IMMUNOLOGICAL STUDIES OF TRYPANOSOMA EVANSI INFECTIONS IN RABBITS

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Title of Thesis Immunological studies of Trypanosoma evansi infections in rabbits.

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The present study evaluated aspects of the class-specific antibody response of rabbits experimentally infected with Trypansoma evansi.

Using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and "Western" blotting adapted to detecting class-specific antibody response and optimized for the T. evansi system, humoral responses to T. evansi were observed to be directed to both the surface and non-surface components of the parasite. Using ^{125}I-iodination, two components with molecular weights of 67,000 and 60,500 daltons were found to be associated with the surface of the parasite with their detection varying between different stocks of T. evansi. Both the surface and non-surface components elicited skin reactions in animals, with the most intense reactions observed with the surface components and in animals drug cured of T. evansi infections.

Class-specific antibody response to the surface components involved the production of the three immunoglobulin classes studied - IgG, IgM and IgA while responses to the non-surface components were predominantly IgG, an observation that was thought to be linked with the role of IgM in bringing about trypanolysis and IgG in neutralising the products of such lysis.

Based on clinical signs, animals were classed as resistant or susceptible with the serum from the resistant animals detecting more antigens of T. evansi and earlier than those of the susceptible animals. The detection of the surface and many non-surface components of the parasite was associated with resistance. Cross-infection studies demonstrated absolute protection of animals to homologous challenge, which was found to be associated with the production of specific antibodies. Partial protection to heterologous challenge was associated with the presence of cross-reacting antibodies to one of the surface and some of the non-surface components. There were also indications that non-antibody factors may be important in the induction of protection while complete absence of antibodies to antigens of the challenge parasites was associated with non-protection.

Studies on complement demonstrated the depletion of C3 during infection with C3 in circulation returning to near preinfection levels after drug treatment and during periods of low parasitaemia. C3 activation was observed in infected animals and using an in vitro system, C3 was activated mainly through the classical and also the alternative pathways, with the complexes formed with IgG being more efficient in activating C3 than those formed with IgM. C3 was also found to be important during secondary responses as rabbits with depleted C3 responded poorly by producing lower levels of antibodies than C3 intact, control animals.

Histopathological studies revealed that changes in organs of infected animals could be the direct effect of the parasites or consequences of the host defence response, some of which could lead to the depression of the host's immune responses. Observations warranting an investigation of a possible transmission of T. evansi through a sexual mode and also the importance of an integrated approach in the investigation of host/parasite relationships in T. evansi infections were made.

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DECLARATION

This thesis has been composed by me and describes my own work.

U.E. Uche

DEDICATION

This thesis is dedicated to Chinma and Chinaza and to the loneliest animal that dies slowly without love and care in Africa from any form of trypanosomiasis and above all to God who created them all and also gave me the blessing and strength to finish and write up this work in a safe place devoid of the deadly and wicked flies that loom about like devils seeking whom to devour.

ABSTRACT

The present study evaluated aspects of the class-specific antibody response of rabbits experimentally infected with *Trypanosoma evansi*.

Using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and "Western" blotting adapted to detecting class-specific antibody response and optimized for the *T. evansi* system, humoral responses to *T. evansi* were observed to be directed to both the surface and non-surface components of the parasite. Using ^{125}I-iodination, two components with molecular weights of 67,000 and 60,500 daltons were found to be associated with the surface of the parasite with their detection varying between different stocks of *T. evansi*. Both the surface and non-surface components elicited skin reactions in animals with the most intense reactions observed with the surface components and in animals drug cured of *T. evansi* infections.

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Histopathological studies revealed that changes in organs of infected animals could be the direct effect of the parasites or consequences of the host defence response, some of which could lead to the depression of the host's immune responses. Observations warranting an investigation of a possible transmission of *T. evansi* through a sexual mode and also the importance of an integrated approach in the investigation of host/parasite relationships in *T. evansi* infections were made.

CHAPTER ONE

LITERATURE REVIEW

HISTORICAL BACKGROUND

Trypanosomes and trypanosomiasis have attracted the attention of investigators since the days of Antony Van Leewenhoek in the latter part of the 17th Century. Since then attempts made to control trypanosomiasis, usually by trying to apply lessons learnt from other diseases and in particular through vaccination have been largely unsuccessful. The principle reason for the failure to develop immunoprophylactic control measures for trypanosome infection stems largely from the trypanosome's ability to alter the biochemical nature of their outer covering through a process known as antigenic variation (Soltys, 1963; Vickerman, 1969; Stephen, 1986).

The scourge of trypanosomiasis on man and his livestock along with the association of the disease with the bite of tsetse flies were known to the indigens of Africa long before the arrival of the first Europeans on the continent. This can be seen from the discussion between the missionary, David Livingstone and Chief Sebituane, when the Chief warned Livingstone that his cattle had already been bitten by the tsetse and that they would certainly die. Later, Livingstone's medical training probably assisted him to describe with admirable accuracy the way that the tsetse fly bites, the progress of the disease in infected cattle and the postmortem appearances (Goodwin, 1978). Two major points were emphasized by Livingstone as important aspects of the disease - namely poisons in the blood which probably refers to anaemia, and the insertion of the proboscis. These aspects continue to be the most important aspects for the establishment of trypanosome infection and pathological effects on the mammalian host.

A wide range of protozoan organisms are classified as trypanosomes. Of these the organisms belonging to the order Kinetoplastida, and Family

Trypanosomatidae are strictly parasitic. Within this family there are nine genera. Most of the parasites in the Genus *Trypanosoma,* are of immense veterinary and medical importance causing widespread disease throughout many tropical and subtropical countries. Hoare (1964) further subdivided trypanosome genera infecting mammalian hosts into two broad sections - the Stercoraria and Salivaria each characterised by their mode of development in their respective vector and the mammalian hosts. The section Stercoraria contains genera in which the parasite completes its developmental cycle in the "posterior station" of the vector, in that the stage infective for the mammalian host appears in the faeces of the vector, and transmission is by the contaminative route. The Salivaria contains genera whose developmental cycle in the vector is completed in the "anterior station" of the vector, in that transmission from vector to host occurs by inoculation of the infective stage of the parasite, metacyclic forms, through the bite of an infected vector.

In consideration of the classification of trypanosomes based on morphological criteria Hoare (1972) concluded that "it is usual to separate trypanosomes differing morphologically as species when the characters distinguishing them do not overlap, so that there is a gap between the species but, if the characters of various populations integrate, they can be regarded as subspecies of the same species." Most of the characteristics of trypanosomes, however, do overlap, presenting many practical problems in the differentiation of trypanosomes. Furthermore, the lack of a clearly defined sexual reproductive cycle in trypanosomes limits the application of conventional Darwinian concepts of speciation in classifying trypanosomes into species.

Many characteristics of the *brucei* subgroup overlap in the *Trypanozoon* group, for example, *T. rhodesiense* and *T. gambiense* are considered as

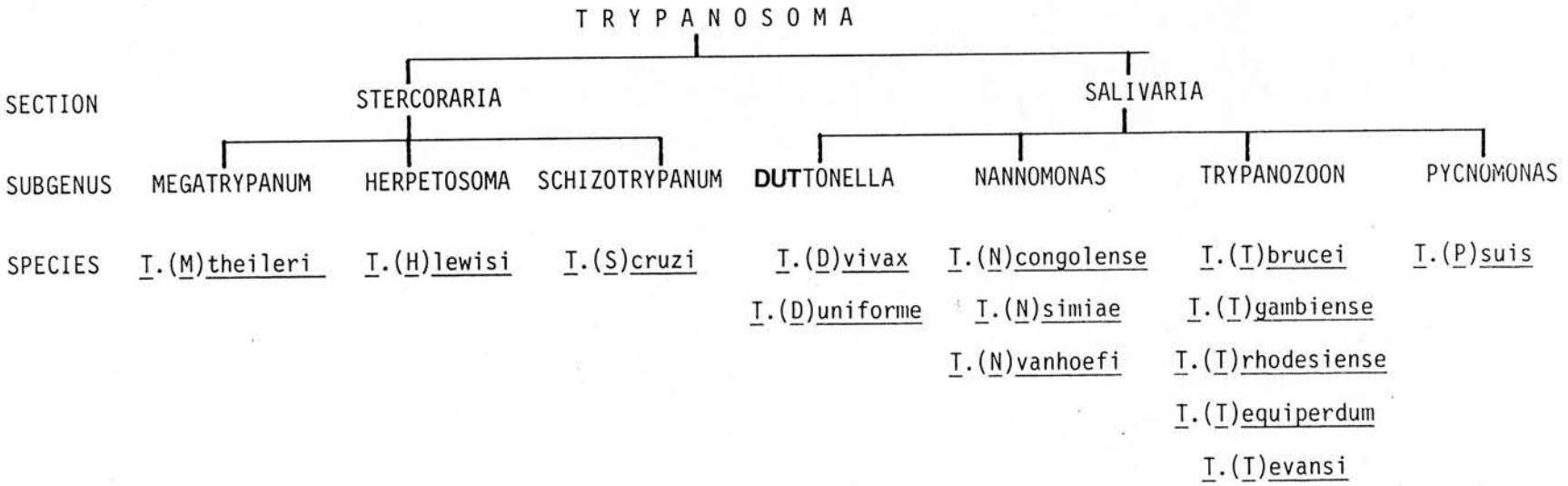
subspecies of *T. brucei,*. *T. evansi* and *T. equiperdum* also belong to the *brucei* subgroup but are considered as separate species because of their mode of transmission (Hoare, 1972). Despite the fact that the metacyclic stages have not been described in *T. evansi* and *T. equiperdum,* they are traditionally retained within the subgenus for historical reasons, based largely on their postulated evolution from *T. brucei.* The separation of the various trypanosome species within the genus *Trypanosoma* is illustrated in Figure 1.1 and is based on the proposal made by Hoare (1964) and later adapted by Stephen (1986).

TRYPANOSOMA EVANSI

Trypanosoma evansi was the first trypanosome to be described from the blood of an infected animal and identified as the causative agent of mammalian trypanosomiasis. The causative agent of the disease now referred to as *T. evansi* was first described by Evans (1880, 1881-1882) who associated the parasite with an endemic disease in equines and camels known as "Surra" in the Derra Ismail Khan in the Punjab in India (Mahmoud and Gray, 1980).

Despite the early description of this parasite and its association with disease in domestic livestock, relatively little research has been conducted specifically on *T. evansi,* the majority of studies on trypanosomiasis of animals in the last 30 years concentrating on the varieties of trypanosomes transmitted by the tsetse flies. It is likely that the relative obscurity of the disease and speculated evolution of *T. evansi* from *T. brucei* as postulated by Hoare (1972) has blinkered research on *T. evansi* with many workers labouring under the notion that the results from studies carried out on organisms such as *T. brucei* would be equally applicable to *T. evansi.*

Figure 1.1 Subgenera and species of mammalian trypanosomes (Hoare, 1964; Stephen, 1986)



Radical differences however, exist between the fundamental properties of *T. brucei* and *T. evansi*. Principally *T. brucei* undergoes a cycle of development in the tsetse, while *T. evansi* does not, being transmitted mechanically through biting flies. Such a difference implies that the two parasites could be exposed to quite different selection pressures for their survival. *T. brucei* is maintained in two hosts, the arthropod vector and the mammalian host, while *T. evansi* only develops in the mammalian host, which reduces its probability of survival and transmission in the event of the death of the host. These differences are likely to affect the parasite at many other levels, particularly those controlling its relationship with the host. The differences could be subtle and only revealed from results of studies conducted specifically on *T. evansi*, hence the need for *T. evansi* specific studies to be conducted.

Following on from the earliest reports of *T. evansi* infections in equines and camels in India by Evans (1880, 1881-1882), several other workers reported outbreaks of diseases in other part of the world with similar clinical signs. According to Lingard (1894) John Henry Steel observed the outbreak of a similar disease in transport mules in Burma in 1885. Other sources (Blanchard, 1888) claim that outbreaks similar to those described by Evans were reported in mules in 1888 in North Vietnam and in Java, Indonesia in 1899. It was Balbiani (1888) however,who first referred the flagellate causing the disease to the genus *Trypanosoma* which he at the time spelt as "*Tripanosoma*". This was amended to *Trypanosoma* after several years because of the confusion it introduced to the nomenclature of this parasite, as many workers referred to it with different names. *Trypanosoma evansi* was finally chosen as the official name of the pathogen causing the disease that was first described by Evans, 1880, 1881-1882.

Other early reports on the outbreak of *T. evansi* infections include those of Schat (1902) concerning an outbreak in Sumatra in 1901 while Yakimoff (1921, 1923) reported outbreaks of *T. evansi* in Russia and claimed that it caused high mortalities in camels. He also attributed the first reported outbreak of *T. evansi* in Russian camels to Feinschmidt in 1912 in the Astrakhan and Saratov regions.

Since the initial reports in the late 19th and early 20th Centuries there have been numerous reports of diseases with similar clinical signs caused by trypanosomes which are morphologically indistinguishable from *T. evansi* in various parts of the New and Old World (Mahmoud and Gray, 1980; Losos, 1980). Diseases attributed to *T. evansi* or *T. evansi*-like parasites are known by more than 33 different names in different parts of the world. A feature common to all of these names is their recognition of the chronic and wasting nature of the disease. The name "Purana", meaning chronic or old; "tibarsa", meaning three-year disease and "dubla", meaning emaciated are some of the names that have been widely used by livestock owners in the eastern block to describe the disease caused by *T. evansi* infection (Luckins, 1988). The "Hindi" term "Surra", meaning rotten or emaciated is, however, the name commonly accepted and now used universally to describe the disease caused by *T. evansi* in all species of domesticated livestock in many different countries (Gill, 1977). Surra has also been used for some other disease syndromes in South America such as "Murina" (*T. hippicum* infections), "Mal de Caderas" (*T. equinum* infections) and "Derrengadera" (*T. venezuelensis* infections) caused by trypanosomes that are morphologically indistinguishable from *T. evansi* (Gutierrez, 1958). Hoare (1940, 1950, 1956, 1965, 1967) considered that there were no scientific grounds for accepting the validity of any of these names other than *T. evansi* and these names are, therefore, used as synonyms

for *T. evansi* and diseases caused by them referred generally to as "American Surra".

The effect of *T. evansi* infections on livestock in some endemic regions of the world is extreme in that a large proportion of animals die from the infection or fail to attain their full economic potential. The recognition of the importance of *T. evansi* infection as a constraint on livestock development stimulated the establishment of centres in countries such as India as far back as 1940 for the monitoring of infections and treatment of infected livestock (Luckins, 1988). Mortalities attributed to *T. evansi* infections can be very high. Reports of 90% mortality were attributed to *T. evansi* infections in buffaloes moved from the mountains to the delta zone of Vietnam's Red River (Wells, 1982). Furthermore, the author estimated the total number of animals killed by Surra in this zone at approximately 20,000 buffaloes annually in the years between 1978 and 1980.

In the case of other species of economically important livestock such as goats, up to 60% mortality has been reported in an infected herd (Mahmoud and Malik, 1978). In horses mortality rates of up to 100% are often reported, with death occurring 4-8 weeks after infection (Rodenwaldt and Doewes, 1921; Bakkar, 1930). Although the high mortalities are spectacular and reportable most animals suffer chronic infection, often going unreported but reducing their economic potential including reduced meat/milk yield, work output and depressed reproduction.

Despite the widespread effect of *T. evansi* infection on different animal species of economic importance, research and control of *T. evansi* have not attracted the degree of co-ordination, discussion and international co-operation that has been a feature of African trypanosomiasis research programmes.

Recently, however, steps have been taken to try to address this anomaly, with recognition of the need to obtain adequate and up-to-date information on the incidence and economic importance of Surra (Luckins, 1988). One of the consequencies of this increased interest in *T. evansi* has been the establishment of an international working group of *T. evansi* by the Office International des Epizooties (OIE), to provide a regular opportunity for discussion between workers in the field. Such collaborative studies are, however, still in their infancy.

ORIGIN OF T. EVANSI

One generally accepted theory of the origin of *T. evansi* is that postulated by Hoare (1972) which suggests that *T. evansi* evolved from *T. brucei* by adapting to a non-cyclical mode of transmission with consequential loss of pleomorphism. Hoare suggested that camels coming into contact with tsetse flies became infected with *T. brucei* but when these camels returned to tsetse-free areas, the infections were perpetuated by other haematophagus insects. Thereafter, camels were thought to have further disseminated the parasite when camel caravans travelled through North Africa into the Middle East and Asia. Curasson (1943) had, however, suggested earlier that the use of dromedaries by the ancient Egyptians in their military campaigns could have been responsible for the introduction of *T. evansi* into Mesopotamia, Persia and Baluchistan. The introduction of *T. evansi* into the New World is often credited to the importation of horses by the Spanish conquistadores for their cavalry (Hoare, 1972). The high mortality due to *T. evansi* infections recorded in these imported animals by the Spanish however, suggests that *T. evansi* was already present, possibly introduced into the region much earlier in cattle, from the Iberian Peninsula during the 16th Century (Rouse, 1977).

GEOGRAPHICAL DISTRIBUTION

Unlike the tsetse-transmitted trypanosomes, the distribution of *T. evansi* is not restricted to that of a particular vector species. *T. evansi* infections, therefore, enjoy a widespread distribution in both the New and Old World. It has an extremely wide geographical range in countries with uniform hot and warm temperate climates. In Africa *T. evansi* infects a range of livestock along the Atlantic and Mediterranean littorals of North Africa where it is reported from Morocco, Algeria, Tunisia, Libya and Egypt. From here its distribution extends across the Sahara into Senegal, Mali, Chad and parts of West Africa lying north of 15^{o}-16^{o}N. In the East, the disease spreads further south to about 13^{o}N in Sudan and almost up to the Equator in northern Kenya and Somalia (Barotte, 1925; Hoare, 1956; 1970).

In the Near and Middle East, *T. evansi* infections have been reported from Israel, Lebanon and Syria, the northern half of the Arabian Peninsula, Asia Minor, Iraq and Iran. In Europe, it has been reported in Turkey, and a few cases in Bulgaria. In the Volga regions of the Soviet Union, the area chiefly affected by the disease reaches the northern-most point of its distribution near Kuibyshev on the Volga (53^{o}N) and the southern-most point in the Caucasus (44^{o}N). From here it passes into Soviet Middle Asia (formerly Turkestan). *T. evansi* is especially prevalent in Iran, India, Burma and presents particular problems to livestock owners in S.E. Asian countries such as those in Malay, Indochina and parts of Southern China. It also occurs in islands of the Indian Ocean, the islands of Indonesia and Philippines (Hoare, 1972; Topacio and Acevado, 1938). In the New World countries such as Mexico, all those of Central America, and Venezuela, Columbia, Brazil livestock can be affected by diseases caused by trypanosomes that are

morphologically indistinguishable from *T. evansi* (Hoare, 1972).

HOSTS OF T. EVANSI

T. evansi infections tend to affect different animal hosts in different parts of the world. Overall it infects a wide range of economically important livestock in particular equids, camels, cattle, buffalo, goats, sheep and pigs (Luckins, 1988). Those that experience high mortalities due to *T. evansi* infections include horses and camels while chronic infections are usually associated with bovines, donkeys, goats and sheep. A range of different wild animals are known to be infected in different parts of the world but the exact impact of *T. evansi* on such wildlife is not known (Losos, 1980).

