to differ noticeably in their innate infectivity for PBM.

Given this variation it would be of significant benefit to develop a sporozoite inhibition assay which uses cryopreserved stabilate as a source of sporozoites. The infectivity could be ascertained by titration beforehand and the same batch could be used for a whole series of inhibition assays. At present, however, cryopreservation of sporozoites in glycerol reduces the infectivity to about 10% of that of the fresh material (Brown, 1987). While this leaves the

stabilate very adequate for the in vivo infection of calves and still allows establishment of in vitro infected cultures, the early infection rates of such cultures are low and they establish slowly. This would make valid assessment of inhibition of sporozoite invasion difficult as early differences between cultures containing test and control antibodies would be minimal.

ii) Antibodies: The concentration of antibody in monoclonal ascites and culture supernatant was not determined and may have fluctuated between preparations although they were checked for anti-sporozoite activity by the IFAT prior to use.

iii) Peripheral blood mononuclear cells: The target cell population, PBM, varies in an individual animal and between animals as they mature and as they encounter pathogens and allergens (Schalm, 1965).

The proportions of various cell subsets changes accordingly. Changes in the number of MHC class II positive antigen presenting cells and B lymphocytes could influence the number of cells becoming infected dramatically, since both these cell types are preferentially infected by T. annulata sporozoites (Spooner et al., 1988). Variation in infectibility of PBM from different animals using the same sporozoite material has been repeatedly observed in this laboratory (C.G.D. Brown, personal communication) and so could be expected to influence the inhibition assays.

b) Reliability

Reliability refers to the degree of certainty with which a reduced level of infection can be considered to be due to inhibition of sporozoite invasion by antibody. Some aspects which influence the reliability of the results are discussed below.

i) Effects independent of antibody: Preston and Brown (1985) described an antibody independent effect of immune bovine serum which caused retardation of trophozoite development into macroschizonts. Investigations into the factors responsible for this effect have incriminated a heat resistant, low molecular weight constituent of serum (P.M. Preston, personal communication) which may be tumour necrosis factor (Cerami and Beutler, 1988). Undoubtedly this effect contributed to the degree of inhibition caused by hyperimmune bovine sera (Section 4.3.1.a) although its importance was not specifically investigated. Whether serum taken from calves immunised with irradiated sporozoites exhibits this effect is not known. Some of the irradiated sporozoites enter lymphocytes (Brown, 1981) as demonstrated for irradiated Plasmodium berghei sporozoites, some of which still entered cultured hepatoma cells but failed to develop further (Sigler, Leland and Hollingdale, 1984). Thus, it is possible that the anti-trophozoite effect described above could be stimulated in these calves also. The immune serum from rabbit 28 which was fed on by infected ticks did not exhibit such an antitrophozoite effect: when serum was added to day 1 assay cultures no inhibition was observed.

Other evidence that non-antibody factors influence results comes from Experiment 4.3.2.c) where heat inactivation of sera from rabbit 28 left the inhibiting activity of day 73 serum unimpaired

but allowed a much greater level of infection to develop in cultures containing heat inactivated day 0 serum. Destruction of complement, and possibly other toxic proteins by heat inactivation was likely to be responsible for this effect which enhanced the difference between the day 0 and day 73 sera.

ii) Effects associated with antibody: This refers to a reduction in the level of infection caused by antibody exerting its effects without preventing sporozoite entry into cells. Mazier et al. (1988) and Hollingdale (1987) observed that in sporozoite inhibition assays, malarial sporozoites evading antibody neutralisation and invading hepatocytes failed to undergo further exoerythrocytic development. They suggested that this was due to the action of antibody bound by the sporozoite and carried into the hepatocyte. Unquantified observations of a similar nature were made during this study. Certainly in cultures where immune sera or monoclonals 4B11/C12 and 1A7/A8 were used, cells containing undeveloped sporozoites were visible in day 4 cytopins even in cultures where no infection established.

There are several possible explanations for these observations. Antibody bound by the parasite before entry which does not have an immediate effect preventing invasion but inhibits further development could do so by causing lysis or structural changes in the sporozoite. Antibody-induced changes were observed by electron microscopy of malaria sporozoites incubated with immune sera (Cochrane, Aikawa, Jeng and Nussenzweig, 1976). Another way in which ant sporozoite antibody could continue to have an inhibitory effect would be if the sporozoite coat, shed during entry to the mononuclear cell (Webster et al., 1985) remains on the cell surface for some time. This was

seen to be the case for the T. parva sporozoite surface coat (Dobbelaere et al., 1985a) and, if true for T. annulata also, could provide an antibody target on the infected cell. Finally, antibody may be able to penetrate the infected cell. Non-parenchymal cells of the liver have been shown to take up immune complexes in vivo in mice by phagocytosis (Hopf, Schaefer, Hess and Meyer zum Büscherfelde, 1981). Since the sporozoites infect cells of the immune system which bear Fc receptors (Spooner et al., 1988; Male, Champion and Cooke, 1987) it is possible that antibodies could gain access to the intracellular parasite.

The other example of an antibody acting to reduce infection levels in these assays was Mab 7B10 which recognises an antigen on the surface of T. annulata-infected mononuclear cells. Mab 7B10 is already known to exert an inhibitory effect on the growth of T. annulata-infected lymphocytes (Preston et al., 1986) although how this is mediated is not known.

iii) Time interval between infection and assessment of inhibition: This interval should be as short as possible to minimise the impact of effects unrelated to inhibition of sporozoite invasion but not so brief that the difference between negative and positive results is not detectable, particularly if only a low level of infection is achieved in control cultures. Producing a high infection rate in control cultures to yield early results was highly desirable but two problems arose with this approach. One was that the sporozoite suspension (GF) contains tick proteins, such as enzymes and gut contents, with potentially toxic effects. When more concentrated GF was used these caused cell death making the assay useless. Also, if cells

are multiply infected with sporozoites they often die (C.G.D. Brown, personal communication). The second problem was that an excessive number of sporozoites could overcome the inhibitory capacity of the antibody present.

Hollingdale (1987) describes a sporozoite invasion inhibition assay where inhibition is assessed after just three hours using a Mab to stain and count intracellular malarial sporozoites which are morphologically distinguishable from free sporozoites. There is no such immediate and obvious change in the morphology of interiorised theilerial sporozoites visible by light microscopy, although using transmission electron microscopy, Jura, Brown and Kelly (1983) describe rapid transformation into the trophozoite stage. In Giemsa-stained preparations confusion existed both in distinguishing intracellular sporozoites from those attached to the exterior of cells and in confidently recognising the trophozoite stage. Figure 17 shows two examples of readily identifiable trophozoites but frequently they were not so clearly seen.

Winger et al. (1978) took smears of erythrocytes at 20 and 40 hours post-infection to assess Babesia divergens merozoite inhibition rates while Dobbelaere et al. (1984) and Musoke et al. (1984) assessed T. parva sporozoite inhibition rates from cytopins taken from days 3 and 5 respectively.

In this study, the majority of inhibition assays were assessed on day 4 after in vitro infection when infection levels were high enough to detect differences but before differences were obscured by secondary effects.

It might appear that the use of, for example, tritiated thymidine incorporation as a measure of proliferation in a culture rather than visual counts of infected cells would be more reliable in assessing inhibition. However, there is a significant degree of lymphoproliferation in an early in vitro infected culture which occurs without the blasting cells being infected. Visual counting of Giemsa-stained cytopins distinguishes these uninfected cells from infected ones, whereas radioactive nucleotide precursor incorporation would not (Preston and Brown, 1985).

It is clear from the results obtained that the in vitro assay used in this study was capable of detecting different levels of sporozoite neutralising activity by antibodies. These are discussed below, bearing in mind the influence of the above points on their interpretation.

All sera from hyperimmunised calves (N53, N61, 54X and 134X) which received repeated doses of live virulent sporozoites, exhibited strong activity inhibiting sporozoite invasion of PBM as previously reported by others for similar antisera raised against both T. annulata (Preston and Brown, 1985) and T. parva (Musoke et al., 1982).

Calves N53 and N61 recovered from primary infection through the use of a theilericidal drug, buparvaquone, administered on day 14 of infection. Taylor (1985) assayed serum levels of buparvaquone after administration at therapeutic doses to calves and found that they were minimal after 16 days and absent 32 days after administration. Thus the inhibitory activity of serum from day 58 post-infection used in the sporozoite inhibition assay would not have been due to the presence of persisting levels of buparvaquone in the serum.

The postimmunisation sera from all calves immunised with irradiated sporozoites appeared to be very effective in neutralising sporozoite infectivity, regardless of the irradiation dose to which the immunising sporozoites had been exposed. Surprisingly, the sporozoite neutralising capacity did not correlate with the susceptibility of these calves to theileriosis on challenge. Calf 102, which appeared fully susceptible to challenge (Section 3.3.1.), exhibited strong neutralising activity in postimmunisation serum after receiving sporozoites irradiated at 840 Gy. The implications of these results must affect the interpretation of inhibition assays involving theilerial sporozoites.

If the protection afforded to calves N44 and L37 by immunisation with irradiated sporozoites was entirely dependent on antibody mediated neutralisation of sporozoites, one would expect serum from calf 102 to have lacked such activity. As this was not the observed result, the calves which survived challenge may have owed their immunity to a secondary mechanism which was not operating in calf 102 and was unrelated to neutralisation of sporozoites. Indeed, compared to immunisation with live sporozoites, immunisation with irradiated sporozoites may be a poor method of eliciting ant sporozoite antibodies which neutralise in vivo. Alternatively, if sporozoite neutralisation was a significant effector mechanism in vivo, the ability of serum from calf 102 to inhibit sporozoite neutralisation in vitro may be an artefact resulting from the conditions of the assay. To consider this point further: the virtual absence of detectable ant sporozoite antibodies in the serum of calf 102 after immunisation when tested by IFAT (Section 3.3.2.) or Western blotting

(Section 5.3.3.) implies either that the in vitro neutralisation observed reflected an antitrophozoite transformation action independent of antibody or that antisporozoite antibodies present were of low affinity and therefore remained undetected by the above methods. In vivo contact of antibody with sporozoites would be brief owing to the rapid entry of sporozoites into their host cells (Fawcett et al., 1982; Jura et al., 1983), whereas the in vitro assay allowed relatively prolonged contact between sporozoites and serum. Thus, antibodies of low affinity in the serum of calf 102 would not have time to neutralise in vivo but might appear effective in vitro. The immunoglobulin class of antisporozoite antibody might also be responsible for differences in the in vivo effectiveness of humoral response between calves. If antisporozoite antibody present in the serum of calf 102 was not only of low affinity but also predominantly IgM, the in vivo neutralising capability of such antibody might be restricted since IgM antibody is largely confined to the intravascular pool (Roitt et al., 1985) and might penetrate to the site of the tick bite less easily than antibody of other immunoglobulin classes. However, Musoke et al. (1982) reported that a change in the predominant immunoglobulin class of bovine sporozoite neutralising antibodies, from IgM to IgG with repeated sporozoite challenge correlated with an increase in the in vitro sporozoite neutralising activity of the antisera. In view of the fact that the in vitro activity of serum from calf 102 was no weaker than other calves' sera, differences in antibody class were probably not involved.

Another explanation for in vitro neutralising activity being observed in serum from calf 102 is that calf 102 might recognise

different sporozoite antigens from the other calves; antigens which are accessible to antibody in the prolonged period of incubation used in the in vitro but not in vivo because they are poorly represented on the sporozoite surface.

Further experiments are required to determine the true explanation and to assess whether calves N44 and L37 were immune to challenge due to in vivo neutralisation of sporozoites, or due to a secondary mechanism perhaps cell mediated, or a combination of the two.

Sporozoite inhibition assays to test the rabbit sera showed that rabbit 28 developed a strong sporozoite neutralising activity after being fed on by infected ticks. That this effect was primarily mediated by a response directed against sporozoite and not tick antigens is shown by the much lower level of inhibition exerted by serum from rabbit 8 which was fed on by uninfected ticks. This slight inhibitory activity became insignificant when the serum was diluted further, whereas the diluted serum from rabbit 28 maintained strong sporozoite neutralising activity. One can speculate that antibody to tick proteins which develops in the serum of rabbits fed on by ticks (Gill, Boid and Ross, 1986) was responsible for the slight inhibition observed. If sporozoites bind small quantities of tick protein to their surface, antibody recognising these tick proteins might cause sporozoite aggregation or interfere with normal sporozoite invasion of cells, thus reducing their infectivity in a non-specific manner.

Sporozoite neutralisation by serum from rabbit 28 was effective against all three T. annulata stocks, as expected since tick batches

infected with each of the three stocks had fed on its ears (see Section 3.2.2.). This neutralising ability was not complement dependent and was unaffected by heat inactivation of the serum. Heat inactivation did have a significant effect in allowing a higher level of infection to establish in cultures containing day 0 serum. This was presumably due to the destruction of other heat labile noxious substances. By performing an experiment in which serum from rabbit 28 added on day 1 of the assay was compared with a normal assay, the sporozoite inhibition was shown to be due to the action of serum within the first 24 hours of the assay. Thus, it is evident that, for this rabbit serum, inhibition of sporozoite invasion rather than of lymphocyte transformation was the mechanism involved. This was not unexpected since the rabbit was considered to have been exposed to the sporozoite stage only.

The assays to test the ant sporozoite monoclonals led to the selection of two Mabs which had shown the most consistent and significant levels of sporozoite inhibition; Mab 4B11/C12 (61-100%) and Mab 1A7/G8 (46-87%). In view of the results with the irradiated sporozoite recipient sera, doubt may be cast on the validity of this assay as a means of selecting Mabs which identify potentially protective antigens. However, significance can be attached to the fact that some ant sporozoite Mabs showed no inhibitory action. Neither monoclonal neutralised sporozoite infectivity as strongly as hyperimmune bovine sera or rabbit 28 serum. This could be because, by definition, they each only recognise a single epitope, whereas the polyclonal sera could be identifying several target antigens to achieve neutralisation. In addition, hyperimmune bovine serum

possesses antilymphocyte transformation activity (Preston and Brown, 1985) which contributes to the final level of inhibition observed.

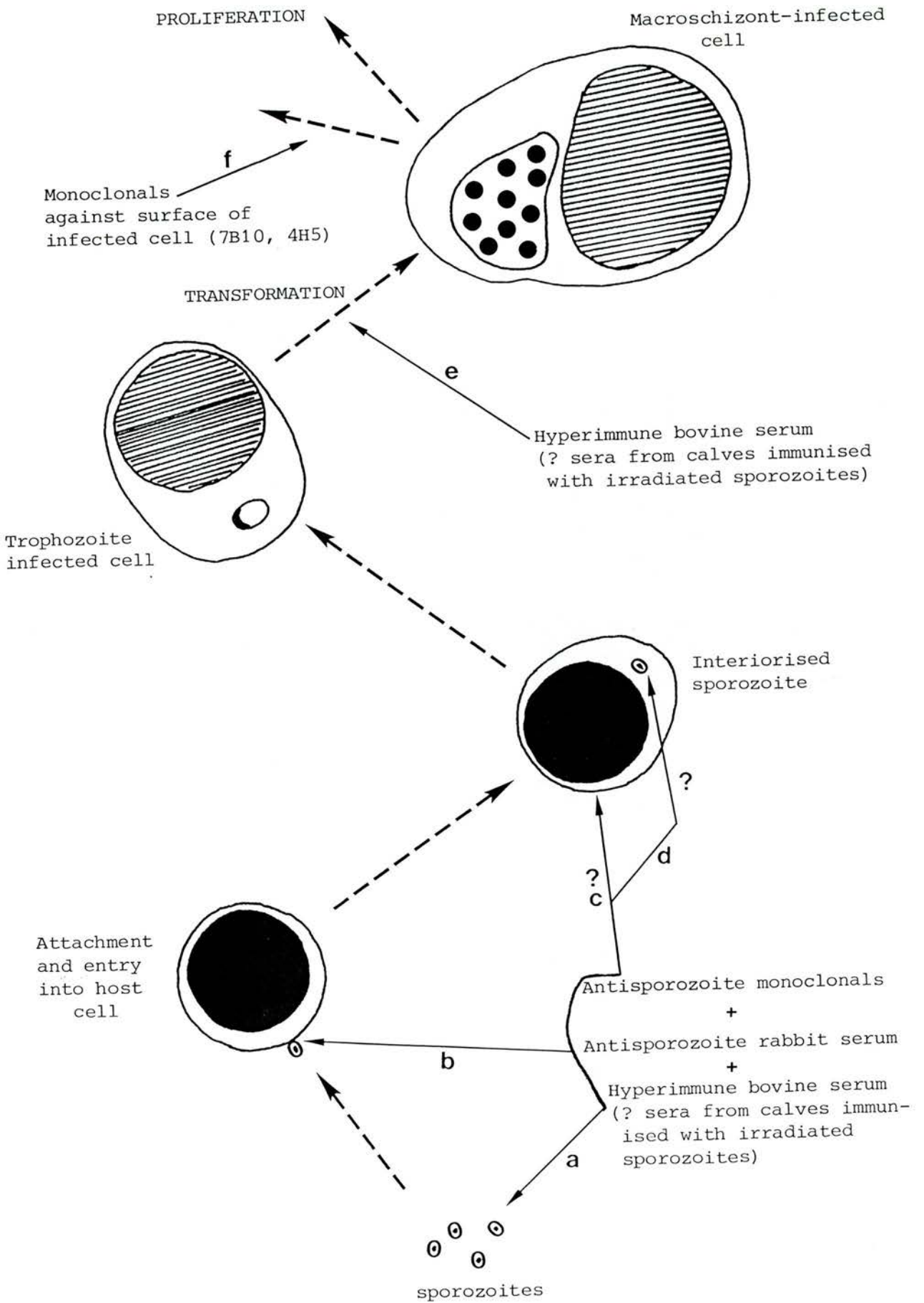
The degree of inhibition by a monoclonal antibody can, in part, be a reflection of the assay sensitivity. Certainly T. annulata is an easier parasite with which to establish infected cell lines than T. parva for which cell feeder layers (Brown, 1983) or filler cells and growth factors (Baldwin, Malu and Grootenhuis, 1988) are required. It follows that it is likely to be harder to demonstrate complete blocking of T. annulata compared to T. parva sporozoites. Both Dobbelaere et al. (1984) and Musoke et al. (1984) show complete blocking of T. parva sporozoite infectivity with high levels of their ant sporozoite Mabs. The merozoite invasion blocking antibody of Winger et al. (1987) showed a maximum of 76% inhibition when calculated in the same manner as in these assays and this was considered to be a significant degree of inhibition. Thus, the inhibition rates obtained with Mabs 4B11/C12 and 1A7/G8 should not be considered as too low to be of significance.

In conclusion, therefore, an in vitro assessment of the functional ability of a range of ant sporozoite antibodies was made and led to the selection of two monoclonal antibodies showing substantial levels of sporozoite neutralising activity.

These monoclonals have advantages over polyclonal sera in that the sporozoite epitope which is recognised by each antibody and which mediates their inhibitory activity can be readily identified. Both the monoclonals and the rabbit 28 serum are particularly useful in that they exert their inhibitory activity entirely through recognition of sporozoite epitopes without the complication of effects

Figure 18 Possible modes of action of antibody or serum factors in sporozoite inhibition assays.

- a antibody binding to sporozoites: aggregation, lysis, structural changes leading to loss of infectivity.
- b antibody binding to sporozoites: interfering with recognition, attachment and/or entry into target cells.
- c antibody entering host cells after sporozoite entry: sporozoite death or failure to develop further.
- d antibody bound to sporozoites carried into cell: sporozoite death or failure to develop further.
- e serum factor (? tumour necrosis factor) acting on trophozoite infected cells: inhibition of transformation.
- f antibody binding surface of macroschizont infected cells: inhibition of cell proliferation.



on lymphocyte transformation observed with polyclonal bovine sera. Figure 18 illustrates the possible modes of action of the different antibodies used in the inhibition assays described in this chapter.

Since monoclonals, 4B11/C12 and 1A7/G8 may identify potentially protective sporozoite surface antigens, the next aim in this study was to characterise these antigens and compare them to those recognised by the polyclonal bovine and rabbit sera.

CHAPTER FIVE
IMMUNOCHEMICAL CHARACTERISATION OF
SPOROZOITE ANTIGENS IDENTIFIED
BY ANTISPOROZOITE ANTIBODIES

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5.1. INTRODUCTION

The indirect fluorescent antibody test reactions and sporozoite neutralising abilities of a range of antisporezoite antibodies have been reported in the previous two chapters. However, these give little indication of how the antigens recognised by these different monoclonals and antisera relate to each other. To determine this, and to define the antigens recognised by Mabs 1A7/G8 and 4B11/C12 (hereafter known as 1A7 and 4B11) more fully, the techniques of protein separation and antigen detection were applied to sporozoite proteins.

Two methods of antigen characterisation were used. The first used the technique known as Western blotting. In this, the proteins of whole sporozoites were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the principles of which are described by Hames and Rickwood (1981) and Walker (1986). Then antigens were detected by antibodies after their transfer to, and immobilisation on, nitrocellulose paper (Towbin, Staehelin and Gordon, 1979; Walker, 1986).

The second method used the techniques of surface labelling and immunoprecipitation and involved radioiodination of sporozoite surface proteins (Fraker and Speck, 1978; Howard, Kaushall and Carter, 1982) prior to their incubation with antibody to form immune complexes. These were precipitated from unbound protein, separated by SDS-PAGE, and their position detected directly using autoradiography to visualise the presence of radioactive iodine molecules.

There are many good reasons for determining the molecular weight of parasite proteins of interest. Statement of molecular weight is

the standard manner in which a protein is described and reported. Once the molecular weight is known, comparisons can be made regarding whether different parasite stocks or parasite stages which react with the antibody by another method of immunodetection, such as immunofluorescence or the enzyme linked immunosorbent assay (ELISA), bear the relevant epitope on proteins of the same size.

Knowledge of molecular weight also reveals whether different antibodies giving identical reactions by other methods are identifying the same antigenic determinants of the parasite, although this can be done without resorting to protein analysis. For example, Musoke et al. (1984) showed that five monoclonal antibodies recognised the same antigenic component of T. parva sporozoites by using a competitive inhibition ELISA. It is of interest to know whether antisera raised in different species or by different immunisation procedures react with different or similar parasite components.

Early work to characterise theilerial antigens by Wagner, Brown, Duffus, Kimber et al. (1974) and Allsopp, Wagner, Matthews and Kariavu (1977) was hampered by two problems; the difficulty in obtaining protein free of bovine contaminants and the considerable range of molecular weights of the antigens obscuring subtle strain differences. Since then, both immune precipitation (Allsopp, 1981; Creemers, 1981, 1983) and immunoblotting techniques (Shapiro, Fujisaki, Morzaria, Webster et al., 1987) have been used with some success to identify macroschizont and piroplasm antigens.

The same methods have been used to analyse theilerial sporozoite proteins of T. parva (Dobbelaere, Shapiro and Webster, 1985b; Iams, Hall and Musoke, 1988) and T. annulata (B. Shiels, personal communication).

The purpose of the work described here was to define the molecular weight of sporozoite antigens recognised by the different anti-sporozoite antibodies, to define how the antibody reactivities related to each other and to investigate the nature of antigens detected by monoclonals 1A7 and 4B11.

5.2. MATERIALS AND METHODS

Formulations of materials mentioned in the text are given in Appendix I.3.

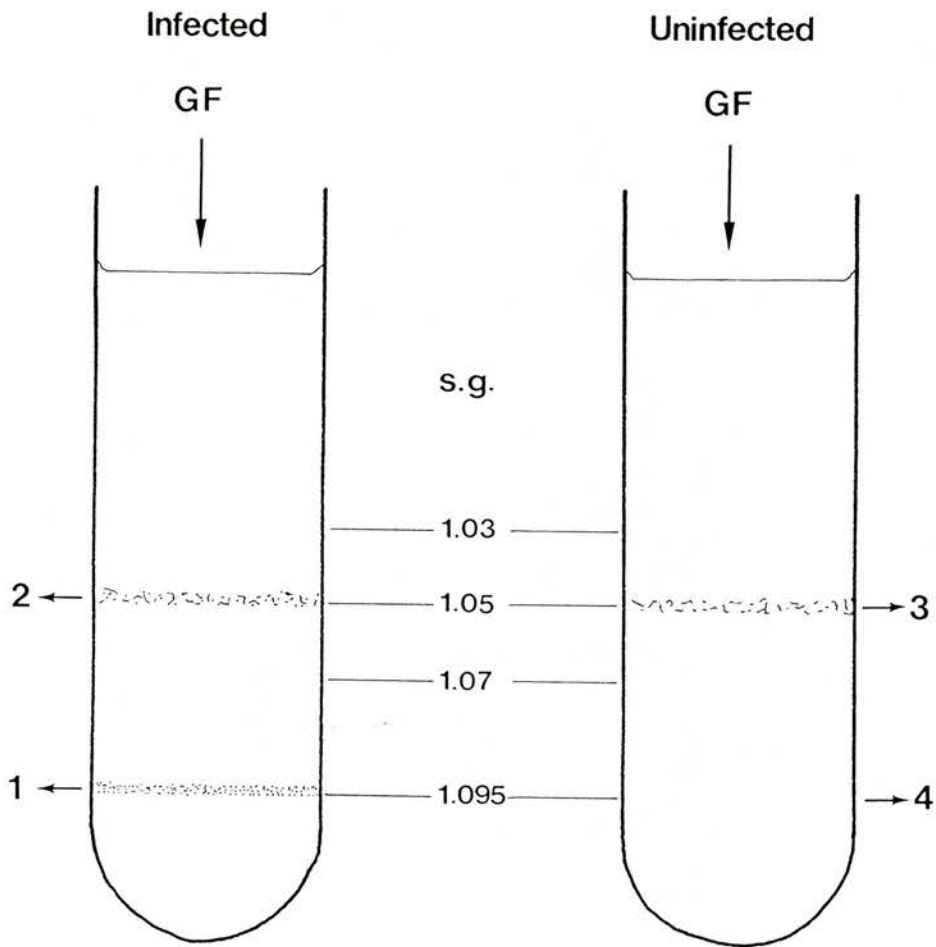
5.2.1. Preparation of protein samples for SDS-PAGE

a) Percoll-purified sporozoites

Thirty two te of sporozoites, prepared using media excluding BPA, were purified on a Percoll gradient (Section 2.2.2.). Sporozoites from all three T. annulata stocks were used. Two bands of material were harvested from the gradient; a band taken from the 1.07/1.095 s.g. interface containing the purified sporozoites (Sample 1) and a band taken from the 1.03/1.05 s.g. interface containing sporozoites, immature sporoblasts and tick debris (Sample 2). Figure 19 shows the samples harvested diagrammatically.

Each was washed once in 20 ml PBS containing protease inhibitors (Appendix I.3.5.) by centrifugation at 1,500 G for 30 minutes at 4°C. The pellets were thoroughly resuspended in 0.75 ml PBS containing protease inhibitors and then 0.25 ml of x 4 reducing SDS sample buffer containing B-ME was added before heating the samples at 100°C for five minutes. Samples were aliquoted and stored at -80°C. Depending on sporozoite density, as assessed from a Giemsa-stained cytospin, 30-100 µl of sample (1-3 te) was used per gel track.

Figure 19 Material harvested from Percoll gradient preparations for immunochemical analysis



Samples :

- 1
- 2
- 3
- 4

Content :

- sporozoites
- sporoblasts, sporozoites, tick debris
- tick debris
- nothing visible

For non-reduced samples, 50 μ l of the resuspended pellet was added to 50 μ l x 1 non-reducing SDS sample buffer containing iodoacetamide. The mixture was heated at 100°C for five minutes and the whole 100 μ l volume used for one gel track. Except where specified otherwise, samples were run in a reduced form.

Early preparations of Percoll-purified sporozoites were made with BPA in the media and without the inclusion of protease inhibitors. For reasons which are explained shortly the protocol was amended to rectify these points.

b) Uninfected Percoll preparation

To act as a control antigen, uninfected GF from 32 x three day fed uninfected H. a. anatolicum adult ticks was separated on a Percoll gradient. Material was collected from exactly the same positions as above and were called samples 3 and 4 (Figure 19). They were treated in exactly the same manner to be run under reducing conditions only.

c) Ground up tick supernatant (GUTS)

Uninfected and T. annulata Hissar infected GUTS were prepared at a concentration of 10 te per ml using MEM without BPA as the grinding medium. The aliquoted samples were stored at -80°C and 1 te separated on each gel track, under reducing conditions.

d) Purified piroplasms

A 0.5 ml pellet of T. annulata Ankara piroplasms prepared in the manner described in Section 2.2.10. was resuspended in 2 ml PBS containing protease inhibitors. An equal volume of x 1 reducing SDS sample buffer was added and the mixture was heated at 100°C for five

minutes. The sample was passed rapidly in and out of a 21 gauge needle to shear the DNA and reduce the viscosity of the sample. Ten μ l was used per gel track.

e) Rabbit and bovine blood

Samples were prepared from rabbit and bovine blood. Whole blood was collected into tubes containing EDTA (Vacutainer, Becton Dickinson) and for each gel track 1 μ l of whole blood was diluted with 23 μ l PBS containing protease inhibitors. Eight μ l of x 4 reducing SDS sample buffer was added to this and the sample was heated at 100°C five minutes before cooling and loading onto the gel.

5.2.2. SDS-PAGE

Separation of proteins in the above samples was achieved using SDS-PAGE on 7-20% acrylamide gel slabs (Studier, 1973). The technique was used to study total protein profiles, to provide proteins separated by molecular weight for immunoblotting and to analyse the products of surface labelling and immunoprecipitation. The practical aspects are described by Zingales (1984), Goding and Handman (1984) and Walker (1986). Briefly, 7-20% polyacrylamide gradient gels were prepared by mixing 20 ml of 7% and 20 ml of 20% acrylamide solutions via a concentration gradient gel pourer and peristaltic pump, having added 26 μ l of 10% ammonium persulphate (BDH) and 6 μ l of N,N,N',N'-tetramethylethylenediamine (TEMED, BDH) to each of the two gel concentrations to cause polymerisation of the acrylamide. The resulting gels (19 cm x 13.7 cm x 0.15 cm) were overlaid with a 3 cm stacking gel. The low acrylamide concentration (4.5%) of this stacking gel allows proteins to concentrate before reaching the lower resolving gel (Laemmli, 1970).

Samples of 10-100 μ l were loaded into wells in the stacking gel and electrophoresis was carried out in electrophoresis tank buffer (Appendix I.3.1.) at 80 volts until the bromophenol blue dye of the sample buffer had reached the end of the gel. This took 16-20 hours.

5.2.3. Protein detection in SDS-PAGE gels

In order to visualise the positions of protein bands after electrophoresis and to fix the proteins in position in the gel, two protein staining methods were used.

a) Coomassie blue stain

Proteins were stained for 2-4 hours in a filtered solution of Coomassie brilliant blue R (Sigma), 0.1% w/v in destain solution, at room temperature. Gels were destained overnight to visualise the proteins.

b) Silver stain (Morrisey, 1981)

This was the main method used to visualise sporozoite proteins in gels. It is more sensitive than Coomassie blue which is unable to detect bands containing less than 100 ng protein (Goding and Handman, 1984).

Gels were incubated for 30 minutes in each of solutions 1, 2 and 3 (Appendix I.3.2.). They were then washed in purified water overnight. After a 30 minute incubation in 0.1% w/v silver nitrate (Sigma), the gels were rinsed in purified water and then developer (Appendix I.3.2.) before being left in developer until bands had appeared (about 20 minutes). The reaction was stopped with 5 ml of 2.3 M citric acid (BDH) per 100 ml developer. After rinsing in purified water over 30 minutes, gels were photographed.

5.2.4. Western blotting

Practical details of performing Western blots are given by Towbin *et al.* (1979), Moriearty (1984) and Walker (1986). Solutions and reagents are detailed in Appendix I.3.3.

Proteins separated by SDS-PAGE were electrophoretically transferred onto 0.45 μm nitrocellulose paper (Schleicher and Schuell) in blotting buffer using an Ancos semi-dry electroblotter (Dako Ltd.) at 200 mA, 12 volts for 90 minutes. When transfer had occurred, the track containing the molecular weight markers was cut off and the proteins visualised by staining in 0.1% amido black (Sigma) in de-stain. The rest of the nitrocellulose paper (blot) was placed in blocking buffer containing 5% skimmed milk (Marvel, Carnation) for four hours to block non-specific protein binding sites on the nitrocellulose paper (Johnson, Gautsch, Sportsman and Elder, 1984). The blot was then divided as necessary and incubated overnight in anti-serum or monoclonal antibody. Rabbit sera were diluted to 1:200 and bovine sera to 1:100 in blocking buffer with 5% skimmed milk, while Mabs were used as undiluted culture supernatants. The blot was then washed in seven changes of PBS over 90 minutes before being incubated for two hours with the appropriate horseradish peroxidase conjugated second antibody at a dilution of 1:1000 (anti-bovine) or 1:2000 (anti-rabbit and anti-mouse) in blocking buffer with 5% skimmed milk. The blot was then washed in PBS as above. The positions of antigen-antibody complexes were detected by incubating the blot in substrate for the peroxidase enzyme. To do this, the blot was placed in substrate solutions 1 and 2 (Appendix I.3.3.) mixed together and colour development allowed to occur over the next 5-20 minutes. The blot was finally rinsed in purified water.

5.2.5. Determination of protein molecular weights

Molecular weights were calculated by reference to the mobilities of the protein standard mixture of the low molecular weight range (Appendix I.3.5.). All molecular weights are given in kilodaltons (kdal, Stryer, 1981) and are estimates.

5.2.6. Radiolabelling of sporozoite surface proteins

The method described here for labelling of surface proteins with radioactive iodine 125 (^{125}I) using a chloroamide, iodogen is based on that followed by Howard et al. (1982) with modifications as noted below.

Forty te of GF from both uninfected and T. annulata Ankara infected ticks were separated on Percoll gradients. BPA was excluded from all the media. Bands were harvested from 1.03/1.05 s.g. and 1.07/1.095 s.g. interfaces of the infected Percoll preparation (samples 1 and 2) and from the 1.03/1.05 s.g. interface of the uninfected Percoll separation (sample 3). The samples were washed as described in Section 5.3.1. except that protease inhibitors were excluded from the PBS. After washing, each pellet was resuspended in 300 μl PBS. Glass bijoux bottles had previously been coated internally with 60 μg iodogen (1,3,4,6-tetrachloro-3-alfa, 6-alfa-diphenylglycoluril, Sigma) by evaporation of 600 μl of 100 $\mu\text{g}/\text{ml}$ iodogen in chloroform.

The three samples (1-3) were each placed in a rinsed iodogen-coated bijou and 300 μCi (3 μl) of ^{125}I (as sodium iodide in dilute sodium hydroxide solution, pH 7-11, free from reducing agents, Amersham International PLC) was added to each. The bijoux were

agitated at room temperature for 10 minutes and then the contents of each were transferred to labelled 1.5 ml Eppendorfs and 1.0 ml PBS containing protease inhibitors, used to rinse the bijou, was added. The samples were centrifuged for 12 minutes at 1,500 G (4,500 rpm) on a microcentrifuge. The supernatants containing free iodine were discarded and each pellet was resuspended in 1.2 ml PBS containing protease inhibitors and recentrifuged. Two further washes were made as above.

Each pellet was then resuspended in 750 μ l lysis buffer (Appendix I.3.5.) and freeze thawed three times in liquid nitrogen. One μ l of each sample was placed in a plastic vial (Sarstedt) and radioactivity was measured using a gamma counter (Nuclear Enterprises NE 1600).

Trichloroacetic acid (TCA, Sigma) precipitation was carried out to see what proportion of the measured radioactivity derived from labelled protein. This involved measuring the radioactive counts per minute (cpm) for 4 μ l of each sample, adding 100 μ l of normal rabbit serum diluted 1:10 in PBS, and then filling the vial with 10% TCA. The tubes were centrifuged for five minutes at 2,500 G and 4°C and the supernatant discarded before counting the pellets. The proportion of radioactivity remaining indicated the efficiency of the radiolabelling process.

To determine which proteins had been labelled with ^{125}I , each sample was separated by overnight SDS-PAGE on a 7-20% acrylamide gel. For each gel track, 100,000 cpm were diluted in 30 μ l PBS containing protease inhibitors, added to 10 μ l x 4 SDS sample buffer and heated at 100°C for five minutes.

5.2.7. Immunoprecipitation of radiolabelled proteins

Four replicate vials were prepared for each sample, all containing 100,000 cpm. For each sample (1-3), each of the four vials was mixed with a different antibody as shown below:

Mab 1A7	400 μ l
Mab 4B11	400 μ l
Normal mouse serum	3 μ l + 397 μ l immunoprecipitation diluent (Appendix I.3.4.)
Mab anti- <u>Taenia saginata</u>	400 μ l

The Mabs were used as undiluted culture supernatants. The contents were mixed, covered in Parafilm (American Can Company) and left at 4°C for three hours. To each vial, 3 μ l normal mouse serum (except to those already containing normal mouse serum), 50 μ l immunoprecipitation diluent and 45 μ l goat anti-mouse antiserum (prepared by Dr. L.J. Harrison and referred to by Harrison and Parkhouse, 1986) were added. Vials were incubated at 4°C overnight to allow precipitate to form.

Five hundred μ l of diluent was added to each vial and they were centrifuged for 10 minutes at 1,200 G, 4°C. The supernatants were discarded, taking care to leave the precipitate intact. A few drops of diluent were added to resuspend the precipitate by vortexing (Whirlimixer, Fisons) before adding another 1.0 ml diluent to wash the precipitate by centrifuging as above. This was repeated three times or until counts of radioactivity remained similar from one wash to the next. At this point each precipitate was resuspended in 20 μ l PBS and 10 μ l of x 1 reducing SDS sample buffer was added. The samples were heated at 100°C for five minutes, cooled and separated by SDS-PAGE on a 7-20% gradient gel.

5.2.8. Gel drying and autoradiography

Polyacrylamide gels on which surface labelled or immunoprecipitated proteins had been separated were stained in 0.1% Coomassie blue and destained to visualise the non-radioactive molecular weight markers. Then the gels were soaked for 45 minutes in 1% glycerol, 10% acetic acid in purified water before being dried for two hours onto porous cellophane using a gel slab dryer (Biorad Labs.). Autoradiographs of the dried gels were obtained by exposing them at -80°C to x-ray film (Agfa-Gevaert Curix RPI [100 NIF]) using fast tungstate intensifying screens (Ilford). They were developed (Kodak D19 developer) and then fixed (Ilford Hypam Fixer, diluted 1 in 4).

5.3. RESULTS

Two problems were encountered in the preparation of sporozoites for SDS-PAGE analysis which were overcome as described. The first was that, if BPA was included in the media during purification, it was bound by the sporozoites to such a degree that washing failed to remove it. This dramatically influenced and obscured the true protein profile of the sporozoites since most of the protein present was BPA (Figure 20a, track 2). Western blots also gave poor results at this stage. Therefore, subsequent sporozoite preparations were made excluding any extraneous protein, in particular BPA, from media and from the Percoll gradient (Figure 20a, track 1). To ensure that sporozoite infectivity was not lost without the BPA, which would imply detrimental effects on sporozoite proteins, infectivity titrations were carried out for GF and Percoll preparations (for in vitro infection method see Section 2.2.4.) and the results are shown in Table 16.

Figure 20 Effects of bovine plasma albumin and protease inhibitors on the analysis of sporozoite antigens.

a) Silver stain of sporozoite proteins separated by SDS-PAGE after purification of sporozoites using:

track 1 media excluding bovine plasma albumin

track 2 media including bovine plasma albumin

b) Western blot of sporozoite proteins detected by monoclonal antibody 1A7 after purification of sporozoites in the:

track 1 absence of protease inhibitors

track 2 presence of protease inhibitors

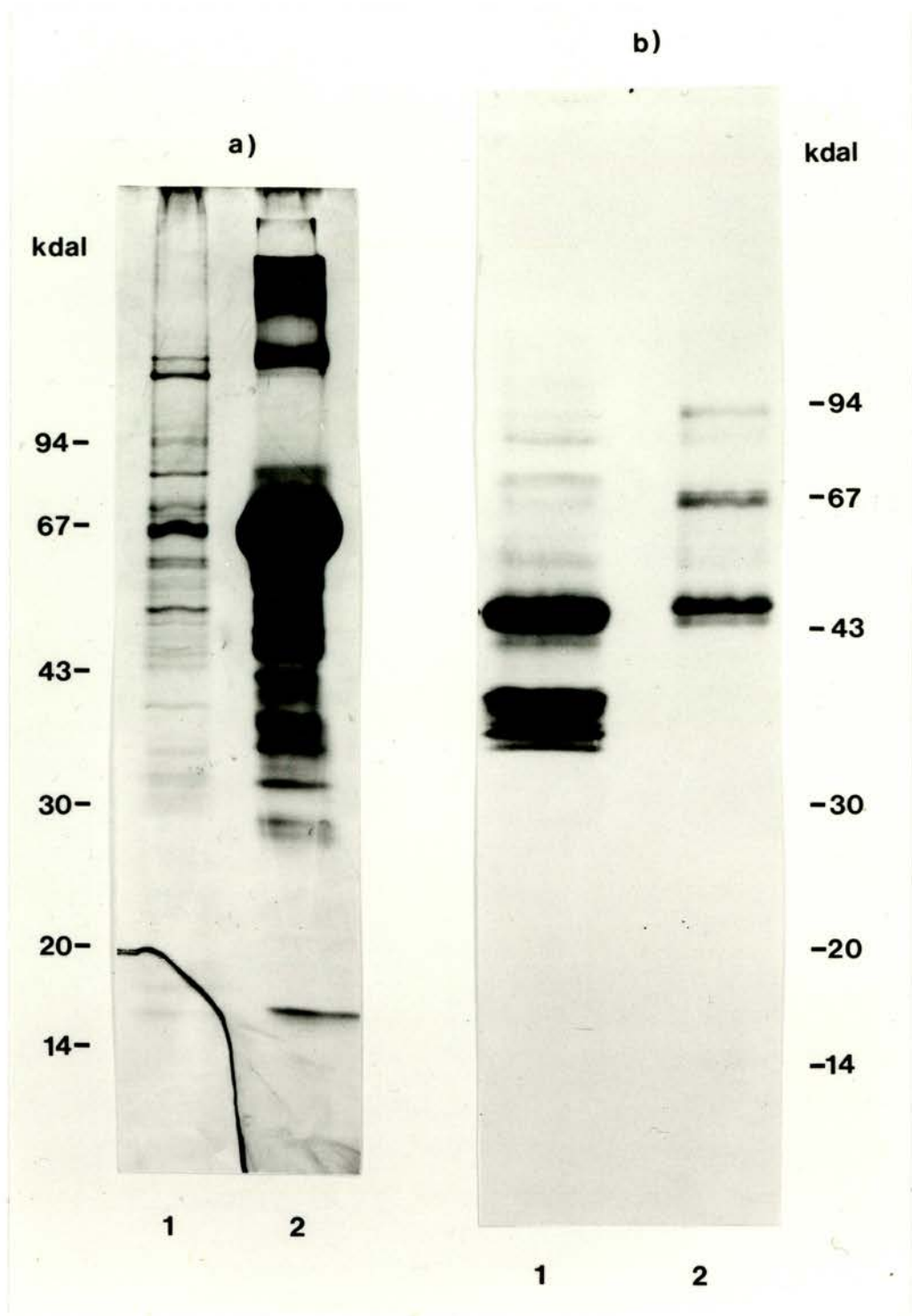


Table 16 In vitro titration of infectivity of sporozoites prepared without BPA.