T. evansi like many other parasites infects a range of hosts that can be considered as reservoir hosts. Such hosts continue to harbour the parasite but do not show any clinical signs of the disease, often remaining as carriers for long periods of time and serving as sources of infection for other animals. Many animals species have been implicated as reservoir hosts of *T. evansi,* in particular wild animals, ranging from animals as diverse as the mongoose *(Herpestes palustris)* to tigers and jaguars (Choudhury and Misra, 1972; Marinkelle, 1976; Reddy *et al.,* 1975; Sinha *et al.,* 1971). Despite the fact that wild animals can be infected with *T. evansi*, it is unlikely that they could serve as important sources of infection for domestic livestock. Successful transmission of *T. evansi* depends on factors such as vector preferences and closeness of donor to recipient host (Payne, 1988) which are unlikely to be satisfied with these wild animals under normal livestock management practices. In South America, the vampire bat *(Desmondus rotundus)* plays a uniquely important role in the transmission of *T. evansi* and is considered to represent a

carrier host (Hoare, 1965) because they carry the parasites in their blood without showing any clinical signs of the disease.

All species of domestic animals, in particular cattle, buffalo, sheep and goats can develop a chronic form of *T. evansi* infections and become carriers and consequently reservoir hosts (Losos, 1980). Experimental evidence of chronic infections in donkeys and cattle (Ilemobade, 1971), goats and sheep (Khasanov and Ivanitskaya, 1974; Chand and Singh, 1971; Malik and Mahmoud, 1978) further confirms that these animals could act as reservoir hosts. It is more likely that conditions necessary for successful transmission of *T. evansi* can be achieved with these animals rather than with wild animals. In reality, therefore, it is more likely that domestic reservoir hosts, such as cattle, buffalo, sheep and goats carrying a chronic *T. evansi* infection are more likely to constitute important sources of infection to other domestic livestock rather than the wild fauna, particularly as they are more likely to be herded together than with wild animals. Animals such as horses and camels often develop acute infections characterised by high parasitaemia and act as a source of highly infective blood for a short period before they die. These animals probably serve as an efficient source of infection to susceptible animals, fuelling short-term epidemics. Domestic livestock running a chronic form of the infection, with parasites circulating in their blood for long periods, are probably the most important sources of parasites maintaining the trypanosome in a particular area.

TRANSMISSION OF T. EVANSI

The greatest difference between *T. evansi* and the tsetse-transmitted trypanosomes infecting livestock such as *T. brucei, T. vivax* and *T. congolense*

is that *T. evansi* can be transmitted in the absence of any form of cyclical development in arthropod vectors. Such a fundamental difference has probably played a significant role in the evolution of *T. evansi* affecting its survival and distribution.

In the absence of any proven cyclical transmission mechanisms, the accepted mode of transmission of *T. evansi* is through the bite of blood-sucking *Diptera* such as the horse-fly *(Tabanus sp.)* which had previously fed on an infected animal. As early as 1899, Lingard suspected that horse-flies could be the vehicle of transmission of *T. evansi* infection from infected to uninfected animals via their blood soiled piercing organs. It was not until 1901, however, that the ability of these flies to transmit *T. evansi* was confirmed experimentally by Rogers (1901) who observed that when flies which had been feeding on a dog suffering from Surra were interrupted in their meal and then allowed to bite a healthy dog, the latter became infected. Rogers (1901) also noted that if the interval between bites was extended to 1-4 days after the initial bite, transmission of the organism did not occur, demonstrating a limited survival time for the parasites on the fly mouthparts.

Stable flies *(Stomoxys sp.)* have also been incriminated as a possible vector of *T. evansi*. Results obtained from attempted experimental transmission between a range of hosts such as horses, guinea pigs and dogs however, have shown that these flies do not appear to be important vectors as the trypanosomes were found to die very rapidly in the mouthparts of the flies. One and a half minutes after a successful bloodmeal all parasites were found either dead or in a form incapable of transmitting infections (Mitzmain, 1913). Other genera of haematophagus insects such as *Haematopota, Chrysops* and *Lyperosia* have been shown to be capable of transmitting the parasite

(Nieschulz, 1927; Nieschulz and Ponto, 1927; Nieschulz, 1930), however, results from attempts to use these flies to transmit infections from horses or buffaloes to susceptible recipient animals showed that none of them were efficient vectors of *T. evansi*.

Other arthropod vectors such as ticks which are important in the transmission of other protozoa are inefficient transmitters of *T. evansi*. Trypanosomes die rapidly in the mouthparts of ticks and no adult engorged ticks have been reported as harbouring viable trypanosomes capable of infecting mammalian hosts (Kirmse and Taylor-Lewis, 1978).

Alternative routes to direct transmission of *T. evansi* via arthropods include the epidemiologically important transmission through the bite of vampire bats *(Desmondus rotundus)* in South America (Hoare, 1965) and through the feeding of carnivores on infected meat particularly dogs feeding on infected wild deer (Adams and Lionett, 1933; Curasson, 1943; Kasansky, 1957; Galuzo and Noviskaja, 1960). This latter mode of transmission could be the method through which predator animals such as tigers and jaguars acquire infection.

EPIDEMIOLOGY OF T. EVANSI

The epidemiology of *T. evansi* like those of other parasite systems depends on a balance between infected and susceptible animals within a particular area. In classical animal disease models, infected animals eventually become immune animals, which in turn can lose their immunity and revert to susceptible animals. The role of the immune system, therefore, can be seen to play an important role in the epidemiology of the parasite system. In the case of trypanosomiasis, animals once infected probably do not become immune in the

classically accepted way due to antigenic variation (Soltys, 1963), a factor that influences the epidemiology of the disease.

The limited time the parasite can survive in the mouthparts of the fly (Rogers, 1901) makes interrupted feeding an important factor in successful transmission. The interruption of feeds is closely linked to the host's response to the fly which in turn is dependent on how the fly disturbs the host. The closeness of the donor and recipient hosts, attractiveness of the host to the flies, the level of parasitaemia in the donor host, chemotherapy and climate are some of the other factors that affect the epidemiology of *T. evansi* discussed in detail by Payne (1988).

Course of Infection

The course of infection of *T. evansi* in the vertebrate host is affected by a number of factors such as the susceptibility of the host, the strain of the parasite and the severity of the disease which differs according to the host species and location (Hoare, 1972).

The most extreme response to *T. evansi* infection can be seen in horses in which high mortality occurs after an incubation period of four to nine days. Chronic forms of the disease characterised by a succession of crises and relapses with infected horses dying after a period ranging between two to three months, have also been reported (Barotte, 1925; Galuzo and Novinskaja, 1960; Gutierrez, 1958). Usually the mortality rate in horses is very high with mortality rates of 100% reported in horses infected with *T. evansi* and death occurring as soon as four to eight days after infection (Rodenwaldt and Doewes, 1921; Doewes, 1923; Bakkar, 1930).

T. evansi infection also develops into an acute infection in dogs with an incubation period of five to ten days. Trypanosomes may appear in the blood periodically or persist throughout the infection (Barotte, 1925; Galuzo and Novinskaja, 1960; Gomez Rodriguez, 1956). The syndrome is usually very acute but in some cases could run up to six weeks. *T. evansi* infection in dogs is fatal, particularly in imported breeds (Hoare, 1972). During the period these animals are alive they could serve as short-time sources of highly infective blood for disease transmission to susceptible animals in a particular environment. The common practice of herdsmen using dogs for guarding their animals suggests that the dogs and livestock are close together. In the presence of an appropriate vector such closeness between a donor dog and the recipient livestock would facilitate disease transmission.

Acute forms of *T. evansi* infection also occur in camels although the disease tends to become chronic more often in these animals than in horses. At the onset of infection the disease may be acute with an incubation period of about seven days with parasitaemia fluctuating at high levels for three to four months. This can end fatally, but in those animals that survive the acute phase, it passes into a chronic phase, where parasite relapses become rare, and the animal can remain in apparently good health for up to several years (Barotte, 1925; Yakimoff, 1931; Boid *et al.*, 1986). Mortality rates in camels range between 50-100% but occasionally the camels are considered to recover spontaneously (Sergant *et al.*, 1918).

The chronic nature of *T. evansi* infections in bovines characterised by sporadic parasite appearances in the blood make it difficult for the course of *T. evansi* infection in these animals to be very well characterised. Trypanosomes are only occasionally detectable in the blood of such animals, their presence

only being revealed by inoculation of susceptible animals with blood samples from animals suspected of carrying infection (Lingard, 1907; Cross, 1921; Edwards, 1926; Gutierrez, 1958; Hoare, 1956). The complex nature of the outcome of infection with *T. evansi* illustrated in studies where trypanosomes causing a particular type of disease in one animal species have been transferred to another species show that severity of the disease varies from one host to another. For example, experimental infections in cattle inoculated with strains of *T. evansi* producing acute diseases in camels and horses and kept under observation for more than two years, only revealed the predominance of chronic infections (Galuzo and Novinskaja, 1960). This result demonstrates the importance of the host factors in determining the outcome of infection in these animals rather than parasite specific factors. However, when these cameline and equine strains of *T. evansi* were injected into calves, the acute form of the disease appeared with high mortalities, a probable indication of existence of an age effect on the course of *T. evansi* infections in bovines. Further evidence of the chronic nature of *T. evansi* infections in cattle came from Musgrave and Clegg (1903) who examined hundreds of cattle for *T. evansi* infection using parasitological techniques and found very few cases of infection. They concluded that the disease in cattle was chronic in nature with a low mortality rate and furthermore, that trypanosomes were so scarce in the peripheral blood that they could only be detected with certainty by inoculating susceptible laboratory animals with suspect blood.

The course of *T. evansi* infections in buffaloes is similar to that reported for cattle, although buffaloes are usually considered to be generally more susceptible. This difference may be due to factors such as stress, as buffaloes are often worked much harder than cattle and are also less likely to be well fed (Payne, 1988). In a survey carried out in the Philippines by Topacio and

Acevado (1938) on the prevalence of *T. evansi* in cattle and buffaloes, the authors used the complement fixation test to examine sera from the animals for the presence of *T. evansi* antibodies. They found that the prevalence of *T. evansi* antibodies was higher in buffaloes than in cattle and noted that although buffaloes were frequently serologically positive, it was nevertheless difficult to find trypanosomes in the peripheral blood of both animals. Trypanosomes were, however, always found to be present in antibody positive buffaloes when blood from these animals was inoculated into white rats compared to blood from cattle. These authors also reported that *T. evansi* infections in buffaloes ran a protracted course and although infected animals remained apparently unaffected by oscillations in parasite numbers, episodes of parasitaemia could continue for several months. This observation led these authors to suggest that buffaloes could serve as an important source of infection to susceptible animals in a particular environment.

IMMUNOLOGY OF T. EVANSI

Antigenic Variation

Antigenic variation is an outstanding and well documented feature of trypanosome species infecting man and domestic livestock. At the cellular level antigenic variation results from the continual changes in the chemical composition of a 12-15 nm thick layer of glycoprotein which covers the surface of the trypanosomes, throughout the course of infection (Soltys, 1963; Vickerman, 1969; Cross, 1975). *T. evansi*, in common with other species of African trypanosomes pathogenic to man and domestic livestock, uses antigenic variation as a means to evade the immune response of their host (Cross, 1975). Apart from ensuring the survival of the parasites and the continuity of the infection, antigenic variation also plays a principal role of rendering the use of

immunoprophylaxis in the control of trypanosomiasis improbable because of the specificity of the host antibody response and the large number of antigenically different parasite populations that can develop during the course of the disease (Vickerman, 1978; Cardoso de Almeida and Turner,1983; Gray and Luckins, 1976).

Trypanosome infections are characterised by successive waves of parasitaemia each one consisting of immunologically distinct parasite populations. The ability of the immune system of the host to eliminate the parasite completely from the blood is compromised by the fact that by the time that specific antibodies have been produced against the major surface component of one particular trypanosome population, other parasites bearing different antigens on their surface emerge, evade the host immune system and proliferate. This emerging parasite population is not affected by the antibodies produced by their host against any previous major parasite population because antibody responses to each parasite population, commonly referred to as Variable Antigenic Types (VAT) (Cross, 1975) are only specific to parasites bearing the homologous variant surface glycoprotein VSG and therefore, confers no protection against antigenically different trypanosomes, (Laveran and Mesnil, 1902; Ehrlich *et al.*, 1909; Weisenhutter, 1970; Wellde *et al.*, 1981; Seed, 1972; Campbell and Phillips, 1976; Murray and Urquhart, 1977; Morrison *et al.*, 1982a). The sequential emergence of antigenically different trypanosome populations often continues until the parasites overwhelm the immune system of the host resulting in death if chemotherapy is not instituted (Gray, 1965; Cross, 1978a; Donelson and Turner, 1985).

The range of individual VATs that a parasite population expresses during the course of the infection constitutes the VAT repertoire of that trypanosome

stock. Although each trypanosomal line has the potential to produce a large number of different VATs, during the course of infection, there is a tendency for stabilisation of the VAT repertoire to occur during the early infection period in naive hosts. In the case of tsetse-transmitted trypanosomes there is a tendency for a certain VAT to occur in the first wave of parasitaemia irrespective of the VAT used to infect the fly. This antigenic type is being referred to as the basic antigen and is characteristic of the particular stock (Gray, 1965). After the appearance of the basic antigen, a tendency exists then for certain VATs from the repertoire to appear regularly during the early stages of infection in different hosts infected with the same stock of parasites. These later VATs are termed the predominant VATs (pVATs) (Lumsden, 1982) and are also characteristic of particular stocks.

In the case of *T. evansi* due to the absence of the metacyclic stage in its life cycle, basic antigen type does not occur by definition but the pVATs develop in the same way as when the tsetse-transmitted species of trypanosomes are propagated by syringe transmission (Jones and McKinnell, 1984). As in the case of tsetse transmitted trypanosomes, particular VAT sequences (VAT repertoire) are associated with particular trypanosome stocks of *T. evansi* . This association offers a potential means for the classification of trypanosome stocks into pVAT serodemes on the basis of their pVAT repertoires (Jones and McKinnell, 1984).

The number of pVAT serodemes of *T. evansi* circulating in nature appears more limited than in the case of tsetse-transmitted trypanosomes such as *T. brucei* where very many serodemes have been recorded (Jones and McKinnell, 1985b). The apparent limited antigenic diversity seen in *T. evansi* could be linked to the lack of cyclical transmission in *T. evansi*. There is

mounting evidence that genetic exchange takes place during cyclical transmission of trypanosomes such as *T. brucei* (Tait, 1980). Such genetic exchange could provide the opportunity for the greater antigenic diversity seen in the case of tsetse transmitted trypanosomes than the non-cyclically transmitted *T. evansi*.

The apparent restriction in the number of pVAT repertoires of *T. evansi* circulating in nature also may be to the advantage of the host and indirectly to the parasite. In situations where the number of serodemes is limited, constant challenge of the animal with the same range of VATs would result in a build-up of immunity to those particular antigenic types. As long as this antigenic stability is maintained and animals continue to receive sufficient challenge to maintain antibody levels to the range of pVATs then it is unlikely that animals would suffer from disease. Should the animals be exposed to trypanosome stocks that express different pVAT sequences or should the antibody levels wane in the absence of a continuous rechallenge, the animals could suffer severely on rechallenge. Such a situation could explain the sporadic nature of *T. evansi* epidemics, in which there are frequent flare-ups of *T. evansi* diseases in the field. This suggests that the physical act of infection need not be the only factor in the development of disease. Some factors influencing the immunological balance within the animal population could be equally or more important.

The limited antigenic diversity amongst *T. evansi* stocks also suggests that the protection of animals from infection with vaccines containing the different pVAT antigen components could be a more realistic possibility in the case of *T. evansi* than with the tsetse transmitted trypanosome species. Prior to the development of such a vaccine, however, specific characterisation of the

dominant pVATs in any area would have to be carried out.

The process of antigenic variation has important consequences for the epidemiology of trypanosomiasis. The antigenically distinct parasite populations, which are continually emerging throughout the infection, renders the infected animal a continuous source of infection to susceptible animals in the environment. Unless the effects of antigenic variation are annulled by the death of the infected animal or the institution of chemotherapy, infected animals will continue to serve as a source of infection as long as transmission conditions are suitable.

The studies conducted specifically on antigenic variation in *T. evansi* have shown that *T. evansi* seems to behave like *T. brucei* with respect to the development of the VAT repertoire (Jones and McKinnell, 1984). Other studies by Jones and McKinnell (1985c) enabled them to classify 15 stocks of *T. evansi* into three distinct serodemes based on their pVAT repertoires. Each representative member of a particular serodeme being associated by the presence of 6-7 particular VATs as a part of their pVAT repertoire. Of these pVATs, two were found to be part of the pVAT repertoire of all 15 stocks and were considered to represent the isoVAT of these *T. evansi* stocks, as have been described from other trypanosome species (Meirvenne *et al.*, 1977; Gray and Luckins, 1976). Host species did not appear to influence the range of VATs that were detected early in infection, although differences were seen in the VAT composition of individual trypanosome populations isolated at similar periods after infection of the different hosts (Jones and McKinnell, 1985b). Horchner *et al.* (1984) made a similar observation in *T. evansi* infections in rabbits and indicated that the parasite populations became antigenically more heterogenous as the infection progressed. This led them to the conclusion that

there may be important differences between the properties of early and late appearing trypanosome populations and that parasite populations developing later during an infection could be less antigenic than those developing earlier in infection.

Few detailed studies have been made on the parasite components involved in antigenic variation in *T. evansi* (Cross, 1978b; M. Onodera, personal communication cited in Richards, 1984), unlike the extensive studies that have been conducted on the surface glycoprotein coats of the tsetse-transmitted trypanosomes such as *T. brucei, T. congolense* (Cardoso de Almeida and Turner, 1983; Ross *et al.,* 1987; Gurnett *et al.,* 1986; Cardoso de Almeida *et al.,* 1984), *T. vivax* (Gardiner *et al.,* 1987). Improved understanding of the role of the parasite surface antigens in host/parasite relationship can come from a detailed understanding of their physical and chemical properties. The fact that surface antigens can also play an important role in diagnosis as reagents in diagnostic tests designed to detect the presence of parasite antibody, provides further impetus for a better understanding of their physical and chemical characteristics. Despite the fact that the surface antigens are very immunogenic (Le Ray, 1975) and antibodies are produced against them early in infection which persist for long periods, their potential use as diagnostic reagents is limited by their variable nature. Under certain circumstances however, the surface components of the trypanosomes are used in some diagnostic techniques, such as the card agglutination test (Magnus *et al.,* 1978). For this test to be effective, the VSG of the most widely distributed isoVAT in the particular environment has to be used as the antigen.

The inherent variability in the composition of the trypanosome surface antigens and its poor potential for use in the diagnosis of trypanosome

infection has stimulated a search for alternative sources of parasite material for diagnostic tests. The fact that animals infected with trypanosomes are known to produce antibodies against both the surface and internal components of the trypanosomes (Le Ray, 1975) and that the composition of internal components are more stable than the surface components makes them better prospects as antigens for diagnostic tests. One major disadvantage of the use of the internal components is that they are made up of a large number of individual components, many of which are common to all species of trypanosomes and are likely to pose problems of cross reactions. Furthermore, it is possible that only a few of these internal components could be antigenic, therefore, the use of crude extracts containing all of them in an assay system would include the many redundant components which could in turn affect the efficiency of the test.