Sporozoites	Dilution	Mean % infection (n = 2)		
		Cytospin day:		
		4	8	12
GF TaH	0	30.5	ND	ND
	10 ⁻¹	9.3	ND	ND
	10 ⁻²	1.8	14.3	ND
	10 ⁻³	0	1.5	55.5
	10 ⁻⁴	0	0.8	39.0
	10 ⁻⁵	0	0.3	8.0
Percoll TaH	0	14.0	ND	ND
	10 ⁻¹	1.3	ND	ND
	10 ⁻²	0.3	10.8	ND
	10 ⁻³	0	1.0	26.5
	10 ⁻⁴	0	0.5	2.5
	10 ⁻⁵	0	0	0
Percoll TaG	0	9.0	ND	
	10 ⁻¹	2.0	24.8	
	10 ⁻²	0.5	5.3	
	10 ⁻³	0	1.0	
	10 ⁻⁴	0	0	
	10 ⁻⁵	0	0	

GF: 2 te per well }
 Percoll: 2 te per well } - ten-fold dilutions made from these

ND = Not done; TaH = T. annulata Hissar; TaG = T. annulata Gharb

Sporozoite infectivity remained high in spite of the absence of BPA, the Percoll preparation (T. annulata Hissar) being about ten-fold less infective than the equivalent GF at the same concentration which is in agreement with previous work (C.G.D. Brown, personal communication).

The other problem was that, initially, samples prepared for SDS-PAGE were susceptible to cleavage by endogenous proteases or, more likely, proteases derived from tick saliva or salivary glands (Gill et al., 1986). To prevent this proteolytic action, samples were always washed, resuspended and diluted in PBS containing protease inhibitors. Figure 20b shows the effect of including protease inhibitors on the sporozoite antigen(s) recognised by Mab 1A7 on a Western blot (track 2). Without the inhibitors 1A7 appeared to recognise proteins of lower molecular weights (track 1).

5.3.1. Sporozoite proteins

Figure 21 shows the protein profiles of material prepared from infected (T. annulata Ankara) and uninfected Percoll preparations. The points of note are:

a) Pure sporozoites (track 1) and sporozoites/sporoblasts contaminated with tick debris (track 3) had a very similar protein content as detected by silver staining. This is in spite of the greater maturity and purity of the sporozoites in track 1 compared to track 2 which is shown by the appearance of each preparation on Giemsa-stained cytopins (Figure 22a and b).

b) Track 4 of Figure 21 contains proteins from the 1.03/1.05 s.g. interace of an uninfected Percoll preparation (Figure 22c).

These were tick derived proteins, many of which can be seen to be present in track 3 and even track 1 also. This preparation acted as a valuable control since protein bands visible in tracks 1 and 3 but absent in track 4 may be considered as sporozoite/sporoblast in origin. The molecular weights of such proteins are given below and their positions are indicated in Figure 21.

<u>Protein</u> (sporozoite/sporoblast)	<u>Molecular weight</u> (kdal)
1	118
2	111
3	74
4	70
5	55
6	22
7	20
8	19
9	17

Thus nine parasite specific proteins are visible, two of which appear to be present predominantly on mature sporozoites (proteins 2 and 4 of molecular weight 111 and 70 kdal respectively), the rest being common to both preparations from an infected Percoll separation.

c) The final feature of Figure 21 is the virtual absence of protein from material harvested from the 1.07/1.095 s.g. interface of an uninfected Percoll separation (track 2).

When protein profiles of infected and uninfected GUTS were compared (Figure 23) there were no visible differences in their proteins. The proteins of sporozoites of each T. annulata stock purified on a Percoll gradient were compared after SDS-PAGE separation and silver staining (Figure 24) and, at this level of sensitivity, no differences could be observed between the different stocks.

Figure 21 Sporozoite and tick proteins harvested from T. annulata Ankara infected and uninfected GUTS filtrate separated on Percoll gradients.

Silver stain of proteins harvested from:

- track 1 1.07/1.095 s.g. interface of infected Percoll preparation; sample 1
- track 2 1.07/1.095 s.g. interface of uninfected Percoll preparation; sample 4
- track 3 1.03/1.05 s.g. interface of infected Percoll preparation; sample 2
- track 4 1.03/1.05 s.g. interface of uninfected Percoll preparation; sample 3

The positions of sporozoite proteins 1-9 are indicated with arrows.

Figure 22 Material harvested from T. annulata Ankara infected and uninfected GUTS filtrate after separation on Percoll gradients.

Giemsa-stained cytopspins showing:

- a) Sample 1: sporozoites from 1.07/1.09 s.g. interface of an infected Percoll preparation.
- b) Sample 2: sporoblasts, sporozoites and tick debris from 1.03/1.05 s.g. interface of an infected Percoll preparation.
- c) Sample 3: tick debris from 1.03/1.05 s.g. interface of an uninfected Percoll preparation.

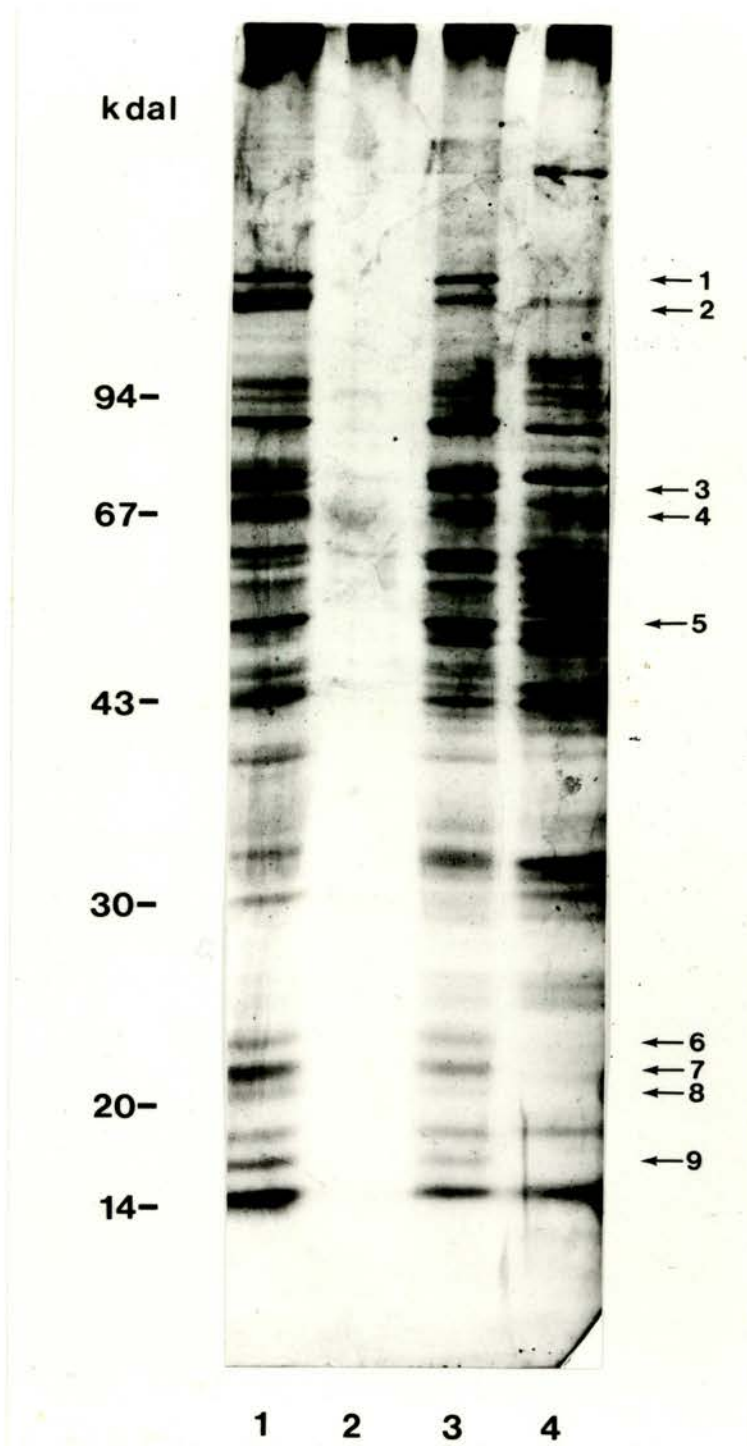
sp sporozoites

sb sporoblasts

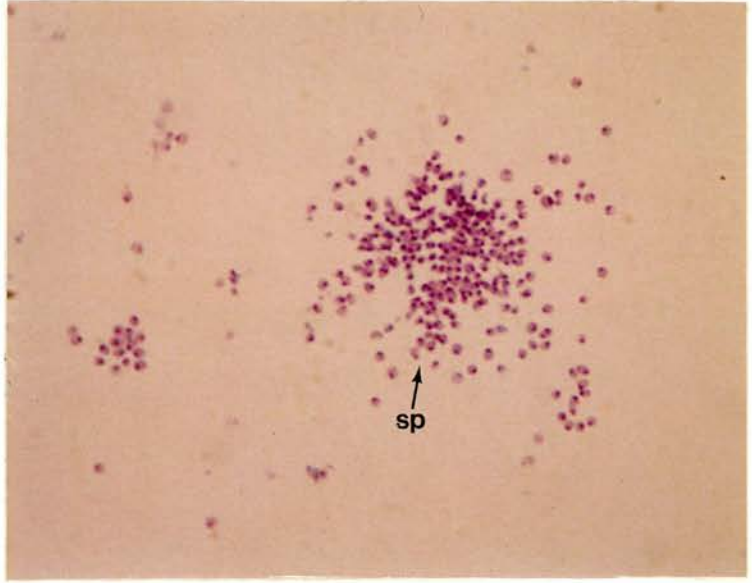
t tick debris

(x 1,100 magnification)

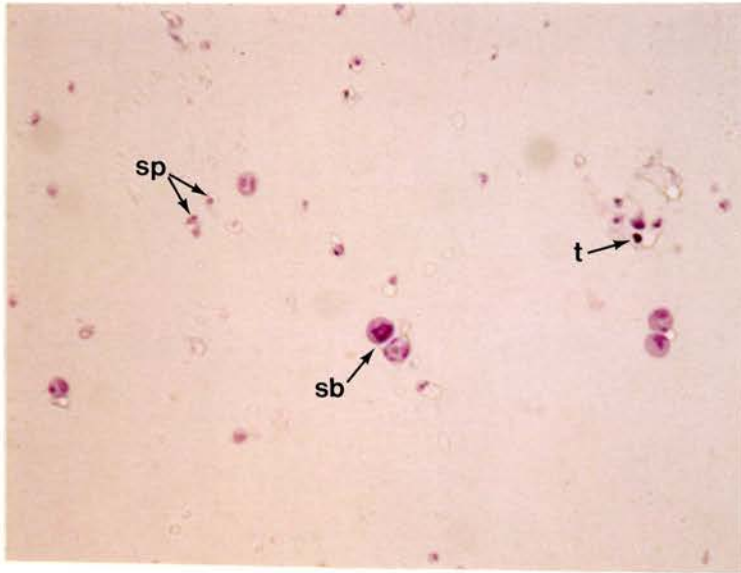
21



22 a



b



c

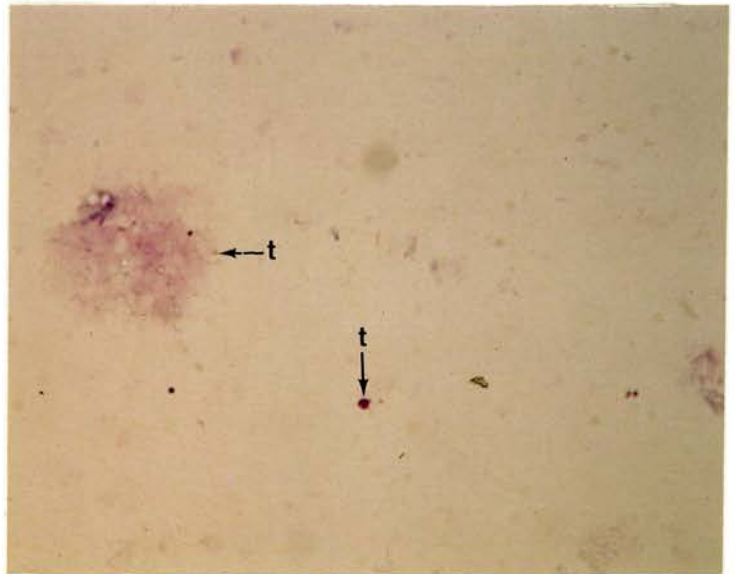


Figure 23 Proteins of crude uninfected GUTS and T. annulata Hissar infected GUTS.

Silver stain of proteins after separation by SDS-PAGE of:

track 1 T. annulata Hissar infected GUTS

track 2 uninfected GUTS

Figure 24 Proteins of Percoll-purified sporozoites from different T. annulata stocks.

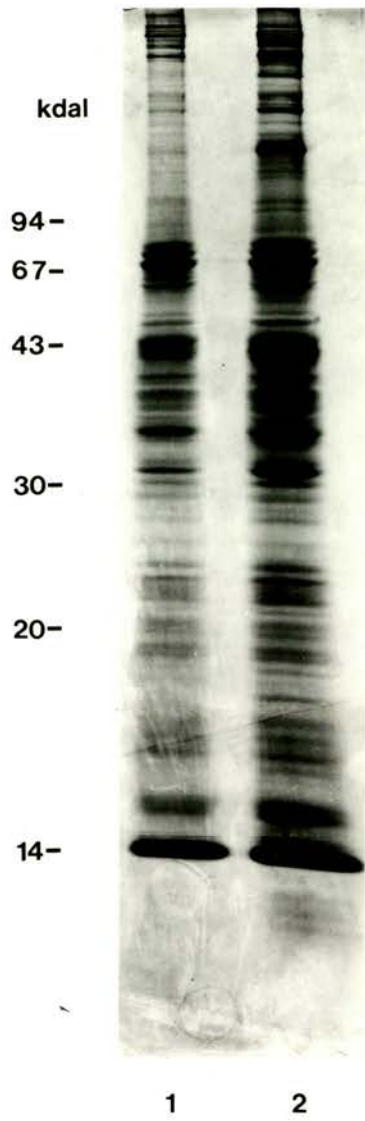
Silver stain of proteins after separation by SDS-PAGE of:

track 1 T. annulata Hissar sporozoites

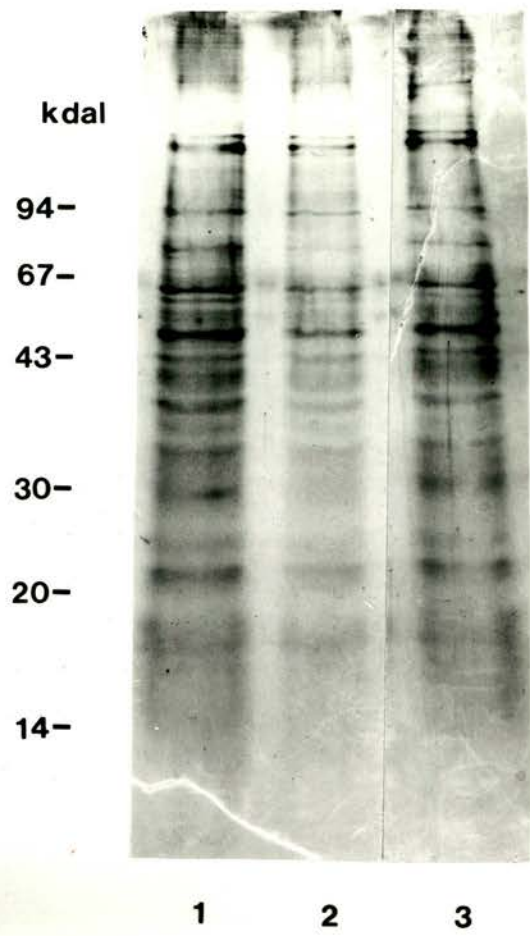
track 2 T. annulata Gharb sporozoites

track 3 T. annulata Ankara sporozoites

23



24



5.3.2. Sporozoite antigens detected by ant sporozoite monoclonal antibodies

Figure 25 summarises the reaction of Mab 4B11 with sporozoite proteins. This monoclonal recognised a sporozoite protein around 17 kdal in size in both Percoll-purified sporozoite and infected GUTS (tracks 1 and 3). This antigen was not sensitive to reduction; the same size protein was detected in non-reduced samples (track 2) and there was no reaction of 4B11 with tick proteins in uninfected GUTS (track 4). The same antigen was recognised in all three stocks of T. annulata (tracks 1, 5 and 6). When larger quantities of sporozoites were used per gel track 4B11 reacted strongly with the 17 kdal antigen and to a minor extent with an antigen of 20 kdal (track 5).

When Mab 1A7 was reacted with Percoll-purified sporozoites four protein doublets were observed at molecular weights of approximately 54, 63, 70 and 85 kdal (Figure 26). These molecular weights are approximate. They were obtained by extrapolation from calibration curves made by plotting the molecular weights of protein standards against their relative mobilities on semilogarithmic paper. Since a "line of best fit" had to be drawn, the molecular weight determined for a band might not concur with that expected from visual inspection of the blot. Apart from this, there were differences between blots, which are discussed later, in the mobility of the protein group detected by Mab 1A7 although the multiple pattern remained very consistent. The same multiple band pattern was detected in purified sporozoites from all three stocks of T. annulata (Figure 26, tracks 2, 3 and 6), the only differences being the 85 kdal doublet which, in the Gharb stock, occurred at 80 kdal. When crude GUTS was used as the

Figure 25 Sporozoite antigen recognised by ant sporozoite monoclonal antibody 4B11.

Western blots of different antigens reacted with monoclonal 4B11. The antigens were:

track 1 Percoll-purified T. annulata Hissar sporozoites: proteins reduced

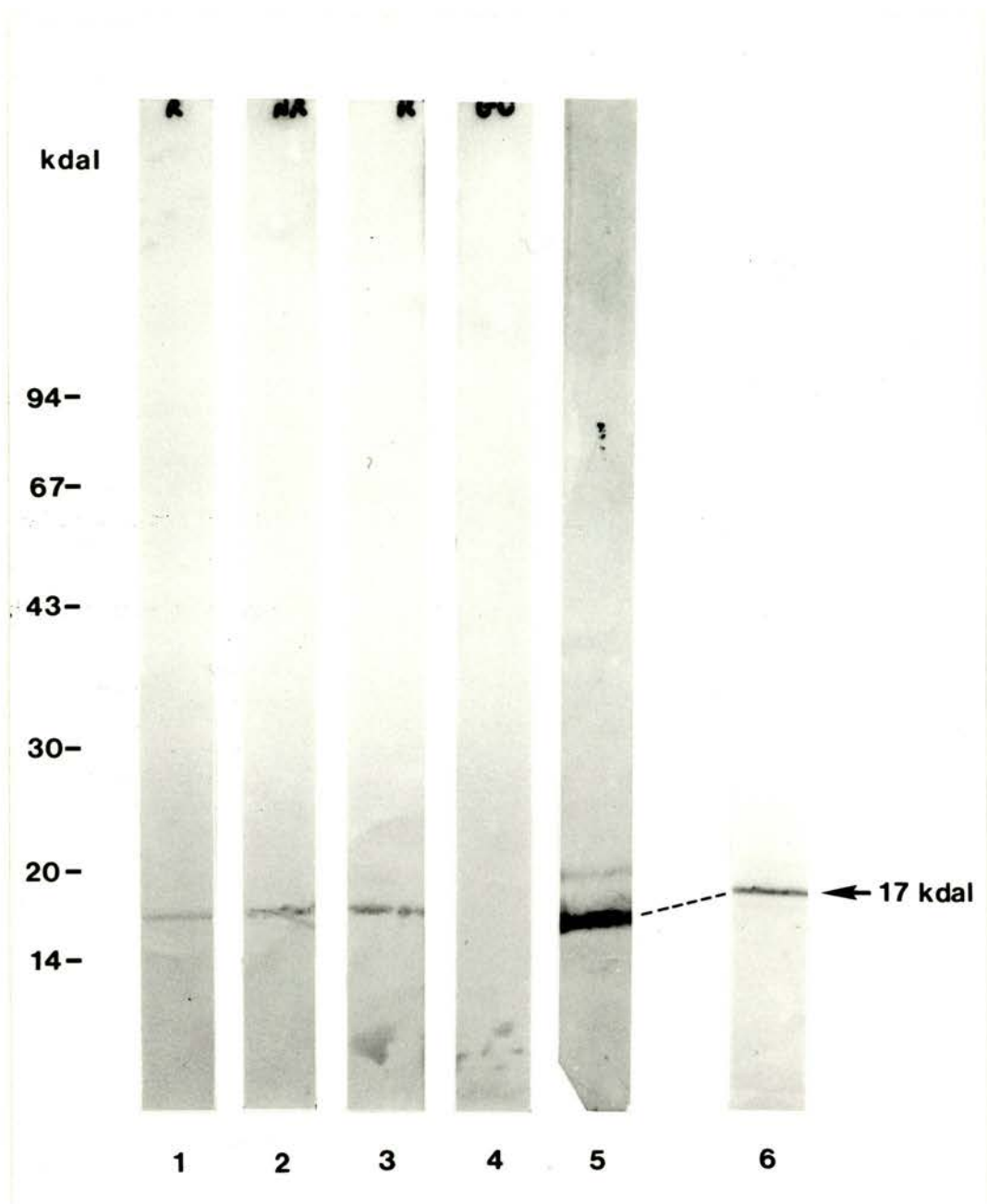
track 2 Percoll-purified T. annulata Hissar sporozoites: proteins non-reduced

track 3 T. annulata Hissar infected GUTS

track 4 uninfected GUTS

track 5 Percoll-purified T. annulata Ankara sporozoites

track 6 Percoll-purified T. annulata Gharb sporozoites



antigen, 1A7 detected the same proteins and an additional protein band of higher molecular weight at 104 kdal (Figure 26, track 4). No antigens were detected when 1A7 was reacted with uninfected T. annulata Hissar GUTS (Figure 26, track 1) or samples from 1.03/1.05 and 1.07/1.095 s.g. interfaces of an uninfected Percoll separation (Figure 27, tracks 1 and 3 respectively).

Figure 27 compares the antigens detected by 1A7 in samples 2 (sporozoites, sporoblasts and tick debris, track 2) and 1 (sporozoites, track 4) of a Percoll separation of T. annulata Ankara sporozoites. The antigens were identical in position, the only difference between them being that the intensity of the three lower molecular weight bands in track 2 is less than in track 4.

The uninfected controls used were true controls in all but one aspect: the nymphs from which the infected adult ticks derived, engorged on infected bovine blood, whereas those providing uninfected adults engorged on rabbit blood. To ensure that the reactions of 1A7 and 4B11 with sporozoites were not, in part, due to traces of bovine blood proteins, a Western blot was carried out to react the two Mabs with rabbit and bovine blood separated on a 7-20% acrylamide gel. Figure 28 (tracks 3-6) shows the complete absence of specific reaction with either antibody. No reaction was seen when proteins of T. annulata Ankara piroplasms were separated by SDS-PAGE (Figure 29, track 1) and blotted before incubating with 1A7 (track 2). Immune bovine serum, from a calf which recovered from sporozoite immunisation, was included as a positive control (track 4) with day 0 serum from the same calf giving no reaction (track 3).

Figure 26 Reactivity of antsporozoite monoclonal antibody 1A7 with sporozoite proteins.

Western blots of different antigens reacted with monoclonal 1A7. The antigens were:

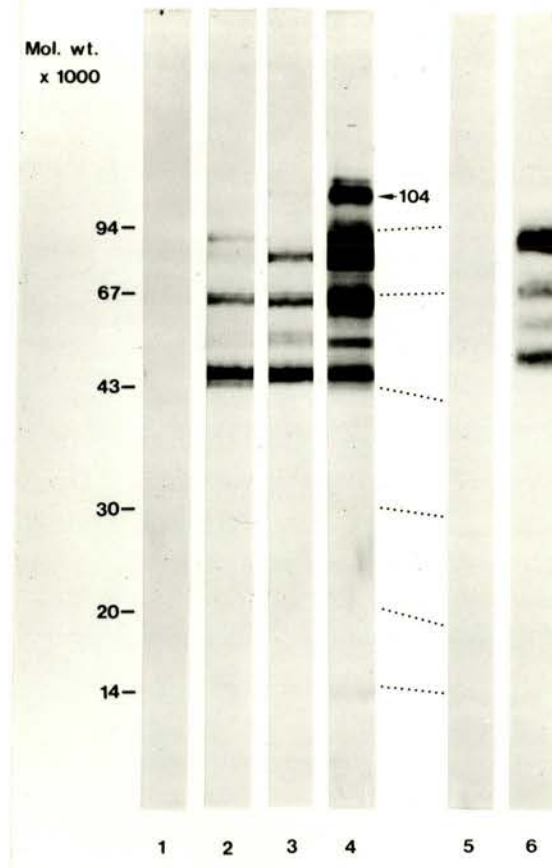
- track 1 uninfected GUTS
- track 2 Percoll-purified T. annulata Hissar sporozoites
- track 3 Percoll-purified T. annulata Gharb sporozoites
- track 4 T. annulata Hissar infected GUTS
- track 5 Material from 1.07/1.095 s.g. interface of an uninfected Percoll preparation
- track 6 Percoll-purified T. annulata Ankara sporozoites

Figure 27 Reactivity of monoclonal antibody 1A7 with material harvested from infected and uninfected Percoll preparations.

Western blot of antigens reacted with monoclonal 1A7:

- track 1 Sample 3 from 1.03/1.05 s.g. interface of uninfected preparation
- track 2 Sample 2 from 1.03/1.05 s.g. interface of infected preparation
- track 3 Sample 4 from 1.07/1.095 s.g. interface of uninfected preparation
- track 4 Sample 1 from 1.07/1.095 s.g. interface of infected preparation

26



27

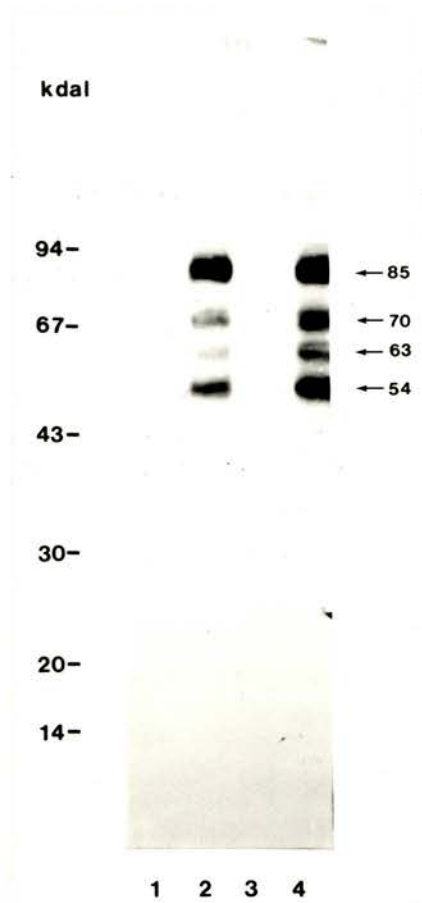


Figure 28 Absence of reactivity of monoclonal antibodies 1A7 and 4B11 with rabbit or bovine blood proteins.

Western blot showing Mabs 1A7 (tracks 3 and 4) and 4B11 (tracks 5 and 6) reacted with:

track 3 rabbit blood

track 4 bovine blood

track 5 rabbit blood

track 6 bovine blood

Tracks 1 and 2 show the Coomassie blue stained proteins of rabbit and bovine blood respectively after separation by SDS-PAGE.

Figure 29 Absence of reactivity of antisporezoite monoclonal antibody 1A7 with piroplasm protein.

Western blot of T. annulata Ankara piroplasm proteins reacted with:

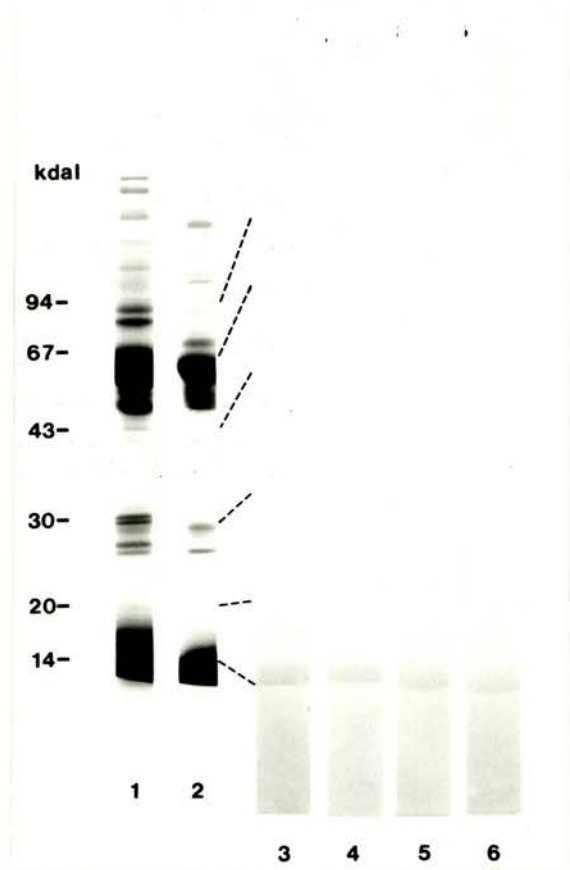
track 2 monoclonal 1A7

track 3 preimmune bovine serum

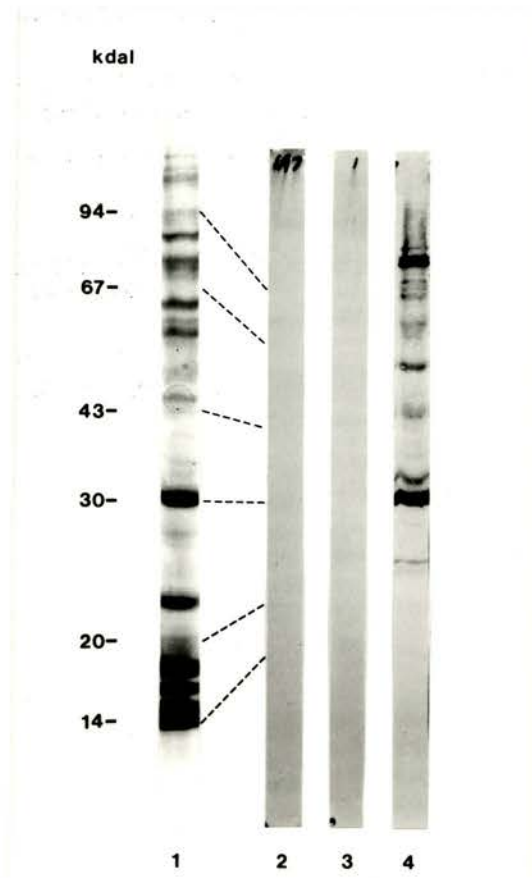
track 4 Theileria immune bovine serum
(positive control)

Track 1 shows Coomassie blue stained piroplasm proteins after separation by SDS-PAGE.

28



29



The anti-Taenia saginata Mab used as a control in previous experiments gave no reaction with Percoll-purified sporozoites on Western blots (data not shown).

5.3.3. Sporozoite antigens recognised by rabbit and bovine antisera

Polyclonal sera from rabbits and calves were reacted in Western blots with T. annulata Ankara Percoll-purified sporozoites. Figures 30-32 illustrate representative samples of the results obtained. Whenever possible, tracks reacted with 1A7 and 4B11 were included so that the relative positions of bands revealed by monoclonal and polyclonal antibody could be determined.

None of the negative controls gave reactions on the blots. These controls included day 0 sera for each calf, serum from calf 45T which was immune to Hyalomma ticks (supplied by Dr. A.R. Walker), serum from rabbit 8 which had been fed on by uninfected ticks and an antimacroschizont Mab, 1C7 (Shiels et al., 1986b). The lack of reactivity of sera from the tick immune calf (Figure 30, track 12) and rabbit 8 (Figure 32, track 4) with the separated proteins suggests that the bands revealed by sera from animals exposed to infected ticks or infected tick material represented sporozoite and not tick antigens.

A 17-20 kdal antigen in a similar position to that recognised by Mab 4B11 was detected by hyperimmune serum from calves 134X (Figure 30, track 10) and N61 (Figure 30, track 5), by serum from calves 102 and L37 (Figure 31, tracks 3 and 4) immunised with irradiated sporozoites and by day 73 serum from rabbit 28 (Figure 32, track 2) which was fed on by infected ticks.

Figure 30 T. annulata Ankara sporozoite antigens recognised by sera from calves hyperimmunised with sporozoites.

Western blots of Percoll-purified T. annulata Ankara sporozoite proteins reacted with antibodies:

track 1	Mab 4B11
track 2	Calf N53 Day 0
track 3	Day 107
track 4	Calf N61 Day 0
track 5	Day 107
track 6	Mab 1A7
track 7	Mab 4B11
track 8	Mab 1C7
track 9	Calf 134X Day 0
track 10	Day 101
track 11	Calf 45T Day 0
track 12	immune to <u>Hyalomma</u> ticks

Details of immunisation of calves is given in Section 3.2.2.

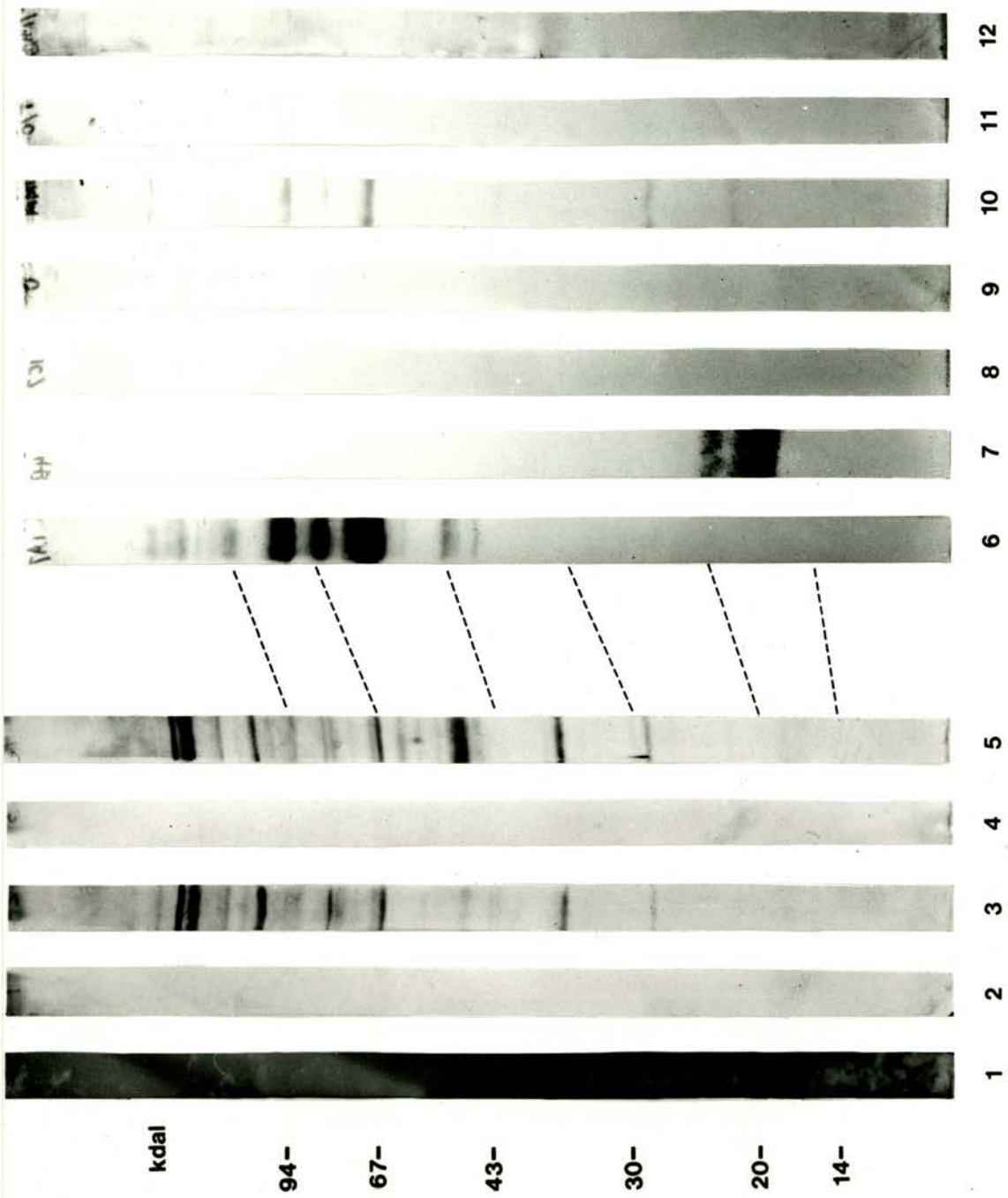


Figure 31 T. annulata Ankara sporozoite antigens recognised by sera from calves immunised with live virulent (54X) or irradiated (L21, N44, L37 and 102) sporozoites.

Western blots of Percoll-purified T. annulata Ankara sporozoite proteins reacted with antibodies:

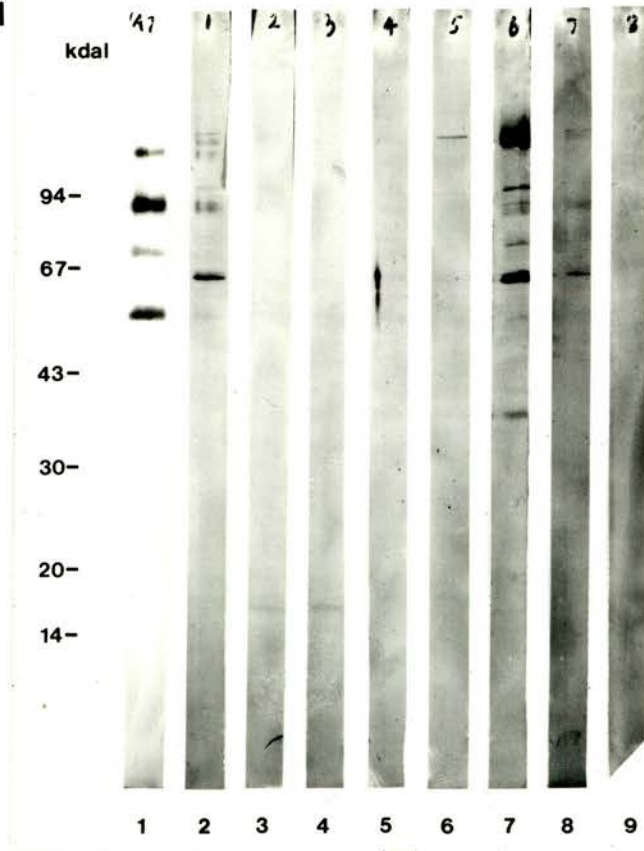
- track 1 Mab 1A7
- track 2 Calf 54X day 95 immunised with live virulent sporozoites
- track 3 Calf L37 day 91 immunised with irradiated sporozoites (640 Gy)
- track 4 Calf 102 day 43 immunised with irradiated sporozoites (840 Gy)
- track 5 Calf N44 day 63 immunised with irradiated sporozoites (500 Gy)
- track 6 Calf N44 day 92 challenged with live sporozoites
- track 7 Calf L21 day 104 challenged with live sporozoites
- track 8 Calf L21 day 49 immunised with irradiated sporozoites (320 Gy)
- track 9 Calf L21 day 0

Figure 32 T. annulata Ankara sporozoite antigens recognised by sera from rabbits fed on by infected (rabbit 28) or uninfected (rabbit 8) Hyalomma ticks.

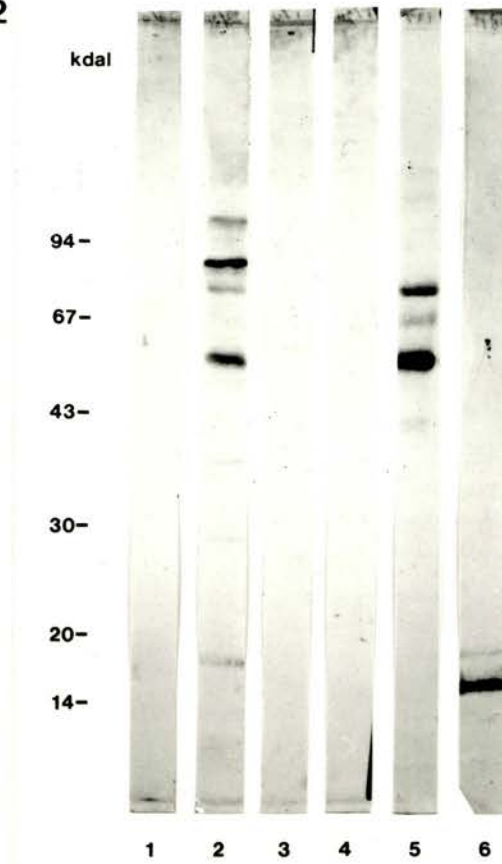
Western blots of Percoll-purified T. annulata Ankara sporozoite proteins reacted with antibodies:

- track 1 rabbit 28 day 0
- track 2 rabbit 28 day 73
- track 3 rabbit 8 day 0
- track 4 rabbit 8 day 71
- track 5 antisporezoite Mab 1A7
- track 6 antisporezoite Mab 4B11

31



32



These blots show that there was variation in the position and relative intensity of the four sporozoite protein doublets detected by Mab 1A7 and in one blot the high molecular weight (104 kdal) protein could be detected in Percoll-purified sporozoites (Figure 31, track 1). In spite of this, the general pattern of reactivity was consistent. The variations were believed to be due to differences between Percoll preparations in maturity and purity of the sporozoites and perhaps also as a consequence of technical factors. They do not cast doubt on the validity of the multiple reaction of 1A7 with sporozoite antigens. When the antigens recognised by calf and rabbit sera are compared with those recognised by Mab 1A7, ant sporozoite serum from rabbit 28 and hyperimmune serum from calf 134X are seen to have detected bands which correlate well with those recognised by 1A7 on the same blot (Figure 32, tracks 2 and 5 and Figure 30, tracks 6 and 10). Serum taken after challenge from calf L21, recipient of 320 Gy irradiated sporozoites (Figure 31, track 7) and hyperimmune sera from calves 54X (Figure 31, track 2), N53 and N61 (Figure 30, tracks 3 and 5) reacted with some but not all of the proteins detected by 1A7.