The basic immunology of *T. evansi* like that of other trypanosome species is complicated by the multicomponent nature of the parasite antigens and the potentially complex nature of the host immune response to the individual antigens. Apart from the surface coat of the trypanosomes which is very immunogenic and usually induces intense immunological reactions from the host, there are numerous internal components of the parasite against which antibodies are produced by the immune system of the host during the course of an infection (Le Ray, 1975). Furthermore, the host immune system could produce antibody belonging to one or more immunoglobulin class, IgG, IgM, IgA, IgE or IgD to each parasite component. Such a complication makes the interpretation of results from tests designed to evaluate host/parasite interactions difficult unless particular immunological techniques, that are capable of resolving the reaction of individual antigenic components with the appropriate host antibody are used.

The general conclusion from the many reviews of studies carried out on the host/parasite relationship in African trypanosomiasis over the last 80 years (Roelants and Williams, 1982; Vickerman and Barry, 1982; Roelants and Pinder, 1984; Nielson, 1985; Bancroft and Askonas, 1986; Shapiro and Pearson, 1986) is that the humoral immune response effected either directly through antibody-dependent phagocytosis or indirectly through complement-mediated lysis is the main method by which trypanosomes are eliminated by the host immune system. Most of these authors of these reviews tended to consider at great lengths the immunological importance of the variable glycoprotein coat of the parasites in ensuring the parasite evasion of the immune system of the host and also as the major obstacle to the use of immunoprophylaxis in the control of trypanosomiasis paying scant attention to the role of internal or non-surface antigens of the parasite. The importance of the host's specific antibody response to the surface glycoprotein coat of the parasite for both the parasite and the host are two aspects of trypanosomal immunobiology that all the authors agreed upon. Less certain however, is the exact role of the host's immune response to the non-surface components of the trypanosomes and the importance of such a response to the host, in terms of protection, resistance and susceptibility to infection. Le Ray (1975) suggested that the internal components or the common antigens stimulate a relatively feeble antibody response in comparison with that to the variable surface antigen. Roelants and Pinder (1984) suggested that the limited response to the internal trypanosome components could be due to the fact that they represented a minor part of the total antigenic make-up of the parasite and in part to their lack of opportunity to contact the relevant immunocytes as they are not usually exposed on the surface of the parasites. Roelants and Pinder (1984) however, suggested that these internal components could play a critical role in the pathogenesis of the

disease by triggering an immune response after the destruction of the parasite by VSG specific antibodies and by the formation of immune complexes. The immune response against the non-surface components of the parasite is one aspect of the *T. evansi* immunology like that of other tsetse-transmitted trypanosome species that has been neglected.

Cell-mediated responses directed against trypanosome antigenic determinants have been demonstrated in trypanosomiasis but the relevance of such a response is unclear not only in the elimination of parasites but also in the protection of animals to reinfection.

The complement system plays an important role in the destruction of microorganisms (Roitt *et al.*, 1985). The importance of the complement system in the host response to trypanosome infection was reviewed by Nielsen (1985). Most reports on the role of complement in trypanosomiasis indicate that complement plays an active role in the elimination of the parasites principally through cell lysis (Murray and Urquhart, 1977). Furthermore, the levels of complement were seen to decline during the course of trypanosome infections in man and animals (Greenwood and Whittle, 1976; Nielsen and Sheppard, 1977; Fruit *et al.*, 1977; Nielsen *et al.*, 1978b; Jarvinen and Dalmasso, 1976). Although the complement system consists of more than 30 components (Lambris, 1988), most of these studies have been conducted on the third complement component (C3) probably because of its pivotal role in the activation of both the classical and alternative complement pathways by which the complement system exerts its effect (Roitt *et al.*,, 1985). Both pathways have been demonstrated to be important in the elimination of trypanosomes (reviewed in Neilsen, 1985). Some studies on other complement components associated with later stages of complement activation such as C8

(Nielsen *et al.*, 1978a) and C6 (Jarvinen and Dalmasso, 1976) indicated that the levels of these components are not affected in trypanosomiasis unlike C3. This observation led these authors to suggest that the completion of the complement cascade might not be required for effective control of trypanosomiasis as in other pathogens. No such studies however, have been conducted specifically with *T. evansi* infected animals.

One feature of trypanosome infection that modifies and compromises the host immune response in laboratory animals (Goodwin, 1970; Goodwin *et al.*, 1972; Murray *et al.*, 1974a,b; Mansfield, 1978; Hudson and Terry, 1979), cattle (Holmes *et al.*, 1974), goats (Griffin, 1978) and man (Greenwood *et al.*, 1973) is immunosuppression. This renders the host susceptible to secondary infection, diminishes their response to viral and bacterial vaccines, and possibly the response to trypanosome infection itself.

The first evidence for trypanosome-induced immunosuppression came from the observation that *T. brucei*-infected mice and rabbits expressed an impaired antibody response to sheep red blood cells (SRBC). (Goodwin, 1970; Goodwin *et al.*, 1972 and Longstaffe *et al.*, 1973). Following this, several other workers evaluated this phenomenon of immunosuppression in greater detail. Hudson *et al.* (1976) demonstrated a reduction in the number of IgG and IgM plaque forming cells in the spleen of trypanosome infected animals while Ackerman and Seed (1976) and Murray *et al.* (1974b) demonstrated a suppression of the ability of B-lymphocytes to respond to helper T cell dependent antigens such as bovine serum albumin and T cell independent antigens such as bacterial lipopolysaccharide. T-cell response induced by oxazolone treatment of ear skin of infected rats and mice was also observed to be depressed in animals infected with *T. b. brucei* and *T. b. gambiense*

(Urquhart *et al.*, 1973; Ackerman and Seed, 1976). Results of these studies indicate that all the components of the host immune response, both humoral and cell mediated, are suppressed during trypanosome infection. In effect, apart from coping with the direct effect of trypanosome infection, trypanosome-infected animals also have to cope with the problems of an impaired immune system.

There is increasing evidence that the degree of immunosuppression is related to the parasite burden of the host in that immunosuppression is most marked in hosts that have high parasitaemias and least marked in hosts that have a fewer parasites in their blood (reviewed in Vickerman and Barry, 1982). Furthermore, the elimination of trypanosomes by drug treatment of the trypanosome infections restores immunocompetence and enables the host to mount a full secondary response (Freeman *et al.*, 1974; Murray *et al.*, 1974c; Wellhausen and Mansfield, 1980). Some of the mechanisms by which trypanosomes are thought to bring about immunosuppression have been reviewed by Vickerman and Barry (1982) and these include amongst other factors, defective antigen handling/presentation and antigenic competition. They suggested that as the induction of a response to T-dependent antigens often depends upon macrophage presentation to T cells, a defect in antigen handling and presentation could be responsible for the development of immunosuppression. Evidence of damage to the macrophage system provided by Mansfield and Bagasra (1978) showed that the serum of *T. b. rhodesiense*-infected mice prevented the uptake of particulate antigens by macrophages, and also that macrophages from infected mice were unable to initiate *in vivo* plaque-forming cell response in syngeneic recipients following exposure to sheep red blood cells (SRBC). A progressive depletion in the number of antigen-presenting macrophage subpopulations has been observed to occur in

the spleen, and lymph nodes and peritoneal cavity of trypanosome-infected animals (Bagasra *et al.*, 1981). Such a development could worsen the state of immunosuppression in trypanosome-infected animals. The progressive depletion of these cells with the progress of the disease could probably account for the more serious forms of immunosuppression associated with the later stages of infection.

Immunological memory, a fundamental property of the immune system responsible for an enhanced response to secondary exposure to an antigen appears to be affected in trypanosomiasis. Evidence is now emerging that trypanosome infection can damage the mechanism of secondary reaction, for example rats initially infected with *Nippostrongylus brasilensis* and then infected with *T. brucei* immediately after the phase of immune expulsion of the worms, lost the ability to expel a subsequent worm challenge. The elimination of the trypanosome infection by chemotherapy, however, resolves the host's secondary response. This result suggests that trypanosome infection blocks the expression of a secondary immune response, rather than abolishing the immunological memory of the infection (Urquhart *et al.*, 1973). Immunological memory may, however, become exhausted as a result of trypanosome infection in that Askonas *et al.* (1979) reported that spleen cells from *T. brucei*-infected mice were unable to confer the ability to mount a response to a secondary infection to T dependent antigens when transferred to irradiated recipient mice.

The five major immunoglobulin classes of antibody interact with the antigens in different ways, therefore a knowledge of the range of immunoglobulin (Ig) isotypes produced in response to trypanosome infection is important in achieving a better understanding of the host/parasite

relationship (Jenni , 1977; Murray and Urquhart, 1977; Nantulya *et al.*, 1980; Morrison *et al.*, 1982a; Greenblatt *et al.*, 1983; Ngaira *et al.*, 1983). The majority of studies on the immunoglobulin classes associated with trypanosome infection have been undertaken on the IgM and IgG classes at the expense of the other classes such as IgA, IgD and IgE. This concentration on IgM and IgG probably stems from the fact that their assays are relatively easily undertaken and the traditional association of these two classes with infections such as those caused by trypanosomes. Each antibody class produced by the host has a potentially different role to play in dealing with the parasites through well established immune defence processes such as, complement fixation, agglutination, neutralisation and cell lysis (Roitt *et al.*, 1985). Results from studies on antibody production in trypanosome-infected animals suggest that early in infection IgM is a more efficient agent than IgG for the *in vitro* neutralisation, agglutination and lysis of trypanosomes and for providing protection *in vivo* (Seed, 1972; 1977; Takayangi and Enriquez, 1973; Luckins, 1976; Musoke *et al.*, 1981). Other studies with different parasite systems such as those of Smith *et al.* (1967) working with *Proteus mirabilis* have suggested that IgG antibody is superior to IgM in enhancing phagocytosis. This finding is different and contradictory to the observation made with trypanosomes considering that processes like agglutination which were found by the group of workers mentioned above to be better effected by IgM than IgG enhance phagocytosis. This difference may be due to the fact that the function of the immunoglobulins is influenced by the nature of the antigens.

Markedly elevated levels of IgM in animals infected with different species of trypanosomes have been reported by several investigators (Seed *et al.*, 1969; Luckins, 1972; 1974; Clarkson *et al.*, 1975; Kobayashi and Tizard, 1976; Zahalsky and Weinberg, 1976; Luckins and Mehlitz, 1976). Kobayashi and

Tizard (1976) observed an increase of 6-24 times the preinfection IgM levels in animals infected with *T. congolense* and only an increase of 2.5 times the preinfection IgG levels. Luckins (1974) in his study of immunoglobulin levels in zebu cattle naturally infected with trypanosomes demonstrated a 2-9 times increase of IgM and a two-fold increase in IgG levels respectively. The mechanism responsible for these marked increased immunoglobulin levels is not understood. Seed *et al.* (1969) attributed the increase in IgM levels to the effect of antigenic variation, in which the continual emergence of new antigenic types stimulate a typical IgM first response. Other workers have suggested that the trypanosome parasitaemia stimulates the synthesis of non-trypanosome specific IgM immunoglobulins. Evidence for this comes from the fact that such IgM from trypanosome-infected hosts have been shown to react with antigens to which the host had not been exposed previously, such as sheep red blood cells and guinea-pig kidney extract (Houba *et al.*, 1969). A similar observation was reported by Hudson *et al.* (1976) who demonstrated polyclonal B cell stimulation during trypanosome infection with a consequential increase in non-specific immunoglobulin production. The production of such immunoglobulins in trypanosomiasis questions the specificity, relevance and importance of the elevated IgM levels in trypanosome infected animals obtained by most of the workers cited above who studied the levels of the different immunoglobulin classes in trypanosomiasis using assay systems that could not relate the antibody specificity to the trypanosome.

Conclusions reached as to the relative importance of the different immunoglobulin isotype in trypanosome-infected animals are often influenced by the technique used for assessing antigen/antibody interaction. Results with the trypanosome agglutination test as reported by Seed *et al.* (1969), Zahalsky and Weinberg (1976) suggested that a predominant trypanosome specific IgM

production during the early period of infection in rabbits infected with *T. b. gambiense* and in rats or cattle infected with *T. b. brucei*. Furthermore, Takayangi and Enriquez (1973) also reported that after primary challenge of mice with *T. b. gambiense,* purified IgM was 200-fold more effective in a neutralization of infectivity assay and 20-fold more effective when used in an agglutination assay than IgG. Other investigators however, reported that the primary trypanosome-specific response of mice and cattle to *T. b. brucei* or mice to *T. b. rhodesiense* was predominantly IgG (Campbell *et al.*, 1978; Nantulya *et al.*, 1979; Sacks and Askonas, 1980; Mansfield *et al.*, 1981). Nantulya and his colleagues used a radioimmunoassay employing purified VSG as the antigen while the other investigators used a fluorescent antibody staining technique with fixed trypanosomes as antigen, which they claim was VSG specific.

The use of an agglutination test to evaluate trypanosome antibody activity would bias the system towards IgM because IgM is considered to act as a more efficient agglutinating agent than IgG (Roitt *et al.*, 1985). Such agglutination is enhanced with particulate antigens (Bowry, 1984) such as when whole trypanosomes are used as antigen in an agglutination test. The use of purified VSG as the antigen in radioimmunoassay or fluorescence on fixed trypanosomes a test that is VSG specific should not bias the system towards any of the immunoglobulin classes unlike the agglutination test. The results obtained by the group that recorded IgG predominance probably reflect more closely the contribution made by each Ig class to the overall trypanosome specific-response than those reporting on IgM predominance.

Of the three other immunoglobulin classes that can be produced by the host in response to infection, only IgA and IgE, have received a cursory

mention in animals infected with trypanosomes. In one study, total serum IgA levels were found to fall in cattle experimentally infected with *T. congolense* (Nielsen *et al.*, 1978a). The authors admitted their difficulty in interpreting the finding but suggested that this might be resolved by evaluating changes in IgA levels at external mucosal surfaces, where IgA is partly responsible for protection against invading pathogens while evaluating changes in IgA levels in the serum. Such invasions are common in trypanosome-infected animals resulting in serious secondary infections which further compromise the host (Losos and Ikede, 1972). The relationship between changes in the IgA levels on the extravascular surfaces and IgA levels in the blood during the course of infection could assist in a better understanding of the importance of IgA in trypanosomiasis.

Studies on the dynamics of antibody levels in *T. evansi* infection are limited in comparison with those that have been undertaken with the tsetse-transmitted trypanosome species. Schoening (1924) used the complement fixation test to demonstrate the presence of antibodies in *T. evansi* infected camels. Following this observation, Bennett (1929) demonstrated the specific increase in euglobulin levels in animals infected with *T. evansi*. Other studies that demonstrated an increase in total antibody levels in animals infected with *T. evansi* include those of Jatkar and Singh (1971) and Boid *et al.* (1981) using standard serodiagnostic tests such as indirect haemagglutination and ELISA.

Studies on the levels of different classes of immunoglobulin production in animals infected with *T. evansi* are even more limited. Results available from such studies have demonstrated that IgM predominates in animals infected with *T. evansi* (Luckins, 1977; Luckins *et al.*, 1978, 1979; Boid *et al.*, 1986) as in the case of infection with other trypanosome species. The usefulness of

results from such studies in helping to understand the dynamics of the host/parasite relationship is, however, limited by the fact that the assay systems used in such studies cannot associate the activities of any of the immunoglobulin classes with any parasite specific antigen.

Despite the different and often conflicting results obtained from studies on the levels of the immunoglobulin isotypes in animals infected with trypanosomes, one aspect of which all workers appear to agree upon is that specific trypanosome antibodies are important not only in controlling an existing parasite infestation but also in protecting animals against reinfections with homologous trypanosomes. Furthermore, all the workers highlighted the importance of non-specific polyclonal B cell stimulation in trypanosomiasis thought to be caused by the mitogenic effect of trypanosomes (Esuruso, 1976; Assoku and Tizard, 1978) which could affect total immunoglobulin assays.

There are hardly any studies of comparable detail with those of the humoral response on the importance of cellular immunity in trypanosomiasis due to *T. evansi* despite the fact that Gomez Rodriguez (1956) suggested that the cellular components of the host immune system could be important in the destruction of *T. evansi*. He emphasized the possible importance of the phagocytic cells found within the leucocyte population, the liver and the spleen in the removal of the parasites.

Other manifestations of host response to trypanosome infection can be obtained from observing changes taking place in organs that may have an important role to play in regulating the immune response of the host. Morphological changes from normality, or rather non-infection, in such organs may not necessarily be pathogenic but could be more related to their role in defence against the invading parasites. Such changes can provide important

information on how effectively the host is coping with the infections. Changes in organs such as the lymph node and the spleen, organs that play an important role in the genesis or development of many effector cells such as the B and T lymphocytes can provide basic information on the nature of the defence mechanism, that is of paramount importance in a particular parasite system. Changes in organs such as the liver may be important in understanding the effect of the infection on accessory systems like the complement system because the liver produces more than 90% of complement component (C3) which is central to the two pathways, classical and alternative, through which the effector functions of the complement system is achieved (Lambris, 1988). Any changes in the liver could therefore, affect the amount of this substance available for the destruction of trypanosomes by the immune system.

Clearly over the past 80 years the role of the immune system of a host suffering from trypanosomiasis has attracted a lot of attention from scientists. Most of the attention has been directed to the humoral immune response, particularly against the surface antigen of the parasite with very little attention paid to the non-surface component. Very little information of practical use has emerged from these studies in reality for the control or eradication of trypanosomiasis, apart from the development of diagnostic techniques. Furthermore, the tendency for studies on the host/parasite relationship in trypanosomiasis have become polarised towards the surface antigen or to responses considering the organism as a single antigenic entity rather than examining the complex multicomponent nature of trypanosome antigen and host response to individual parasite components. This experimental trend could compromise other parts of the parasite that are non-surface in origin and other aspects of the host response that are not antibody-dependent that could

provide important alternative strategies to solve the problems of trypanosomiasis. The tendency to isolate the immune response to specific effector functions such as agglutination, or systems such as antigen/antibody interaction at the expense of changes in important accessory systems such as complement, may further hinder our better understanding of host/parasite relationships in trypanosomiasis. Very few systems in the host act in isolation, particularly the immune system, therefore future studies need to broaden their baseline encompassing an integrated approach in evaluating the effects of trypanosomes on their hosts.

CHAPTER TWO

OPTIMIZATION OF IMMUNOBLOTTING TECHNIQUE FOR THE DETECTION OF ANTIGENIC COMPONENTS OF TRYPANOSOMA EVANSI

INTRODUCTION

By virtue of their intracellular location and chemical structure, the different subcellular components of the trypanosome engender a complex range of host reactions to their presence. Some components may not evoke any response while others stimulate a range of responses that may or may not be protective against further challenge with the organism. Most studies conducted on the role of the immune system in host/parasite relationships have used assay systems such as ELISA (Voller *et al.*, 1975, 1976, 1977; Luckins, 1977) employing crude extracts of the parasite as antigen. Results from such assays are incapable of providing information on the way in which the host responds to individual trypanosome components. Apart from obtaining information on host/parasite relationships serological assays are being used increasingly as diagnostic aids. The use of crude parasite extracts as antigen also often complicates the interpretation of results from such assays with the multicomponent nature of the antigens often contributing to reduced performance of potentially sensitive and specific assay techniques such as ELISA. There is the need therefore, to identify the important components of the parasite that could satisfy specific needs, such as reagents in immunoassays, starting materials for the development of vaccines and also for a better overall understanding of the host/parasite relationship.

Trypanosomal antigens can be divided into surface and non-surface or internal components. The surface component consists of a 12-15 nm thick layer of glycoprotein (Vickerman, 1969), the chemical composition of which continually changes by a process known as antigenic variation throughout the course of the infection (Soltys, 1963; Cross, 1975). The host generates a specific immune response to each new antigenic type or variable antigen type

VAT (Cross, 1975) which is only protective against a challenge with a homologous VAT (Wellde *et al.*, 1975, 1981; Emery *et al.*, 1980; Morrison *et al.*, 1982a; Hall and Esser, 1984). The variable nature of the surface antigens within the same stock of trypanosome reduces their value for use in serodiagnostic tests as any one VAT will only detect antibodies produced against itself unless a particular widespread VAT is used as antigen such as in the card agglutination test CAT (Magnus *et al.*, 1978). Surface antigens of trypanosomes have attracted a lot of attention, however, for experimental studies probably due to the practical ease with which such studies can be conducted often with intact trypanosomes.

Internal antigens are components common not only to all VATs of a trypanosome stock but also to other trypanosome stocks and species. These antigens unlike the surface antigens, consist of a large number of components and only become available to the immune system of the host after the intact trypanosomes have been broken down by specific antibodies directed against the surface antigen of the parasite (Roelants and Pinder, 1984). Antibody responses to internal antigens characteristically, therefore, develop later than those to the surface. The common nature of the internal antigens of trypanosomes probably explains the high degree of cross reactions seen between serum samples from animals infected with different species of trypanosomes when tested using assays based on crude trypanosome extracts as antigen. Cross reactions are important where more than one trypanosome species is prevalent in an area each of which requires different control strategies. In areas where there is only a single trypanosome species present the potentially high level of redundancy of most of the trypanosome components in the crude antigen extracts probably reduces the overall efficiency of assays such as ELISA.

The host response to trypanosome infection is considered to play a key role in determining the outcome of infection. Many studies have been conducted on the interaction between host immune system and the surface antigen (Wellde *et al.*, 1975, 1981; Emery *et al.*, 1980; Morrison *et al.*, 1982a; Hall and Esser, 1984), while few studies have been undertaken with the response to non-surface components (Le Ray, 1975). Surface antigen studies are relatively easy to carry out as only a single component of the parasite is involved and techniques for such assays are often not technically demanding. The multicomponent nature of non-surface antigen and their location within the body of the trypanosome make it difficult to assay the response to individual components when employing commonly used methods such as enzyme-linked immunosorbent assays (Voller *et al.*, 1975; 1976; 1977; Luckins, 1977) with crude protein extracts as antigen.

The introduction of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Towbin *et al.*, 1979), provided a powerful and rapid method for the separation and identification of individual parasite components based on their mobility in an electric field and molecular weight. The identification of individual components as antigen, however, requires their reaction with specific antibodies. Unfortunately, this cannot be undertaken directly on the acrylamide gel, therefore the separated parasite components have to be transferred onto a solid matrix such as nitrocellulose where they can be probed for antigenicity. The process of transferring the component from the SDS-PAGE gels onto the solid matrix is referred to as blotting; the commonest variant used for antigenicity studies being referred to as "Western" blotting (Burnette, 1981). Once transferred, the individual protein bands can then be probed for antigenic activity with specific antibodies. The combination of SDS-PAGE and "Western" blotting, therefore offers the possibility of

dissecting the host's response to individual parasite antigens to evaluate their association with particular stages of the infection.

The identification of individual parasite antigens using a combination of SDS-PAGE and "Western" blotting, however, presents a number of practical problems that have to be overcome to ensure that the results obtained are a true reflection of the antigen-antibody reactions and not a procedural artifact. Commonly encountered problems are mainly associated with the processing of blots and the antibody preparation used to probe the blot. Some of these problems include:

(1) The non-specific binding of reagents to areas of the solid matrix that do not contain any parasite material. This arises from the high binding affinity of the nitrocellulose membrane to any protein. This problem is usually overcome by blocking off any such sites with excess proteins from other animal species after transfer of the parasite components. The introduction of these proteins could, however, interfere with subsequent reactions for the detection of antigens, so the conditions for use of blocking reagents have to be well established, usually for individual parasite system, to ensure reproducible results.

(2) "Western" blotting also shares many of the problems encountered with other immunoassays such as the non-specific binding of immunoglobulins or normal serum components to the antigen. This problem is usually overcome by the use of appropriate dilutions of the serum and other reagents.

The combination of SDS-PAGE and "Western" blotting has been used extensively for the study of parasite systems (Almond and Parkhouse, 1986; Cabrera *et al.*, 1986; Cabrera and Parkhouse, 1986) including several trypanosome species. Up to now the main applications of this technique has

been in the study of the surface antigen of trypanosomes. Cardoso de Almeida and Turner (1983) used the technique to investigate the properties of the membrane form of the variant surface glycoprotein (VSG) of *T. brucei*, while Ross *et al.* (1987) used it to evaluate the nature of attachment of the VSG to the surface of *T. congolense*. Araujo and Tighe (1984) investigated the expression of a surface coat component of *T. cruzi* in the bloodstream forms of the parasite using SDS-PAGE/"Western" blotting, while Gardiner *et al.* (1987) used the technique for the identification and isolation of a variant surface of glycoproteins from *T. vivax* along with Cross (1978b) who used it for the identification of the surface components of *T. evansi*.

The practical application of "Western" blotting for the analysis of immune responses involve several procedure steps, each of which often has to be optimized for the particular parasite systems. Such optimisation ensures that the system itself does not influence the results of the antigen/antibody interaction (R. Boid, personal communication) and also ensures reproducibility of results:an overriding requirement in any comparative study.

The major steps that are involved in any "Western" blotting procedure are:-

(a) The solubilisation of parasite proteins.

(b) The electrophoretic separation of crude parasite extract into individual components.

(c) The transfer of separated parasite proteins onto a solid matrix such as nitrocellulose membrane on which the antigen/antibody reactions take place.

(d) The identification of the parasite antigens by probing of parasite proteins with appropriate antibody.

The experiments in this chapter were designed to develop a suitable protocol that could be used comparatively for the study of *T. evansi* antigens throughout the study.

SOLUBILISATION OF PARASITES

MATERIALS AND METHODS

Trypanosomes

T. evansi TREU 2113 used for the study was obtained after a single passage through mice of stabilated parasites from *T. evansi* TREU 1840. *T. evansi* TREU 1840 was acquired by the Centre for Tropical Veterinary Medicine (CTVM) from the University of Berlin in 1983 as a stabilated material with a history of characterisation as *T. evansi* and isolation from a buffalo in Java, Indonesia. The morphological appearance of the parasites on Giemsa stained smears agreed with the characteristics of *T. evansi* listed by Hoare (1972). The population of *T. evansi* (TREU 2113) used for the studies is known to consist of trypanosomes of a single variant antigen type (VAT) (T.W. Jones, personal communication).

Infections

Trypanosome infections were initiated from cryopreserved material injected into mice. Once fulminating infections were established, the trypanosomes were passaged into white albino rats. In turn, when the rats developed fulminating infections they were exsanguinated by cardiac puncture under general anaesthesia and about 10 ml of blood collected from each rat into syringes containing 50 iu of heparin in 1 ml of phosphate buffered saline (PBS) (Appendix 1). Infections in mice/rats were not continued beyond three

days to minimise the possibility of antigenic change in the parasite population.

Preparation of T. evansi Soluble Antigens

Trypanosomes were separated from the host's blood cells by anion-exchange chromatography on DEAE cellulose (DE52, Whatman Biochemicals) by the method described by Lanham and Godfrey, (1970). The separated trypanosomes were washed three times by centrifugation at 2,500 g for 10 minutes with phosphate saline glucose (PSG) pH 8.0 (Appendix 2). Parasite washes were carried out in a 12 ml graduated centrifuge tube and after the third centrifugation the pelleted trypanosomes were resuspended in twice their volume of an enzyme protectant solution (Appendix 3) containing 0.25 mM TLCK (N-p-tosyl-L-lysine chloromethyl ketone, Sigma Ltd.) as proteinase inhibitor. Soluble antigens of the parasite were released by disrupting the trypanosomes by freeze-thaw or sonication (Auffret and Turner, 1981). For freeze-thaw disruption, trypanosomes suspended in enzyme protectant (Appendix 3)/0.25 mM TLCK solution were subjected to three cycles of freezing to -80^{O}C for 10 minutes with solid carbon dioxide followed by thawing to room temperature for 10 minutes. For sonication trypanosomes were subjected to five cycles of ultrasound using an MSE ultrasonic disintegrator at maximum amplitude (10 microns peak to peak) with a 2 mm titanium probe at 4^{O}. Each sonication cycle lasted for 10 seconds with a 10-second interval between cycles.

The resulting trypanosome lysate from both methods of disruption was centrifuged at 10,000 g for 45 minutes at 4^{O}C to separate the solubilise components of the parasite from the non-solubilised components. The total protein concentration of each supernate fraction was determined

photometrically using BCA^R technique undertaken according to the manufacturer's instruction (Pierce Chemical Company, USA). Sufficient SDS-sample buffer was then added to the supernate to ensure that the final solution contained three times as much SDS as there was trypanosomal protein. The solution was then heated to 100°C for three minutes. The resulting solution was aliquoted into 100 µl volumes and stored at -20°C until required. Immediately before use, the samples were heated to 100°C again for one minute to dissolve any SDS that might have crystallised out.

Electrophoretic Separation of T. evansi Proteins using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Vertical glass plates (160 mm x 180 mm) separated with 1.5 mm spacers were filled with 7-20% continuous gradient polyacrylamide gel (see Appendix 4 for formulae). Each gel was cast using 20 ml of each polyacrylamide gel component (7% and 20%) poured into one of the two units of a gradient mixer. 26 µl of 10% ammonium persulphate and 6 µl of $N_1N_1N_1N$-tetra-methylene diamine (TEMED, BDH) were added to each unit of the gradient mixer and the contents pumped into a glass mould at room temperature. After polymerisation, a 4.5% stacking gel (Laemmli, 1970) containing 25 µl of TEMED and 100 µl of 10% ammonium persulphate was layered on top of the continuous gradient gel and 10 x 20 mm sample wells cast in the stacking gel with a plastic comb.

For electrophoresis each gel was placed in a vertical gel tank apparatus containing electrophoresis tank buffer (Appendix 5) in both top and bottom reservoirs before the samples and proteins of known molecular weights (Molecular weight markers, Pharmacia, Appendix 20) were loaded in the wells

formed by the plastic comb in the polymerised stacking gel. Electrophoresis was performed at room temperature, initially with a constant voltage of 150V for one hour, to hasten the migration of the proteins through the stacking gel, thereafter at a constant voltage of 80V until the bromophenol blue dye of the SDS-sample buffer reached the bottom of the gel, a process that took between 16 and 20 hours.

Identification of Individual Trypanosome Proteins on SDS-PAGE Gels

After electrophoresis the gels were removed from the glass moulds and stained either with Coomassie Brilliant Blue R-250 (Sigma Ltd., U.K.) or with silver stain.

For Coomassie Blue staining, the gels were immersed in 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma Ltd., U.K.) in destain solution (Appendix 6) at room temperature with continuous mild shaking for 2-4 hours. Thereafter, the gels were destained with destain solution overnight. For silver staining of trypanosome proteins in SDS-PAGE gels, the method described by Morrissey (1981) was used. Briefly, the gels were incubated sequentially for 30 minutes in solutions 1, 2 and 3 (Appendix 7) to fix the proteins in the gels. The gels were then washed with several changes of distilled water overnight. Thereafter, the gels were incubated for 30 minutes in 0.1% (w/v) silver nitrate (Sigma Ltd., U.K.), subsequently washed and then developed with an alkaline solution of formaldehyde (Developer, Appendix 8) for about 20 minutes before the reaction was stopped with 5 ml of 2.3M citric acid (BDH, U.K.) added per 100 ml developer. After thorough rinsing of the stained gels in distilled water for about 30 minutes the gels were photographed in order to have permanent records of the results. The calibration curve from the molecular

weight markers and calculation of molecular weight of individual trypanosome proteins were based on measurements obtained from such photographs.

RESULTS

The overall protein profiles of the soluble antigens of *T. evansi* obtained after freeze-thawing (Figure 2.1, No. 1) and sonication (Figure 2.1, No. 2) were similar in that the same pattern of bands was seen when SDS-PAGE gels were stained with both silver stain and Coomassie Blue. A greater number of bands were, however, seen when the gels were stained with the silver stain than with Coomassie Blue. Some differences were also seen between the range of molecular weight of the components obtained by freeze-thaw and those by sonication. The differences were confined to the lower molecular weight regions in that the components identified from the freeze-thawed sample had a molecular weight range of between 123800-11000 daltons (Figure 2.1, No. 1) while those released by sonication were in the range of 123800 to 12500 daltons (Figure 2.1, No. 2). Certain protein bands such as those with molecular weights of less than 20,000 were better resolved and more intensely stained with both staining methods in the extracts obtained by freeze-thawing rather than by sonication.

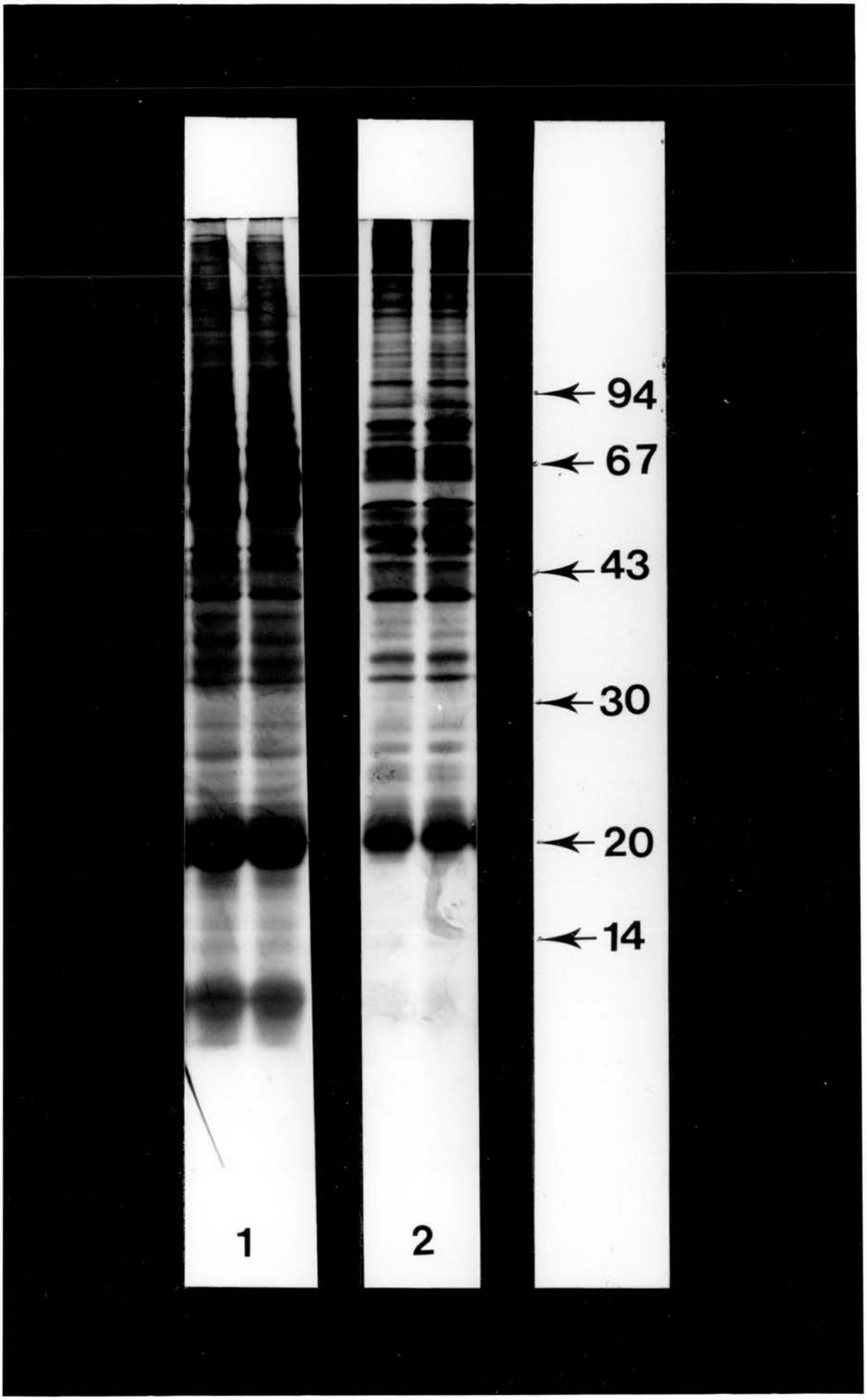
Furthermore, twice as much total protein obtained after sonication (Figure 2.1, No. 2) had to be applied to the gel to produce results equivalent to those obtained by the freeze-thaw method (Figure 2.1, No. 1).

FIGURE 2.1 (Nos. 1 and 2)

Supernate fraction of *T. evansi* lysate separated on SDS-PAGE and stained with silver stain.

1. Sample prepared by freeze-thawing (39 μg of protein material/well).

2. Sample prepared by sonication (56 μg of protein material/well).


94
67
43
30
20
14
1
2

DISCUSSION

Solubilisation releases the parasite antigens from intact organisms into a state they can be easily used to investigate antigen/antibody reactions. Furthermore, it is likely that the cell lysis resulting from treatments adopted in the present study is closer to that occurring in the host when the parasite is destroyed by the immune system (Murray and Urquhart, 1977), than if parasite extracts had been obtained by other methods such as mixing the parasites with sample buffer and boiling to release the proteins.

Disruption by freeze-thaw and sonication both released a broadly similar range of proteins (Figure 2.1, Nos. 1 and 2). Differences were however, seen in the intensity with which individual components were stained with the intensity being greatest in samples obtained by freeze-thawing (Figure 2.1, No. 1). This was particularly noticeable with the lower molecular weight components. Differences between the two techniques used for parasite disruption were amplified by the staining method due to the sensitivity of the silver staining method which is capable of detecting components present in 100 ng of protein amounts or less (Goding and Handman, 1984).

The differences seen in the amount and composition of proteins released by freeze-thaw compared with sonication is probably related to the amount of cell destruction taking place during each process. It would appear that the extremes of temperature encountered by the trypanosome during freeze-thaw were more effective in releasing soluble antigens from the trypanosomes than the waves of ultrasound encountered during sonication.

TRANSFER OF T. EVANSI PROTEINS ONTO NITROCELLULOSE MEMBRANE

INTRODUCTION

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) provides a very rapid and refined way of separating the many parasite proteins into individual components. The relatively small pore size of acrylamide in the gel, however, presents practical problems in ensuring the penetration of antibody into the gel when attempting to probe for antigenicity using specific antibody.