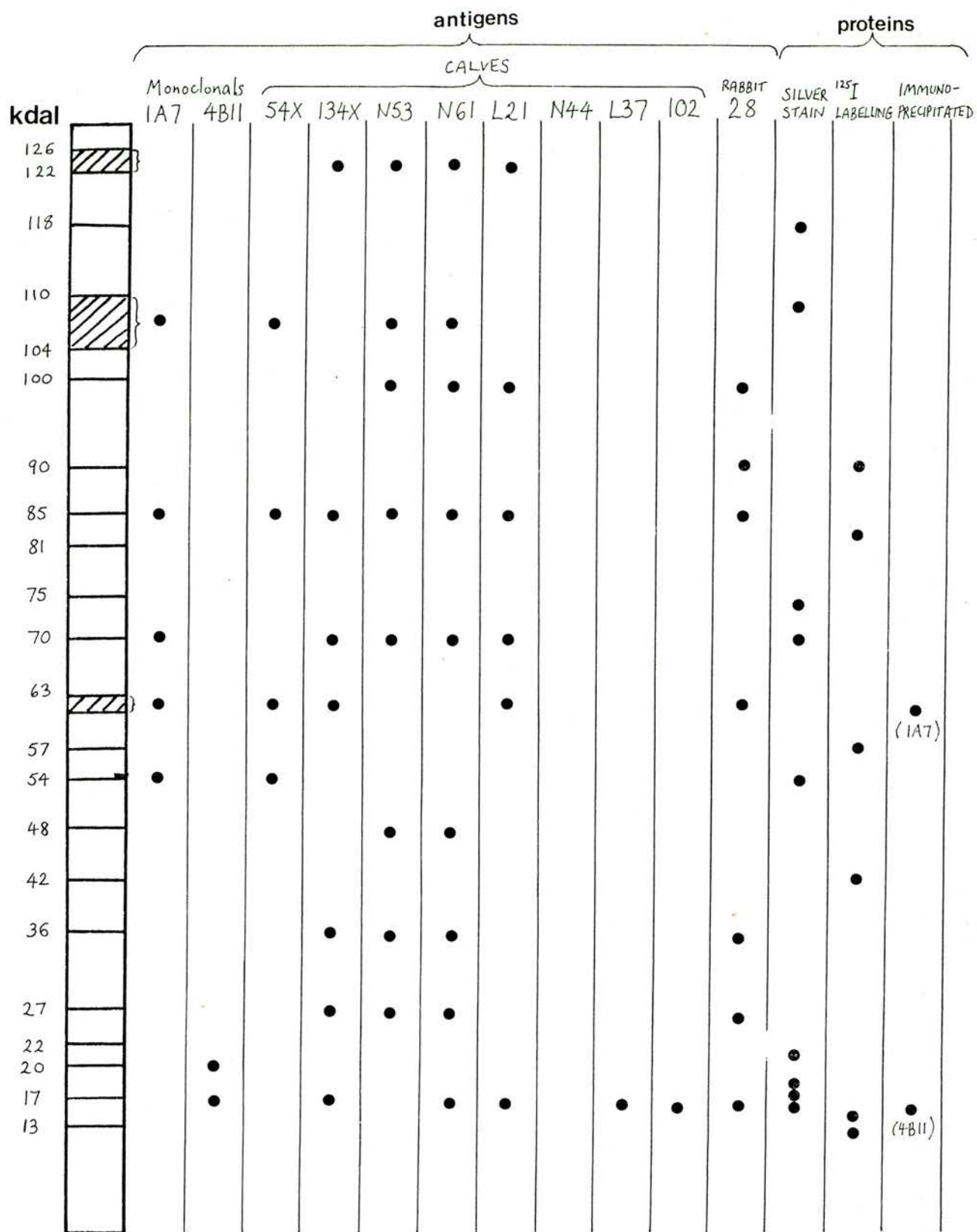
Of the other antigens recognised by the ant sporozoite antisera, summarised in Table 17, those of molecular weight 122-126, 100, 36 and 27 kdal are of particular interest in that they were detected by at least four different immune antisera.

5.3.4. Surface labelling and immunoprecipitation

Five major and three minor proteins were labelled by ^{125}I in sample 1, the sporozoite preparation (Figure 33a, track 1). Their molecular weights are given in Table 18 together with an indication of their origin.

Table 17

Summary of sporozoite proteins and antigens detected.



All sera are postimmunisation

● = band detected

Figure 33 a) 125 Iodine labelled surface proteins of samples 1, 2 and 3 from Percoll gradients of GUTS filtrate.

Autoradiograph of proteins separated by SDS-PAGE

- track 1 sample 1 from 1.07/1.095 s.g. interface of infected Percoll gradient
- track 2 sample 2 from 1.03/1.05 s.g. interface of infected Percoll gradient
- track 3 sample 3 from 1.03/1.05 s.g. interface of uninfected Percoll gradient.

b) Immunoprecipitation of radiolabelled proteins by antisporezoite and control monoclonal antibodies.

Autoradiography of proteins separated by SDS-PAGE after immunoprecipitation by:

<u>Track</u>	<u>Antibody</u>	<u>Antigen</u>
1	anti <u>T. saginata</u> Mab	} - Sample 1
2	normal mouse serum	
3	antisporezoite Mab 1A7	
4	antisporezoite Mab 4B11	
5		} - Sample 2
6	as above	
7		
8		} - Sample 3
9		
10	as above	
11		
12		

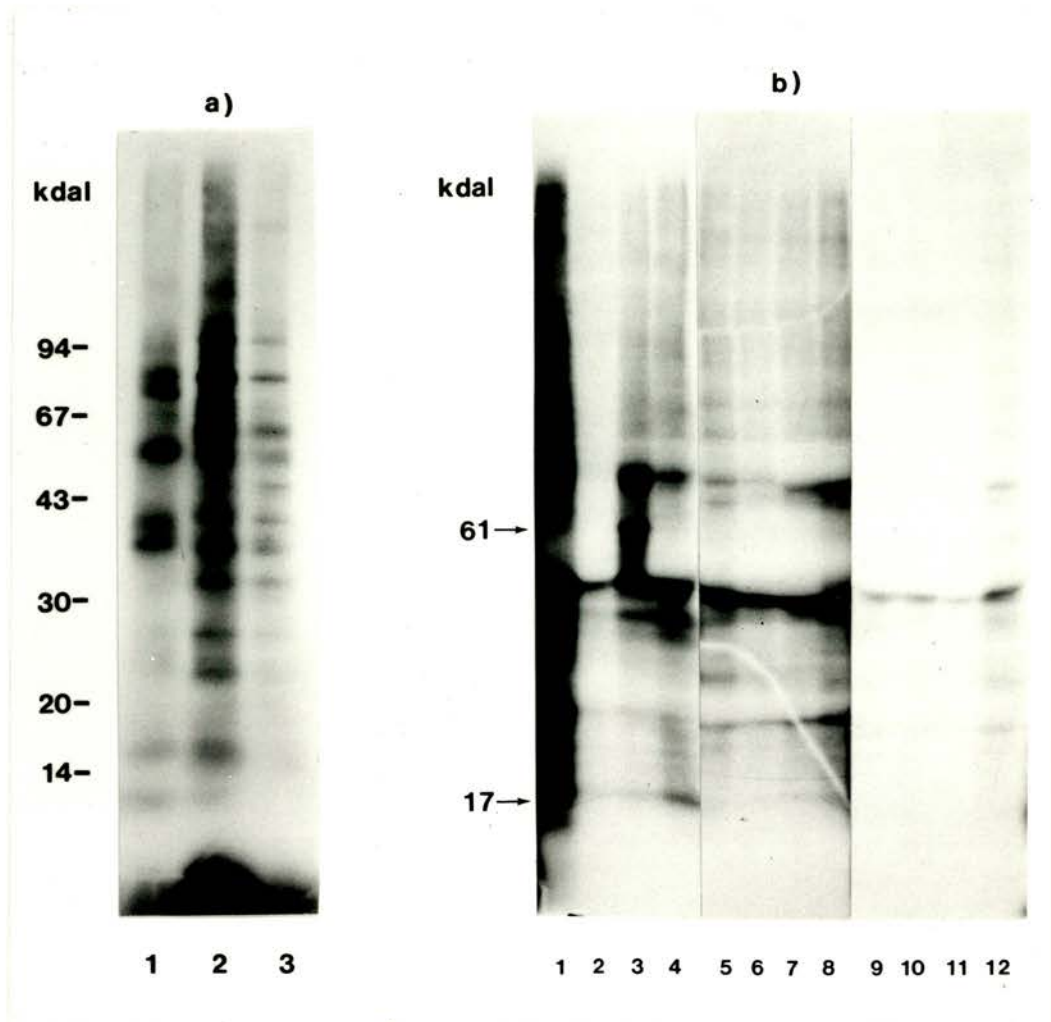


Table 18 Molecular weights and likely origin of radiolabelled proteins in Percoll preparations (Figure 33a)

Molecular weights (kdal) of proteins in sample 1	Present in samples		Likely origin
	2	3	
(90)	-	-	sporozoite
81	+	+	tick
75	-	-	sporozoite
57	-?	-?	sporozoite?
42	-?	-?	sporozoite?
38	+	+	tick
(15)	+	-	sporozoite/sporoblast
(13)	+	-	sporozoite/sporoblast

() = minor; + = present; - = absent

The efficiency of radioiodination by the iodogen method was measured by TCA precipitation. The percentage of radioactivity due to labelled protein in each sample was:

<u>Sample</u>	<u>Content</u> (predominant)	<u>%</u>
1	Sporozoite proteins	81
2	Sporozoite, sporoblast and tick proteins	74
3	Tick proteins	68

Immunoprecipitation of the radiolabelled proteins showed a number of proteins to be non-specifically precipitated even by controls (anti-Taenia saginata Mab and normal mouse serum, Figure 33b, tracks 1 and 2). However Mab 1A7 can be seen to have specifically precipitated a protein of 61 kdal from sporozoite material (Figure 33b, track 3). This may correspond to the 57 kdal radiolabelled protein

observed in track 1, Figure 33a. Mab 4B11 precipitated a protein of around 17 kdal in both sporozoite (Figure 33b, track 4) and impure sporoblast/sporozoite material (Figure 33b, track 8). This precipitated protein probably corresponds to the 15 kdal radio-labelled protein observed in tracks 1 and 2, Figure 33a.

5.4. DISCUSSION

In the studies described it was possible to identify sporozoite specific proteins in both lysates of whole sporozoites and surface labelled sporozoite preparations. Certain of these sporozoite proteins were specifically identified by Mab 4B11 and 1A7 in both types of preparation. Sporozoite proteins were shown to be recognised by antisporezoite rabbit serum and certain immune bovine sera, some of which identified proteins of similar molecular weights to those detected by the two Mabs.

Nine sporozoite specific proteins were visible on a silver stained polyacrylamide gel on which whole sporozoites had been separated, while between four and six proteins were radiolabelled on the sporozoite surface. The molecular weights of sporozoite proteins detected by these two methods do not correlate exactly. One reason for this may be that the purified sporozoite preparation contained a number of tick proteins which appeared to bind to the sporozoite surface in the same manner as observed for BPA. These tick contaminants probably obscured the visualisation of some sporozoite proteins while other sporozoite proteins may have been present in small amounts and remained undetected even by silver staining. Proteins which possessed similar but not identical weights as assessed

by the two methods (e.g. the 54 kdal silver stained protein and the 57 kdal radiolabelled protein, see Table 17) may actually represent the same sporozoite protein. The estimations of molecular weight of a single protein on different occasions may give slightly different results. This should be borne in mind for all the results obtained and is a good reason for comparisons between antibody reactions to be made only between results obtained on the same blot. Fish (1975) details a number of technical causes of variation and inaccuracy in molecular weight estimation by SDS-PAGE and suggests that the SDS itself can introduce variability if different commercial preparations of the chemical are used.

The silver stained proteins of the infected and uninfected GUTS were predominantly tick and rabbit (from the tick's blood meal) in origin and obscured any sporozoite proteins which may have been present.

No differences could be observed between the proteins of Percoll-purified sporozoites from different T. annulata stocks separated by SDS-PAGE and silver stained. This ties in with the close similarity of the reaction of Mabs 1A7 and 4B11 with different stocks.

The Western blot results show that the epitopes recognised by the two ant sporozoite Mabs, 1A7 and 4B11 were different from one another, were both present on sporozoite proteins, and were absent from uninfected material. Both Mabs recognised antigenic determinants common to all three stocks of T. annulata.

This correlates with the cross-reactivity between T. annulata stocks when the monoclonals were assessed by IFAT and suggests that there are antigens present on the surface of the sporozoite which may

be cross-protective. This possibility has been found to exist for T. parva also and was demonstrated by cross-reactivity in the IFAT and by neutralisation of the infectivity of sporozoites from different stocks (Dobbelaere et al., 1984; Musoke et al., 1984). The major 17 kdal antigen recognised by Mab 4B11 (Figure 25) probably corresponds to protein 9 on silver stained gels (Figure 21) and to the 15 kdal radiolabelled sporozoite protein (Figure 33). The latter is further evidence that 4B11 is directed against a sporozoite surface protein. The 4B11 17 kdal antigen was recognised by certain antisera. It appears to have been the only protein detected by post-immunisation serum from calves L37 (640 Gy sporozoites) and 102 (840 Gy sporozoites, Figure 31). Calf 102 was fully susceptible to challenge with virulent sporozoites while calf L37 underwent a mild reaction and recovered. Thus, presence of antibody to the 4B11 antigen did not in this instance necessarily indicate immune status although it was an antigen recognised by sera from two calves hyperimmune to sporozoites (N61 and 134X, Figure 30) and from rabbit 28 which was immunised by repeated feeding of infected ticks (Figure 32). Nor does presence of antibody to the 4B11 antigen correlate with sporozoite neutralising activity since sera from some calves which exhibited potent sporozoite blocking activity (54X, N53, N44, Figures 30 and 31) failed to recognise this antigen.

Mab 1A7 recognised a more complex antigen than Mab 4B11 and showed a multiple reaction with sporozoites. This indicates that 1A7 recognises an epitope which resides on each member of a group of proteins of different molecular weights. There are several possible explanations of the nature of the proteins bearing the 1A7 epitope which are illustrated in Figure 34:

a) The epitope may reside, and may be repeated, on a protein which undergoes processing as the sporoblast, and then sporozoite, matures. Evidence which supports this explanation includes the detection of a higher molecular weight protein (104 kdal) in infected GUTS. GUTS contains a proportion of the least mature sporozoites and sporoblasts in which the 104 kdal protein may be the precursor of the lower molecular weight proteins. There is evidence that immature sporozoites and sporoblasts express the T. parva sporozoite antigen recognised by monoclonal antibody with sporozoite neutralising activity, at low levels on day 0-2 of the tick feed. From day 3 (sporozoites are harvested on day 4 or 5 from T. parva-infected Rhipicephalus appendiculatus) the antigen, or its precursors, are synthesised at a rapid rate (Dobbelaere et al., 1985a) which coincides with the time when maximal parasite proliferation occurs (Dobbelaere et al., 1983). A similar situation may exist for T. annulata. When infected ticks were removed on day 3, sporoblasts and sporozoites of this parasite may have contained protein bearing the 1A7 epitope at differing degrees of maturity. This has been observed to be the case for a sporozoite surface antigen of Plasmodium knowlesi (P. knowlesi) where the relevant monoclonal antibody recognises three proteins of different molecular weights when P. knowlesi sporozoites are immunoprecipitated (Ellis, Ozaki, Gwadz, Cochrane et al., 1983). The two higher molecular weight proteins were found by immunoelectron microscopy to be intracellular precursors, while the lower molecular weight protein was present on the surface of sporozoites as the mature form of the antigen (Fine, Aikawa, Cochrane and Nussenzweig, 1984). The immunoprecipitation of a single 61 kdal

protein by Mab 1A7 also supports this explanation although this was only a preliminary result. The 61 kdal protein is likely to be the same protein as that represented by the 63 kdal band observed in blots with 1A7. This was, therefore, the mature form of this sporozoite antigen and, as such, was present on the sporozoite surface. Differences in intensity of the protein bands detected by 1A7 between blots might have been due to different levels of sporozoite maturity in different Percoll separations. It is, however, surprising that a blot of sporoblast containing material reacted with 1A7 did not differ more from that obtained with purified sporozoites (Figure 27), and that the 104 kdal protein was not apparent in the sporoblast containing preparation.

b) Since the parasite stocks were not cloned, the possibility exists that the multiple reaction of 1A7 was due to the presence of a mixed population of sporozoites. Sporozoites in each population may bear the epitope on a protein of a different size. If this were the whole explanation, one would not have expected to see such similar patterns of reactivity with 1A7 as was evident between the different stocks (Figure 26) and the higher molecular weight protein observed in GUTS remains unexplained. However, Shapiro *et al.* (1987) and Hall, Williamson, Shiels, Brown and Tait (1988) report that Mabs recognising macroschizonts of *T. parva* and *T. annulata* respectively, react with different sized proteins in different parasite stocks and there may be a similar phenomenon in sporozoites.

c) The different sized proteins detected by 1A7 could represent independent but related gene products within a parasite population

which are expressed together in sporozoites, each containing an amino acid sequence which forms the 1A7 epitope.

d) There may be partial epitope identity between different proteins or additional binding capacity of 1A7 causing cross-reactivity. Ghosh and Campbell (1986) have reviewed multispecificity in monoclonal antibodies and while this is a possible explanation, the fact that there was no reaction with uninfected material and only one surface labelled protein was immunoprecipitated by 1A7 points to this monoclonal being specific.

e) The group of proteins recognised by Mab 1A7 may represent glycoproteins, related only as a result of the attachment of similar, or identical, oligosaccharide chains rather than shared amino acid sequences (Ramasamy and Reese, 1985).

However, the T. parva sporozoite antigen recognised by neutralising Mab has been found to be almost certainly protein in nature, being susceptible to proteolytic but not exoglycosidase digestion (Dobbelaere et al., 1985b).

f) Sporozoites purified for SDS-PAGE were washed and resuspended in the presence of protease inhibitors and were immediately and rapidly solubilised under denaturing conditions, before being separated by SDS-PAGE or stored at -80°C . Under such conditions it was hoped that the action of proteases could be discounted as a reason for the multiple bands detected by Mab 1A7.

Additional experiments would be required to distinguish the true reason for the multiple reactivity of Mab 1A7 with sporozoite proteins. Information derived from work described later in this thesis helps to indicate the most likely explanation.

Figure 34 Possible explanations for multiple reaction of Monoclonal 1A7 with sporozoite proteins.

Key:







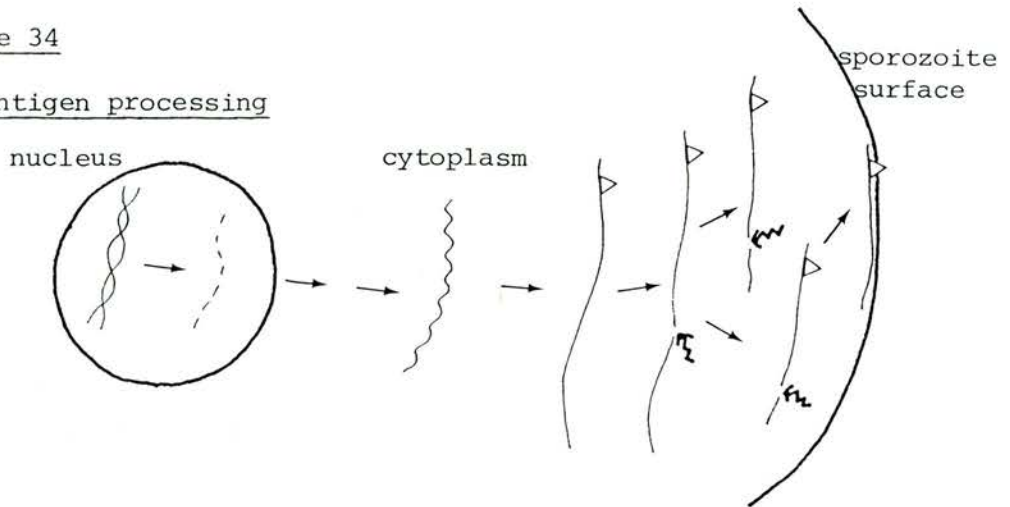
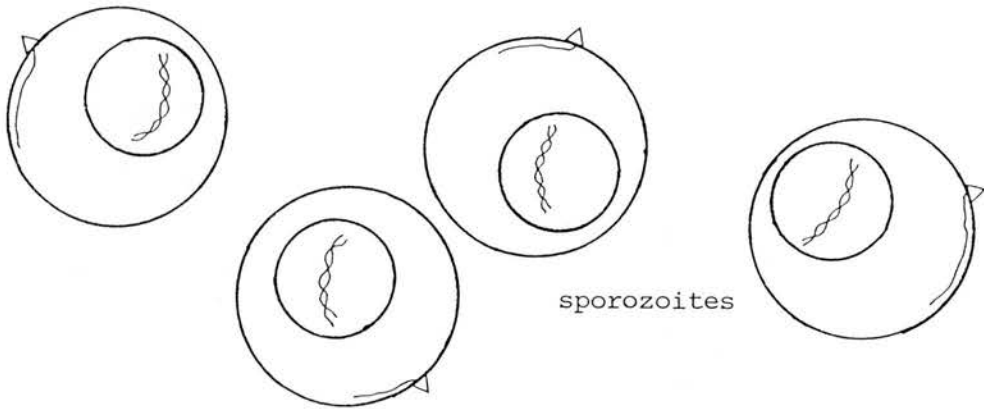
DNA	
complementary DNA	
messenger RNA	
protein	
Mab 1A7 epitope	
processing	

Figure 34

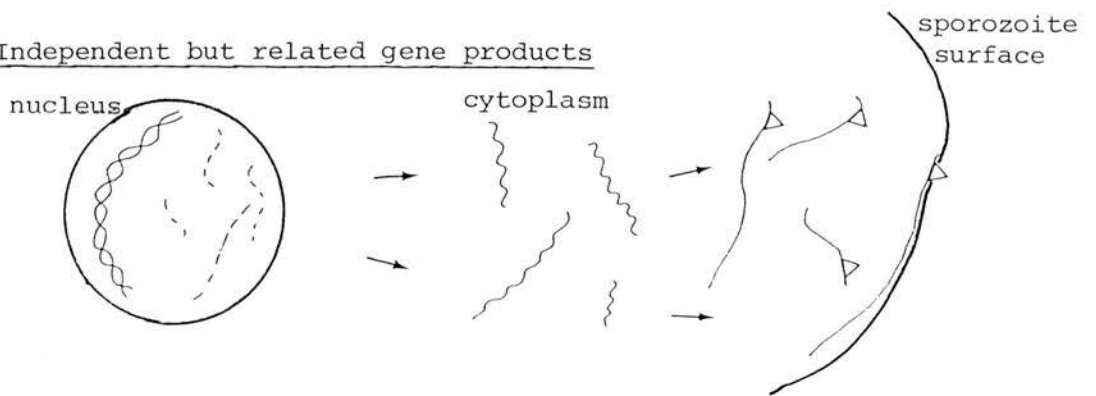
(a) Antigen processing



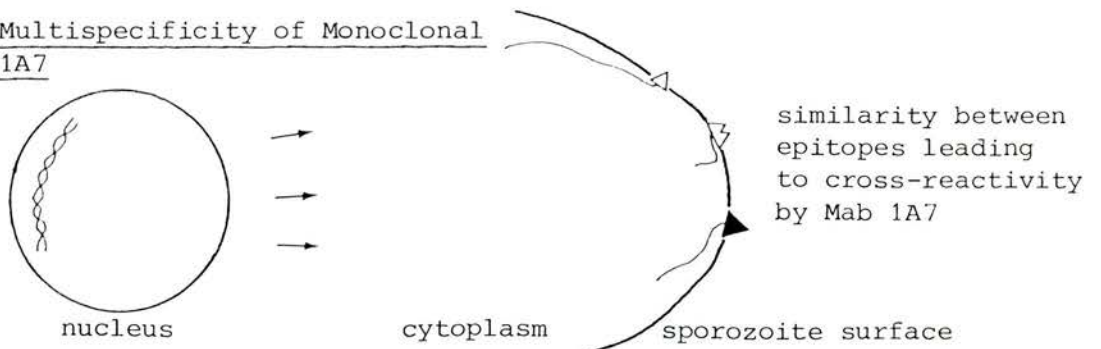
(b) Uncloned parasite population



(c) Independent but related gene products



(d) Multispecificity of Monoclonal 1A7



The iodogen method was chosen for radioiodination of the surface molecules of the sporozoite because it is relatively rapid and simple. In addition, there is no requirement for extraneous protein or damaging chemicals such as hydrogen peroxide or reducing agents, as when the lactoperoxidase technique is employed (Howard et al., 1982). In addition, the iodogen is a large, sparingly water-soluble molecule (Zingales, 1984) which acts to catalyse the radioiodination of tyrosyl residues of proteins from its position on the inner surface of the reaction vessel. Therefore, over the short period (10 minutes) for which the iodogen was in contact with the sporozoites, labelling of surface molecules should have occurred with minimal penetration and labelling of cytoplasmic proteins (Howard et al., 1982). The iodogen method achieved a good level of radioiodination with at least 68% of the radioactivity present being due to labelled protein.

The proteins immunoprecipitated by Mabs 1A7 and 4B11 have been discussed above. Dr. B. Shiels (personal communication) detected three sporozoite proteins of different sizes with a single anti-sporozoite monoclonal antibody, 4E5, by surface labelling and immunoprecipitation. These three proteins were of a higher molecular weight than those detected by 1A7 but suggest that the sporozoite may be composed of groups of immunologically related molecules. Similarly, Iams et al. (1988) provide evidence that the same is true for the proteins of T. parva sporozoites. They show that the reaction of ant sporozoite bovine serum with sporozoites in Western blots is the result of antibodies identifying several groups of proteins, all members within a group containing a similar amino acid sequence within their structure.

The 1A7 epitope was recognised by serum from calf 134X, hyper-immune to sporozoites (Figure 30), and serum from rabbit 28, exposed to infected ticks (Figure 32). Sera taken after challenge from calf L21, recipient of 320 Gy irradiated sporozoites, and hyperimmune sera from calves 54X, N53 and N61, all immunised with virulent sporozoites, are more difficult to assess with respect to their reaction with the epitope recognised by 1A7. They reacted with some, but not all, of the proteins detected by 1A7. Whether this means that they were detecting different epitopes which happen to reside on the same proteins or whether these sera did contain antibody to the 1A7 epitope but exhibited variable affinity for the epitope depending on the particular protein on which it is located, is not clear.

What is clear is that the antibody reactions between calves immunised in a similar manner may differ markedly; 54X and 134X show different reactions from those of N53 and N61 as well as differing from each other. Except for calf L21, calves immunised with irradiated sporozoites showed little or no reaction with sporozoites by Western blotting. This lack of reactivity may be due to the Western blot technique or to low antibody affinity or simply to the absence of adequate ant sporozoite antibody, in spite of the fact that calf N44 developed an ant sporozoite IFAT titre of $1/160$ after immunisation. Assuming that the method of antibody detection was not at fault, it seems that immunisation with irradiated sporozoites is a much less effective method of raising ant sporozoite antibodies than the use of live virulent sporozoites.

The small numbers of calves in these experiments make it difficult to be very conclusive, not only about the irradiated sporozoite

experiments, but also about the reaction of different hyperimmune sera with sporozoite proteins. Regarding the latter, it seems that there is variation between calves' responses when exposed to the same sporozoites. In view of the outbred genetic background of the calves, they were likely to be of different BoLA (bovine MHC) types (Spooner, Oliver, Sales, McCoubrey et al., 1979). When compared to, for example, inbred laboratory mice in which the H2 (murine MHC) type is much more confined, it is perhaps not surprising that the potential of calves to respond to a particular antigenic determinant could prove less predictable.

The observation that the epitopes recognised by 1A7 or 4B11 may not be consistently recognised by immune bovine sera does not preclude their development as potential protective proteins. The presentation of one or both of these antigens in an appropriate manner, purified and in adequate amounts, in excess of the quantity of the antigen which would be presented in sporozoites, could elicit a sporozoite neutralising response. If this response could be shown to play a role in protection, a viable alternative to vaccination with live parasite would be available. In order to be able to test this possibility, large quantities of the epitopes recognised by 1A7 and 4B11 were required. Therefore work was initiated to obtain one or both of the epitopes as a recombinant protein. This is described in the next chapter.

CHAPTER SIX

SELECTION AND ANALYSIS OF RECOMBINANTS

EXPRESSING A SPOROZOITE EPITOPE

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6.1. INTRODUCTION

The application of antibody reagents and an in vitro screening assay to identify sporozoite antigens which are, or could be, targets for a protective immune response has been described in earlier chapters. This led to the selection of two ant sporozoite monoclonal antibodies, 4B11 and 1A7 with sporozoite neutralising activity and the characterisation of the epitopes they recognise. While information is only available from an in vitro sporozoite neutralisation assay, the relevance of these sporozoite epitopes to the stimulation of protective immunity in the bovine host remains a matter for speculation. Therefore, it was considered important to direct work towards the production of sufficient material bearing the sporozoite epitopes to use in immunisation trials in cattle.

Two alternatives were available by which to obtain such immunising material. One was to purify the relevant antigens directly from sporozoites. This approach has been used to isolate antigens from Babesia bovis (Wright, Mirre, Rode-Bramanis, Chamberlain et al., 1985) and Anaplasma marginale (Palmer, Barbet, Davis and McGuire, 1986) to test in cattle. The limitations of this approach are mainly concerned with the large number of infected ticks that would be required to provide sufficient sporozoites from which to purify antigens. The nature of the 1A7 antigen(s) would also tend to complicate this approach. Since several proteins bear the relevant epitope a decision would have to be made whether to extract all these proteins or just one representative. The stability of the sporozoite antigens is not known although they appear to be susceptible to proteases. Obviously protein degradation would further decrease antigen yield.

A second alternative by which to obtain proteins in quantity has become available with the development of techniques in gene manipulation. This alternative is the use of DNA recombinant technology to clone and express the theilerial DNA coding for the sporozoite epitope. The recombinant proteins expressed are then available in large amounts for testing in cattle.

The use of lambda phage gt11 (λ gt11) as a cloning vector for DNA fragments and the expression of these fragments in E. coli (Young and David, 1983a and b) has been reviewed already. A λ gt11 expression library constructed using genomic DNA from T. annulata (Dr. F.R. Hall, personal communication) was available for use. Such libraries expressing recombinant parasite antigens have been screened with monoclonal (Dame et al., 1984) or polyclonal (Kemp et al., 1983; Lanar et al., 1985; Gill et al., 1987) antibodies. The value of selecting recombinants by immunological screening of the expressed proteins over screening by nucleic acid hybridisation is two-fold. The antibody reagents are readily available and, by screening for the desired end product, selected recombinants are known to contain the parasite DNA insert in frame and in the correct orientation. Also, the amino acid sequence expressed must have retained epitope identity and reactivity with the screening antibody.

Both complementary DNA (cDNA, Enea et al., 1984) and genomic DNA (Dame et al., 1984) have been used to prepare expression libraries from which Plasmodium falciparum sporozoite genes have been cloned. A cDNA library has the advantage of being parasite stage specific since it only represents the DNA which is expressed in the life cycle stage from which it was prepared. This biases the library towards

expression of stage specific antigens and so increases the chances of cloning such an antigen gene. However, the same property diminishes the general value of a cDNA library, as a separate library would be required for each parasite stage being studied. In view of this constraint and the consideration that it might be difficult to obtain adequate amounts of messenger RNA from sporozoites to make a cDNA library, a genomic expression library of T. annulata was screened with the ant sporozoite Mabs, 4B11 and 1A7.

The prime objective of the work described in this chapter was to isolate recombinants containing theilerial DNA sequences coding for the sporozoite epitopes recognised by ant sporozoite monoclonals 4B11 and 1A7. In doing this, a thorough analysis was made of the nature of the recombinants obtained in order to define them and to ensure their validity. This analysis is described.

It is of interest both in relation to the production of immunising material and also in furthering the understanding of the DNA encoding the sporozoite epitope, the RNA transcribed from it and the polypeptide translation products.

6.2. MATERIALS AND METHODS

The majority of methods used followed established protocols detailed by Maniatis, Fritsch and Sambrook (1982) and Huynh, Young and Davis (1985) with modifications or additions as described below.

6.2.1. Equipment, buffers and solutions

Any stock solutions or buffers not detailed in the text are listed in Appendix I.4.

Glassware and other equipment was sterilised as described in Section 2.1.2. except when being used for work with RNA. In RNA experiments, all glassware and equipment was baked in a hot air oven (180°C, 3 hours). Where baking was not feasible, the equipment was soaked in purified water containing 0.1% diethylpyrocarbonate (DEP, Sigma) then autoclaved. Solutions and buffers were sterilised by autoclaving (15 p.s.i., 15 minutes) or filtration (0.22 μ m, Millipore). For RNA experiments, solutions and buffers were made up using DEP treated purified water and autoclaved.

6.2.2. Bacterial strains and cloning vectors

a) Bacterial strains

i) Y1090: A restriction negative, ampicillin resistant strain of E. coli described by Young and Davis (1983b) was used as the host cell for λ gt11 for screening and purifying recombinant phage.

ii) Y1089: An ampicillin resistant strain of E. coli was used to prepare lysogens of wild type λ gt11 and recombinant λ gt11 clones. Y1089 was particularly suitable for generating lysogens because it contains a deficiency in the lon protease which increases the stability of the recombinant fusion protein. It also has a mutation (hfl A 150) which enhances the frequency of phage lysogeny (Young and Davis, 1983b; Huynh et al., 1985).

iii) JM 83: An E. coli K-12 strain developed by Messing (1979) was used as the host cell for transformation by the plasmid vector, puC 18.

b) Cloning vectors

i) Lambda phage gt11: Young and Davis (1983a) detail the construction of λ gt11 which contains the lac Z gene and a unique EcoR1 site.

ii) Plasmid puC 18: Norrander, Kempe and Messing (1983) describe the construction of this bacterial plasmid which also contains the lac Z gene and a single EcoR1 site.

c) Storage

Bacterial host strains and plasmid transformed cells taken from the logarithmic phase of growth were mixed with an equal volume of sterile glycerol. These were stored at -80°C . Short term storage of Y1090 cells was at 4°C in 10 mM magnesium sulphate. Recombinant phage were stored in phage medium (SM) saturated with chloroform at 4°C . For long term storage at -80°C , phage were snapfrozen while in SM containing 7% dimethylsulphoxide (DMSO).

6.2.3. Genomic library of *T. annulata*

The genomic library screened was prepared by Dr. Roger Hall of the Wellcome Unit for Molecular Parasitology, University of Glasgow. The library was made from genomic DNA isolated from purified piroplasms of *T. annulata* Hissar. DNA was mechanically sheared to obtain random fragments of approximately 300 basepairs (bp) to four kilobases (kb) in size. The DNA was methylated with EcoR1 methylase to protect it from restriction endonuclease digestion. EcoR1 linkers were then ligated to the theilerial DNA and digested with EcoR1. These DNA fragments (representing approximately 0.2 μg theilerial DNA) were

ligated using T4 DNA ligase into the EcoR1 site of λ gt11. Phage DNA was packed in vitro into phage particles and the presence of 70% recombinant plaques was detected by their lack of B-galactosidase activity (Miller, 1972). This was confirmed by the presence of DNA inserts on EcoR1 digestion.

6.2.4. Genomic library screening

The enzyme linked immunoassay method used essentially followed a protocol described by Young, Bloom, Grossinsky, Ivanyi, Thomas and Davis (1985).

a) Unamplified library screening

Y1090 cells were grown to mid-logarithmic phase (optical density 0.5 at 600 nm wavelength) in LB medium containing 100 μ g/ml ampicillin, 10 mM magnesium sulphate (MgSO_4) and 0.2% maltose (all from Sigma). The cells were pelleted by centrifugation at 1,500 G for 10 minutes at 4°C and resuspended in one tenth of the original culture volume of 10 mM MgSO_4 .

Four $\times 10^4$ phage particles from the unamplified library were mixed with 0.9 ml Y1090 cells in four replicate tubes and incubated at 42°C for 20 minutes. To each tube, 50 ml of liquid (37°C) top agar containing 10 mM MgSO_4 and 100 μ g/ml ampicillin were added. After mixing gently each was poured onto the dry, level surface of a 24 x 24 cm tray containing bottom agar. Once the top agar had set the four plates were incubated at 42°C for four hours until phage plaques were clearly visible. Nitrocellulose paper filters previously soaked for two hours in 10 mM isopropyl B-D-thiogalactopyranoside

(IPTG, Sigma), dried and labelled, were overlaid onto the plates. The plates were incubated for a further two hours at 37°C. Then the filters were carefully removed and rinsed briefly in Tris/saline buffer (TS) pH 8.0 before being blocked for one hour using 5% ovalbumin (Sigma) in TS. Meanwhile the agar plates were stored at 4°C. The filters were incubated overnight at 20°C in a mixture of the two antisporozoite monoclonal antibodies, either as ascites diluted 1 in 200 in TS/5% ovalbumin containing 0.01% sodium azide (Sigma) or as undiluted culture supernatant. Next, filters were worked in four changes of TS/0.05% Tween 80 and two changes of TS, for five minutes each wash. Second antibody (sheep anti-mouse immunoglobulin, SAPU) conjugated to horseradish peroxidase was used at a 1:300 dilution in TS/5% ovalbumin and filters were incubated in this for two hours at 20°C. They were then washed in the same manner as described previously. Finally, to visualise any recombinant phage plaques binding monoclonal antibody, peroxidase substrate solution was added to the filters. After colour had developed, filters were washed in two changes of purified water and allowed to dry. Positively staining plaques were distinguished from background negative plaques by the darker brown colour they developed. Using the filter as a template guide, the plaques on the agar plates corresponding to the positive plaques on the filters were removed using the butt end of a sterile Pasteur pipette and placed in 1 ml SM containing 25 µl chloroform (BDH) for storage at 4°C.

b) Cloning positive phage

The phage plaques which had been picked were rescreened three times until pure phage clones were obtained which gave positive reactions with Mab 1A7. To do this, 100 μ l SM containing approximately 10^3 phage particles was mixed with 100 μ l Y1090 cells, incubated at 42°C for 20 minutes and plated onto circulate agar plates (Petri dishes, Sterilin, 8.0 cm diameter) using 2.5 ml top agar. The plaque immunoassay then followed the protocol described above. Once cloned phage were available, they were screened with a panel of anti-theilerial monoclonal antibodies.

6.2.5. DNA extraction methods

a) Parasite DNA

To avoid mechanical shearing of parasite DNA during extraction all mixing was carried out by gentle tube rotation and pipette ends were cut off to give a wide bore opening.

i) Piroplasm: A number of different methods were used to extract and purify DNA from piroplasms of Theileria. The method described here is simple but was found to yield high molecular weight DNA of adequate purity for restriction endonuclease digestions and Southern blotting.

A 0.6 ml pellet of theilerial piroplasms purified from red and white bovine blood cells as described in Section 2.2.10. was resuspended in 8.9 ml TE pH 7.6. SDS was added to 1% and the mixture was slowly homogenised with three strokes of the homogeniser. The volume of the homogenate was made up to 50 ml with TE and mixed gently. An equal volume of phenol was added and mixed for 20 minutes

followed by centrifugation at 20°C, 8,000 G (Sorvall Superspeed RC-2, Dupont Instruments) for 15 minutes. The aqueous phase was removed and mixed with an equal volume of phenol:chloroform (2:1) for 20 minutes and recentrifuged as above. This time the aqueous phase was mixed with an equal volume of chloroform for five minutes and centrifuged at 15°C, 2,000 G for 10 minutes. The final volume of the aqueous phase was measured after its removal to a clean tube and 4 M sodium chloride solution was added to a final concentration of 0.1 M. Two and a half volumes of ice-cold 100% ethanol were immediately added and mixed by swirling. Using a Pasteur pipette the precipitated DNA was spooled out of the ethanol into a glass centrifuge tube and washed in 70% ethanol. The DNA was dried, redissolved in 5 ml TE pH 7.6 and stored at 4°C.

ii) Macroschizont-infected lymphoblastoid cell lines: Cells from 50 ml of T. annulata Hissar and Ankara infected cell lines in the logarithmic phase of growth were pelleted by centrifugation at 800 G, 4°C for 15 minutes. Three times the pellet volume of lysis buffer (100 mM Tris, 240 mM potassium chloride, 35 mM magnesium chloride) was added to each and they were resuspended by gentle vortexing. Twenty percent SDS was added to a final concentration of 4% and the cell lysate was mixed with the end of a pipette. Four ml TE pH 7.6 was added to each and they were left mixing for 30 minutes. Phenol/chloroform extraction and ethanol precipitation were carried out as above.

b) Recombinant phage

One hundred μl SM containing approximately 10^5 phage particles were incubated with 100 μl Y1090 cells (prepared as described for genomic library screening) for 20 minutes at 42°C . 2.5 ml top agarose was added and the cells plated onto LB medium solidified with 1% agarose and incubated overnight at 42°C to obtain confluent lysis of the bacterial cells by the phage. SM (5 ml per plate) was added and the plates left shaking at 20°C for two hours. The SM containing phage was removed and centrifuged at 8,000 G, 10 minutes, 4°C (Highspeed 18 MSE). RNase A (DNase free) and DNase 1 were added to the decanted supernatant to a concentration of 1 $\mu\text{g}/\text{ml}$ and the solution was incubated at 37°C for 30 minutes. An equal volume of SM containing 20% polyethylene glycol (molecular weight 6,000) and 2 M sodium chloride was added and, after mixing, allowed to stand for one hour at 0°C . To recover the precipitated phage, the solution was centrifuged at 10,000 G, 20 minutes, 4°C . The supernatant was discarded and the drained pellet resuspended in 0.5 ml SM by vortexing. Having transferred the solution to an Eppendorf tube, 5 μl of 10% SDS and 5 μl of 0.5 M EDTA pH 8.0 were added and incubation carried out at 68°C for 15 minutes. The solution was extracted twice with phenol:chloroform (1:1) and once with chloroform, retaining the aqueous phase each time. To the last aqueous phase, an equal volume of isopropanol (May and Baker) was added. The mixture was stored at -70°C for 20 minutes, thawed and centrifuged for 15 minutes at 15,000 G. The resulting pellet was chained and washed with 70% ice-cold ethanol and then dried. Fifty μl TE pH 8.0 was added to redissolve the pellet which was then stored at -20°C .

c) Recombinant plasmid

The alkaline lysis method of DNA extraction was used (Maniatis et al., 1982).

i) Small-scale: The cells from 1.5 ml of an overnight plasmid culture (37°C in LB medium containing 100 µg/ml ampicillin) were pelleted by centrifugation. One hundred µl of ice-cold 50 mM glucose, 10 mM EDTA, 25 mM Tris Cl pH 8.0 (Solution 1) was added and the pellet was resuspended by vortexing. After five minutes at 20°C, 200 µl 0.2 N sodium hydroxide, 1% SDS (Solution 2) was added and the mixture was gently mixed by inversion. After five minutes at 0°C, 150 µl ice-cold 5 M potassium acetate was added and the sample was incubated for five minutes at 0°C. After centrifugation at 10,000 G for five minutes, the supernatant was extracted with an equal volume of phenol: chloroform (1:1) and the phases were separated by centrifugation for two minutes at 10,000 G. The aqueous phase was precipitated with two volumes of ethanol, then washed and dried as above. Fifty µl TE pH 8.0 containing DNase free pancreatic RNase A (20 µg/ml) was added and the pellet was resuspended by vortexing for storage at -20°C.

ii) Large-scale: To obtain large amounts of recombinant plasmid, amplification in rich medium was carried out. Twenty five ml LB medium containing 100 µg/ml ampicillin was inoculated with 0.1 ml of a plasmid culture grown overnight. This culture was grown at 37°C to late logarithmic phase (OD 0.6 at 500 nm) and was then used to inoculate 500 ml prewarmed (37°C) LB medium containing ampicillin. After exactly 2½ hours growth at 37°C, 2.5 ml chloramphenicol (34 mg/ml) were added to give a working concentration of 170 µg/ml. This

culture was incubated at 37°C for 16 hours and then cells were harvested by centrifugation at 2,000 G, 4°C for 10 minutes. The pellet was resuspended in 100 ml ice-cold STE and pelleted again as above. The pellet was resuspended in 10 ml solution 1 (see above) containing 5 mg/ml lysozyme (Sigma) and left to stand for five minutes at 20°C. Twenty ml fresh solution 2 was added and mixed by gentle inversion then left to stand for 10 minutes on ice. To this, 15 ml of ice-cold 5 M potassium acetate was added and mixed by inverting sharply, then left on ice for 10 minutes. The mixture was centrifuged at 12,000 G, 4°C for 30 minutes and the supernatant obtained was mixed with 0.6 volumes of isopropanol. After 15 minutes at 20°C, this was centrifuged at 12,000 G, 20°C for 30 minutes and the supernatant discarded. The pellet was washed with 70% ethanol at room temperature then dried and redissolved in 8.0 ml TE pH 8.0.