A number of methods have been used to detect proteins directly after fractionation on polyacrylamide gels such as using enzyme substrates (Dulaney and Touster, 1970) or specific antibody (Showe *et al.*, 1976) but the recent innovation of transferring the gel-fractionated proteins onto some form of solid phase (Bowen *et al.*, 1980; Renart *et al.*, 1979; Bittner *et al.*, 1980) has greatly assisted in the characterisation of the parasite components initially separated by SDS-PAGE. Many of the techniques, however are time consuming, complicated and technically demanding. The method described by Towbin *et al.* (1979), however, overcomes many of these problems and involves the electrophoretic transfer of gel-fractionated parasite proteins onto nitrocellulose paper. Once transferred, the antigenicity of the individual parasite components can be assessed with specific antisera. This process has been found to be an elegant and straightforward method for antigenic analysis of parasite proteins and has been used by many workers studying different parasite systems (Burnette, 1981). The use of an electric current to effect the protein transfer makes this process much faster than other techniques employing passive diffusions or requiring the chemical activation of paper such

as diazobenzyloxyl-methyl (DBM) paper before use. With the nitrocellulose paper technique (Towbin *et al.*, 1979) it is possible to routinely achieve what is essentially a complete and quantitative elution of most proteins from SDS-PAGE gels with no loss in electrophoretic resolution, which is not possible with some of the other techniques (Burnette, 1981). Furthermore, it is also possible to correlate specific immunological activity with individual protein bands separated on the gel, since a perfect replica of the proteins on the gel is produced after the electrophoretic transfer of proteins onto nitrocellulose paper.

The principal factor influencing the transfer of proteins from SDS-PAGE gels onto nitrocellulose paper is the length of time that the system is exposed to electric field. Too short a time will result in too few proteins being eluted while too long a time may result in the denaturation of the proteins and possible damage of the gels due to excessive heat generated during the process of electrophoresis. Recommended times for optimum transfer, however differ. Burnette *et al.* (1981), for example, suggested a protein transfer (blotting) time of between 16-22 hours at 6-8 V/cm of gel while Towbin *et al.* (1979) suggested that a transfer time as short as one hour at the same field strength, 6 V/cm of gel,was sufficient.

Experiments in this part of Chapter Two were designed to determine the appropriate time interval that would provide the maximum transfer of *T. evansi* proteins from the gel to the nitrocellulose paper with respect to the number of bands resolved and their intensity of staining.

MATERIALS AND METHODS

Soluble antigens of *T. evansi* were prepared as described previously using the freeze-thaw method and fractionated into individual components by SDS-PAGE as described in the previous section.

Electrophoretic Transfer of T. evansi Proteins onto Nitrocellulose Paper (Electroblotting Conditions)

After fractionation of *T. evansi* proteins by SDS-PAGE, the gel was sectioned into four equal vertical pieces and each section laid, side by side, on four strips of nitrocellulose paper previously cut to the size of the four gel sections. A filter paper:nitrocellulose membrane:gel:filter paper "sandwich" was then formed and the electrophoretic transfer of the proteins to the nitrocellulose membrane undertaken following the instructions of the makers of Ancos semi-dry electroblotter (Dako Ltd., U.K.) using the blotting buffer (Appendix 19). Electroblotting was conducted for a maximum of 90 minutes at room temperature with a voltage of 100V and a current of 200 mA. The effect of transfer time on protein transfer was evaluated by removing one of the four strips of gels with their corresponding underlying nitrocellulose membranes, after 15, 45, 60 or 90 minutes elution. The residual proteins left in the gel after a particular transfer time were detected by staining the gel with Coomassie Blue as described previously. The amount of proteins transferred onto nitrocellulose paper was determined by staining the nitrocellulose paper with a 1% Amido black (Sigma Ltd., U.K.) in destain solution (Appendix 6) for 15 minutes after which the nitrocellulose paper was destained with destain solution for 30 minutes at room temperature to enhance the appearance of the protein bands.

RESULTS

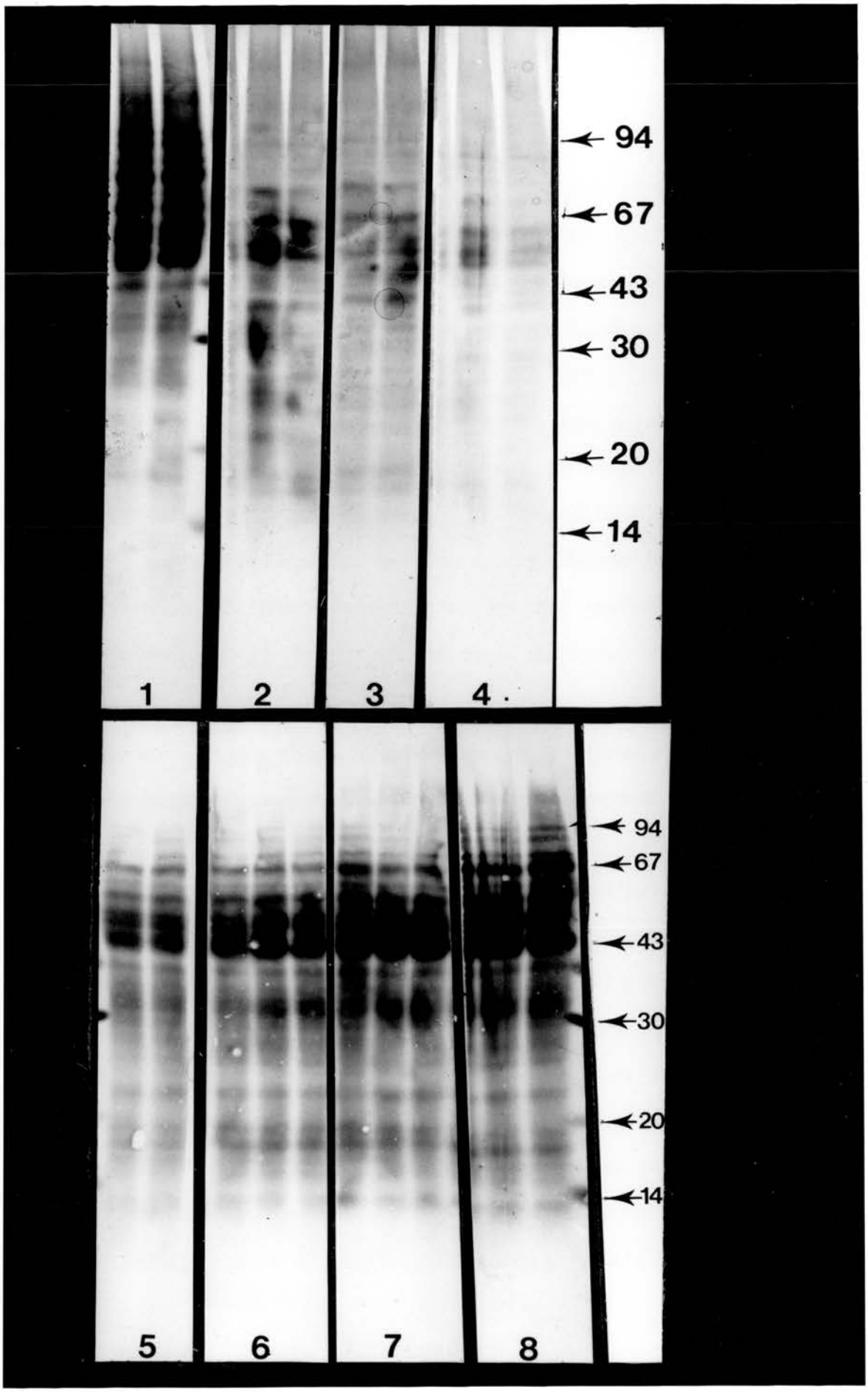
Increasing amounts of *T. evansi* proteins were transferred from the gel onto the nitrocellulose membrane with time. Maximum amounts of parasite proteins were eluted by 90 minutes of blotting (Figure 2.2, No. 4) while the least amount of proteins was transferred after 15 minutes of blotting (Figure 2.2, No. 5).

The intensity of staining of individual bands was also time dependent with the lowest intensity of staining observed after 15 minutes and the highest after 90 minutes of blotting respectively. Protein bands with a lower molecular weight, such as those below 43,000 daltons, were observed to elute faster from the gels than those with higher molecular weights (Figure 2.2, Nos. 1-4). By 90 minutes of blotting however, small amounts of the lower molecular weight bands were detected on the gel while slightly more of the higher molecular weight components were left on the gel (Figure 2.2, No. 4).

FIGURE 2.2 (Nos. 1 to 8)

Transfer of *Trypanosoma evansi* proteins onto nitrocellulose membrane. (SDS-PAGE gel stained with Coomassie blue and nitrocellulose membrane with amido black.)

1. Polyacrylamide gel after 15 minutes of blotting.
2. Polyacrylamide gel after 45 minutes of blotting.
3. Polyacrylamide gel after 60 minutes of blotting.
4. Polyacrylamide gel after 90 minutes of blotting.
5. Nitrocellulose membrane after 15 minutes of blotting.
6. Nitrocellulose membrane after 45 minutes of blotting.
7. Nitrocellulose membrane after 60 minutes of blotting.
8. Nitrocellulose membrane after 90 minutes of blotting.


94
67
43
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1
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3
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7
8

DISCUSSION

The principal results from this study clearly demonstrated that the elution of *T. evansi* proteins from the gels to the nitrocellulose membrane was time dependent, and that smaller molecular weight components appeared to be eluted faster from the gel than those with higher molecular weights. These observations are similar to the findings of Burnette (1981) who also demonstrated that the rate of protein transfer was time dependent and that smaller molecular weight components are eluted faster from the gel than the higher molecular weight ones. The effect of component molecular size on transfer time is probably associated with the sieving effect of the gel, in which those components of large molecular weights are presented with more resistance leaving the gel than those of small molecular weight.

Despite the fact that differences were seen in the transfer rates between molecules of large and small molecular weight during the present study, the fact that sufficient material had been transferred from the gel to the nitrocellulose membrane by 90 minutes to provide an accurate representation of the proteins in the gel was considered sufficient reason to adopt 90 minutes transfer time for all future studies. The transfer time of 90 minutes using the present semi-dry system is roughly in agreement with that recommended by Towbin *et al.* (1979) for a wet system although markedly different from that of Burnette (1981), who recommended a time of 20-22 hours for complete transfer of all proteins up to 100,000 daltons using a wet system. The lack of adequate cooling in the semi-dry system used in the present investigation would, however, probably preclude blotting for unduly long periods of time.

DETECTION OF ANTIGENIC COMPONENTS OF T. EVANSI ON NITROCELLULOSE BLOTS

The principal requirement of any experimental system involving antigen/antibody reactions is that the results obtained are a true reflection of antigen/antibody interaction and not an artifact arising from factors such as background or non-specific reactions (Tsang *et al.*, 1985). The problem of background staining is of particular importance in the case of nitrocellulose membrane due to its high affinity for proteins which enhances the non-specific staining problems which are common to all immunological assay systems.

Traditionally, the problem of background staining of the nitrocellulose membrane has been overcome by a variety of methods including blocking and/or inactivation of the binding sites of the nitrocellulose matrix after the transfer of protein by blotting. Initially, inactivation and blocking with substances such as tris (hydroxymethyl) aminoethane and ethanolamine proved to be effective in reducing or eliminating non-specific reactions (Renart *et al.*, 1979). Lately, however, blocking procedures involving the use of excess proteins such as bovine serum albumin (BSA) (Towbin *et al.*, 1979; Aubertin *et al.*, 1983), foetal bovine serum (De Blas and Cherwinsk, 1983; Ramirez *et al.*, 1983), haemoglobin (Gershoni and Palade, 1982), gelatin (Lim and Kasamatsu, 1983), milk (Johnson *et al.*, 1984) and normal goat serum (Grogl *et al.*, 1985) have been used extensively for the control of background staining (reviewed by Mohammad and Esen, 1989).

Clearly it is important to ensure that any blocking agent used to control background staining does not influence the outcome of any antigen/antibody reaction. In the present study the efficiency of two commonly used blocking agents -lyophilised skimmed milk and goat serum have been evaluated under a

number of conditions as part of an overall study designed to develop a suitable protocol for the study of *T. evansi* antigens.

MATERIALS AND METHODS

Soluble antigens of *T. evansi* used were prepared as described previously using the freeze-thaw method, fractionated into individual components by SDS-PAGE and transferred onto nitrocellulose paper as described previously using a 90 minute transfer time.

T. evansi-specific serum was prepared by infecting two rabbits with *T. evansi* by injecting 4 x 10^5 trypanosomes of TREU 2113 through an ear vein. Seventeen days later the rabbits were bled for serum. Serum samples were also collected from one uninfected rabbit.

Goat serum for blocking were obtained from a locally purchased goat. Blood samples for serum were collected from both the goat and rabbits into bottles without any anticoagulant. The blood samples were then held at 37°C for 20 minutes and then at 4°C for two to three hours after which the separated serum was carefully aspirated into 10 ml centrifuge tubes. The serum samples were centrifuged at 2,500 g for 35 minutes at 4°C and subsequently aliquoted into small volumes and stored at -20°C until required. Lyophilised skim milk powder (Marvel) was dissolved in blocking buffer (Appendix 9) to the appropriate w/v concentration and gently mixed until completely solubilised.

Comparative studies on the efficiency of the two blocking agents were initially undertaken by using both agents at a single concentration of 5% and a range of anti-trypanosome serum dilutions of 1:200, 1:400 and 1:800. Later studies were conducted using several concentrations of both blocking agents in

conjunction with a single 1:200 dilution of the anti-trypanosome serum.

Processing of Blots

After the separation of the *T. evansi* soluble antigens with SDS-PAGE and transfer onto nitrocellulose paper, the blot was blocked overnight with the appropriate blocking agents made up in blocking buffer (Appendix 9). After blocking, the blots were washed in PBS (Appendix 1) for 30 minutes with three changes of the buffer (Grogl *et al.*, 1985). Blots were then incubated with the appropriate dilution of the *T. evansi* specific rabbit serum in blocking buffer overnight. Unreacted serum protein was then removed by washing in PBS as described above. The blots were finally incubated for three hours in a 1:4000 dilution of a peroxidase-labelled conjugate to goat anti-rabbit IgG heavy and light chains (Nordic) made up in blocking buffer. The blots were washed in PBS as previously described and any antigen/antibody complex was then visualised by incubating the blots in 4-chloro-1-napthol substrate solution (Sigma) (Appendix 10). Colour development was allowed to occur over the next 15 to 20 minutes, blots finally rinsed in distilled water and permanent records made by photography. Each test system was also tested with control units in which the trypanosome-specific serum was replaced with serum collected from an uninfected rabbit at the equivalent dilution.

RESULTS

(a) Effect of blocking agent and anti-trypanosome serum dilution

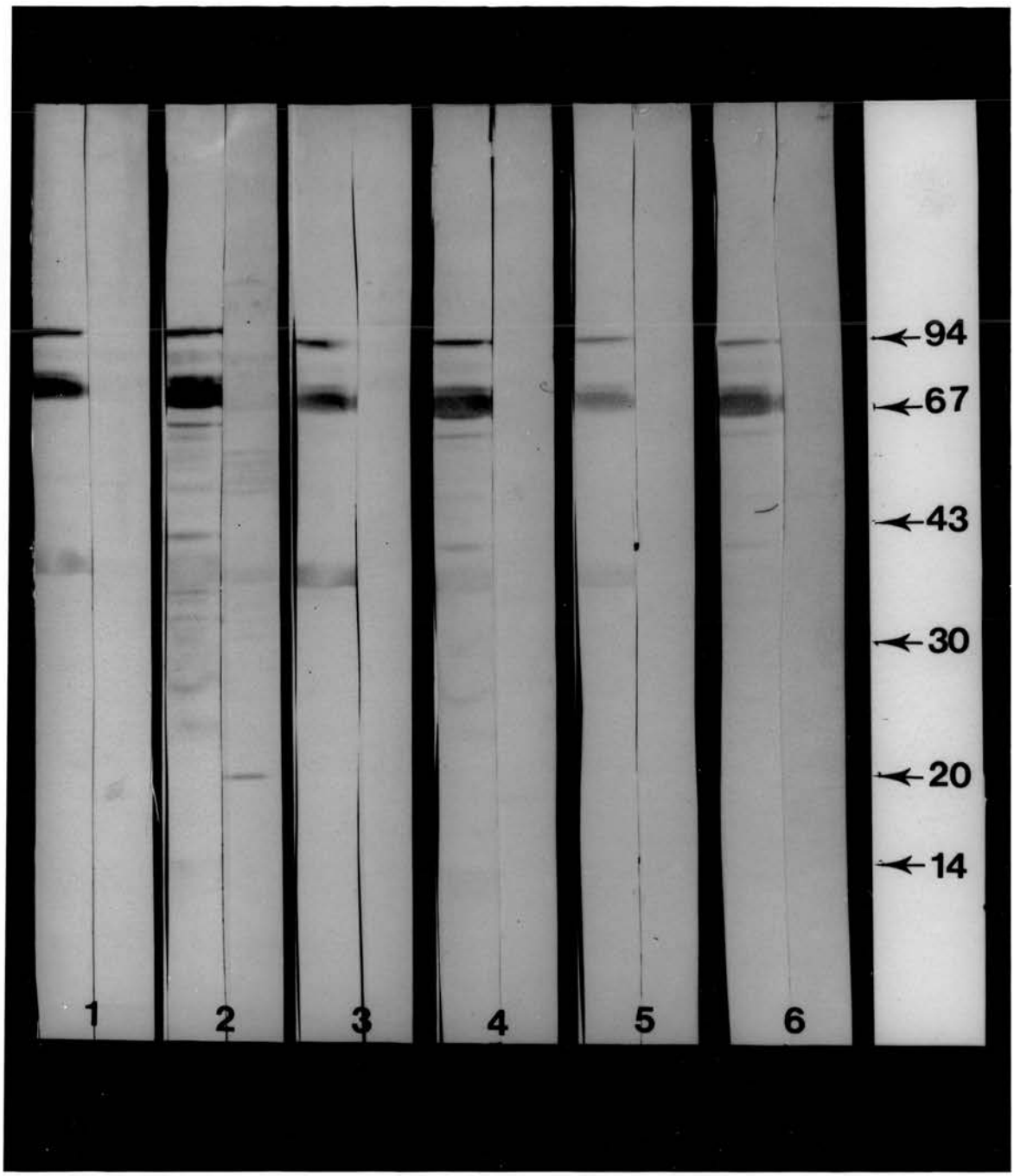
More antigen components were visualised when 5% goat serum (v/v) was used as the blocking agent (Figure 2.3, Nos. 2, 4 and 6) than when 5% lyophilised milk (v/v) was used (Figure 2.3, Nos. 1, 3 and 5) at all dilutions of

the trypanosome specific serum. With both blocking agents the total number and intensity of staining of individual components visualised was dependent on the dilution of the trypanosome specific serum. The greatest number of components and the most intense staining were obtained with a serum dilution of 1:200 (Figure 2.3, Nos. 1 and 2). The least number of components and the lowest level of staining were obtained with a serum dilution of 1:800 (Figure 2.3, Nos. 5 and 6). In the absence of either blocking agents high background staining of the nitrocellulose membrane was obtained which made it difficult to identify the individual trypanosome components from the background (details not shown).

FIGURE 2.3

Effect of anti-trypanosome serum dilution on antigen/antibody reactions.

1. Blots incubated with anti-trypanosome and normal rabbit sera diluted 1:200 respectively and blocked with 5% milk.
2. Blots incubated with anti-trypanosome and normal rabbit sera diluted 1:200 respectively and blocked with 5% goat serum.
3. Blots incubated with anti-trypanosome and normal rabbit sera diluted 1:400 respectively and blocked with 5% milk.
4. Blots incubated with anti-trypanosome and normal rabbit sera diluted 1:400 respectively and blocked with 5% goat serum.
5. Blots incubated with anti-trypanosome and normal rabbit serum diluted 1:800 respectively and blocked with 5% milk.
6. Blots incubated with anti-trypanosome and normal rabbit serum diluted 1:800 respectively and blocked with 5% goat serum.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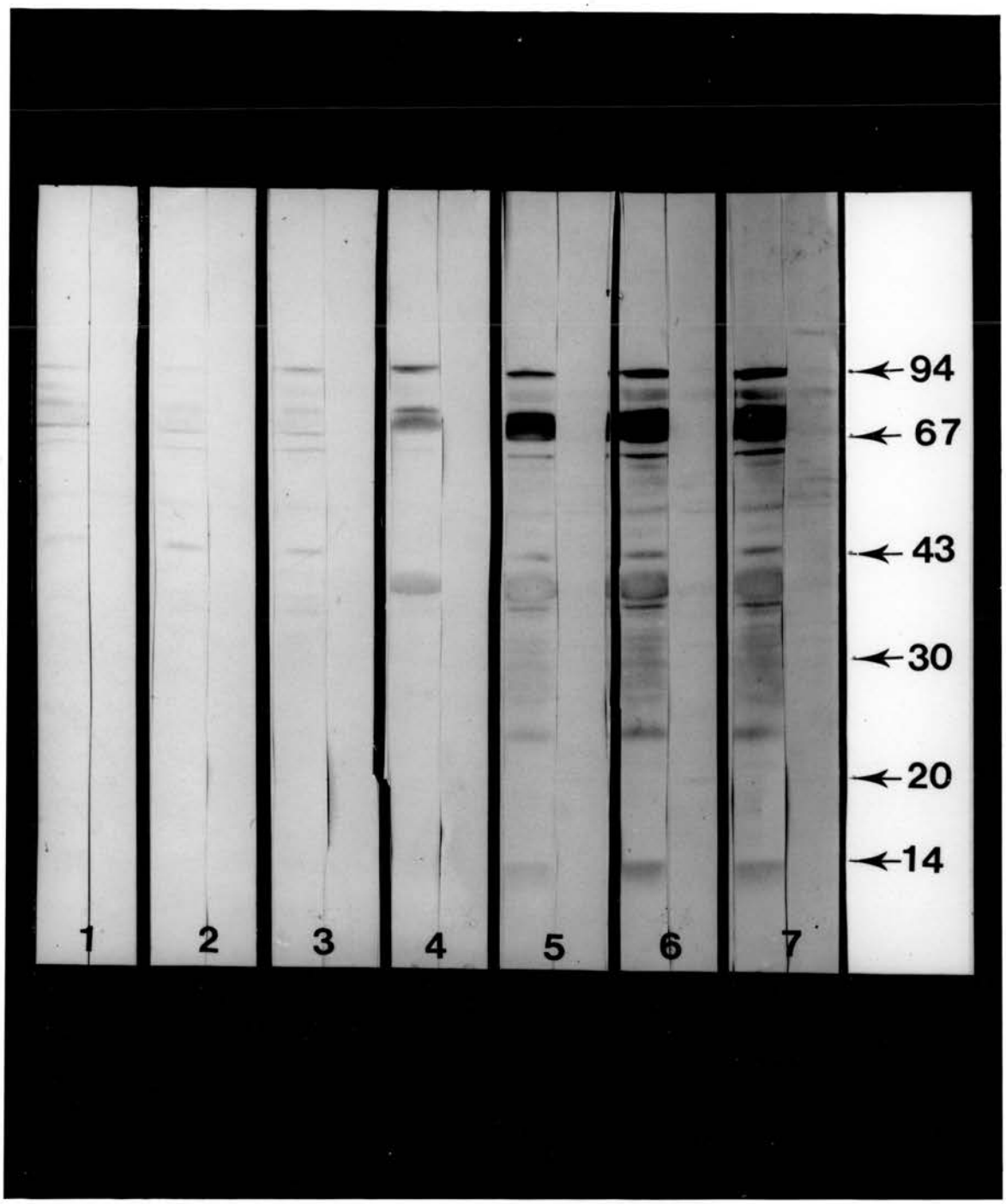
(b) Effect of blocking agent concentration on antigen/antibody reactions

At each blocking agent dilution more antigenic components were resolved and were better stained when goat serum rather than milk was used as the blocking agent (Figure 2.4, Nos. 5 to 7 and Nos. 1 to 4). Further, more variation in results was obtained when milk rather than goat serum was used as a blocking agent at each dilution. This variation appeared in the form that more components were visualised when higher concentrations of milk were used for blocking (Figure 2.4, No. 4) whilst on some occasions fewer components were resolved at higher concentrations (Figure 2.4, Nos. 1 and 2). Occasionally little difference was seen in the number of components seen and in their degree of staining with widely varying milk concentrations (Figure 2.4, Nos. 1, 2 and 3). On one occasion very intensely stained components were seen when 20% (w/v) milk was used as the blocking agent (Figure 2.4, No. 4). In general, results obtained using goat serum as the blocking agent were easily reproduced with little variation between repeated runs, in terms of the number of bands and their degree of staining. In the case of goat serum maximum differentiation of bands was obtained when the system was blocked with either 4% or 8% (v/v) goat serum (Figure 2.4, Nos. 6 and 7).

FIGURE 2.4

Effect of blocking agent concentration on antigen/antibody reactions.

1. Blots incubated with anti-trypanosome and normal rabbit sera diluted 1:200 respectively and blocked with 2.5% milk.
2. Blots incubated with anti-trypanosome and normal rabbit sera diluted 1:200 respectively and blocked with 10% milk.
3. Blots incubated with anti-trypanosome and normal rabbit sera diluted 1:200 respectively and blocked with 15% milk.
4. Blots incubated with anti-trypanosome and normal rabbit serum diluted 1:200 respectively and blocked with 20% milk.
5. Blots incubated with anti-trypanosome and normal rabbit serum diluted 1:200 respectively and blocked with 2% goat serum.
6. Blots incubated with anti-trypanosome and normal rabbit serum diluted 1:200 respectively and blocked with 4% goat serum.
7. Blots incubated with anti-trypanosome and normal rabbit serum diluted 1:200 respectively and blocked with 8% goat serum.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DISCUSSION

The erratic nature of the results obtained in the present study when milk was used as the blocking agent made milk unsuitable for further use in the *T. evansi* system. Other workers have also reported difficulties with milk as a blocking agent (Mohammad and Esen, 1989). These authors observed a diminished antigen-antibody reaction when their system was blocked with milk and they attributed this lowered sensitivity of their reactions to the possible non-specific blocking of the antigen sites by milk proteins. They considered that this was probably brought about by steric hinderance caused by proteins in milk that bound to the sites not occupied by the antigens on the solid matrix and physically preventing the antibodies from binding to specific antigen sites. A similar observation was made in the present study where fewer *T. evansi* components were observed when the system was blocked with milk (Figure 2.4, Nos. 1 to 4) than with goat serum (Figure 2.4, Nos. 5 to 7). This observation coupled with an unexpected result of the boosting of both the intensity and number of components visualised when a higher concentration of milk was used for blocking, made the use of milk in the *T. evansi* system unreliable. Consequently goat serum was chosen as a suitable blocking agent for further studies on antigenic components of *T. evansi* and used at a dilution of 4% as no differences were seen between the systems blocked with 4% or 8% goat serum.

OPTIMIZATION OF BLOCKING, SERUM AND CONJUGATE REACTION TIMES

INTRODUCTION

In common with many chemical reactions, the rate at which the individual components combine in an immunoassay is often time dependent. In most immunoassays incubation times are designed to ensure saturation of appropriate reaction sites which ensures that reagent specificity or its concentration limits the assay and not the methodology. Too short an incubation time could prevent maximum reactivity while too long an incubation time could result in non-specific reactivity. Incubation times for the individual steps in multistep immunoassay systems such as immunoblotting also need to be realistically as short as possible to ensure that results can be obtained in a reasonable period of time.

The previous sections of this chapter established most of the key factors influencing the several stages involved in immunoblotting, but without regard to the minimum time intervals required, for each stage. This final part of the study was designed, therefore, to establish the practical incubation times for the various stages of immunoblotting.

MATERIALS AND METHODS

Antigens and Antibodies

The combination of soluble antigen and anti-trypanosome serum at a dilution of 1:200 shown to be optimum in previous studies were used in the present experiment. After fractionation of *T. evansi* proteins using SDS-PAGE and immunoblotting, the blot was blocked with 4% goat serum made up in blocking buffer (v/v). The effect of different time intervals of 0.5, 1, 2 and 4

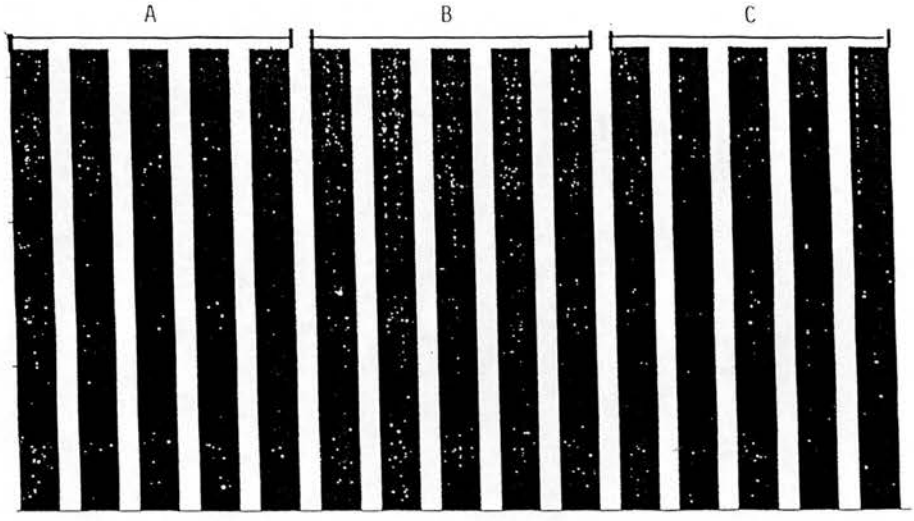
hours, and overnight incubation at different stages in the antigen detection protocol were studied to establish the appropriate combinations of incubation times that gave the optimum immunoblotting results, as defined by the number of bands visualised and their intensity of staining. The details of the procedure followed for the analysis of the parameters are shown in Figure 2.5.

After the transfer of the *T. evansi* proteins onto the nitrocellulose membrane, the blot was divided into three equal sections A, B and C (Figure 2.5), each section containing five columns of the transferred proteins. Strips of the blot in Section A (Figure 2.5) were individually blocked (Strips 1-5) for 0.5, 1, 2 and 4 hours and overnight respectively. Each of these blots were later incubated in the trypanosome-specific serum for four hours and in the conjugate solution for two hours respectively. In between the different steps, blots were washed in PBS.

The five strips from the blot in Section B (Figure 2.5) were blocked for four hours. They were then incubated individually in the trypanosome-specific serum (Strips 6-10) for 0.5, 1, 2 and 4 hours and overnight respectively. Thereafter, all the strips were incubated in the conjugate solutions for two hours. In between the different stages, the blots were washed in PBS.

The strips from the blot in Section C (Figure 2.5) were blocked and incubated in the trypanosome specific rabbit serum for four hours. They were then incubated individually in the conjugate solution starting from 0.5, 1, 1.5, 2 and 4 hours (Strips 11-15) respectively. In between the different stages, the blots were washed in PBS.

Figure 2.5 Schematic representation of procedure used to optimise blocking, serum and conjugate incubation times.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blocking times in hours.	0.5	1	2	4	O/N*	4	4	4	4	4	4	4	4	4	4
Serum reaction times in hours	4	4	4	4	4	0.5	1	2	4	O/N*	4	4	4	4	4
Conjugate reaction time in hours	2	2	2	2	2	2	2	2	2	2	0.5	1	1.5	2	4

A: Blots used to ascertain the effect of different blocking times on antigen/antibody reactions.

B: Blots used to ascertain the effect of different serum reaction times on antigen/antibody reactions.

C: Blots used to ascertain the effect of different conjugate reaction times on antigen/antibody reactions.

*: O/N - Overnight incubation.

RESULTS

(a) Effect of Blocking Time

Least background staining was observed in the strips of blots blocked for between four hours and overnight (Figure 2.6, Strips 4 and 5).

(b) Effect of Serum Reaction Time

More components that were also more intensely stained were visualised in the part of the blot that was incubated in the trypanosome specific rabbit serum overnight (Figure 2.6, Strip 10).

(c) Effect of Conjugate Reaction Time

The optimum result, in terms of the degree of staining and number of the components identified was achieved when the strips from the blot was incubated in the conjugate solution for between two and four hours (Figure 2.6, Strips 14 and 15) respectively.

DISCUSSION

A combination of a blocking time of four hours, overnight incubation with anti-trypanosome serum and a two-hour conjugate incubation time was considered to provide optimum antigen detection; in that minimum amounts of background staining and more numbers of individual *T. evansi* components that were antigenic and also well stained were obtained using these time combinations. Effectively, with this protocol the identification of trypanosome antigenic proteins can be easily done within three days with the worker still carrying on his routine laboratory activities and only returning to the "Western" blotting procedure at stipulated intervals without interfering with the efficiency of the experiment.

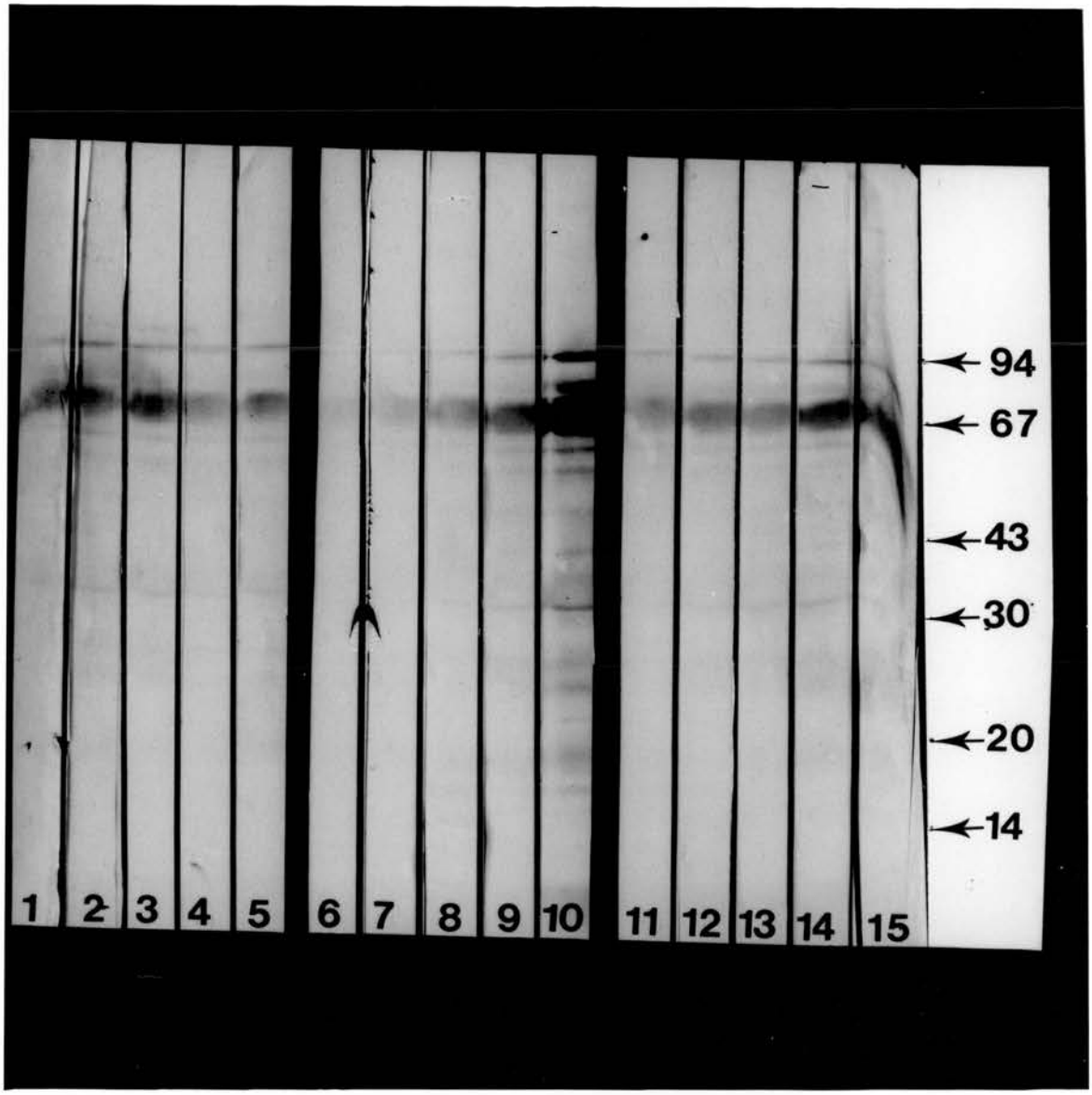
FIGURE 2.6

Optimization of blocking, serum and conjugate incubation times.

Numbers 1-5 Blots blocked for 0.5, 1, 2, 4 hours and overnight respectively.

Numbers 6-10 Blots incubated with anti-trypanosome rabbit serum for 0.5, 1, 2, 4 hours and overnight respectively.

Numbers 11-15 Blots incubated in conjugate solution for 0.5, 1, 1.5, 2, and 4 hours respectively.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PROTOCOL

Based on the overall results from the studies reported in this chapter, a final protocol was established for future studies on the antigenic composition of *T. evansi*. The final combination of conditions that permitted the resolution of the maximum possible number of antigenic components that could be discerned easily and reliably were accepted for use. The following final protocol was therefore adopted.

(a) The preparation of *T. evansi* soluble antigens by freeze-thawing.

(b) Protein transfer (blotting) time of 90 minutes onto nitrocellulose membrane.

(c) The blocking of active sites on the solid matrix (nitrocellulose membrane) with 4% goat serum in blocking buffer (Appendix 9) for a minimum time of four hours.

(d) Overnight incubation of transferred trypanosome components with 1:200 dilution of trypanosome specific sera followed by a two-hour incubation of the nitrocellulose strips with peroxidase-labelled conjugate solution.

This basic protocol was adopted in all the assays conducted in the later stages of this study and any modifications included as appropriate.

CHAPTER THREE

CHARACTERISATION OF THE SURFACE COAT OF TRYPANOSOMA EVANSI AND THE CLASS-SPECIFIC ANTIBODY RESPONSE TO IT IN RABBITS EXPERIMENTALLY INFECTED WITH TRYPANOSOMA EVANSI

INTRODUCTION

Each individual Salivarian trypanosomes is enveloped by a 12-15 nm thick surface coat consisting of a matrix of identical glycoprotein molecules (Vickerman, 1969). As in all parasite infections the surface of the organism constitutes the first point of contact between the parasite and the host immune system. Consequently the host's response to the surface of the parasite is often of paramount importance in determining the outcome of the infection. Trypanosomes differ from other parasites in their ability to change the chemical composition of their surface glycoprotein coat (VSG) extensively which leads to the appearance of successive waves of antigenically different parasites throughout the course of infection. Due to the extreme specificity of the host's response to each antigenically distinct population, the emergence of new variants allows the parasite to escape the host's immune system. The host's immune response to the VSG of trypanosomes has been extensively studied (Wellde *et al.*, 1975, 1981; Emery *et al.*, 1980; Morrison *et al.*, 1982a; Hall and Esser, 1984) possibly to the detriment of other features of the parasite, such as responses to the non-surface component of the parasite.