The purification of closed circular DNA was achieved by centrifugation to equilibrium in caesium chloride - ethidium bromide gradients. One g of Caesium chloride (Boehringer Mannheim) was dissolved into each ml solution. In order to stain the DNA, 0.8 ml of a 10 mg/ml solution of ethidium bromide (Sigma) was added to each 10 ml of solution and mixed well. The tubes were kept covered with foil. Centrifugation to equilibrium was performed in an MSE Superspeed 65 Ultracentrifuge using a fixed rotor (MFT 70.10 43114-125) for 36 hours at 45,000 rpm, 20°C.

After centrifugation the gradient was examined by ultraviolet light. The lower of two visible bands was harvested by inserting a 20 gauge, one-inch needle through the tube wall. An equal volume of isoamyl alcohol (Sigma) was added to the harvested DNA and centrifuged

at 1,500 G, 20°C for three minutes. The bottom aqueous phase was retained and re-extracted four times with isoamyl alcohol until the pink colouration of ethidium bromide was no longer visible. The final aqueous phase was dialysed overnight against several changes of TE pH 8.0.

The volume of the dialysed DNA solution was measured and 0.1 volume of 3 M sodium acetate (pH adjusted to 5.2 using glacial acetic acid) was added followed by precipitation with two volumes of 100% ethanol. The precipitate was recovered by centrifugation, washed with 70% ethanol and then dried. The pellet was redissolved in 200 μ l TE pH 8.0 and the DNA was stored at -20°C.

d) Estimation of DNA concentration

DNA concentrations were estimated by measuring the absorbance of the DNA sample at 260 nm using a spectrophotometer (Pye Unicam 8-100), an absorbance of 1 OD representing a concentration of 40 μ g/ml.

6.2.6. Subcloning DNA inserts

The theilerial DNA inserts present in selected recombinant λ gt11 were subcloned into the plasmid vector puC 18.

a) Ligation

For a target:vector ratio of 3:1, 600 ng of recombinant λ gt11 were mixed with 10 ng of plasmid puC 18 after both had been digested with restriction endonuclease EcoR1 (see Section 6.2.7.). The mixture was diluted to 50 μ l using TE pH 8.0, having adjusted the salt concentration to 0.2 M using 4 M sodium chloride. Two and a half volumes

of ice-cold 100% ethanol were added and the DNA mixture was left for two hours at -70°C . Then the precipitated DNA was pelleted by centrifugation at 10,000 G for 10 minutes in a microcentrifuge. The pellet was washed with 70% ethanol and dried well before resuspending in 3.5 μl sterile water, 1 μl x 5 ligase buffer (BRL) and 0.5 μl (0.5 units) T4 DNA ligase enzyme (BRL) and incubated at $12-16^{\circ}\text{C}$ for two hours.

b) Preparation of competent bacteria

JM83 cells were made competent for transformation with plasmid DNA by incubation in calcium chloride. Cells in the logarithmic phase of growth at 37°C at a low cell density (5×10^7 cells/ml), were chilled on ice and pelleted by centrifugation (4,000 G, 5 minutes, 4°C). The cell pellet was resuspended in half the original culture volume of ice-cold 50 mM calcium chloride and 10 mM Tris Cl pH 8.0 and incubated at 0°C for 15 minutes. The cells were pelleted as above and resuspended in $1/15$ of the original culture volume of the same calcium chloride solution, then stored at 4°C for 12-24 hours.

c) Transformation

Two hundred μl of competent JM83 cells were mixed with the ligation reaction mixture and incubated on ice for one hour. They were heat-shocked at 42°C for exactly two minutes and immediately chilled for two minutes. Then, 0.5 ml LB medium (without ampicillin) was added and incubated at 37°C for 30 minutes. Ten μl 1 M IPTG and 50 μl 2% X-gal (5-bromo-4-chloro-3-indolyl B-D-galactopyranoside, Sigma) in N,N-dimethyl formide (Sigma) were added to the cells before plating onto four LB agar plates containing 100 $\mu\text{g}/\text{ml}$ ampicillin.

These were incubated at 37°C for 16 hours. Blue colonies represented cells which had been transformed by non-recombinant plasmid as they were still able to utilise X-gal, the chromogenic substrate for the B-galactosidase enzyme (Miller, 1972). White colonies were selected as containing possible recombinant plasmids.

6.2.7. Restriction endonuclease digestion and agarose gel eletrophoresis

The conditions required for digestion by each restriction endonuclease are detailed in Appendix I.4.10.

In general digestions were carried out in small buffer volumes (less than 30 µl) for five hours, or, occasionally overnight for about 16 hours. Endonucleases were used in excess; 2-10 units per reaction. The DNA contents per reaction (one reaction per agarose gel track) were approximately 1 µg for recombinant phage and plasmid, 2 µg for Theileria piroplasm DNA and 10 µg for Theileria macroschizont infected cell line DNA and bovine DNA (from calf thymus, Sigma). Twenty µg/ml pancreatic RNase A (free of DNases) was added to digestions of phage and plasmid DNA to improve the action of the endonuclease.

Agarose gel electrophoresis was used to separate DNA fragments to analyse the products of endonuclease digestion or prior to Southern blotting or extraction of an insert from the gel. A horizontal slab gel system was used as described by McDonnell, Simon and Studier (1977). In this system good resolution and accurate relative mobilities of DNA are obtained. 0.8-1.0% agarose gels were prepared by melting agarose (Type 1, low EEO, Sigma) in TBE x 1 (Appendix I.4.6.) containing 1 µg/ml ethidium bromide (Sigma).

Samples (10-30 μ l per track) were prepared for electrophoresis by addition of loading buffer (x 6, Appendix I.4.8.). Standard DNA markers (1 kb ladder, BRL), diluted in TE pH 8.0, were prepared in the same way. One μ g standard DNA was used per track except for Southern blotting when 0.02 μ g was used.

Submarine gels were electrophoresed in TBE x 1 containing 1 μ g/ml ethidium bromide.

Electrophoresis of large gels for Southern blotting was carried out for 16-20 hours at 20 mA while gels for the separation of inserts from recombinant phage or plasmid were run at 60 mA, 100 V for 1-1½ hours. Visualisation of DNA bands was possible under ultraviolet light illumination (312 nm, U-V transilluminator, Vilber Lourmat) because of the presence of fluorescent ethidium bromide which intercalates with DNA; as little as 1 ng of DNA can be detected by direct examination of a gel in ultraviolet light (Sharp, Sugden and Sambrook, 1973). DNA size in gels or blots was estimated by reference to DNA markers of known size in a standard 1 kb DNA ladder (BRL) electrophoresed in the same gel as the sample DNA.

6.2.8. Radiolabelling DNA inserts

The inserts subcloned into plasmid pUC 18 were used to probe Southern and Northern blots. They were extracted free of plasmid DNA and labelled using a commercial kit (Polymeraid - RH (TM), P and S Biochemicals) by a technique described by Feinberg and Vogelstein (1983, 1984). EcoRI digested recombinant plasmid DNA was separated using a 0.9% low melting point agarose gel (Sigma). The desired bands were visualised under U-V illumination and removed with a minimum amount of extraneous agarose. The samples were diluted in water (1.5 ml/g agarose) and boiled for seven minutes to denature the

DNA and melt the agarose. After incubating at 37°C for 10 minutes, 25 ng DNA was radiolabelled using 25 μCi ^{32}P phosphorus-dCTP (3000 Ci/mmmole, Amersham International). The reaction was incubated at 20°C for five hours and was stopped by the addition of reaction termination buffer (dCTP, EDTA). Ninety μl STE (Appendix I.4.7.) and 10 μg sonicated salmon sperm DNA (Sigma) as carrier DNA were added and the total sample loaded onto a 1.0 cm high spun column (Biogel P6DG, Buorad, in STE) and centrifuged to separate unincorporated nucleotides from labelled DNA. The column was washed once with 100 μl STE and the radioactivity of the collected sample was measured in a liquid scintillation counter (Packard Tri-carb, 4430). DNA fragments were labelled to an activity of $4-6 \times 10^8$ cpm/ μg DNA.

6.2.9. Southern blot analysis

The technique of Southern blotting was developed by Southern (1975) and allows the identification of specific DNA sequences within a complex mixture of DNA fragments. Fragments separated by agarose gel electrophoresis are transferred to nitrocellulose paper which is then probed with a radioactively labelled defined DNA sequence which will hybridise to homologous sequences.

Following electrophoresis, agarose gels were treated as follows (Appendix I.4.16. for solutions). To depurinate the DNA and so aid the transfer of larger DNA fragments, the gel was soaked in 0.25 M hydrochloric acid for 15 minutes. The DNA was denatured by soaking for 60 minutes in three changes of alkali denaturing solution and then soaked for 60 minutes in three changes of neutralising solution pH 7.5. Transfer of the DNA to nitrocellulose paper or nylon membrane (Hybond-N,

Amersham International) was achieved using the Southern blotting technique detailed by Maniatis et al. (1982). Blotting occurred by the capillary movement of a high salt buffer from the gel to the nitrocellulose paper or nylon membrane over five hours. When transfer was complete the nitrocellulose or nylon blot was dried at 37°C for 20 minutes and then baked in a vacuum oven at 80°C for one hour, to bind the DNA.

The nitrocellulose or nylon blot was prehybridised for 18 hours at 65°C by incubation in Southern blot hybridisation buffer containing 100 µg/ml denatured sonicated salmon sperm DNA to block non-specific binding sites. Hybridisation was carried out by incubating the blot for at least 18 hours at 65°C in the same buffer containing the appropriate ³²P labelled DNA insert. Before addition, labelled probes were denatured by boiling for 10 minutes. Enough labelled probe was added to give a radioactivity of 10⁶ cpm/ml hybridisation buffer and 4 ml buffer was used per 100 cm² of blot. Standard DNA markers (1 kb ladder) radiolabelled in exactly the same manner as the DNA inserts were also added to each blot.

Blots were washed at 65°C for one hour in two changes of 2 x SSC, 0.1% SDS and for one hour in two changes of 0.2 x SSC, 0.1% SDS. At this temperature and at the salt concentrations used during hybridisation and washing, conditions were suitable for the hybridisation of homologous sequences. One blot of T. parva DNA was hybridised at 59°C and the one hour wash in 0.2 x SSC was omitted to provide lower stringency conditions.

When washed, blots were examined for hybridisation by autoradiography using x-ray film (Kodak XOMAT AR5) and fast tungstate

intensifying screens (Ilford), films being exposed at -70°C . Films were developed as described in Section 5.2.8.

6.2.10. Northern blot analysis

Northern blotting is based on the same principle as Southern blotting except that it is RNA which is electrophoresed, transferred to nitrocellulose paper and probed (Thomas, 1980). The salivary gland RNA used for the Northern blot was extracted by Dr. Roger Hall from 100 uninfected and 100 infected (T. annulata Ankara) tick salivary glands from H. a. anatolicum ticks which had been feeding on a rabbit for two days (Walker et al., 1985). Briefly, salivary glands were dissected directly into 4 M guanidium thiocyanate, 50 mM Tris pH 7.5, 10 mM EDTA, 1% B-mercaptoethanol. The glands were homogenised and precipitated with 0.75 volumes of 100% ethanol. The material was pelleted, resuspended in the above solution and reprecipitated with ethanol. The pellet was then homogenised in 50 mM EDTA, phenol, chloroform and the aqueous phase extracted with ether. The RNA was isolated by overnight precipitation on ice in 3 M sodium acetate pH 5.5. RNA concentration was measured using a spectrophotometer as for DNA. For Northern blot analysis the method described by Gehring, Shiels, Northemann, de Bruijn et al. (1987) was followed. Solutions are detailed in Appendix I.4.17. Twenty μg of total RNA was used for each gel track. This RNA was denatured at 55°C for 15 minutes in a solution of 50% formamide (BRL), 2.2 M formaldehyde (BDH), 20 mM MOPS, 5 mM sodium acetate (Sigma), 0.5 mM EDTA, pH 7.0 before being separated in a 1.4% agarose gel containing 0.74% formaldehyde in MOPS buffer. RNA was transferred to a nylon membrane by electroblotting followed by UV fixation.

The Northern blot was treated for 30 minutes in Northern blot hybridisation buffer at 55°C before adding ^{32}P -labelled $\lambda_{\text{gt11-SR1}}$ insert (10^6 cpm/ml buffer). Hybridisation was performed overnight at 55°C. The blot was washed for 60 minutes in four changes of hybridisation buffer at 55°C and exposed to x-ray film as described above.

6.2.11. Generation of lysogens

In order to obtain larger quantities of the recombinant proteins expressed by $\lambda_{\text{gt11-SR1}}$ and $\lambda_{\text{gt11-SR2}}$, recombinant lysogens were prepared in E. coli strain Y1089 as follows.

Y1089 cells were grown to mid-logarithmic phase of growth (OD 0.6 at 600 nm) in LB medium containing 100 $\mu\text{g/ml}$ ampicillin (LBm + amp), 10 mM MgSO_4 , 0.2% maltose, and then harvested by centrifugation at 1,500 G for 10 minutes at room temperature. The cell pellet was resuspended to its original volume in 10 mM MgSO_4 and incubated at 37°C for five minutes. To enhance the chances of lysogeny occurring, cells were mixed with recombinant phage at a ratio of 1:1. Thus in each case, 1 ml of Y1089 cells containing 5×10^8 cells was added to 5×10^8 phage particles and they were incubated together for one hour at 32°C to allow adsorption to occur. Cells were pelleted by centrifugation for 10 minutes at 1,200 G and resuspended in 2 ml LBm + amp containing 10 mM MgSO_4 . After incubating at 32°C for 30 minutes the cells were pelleted again and this time were resuspended in 1 ml 10 mM MgSO_4 to which 5×10^8 λ_{C1} phage particles were added. λ_{C1} is a phage which disregards the hfl mutation and will infect and lyse cells which are not already infected by other

phage. Its inclusion during lysogen generation reduced the number of unwanted non-lysogens growing. λ C1 was kindly provided by Dr. M. Goman.

The cells were incubated with λ C1 at 32°C for another 30 minutes before pelleting once again and resuspending in LBm + amp. The cells were diluted using LBm + amp and spread onto LB agar plates at a density of approximately 200 cells per plate having included λ C1 in the medium at a 1:1 ratio.

Plates were incubated overnight at 32°C.

Single colonies which had grown overnight were streaked onto two replicate agar plates (containing ampicillin), one of which was incubated overnight at 32°C and the other at 42°C, in order to test for temperature sensitivity. At 32°C the temperature sensitive phage repressor is functional, therefore cells growing at 32°C but not at 42°C were assumed to be lysogens.

Lysogens of both recombinant and wild type λ gt11 were generated in this way.

6.2.12. Lysogen colony screening

To check that the recombinant λ gt11 lysogens produced expressed the recombinant protein recognised by Mab 1A7, a rapid colony screen was carried out.

Lysogen clones were grown at 32°C to mid-logarithmic phase from an overnight culture in LBm + amp. Cells were then plated onto agar at a density of 200 cells per plate. Two plates of each lysogen were grown overnight.

For each lysogen, two agar plates were prepared; one with a layer of top agar containing ampicillin (100 µg/ml) and 20 mM IPTG, the other with a layer of top agar containing ampicillin only. Untreated nitrocellulose paper filters were laid on the lysogen colonies and then lifted off and placed, with the colonies uppermost, on the prepared agar plates. One filter for each lysogen was placed on a plate containing 10 mM IPTG, the other filter on a plate without IPTG. Plates containing IPTG were grown at 42°C while the others were grown at 32°C, both for two hours. Then all filters were removed and the colonies lysed in situ by incubating them in chloroform vapour for 15 minutes.

The filters were then treated in exactly the same manner as were the filters used for genomic library screening, in order to react the colonies with Mab's 1A7 and 4B11 and then visualise this reaction.

6.2.13. Recombinant protein analysis

To determine the molecular weights of recombinant proteins produced by the recombinant λ gt11 clones selected, lysogens were used to prepare larger quantities of the proteins for SDS-PAGE and Western blotting.

Two ml of an overnight lysogen culture was added to 8 ml fresh LBm + amp and grown at 32°C to mid-logarithmic phase. The lysogen was then induced by raising the temperature to 42°C and adding IPTG to a 10 mM concentration. After 80 minutes of induction the cells were pelleted by centrifugation at 2,000 G, 30°C for six minutes. A time course induction experiment had been carried out to determine

the optimal time for harvesting the induced lysogens (see Section 6.3.5.). The pellet was resuspended in 0.75 ml PBS containing 1 mM PMSF and 0.25 ml x 4 SDS sample buffer and then lysed by boiling for five minutes. The induced lysates were separated by SDS-PAGE on 7% or 10% gels, 50-100 μ l lysate per gel track. Lysates not used immediately were stored at -20°C .

Transfer of the separated proteins to nitrocellulose paper, reaction with antibody and detection of the reaction were carried out as described previously (Section 5.2.4.). Wild type λ gt11 lysogen was used as a control antigen while antisporezoite monoclonal 4B11 and anti-B-galactosidase serum acted as antibody controls.

As part of the study of the recombinant proteins, the reactivity of lysogens with the antisporezoite antisera used in previous chapters was examined by Western blotting. Again, the techniques used are fully described in Section 5.2.4.

6.3. RESULTS

6.3.1. Genomic library screening

After cloning and rescreening a number of plaques which appeared positive in the initial screen, two recombinant phage clones were purified which reacted positively with Mab 1A7. These were named λ gt11-SR1 and λ gt11-SR2. Both clones gave positive staining plaques with Mab 1A7 but not with 4B11 (Figure 35) or any other anti-theilerial monoclonal antibodies raised against the sporozoite and other stages.

Figure 35 Genomic library screening: detection of a T. annulata sporozoite antigen gene fragment cloned in λ gt11 and expressed in E. coli.

Plaque immunoassay of pure or impure λ gt11-SR1 phage. Plaques gave positive reactions with Mab 1A7 and negative reactions with Mab 4B11.

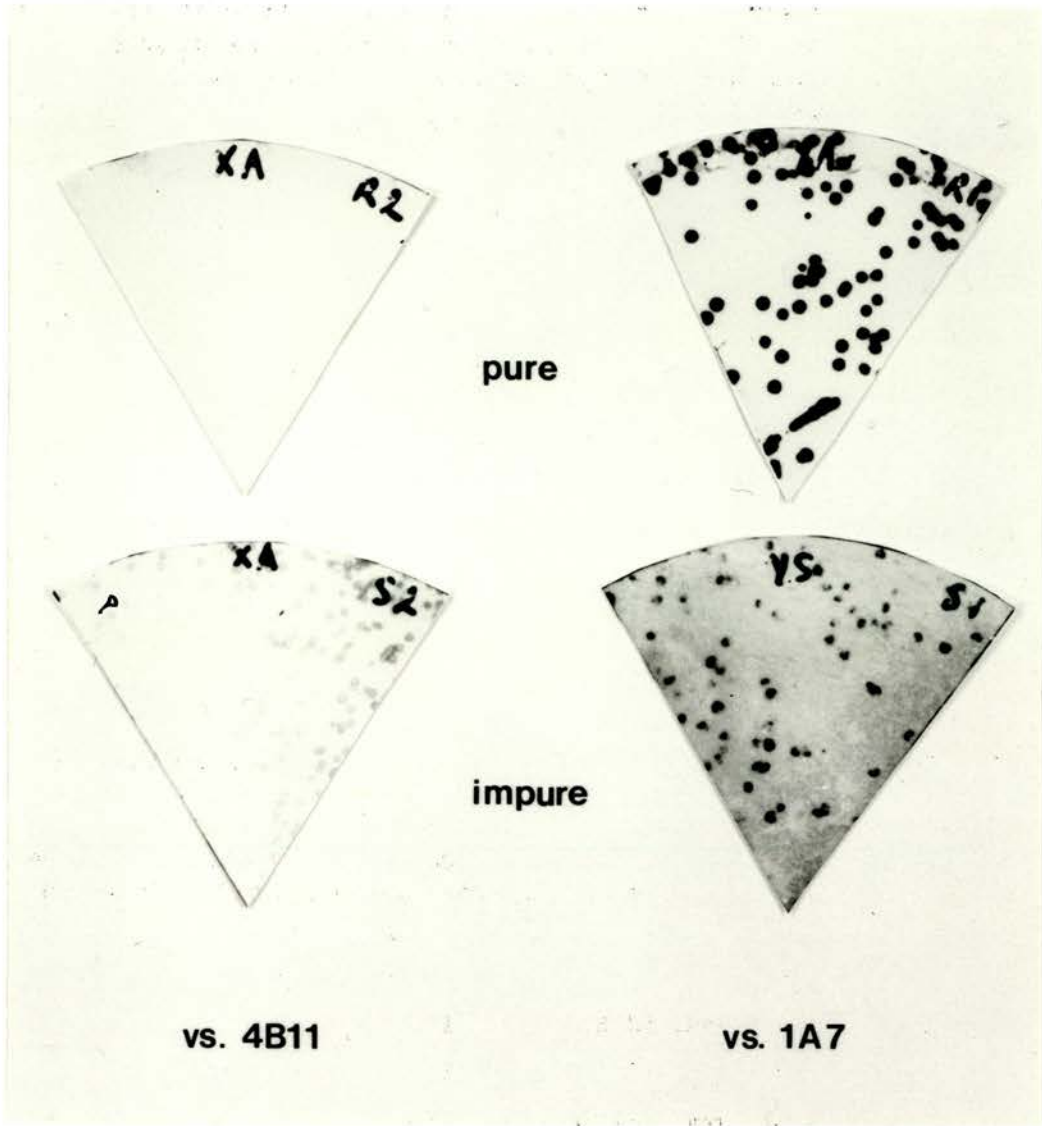
Figure 36 DNA insert sizes of recombinant plasmid or phage.

EcoR1 digestions of recombinant plasmid or phage DNA separated by agarose gel electrophoresis to show insert sizes of:

<u>Track</u>		<u>Insert size</u>
1	puC 18-SR2a	1.3 kb
2	puC 18-SR2b	1.7 kb + 100 bp
3	puC 18-SR1	330 bp
4	λ gt11-SR2	1.3 + 1.7 kb + 100 bp
5	λ gt11-SR1	330 bp

Insert sizes are indicated with arrows and were estimated with reference to standard kb ladder markers run simultaneously.

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6.3.2. Insert sizes in recombinant phage and plasmid

EcoR1 digestions of DNA purified from recombinant clones λ gt11-SR1 and λ gt11-SR2 when separated by agarose gel electrophoresis, revealed that λ gt11-SR1 contained a single insert of 330 base pairs (Figure 36, track 5) while λ gt11-SR2 contained three inserts of 1.7 kb, 1.3 kb and 100 base pairs (Figure 36, track 4), indicating the presence of two internal EcoR1 digestion sites. The inserts were successfully cloned from λ gt11 to puC 18 (Figure 36, tracks 1-3) with the 1.3 and 1.7 kb inserts of λ gt11-SR2 being cloned separately into two different plasmid clones named puC 18-SR2a and b respectively. By chance, the 100 bp insert was randomly ligated into the same clone as the 1.7 kb insert.

6.3.3. Southern blot analysis

Figure 37 shows ethidium bromide stained DNA fractionated in an agarose gel. Figure 38 shows the corresponding Southern blots derived from three replicates of the gel probed with the inserts from the recombinants puC 18-SR1 (100 bp insert, C i) and ii)), puC 18-SR2a (1.3 kb insert, A) and puC 18-SR2b (1.7 kb insert, B). There was no hybridisation to bovine DNA by any of the inserts (track 5). All three inserts hybridised to both stocks (Ankara and Hissar) of T. annulata (tracks 3 and 4). The hybridisation to genomic Theileria DNA was similar between SR2b and SR1, while SR2a and SR2b hybridised to different bands. Hybridisation to the gt11-SR1 and gt11-SR2 inserts (tracks 1 and 2) shows that SR1 and SR2b cross-hybridised to each other but SR2a hybridised only to itself. There was no hybridisation to the small 100 bp insert by any of the three probes.

36

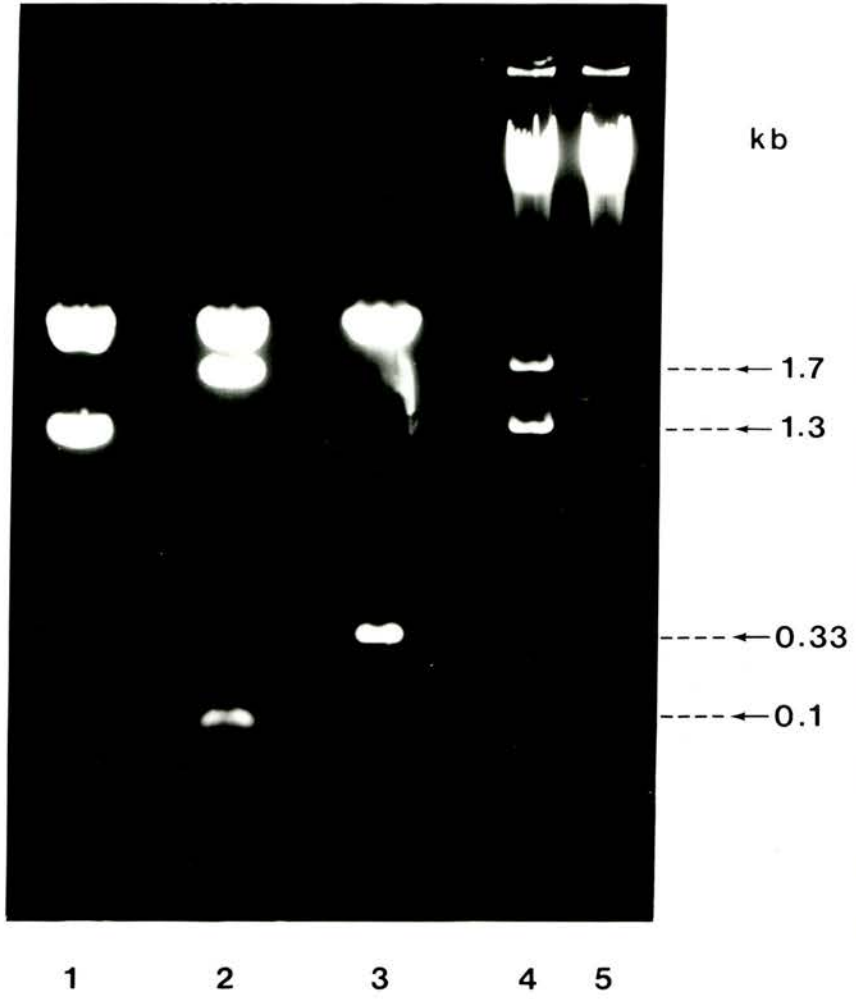


Figure 37 EcoR1 digestions of recombinant phage, theilerial and bovine DNA separated by agarose gel electrophoresis.

Tracks represent DNA from:

track 1 λ gt11-SR2

track 2 λ gt11-SR1

track 3 T. annulata Ankara piroplasms

track 4 T. annulata Hissar piroplasms

track 5 bovine thymus gland

Track 6 contains kb markers (20 ng).

The adjacent markers visible (1 μ g) were present to indicate that the separation of DNA fragments had occurred and were removed before the DNA was transferred from this agarose gel to nitrocellulose paper (Figure 38).

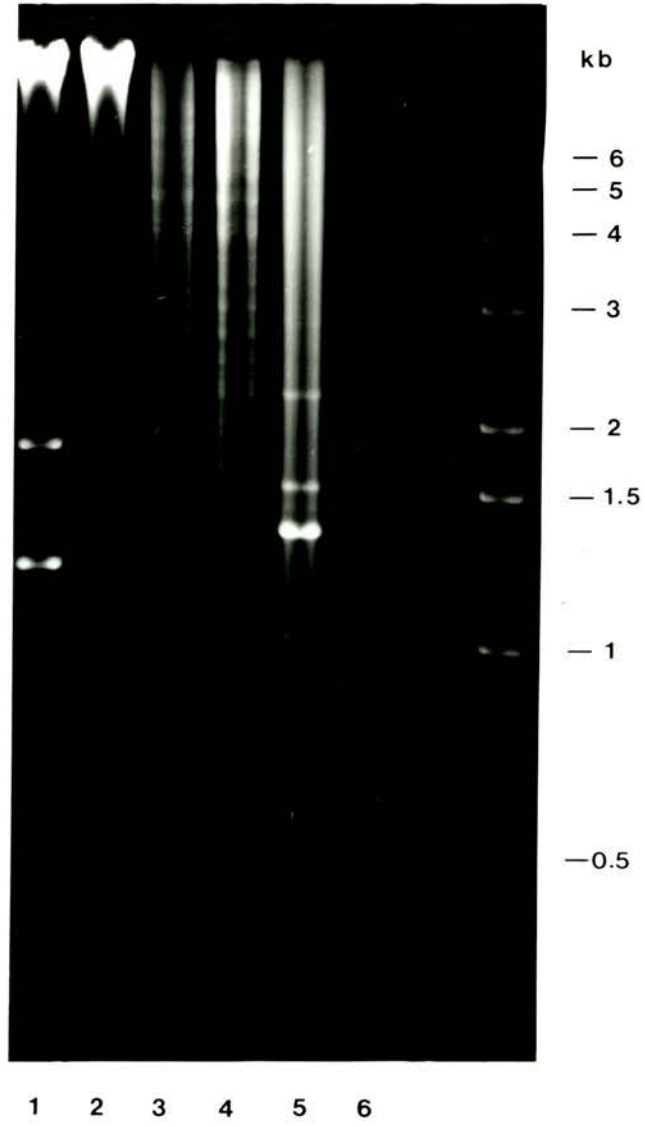


Figure 38 Southern blot hybridisation of recombinant plasmid clone inserts to bovine, theilerial and phage DNA.

Plasmid inserts SR1 (Ci and Cii), SR2a (A) and SR2b (B) hybridised to EcoR1 digested DNA blotted from three replicates of the agarose gel shown in Figure 37.

track 1 λ gt11-SR2

track 2 λ gt11-SR1

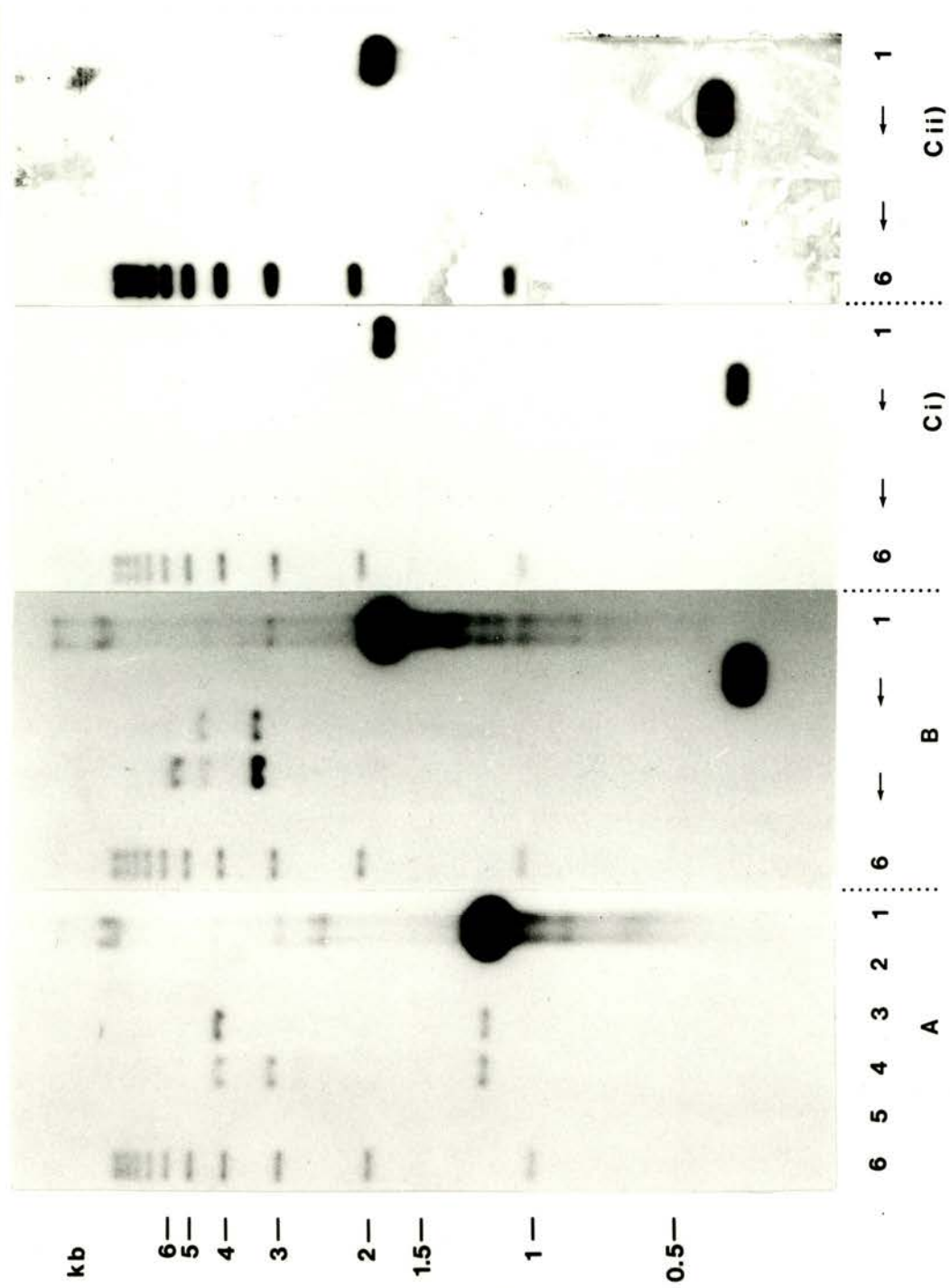
track 3 T. annulata Ankara piroplasms

track 4 T. annulata Hissar piroplasms

track 5 bovine thymus gland

Track 6 contains kb ladder markers.

Cii) represents Ci) exposed to X-ray film for 10 days rather than 24 hours to reveal hybridisation of the SR1 insert to theilerial DNA.



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27

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Figure 39 shows that the puC 18-SR1 insert hybridised to three bands of DNA in EcoR1 digested T. annulata piroplasm DNA from both Ankara and Hissar stocks at about 3.3, 4.8 and 6.0 kb (tracks 1 and 2) but did not show any reaction with T. parva piroplasm DNA (track 3). The T. parva containing track was probed at 59°C and washed at a lower stringency (2 x SSC) than the blots with T. annulata DNA. Although the three DNA bands hybridised are similar in position in Ankara and Hissar stocks, their relative intensity differs between the stocks.

When different restriction endonucleases were used to digest Ankara (Figure 40, tracks 1-3) or Hissar (Figure 40, tracks 4-7) piroplasm DNA, different patterns of hybridisation are observed. Hind III digested DNA probed with the puC 18-SR1 insert (Figure 40, tracks 1, 2 and 6) reveals four hybridisation bands of 3.5, 4.8, 5.8 and 6.3 kb. The bands are the same size in Ankara and Hissar DNA. When Pst 1 digested Hissar DNA (Figure 40, tracks 4 and 5) was probed three bands are observed at 3.1 and 3.5 kb and a high band at greater than 10 kb.

A Southern blot was performed using EcoR1 digested DNA from cloned T. annulata Ankara and Hissar infected cell lines (Section 2.2.7.) to compare the hybridisation pattern with that obtained with parent cell lines and piroplasm DNA when probed with the puC 18-SR1 insert. T. annulata Ankara DNA contained three hybridising bands in both macroschizont-infected cell line (Figure 41, track 12) and piroplasm (track 5) derived DNA. All of the Ankara cloned cell lines gave just a single band. The 3.3 kb band was represented in four clones; 139-D4, 139-E5, 46-A and 46-2 (tracks 7, 8, 9 and 11); the

Figure 39 Southern blot hybridisation of recombinant DNA insert from puC 18-SR1 to T. annulata and T. parva piroplasm DNA.

EcoR1 digested piroplasm DNA from:

track 1 T. annulata Ankara

track 2 T. annulata Hissar

track 3 T. parva Muguga

probed with ^{32}P labelled 330 bp insert of puC 18-SR1.

Figure 40 Southern blot hybridisation of recombinant DNA insert from puC 18-SR1 to T. annulata piroplasm DNA digested with different restriction endonucleases.

T. annulata Ankara (tracks 1-3) and Hissar (tracks 4-7) piroplasm DNA digested with:

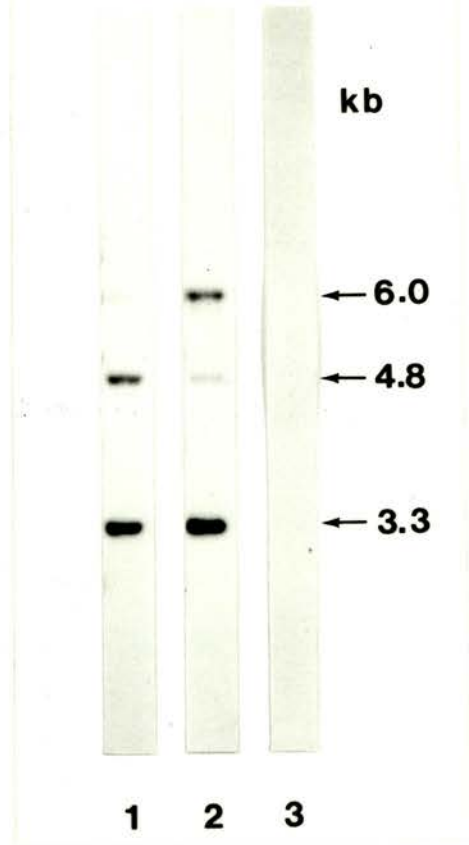
Hind III tracks 1, 2 and 6

EcoR1 tracks 3 and 7

Pst I tracks 4 and 5

and probed with ^{32}P labelled puC 18-SR1 insert.

39



40

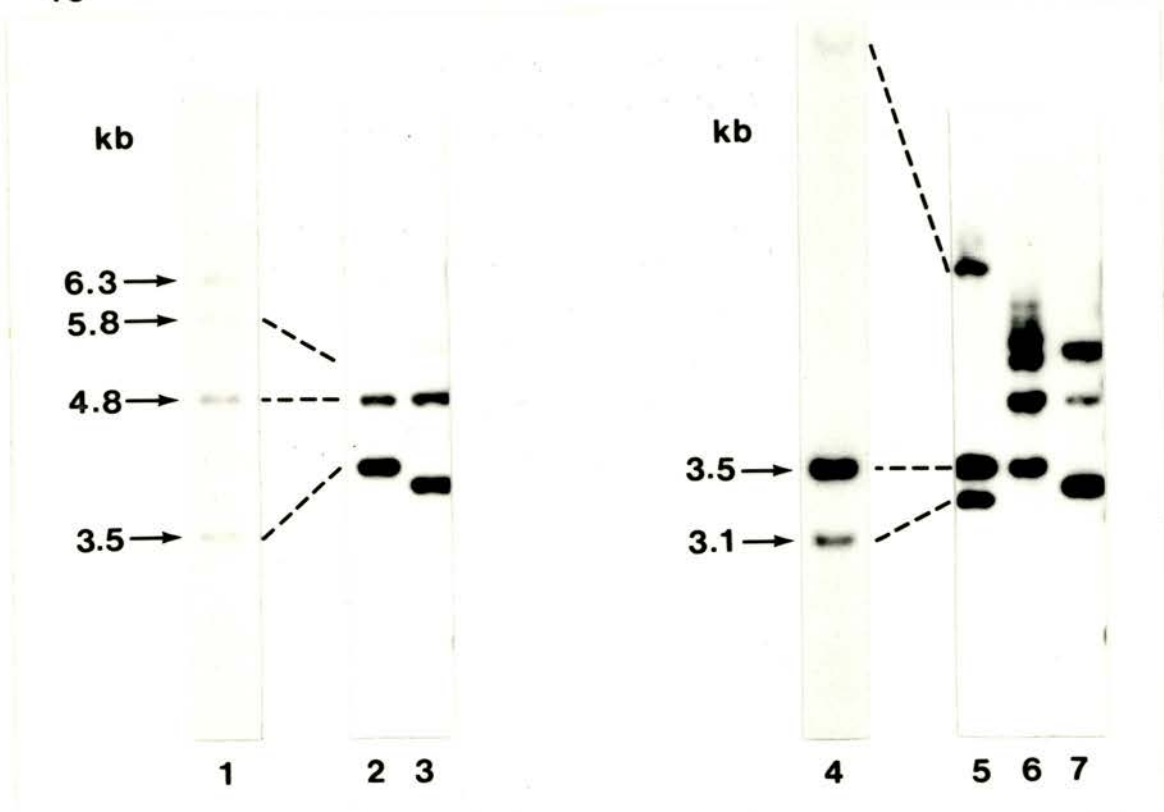


Figure 41 Southern blot hybridisation of puC 18-SR1 insert to genomic DNA from cloned and uncloned parasite material.

EcoR1 digested DNA from T. annulata:

track 1	Hissar 46-4	}	- cloned cell lines
track 2	Hissar 46-3		
track 3	Hissar 46-2		
track 4	Hissar 46-parent cell line		
track 5	Ankara piroplasms		
track 6	Ankara 139-D6	}	- cloned cell lines
track 7	Ankara 139-D4		
track 8	Ankara 139-E5		
track 9	Ankara 46-A		
track 10	Ankara 46-3		
track 11	Ankara 46-2		
track 12	Ankara 46-parent cell line		

probed with ^{32}P labelled puC 18-SR1 insert.

Figure 42 Northern blot hybridisation of puC 18-SR1 insert to total RNA from infected and uninfected tick salivary glands.

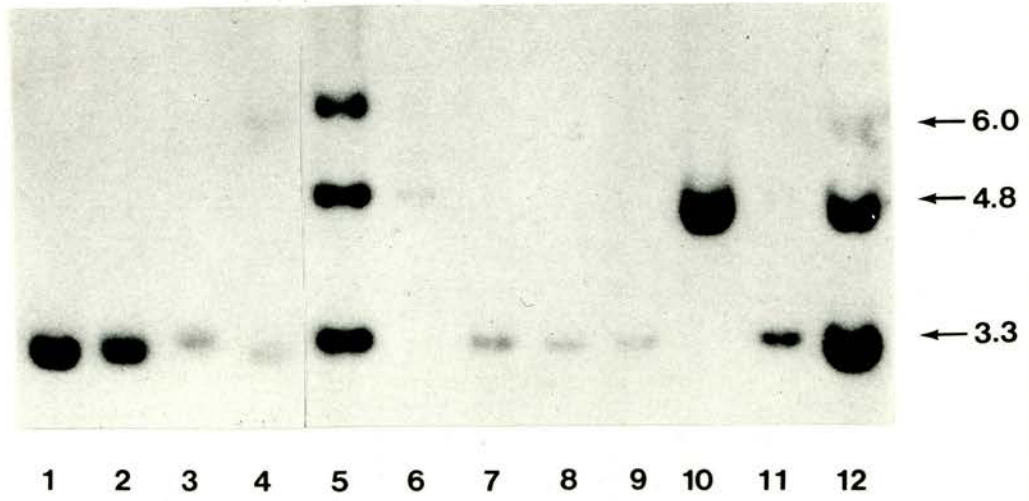
Total RNA separated by agarose gel electrophoresis, blotted and probed with ^{32}P labelled puC 18-SR1 insert. RNA was obtained from:

tracks 1 and 3 uninfected H. a. anatolicum salivary glands

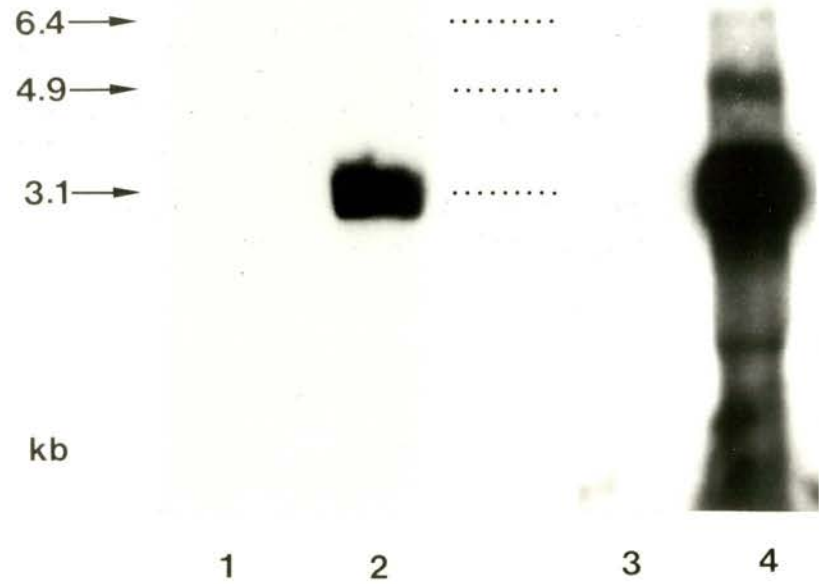
tracks 2 and 4 T. annulata Ankara infected H. a. anatolicum salivary glands

Tracks 3 and 4 represent tracks 1 and 2 exposed to X-ray film over a longer period.

41



42



4.8 kb band by two clones; 139-D6 and 46-3 (tracks 6 and 10) and the 6.0 kb band by none. The Hissar reaction is less clear, the 4.8 kb band is barely visible in the Hissar infected parent cell line DNA (track 4). However the Hissar clones, 46-4, 46-3 and 46-2 clearly show only one band of 3.3 kb to have hybridised with no evidence of a higher band (tracks 1-3). The blot shown was performed by Len Metcalfe while working at the Wellcome Unit for Molecular Parasitology.

6.3.4. Northern blot analysis

Northern blot analysis (Figure 42) revealed a major RNA band of 3.1 kb to be expressed specifically in infected tick salivary glands (track 2). No hybridisation is seen in RNA from uninfected glands (track 1). On exposing the Northern blot longer, higher molecular weight bands of 6.4 and 4.9 become visible in infected salivary gland RNA (track 4), still with no reaction in uninfected salivary gland RNA (track 3).

6.3.5. Lysogen generation and recombinant protein analysis

Lysogens harbouring recombinant λ gt11 phage and wild type λ gt11 phage were successfully produced in Y1089. Of 100 single colonies tested for temperature sensitivity for each recombinant λ gt11, 42% and 36% were lysogens of λ gt11-SR1 and λ gt11-SR2 respectively and failed to grow at 42°C. The lysogen colony screen confirmed the reactivity of the recombinant lysogens with Mab 1A7 when induced to express recombinant protein with IPTG. Figure 43 shows an example from this screen. Induced wild type λ gt11 lysogen and uninduced

Figure 43 Colony screen of recombinant lysogen λ gt11-SR1.

The upper segment shows induced λ gt11-SR1 lysogen colonies reacting positively with monoclonal antibody 1A7.

The lower segment shows uninduced λ gt11-SR1 lysogen colonies failing to react with monoclonal 1A7.

An enzyme linked immunoassay was used to reveal positive or negative antigen-antibody reactions.

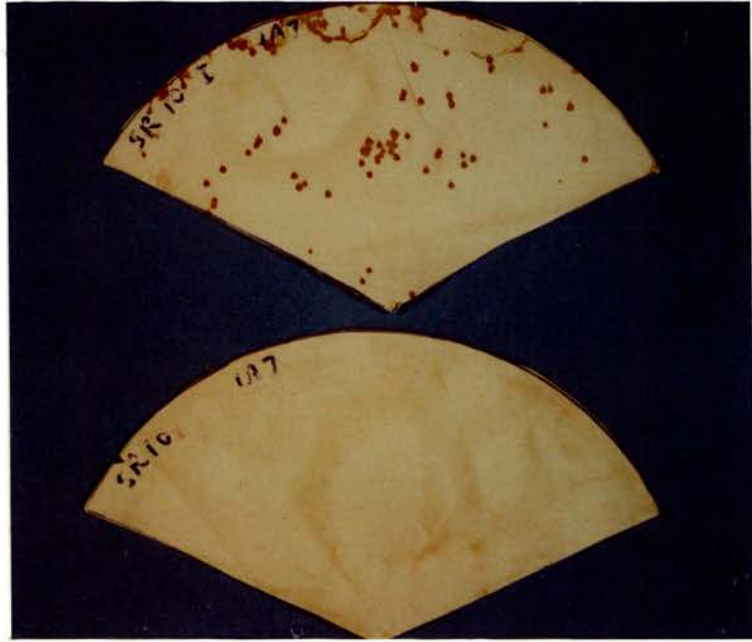
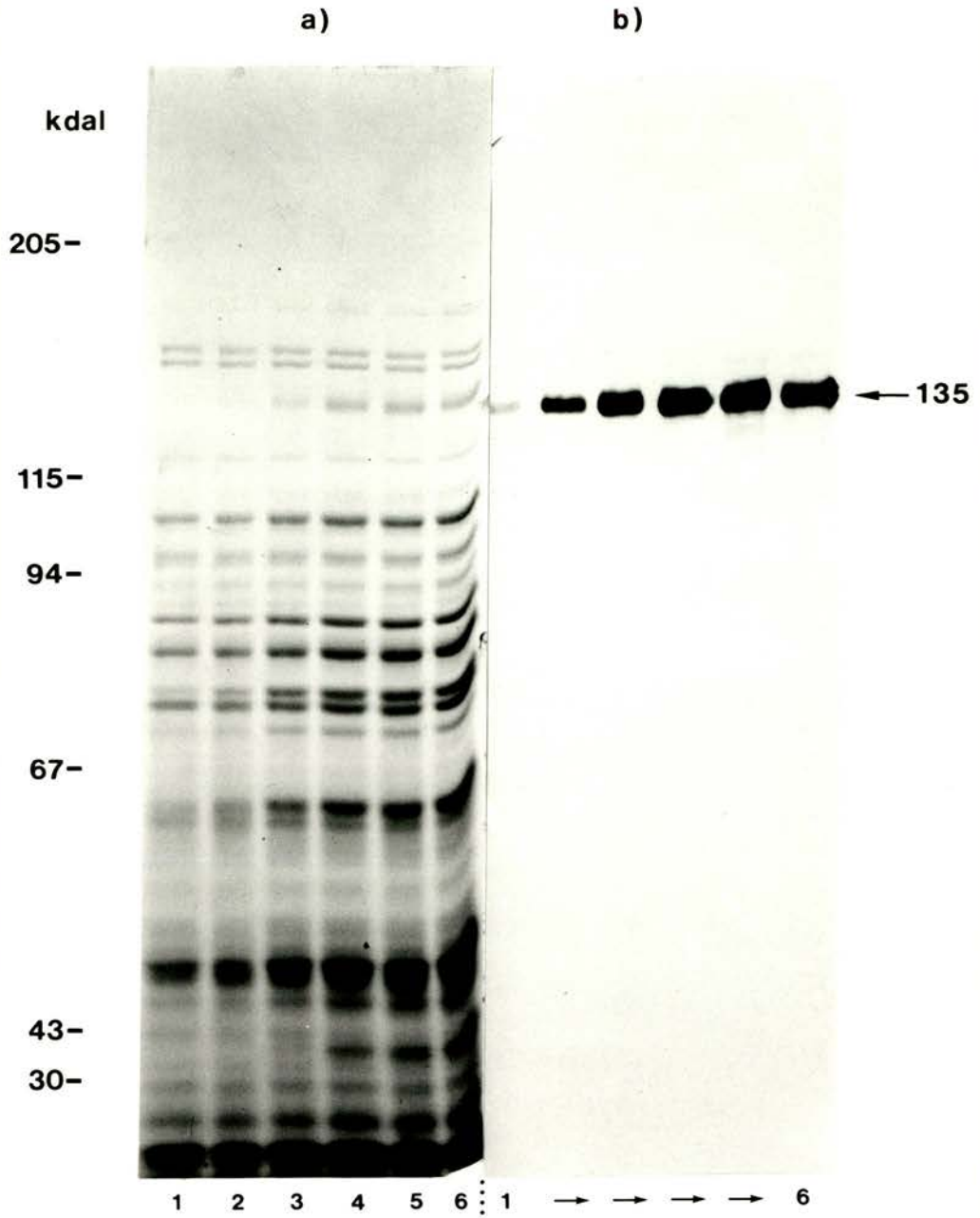


Figure 44 Time course of induction for lysogen λ gt11-SR1.

- a) Coomassie blue stained acrylamide gel of λ gt11-SR1 lysogen lysates from cultures induced for 15, 30, 45, 60, 75 and 90 minutes (tracks 1-6).
- b) Western blot of a replica gel reacted with monoclonal antibody 1A7.



recombinant lysogens failed to react with Mab 1A7 and no reaction was obtained when induced recombinant lysogens were incubated with other Mabs, 4B11, 7B10 and 4H5 (data not shown). The time course for recombinant lysogen induction showed that the level of recombinant protein increased sharply in the first 60 minutes and did not change appreciably in the following 30 minutes. An 80 minute incubation time was selected for use in subsequent experiments. Figure 44 shows a Coomassie stained acrylamide gel (a) and corresponding Western blot (b) of lysogen samples taken during the induction process.

Figure 45 shows induced lysogen and Y1089 lysates analysed by protein stains of SDS-PAGE gels and by Western blotting. Specific protein bands were induced of 135 kdal in λ gt11-SR1 lysogen (tracks 2 and 10) and of 115 kdal in wild type λ gt11 lysogen (track 11), the latter representing induction of the B-galactosidase enzyme. In λ gt11-SR2 lysogen (track 1) and Y1089 (track 3) no unique bands were visible. Western blots of the same material show several features. Mab 1A7 reacted with a specific protein in both λ gt11-SR1 and λ gt11-SR2 lysogens of 135 and 147 kdal respectively (tracks 5 and 4) but recognised no proteins in wild type λ gt11 lysogen (track 13) or Y1089 (track 6). Mab 4B11, acting as a negative antibody control, gave no such reaction (tracks 7-9). When λ gt11-SR1 and wild type lysogens were incubated in serum possessing anti-B-galactosidase activity, bands were detected at 135 and 115 kdal respectively (tracks 14 and 15).

The reactivity of the bovine and rabbit antisera used in earlier chapters, with the recombinant protein expressed by λ gt11-SR1 lysogen is illustrated in the Western blots in Figures 46 and 47a and compared with their reactivity with B-galactosidase expressed by wild type

Figure 45 Analysis of recombinant proteins of λ gt11-SR1 and λ gt11-SR2 expressed in E. coli.

Coomassie blue stained gels showing induced lysates of lysogens of:

track 1 λ gt11-SR2
track 2 λ gt11-SR1
track 3 Y1089
track 10 λ gt11-SR1
track 11 wild type λ gt11

Western blots of the same material reacted with antibody:

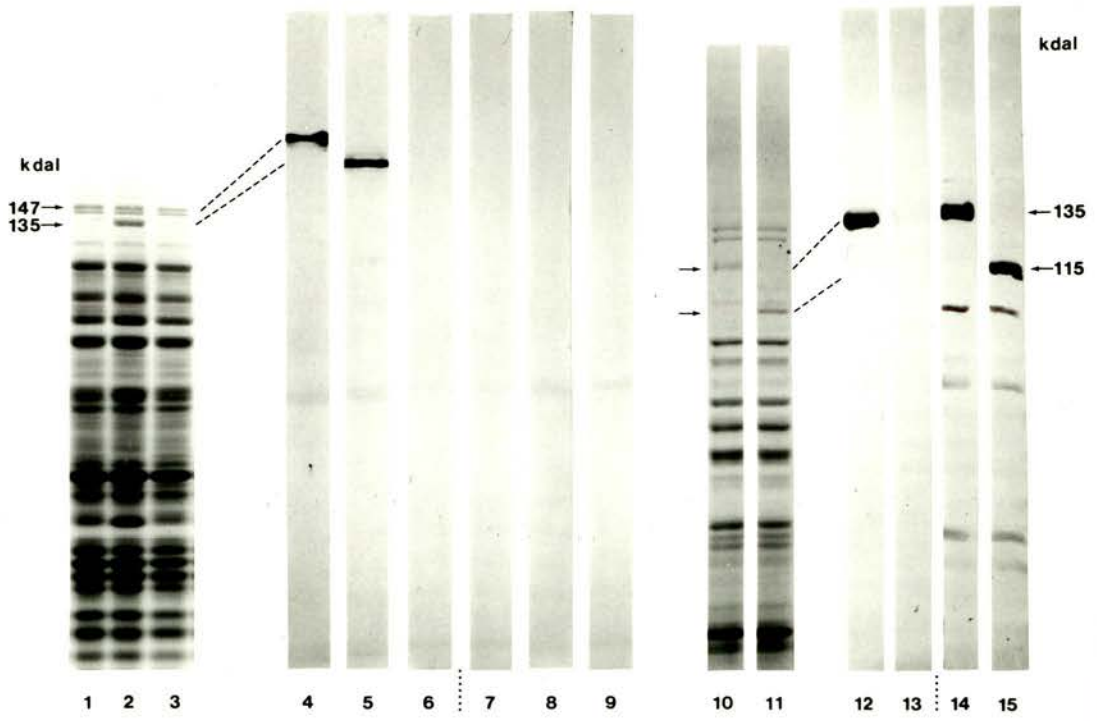
track 4 λ gt11-SR2
track 5 λ gt11-SR1 } - vs. monoclonal 1A7
track 6 Y1089 }
track 7 λ gt11-SR2
track 8 λ gt11-SR1 } - vs. monoclonal 4B11
track 9 Y1089 }
track 12 λ gt11-SR1 }
track 13 wild type λ gt11 } - vs. monoclonal 1A7
track 14 λ gt11-SR1 }
track 15 wild type λ gt11 } - vs. anti-B-galactosidase serum

Figure 46 Reactivity of selected antibodies with λ gt11-SR1 recombinant protein.

Western blot of induced lysates of λ gt11-SR1 lysogen reacted with antibodies:

- track 1 Mab 1A7
- track 2 Mab 4H5
- track 3 Mab 1C7
- track 4 Calf 53 day 0
- track 5 Calf 53 day 107 hyperimmunised with live virulent sporozoites
- track 6 Calf 61 day 0
- track 7 Calf 61 day 107 hyperimmunised with live virulent sporozoites
- track 8 Calf N44 day 0
- track 9 Calf N44 day 63 immunised with irradiated sporozoites (500 Gy)
- track 10 Calf N44 day 92 challenged with live virulent sporozoites
- track 11 Calf 45T day 0
- track 12 Calf 45T immune to Hyalomma ticks

45



46

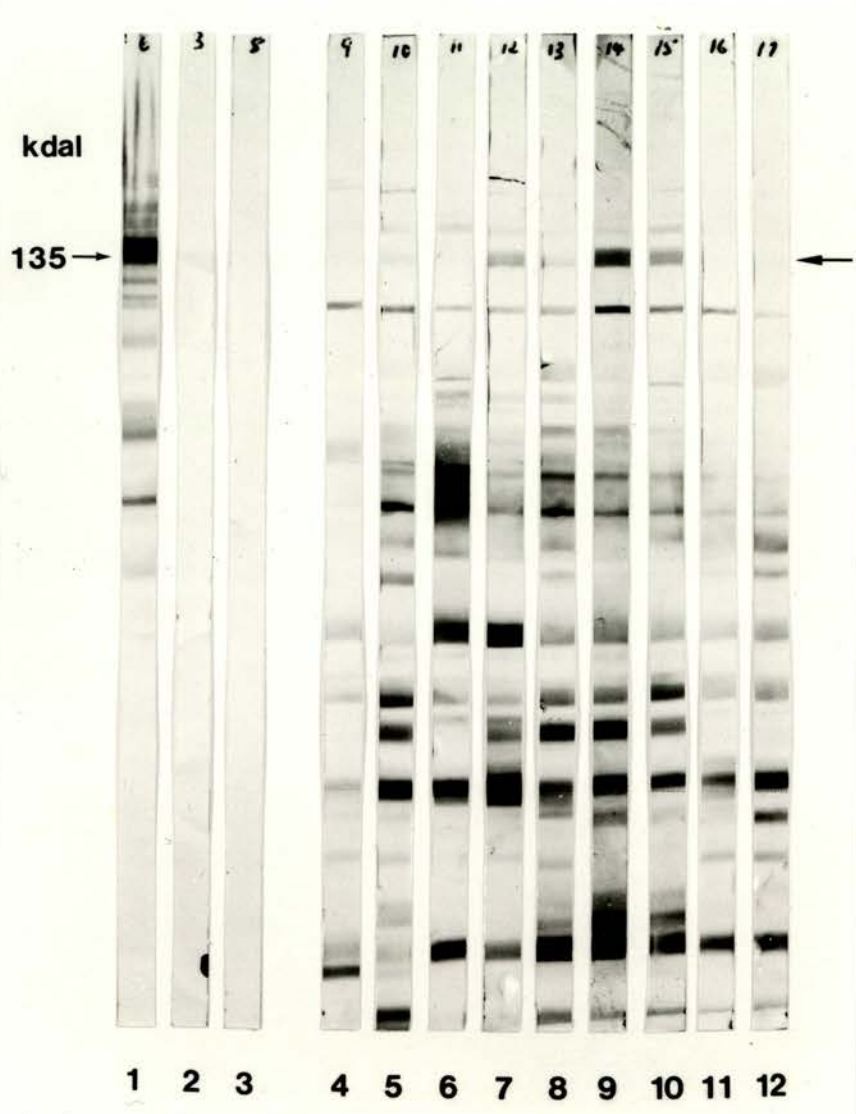


Figure 47 Reactivity of antisporozone antibodies with λ gt11-SR1 recombinant protein and wild type λ gt11 B-galactosidase

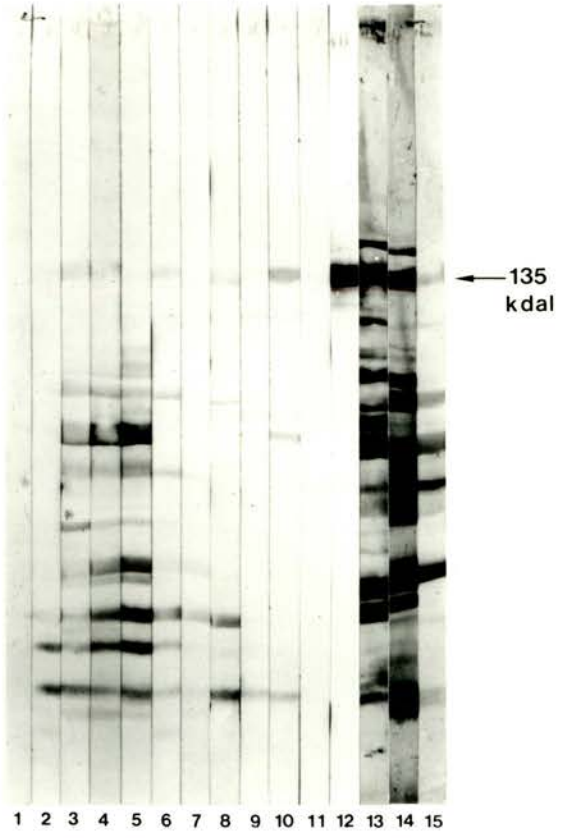
a) Western blot of induced λ gt11-SR1 lysogen reacted with antibodies:

- track 1 Calf L37 day 63 immunised with irradiated sporozoites (640 Gy)
- track 2 Calf L37 day 0
- track 3 Calf L21 day 91 challenged with live virulent sporozoites
- track 4 Calf L21 day 63 immunised with irradiated sporozoites (320 Gy)
- track 5 Calf L21 day 0
- track 6 Calf 102 day 43 immunised with irradiated sporozoites (840 Gy)
- track 7 Calf 102 day 0
- track 8 Calf 134X day 101 hyperimmunised with live virulent sporozoites
- track 9 Calf 134X day 0
- track 10 Calf 54X day 95 hyperimmunised with live virulent sporozoites
- track 11 Calf 54X day 0
- track 12 Mab 1A7
- track 13 Rabbit 77 day 66 (see Section 7.3.3.) anti-B-galactosidase serum
- track 14 Rabbit 28 day 73 after fed on by infected ticks
- track 15 Rabbit 28 day 0

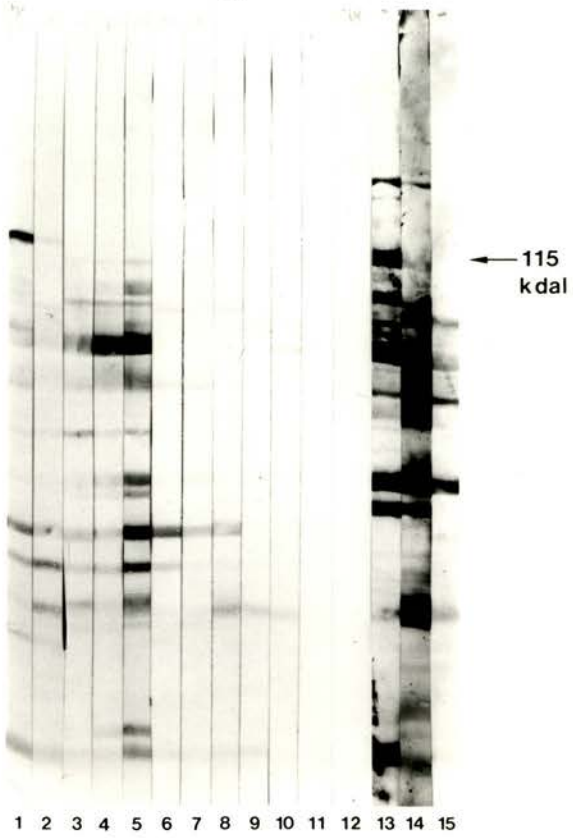
b) Western blot of induced wild type λ gt11 reacted with the same antibodies.

Tracks 1-15 as above.

a)



b)



λ gt11 lysogen (Figure 47b). The essential points of interest are that all calves, except L37, immunised with sporozoites by repeated inoculation of either virulent live sporozoites or irradiated sporozoites developed a reaction to the same 135 kdal protein expressed in λ gt11-SR1 lysogen as recognised by Mab 1A7, albeit less strongly than Mab 1A7. This reactivity was not observed in day 0 sera, nor in serum from a calf shown to be immune to uninfected ticks (Figure 46, track 12). Rabbit 28 developed very strong recognition of the 135 kdal protein (Figure 47a, track 14); day 73 serum taken after immunisation by infected ticks feeding gave as strong a reaction to this protein as Mab 1A7 (Figure 47a, track 12). The corresponding serum from rabbit 8, which was immunised by uninfected ticks feeding, gave no reaction at all with this protein (data not shown).

Figure 47b shows that identical sera to those used in Figure 47a, when reacted with the induced wild type λ gt11 lysogen, do not recognise the B-galactosidase protein at 115 kdal.

Monoclonals raised against the infected lymphocyte surface (4H5) and the macroschizont (1C7) also fail to recognise the 135 kdal recombinant protein (Figure 46, tracks 2 and 3).

6.4. DISCUSSION

The two recombinants, λ gt11-SR1 and λ gt11-SR2, selected from the genomic library expressed proteins which contained the epitope recognised specifically by ant sporozoite Mab 1A7. There was no cross-reaction with 4B11 or other monoclonals raised against the sporozoite, nor with monoclonals raised against other stages of the parasite.

No recombinants expressing the epitope recognised by 4B11 were detected. This may have been due to the absence of any recombinants containing the appropriate gene sequence in the correct orientation and reading frame. Another reason might be that a recombinant may have been expressing the appropriate amino acid sequence but either essential protein folding or post translational modification, absent in E. coli, were required to form the epitope showing reactivity with 4B11.

Although λ gt11-SR1 and λ gt11-SR2 both contain theilerial DNA coding for the 1A7 epitope they are not identical; the insert sizes show that λ gt11-SR2 has a greater theilerial DNA content.

Since the genomic library was constructed from piroplasm DNA there was a small risk that a few bovine white blood cells not removed during piroplasm purification might have contaminated the library with fragments of bovine DNA.

The bovine genome (10^9 kb) is approximately 100 times larger than the Theileria genome (10^7 kb) so very few white cells could introduce some bovine DNA recombinants. To ensure that the selected recombinants did not contain bovine DNA which, due to some artefact, coded for a protein reacting positive with 1A7, bovine DNA was included in a Southern blot probed by the puC 18 inserts (Figure 38). None of the inserts hybridised to bovine DNA, confirming that their DNA is parasite and not host derived. The agarose gel on which the digested DNA was separated (Figure 37) shows that the piroplasm DNA did not have bands in common with bovine DNA. Thus, at this level of sensitivity, the piroplasm DNA was not significantly contaminated with bovine.

The same Southern blot as above shows that the 330 bp sequence of λ gt11-SR1 must be contained in the large 1.7 kb insert of λ gt11-SR2 since these two inserts cross-hybridised with each other. This correlates with their similar hybridisation pattern on probing theilerial DNA, which differed from that of the 1.3 kb insert. Figure 48 shows the interrelationship of λ gt11-SR1 and λ gt11-SR2 diagrammatically.

The genomic library from which these recombinants were selected was constructed using DNA fragments created in a random fashion (Section 6.2.3.). This makes it possible for just fragments of a gene to be cloned and also unlikely that whole genes will be cloned discrete from adjacent theilerial DNA from a non-coding region or another gene. The recombinant protein analysis shows that the expressed fusion proteins contain theilerial portions which are smaller than any of the native sporozoite proteins bearing the 1A7 epitope. It is clear from this that the 1.7 kb and 330 bp gene sequences coding for this epitope are only portions of a larger gene.

Since there are no internal EcoR1 digestion sites in the puC 18-SR1 insert, the presence of three bands in Ankara and Hissar DNA probed with this insert indicates that there are three copies of the DNA sequence in both stocks. These parasite stocks are not cloned so the three gene fragment copies could represent the presence of a mixed population with three different genomes. Alternatively there could be three copies of this gene fragment within a single genome, or even a combination of the two situations.

The hybridisation pattern of cloned macroschizont-infected cell line DNA probed with the puC 18-SR1 insert (Figure 41) affords

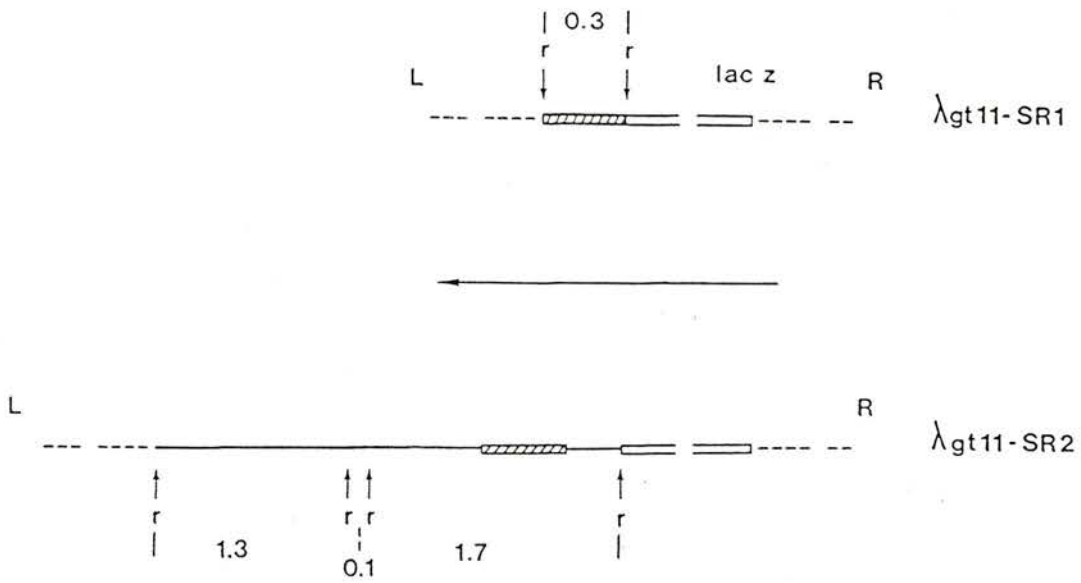
Figure 48 Relationship between the inserts in clones lambda gt11-SR1 and lambda gt11-SR2 which both express the epitope recognised by Mab 1A7.

The numbers refer to lengths in kb.

The letter r designates EcoRI restriction sites.

The arrow denotes the direction of transcription.

The shaded segment represents the gene sequence of the λ gt11-SR1 insert.



preliminary evidence that the former is true and that the three bands observed in parent cell line and piroplasm DNA are the result of restriction fragment polymorphism in a mixed parasite population and are not multiple gene copies. The bands were segregated to single copies in the clones. The 6.0 kb band was not present in any clone of either Ankara or Hissar. This may just reflect its apparently low representation in the uncloned parent cell lines (Figure 41, tracks 4 and 12), if more clones had been available for testing it would probably have been detected. Restriction fragment polymorphism is a distinguishing feature of some repetitive antigens of Plasmodium falciparum (Coppel, Saint, Stahl, Langford et al., 1985a; Kemp, Coppel, Stahl, Bianco et al., 1986). Workers cloning and expressing Plasmodium circumsporozoite epitopes in a similar manner to this study have found single gene copies of the DNA coding for this epitope on Southern blots in Plasmodium cynomolgi (Enea et al., 1984a), Plasmodium knowlesi (Ellis et al., 1983) and Plasmodium falciparum (Dame et al., 1984). In a similar manner to the λ gt11-SR1 insert, a cDNA clone encoding a Babesia bovis antigen detected two bands in genomic DNA from an uncloned isolate while two other isolates showed a single band (Gill et al., 1987). This suggests that the cDNA clone gene sequence may belong to a polymorphic locus of Babesia bovis.

The DNA sequence of the SR1 insert was originally derived from T. annulata Hissar genomic DNA. The Southern blots show that the sequence is also present in Ankara DNA and further work has confirmed that Gharb DNA shows the same hybridisation pattern (data not shown). This correlates with the observation that Mab 1A7 reacts with all three stocks by IFA and Western blotting. Use of this gene sequence

as a DNA probe would not differentiate uncloned T. annulata stocks and would group clones derived from these stocks differently from groups resulting from GPI analysis. However, T. parva DNA did not appear to contain the DNA representing the SR1 insert even when hybridised under conditions allowing hybridisation of a sequence showing incomplete homology. This feature is in common with observations that the DNA sequence coding for the repetitive epitope of the circumsporozoite protein of Plasmodium is not shared between species of that parasite (Enea et al., 1984b).

The Northern blot analysis (Figure 42) gave good evidence to indicate that the DNA sequence of the puC 18-SR1 insert was specifically expressed in the infected and not the uninfected tick salivary glands. A very strong signal was apparent from the 3.1 kb RNA transcript. By day 2 of a tick feed, which is when salivary glands were harvested for RNA extraction, sporogony will have occurred under the stimulus of the tick feeding (Schein and Friedhoff, 1978) resulting in peak synthesis of the sporozoite surface antigens. During this short time there will have been sudden and rapid expression of the DNA encoding these antigens and therefore the mRNA species responsible for the protein synthesis will be well represented. Indeed, the methyl green and pyronin stain demonstrates the maximal presence of RNA on day 2, since RNA stains a blue colour with this technique (Walker et al., 1979). The higher molecular weight RNA bands of 6.4 and 4.9 (Figure 42) visible on longer exposure may represent precursors of the 3.1 kb sequence or may, since the RNA was extracted from uncloned parasite, be transcripts from other copies of the sequence.

The analysis of the in vivo RNA transcripts has been extended by hybridising the insert of λ gt11-SR1 to RNA from macroschizont and piroplasm stages of the life cycle. The results indicate that the expression of this gene is confined to sporoblast and sporozoite stages (Dr. F.R. Hall, personal communication). Dr. Roger Hall has also performed preliminary experiments to study the translation products of infected salivary gland RNA. Immunoprecipitation of these products with serum raised against the λ gt11-SR1 fusion protein (see Section 7.3.3.) revealed a single high molecular weight protein band of over 100 kdal. This could represent the 104 kdal sporozoite protein observed in Western blots of crude infected tick homogenates (Figure 26). Its presence suggests that the multiple reaction of Mab 1A7 with sporozoites is, at least partly, due to processing of a high molecular weight precursor molecule.

The validity of the two recombinants selected by Mab 1A7 from the genomic library has been confirmed by showing the presence of the cloned theilerial DNA sequence in the genome (Figure 39) and by demonstrating stage specific expression of the DNA (Figure 42). This analysis of the DNA cloned in the two recombinants was carried out concurrently with an analysis of the recombinant proteins they express in E. coli.

Since the initial selection of λ gt11-SR1 and λ gt11-SR2 was conducted by screening for expression of the recombinant protein, the selected recombinants were known to contain the theilerial DNA sequence in the correct transcriptional orientation and translational frame (Young and Davis, 1983a).

Lysogens were generated in E. coli strain Y1089 in order to obtain larger quantities of the recombinant proteins. As the results show (Figure 45), $\lambda_{gt11-SR1}$ and $\lambda_{gt11-SR2}$ express recombinant proteins of 135 and 147 kdal respectively, each of which bear the sporozoite epitope recognised specifically by Mab 1A7. $\lambda_{gt11-SR2}$ recombinant protein was not visible on Coomassie blue-stained gels. However, it may have been marked by a host cell protein occurring at the same site. Partly for this reason, $\lambda_{gt11-SR1}$ was used for all future work. These recombinant proteins are expressed fused to B-galactosidase which has a molecular weight of 115 kdal. This means that a 115 kdal portion of the expressed fusion protein is bacterial in origin, leaving the eukaryotic peptide portions of 20 ($\lambda_{gt11-SR1}$) and 32 ($\lambda_{gt11-SR2}$) kdal. The DNA insert isolated from $\lambda_{gt11-SR1}$ which was described earlier was only 330 bp in size, with a theoretical coding capacity of only about 11-12 kdal. An anomaly thus exists between the sizes of the DNA insert and the eukaryotic peptide portion. This has been investigated by Dr. Roger Hall who sequenced the DNA of the $\lambda_{gt11-SR1}$ insert and obtained the predicted amino acid sequence (Figure 49). This amino acid sequence contains a protein coding region relatively high in proline (10% overall with one stretch of 25% between amino acids 20 to 64). Such high proline content has previously been reported to retard protein migration on SDS-PAGE (Young et al., 1985; Carrol and Laughon, 1987) and may explain the apparently large size of the fusion protein.

The reactivity of the rabbit and bovine antisera with the $\lambda_{gt11-SR1}$ fusion protein (Figures 46 and 47) was interesting since it indicates that animals exposed to sporozoites produce antibodies which

Figure 49 Nucleotide sequence of the 330 base pair SR1 insert and the predicted amino acid sequence

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-----
GAA  TTC  CTA  GTG  AAG  GAT  GTA  AGC  GAA  GAA  CAG  GDD  GGA
Glu  Phe  Leu  Val  Lys  Asp  Val  Ser  Glu  Glu  Gln  Val  Gly

ATA  GGA  GAT  TTA  AGT  GAC  CCG  AAT  AGC  AGA  ACA  CCA  AAT
Ile  Gly  Asp  Leu  Ser  Asp  Pro  Asn  Ser  Arg  Thr  Pro  Asn

GCA  AAA  CCA  GCC  GAA  CTT  GGA  CCT  TCA  CTA  GTG  ATA  CAA
Ala  Lys  Pro  Ala  Glu  Leu  Gly  Pro  Ser  Leu  Val  Ile  Gln

AAT  GTA  CCA  TCA  GAC  CCC  TCA  AAA  GTG  ACA  CCA  ACA  GAG
Asr  Val  Pro  Ser  Asp  Pro  Ser  Lys  Val  Thr  Pro  Thr  Glu

CCT  TCA  AAT  TTG  CCA  CAA  GTA  CCA  ACA  ACA  GGG  CCG  GGG
Pro  Ser  Asn  Leu  Pro  Gln  Val  Pro  Thr  Thr  Gly  Pro  Gly

AAC  GGG  ACG  GAT  GGA  ACA  ACA  ACA  GGA  CCA  GGT  GGA  AAC
Asn  Gly  Thr  Asp  Gly  Thr  Thr  Thr  Gly  Pro  Gly  Gly  Asn

GGG  GAA  GGA  GGC  AAA  GAT  TTG  AAG  GAA  GGA  GAA  AAG  AAA
Gly  Glu  Gly  Gly  Lys  Asp  Leu  Lys  Glu  Gly  Glu  Lys  Lys

GAA  GGA  TTA  TTT  CAA  AAG  ATC  AAA  AAC  AAA  CTC  TTG  GGC
Glu  Gly  Leu  Phe  Gln  Lys  Ile  Lys  Asn  Lys  Leu  Leu  Gly