Mammals defend themselves against infection with parasites such as trypanosomes by producing specific antibodies directed against particular components or the organism (antigens) or in reality molecules that the host recognises as not belonging to itself ("nonself" molecules). The usual outcome of such a response is the elimination of the parasite from the host. Equally important as parasite elimination is the role of the host's immune response in protecting the host against reinfection with the same parasite. Protection to reinfection stems from the fact that some antibody-secreting cells (B memory cells) produced during the primary infection can persist for many years and

provide lasting immunity to reinfection. A similar type of immunity can also be elicited by vaccines which mimic the host's response to natural infections.

In the case of trypanosomes, however, neither the primary immune response to infection nor vaccination can effectively eliminate or protect against subsequent trypanosomal infections unless in cases of homologous challenge due to the process of antigenic variations. The effect of antigenic variation being that by the time the host's immune system has produced antibodies to the antigen on the surface of a particular trypanosome population, a proportion of the parasites will already be expressing a range of different surface antigens. The parasites expressing these different surface antigens then multiply and give rise to antigenically different parasite populations which the existing antibodies produced by the host will not recognise. This process continues throughout the infection with the host's overworked immune system failing to eliminate the infection, and so the parasites survive and proliferate (Donelson and Turner, 1985) in what appears to be an immune host.

The total number of serologically different variant surface antigens that can be expressed by any given trypanosome stock is unknown but studies with *T. b. equiperdum* indicate that it is probably in excess of 100 (Capbern *et al.*, 1977) and could be as great as 1,000. Establishing serological relationships between trypanosome isolates is a very complex process arising from the fact that an infection initiated from a single trypanosome can give rise to a large number of different antigenic types and that different stocks of trypanosomes give rise to different sets of antigenic types. The serological identity of any particular bloodstream trypanosome population, is determined by the antigens on the surface of the organisms constituting that trypanosome population. The

antigenic identity of any one trypanosome within a given population is termed the Variant Antigen Type (VAT) of that trypanosome. At any one time during the course of infection a trypanosome population can consist of one or more VATs. All of the VATs potentially expressible by infection with a trypanosome stock are referred to as the VAT repertoire of that particular stock. Stocks of trypanosomes, which express the same VAT repertoire, are considered to belong to the same serodeme (Gray and Luckins; 1976; Shapiro and Pearson, 1986).

During the course of a trypanosome infection there is a tendency for certain VATs of a serodeme to appear more frequently than others, particularly during the early stages of infection. These VATs are termed predominant VATs (pVATs) (Gray and Luckins, 1976) and are characteristic of particular stocks of trypanosomes. In the case of tsetse-transmitted trypanosomes there is also a tendency for a certain VAT to appear in the first wave of parasitaemia after cyclical transmission by the tsetse fly (Gray, 1965) irrespective of the VAT which infected the fly. This antigen type has been termed the "basic" antigen, and it is likely different basic antigens could exist for each trypanosome stock.

The VSG molecule of the trypanosome coat is highly immunogenic in that it induces a strong antibody response from the host (Le Ray, 1975). The action of this antibody probably represents the main mechanisms by which trypanosomes are destroyed in the host (Wellde *et al.*, 1975, 1981; Emery *et al.*, 1980; Morrison *et al.*, 1982a; Hall and Esser, 1984). The antibody response to the VSG of the individual trypanosome is considered to control each wave of parasitaemia with at least 99.9% or more of the particular trypanosome population being destroyed by specific antibodies. New parasite

populations are then established from the surviving antigenically different parasites (Doyle, 1977; Vickerman, 1978).

Antibodies produced by animals during the course of an infection have been shown to be very effective in protecting animals against reinfection with an antigenically homologous trypanosome population but confer no protection against challenge by antigenically different trypanosomes. The net effect of such specificity is that host antibodies developed against one particular VAT do not react efficiently with other VATs in the repertoire, a phenomenon which has intrigued workers since the turn of the century (Laveran and Mesnil, 1902; Ehrlich *et al.*, 1909; Browning and Gulbransen, 1936; Hornby, 1941; Fulton and Lourie, 1946; Weisenhutter, 1970; Wellde *et al.*, 1975; Nantulya *et al.*, 1980; Wellde *et al.*, 1981; Morrison *et al.*, 1982a).

Despite the extreme immunogenicity of the VSG molecule the range of VATs produced coupled with the specificity of the host antibody response virtually eliminates the potential of using the VSG as an immunogen in the development of a single VAT-component for a vaccine. The stabilising effect of transmission on VAT expression that is linked to the appearance of basic and predominant antigen types and the fact that metacyclic trypanosome populations have a limited VAT repertoire has led some investigators to suggest that some protection could be elicited with a multicomponent vaccine containing a range of VATs (Hajduk and Vickerman, 1981; Esser *et al.*, 1982; Jenni, 1977; Nantulya *et al.*, 1980). Outside of experimental systems, however, this has not been achieved. Furthermore, the variable nature of the VSG even within the same stock of trypanosome makes it of limited value as a reagent in the serological assays for trypanosome infection. VSG specific tests have however, a limited application in some diagnostic tests such as the card

agglutination test (CAT) (Magnus *et al.*, 1978) which depends on the use of widespread predominant variable antigen types as antigen.

At present, most of the information on the properties of trypanosome VSG and the equivalent host response has been obtained from studies conducted using tsetse transmitted trypanosomes with *T. brucei* receiving most attention (Cross, 1975; Johnson and Cross, 1979; Cardoso de Almeida and Turner, 1983 etc.). Other authors have undertaken VSG-characterisation studies with *T. congolense* (Rovis *et al.*, 1978; Reinwald *et al.*, 1978; Ondera *et al.*, 1982; Ross *et al.*, 1987) and *T. vivax* (Gardiner *et al.*, 1987). Studies on non-tsetse-transmitted trypanosomes such as *T. evansi* are very limited. These were often conducted as an adjunct to studies on tsetse transmitted trypanosomes, consequently receiving cursory mention, and include those of Cross (1978b) and Onodera (1984) with the latter making only a passing reference to differences in the properties of the VSG molecules of relapsing *T. evansi* populations.

Results from the biochemical characterisation of the VSG of a range of Salivarian trypanosomes reported by Richards (1984) indicated that the VSG of trypanosomes have a molecular weight range of 53,000-63,000. Results from other studies, however, indicated that there are very wide variations in the molecular weight of VSGs not only between different trypanosome species but also between different stocks of the same species. Studies conducted on the VSG of *T. brucei* by Cross (1975; 1977), Johnson and Cross (1977) and Hoeijmakers *et al.* (1980) showed that the molecular weight of *T. brucei* VSG ranged from 53,000-65,000 daltons as determined by sodium dodecyl sulphate gel electrophoresis. Similar studies conducted on *T. congolense* also established considerable difference between the molecular weight of VSG reported by

several authors. Ross *et al.* (1987) reported that the VSG of a *T. congolense* stock consists of a single component of molecular weight 54,000 while Reinwald *et al.* (1978) identified two components of molecular weight 57,000 and 50,000 daltons with Rovis *et al.* (1978) reporting a single 56,000 dalton component. Ondera *et al.* found a wider range of molecular weight of 53,000-63,000 for the surface component of different *T. congolense* stocks. Only limited studies have been conducted on the VSG of other economically important tsetse transmitted trypanosomes infecting livestock such as *T. vivax*. The results from these studies showed that the VSG of *T. vivax* has a molecular weight of 46,000 daltons (Gardiner *et al.*, 1987), considerably lower than that reported for other trypanosome species.

In the case of non-tsetse-transmitted trypanosomes such as *T. evansi* information on the surface components is very limited, most of which is unfortunately so vague and incomplete as to be of little use in furthering the understanding of the nature of the VSG of this group of trypanosomes. Cross (1978) applying the method used for characterising the VSG of *T. brucei* found that the purified glycoprotein of *T. evansi* when examined by SDS-PAGE under non-reducing conditions showed a single component of 96,000 molecular weight. Under reducing conditions, however, two major components of molecular weights of either 65,000 or 45,000 daltons were revealed which differed from the single band he reported for *T. brucei*. The only other information referring specifically to the VSG of *T. evansi* appears to be that credited to Onodera (cited by Richards, 1984), who observed changes in the molecular weight of the VSG of *T. evansi* from 53,000 to 63,000 daltons between different relapsing *T. evansi* populations.

The first detailed studies on the biochemistry of the VSG of trypanosomes were those reported by Cross (1975) who extracted, purified and labelled the VSG of *T. brucei* and found that it consisted of a single polypeptide chain with a molecular weight range of 53,000-65,000 daltons. Later, Johnson and Cross (1979) using limited tryptic digestion, showed that each VSG molecule was made up of two regions. A large N-terminal fragment with a molecular weight range of 40,000-52,000 daltons and a smaller C-terminal fragment with a molecular weight range of 13,000-17,000 daltons. Subsequent studies by Holder and Cross (1981) established that the C-terminal domain contained an immunogenic oligosaccharide common to VSG molecules from different stocks and species which they referred to as the cross-reacting determinant (CRD). Cardoso de Almeida and Turner (1983) were able to demonstrate that the form of the VSG molecule of *T. brucei* that was bound to the membrane of the intact trypanosome differed biochemically and immunochemically from the released form of VSG that existed in soluble extracts normally used for *in vitro* biochemical studies. By using an antibody raised to the CRD they showed that after the rupture of the cell, membrane-bound form of VSG (mfVSG) becomes enzymatically transformed into the soluble or released form of VSG (sVSG), with important differences between their C-terminal domain. In the membrane form VSG (mfVSG), the CRD is not exposed and is not, therefore, recognised by the antibodies to the CRD while in the released or soluble form VSG (sVSG) the CRD is exposed and can be recognised by the anti-CRD serum. Many workers have used this difference between the two forms of the VSG to carry out detailed studies of the VSG of many trypanosomes (Gurnett *et al.*, 1986; Bulow *et al.*, 1989).

In view of the importance of the surface coat of the trypanosome in the general immunobiology of the parasite coupled with the lack of information

specific to the surface antigen of *T. evansi* (Cross, 1978), the studies in this chapter were designed to identify and characterise the surface antigen component of *T. evansi* and also to evaluate the host's antibody response to this component over time. The results from such studies could provide information of importance in better understanding of the relationship between *T. evansi* and its mammalian host.

MATERIALS AND METHODS

History of Stocks of Trypanosomes Used

Trypanosoma evansi TREU 2113: The history of this stock of *T. evansi* was presented earlier in Chapter 2.

T. evansi TREU 2147: *T. evansi* TREU 2147 was obtained after a single direct passage of TREU 2113 through mice.

T. evansi TREU 1944: *T. evansi* TREU 1944 was cloned from stabilated trypanosomes, TREU 1839, which was acquired by CTVM from the University of Berlin. The stock was originally isolated from a camel in Cairo, Egypt in 1985.

T. evansi TREU 1773: *T. evansi* TREU 1733 was cloned from TREU 1445 which was a stock originally isolated from a camel from the Kassal Province of the Sudan in 1978.

T. evansi TREU 1380: *T. evansi* TREU 1380 was obtained after TREU 381 was passaged through mice five times. The stock from which TREU 381 was derived was originally isolated from a horse from Arauca Province of Columbia in 1967.

T. evansi TREU 1834: *T. evansi* TREU 1834 was obtained after TREU 1804 was passed through mice once. TREU 1804 was a stock isolated from a

camel from Wadi Jauf in Yemen in 1983.

All the stocks in Giemsa stained smears conformed with the morphological description of *T. evansi* listed in Hoare (1972).

SURFACE IODINATION OF TRYPANOSOMES

Trypanosome surface proteins of all the *T. evansi* stocks used in this study were radioactively labelled with ^{125}I (Amersham) by a modification of the method used by Lanar and Manning (1984) as described by Ross *et al.* (1987).

Briefly, bloodstream forms of trypanosomes obtained from mice with fulminating infections were separated from host blood cells by anion exchange chromatography on DEAE cellulose (DE52, Whatman Biochemicals) as described by Lanham and Godfrey (1970). After separation, trypanosomes were washed three times in phosphate buffered saline pH 8.0 containing 1% (w/v) glucose (BDH Chemicals Ltd.) (PSG, Appendix 2) by centrifugation at 2,500 g for 10 minutes at 4°C to remove any remaining host component.

The final trypanosome pellet was then resuspended to a final concentration of 5×10^7 ml^{-1} in PSG. Six hundred μl of the trypanosome suspension were then placed into a glass bijou bottle containing 200 μg of 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (IODO-GEN, Sigma Ltd.) which had been previously dried down from a 2 μg/200 μl solution of the reagent in chloroform. Three hundred μCi of NaI^{125} (Amersham) were then added to the bijou bottle and the mixture was incubated at 4°C or at room temperature for 10 minutes.

Non-detergent Lysis of ^{125}I-labelled Trypanosomes

Following several washes with PSG containing 0.25 mM n-tosyl-1-lysyl-chloromethyl ketone (Sigma Ltd., PSG/TLCK) to remove excess radiolabelling reagents, the iodinated cells were lysed by freezing and thawing as described in Chapter 2. The resulting trypanosome lysate was then separated into pellet and supernatant fractions by centrifuging at 2,500 g for 10 minutes at 4^{o}C.

Detergent-lysis of ^{125}I-labelled Trypanosomes

The method used for the preparation of the ^{125}I-labelled trypanosome detergent lysate was that described by Cardoso de Almeida and Turner, 1983; with modifications introduced by Ross *et al.*, 1987). Briefly, about 3 x 10^{7} ^{125}I-labelled trypanosomes were resuspended in 600 μl of PBS (Appendix 1) containing 2% SDS and heated to 100^{o}C. After cooling to room temperature a sufficient amount of 2.5% Triton X-100 in PBS was added to give a final concentration of 2% TX-100 and 0.4% SDS in the lysate.

Analysis of ^{125}I-labelled Trypanosome Components

The supernatant and precipitate fractions obtained from ^{125}I-labelled trypanosome non-detergent lysate, and ^{125}I-labelled trypanosome detergent lysate were subjected to SDS-PAGE analysis on 7-20% polyacrylamide gels as described in Chapter 2. Before applying the samples on the gels, 15μl of each ^{125}I-labelled preparation containing 100,000 counts/min was mixed with 15 μl of an equivalent unlabelled *T. evansi* homologous protein preparation containing 90 μg of protein. The mixture was then heated at 100^{o}C for three minutes. The unlabelled *T. evansi* material served as a source of extra proteins which would enhance the visibility of the protein bands after staining the gels

with Coomassie Blue.

Proteins of known molecular weight ranging from 94,000-14,000 daltons (Pharmacia) were included in each electrophoresis run. Results from these were used to construct a standard curve which was then used to calculate the molecular weight of individual trypanosome protein components visualised after the gels were stained with Coomassie Blue. After Coomassie Blue staining the gels were placed in a gel drying apparatus before being exposed for 36 hours to Fuji RX-ray film (Fuji Photo Ltd., Japan) at -70^{o}C in order to visualise the radioactively labelled proteins. The molecular weight of radiolabelled trypanosome components was determined by superimposition of the autoradiograph onto the equivalent Coomassie Blue-stained gel.

T. evansi Specific Antibodies

Two female adult New Zealand White rabbits were used for the production of *T. evansi* specific antibody. Each rabbit was infected through the ear vein with 4 x 10^5 trypanosomes of *T. evansi* TREU 2113. The trypanosomes were initially grown in mice until fulminating infections developed before the parasites were separated using anion-exchange chromatography on DEAE cellulose (DE52, Whatman Biochemicals, UK) as described in Chapter 2. After washing to remove host components, the separated trypanosomes were resuspended in ice-cold PSG before they were used to infect the rabbits.

Twenty-eight days after infection each rabbit was treated with 7 mg (active principle) of diminazene aceturate (Berenil^{R}, Hoechst Lab, Germany) per kg body wt. Rabbits were bled for serum immediately before infection (day 0) and on days 7, 14 and 28 after infection. The rabbits were also bled on days 7, 28 and 39

after drug treatment. Serum was prepared from the blood samples as described in Chapter 2.

Anti-cross Reacting Determinant (CRD) Serum

The anti-CRD serum was a kind gift from Dr. C.A. Ross. The antisera was prepared from the serum of a rabbit immunised with *T. b. brucei* as described by Cardoso de Almeida and Turner (1983). The antiserum is known to cross-react with the cross-reacting determinant of VSG from several other trypanosome species (C.A. Ross, personal communication).

Identification of CRD in T. evansi VSG

After SDS-PAGE of unlabelled soluble antigen of the appropriate trypanosome population prepared according to the protocol in Chapter 2, the separated *T. evansi* proteins were transferred onto nitrocellulose membrane (Anderman and Co., U.K.) using the semi-dry electroblotter (Ancos Electroblotter, Dako Ltd., UK) according to the final protocol developed during the study described in Chapter 2.

After blocking with excess proteins containing 4% goat serum (v/v) in XI blocking buffer (Appendix 9), the presence of the CRD moiety on the blot was probed by incubating the membrane with undiluted anti-CRD serum. Any antibodies that bound to the trypanosome proteins were subsequently detected by overnight incubation with ^{125}I-labelled donkey anti-rabbit immunoglobulin (Amersham, U.K.) (used at the rate of 50 µl of the radiolabelled antiserum containing 3 x 10^6 cpm (counts minute^{-1}) added to 30 to 40 ml of the blocking buffer). Subsequently any components of *T. evansi* that had reacted with the anti-CRD serum were revealed by autoradiography at -70^{o}C for 24 hours using

Fuji X-ray film (Fuji Photo Ltd., Japan).

CLASS-SPECIFIC ANTIBODY COPRECIPITATION ASSAY OF T. EVANSI PROTEINS

Class-specific antibody immunoprecipitation of *T. evansi* ^{125}I-labelled proteins was performed by a modification of the method described by Almond and Parkhouse (1986). Serum samples that were examined in this way had been collected from the rabbits before infection (day 0) and on days 7, 14 and 28 after infection and days 7, 28 and 39 after drug treatment. Each serum sample was analysed for anti-trypanosome IgG, IgM and IgA activity using appropriate heavy chain (Fc) specific antisera raised in goats against rabbit IgG, IgM or IgA (Nordic^{R}) to precipitate the equivalent class-specific fraction of antigen/antibody complexes formed between radiolabelled trypanosome proteins and serum from the trypanosome-infected rabbits.

Briefly, co-precipitation was undertaken by mixing 10 µl of the appropriate serum from the rabbits exposed to infection with *T. evansi* with 20 µl of the supernate fraction of ^{125}I-labelled *T. evansi* TREU 2113, non-detergent lysate adjusted with PBS to 10^5 counts per minute at 4^{o}C. The mixture was then made up to a final volume of 400 µl for the IgG assay and 200 µl for the IgM and IgA assays with co-precipitation diluent (Appendix 11). These volumes had been found to give optimum results from an initial study.

After incubation of the ^{125}I-labelled *T. evansi* antigen and rabbit serum for four hours on ice, any trypanosome antibody complex formed was precipitated from the mixture using an appropriate antiserum raised in goats to

the heavy chain (Fc) portion of each class of rabbit immunoglobulin (Nordic^{R}). In the case of anti-trypanosome IgG precipitation 10 μl of the antiserum was used and the mixture incubated for 24 hours. In the case of anti-trypanosomes IgM and IgA 30 μl of the appropriate antisera was used and the mixture incubated for 72 hours. The volumes of antisera and incubation times had been found to be optimum from a preliminary assay.