TCA  GGA  TTC  GAA  GTC  ACA  AGA  ATTC
Ser  Gly  Phe  Glu  Val  Thr  Arg  Ile

```

The two EcoRI sites are indicated by a dotted line above the sequence. The proline residues are underlined to emphasise their high frequency. The reading frame is shown in phase with B-galactosidase.

Sequence determination was entirely the work of Dr. Roger Hall.

recognise this fusion protein. This response was not observed in animals exposed to uninfected ticks. Since the fusion protein contains a major bacterial component the same sera were reacted with B-galactosidase from wild type λ gt11 to see if the reactivity was directed against the theilerial or bacterial component of the fusion protein. It would not be surprising for calves to develop antibodies to a range of E. coli proteins since this is a major inhabitant of the gastrointestinal tract and pathogenic E. coli strains are one of the most common causes of enteritis in young calves (Blood, Henderson and Radostits, 1979). In fact, the calves and rabbit specifically identified the sporozoite portion of the fusion polypeptide. This was surprising since in Western blots in which the same sera were reacted with purified sporozoites only a few of the calves and rabbit 28 identified the same sporozoite epitope as Mab 1A7. The reaction of calf sera with this epitope was weak compared to that of Mab 1A7 and rabbit 28. This may be reason why it was not observed in Western blots with sporozoites where, proportionally, the quantity of epitope present would have been much smaller.

Uncertainty must still surround the role of this sporozoite epitope as a component of an antigen stimulating protective immunity. Calf 102, immunised by repeated inoculation of irradiated sporozoites, was shown to be susceptible to challenge infection, yet shows some reactivity with the 135 kdal protein as well as in vitro sporozoite neutralisation. Whether this response was not adequately stimulated in calf 102 to confer protection or whether, in other calves, the response was too weak and occurred incidental to protection is open to question.

The obvious means of resolving these questions was first to immunise animals with the λ gt11-SR1 fusion protein in order to obtain antibody responses which would react with the sporozoite epitope as strongly as Mab 1A7; then to challenge the immunised calves with virulent sporozoites and observe whether any degree of protection was afforded. The next chapter describes work towards and including such an immunisation trial.

CHAPTER SEVEN

IMMUNISATION TRIALS TO TEST A
RECOMBINANT SPOROZOITE EPITOPE

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7.1. INTRODUCTION

Prior to cloning and expression of parasite antigen genes with a view to producing material for an effective subunit vaccine, preliminary work will normally have been performed to suggest that the antigen has the potential to stimulate an effective immune response. Evidence of the latter may result directly from immunisation trials with the purified antigen (Hall, Hyde, Goman, Simmons et al., 1984) or indirectly from experiments which indicate that antibody recognising the antigen is, or could be, protective (Potocnjak et al., 1980; Nardin, Nussenzweig, Nussenzweig, Collins et al., 1982; Hollingdale et al., 1984; Dobbelaere et al., 1984; Musoke et al., 1984; Winger et al., 1987). Another feature used to indicate an antigen's importance, which is perhaps the least reliable, is detection of reactivity with polyclonal immune serum (Iams et al., 1988; Goff, Davis, Palmer, McElwain et al., 1988).

Once selected antigens have been produced as genetically engineered recombinant proteins they are available in large quantities for immunisation trials. These trials have two purposes; firstly to determine whether the purified recombinant protein is immunogenic when inoculated, and secondly, to assess whether the response, if one is obtained, is protective.

In parasite systems where a laboratory animal model is readily available, preliminary trials can combine these two activities with those animals responding to the recombinant protein being challenged with live parasite. For some parasites there is no laboratory animal model and the definitive host would be either too costly, or too controversial to use without considerable justification from

preliminary experiments (for example: cattle, chimpanzees, human volunteers). In such situations the assessment of immunogenicity and of methods of antigen presentation can be initially investigated in an alternative species. In these species some idea can be gained of the potential protective function of the recombinant protein by coupling the in vivo response with in vitro methods similar to those described for assessing the role of a parasite antigen. One such example is the work carried out in mice to test a potential Plasmodium falciparum recombinant sporozoite vaccine for use in man. The serum from immunised mice was first tested for reactivity with sporozoites in the IFAT and then tested for its ability to neutralise sporozoite infectivity for human hepatocytes in vitro (Mazier et al., 1986).

The aim of the work described in this chapter was to carry out immunisation trials in mice and rabbits using the λ gt11-SR1 fusion protein and then to extend the work in vivo by immunising the natural bovine host of T. annulata with the protein and challenging with a lethal dose of homologous sporozoites to test whether any degree of protective immunity has resulted.

The experiments described include a detailed assessment of the response of immunised mice and rabbits in order to select an appropriate method for presenting the material to calves. This assessment included the use of IFAT, Western blotting and in vitro sporozoite inhibition assays. It was intended to determine whether animals had responded to the immunising material; if they had, whether the response was directed towards sporozoite and/or bacterial portions of the fusion protein and, lastly, whether their response demonstrated functional ability in blocking sporozoite infectivity in vitro.

A method for immunising calves was only selected after satisfactory results had been obtained in all these aspects. Likewise, not until similar results had been obtained in immunised calves, were they subjected to challenge with virulent parasite.

In this way it was hoped that an objective and meaningful assessment could be made of the immunising and protective ability of this recombinant sporozoite epitope.

7.2. MATERIALS AND METHODS

7.2.1. Experimental animals

a) Mice

Ten Balb/c female mice over eight weeks old. Mice were identified as 1-10.

b) Rabbits

Six New Zealand White female rabbits aged at least three months old. Rabbits were identified as 1608, 1609, 73, 75, 77 and 11.

c) Calves

Six castrated male calves of Friesian x Holstein or Friesian x Ayrshire breeds, aged at least two months old at the start of the experiment. Ear tag numbers were 10T, 34A, 14T, 37A, 41P and 38A.

7.2.2. Immunising material

a) Extraction

Animals were immunised with either the λ gt11-SR1 recombinant protein or with type λ gt11 B-galactosidase (except rabbit 11).

Both these proteins were extracted in an identical manner from SDS-PAGE gels. Thirty ml of induced lysogens (for induction method see Section 6.2.13.) of λ gt11-SR1 or wild type λ gt11 were harvested by centrifugation for six minutes at 2,200 G, 30°C (ME Mistral 3000). The pellets were drained and resuspended in 2.25 ml PBS containing 1 mM PMSF. 0.75 ml SDS sample buffer (x 4) was added and the lysogens were heated at 100°C for five minutes and then stored at -20°C.

After running a gel to ensure that good lysogen induction had been achieved, two 7% polyacrylamide gels were loaded with each lysogen. 1.5 ml lysogen was used per gel. After overnight electrophoresis to separate the reduced proteins, a 1 cm wide longitudinal strip was cut from the centre of each gel and stained for one hour in 0.1% Coomassie blue. After destaining for one hour these acted as templates so that a horizontal strip (approximately 0.1 x 12.5 x 0.9 cm) containing the desired protein (λ gt11-SR1 at 135 kdal or λ gt11-B-galactosidase at 115 kdal) could be cut out of each gel.

The gel strips were macerated, then 4-8 ml PBS was added and the mixture was left to elute overnight at 4°C. The acrylamide was spun down at 2,500 G, 10°C for 30 minutes and the supernatant was removed. This undialysed material was used to immunise mice and rabbits 1608 and 1609.

A more efficient method was used for protein elution to obtain material for the second and third calf immunisations using an electroeluter (Model 422 Electro-eluter, Biorad). In this, gel strips were subjected to electroelution for five hours at 10 mA per electroelution tube in ammonium bicarbonate buffer (0.395% NH_4HCO_3 , 0.1% SDS). The eluted and concentrated protein was collected in buffer from just

above a dialysis membrane (35 kdal pore size). Material, from either elution technique, used to immunise rabbits 73, 75 and 77 and calves was dialysed overnight. The eluted protein in solution was dialysed against two litres of PBS at 4°C (if extracted mechanically) or 20°C (if extracted by electroelution to prevent the NH_4HCO_3 crystallising out of solution).

The dialysed solution was then used for immunisation.

b) Protein estimation

One hundred μl of each protein extraction was taken before or after dialysis to measure protein concentration using the BCA Protein Assay Reagent microprotocol method (Pierce Chemical Company).

c) Adjuvant

When adjuvant was used, an equal volume of sterile complete or incomplete Freund's adjuvant (Gibco) was mixed with the dialysed aqueous protein solution until an emulsion formed.

7.2.3. Immunisation regimes

Table 19 summarises the details of the immunisation protocol followed for each experiment.

Rabbit 11 was immunised with Dermatophilus congolensis antigen combined with Freund's adjuvant as indicated in Table 19. This immunisation was performed by Ian Heron and Dr. Alex Morrow who kindly donated serum from before and after the rabbit was immunised.

Table 19 Immunisation of mice, rabbits and calves to test gt11-SR1 recombinant protein

Experimental animals x number	Identification	Immunising material	Adjuvant (Dose No.)	No. of doses	Time interval between doses	Route of administration	Dose µg	Dose volume ml	Remarks
Mice x 8	1-8	SR1 (non-dialysed)	(SDS)	3	10 days	Intraperitoneal	10-20	0.1-0.2	SDS eluted from gel may have some adjuvant action
x 2	9 and 10	Unimmunised	-	0	-	-	-	-	
Rabbits x 2	1608 and 1609	SR1 (non-dialysed)	(SDS)	3	18 days (1-2) 10 days (2-3)	Intradermal Multiple	50	1.0 (50 µl per site)	As above Inoculations into skin of back
Rabbits x 3	73, 75, 77	SR1	CFA (1+2) IFA (3)	3	4 weeks	Intramuscular	50	0.75-1.0	L and R quadriceps muscles used alternately
x 1	11	D.c.	CFA (1) IFA (2)	2	4 weeks	Intramuscular	500	0.75-1.0	
Calves x 2	10T and 34A	SR1	CFA (1+2)	3	8 weeks (1-2)	Intramuscular	50	1.5-5.0	L and R quadriceps muscles used alternately
x 2	14T and 37A	B-gal	IFA (3)	3	4 weeks (2-3)	Intramuscular	50	1.5-5.0	
x 2	41P and 38A	Unimmunised	-	0	-	-	-	-	

SR1 = gt11-SR1 fusion protein; CFA = Complete Freund's adjuvant; IFA = Incomplete Freund's adjuvant;

D.c. = *Dermatophilus congolensis* antigen; B-gal = gt11-B-galactosidase

7.2.4. Routine monitoring

a) Mice and rabbits

Day 0 serum and serum from day 10 after second and third doses were obtained from all mice and rabbits. Rabbits were bled from the marginal ear vein and mice from the tail vein. On day 10 after their third immunising dose mice were killed and bled out from the heart.

b) Calves

Blood was taken for serum once every two weeks from day 0 and on day 94, 10 days after the last immunising dose. EDTA blood was taken once weekly from day 0 for routine haematology. At the same time, rectal temperatures were measured and the calves were given a general clinical examination during which the reaction at the site of the injection was monitored.

7.2.5. Assessment of response to immunisation

Sera from all animals were tested in an identical manner. Four methods of assessing the animals' responses to immunisation were used:

1. Western blots using λ gt11-SR1 or wild type λ gt11 lysogens as antigen.

2. Western blots using Percoll-purified T. annulata Ankara sporozoites as antigen.

3. IFAT using formalin-fixed T. annulata Ankara sporozoite antigen.

4. In vitro sporozoite inhibition assays.

The methods for all the above have been described in detail in previous chapters.

7.2.6. Challenge of calves

All six calves were challenged 18 days (day 102 of the experiment) after the third immunising dose by subcutaneous inoculation anterior to the left shoulder of 1.0 tick equivalents (0.5 ml) from pooled stabilates of T. annulata Hissar. The order of calf inoculation was as follows:

14T, 10T, 41P, 37A, 34A, 38A

A series of experiments to titrate the infectivity of a T. annulata Hissar sporozoite stabilate in vivo had already been performed at CTVM during the previous year (C.G.D. Brown, personal communication). Table 20 shows the dose dependent effect on various clinical parameters which occurred. Since the infectivity of this pool of stabilates was proven and titrated it was selected as challenge material for this experiment. On the basis of the previous results obtained (Table 20) calves were given a 1.0 te dose which was considered likely to be a lethal challenge to at least 50% of the susceptible calves.

7.2.7. Monitoring reaction to challenge

All measurements and observations were made from day -1 of challenge (day 101 of experiment).

a) Rectal temperature

Rectal temperatures were recorded daily.

b) Haematology and piroplasm parasitaemia

EDTA blood was taken on alternate days up to day 8 when it was taken daily until the first piroplasms and peak piroplasm parasitaemia were observed after which alternate day sampling was resumed.

Table 20 In vivo infectivity titration of the T. annulata Hissar sporozoite stabilate used as challenge material

Dose (t.e.)	Breed	Animal number	Day of			Death
			1st Ma	1st Piro	1st Temp.	
10	Friesian	S24	7	9	8	15
	Friesian	S21	3	9	7	17
	Friesian	S33	5	9	6	21
	Friesian	1P	4	9	6	17
0.1	Hereford	910	7	11	10	-
	Hereford	908	7	11	10	-
	Friesian	S39	9	9	15	22
	Friesian	143N	7	10	10	21
0.001	Friesian	909	14	11	17	26
	Ayrshire	S56	14	12	15	-
	Ayrshire	S57	13	12	18	-
	Friesian	144N	13	10	13	24

- = survived

c) Lymph nodes

The left prescapular lymph node was palpated daily. From day 4, lymph node biopsies of the left prescapular lymph node were taken daily until the first macroschizonts had been detected and confirmed the following day. Thereafter sampling was carried out on alternate days.

d) Clinical examination

The clinical condition of calves was assessed at the same time as samples were taken as described in Section 2.2.8.

The parameters measured included:

rectal temperature	
hyperplasia	} - in lymph node biopsy smears
macroschizonts	
microschizonts	
piroplasm parasitaemia	
packed cell volume	
white blood cell count	
red blood cell count	

7.3. RESULTS

7.3.1. Immunising material

Figure 50a shows Coomassie stains and the corresponding Western blots of representative samples of extracted λ gt11-SR1 fusion protein used to immunise rabbits and mice. Both λ gt11-SR1 protein and λ gt11-B-galactosidase extracted to immunise calves are illustrated in Figure 50b.

These proteins were obtained free of the vast majority of host cell proteins and represented about 90% of the total protein presented to animals at immunisation. The Western blots show that extracted

Figure 50 Purity and immunoreactivity of extracted λ gt11-SR1 fusion protein and wild type λ gt11 B-galactosidase

Coomassie stained gels:

a) tracks 1 and 2

b) tracks 1-4

and corresponding Western blots:

a) tracks 3 and 4

b) tracks 5-8

a) Material used to immunise mice and rabbits:

tracks 1 and 3 crude λ gt11-SR1 lysogen lysate

tracks 2 and 4 extracted λ gt11-SR1 fusion protein.

b) Material used to immunise calves:

tracks 1 and 5 crude λ gt11 lysogen lysate

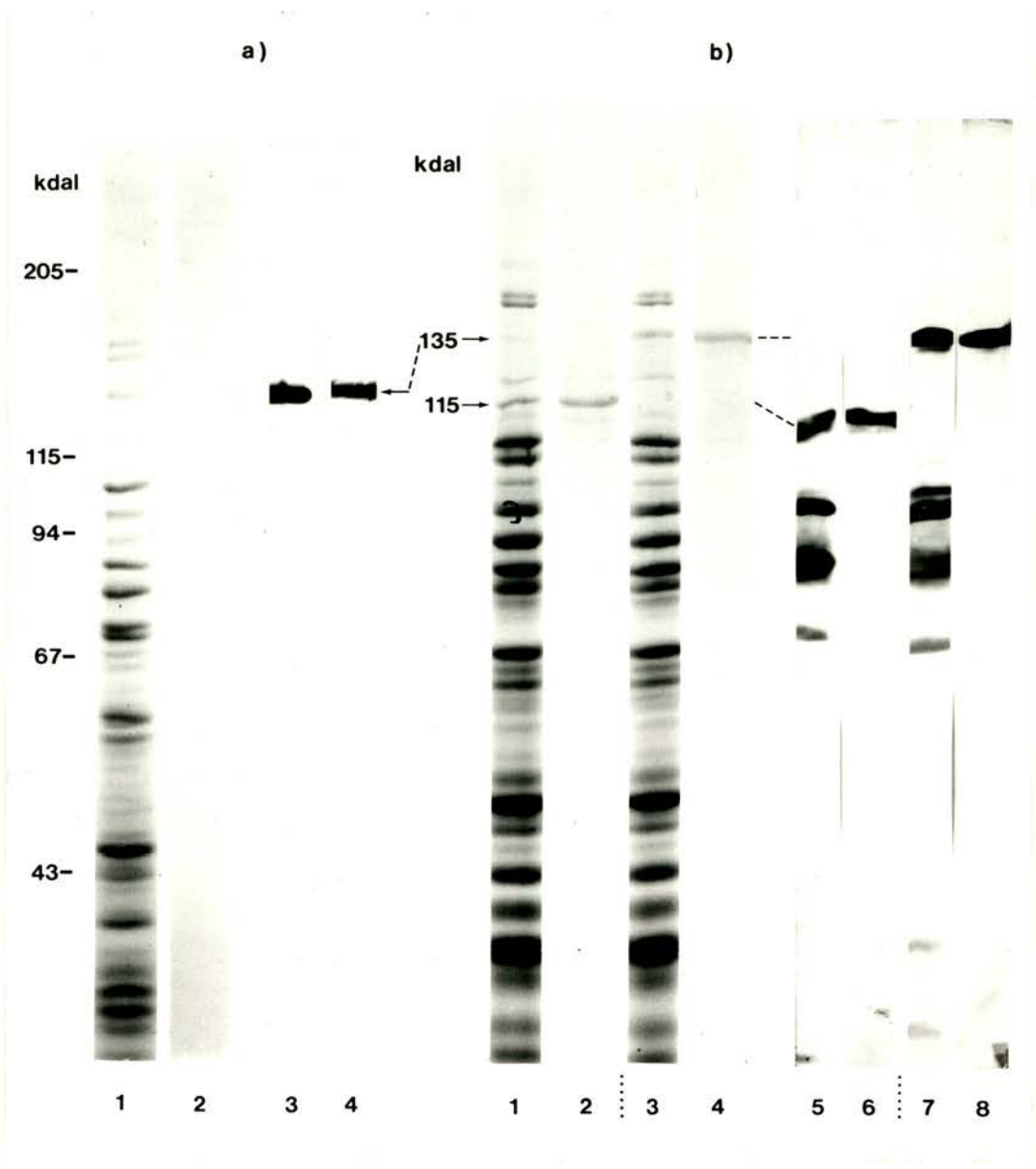
tracks 2 and 6 extracted λ gt11 B-galactosidase

tracks 3 and 7 crude λ gt11-SR1 lysogen lysate

tracks 4 and 8 extracted λ gt11-SR1 fusion protein

Tracks 3 and 4 were reacted with Mab 1A7

Tracks 5-8 were reacted with anti λ gt11-SR1 fusion protein serum (see Section 7.3.3.).



λ gt11-SR1 retained its reactivity with Mab 1A7 (Figure 50a, track 4) and λ gt11-B-galactosidase retained reactivity with serum possessing anti-B-galactosidase activity (Figure 50b, track 6).

7.3.2. Mice

Mice 1-8 were immunised with λ gt11-SR1 fusion protein while mice 9 and 10 remained as unimmunised controls.

a) Reaction with immunising material

After two immunising doses, 4/8 mice had produced antibodies to the λ gt11-SR1 fusion protein used as immunising material (data not shown) and after three doses, 6/8 mice had responded as judged by reactivity with the same 135 kdal protein recognised by Mab 1A7 on a Western blot (Figure 51a). Sera taken from non-immunised mice (Figure 51a, tracks 2 and 3) or mice before immunisation (data not shown) showed no reactivity with this protein.

b) Reaction with sporozoites

The same sera failed to show significant reactivity with T. annulata Ankara sporozoites either by IFAT or Western blotting. Figure 51b illustrates the extremely weak reaction of the serum of mouse 7 after immunisation with sporozoites (track 2). No other sera detected any sporozoite proteins and no sera developed a detectable ant sporozoite IFAT titre (data not shown).

c) In vitro sporozoite inhibition

Immunised and control mice sera were used at dilutions of $1/1$, $1/4$ and $1/16$ in inhibition assays carried out in microtitre wells (see Section 4.3.3.).

Figure 51 Reactivity of mice sera with λ gt11-SR1 fusion protein and sporozoites.

- a) Western blot of induced λ gt11-SR1 lysogen lysate reacted with antibodies:
track 1 Mab 1A7
tracks 2-11 postimmunisation sera from mice 1-10
- b) Western blot of T. annulata Ankara sporozoites reacted with antibodies:
track 1 Mab 1A7
track 2 mouse 7 before immunisation
track 3 mouse 7 after immunisation

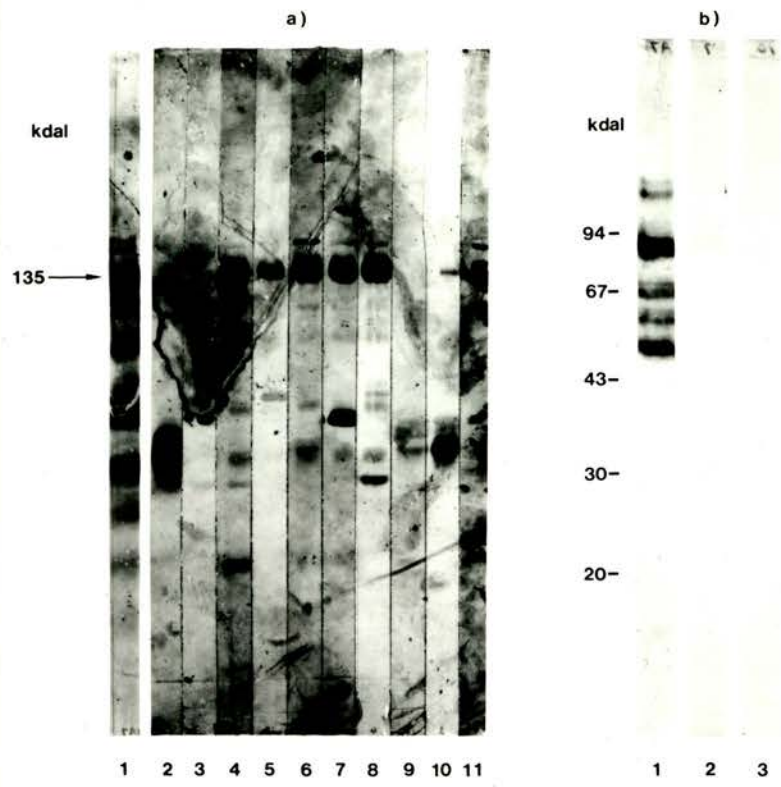
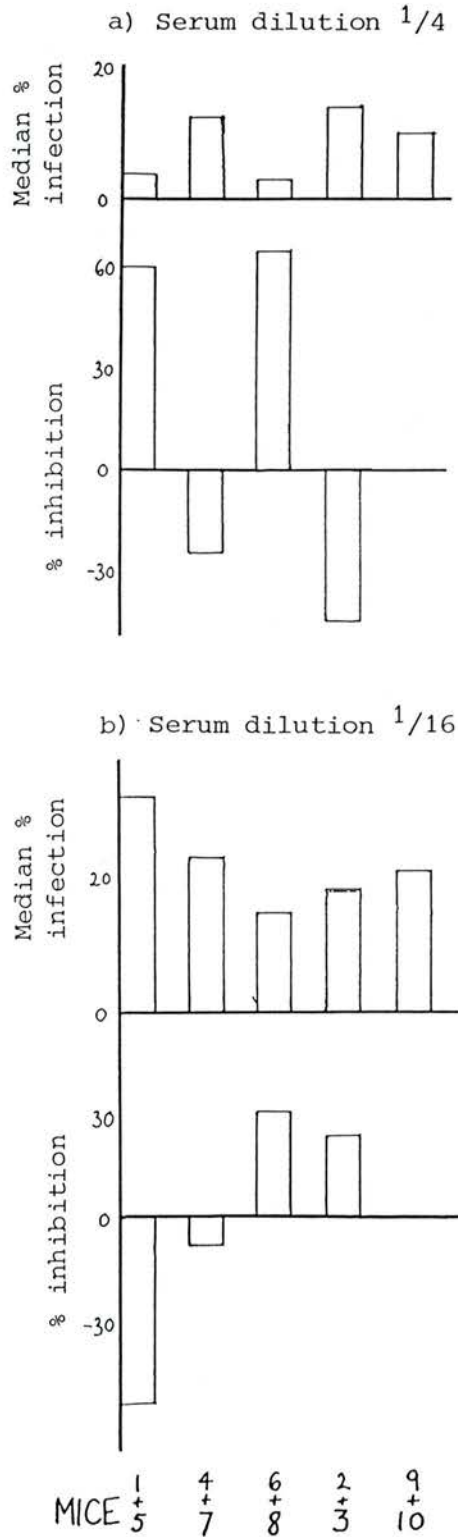


Figure 52 Sporozoite inhibition assay to test sera from mice immunised with λ gt11-SR1 fusion protein. Bar charts showing median % infection and % inhibition levels in assays to test sera from pairs of mice.



Mice were paired and their sera mixed for testing for sporozoite blocking activity as only small amounts of serum were available. Figure 52 shows the results obtained.

Of the mice which produced antibodies to the λ gt11-SR1 fusion protein, only pairs 1+5 and 6+8 showed significant inhibition of sporozoite infectivity; inhibiting 60 and 65% infectivity respectively at $1/4$ serum dilution. This effect was not observed by the same sera diluted further to $1/16$. Pair 4+7 had no significant effect on sporozoite invasion even though mouse 7 serum was the only one with any sign of reactivity with sporozoites on a Western blot. Mouse pair 2+3 which produced no detectable antibody response to the immunising material also lacked sporozoite neutralising activity. Undiluted serum in the assay proved too toxic in all cultures to be of use.

7.3.3. Rabbits

a) Intradermal inoculation

Rabbits 1608 and 1609 were immunised with the extracted λ gt11-SR1 fusion protein on three occasions by multiple intradermal inoculations. Sera taken from these rabbits after two and three immunising doses failed to show a response to the fusion protein at 135 kdal in a Western blot (Figure 53) or to sporozoites by IFAT (data not shown). In view of this lack of response no Western blot or inhibition assay with sporozoites was performed.

b) Intramuscular inoculation with Freund's adjuvant

Rabbits 73, 75 and 77 all received intramuscular inoculations of the extracted λ gt11-SR1 fusion protein combined with complete or

incomplete Freund's adjuvant on three occasions. Rabbit 11 received two doses of Dermatophilus antigen in complete or incomplete Freund's adjuvant.

i) Reaction with immunising material: Serum taken 10 days after the third immunisation (second immunisation of Dermatophilus antigen in rabbit 11) demonstrated anti- λ gt11-SR1 fusion protein activity as strong as that of Mab 1A7 in rabbits 73, 75 and 77 (Figure 54a, tracks 1, 3, 5 and 7). This activity was absent in day 0 sera from the same rabbits (Figure 54a, tracks 2, 4 and 6) and in postimmunisation serum from rabbit 11 (Figure 56b, track 8). Some of the antibody response in rabbits receiving λ gt11-SR1 fusion protein was directed against the bacterial component of the fusion protein since they all reacted with the 115 kdal B-galactosidase protein in Western blots of induced wild type λ gt11 lysogen (Figure 50, tracks 5 and 6; Figure 56a, track 7, data shown for rabbit 77 only).

ii) Reaction with sporozoites: Postimmunisation sera from rabbits 73, 75 and 77 reacted with the same four protein doublets on sporozoites as Mab 1A7 (Figure 54b, tracks 2-4). Again the recognition of the bands was as strong as Mab 1A7 showed with sporozoites on the same blot (Figure 27, track 4). An IFAT using these sera against formalin-fixed T. annulata Ankara sporozoite antigen confirmed that postimmunisation sera from rabbits 73, 75 and 77 recognised sporozoites. The postimmunisation IFAT titre from the three rabbits had risen from $1/10$ to $1/160$ giving a titre comparable to that of rabbit 28 after it was fed on by infected ticks as Table 21 indicates. Rabbit 11 serum had no detectable IFAT titre against sporozoites. Table 21 shows the lack

Figure 53 Reactivity of sera from rabbits inoculated intradermally with λ gt11-SR1 fusion protein.

Western blot of λ gt11-SR1 induced lysogen lysate reacted with antibodies:

track 1 Mab 1A7
track 2 Rabbit 1608 day 0
track 3 Rabbit 1608 day 28
track 4 Rabbit 1608 day 38
track 5 Rabbit 1609 day 0
track 6 Rabbit 1609 day 28
track 7 Rabbit 1609 day 38

Figure 54 Reactivity of sera from rabbits inoculated intramuscularly with λ gt11-SR1 fusion protein in Freund's adjuvant.

Western blots of:

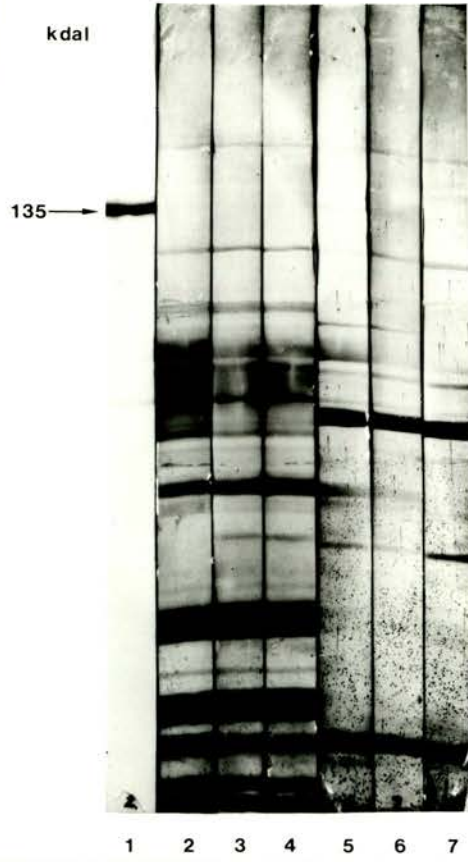
a) λ gt11-SR1 lysogen reacted with:

track 1 Mab 1A7
track 2 Rabbit 73 day 0
track 3 Rabbit 73 day 66
track 4 Rabbit 75 day 0
track 5 Rabbit 75 day 66
track 6 Rabbit 77 day 0
track 7 Rabbit 77 day 66

b) T. annulata Ankara sporozoites reacted with:

track 1 Rabbit 73 day 0
track 2 Rabbit 73 }
track 3 Rabbit 75 } - day 66
track 4 Rabbit 77 }

53



54

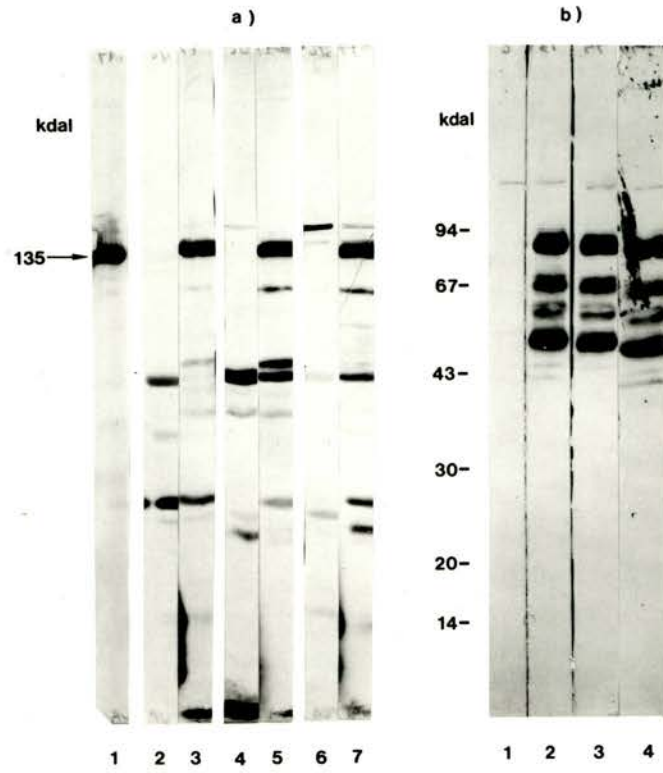


Table 21 Reciprocal IFAT titres of sera from rabbits immunised with λ gt11-SR1 fusion protein, sporozoites or Dermatophilus antigen.

Rabbit	Immunising material	Serum day	Reciprocal IFAT titre			
			Sporozoite antigen		Macroschizont antigen	Piroplasm antigen
			Total Ig	IgG*		
73	λ gt11-SR1	0 66	10 160			
75	"	0 66	10 160			
77	"	0 66	10 160	10 160	- 40	- 10
11	<u>Dermatophilus</u> antigen	0 38	- -			
28	Infected tick feeding	0 73	- 160			

All antigens were prepared from the Ankara stock of T. annulata.

All antigens were formalin-fixed except piroplasm antigen which was acetone-fixed.

- titre less than 1/10

*Class specific fluorescein labelled second antibodies were used to identify antibody class possessing ant sporozoite activity.

of significant reactivity of day 0 and postimmunisation sera from rabbit 77 against macroschizont and piroplasm antigens of T. annulata Ankara in the IFAT, thus the anti- λ gt11-SR1 fusion protein response appeared to be specific to sporozoites. The response predominantly consisted of antibodies of immunoglobulin class IgG with a less marked but detectable IgM component. This was assessed by using two second antibodies, one goat antirabbit (GAR) IgG (Fc) and goat antirabbit IgM (Fc), before a third rabbit antigoaat antibody conjugated with fluorescein.

iii) Sporozoite inhibition assays: Figure 55a shows that all three rabbits immunised with the λ gt11-SR1 fusion protein exhibited strong sporozoite inhibiting activity such that up to 100% inhibition was achieved by serum diluted to $1/4$. Even serum diluted to $1/8$ caused marked neutralisation of sporozoite infectivity, the levels of blocking at both dilutions were similar to those obtained with serum from rabbit 28 which was fed on by infected ticks. Control rabbit 11 which was immunised with Dermatophilus antigen failed to affect sporozoite invasion to any significant degree. To investigate the nature of the sporozoite inhibiting activity of these anti- λ gt11-SR1 fusion protein sera, further assays were performed. Figures 55b and c illustrate the results of these assays, all of which used serum from rabbit 77 since this rabbit had shown the strongest sporozoite neutralising action. This neutralisation was active against all three stocks of T. annulata; Ankara, Gharb and Hissar with no significant differences between the degree of action on each stock. Control rabbit 11 had no effect on sporozoite invasion in any of the stocks. Heat inactivation of rabbit 77 serum reduced the strength of the neutralising activity

Figure 55 Sporozoite inhibition assays to test sera from rabbits immunised with the λ gt11-SR1 fusion protein combined with Freund's adjuvant.

Bar charts showing median % infection and % inhibition levels in assays to test sera from:

a) Rabbits 73, 75, 77 and 11 against T. annulata Ankara sporozoites

Open bars: serum dilution 1/4

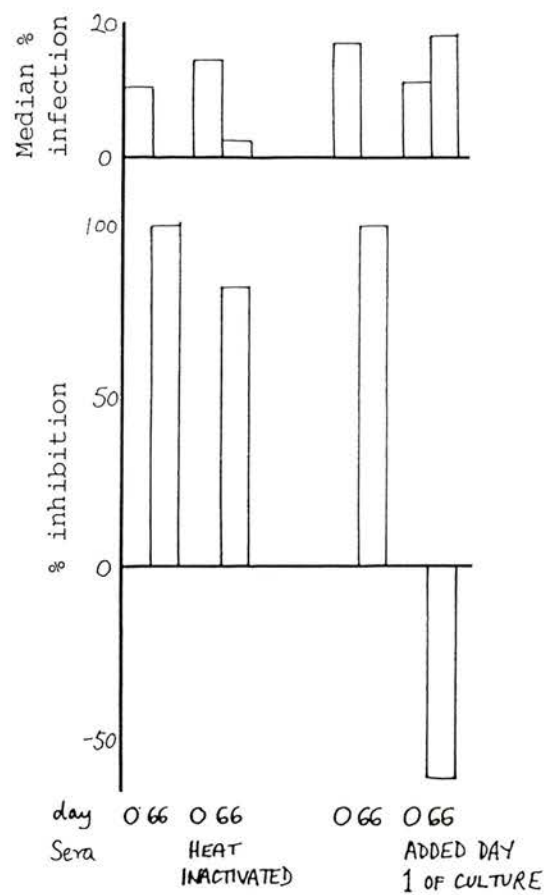
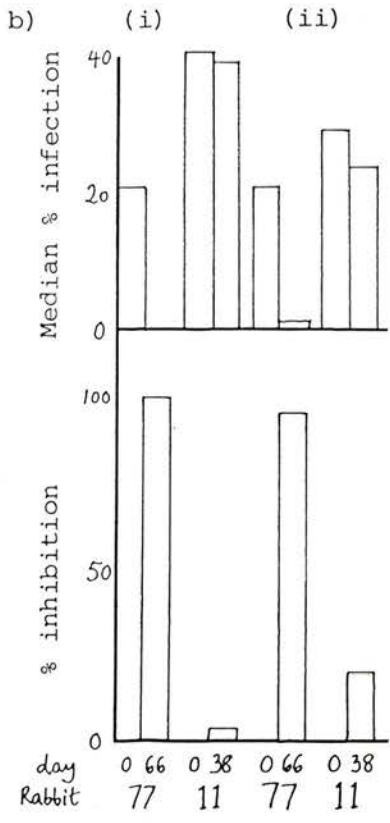
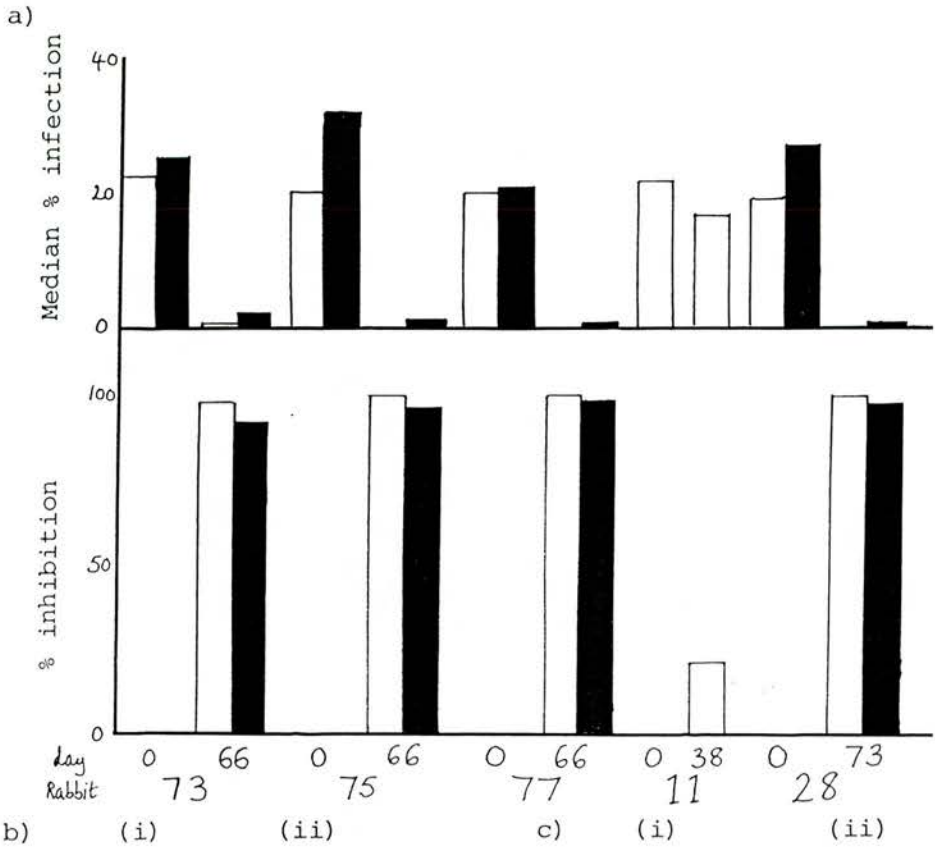
Closed bars: serum dilution 1/8

b) Rabbits 77 and 11 against T. annulata Gharb (i) and Hissar (ii) sporozoites

c) Rabbit 77 against T. annulata Hissar sporozoites.

i) Sera used: normal and heat inactivated

ii) Sera added: day 0 and day 1 of culture



but still left it highly significant (Figure 55c). When serum from rabbit 77 was added 24 hours after in vitro infection of PBM with sporozoites, there was no effect on the level of infection established (Figure 55c).

7.3.4. Calves

Six calves were used in a preliminary immunisation trial. Two calves (10T and 34A) were immunised with λ gt11-SR1 fusion protein, two (14T and 37A) were immunised with λ gt11-B-galactosidase and two (41P and 38A) were kept as unimmunised controls.

a) Reaction to immunising material

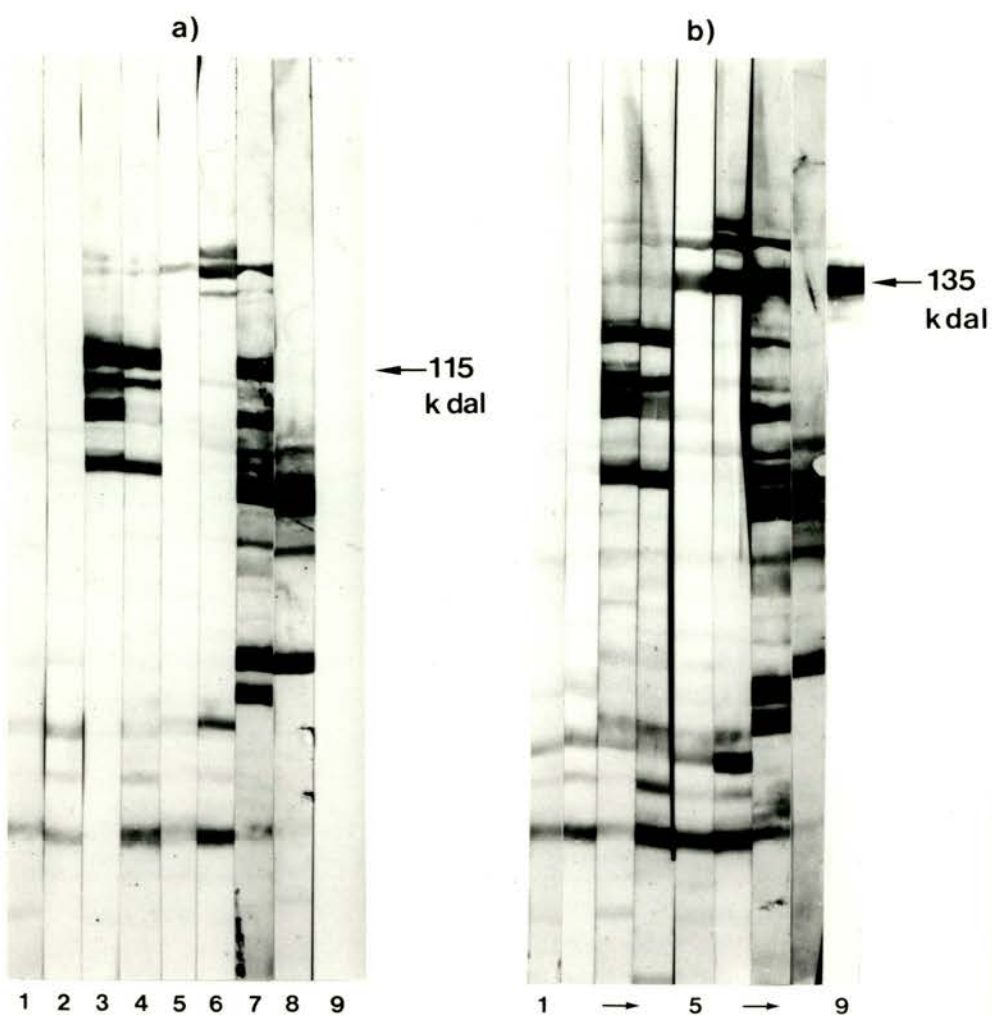
Western blots showing the reaction of λ gt11-SR1 (Figure 56b) and wild type λ gt11 (Figure 56c) lysogens with sera taken from the six calves on day 94 give results which correlate with the material each calf had received. Thus, calves 10T and 34A detected the 135 kdal band representing the λ gt11-SR1 fusion protein (Figure 56b, tracks 5 and 6); calves 14T and 37A detected the 115 kdal band representing B-galactosidase (Figure 56a, tracks 3 and 4) and calves 41P and 38A detected neither of these (Figure 56a and b, tracks 1 and 2). It is interesting to note that calves 10T and 34A did not recognise the 115 kdal protein in wild type λ gt11-SR1 in addition to the 135 kdal protein in λ gt11-SR1. Also the anti-B-galactosidase activity of calves 14T and 37A only gave a faint reaction with the 135 kdal protein in λ gt11-SR1. The extracted B-galactosidase used to immunise calves 14T and 37A, obviously contained some contaminating proteins since these calves detect some other bacterial proteins of similar molecular weight to B-galactosidase in both wild type and recombinant λ gt11 lysogens.

Figure 56 Reaction of calf sera with immunising material.

- a) Western blot: calf sera vs. wild type λ gt11 lysogen.
- b) Western blot: calf sera vs. recombinant λ gt11-SR1 lysogen.

<u>Track</u>	<u>Antibodies</u>
1	Calf 38A}
2	Calf 41P} - non-immunised controls
3	Calf 37A}
4	Calf 14T} - immunised controls (B-gal)
5	Calf 34A}
6	Calf 10T} - immunised (λ gt11-SR1)
7	Rabbit 77 immunised (λ gt11-SR1)
8	Rabbit 11 immunised control (<u>Dermatophilus</u> antigen)
9	Mab 1A7

Calf and rabbit sera were all taken 10 days after the final immunisation.



The intramuscular inoculation sites became palpably and visibly swollen, the degree of swelling was maximal between days 7 and 14 after each immunising dose was administered. No skin ulceration occurred and the calves were not otherwise affected.

b) Reaction to sporozoites

Although the Western blot in Figure 57 is of poor quality, it clearly shows that, like rabbits 73, 75 and 77, postimmunisation sera from calves 10T and 34A (tracks 3 and 4) which received λ gt11-SR1 fusion protein reacted with the same group of sporozoite proteins as Mab 1A7 (track 6). Control immunised calves (tracks 1 and 2) or non-immunised calves (track 5, data shown for calf 41P only) did not recognise any sporozoite bands. Interestingly, there is some variation in the relative intensity of bands detected between calves 10T, 34A and Mab 1A7. In particular, the 104 kdal protein, which was previously a feature of Western blots using crude infected tick homogenate, was stained very intensely by serum from calf 34A (Figure 57, track 3).

Calves 10T and 34A were also shown to recognise sporozoites by using the IFAT. Antisporozoite titres against formalin fixed T. annulata Ankara sporozoites rose from $1/10$ to $1/160$ compared to $1/40$ in calves after immunisation with B-galactosidase and $1/10$ in unimmunised controls.

c) Sporozoite inhibition assay

Again, as demonstrated by anti- λ gt11-SR1 fusion protein sera from rabbits 73, 75 and 77, postimmunisation serum from calves 10T and 34A alone had a marked sporozoite neutralising ability which was

Figure 57 Reactivity of calf sera with T. annulata Ankara sporozoites

Western blot of sporozoite proteins reacted with antibodies:

track 1 Calf 37A }
track 2 Calf 14T } - immunised controls (B-gal)
track 3 Calf 34A }
track 4 Calf 10T } - immunised (λ gt11-SR1)
track 5 Calf 41P non-immunised control
track 6 Mab 1A7

Calf sera all day 94.

The arrows indicate the positions of sporozoite proteins recognised by Mab 1A7 (track 6).

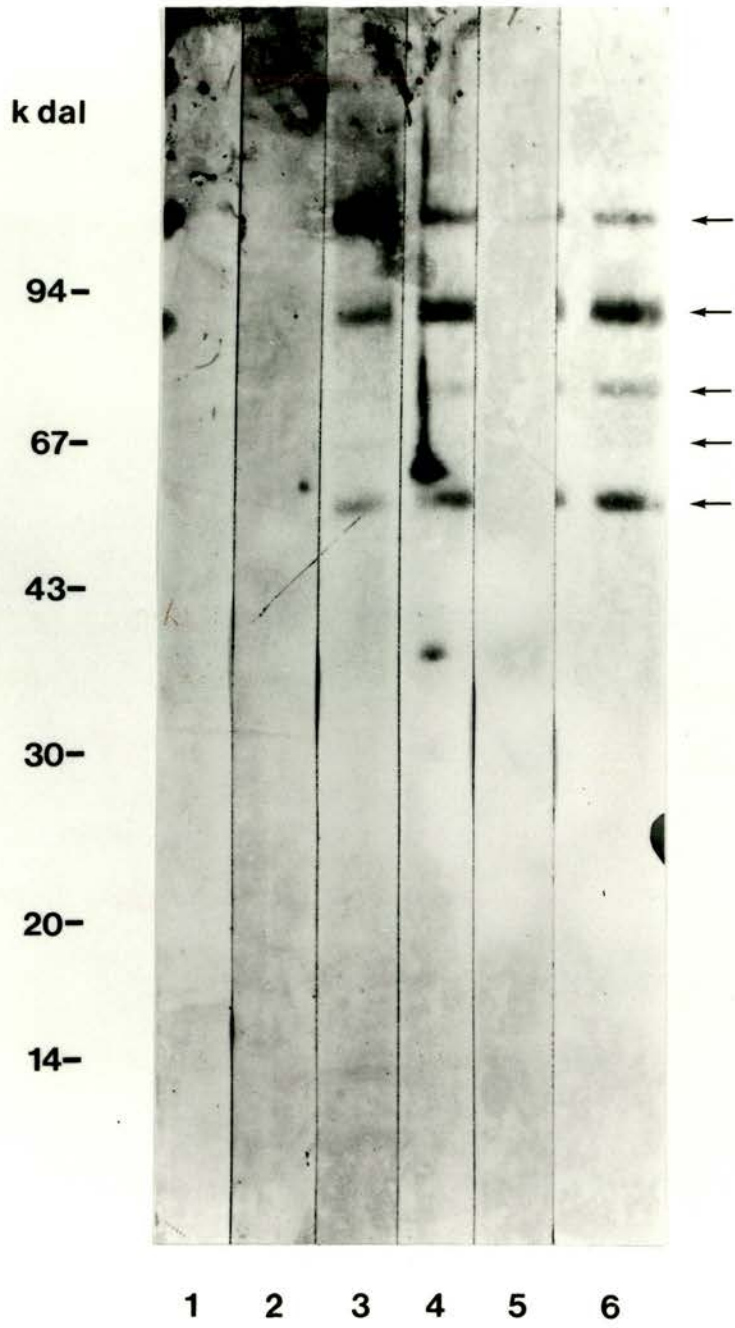
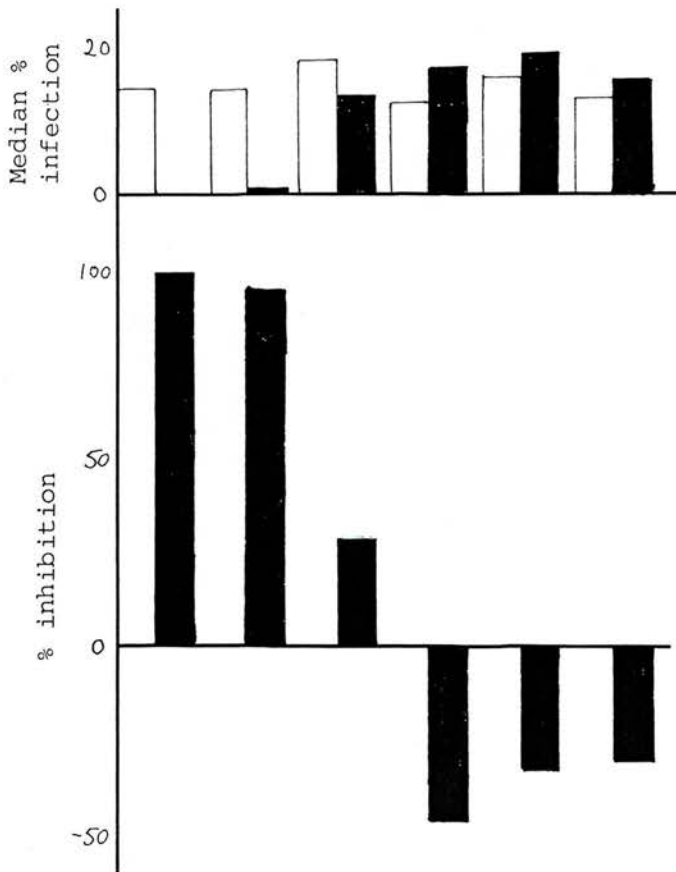


Figure 58 Sporozoite neutralisation assay to test sera from immunised and control calves.

Bar charts showing median % infection and inhibition levels.



day 0 94 0 94 0 94 0 94 0 94 0 94
 CALF 10T 34A 14T 37A 41P 38A

absent from the other four calves' sera also taken on day 94. This is illustrated in Figure 58.

d) Reaction to challenge

On day 102 of the experiment, 18 days after four of the calves had received their final immunising doses, all six calves were challenged with virulent T. annulata Hissar sporozoites and their subsequent reaction was monitored (see Sections 7.2.6. and 7.2.7.).

Figure 59 shows the clinical reactions of the calves illustrated graphically and Table 22 summarises the salient comparative features of these reactions.

All six calves became infected with T. annulata with the appearance of macroschizonts in the local lymph node between 4-7 days and piroplasm infected red blood cells between 9-10 days postinfection. Three calves underwent a severe reaction and were destroyed in extremis (calves 34A, 14T and 37A). The other three calves all survived, two (41P and 38A) having suffered a severe reaction and one (10T) only a moderate clinical reaction.

The calves which were destroyed had been immunised with either B-galactosidase (14T and 37A) or λ gt11-SR1 fusion protein (34A) combined with Freund's adjuvant. At post mortem, 35-39 days after the last immunisation, the intramuscular injection sites were examined. They consisted of localised areas of fibrosis with no significant abscessation or necrosis.

Figure 59 Clinical reactions of immunised and control calves on challenge with live virulent sporozoites.

a) Rectal temperatures (T°C) and presence of macroschizonts and microschizonts in the regional lymph node (heavy lines).

The upper of the heavy lines represents macroschizonts.

The lower of the heavy lines represents microschizonts.

b) White blood cell counts (wbc)

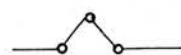
c) Packed cell volumes (% PCV) and piroplasm parasitaemias (% Piro)

% PCV ———

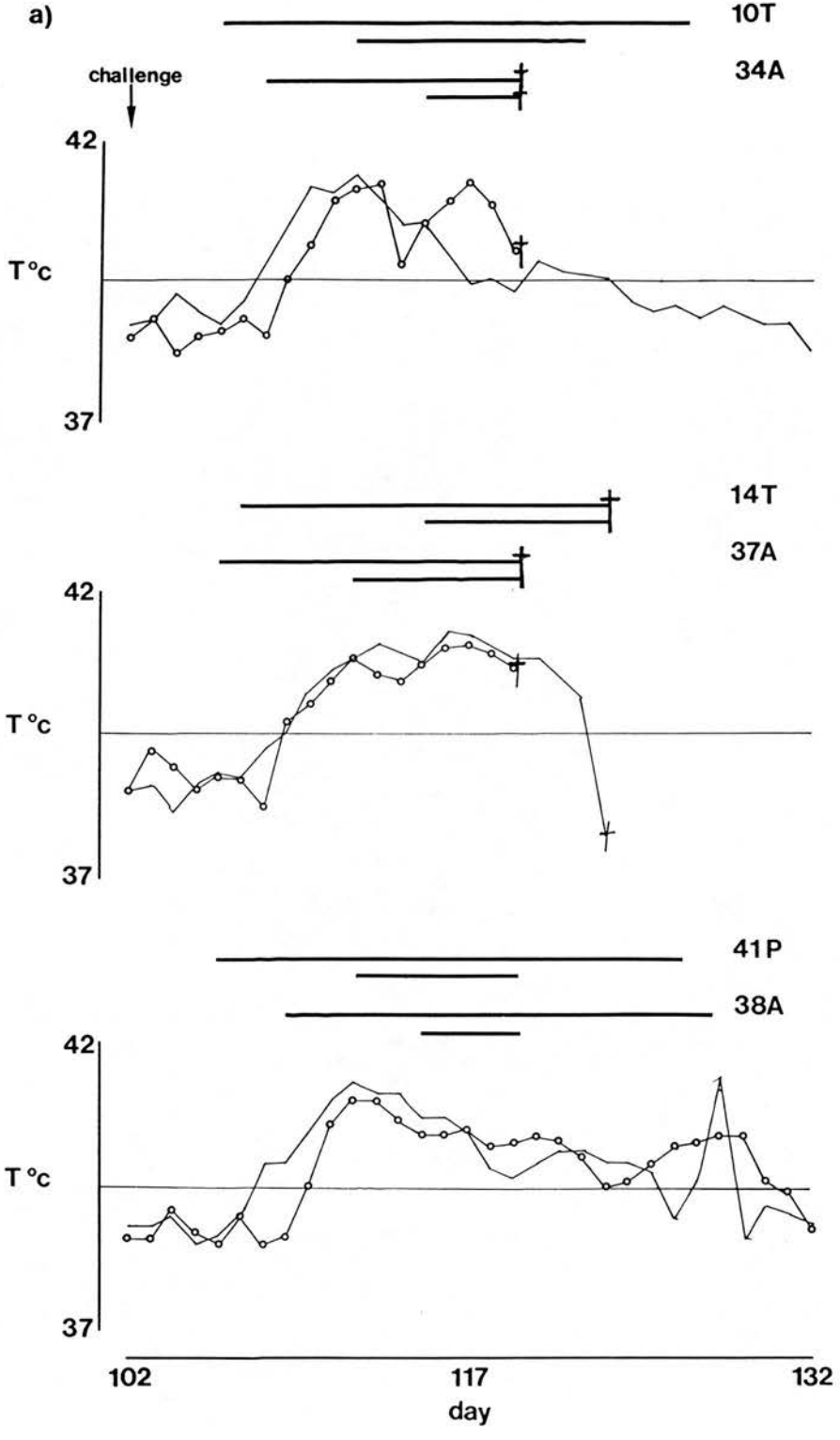
% Piro - - - -

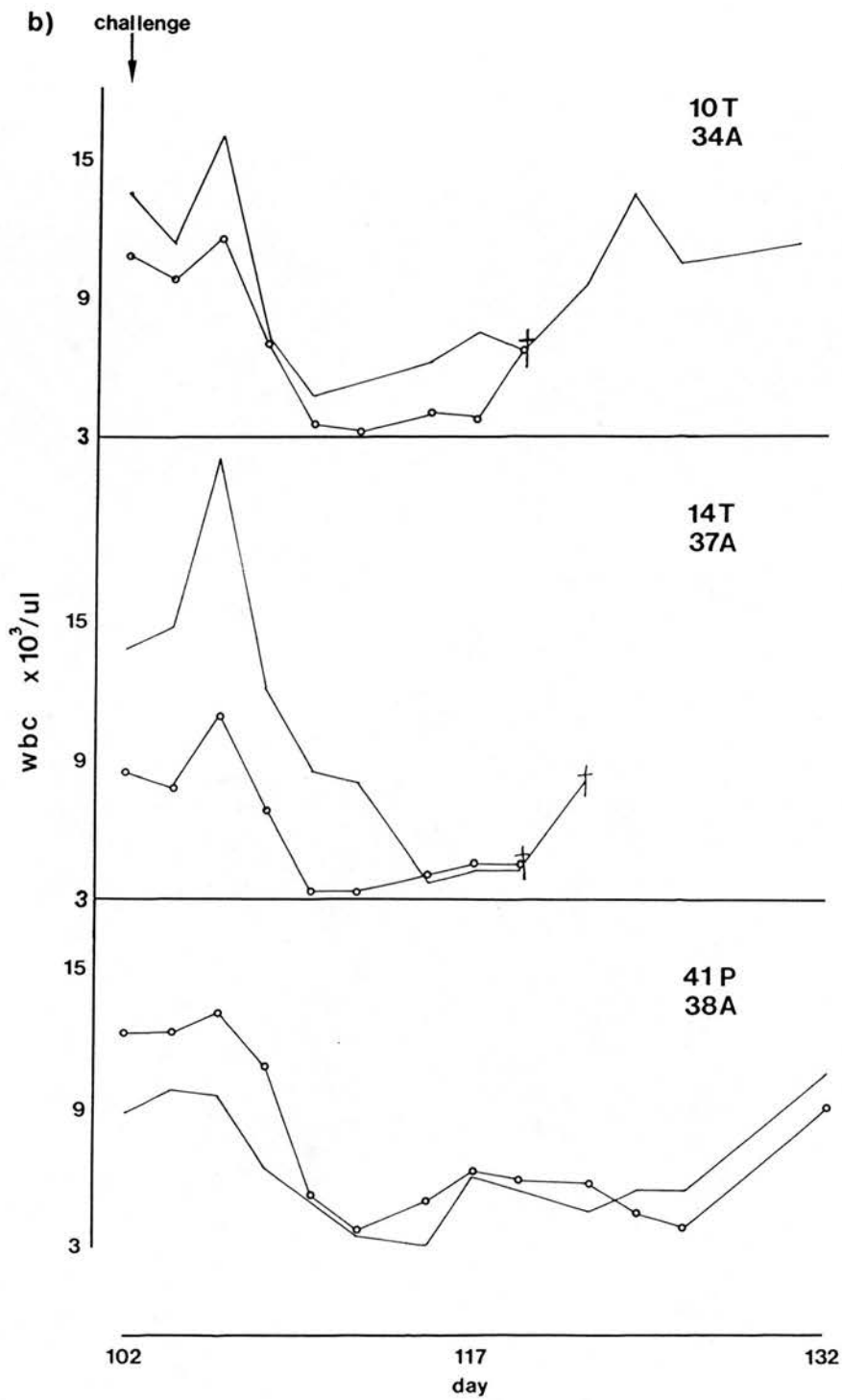
In each diagram:

10T }
 14T } - are represented as 
 41P }

34A }
 37A } - are represented as 
 38A }

† indicates death of the calf





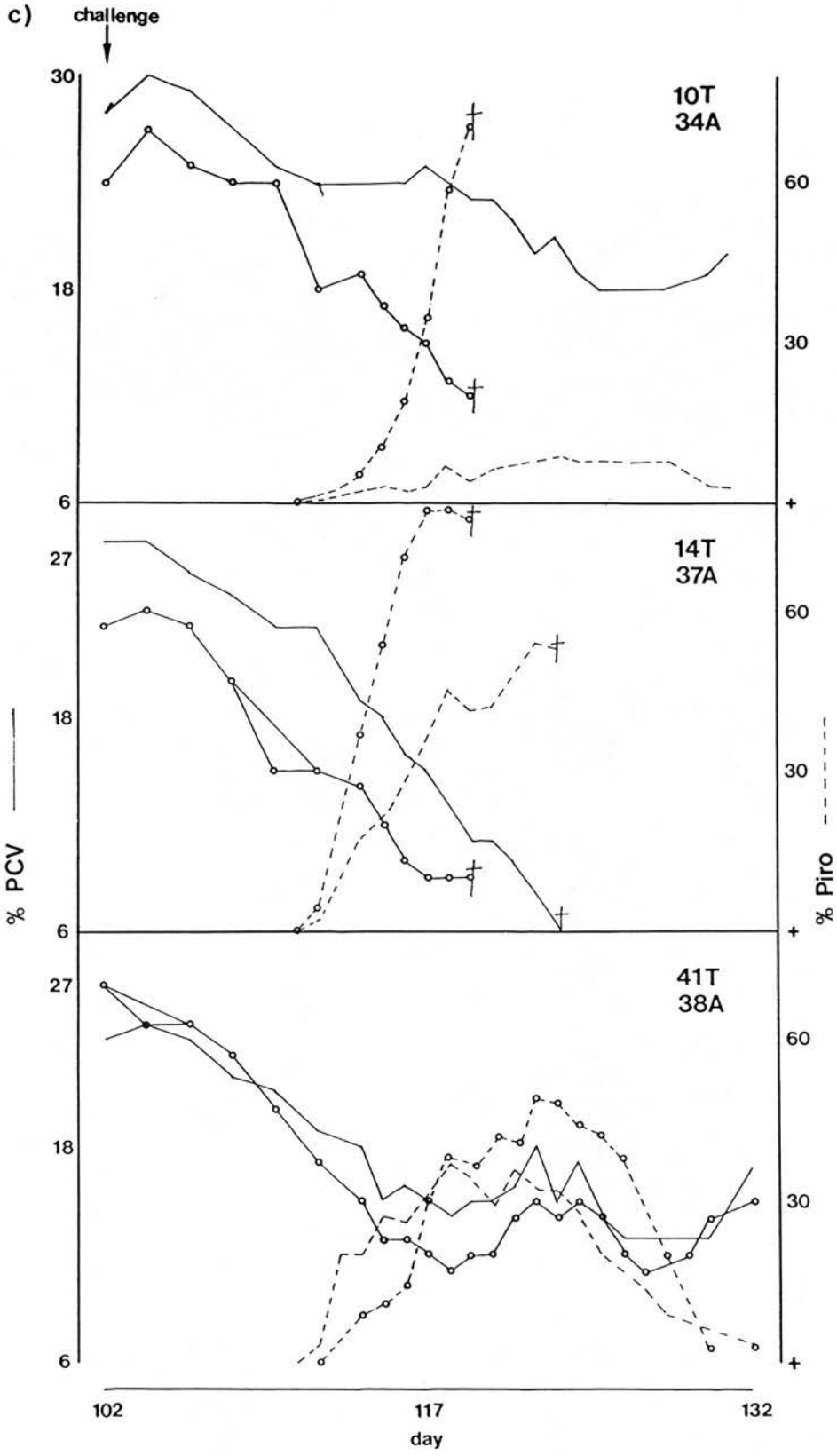


Table 22 Summary of salient comparative features of the calves' clinical reactions to live sporozoite challenge

Calves	Days to							Maximum piro parasitaemia %	% drop PCV	% drop WBC
	First Ma	T°C above 39.5	Ma +++	First piro	Maximum piro parasitaemia %	Death				
Test immunised with λ gt11-SR1										
10T	4	7	8	9	21	-	9	36	65	
34A	6	8	10	9	17	17*	70	50	71	
Control immunised with B-gal										
14T	5	8	10	9	20	21*	54	79	73	
37A	4	7	10	9	16	17*	85	61	61	
Control nonimmunised										
41P	4	6	10	9	17	-	37	46	65	
38A	7	9	13	10	20	-	49	59	69	

Ma macroschizont; T°C rectal temperature; piro piroplasm; PCV packed cell volume
 wbc white blood cell count; - survived; * destroyed in extremis.

7.4. DISCUSSION

The experiments described have assessed the immunogenicity of the λ gt11-SR1 fusion protein in both naturally resistant and susceptible hosts of T. annulata. When administered to calves and rabbits in Freund's adjuvant, a strong antibody response was stimulated which recognised sporozoites and neutralised their infectivity in vitro. However, the calves remained susceptible to challenge with live virulent sporozoites.

The fusion protein used as immunising material remained immunoreactive after extraction and separation from the majority of host cell proteins. High levels of purity were not considered an essential requirement for raising high titres of monospecific antibody against the sporozoite epitope. Indeed, Coppel, Brown, Mitchell, Anders and Kemp (1984) used crude bacterial lysates of induced lysogens to raise antibodies to a mature blood stage antigen of Plasmodium falciparum. However, others recommend that small quantities of a single immunogen are more likely to induce populations of antibody with high specificity and affinity, and consequently, sensitivity for the injected substance (Eisen and Siskind, 1974). Therefore fused polypeptides are more usually purified before being used to prepare polyclonal antisera (Coppel, Cowman, Lingelbach, Brown et al., 1983).

In each experiment different host responses were obtained. Without the use of an adjuvant, rabbits inoculated intradermally failed to mount an antibody response at all and mice inoculated intraperitoneally responded to bacterial but not sporozoite portions of the fusion protein. When the protein solution was dialysed against PBS, combined with Freund's adjuvant and inoculated into an intramuscular

site, strong antibody responses were obtained in both rabbits and calves. Interestingly, sera from these rabbits contained antibodies against both bacterial and sporozoite portions of the fusion protein while the calves only recognised the smaller sporozoite portion. The IFAT confirmed the Western blot data and significant titres against sporozoite antigen were only observed in sera which detected a sporozoite band pattern similar to the reaction of Mab 1A7.

To explain these differences there are several aspects to consider which include: the species of animal immunised, the route of administration, the immunising dose and the use of adjuvant.

The properties of the expression system used in this study were detailed in the literature review. One of several reasons for selecting the system was that the theilerial peptide would be expressed fused to a bacterial protein. The latter could then act as a protein carrier to enhance the immunogenicity of the smaller peptide by stimulating an antihapten response (Lachmann et al., 1986). The failure of rabbits inoculated intradermally to mount a humoral response against the fusion protein most likely reflected a problem in the method of antigen presentation, in particular, the absence of adjuvant. Although inoculation into an intradermal site exposes the immunogen to antigen presenting dendritic cells and macrophages, Vaitukaitis (1981) who described the intradermal immunising technique employed in this study, did recommend the inclusion of Freund's adjuvant. Alternatively, it is possible that only a cellular response was made or that T suppressor cells were activated and reduced the B cell response stimulated (Roitt et al., 1985).

There are a number of possible explanations for the restricted humoral response of the mice which failed to recognise the sporozoite epitope, even though they exhibited a strong response to the bacterial portion of the fusion protein. The principle behind the role of a carrier protein involves linked recognition of the carrier protein by T cells and of the smaller peptide or hapten by B cells (Roitt et al., 1985). Mice as a species only recognise a restricted number of T helper cell determinants on B-galactosidase in spite of the large size of this protein (Krzych, Fowler, Miller and Sercarz, 1982). If the sporozoite epitope was not optimally placed in relation to these determinants, the anti-hapten response may not occur. If this was the case, the hope would be that this phenomenon occurs in mice only. However, there is already a precedent in cattle for antibodies raised against a fusion protein failing to recognise the foreign peptide portion. The failure of a recombinant Babesia bovis antigen to protect cattle against challenge with virulent parasite was attributed to this (Timms, Barry, Gill, Sharp and de Vos, 1988). Whether the cattle were exhibiting the same phenomenon as the mice or whether the method of antigen presentation was at fault was not known.

A second consideration to explain the results is the genetic background of the animals. This can restrict the combination of determinants recognised at either the T or B cell level and can limit the antibody responses of mice (Katz, Maizells, Wicker, Miller and Sercarz, 1982). Winther, Allen, Bomford and Brown (1986) thought this an unlikely reason for their failure to obtain an antibody response to recombinant foot and mouth disease viral protein 1 fused to B-galactosidase. They had used a variety of mouse H₂ haplotypes and, in all,

a proportion failed to recognise the viral portion of the fusion polypeptide. Epitope specific suppression has been described by Herzenberg, Tokuhisa and Herzenberg (1980) who found that priming animals with the carrier protein before inoculation of the carrier-hapten, led to hapten specific suppression. If naturally occurring anti-B-galactosidase antibodies were present in the mice prior to immunisation, a similar effect could occur, however, none of the day 0 mice sera contained detectable levels of such antibody.

Given that an antibody response to the B-galactosidase portion was obtained, the mouse results were unlikely simply to be due to an inadequate immunising dose. Certainly, increasing the dose level had no effect on the response to the foot and mouth disease viral protein (Winther et al., 1986).

The explanation for the mouse response is, therefore, unresolved and could simply be due to an inappropriate method of antigen presentation. An entirely different method used for immunising calves and rabbits did result in a strong and specific antibody response to the sporozoite epitope.

Although Freund's adjuvant is not a realistic alternative for use in a commercial vaccine it was chosen because of its proven ability to potentiate immune responses in cattle. Wells, Emery, Hinson, Morrison and Murray (1982) demonstrated that, of nine different adjuvants, complete Freund's stimulated antibody production and delayed type hypersensitivity against a variant specific surface antigen of Trypanosoma brucei most effectively. Also, successful immunisation trials to protect cattle against Anaplasma marginale infection (Palmer et al., 1986) and virulent Babesia bovis (Wright et al., 1985) both used purified antigens combined with Freund's adjuvant.

Several beneficial properties of Freund's adjuvant may have helped stimulate the antibody response observed in the rabbits and calves. The antigen in emulsion is stabilised and resistant to dispersal and so acts as a depot from which antigen is slowly released, exposing the antigen to the immune system over a prolonged period of time. The oil adjuvant stimulates an inflammatory response at the inoculation site increasing vascular and lymphatic flow, attracting cells of the immune system and increasing the circulation of lymphoid cells. In addition, the heat killed Mycobacterium tuberculosis in complete Freund's adjuvant activates macrophages to produce factors enhancing the immune response (Allison and Byars, 1986).

The calves' antibody response was directed entirely towards the sporozoite portion of the fusion protein, suggesting that the B-galactosidase was being recognised by T helper cells and was acting as a carrier protein without stimulating the production of antibodies to itself. This is a valuable characteristic for a carrier protein. It is observed to occur with the purified protein derivative of tuberculin and repeated intradermal injections of tuberculin in the diagnostic Mantoux test do not give rise to tuberculin sensitivity allowing the test to be used on multiple occasions (Lachmann et al., 1986).

The sporozoite inhibition assay results were particularly exciting since sera from rabbits and calves immunised with the λ gt11-SR1 protein retained the functional ability of Mab 1A7 in neutralising sporozoite infectivity for bovine PBM in vitro. This neutralising action was of a similar order of strength as that of serum from

hyperimmune cattle and was both stronger and more consistent than exhibited by Mab 1A7. Thus, specific interaction of antibody with the sporozoite epitope recognised by Mab 1A7 was confirmed to be responsible for the inhibition of sporozoite infectivity in vitro. The activity was found to operate against all three stocks of T. annulata used in this study. This is of importance because, if functional in vivo, the protective mechanism may afford broad spectrum immunity.

Additional inhibition assays using the rabbit antisera showed the main inhibitory action to be complement independent: the degree of inhibition was only slightly reduced by heat inactivation. Addition of serum on day 1 of the assay had no effect on the number of infected cells establishing and suggests that, as expected, the inhibitory effect was directed at the invasive sporozoite stage rather than at transforming infected cells. When Mazier et al. (1986) used antibody against recombinant peptides of Plasmodium falciparum sporozoites in an in vitro inhibition assay they detected a continued inhibitory effect on the development of exoerythrocytic forms from sporozoites which had managed to penetrate cultured hepatoma cells. Some of the inhibitory effect in these experiments may result from the action of antibody carried into the cell when the sporozoite invades. This possibility was discussed in Section 4.4.

Gysin, Barnwell, Schlesinger, Nussenzweig and Nussenzweig (1984) also found that antibody produced artificially to the sporozoite surface of the monkey parasite, Plasmodium knowlesi, by immunising rabbits with a synthetic peptide, possessed sporozoite neutralising activity. They demonstrated this by inoculating monkeys with sporozoites which

had been incubated in serum containing the antipeptide antibody, these monkeys then failed to develop patent infections.

T. annulata Hissar sporozoites were chosen as the challenge stock for the calf immunisation trial. Strictly speaking, they represented a homologous challenge since the recombinant protein λ gt11-SR1 was derived from a genomic library constructed using T. annulata Hissar piroplasm DNA, although Mab 1A7, which was used to screen the library, was raised against T. annulata Ankara sporozoites.

Prior to challenge, only calves 10T and 34A possessed anti-sporozoite antibody and strong sporozoite neutralising activity. Disappointingly, on challenge there was no difference between their clinical reactions and those of the control calves which could not be put down to the normal variation observed between individual susceptible calves (Sergent et al., 1945). Calf 34A exhibited a very similar pattern of disease to calves 14T and 37A. While calf 10T showed a much lower level of parasitaemia and a relatively minor fall in PCV compared to the other five calves. Little significance can be attached to its recovery in that calves 38A and 41P also survived. This 50% survival rate was unexpectedly high having selected an infective dose judged from previous experience to be between 10-1000 x LD50. Indications that calf 10T was going to suffer only a moderate clinical reaction did not appear until after the first piroplasms were detected. Since the immune mechanism being studied was expected to act principally at the sporozoite stage, one would predict that evidence of a protective effect would be seen soon after infection as a delayed prepatent period (days to first macroschizonts in the regional lymph node and days to temperature rise above 39.5°C) and

fewer macroschizonts in the regional lymph node. Such observations would have indicated that the infective dose had been reduced by the neutralising antibody and would reflect the dose-dependent nature of Theileria infection in cattle (Cunningham, Brown, Burridge, Musoke et al., 1974; Radley, Brown, Burridge, Cunningham et al., 1974; Purnell, 1974). Unfortunately this dose dependency is not always a consistent feature of T. annulata and sporozoite stabilate can still kill individual cattle at doses which fail to infect others (Samish and Pipano, 1981). However, Gill, Bhattacharyulu, Kaur and Singh (1981) monitored host responses in cattle infected with graded stabilate doses or tick numbers and were confident that the severity of reaction to T. annulata was dose dependent.

Obviously it is difficult to draw general conclusions from an immunisation trial with only two calves in each experimental group and using a single method of immunisation and challenge. Under the conditions of this experiment, immunisation with the λ gt11-SR1 recombinant protein was not successful in protecting calves against tropical theileriosis. Whether this failure reflects the fact that antibody responses to sporozoites only play a minor role in protective immunity or whether modifications in the methods of immunisation or challenge would demonstrate the λ gt11-SR1 protein as an effective immunogen with protective capacity are matters for discussion in Chapter 8.

CHAPTER EIGHT

GENERAL DISCUSSION

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The identification of an epitope on the surface of Theileria annulata sporozoites and its production as a recombinant protein, culminated in the failure to stimulate a detectable degree of protective immunity in calves immunised with this protein, in spite of the strong sporozoite neutralising activity developed by their sera.

The identification of sporozoite antigens and the cloning and expression of the gene sequence coding for a selected sporozoite epitope were described and discussed in earlier chapters. For this reason, the discussion here concentrates on the influence of antigen presentation on the success or failure of a potential recombinant DNA vaccine and also on the role of ant sporozoite immunity and whether such immunity is unable to confer protection in vivo.

A review of the literature revealed the uncertainty which surrounds the role of ant sporozoite immunity. Early reports of protection conferred by passive transfer of serum (Koch, 1930b; Robson et al., 1961) have not been repeated successfully (Theiler, 1970; Muhammed et al., 1975). Cell mediated immunity has been shown to play a major role in conferring protection to infection with Theileria (Emery, 1981; Emery et al., 1981; Preston et al., 1983). However, since the discovery that hyperimmune bovine serum blocks sporozoite infectivity in vitro very effectively (Gray and Brown, 1981; Musoke et al., 1982), the sporozoite stage has been the subject of much interest. Techniques for maximising sporozoite yields from infected ticks (Walker et al., 1985) and for their purification (Walker and McKellar, 1983) have made sporozoite material accessible in sufficient quantities to consider, for example, the preparation of sporozoite IFAT antigen

This is important; no such antigen was available for the antisporezoite status of bovine sera used in the passive transfer experiments mentioned above to be assessed. Together with the development of hybridoma technology and genetic engineering, new tools have become available and have been applied to a study of the sporozoite stage, allowing defined antigenic material to be assessed and produced in order to investigate the possibility of developing a subunit non-infective vaccine based on this stage.

8.1. SELECTION OF A SPOROZOITE ANTIGEN

In vitro assays similar to that employed in this study have been used as the basis for selection and assessment of antibody recognising invasive stages of other protozoan parasites (Musoke et al., 1984; Dobbelaere et al., 1984; Hollingdale et al., 1984; Winger et al., 1987; Whitmire et al., 1988). Monoclonal antibodies or antisera which reduce, or completely block, the infectivity of the parasite they recognise are considered to be recognising antigens of potential importance in stimulating an immune response. This was the rationale behind the use of such an assay in this study, particularly as hyperimmune bovine serum had previously been shown to neutralise sporozoite infectivity in vitro (Gray and Brown, 1981; Musoke et al., 1982; Preston and Brown, 1985). Considerable importance has been attached to this sporozoite blocking activity of Theileria immune serum. It was thus disconcerting to observe, when testing serum from calves receiving multiple doses of irradiated sporozoites, that the serum of calf 102 which had been immunised with sporozoites irradiated at the highest dose but which was susceptible to challenge, was highly effective in

blocking sporozoite infectivity in vitro. The implications and possible explanations of this result were fully discussed in Chapter 4. Serum from calves which had been immunised with irradiated sporozoites without becoming patently infected recognised only a single sporozoite antigen (Calves L37 and 102), or none at all (Calf N44), in Western blots. If this was not a fault of the Western blot technique, the implication is that irradiated sporozoites are a relatively poor method of eliciting ant sporozoite antibodies compared to exposure to fully virulent live sporozoites: sera from the rabbit which was fed on by infected ticks and from hyperimmunised calves gave strong multiple reactions with sporozoite proteins on Western blots. It is clear that in vitro sporozoite neutralisation alone does not necessarily indicate prediction of an effective immune response. Nevertheless Mabs 1A7 and 4B11 were selected on the basis of in vitro sporozoite neutralisation since they appeared to be potentially of greater interest than other Mabs which failed to block sporozoite infectivity. Inhibition assays using the anti- λ gt11-SRI sera showed the inhibitory effect to act within the first 24 hours, presumably on sporozoite invasion. Extrapolating from this, the assumption was that, by binding to the sporozoite antigen, Mabs 1A7 and 4B11 interfered with the sporozoites' recognition, attachment or entry into the host cells. However, in vivo testing of the antigens recognised by these Mabs was made a priority early on. As described in Chapter 5, recognition of the epitopes detected by Mabs 1A7 and 4B11 by immune serum was variable. Recognition by immune serum raised in the definitive host, in this case, cattle, is not a prerequisite for a parasite antigen to be chosen for testing as vaccine material, although it is a useful

feature as it indicates that the natural host of the parasite is capable of responding to the epitope involved. Goff et al. (1988) identified Babesia bovis merozoite surface antigens with Mabs and considered the recognition of these antigens by immune bovine serum to be evidence that the antigens could be considered important in stimulating protective immunity. Mitchell (1984) points out that novel parasite antigens of negligible immunogenicity to the natural host when presented as a component of the parasite during infection, might be recognised by monoclonal antibodies. If such antigens were isolated from the parasite and presented in a suitable manner to the host, a response absent during natural infection might be elicited and consequently a novel effector mechanism might confer protection to challenge with live parasites.

It was fortuitous that it was the sporozoite epitope recognised by Mab 1A7 which was obtained as a recombinant protein. This recombinant was shown to contain a gene sequence which was sporozoite specific in expression and highly conserved; as such it was common to sporozoites from all stocks of T. annulata which were tested.

These features of the 1A7 epitope, together with the strong sporozoite neutralising activity of sera from rabbits immunised with the expressed recombinant protein bearing the epitope, meant that it was considered worthwhile and necessary to determine whether immunisation of calves with this protein would protect them against a lethal challenge with sporozoites of Theileria annulata.

8.2. ANTIGEN PRESENTATION

In this study, cattle were presented with a polypeptide bearing a sporozoite epitope capable of stimulating a sporozoite neutralising antibody response. Given alone such a vaccine would have no means of amplifying either itself or the immune response mounted against it. In addition, even if a protein fragment, such as this sporozoite protein possesses specificity as demonstrated by its reactivity with specific antibody, it may lack essential antigenic determinants which confer immunogenic potency on the native protein structure from which it derives.

For these reasons, which apply to many new forms of immunogen being tested as vaccines, the features of antigen presentation which influence whether and how an effective and durable immune response is induced are coming under closer scrutiny (Kaye, 1987).

Different strategies can be adopted to manipulate the immunogenic properties of an immunogen and the application of these to the recombinant protein used to immunise calves is discussed below.

Soluble small proteins or peptides bearing few, or even single, epitopes administered alone are generally non-immunogenic and require the use of adjuvant (Deans, Knight, Jean, Waters et al., 1988) or conjugation to carrier proteins (Itoh, Takai, Ahnuma, Kitajima et al., 1986) for an immune response to be stimulated.

Adjuvants particularly influence whether the immune response elicited is predominantly cell mediated or humoral and the isotype of antibody produced (Allison and Byars, 1986). The use of a carrier protein is important in directing the attention of the immune system to particular epitopes, in stimulating linked T and B cell responses and in conferring memory (Zanetti et al., 1987).

The isotype of antisporezoite antibody in the immunised calves may have influenced the effectiveness of the antibodies in vivo. IgG was certainly stimulated since the peroxidase conjugate used in Western blots was antibovine IgG, but the IgG subclass was not determined. Whether other antibody isotypes were present was also not determined. Musoke et al. (1982) found that the IgG2 containing fraction of T. parva hyperimmune bovine serum possessed most of the neutralising activity. Opsonisation by antibody, without the involvement of complement is mediated particularly efficiently by IgG2 antibody (McGuire, Musoke and Kurtti, 1979) which is also able to fix bovine complement. These effector mechanisms would tend not to be detected in in vitro assay because of the absence of phagocytic cells but might afford greater activity in vivo than that predicted from the in vitro assay. Complete Freund's adjuvant (CFA) stimulates predominantly IgG2 antibody in the guinea pig while incomplete FA stimulates mainly IgG1 (White, 1976). If the same is true in cattle, one would have expected both IgG1 and IgG2 to have been elicited since both CFA and IFA were used during immunisation. Thus the appropriate immunoglobulin isotype should have been present.

The carrier protein used can also influence antibody isotype. Bacterial polysaccharides, such as tetanus toxin, have been conjugated to proteins from organisms such as Haemophilus influenzae type b (Chu, Schneerson, Robins and Rastogi, 1983) with the specific intention of changing the isotype of antibody. Use of tetanus toxin induced the formation of protective IgG antibody to the Haemophilus influenzae component and also induced immunological memory whereas, without this carrier protein, IgM antibody providing poor protection was elicited and memory was poor.

Reasons for the humoral response of the immunised calves failing to confer protection may relate to the absence of accessory immune responses resulting in a lack of cooperation between B, T and antigen presenting cells. The antibody response detected in the calves could be interpreted as being the result of highly regulated collaboration between T helper and B cells, possibly together with modulation from T suppressor cells (Zaretti et al., 1987). Without assaying for T helper cell function one can only speculate on how the response was obtained and how modifying the presentation of the epitope might have made the immune response more effective in protection. T helper cell determinants on the B-galactosidase molecule were presumably recognised by the calves and gave rise to the antihapten response to the sporozoite epitope. Different carriers fused to a purified recombinant protein encoding the immunodominant region of the Plasmodium falciparum circumsporozoite protein were tested in rabbits (Que et al., 1988), Cholera toxin and Pseudomonas aenigihosa toxin A conjugates to the protein made the circumsporozoite portion significantly more immunogenic (ibid). Previous trials in human volunteers using reported inoculations of the same purified recombinant protein had only generated partial protection in those given high doses (Ballou et al., 1987). Increased immunogenicity using different non-toxic conjugated carrier proteins may be an aim for future immunisation trials using the λ gt11-SR1 fusion protein. Immunogenicity may be increased through more favourable spacing of T helper and B cell determinants or simply by providing a greater number of determinants for interaction.

Inadequate T cell responses to the carrier protein were not thought to be a problem in the calves in that they produced antibodies reacting strongly with the sporozoite epitope. Lack of T cell determinants on the sporozoite portion of the molecule could, however, have had profound effects on the effector mechanisms stimulated (Zaretti et al., 1987). No work has been reported on the cell mediated responses to sporozoites of Theileria. Extrapolating from studies of the immune response of humans to the circumsporozoite protein of Plasmodium falciparum (Campbell, Paleologo, Franke, Ratiwayanto et al., 1988), it is likely that a recombinant protein, such as λ gt11-SRI which represents only a small portion of the larger antigenic molecule on the sporozoite from which it derives may lack B and T cell determinants crucial to the development of a significant immune response. The fewer T helper cell determinants on the sporozoite portion of the fusion protein, the more likely one is to see genetic restriction of the bovine T cell response to the immunising material. Such genetic restriction has been shown to occur in the response of mice to the repeat region of the circumsporozoite protein of Plasmodium falciparum (Del Giudice, Cooper, Merino, Verdini et al., 1986) and there is circumstantial evidence that the same is true of man (Campbell et al., 1988). Absence of crucial T helper determinants may have limited the magnitude or affinity of the antibody response to the λ gt11-SRI sporozoite epitope making neutralising antibody less likely to be functional in vivo, establishment of memory may also have been poorer. An in vitro assay for detecting T helper determinants on proteins has been developed by Lamb and Young (1987). In this, native sporozoites or recombinant proteins separated by SDS-PAGE

and blotted onto nitrocellulose paper could be tested individually for their ability to stimulate proliferation of T helper cell lines established from Theileria immune cattle. By using a larger portion or the whole of the native molecule to immunise, a more effective immune response might be elicited, although care may be required to avoid determinants recognised by T suppressor cells. The co-existence of T suppressor and T helper determinants in an antigen can lead to non-immunogenicity as the T suppressor cells stimulation prevents establishment of all the T helper cells (Jensen, Pierce and Kapp, 1984). Fortunately, the two types of determinant are usually well separated (Sercarz, Yowell, Turkin et al., 1978) allowing the portions containing suppressor determinants to be cut out.

Even when the appropriate B and T cell determinants are present on the immunogen, the correct balance of humoral and cell mediated responses still needs to be recruited. CFA is capable of stimulating both humoral and cell mediated responses while IFA tends to result in antibody production only (Allison and Byars, 1986),

The physico-chemical properties of the recombinant protein itself may be important. Proteins which have been denatured are frequently more immunogenic than the native molecules, assuming the epitopes remain intact, because the hydrophobic inner domains of the molecules are exposed and the molecules aggregate (Dresser and Mitchison, 1968).

The use of a virus which replicates in mammalian cells as the expression vector is another alternative for production of this sporozoite recombinant. For example, if vaccinia virus were used for this

purpose it would provide an efficient method of antigen delivery and circumvent the need for carrier proteins and adjuvants (Smith, Cheng and Moss, 1986). Obviously the protective capacity of immune response would still be influenced by the availability of T and B cell determinants on the recombinant protein produced.

Recombinant vaccinia virus contains foreign DNA spliced onto a vaccinia promoter and introduced into a region of the vaccinia genome not essential for virus replication (Panicali and Paoletti, 1982; Mackett, Smith and Moss, 1982). Vaccinia virus holds certain attractions as a vector for a recombinant vaccine. Firstly, the vaccinia virus can accept a relatively large quantity of foreign DNA (Smith and Moss, 1983), thus the whole sporozoite antigen gene could be cloned together with antigen genes from other stages of the parasite, or even other pathogens (Perkus, Piccini, Lipinskas and Paoletti, 1985). Secondly, the replication of recombinant vaccinia virus in cells of the host not only amplifies the quantity of recombinant protein presented but can also result in expression of the recombinant protein on the surface of the infected cells (Smith et al., 1986). Immunisation with vaccinia virus recombinants has already been shown to stimulate production of protective antibody against hepatitis B (Moss, Smith, Gerin and Purcell, 1984) and rabies (Wiktor, McFarlan, Reagan, Dietzschold et al., 1984) viruses. Expression on the surface of infected cells means that protective antigens can be identified together with the MHC of the host, an ideal situation for stimulating cell mediated immune responses. This would, perhaps, make vaccinia a suitable choice for cloning the gene coding for the 4H5 antigen on

the surface of T. annulata-infected cells (Shiels et al., 1985a; Preston et al., 1986). With these advantages in mind, the circumsporozoite genes of Plasmodium knowlesi and Plasmodium falciparum have been cloned and expressed in vaccinia virus and the recombinant products elicited specific antibody to the native circumsporozoite protein in rabbits (Smith, Godson, Nussenzweig, Nussenzweig et al., 1984; Smith et al., 1986).

8.3. ROLE OF ANTISPOROZOITE HUMORAL IMMUNITY

If one accepts that a suitable sporozoite epitope was selected for the immunisation trial and presented to the calves in an adequate manner and, assuming crucial T cell responses were not missing, the immune response was potentially protective, then the factors which may have prevented the response from being effective must be considered.

The main action of ant sporozoite antibody in theileriosis is believed to be inhibition of sporozoite invasion (Preston and Brown, 1985). If effects mediated by antibody occur after sporozoite invasion through recognition of antigen shed during invasion but remaining on the surface of the infected cell (Dobbelaere et al., 1985a) or by the action of antibody bound to sporozoites which still managed to enter cells (Mazier et al., 1986; Hollingdale, 1987), these are shortlived and have not actually been demonstrated as effector mechanisms in bovine theileriosis. Thus, for the ant sporozoite immunity to operate, the sporozoites must be exposed to antibody before they can enter cells. The length of time sporozoites are exposed to antibody in part depends on where the target cell becomes infected since,

wherever it is, the process is rapid once they have made contact with the cells (Fawcett et al., 1982; Jura et al., 1983).

By the time the number of sporozoites entering the bovine host is reaching its maximum, a cellular reaction has developed at the site of the Hyalomma tick bite. Gill and Walker (1985) describe this reaction in rabbits and noted that the nature of the cells present depended on whether the animal had been exposed to ticks on previous occasions; with repeated tick infestation the proportion of mononuclear cells, some of which can be infected by T. annulata sporozoites, increases. Thus, the tick attachment site may provide readily available host cells in the immediate vicinity of sporozoite entry, again leaving little opportunity for antibody to act. The presence of infectable cells at the tick attachment site should, however, be interpreted with caution. The cells recruited may include stimulated B cells and plasma cells actively secreting ant sporozoite antibody; an anamnestic response only taking three days to develop (MacLeod, 1953).

Thus tick feeding may decrease or increase the likelihood of ant sporozoite antibody being effective. If the latter, subcutaneous inoculation of sporozoite stabilate might have been an unrealistic method of challenging the immunised calves. Establishment of a tick bite lesion and a slow "trickle" of sporozoites into the host over 5-6 days (Bhattacharyulu et al., 1975) may be necessary to allow ant sporozoite antibody to neutralise sporozoite infectivity in vivo. In this respect, ant sporozoite immunity in theileriosis may have an advantage over the same in malaria where the mosquito inoculates sporozoites directly into the bloodstream over just a few seconds

(Manwell, 1968). In the calf immunisation trial described in this thesis, sporozoite stabilate was chosen as the method of challenge because, in view of the small number of calves in each group, the challenge needed to be as uniform as possible. Tick challenge is inevitably more variable than stabilate challenge as individual ticks vary in infectivity. This was clearly shown by Cunningham, Brown, Burridge, Irvin et al. (1973) when they compared the infectivity and virulence of a T. parva sporozoite stabilate with the classical 10 tick challenge which had been used previously.

Where bovine mononuclear cells become infected with sporozoites is still open to speculation and it is possible that sporozoites reach the lymph node draining the site of the tick bite before entering host cells. Profuse amounts of saliva are produced by the feeding tick and the excess fluid is drained by the lymphatic system via the regional lymph node (Theis, Franti, Engel and Littrell, 1976). Large numbers of lymphoid cells are available for sporozoites to infect within the lymph node, although if a proportion of these are secreting ant sporozoite antibody, the sporozoites may be exposed to higher levels of inhibitory antibody in this environment.

Alternatively, it is conceivable that PBM are predominantly infected while in the tick mouthparts. The tick feeds using a pumping action such that mononuclear cells could enter the mouthparts as the tick ingests blood, become infected and then be returned to the bovine host with a pulse of saliva. Tatchell, Carnell and Kemp (1972) describe this feeding action in ticks. Such an infection mechanism, if it occurs, would severely restrict the access of antibody to sporozoites.

There are, therefore, a number of reasons why antisporozoite antibody may have failed to inhibit sporozoite invasion in vivo even when very effective in vitro blocking had been obtained.

If challenge with ticks instead of sporozoite stabilate inoculation allowed a proportion of the sporozoites entering the bovine host to be neutralised, this would serve to reduce the infective dose. Given the dose dependent nature of Theileria infection discussed in Chapter 7 (Cunningham et al., 1974; Gill et al., 1981), this could permit a successful immune response and recovery.

8.4. FUTURE PROSPECTS

Further work is necessary to define the contribution of anti-sporozoite immunity to protection against tropical theileriosis. An assessment of whether antisporozoite immunity becomes more effective in cattle under heavy tick challenge in an endemic area would be of great interest and analysis of sera from such cattle would be valuable in this respect. At the same time, the use of inactivated sporozoites to immunise calves, followed by correlation of their IFAT and Western blot reactions and in vitro sporozoite neutralising activity with their reaction to challenge would help elucidate the role of antisporozoite immunity. Serum from cattle repeatedly immunised with freeze thawed T. parva sporozoites was used to select recombinants coding for sporozoite epitopes considered as potentially protective (Iams et al., 1988). This serum was described as possessing in vitro sporozoite neutralising activity but no indication was given of the immune status of the cattle to ECF (*ibid*).

It may be worthwhile performing further immunisation trials using the λ gt11-SR1 protein, and also the polypeptide expressed by the whole gene which has now been cloned (Dr. F.R. Hall, personal communication). Larger numbers of cattle, a variety of immunisation regimes and challenge with ticks or titrated doses of sporozoites, may allow a more realistic assessment of protective capacity of the response stimulated and may reveal a degree of protective immunity resulting from the action of antibody directed against this particular sporozoite epitope.

To stimulate more complete immunity may then require the use of the sporozoite recombinant as part of a "cocktail" vaccine together with antigens from other stages. For example, antigens on the surface of parasitised mononuclear cells recognised by genetically restricted cytotoxic T lymphocytes (Preston et al., 1983; Hall, 1988). Such an antigen might be the 4H5 antigen on the surface of infected cells (Shiels et al., 1986a) which mediates complement lysis of these cells (Preston et al., 1986). In this way, a number of immune mechanisms would be activated to simulate the protective immune responses elicited during active infection with T. annulata.

Particularly in the case of tropical theileriosis in which anaemia is a component of the pathogenesis, it would also be exciting to attempt to immunise against the merozoite stage of the parasite. An approach could be used similar to that adopted in this study. The merozoite is the only stage in the bovine host, other than the sporozoite, which is invasive and extracellular for a period of time. Coppel, Favaloro, Crewther, Burkot et al. (1985b) describe a blood stage antigen of Plasmodium falciparum which shares determinants with

the sporozoite coat protein. Whether similar antigens common to sporozoite and merozoite stages occur in T. annulata and whether they would be relevant to stimulating protective immunity has yet to be investigated.

Bearing in mind that epidemiological tools may contribute to disease control, the work described in this study may have yielded reagents of use in this area. Monoclonal antibody 1A7 or the gene fragment cloned in λ gt11-SR1 may prove useful for other purposes, such as a gene probe for identification of infection in tick salivary glands. This depends on whether a detection system can be established which is less cumbersome and more accurate than existing methods for assessing the rate of tick infection (Walker et al., 1979).

Even if this study has not resulted in the production of an effective subunit sporozoite vaccine for tropical theileriosis, some insight has been afforded into the role of ant sporozoite immunity, the sporozoite antigens involved and the dubious relevance of in vitro neutralisation assays to in vivo protection. In addition, the analysis of the cloned λ gt11-SR1 recombinant demonstrated the presence of a mixed parasite population showing polymorphism of the SR1 locus. The expression of the gene fragment was stage specific, occurring only in sporoblast and sporozoite stages and thus affords a model system for studying the control of differential gene transcription in this important parasite.

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APPENDIX I

REAGENTS AND SOLUTIONS

I.1. REF. CHAPTER 2

I.1.1. Anticoagulantsa) Heparin

Preservative free heparin (Porcine intestinal mucosa Grade I, Sigma) was dissolved in sterile phosphate buffered saline, pH 7.3, at 1,000 units per ml. The solution was filter sterilised (0.22 μ m, Millipore) and stored at 4°C. Heparin was used at a concentration of 20-40 units per ml blood.

b) Acid citrate dextrose solution (ACD, Dacie and Lewis, 1975)

Trisodium citrate dihydrate (Analar, BDH)	22 g
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Citric acid, monohydrate (BDH)	8 g
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Dextrose (D-glucose, Analar, BDH)	25 g
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Purified water up to one litre

The solution was autoclaved (15 p.s.i., 15 minutes) and stored at 20°C. For use, five volumes of blood were added to one volume of ACD.

I.1.2. Phosphate buffered saline (PBS)

Phosphate buffered saline, pH 7.3 and free of calcium and magnesium ions, was prepared according to the formulation below (Oxoid, 1979) and autoclaved at 15 p.s.i. for 15 minutes.

Sodium chloride (Analar, BDH)	8.0 g
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Potassium chloride (BDH)	0.2 g
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Disodium hydrogen phosphate, anhydrous (BDH)	1.15 g
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Potassium dihydrogen phosphate (BDH)	0.2 g
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Purified water to one litre

pH 7.3

I.1.3. Giemsa's stain

Ten g of Giemsa powder (Azur-eosin-methylene blue, Merck) was ground with 540 ml glycerol (Analar, BDH) and kept at 60°C for one hour with intermittent shaking. Once cooled to room temperature, 840 ml methanol (Analar solvent methanol, BDH) was added and the mixture was shaken vigorously and then left stirring overnight. 2.75 g Azur II (Merck) was added and stirring continued for another night. This concentrated solution was filtered (Whatman No. 4) and stored in a dark bottle. For use, a 5% solution was made up in phosphate buffer (GURR 6550, BDH) pH 7.2.

I.1.4. Penicillin - Streptomycin

A stock antibiotic solution (x 1,000) containing 100,000 units per ml of sodium benzylpenicillin (Crystapen, Glaxovet) and 100,000 µg per ml of streptomycin sulphate (Glaxo) in purified water was stored in aliquots at -20°C. The working concentration was 100 units/µg per ml solution.

I.1.5. Nystatin

A stock fungistat solution (x 100) containing 10,000 units per ml of nystatin (Gibco) in purified water was stored in aliquots at -20°C. The working concentration was 100 units per ml solution.

I.2. REF. CHAPTER 3

I.2.1. IFAT materialsa) Formalin fixative

A 1:10 formalin (3.7% formaldehyde solution) was made by adding 10 ml of 37% formaldehyde solution (Analar, BDH) to 90 ml PBS pH 7.3 and storing at 4°C.

b) Fluorescein conjugated second antibody

Second antibody conjugated to fluorescein (FITC) was supplied (Nordic Immunologicals) as a lyophilised powder and was reconstituted in purified water. The conjugate was stored in 50 µl aliquots at -20°C. The conjugates used were:

rabbit antiovine IgG (Fc and Fab) = RAB/FITC

goat antirabbit Ig (IgG1, IgG2, IgA, IgM, Fc and Fab) = GAR/Ig/FITC

rabbit antimouse Ig (IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, Fc and Fab)
= RAM/Ig/FITC

These were diluted in PBS before use at 1:80 dilution (RAB/FITC, GAR/Ig/FITC) or 1:40 (RAM/Ig/FITC).

c) IFAT Mountant

A 66% glycerol mountant was used to mount slides to be examined for fluorescence. This was prepared by mixing 10 ml 50 mM Tris Cl buffer (pH 9.2) with 20 ml glycerol.

I.2.2. Hybridoma culture medium constituentsa) B-mercaptoethanol

A sterile 0.1 M stock B-mercaptoethanol (B-ME) solution was prepared by adding 0.7 ml commercial B-ME (Sigma) to 99.3 ml purified water. As supplied, 1 ml B-ME is equivalent to 1.12 g, thus the

molar weight 78.13 g is equivalent to 69.76 ml. This stock B-ME was stored in aliquots at -20°C and diluted x 2,000 in medium for use at the working concentration of 5×10^{-5} M.

b) Amphotericin B

Amphotericin B (fungizone, Gibco) was supplied as a stock solution of 1 mg/ml and was stored at -20°C . For use it was diluted x 500 in medium to achieve the working concentration of 2 $\mu\text{g}/\text{ml}$.

c) Foetal calf serum

Myoclone (Gibco), a selected foetal calf serum, was heat inactivated at 56°C , 30 minutes and stored at -20°C . It was used to supplement RPMI 1640 medium at a level of 16.7%.

d) HAT and HT supplements

Hypoxanthine - Aminopterin - Thymidine (HAT) and Hypoxanthine - Thymidine (HT) supplements (Gibco) were supplied as sterile 50 x concentrated solutions containing:

hypoxanthine	65 mg
aminopterin (HAT only)	0.95 mg
thymidine	19.5 mg

per 100 ml

The aminopterin, once diluted to 1 x in medium was at its active concentration of 4×10^{-7} M.

I.2.3. Fusion solutions

a) PEG 1

Polyethylene glycol solution 1 (PEG 1) was made up of 41.6% PEG and 15% DMSO in incomplete medium (no FCS). 20.8 g PEG (molecular

weight 6,000, BDH) was diluted in 20 ml warm (37°C) incomplete RPMI 1640 medium and made up to 42.5 ml. The solution was autoclaved and, once cool, 7.5 ml DMSO was added.

b) PEG 2

PEG 2 was a 25% PEG solution made up by dissolving 6.25 g PEG in 12 ml warm incomplete medium and making up the volume to 25 ml before autoclaving.

PEG solutions 1 and 2 were made up the day before the fusion.

I.2.4. Counting fluids

a) White blood cell counting fluid (Dacie and Lewis, 1975)

2% acetic acid (BDH) was used as a diluent for the mouse spleen cell count prior to fusion since it lyses red blood cells.

b) Trypan blue

0.2% Trypan blue (Sigma) in PBS pH 7.3 was used to stain non-viable cells for myeloma and hybridoma cell counts.

I.3. REF. CHAPTER 5

I.3.1. SDS - Polyacrylamide gel electrophoresis

a) Stock 30% acrylamide solution

Acrylamide (Sigma)	154 g
N,N methylene bisacrylamide (Sigma)	4 g
Mixed bed ion exchanger (Duolite MB6113, BDH)	10 g
Purified water to 500 ml	
Filtered to remove mixed resin.	

b) Lower Tris x 4

*Tris (Sigma) 36.4 g

+SDS (Sigma) 0.6 g

Purified water to 200 ml

pH changed to 8.8 using concentrated HCl (BDH)

*Tris = trishydroxymethylamine

+SDS = sodium dodecyl sulphate

c) Upper Tris x 4

Tris 12.12 g

SDS 0.8 g

Purified water to 200 ml

pH changed to 6.8 using concentrated HCl

d) Stacking gel (4.5% acrylamide)

Upper Tris x 4 50 ml

30% acrylamide 30 ml

Purified water to 200 ml

e) Separating gels

	<u>7</u>	<u>% acrylamide</u> <u>10</u>	<u>20</u>
Lower Tris x 4	50	50.0	50 ml
30% acrylamide	46	66.6	132 ml
Sucrose (Analar, BDH)	-	-	30 g
Purified water to 200 ml			

f) Sample buffers

i) Reducing:

	<u>x 1</u>	<u>x 4</u>
Tris	45 mg (18.5 mM)	180 mg
EDTA (disodium salt, Sigma)	223 mg (30 mM)	892 mg
SDS	1.0 g (5% w/v)	4.0 g
B-ME (Koch Light Labs)	0.45 ml (2.5% w/v)	1.8 ml
Glycerol (Analar, BDH)	5.0 ml (25% w/v)	5.0 ml
Bromophenol blue (Sigma)	Spatula tip	Spatula tip
Purified water	to 20 ml	to 20 ml

ii) Non-reducing:

	<u>x 1</u>
Tris	45 mg
EDTA	223 mg
SDS	1.0 g
Iodoacetamide (Sigma)	95 mg
Glycerol	5.0 ml
Bromophenol blue	Spatula tip
Purified water	to 20 ml

g) Electrophoresis tank buffer (pH 8.3)

Tris	3.03 g	25 mM
Glycine (BDH)	14.14 g	0.192 M
SDS	1.0 g	0.1%
Purified water	to 1,000 ml	

All the solutions above were stored at -20°C except tank buffer which was freshly prepared when required.

I.3.2. Protein staininga) Destain solution

Methanol (BDH) 25%

Glacial acetic acid (BDH) 10%

in purified water

b) Silver stain solutions

i) Solutions 1 and 2:

	<u>Solution 1</u>	<u>Solution 2</u>
Methanol	50%	5%
Glacial acetic acid	10%	7%

in purified water

ii) Solution 3:

Gluteraldehyde (Taab Labs) 5.0%

Sodium tetraborate (BDH) 1.9%

in purified water

iii) Developer:

Sodium carbonate (BDH) 3 g

37% formaldehyde (Analar, BDH) 50 μ l

Purified water to 100 ml

I.3.3. Western blottinga) Blotting buffer

For use with semi-dry Ancos blotter (Dako Ltd.)

Electrophoresis tank buffer 100 ml

Technical methanol (BDH) 100 ml

Purified water 300 ml

b) Blocking buffer x 10

Tris	60.5 g	50 mM
Sodium chloride (BDH)	87.0 g	150 mM
EDTA	3.72 g	1 mM
Nonidet P40 (Sigma)	5.0 ml	0.005%
Gelatin (BDH)	25.0 g	2.5%
Thiomersal	2.0 g	0.2%

Purified water to 1,000 ml

pH changed to 7.4 using concentrated HCl

c) Peroxidase conjugated second antibody

Horseradish peroxidase conjugated second antibody was supplied (Nordic Immunologicals) as a lyophilised powder. This was reconstituted in 1 ml water and then diluted with 4 ml PBS containing 0.05% Tween 20 (Sigma). 75 µl aliquots were stored at -20°C. The conjugates used were:

goat antiovine IgG (heavy and light chains) = GAB/PO

goat antirabbit IgG (heavy and light chains) = GAR/IgG (H+L)/PO

goat antimouse Ig (IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, Fc and Fab)
= GAM/Ig/PO

d) Substrate solutions

i) Tris buffered saline (TBS):

Tris	4.85 g	20 mM
Sodium chloride	58.48 g	500 mM

Purified water to 2,000 ml

pH changed to 7.5 with concentrated HCl

ii) Solution 1: 60 μ l of ice-cold 30% hydrogen peroxide (Analar, BDH) added to each 100 ml TBS just prior to use.

iii) Solution 2: 60 mg 4-chloro-1-naphthol (Sigma) dissolved in 20 ml ice-cold technical methanol.

I.3.4. Surface radioiodination and immunoprecipitation

a) Lysis buffer

PBS (pH 7.2) containing protease inhibitors and 1% Nonidet P40 (non-ionic detergent).

b) Immunoprecipitation diluent buffer

Prepared from stock solutions

Tris buffer 2.0 M pH 8.0	10 ml	20 mM
Sodium chloride 0.5 M	100 ml	50 mM
Nonidet P40 10%	20 ml	0.2%

I.3.5. General

a) PBS containing protease inhibitors

N-tosyl-l-lysine chloromethylketone (TLCK, Sigma)	2.5 mg
N-tosyl-l-phenylalanine chloromethylketone (TPCK, Sigma)	5.0 mg
PBS pH 7.2	99 ml

PBS was stirred overnight and then stored at 4°C. Just prior to use 1 ml 100 mM phenylmethyl-sulphonylfluoride (PMSF, Sigma) was added.

b) Molecular weight markers

A commercial mixture of six molecular weight protein standards were used covering the low molecular weight range (Pharmacia). The

contents of one vial were reconstituted in 1.0 ml 1 x SDS sample buffer and heated at 100°C for five minutes. Aliquots were stored at -20°C and 10 µl was used per gel track.

The protein standards present were phosphorylase b (94 kdal), bovine serum albumin (67 kdal), ovalbumin (43 kdal), carbonic anhydrase (30 kdal), soyabean trypsin inhibitor (20 kdal) and -lactalbumin (14 kdal).

I:4 REF. CHAPTER 6

I.4.1. Antibiotics

a) Ampicillin (Sigma)

Stock solution of 100 mg/ml of the sodium salt of ampicillin in water. Sterilised by filtration and stored at -20°C. Working concentration of 100 µg/ml.

b) Chloramphenicol (Sigma)

Stock solution of 34 mg/ml in 100% ethanol stored at -20°C. Working concentration of 170 µg/ml.

I.4.2. Media

a) Luria-Bertani (LB) medium

Per litre:

Bactotryptone (Difco) 10 g

Sodium chloride (BDH) 10 g

Yeast extract (Difco) 5 g

pH to 7.5 with sodium hydroxide

b) Solid media

"Bottom" agar or agarose plates were prepared by the addition of 15 g agar (Bactoagar, Difco) or agarose (type 1, Sigma) per litre of LB medium prior to autoclaving. After cooling to 55°C, ampicillin was added to a concentration of 100 µg/ml and the plates were poured.

"Top" agar or agarose was prepared by adding 7 g of agar or agarose to each litre of LB medium.

I.4.3. For working with bacteriophagea) Maltose (Sigma)

20% maltose prepared in water and filter sterilised was stored at -20°C and used at a working concentration of 0.2%.

b) SM (phage buffer)

Per litre:

Sodium chloride		5.8 g
Magnesium sulphate ($\text{MgSO}_4, 7\text{H}_2\text{O}$, BDH)		2.0 g
1M Tris buffer pH 7.5		50 ml
2% gelatin (BDH)		5 ml

I.4.4. Genomic library screeninga) Tris-saline buffer (TS)

Per litre:

Sodium chloride	8.77 g	0.15 M
Tris base	1.21 g	10 mM
pH 8.0		

b) Substrate solution

Imidazole buffer pH 7.4 10 mM (Sigma)	4.5 ml
Dianisidine dihydrochloride 5 mg/ml (Sigma)	2.25 ml
Hydrogen peroxide (Analar, BDH)	0.45 ml
Purified water	38.0 ml

The reagents were mixed together in the above order immediately prior to use.

I.4.5. TE

pH 7.6

Tris buffer pH 7.6 10 mM

EDTA pH 8.0 1 mM

pH 8.0

Tris buffer pH 8.0 10 mM

EDTA pH 8.0 1 mM

I.4.6. TBE (Tris-borate electrophoresis buffer)

Working solution:

Tris - borate 0.089 M

boric acid 0.089 M

EDTA 0.002 M

x 10 stock solution, per litre:

Tris base 108 g

boric acid (Sigma) 55 g

0.5M EDTA pH 8.0 40 ml

I.4.7. STE

Tris buffer pH 8.0	10 mM
Sodium chloride	100 mM
EDTA pH 8.0	1 mM

I.4.8. Gel loading buffer (type III)

6 x buffer:

bromophenol blue (Sigma)	0.25%
xylene cyanol (Sigma)	0.25%
glycerol	30%

in purified water

Stored at 4°C

I.4.9. Buffers for restriction endonuclease digestion

	Strength of salt buffer		
	Low	Medium	High
Sodium chloride	-	50 mM	100 mM
Tris buffer pH 7.5	10 mM	10 mM	50 mM
Magnesium chloride	10 mM	10 mM	10 mM
Dithiothreitol	1 mM	1 mM	1 mM

I.4.10. Conditions for restriction endonuclease digestion

<u>Restriction enzyme</u>	<u>Temperature</u>	<u>Buffer salt concentration</u>
Eco R1	37°C	High
Pst 1	37°C	Medium
Hind III	37°C	Medium

I.4.11. Phenol

Phenol was either obtained as a liquid commercial preparation (BRL Ultrapure) or from crystalline phenol by redistillation at 160°C. Liquid phenol was stored wrapped in foil at -20°C under 1.0 M Tris buffer pH 8.0. Antioxidant, 8-hydroxyquinoline (Sigma) was added to a concentration of 0.1%.

Before use the phenol was extracted several times with 0.1 M Tris buffer pH 8.0 or was equilibrated with the buffer to be used, e.g. TE pH 7.6, TE pH 8.0.

I.4.12. DNase free RNase

A stock solution of pancreatic RNase (RNase A, Sigma) at 10 mg/ml was prepared in 10 mM Tris buffer pH 7.5 and 15 mM NaCl. To destroy any DNase activity this was heated to 100°C for 15 minutes, cooled and then stored at -20°C.

I.4.13. DNase

A stock solution of DNase (DNase 1, Sigma) at 1 mg/ml was prepared in sterile deionised purified water and stored at -20°C.

I.4.14. 5M Potassium acetate pH 4.8

5M Potassium acetate (Sigma)	60.0 ml
Glacial acetic acid (BDH)	11.5 ml
Purified water	28.5 ml

This mixture results in a 3 M solution with respect to potassium and a 5 M solution with respect to acetate.

I.4.15. Preparation of dialysis tubing

Dialysis tubing (Medicell International Ltd.) cut into suitable lengths was boiled in two litres of 2% sodium bicarbonate, 1 mM EDTA for 10 minutes, rinsed thoroughly and boiled again with purified water. The tubing was cooled and stored at 4°C. Before use the tubing was rinsed with purified water.

I.4.16. Southern blot hybridisationa) Acid depurinating solution

0.25 M hydrochloric acid (HCl, BDH) made by the addition of 10.4 ml concentrated HCl to 490 ml purified water.

b) Alkali denaturing solution

		Per 2 litres:
Sodium chloride	1.5 M	175.32 g
Sodium hydroxide (BDH)	0.5 M	40 g

c) Neutralising solution

		Per 2 litres:
Tris buffer pH 8.0	1 M	242.2 g
Sodium chloride	1.5 M	175.32 g

d) 20 x SSC

		Per litre:
Sodium chloride		175.3 g
Sodium citrate (Sigma)		88.2 g
pH adjusted to 7.0 with 10 N sodium hydroxide		

e) Hybridisation buffer (Southern blots)

	For 100 ml	Working concentration
1M Sodium phosphate buffer pH 7.4	50 ml	0.5 M
20% SDS	35 ml	7%
Purified water	14 ml	
Denatured (boiled) sonicated salmon sperm DNA 10 mg/ml	1 ml	100 µg/ml

I.4.17. Northern blot hybridisationa) MOPS buffer

MOPS ⁺ pH 7.0 (Sigma)	40 mM
Sodium acetate (Sigma)	10 mM
EDTA	1 mM

b) Hybridisation buffer (Northern blot)

PIPES* pH 6.8 (Sigma)	50 mM
Sodium chloride	1 M
Sodium phosphate (Sigma)	50 mM
EDTA	1 mM
SDS	5%

⁺MOPS = morpholinopropanesulphonic acid

*PIPES = 1,4-piperazine diethanesulphonic acid

APPENDIX II

Median % infection and range values for sporozoite
inhibition assays in Chapters 4 and 7

The bar histogram figure numbers to which these values correspond are indicated

Figure No.	Calf	Day of culture	GE te/well	Serum (undiluted)	Median % infection (range)	n = 4
13a	Calf N53	4	0.5	-1	8.5	(7.0-12.5)
				58	0.8	(0.5-1.0)
				90	0.5	(0.5-0.5)
				107	0.8	(0.5-3.0)
13a	Calf N61	4	0.5	-1	21.3	(20.0-29.0)
				58	2.8	(2.0-3.5)
				90	0.5	(0.5-0.5)
				107	0.8	(0.5-1.0)
13b	Calf 54X	4	0.25	0	25.0	(23.5-31.5)
				94	0.0	(0.0-0.5)
13b	Calf 134X	4	1/4	0	23.5	(21.0-24.5)
				101	0.0	(0.0-0.0)
		7	1/16	0	31.0	(22.5-33.0)
				101	0.0	(0.0-0.0)
		11	1/64	0	19.5	(18.5-24.0)
				101	0.0	(0.0-0.0)

n = 3

Sporozoites: T. annulata Ankara

Figure 13c

Calf	Day of culture	Serum	Median % infection (range) n = 4		
			1/1	Serum dilution: 1/4	1/16
L21	8	0	70.8 (63.5-77.0)	59.3 (43.0-60.5)	37.0
		49	0.0 (0.0-0.0)	12.8 (8.0-16.5)	4.0 (1.0-9.0)
		104	0.0 (0.0-0.0)	5.3 (4.0-11.5)	9.3 (4.5-14.5)
N44	8	0	53.5 (46.0-78.0)	33.8 (21.5-51.5)	24.0 (19.0-24.0)
		63	0.0 (0.0-0.5)	6.3 (0.5-9.0)	11.3 (5.5-14.5)
		92	0.0 (0.0-2.0)	3.0 (0.05-5.5)	5.0 (4.0-5.0)
L37	8	0	67.3 (48.5-81.5)	22.0 (19.0-25.0)	23.0 (11.5-25.0)
		91	0.0 (0.0-0.0)	3.3 (1.0-7.5)	4.0 (3.0-6.0)
N53	8	0	63.0 (54.0-68.0)	43.0 (26.0-50.0)	36.0 (24.0-49.0)
		107	0.0 (0.0-0.0)	4.5 (1.0-8.0)	7.0 (4.0-9.0)
102	3	0	21.0 (10.5-27.0)	29.5 (24.0-35.0)	ND
		42	0.0 (0.0-0.5)	5.0 (3.0-10.5)	ND
N53	3	0	18.8 (17.0-35.0)	48.0 (43.0-54.0)	ND
		107	0.8 (0.0-1.0)	21.3 (13.0-25.0)	ND

Sporozoites: T. annulata Ankara, 0.25 te GF per well

ND = not done

Figure No.	Rabbit	Day of culture	GF te/well	Serum	Median % infection (range)			n = 4	
					1/4	1/8	1/16		
14a	28	9	A 1/16	0 73	38.5	(34.0-41.0)	ND	37.5	(28.0-46.0)
					0.0	(0.0-0.0)	ND	0.0	(0.0-0.0)
			G 1/16	0 73	6.0	(3.0-16.0)	ND	15.0	(12.0-17.0)
					0.0	(0.0-0.0)	ND	0.0	(0.0-0.0)
14b	28	4	A 1/4	0 73	19.5	(16.0-26.5)	27.3	(20.5-29.5)	ND
					0.0	(0.0-0.0)	0.3	(0.0-1.5)	ND
	8	4	A 1/4	0 71	29.8	(21.0-40.5)	24.0	(9.0-33.0)	ND
					17.5	(13.0-18.0)	19.0	(17.0-21.5)	ND
14c	28	2*	H 1/3	0 73	19.3	(19.0-25.5)			
					0 HI	(37.0-46.5)			
				73 HI	0.0	(0.0-0.0)			
14d	28	3	H 1/4	0 73	24.5	(18.5-32.0)			
					0.0	(0.0-0.0)			
				0	33.3	(31.5-40.0)			
				73	42.8	(35.5-53.0)			

*Median % infection here includes trophozoite and macrochizont infected cells.

HI = heat inactivated; ND = not done;

H = Hissar

A = Ankara } - stocks of T. annulata sporozoites

G = Gharb }

Sera added to cultures on day 1

Figure 15a

Monoclonal antibody		Median % infection n = 3
<u>GF 1/4 te/well</u>		
4B11/C12	A 1:10	3.0 (2.0-5.0)
	A 1:100	3.0 (3.0-4.5)
	S	3.0 (1.5-7.0)
1A7/G8	A 1:10	7.5 (4.5-8.0)
	A 1:100	8.5 (7.0-14.0)
	S	20.5 (16.0-23.5)
4B11/C12 +	A 1:10	2.0 (2.0-3.5)
	A 1:100	6.0 (4.5-7.0)
	1A7/G8 S x 2	4.0 (1.5-6.5)
7B10	A 1:10	15.0 (14.0-20.5)
	A 1:100	15.5 (15.0-37.0)
	S	10.0 (8.0-11.0)
	S x 2	4.0 (4.0-5.0)
Calf 134X sera	day 0	23.5 (21.0-24.5)
	day 101	0.0 (0.0-0.0)
<u>GF 1/16 te/well</u>		
4B11/C12	A 1:10	1.5 (1.0-2.5)
	A 1:100	0.5 (0.5-1.5)
	S	0.5 (0.5-1.5)
1A7/G8	A 1:10	1.0 (0.5-1.5)
	A 1:100	1.0 (0.5-1.5)
	S	10.5 (8.0-23.5)
4B11/C12 +	A 1:10	0.5 (0.5-0.5)
	A 1:100	0.5 (0.0-1.0)
	1A7/G8 S x 2	1.0 (0.5-1.0)
7B10	A 1:10	7.5 (4.5-8.0)
	A 1:100	7.5 (7.5-11.0)
	S	1.0 (0.5-1.5)
	S x 2	0.5 (0.5-4.5)
Calf 134X sera	day 0	2.5 (1.0-6.0)
	day 101	0.0 (0.0-0.0)

Sporozoites: T. annulata Ankara

Infection rates assessed on day 4 of culture

A = ascites; S = undiluted supernatant

Figure 15b

Monoclonal antibody culture supernatant	Median % infection (range) n = 4
4B11/C12	1.0 (1.0-2.0)
1A7/G8	9.0 (4.0-10.0)
1C7	25.5 (15.0-34.0)
anti <u>Taenia saginata</u>	39.5 (25.0-44.0)

Sporozoites: T. annulata Ankara, 0.05 te GF per well

Infection rates assessed on day 4 of culture

Figure 52

Mouse	Median % infection (range)		n = 4	
	Serum dilution: 1/4		1/16	
Mouse 1 + 5	4.0	(1.0-6.0)	32.3	(27.0-34.0)
4 + 7	12.5	(5.0-15.0)	22.8	(20.0-30.0)
6 + 8	3.5	(2.0-7.0)	14.5	(5.0-28.5)
2 + 3	14.5	(10.0-25.0)	18.0	(11.0-28.0)
9 + 10	10.0	(9.0-18.0)	21.0	(9.0-33.0)

Sporozoites: T. annulata Ankara, 0.05 te per well

Infection rates assessed on day 4 of culture

Figure 55a

Rabbit	Serum day	Median % infection (range) n = 4			
		1/4		1/8	
73	0	22.3	(16.0-30.5)	25.5	(21.5-36.5)
	66	0.5	(0.0-0.5)	2.0	(2.0-3.0)
75	0	20.3	(13.5-25.5)	32.0	(23.0-50.0)
	66	0.0	(0.0-0.5)	1.0	(0.5-2.0)
77	0	20.5	(15.5-29.5)	21.0	(13.5-25.5)
	66	0.0	(0.0-0.0)	0.3	(0.0-2.0)
11	0	22.0	(17.0-32.0)	ND	
	38	17.3	(11.5-25.5)	ND	
28	0	19.5	(16.0-26.5)	27.3	(20.5-29.5)
	73	0.0	(0.0-0.0)	0.3	(0.0-1.5)

Sporozoites: T. annulata Ankara, 0.25 te per well

Infection rates assessed on day 4 of culture

Figure 55b

Rabbit	Serum day'	<u>T. annulata</u> stock 0.25 te per well	Median % infection (range) n = 4			
			Day 4 of culture		Day 7 of culture	
77	0	Gharb	21.0	(13.0-41.5)	59.0	(49.5-63.5)
	66		0.0	(0.0-0.0)	0.0	(0.0-0.5)
11	0	Gharb	40.8	(25.5-42.5)	55.0	(52.0-71.0)
	38		39.8	(24.0-47.0)	63.5	(43.0-73.5)
77	0	Hissar	21.0	(17.5-28.0)	60.0	(55.5-64.5)
	66		0.5	(0.0-0.5)	1.3	(1.0-2.0)
11	0	Hissar	29.8	(26.5-30.5)	46.0	(45.5-62.5)
	38		23.8	(18.0-49.5)	51.0	(46.5-52.5)

Figure 55c

Rabbit	Serum day	Treatment	Day of culture	Median % infection (range)	n = 4
77	0	Normal		10.5	(8.5-23.0)
	66		2*	0.0	(0.0-1.0)
77	0	Heat inactivated		14.5	(11.5-25.0)
	66			2.5	(2.0-6.0)
77	0	Added as usual on day 0 of assay		17.0	(10.0-22.0)
	66		3	0.0	(0.0-0.0)
77	0	Added to cultures on day 1 of assay		11.0	(7.5-18.5)
	66			17.8	(16.0-20.0)

Sporozoites: T. annulata Hissar, 0.3 te per well

*Median % infection includes trophozoite and schizont infected cells as counts were made on day 2 of the assay

Figure 58

Calf	Serum day	Median % infection (range) n = 4
10T	0	13.8 (10.5-19.0)
	94	0.0 (0.0-0.0)
34A	0	14.0 (10.5-20.0)
	94	0.5 (0.0-0.5)
14T	0	17.8 (14.0-22.5)
	94	12.5 (8.5-26.5)
37A	0	11.8 (10.0-13.5)
	94	17.3 (11.5-20.0)
41P	0	14.3 (11.5-15.0)
	94	18.8 (16.5-24.0)
38A	0	12.0 (10.5-13.5)
	94	15.5 (13.0-16.0)

Sporozoites: T. annulata Ankara

Infection rates assessed on day 2 of culture

Median % infection includes trophozoite and schizont infected cells

APPENDIX III

Manufacturers' and suppliers' names and addresses

- Agfa Professional Photographic Division, 27, The Great West Road, Brentford, Middlesex, T88 9AX, England.
- Alpha Laboratories, 40, Parkham Drive, Eastleigh, Hampshire, SP5 4NV, England.
- American Can Company. Suppliers: Gallenkamp Ltd., Belton Road West, Loughborough, Leicestershire, England.
- Amersham International PLC, Lincoln Place, Green End, Aylesbury, Berkshire, England.
- Anderman and Company Ltd., 145, London Road, Kingston-on-Thames, Surrey, KT2 6NH, England.
- BDH Ltd., Broom Road, Poole, Dorset, BH12 4NN, England.
- Becton Dickinson UK Ltd., York House, Empire Way, Wembley, Middlesex, England.
- Bethesda Research Laboratories. Suppliers: Gibco Ltd.
- Biorad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Hertfordshire, England.
- BOC Ltd., 150, Polmadie Road, Glasgow, G5 OHN, Scotland.
- Boehringer Mannheim (BCL), Bell Lane, Lewes, East Sussex, BN7 1LG, England.
- Carnation, 36, Park Street, Croydon, Surrey, CR9 1TT, England.
- Costar. Suppliers: Northumbria Biologicals Ltd., South Nelson Industrial Estate, Cramlington, England.
- Dako Ltd., 22, The Arcade, The Octagon, High Wycombe, Buckinghamshire, England.
- Decon Laboratories Ltd., Ellen Street, Portslade, Brighton, BN4 1EQ, England.
- Difco Laboratories, P.O. Box 149, Central Avenue, West Moseley, Surrey, England.
- Fisons Scientific Apparatus Ltd., Bishop Meadow Road, Loughborough, Leicestershire, England.

- Gibco Ltd., P.O. Box 35, Washington Road, Abbotsinch Industrial Estate, Paisley, PA3 4EP, Scotland.
- Glaxovet Ltd., Greenford, Middlesex, UB6 OHE, England.
- Heraeus Equipment Ltd., Unit 9, Wates Way, Brentwood, Essex, CM15 9TB, England.
- Ilford Ltd., Mobberley, Cheshire, England.
- Koch Light Laboratories Ltd., Poyle, Colbrook, Buckinghamshire, England.
- Kodak Ltd., Hemel Hempstead, England.
- LEEC Ltd., Private Road 7, Colwick, Nottingham, NG4 2AJ, England.
- Leitz Instruments Ltd., 48, Park Street, Luton, LU1 3HP, England.
- Merck, Darmstadt, West Germany. (Suppliers: BDH).
- Millipore (UK) Ltd., The Boulevard, Ascot Road, Croxley Green, Watford, WO1 8YW, England.
- MSE Scientific Instruments, Manor Royal, Crawley, Sussex, RH10 2QQ, England.
- Nordic Immunological Laboratories Ltd., P.O. Box 544, Maidenhead, Berkshire, England.
- Nuclear Enterprises Ltd., Bankhead Meadway, Edinburgh, EH11 4EY, Scotland.
- Nunclon. Suppliers: Gibco Ltd.
- Oxoid Ltd., Wade Road, Basingstoke, RG24 OPW, England.
- P and S Biochemicals Ltd., 38, Queensland Street, Liverpool, L7 3JG, England.
- Packard Instruments Ltd., Manulife House, 13-17, Church Road, Caversham, Berkshire, RG4 7AA, England.
- Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, England.
- Pierce Chemicals. Suppliers: Life Science Laboratories Ltd., Sedgewick Road, Luton, LU4 9DT, England.
- Promega Biotech. Ltd. Suppliers: P and S Biochemicals Ltd.

Pye Unicam Ltd., York Street, Cambridge, CB1 2PX, England.

Sarstedt Ltd., 68, Boston Road, Leicester, LE4 1AW, England.

Schleicher and Schuell. Suppliers: Anderman and Company Ltd.

Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke,
Lanarkshire, ML8 5BS, Scotland.

Serotec, 22, Bankside Station Approach, Kidlington, Oxford, OX5 1JE,
England.

Shandon Southern Products Ltd., 93, Chadwick Road, Astmoor, Runcorn,
Cheshire, England.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7NH,
England.

Sorvall. Suppliers: Dupont (UK) Ltd., Wedgewood Way, Stevenage,
Hertfordshire, SG1 4QN, England.

TAAB Laboratory Equipment, 3, Minerva House, Calleva Industrial Park,
Aldermaston, England.

Whatman Labsales Ltd., Unit 1, Coldred Road, Parkwood, Maidstone,
Kent, ME15 9XN, England.

Winthrop Laboratories, Winthrop House, Surbiton-upon-Thames, Surrey,
England.