Complexes formed with the class-specific antisera were precipitated by centrifugation at 2,000 g for 10 minutes. Any unbound reactants were removed by washing with coprecipitation diluent. Washing of the pellet was continued until the radioactivity content of the pellet stabilised as monitored with a gamma counter (Nuclear Enterprises NE1600). This happened by the third wash.

Quantification of the Class-specific Antibody Response to the Iodinated (^{125}I) components of T. evansi

After the third wash, the pellets produced after precipitation with class-specific antisera were resuspended in 100 μl of co-precipitation diluent (Appendix 11). The degree of radioactive incorporation in each of the pellets was then estimated by measuring the amount of radioactivity in two 20 μl aliquots, of the suspension obtained from each sample with a gamma counter (Nuclear Enterprises NE 1600).

Analysis of Co-precipitation Precipitates for Class-specific Antibody Activity

The composition of precipitates obtained after co-precipitation were analysed by SDS-PAGE after mixing with an appropriate amount of cold protein and SDS sample buffer as described above using the final protocol

described in Chapter 2 with a 7-20% continuous gradient polyacrylamide gel for 18 hours. The total protein profile of *T. evansi* TREU 2113 was examined after staining the gel with Coomassie Blue as described previously. The gels were then dried down and any ^{125}I-labelled *T. evansi* antigen/class specific Ig reactions visualised by autoradiography at -70^{o}C for 36 hours using Fuji RX-ray film (Fuji Photo Ltd., Japan).

Electron Microscopal Examination of Surface Coat of T. evansi

Trypanosomes derived from *T. evansi* TREU 1944, 2147 and 1834 were fixed in suspension with 2.5% gluteraldehyde in 0.1M cacodylate buffer, pH 7.3 (Appendix 12) for periods up to two hours. The cells were then washed in 0.1M cacodylate buffer by centrifugation at 2,000 g over 20 minutes with the buffer changed three times. The specimens were post-fixed in 1% osmium tetraoxide (TAAB Lab. Equipment Ltd., UK) in 0.1M cacodylate buffer for 45 minutes and dehydrated in graded acetone (50%, 70%, 90% and 100%) before being infiltrated with equal volumes of araldite mix (Agar Scientific, UK) and Analar acetone (BDH) for 30 minutes at room temperature with slight agitation and then overnight at 60^{o}C. Further infiltration was carried out with the araldite mix alone for one hour followed by three changes of araldite mix and accelerator (Appendix 13) (19:1, v/v) for one hour per infiltration. Embedding was undertaken with araldite mix and accelerator (19:1, v/v) for another 48 hours at 60^{o}C. Sixty nm thick sections were produced from the blocks with Reichert OmU4 ultramicrotome fitted with a diamond knife. Sections were then picked up on copper grids and examined with a Phillips 400 TEM electron microscope operated at 100 KV.

RESULTS

Non-detergent Lysis of T. evansi

More than 30 protein bands with a molecular weight range of 123,800 to 13600 daltons (d) were seen when both the supernate (Figure 3.1, No. 2) and precipitate (Figure 3.1, No. 4) fractions of the *T. evansi* TREU 2113 non-detergent lysate were subjected to SDS-PAGE gels and then stained with Coomassie Blue (Sigma). Two protein bands with molecular weights of 67,000 and 60,500 d were visualised after radioactive labelling, from both the supernate and precipitate fractions of TREU 2113 (Figure 3.1, Nos. 1 and 3 respectively), 2147 and 1733 (data for the last two stocks not shown). A single component with a molecular weight of 60,500 d was seen in autoradiographs from the supernate and precipitate fractions of ^{125}I-labelled TREU 1944 (Figure 3.2, Nos. 4a and 4b respectively) and TREU 1380 (data not shown). No radiolabelled bands were seen on an autoradiograph from both the supernate and precipitate fractions of lysates of radiolabelled proteins of TREU 1834 (Figure 3.2, Nos. 3a and 3b respectively).

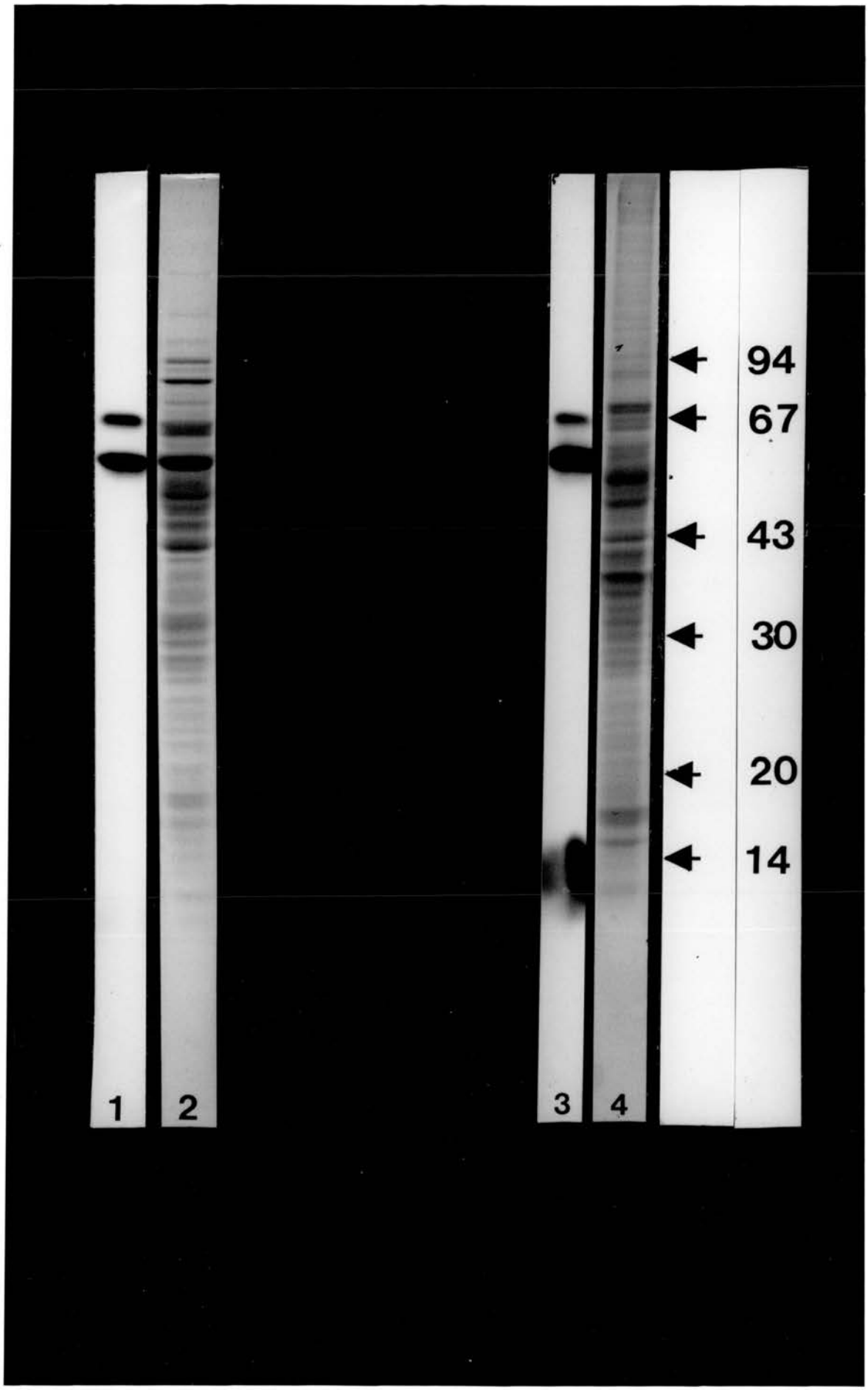
Detergent Lysis of T. evansi

A single band with a molecular weight of 60,500 d was seen on an autoradiograph from the detergent lysate of ^{125} I-labelled TREU 1944 (Figure 3.2, No. 2) with no radioactivity detected from the lysate of TREU 1834 (Figure 3.2, No. 1). In each case, cells were observed to incorporate more radioactivity at 4^{o}C than at room temperature. The incorporation of radioactivity into many bands was observed during some labelling procedures carried out at room temperature. This was particularly evident in samples in which many dead trypanosomes were observed at the end of the labelling procedure.

FIGURE 3.1

Surface iodination of *Trypanosoma evansi* TREU 2113.

1. Supernate fraction of *T. evansi* TREU 2113 non-detergent lysate after iodination.
2. Supernate fraction of *T. evansi* TREU 2113 non-detergent lysate, non-iodinated stained with Coomassie Blue.
3. Precipitate fraction of *T. evansi* TREU 2113 non-detergent lysate after iodination.
4. Precipitate fraction of *T. evansi* TREU 2113 non-detergent lysate, non-iodinated stained with Coomassie Blue.


1
2
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4
94
67
43
30
20
14

FIGURE 3.2

Proteins of *T. evansi* TREU 1834 and 1944 iodinated and lysed in the presence and absence of detergents.

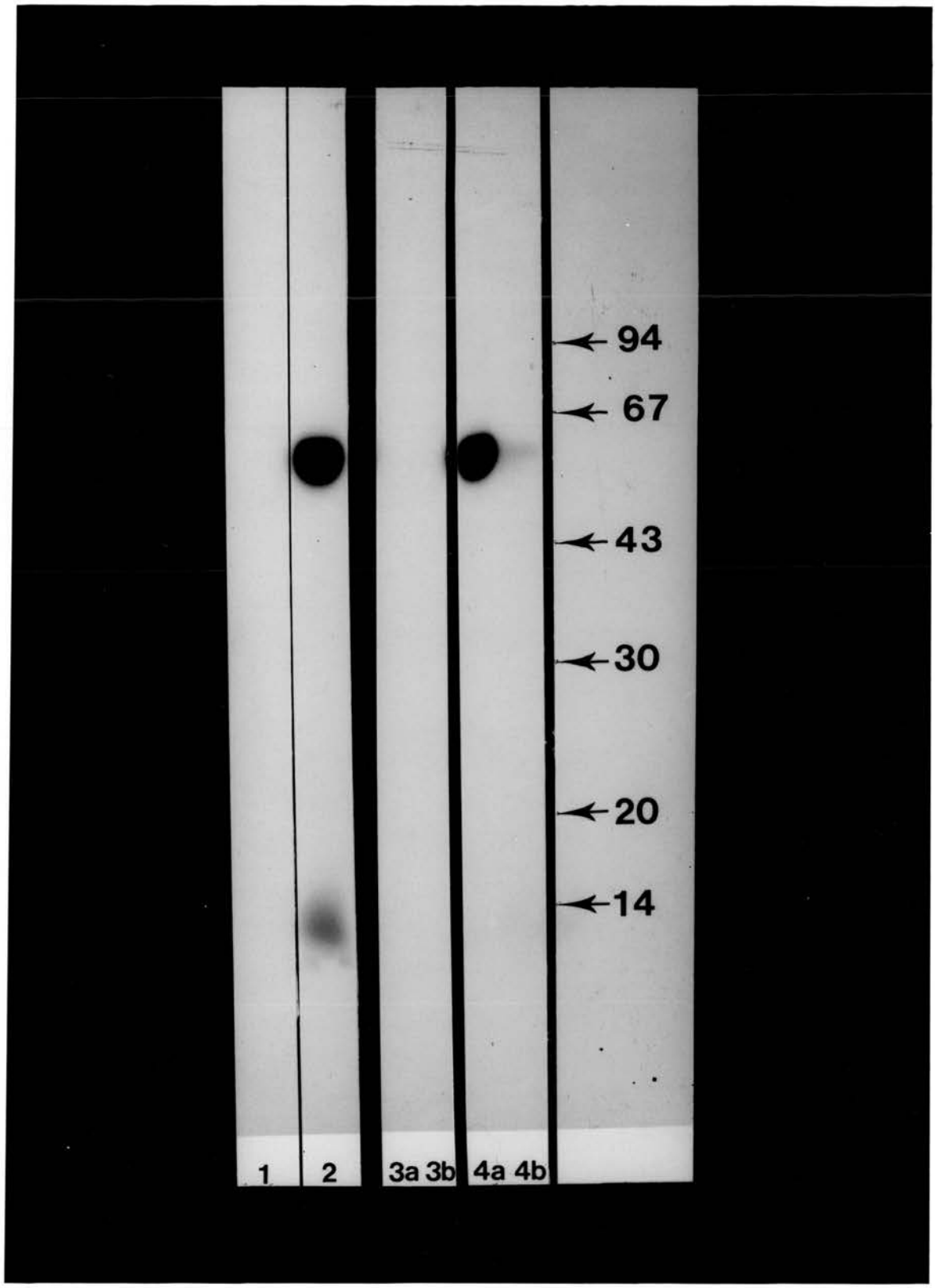
1. Blot onto which proteins of TREU 1834 were transferred after iodination and lysis in the presence of a detergent.
2. Blot onto which proteins of TREU 1944 were transferred after iodination and lysis in the presence of a detergent.

3a. Blot onto which the supernate fraction of *T. evansi* TREU 1834 was transferred after iodination and lysis in the absence of a detergent.

3b. Blot onto which the precipitate fraction of *T. evansi* TREU 1834 was transferred after iodination and lysis in the absence of a detergent.

4a. Blot onto which the supernate fraction of *T. evansi* TREU 1944 was transferred after iodination and lysis in the absence of a detergent.

4b. Blot onto which the precipitate fraction of *T. evansi* TREU 1944 was transferred after iodination and lysis in the absence of a detergent.


94
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3a 3b
4a 4b

Components of T. evansi Recognised by the Anti-CRD Serum

Two radiolabelled components of molecular weights 67,000 and 60,500 d were seen on an autoradiograph of the nitrocellulose membrane, onto which the separated proteins of the supernate fractions of *T. evansi* TREU 1944 and 1380 produced by non-detergent lysis had been transferred and then probed with anti-CRD serum (Figure 3.3, Nos. 2 and 10 respectively). One component of molecular weight 60,500 d was recognised on a similarly processed autoradiograph when the total proteins of TREU 2113 (Figure 3.3, No. 4), 2147 (Figure 3.3, No. 6) and 1733 (Figure 3.3, No. 8) were probed with the antiserum. No bands were seen when the supernate fraction of the non-detergent lysate of TREU 1834 was probed with the anti-CRD serum (Figure 3.3, No. 12). A 60,500 d band was, however, recognised by the anti-CRD serum in the precipitate fraction from the non-detergent lysate of TREU 1834 (Figure 3.4, No. 2).

For clarity, the total protein profile of the supernate fraction from the non-detergent lysate of each trypanosome stock after separation on SDS-PAGE and Coomassie Blue staining is shown as odd numbers in Figure 3.3, adjacent to each autoradiograph from the equivalent blots probed for reactivity with anti-CRD serum.

FIGURE 3.3

Components of different stocks of *T. evansi* recognised by the anti-CRD serum.

(a) Odd number figures represent the protein profiles of the supernate fraction of the lysate of the different stocks of *T. evansi* produced by non-detergent lysis, separated with SDS-PAGE and stained with Coomassie Blue.

(b) Even number figures represent the proteins as in (a) above of the different stocks of *T. evansi* probed with anti-CRD serum on "Western" blotting.

Numbers 1 and 2: TREU 1944

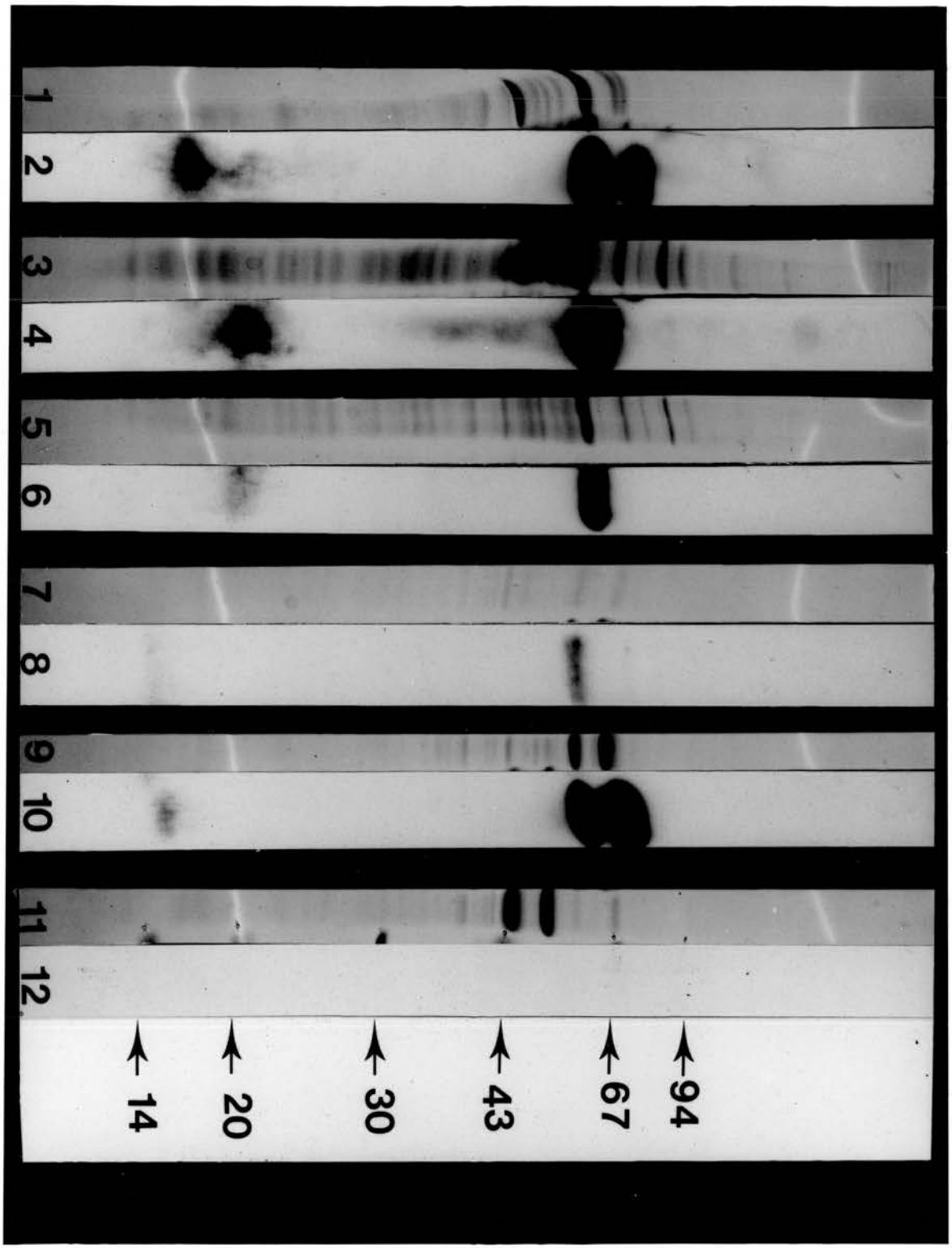
Numbers 3 and 4: TREU 2113

Numbers 5 and 6: TREU 2147

Numbers 7 and 8: TREU 1733

Numbers 9 and 10: TREU 1380

Numbers 11 and 12: TREU 1834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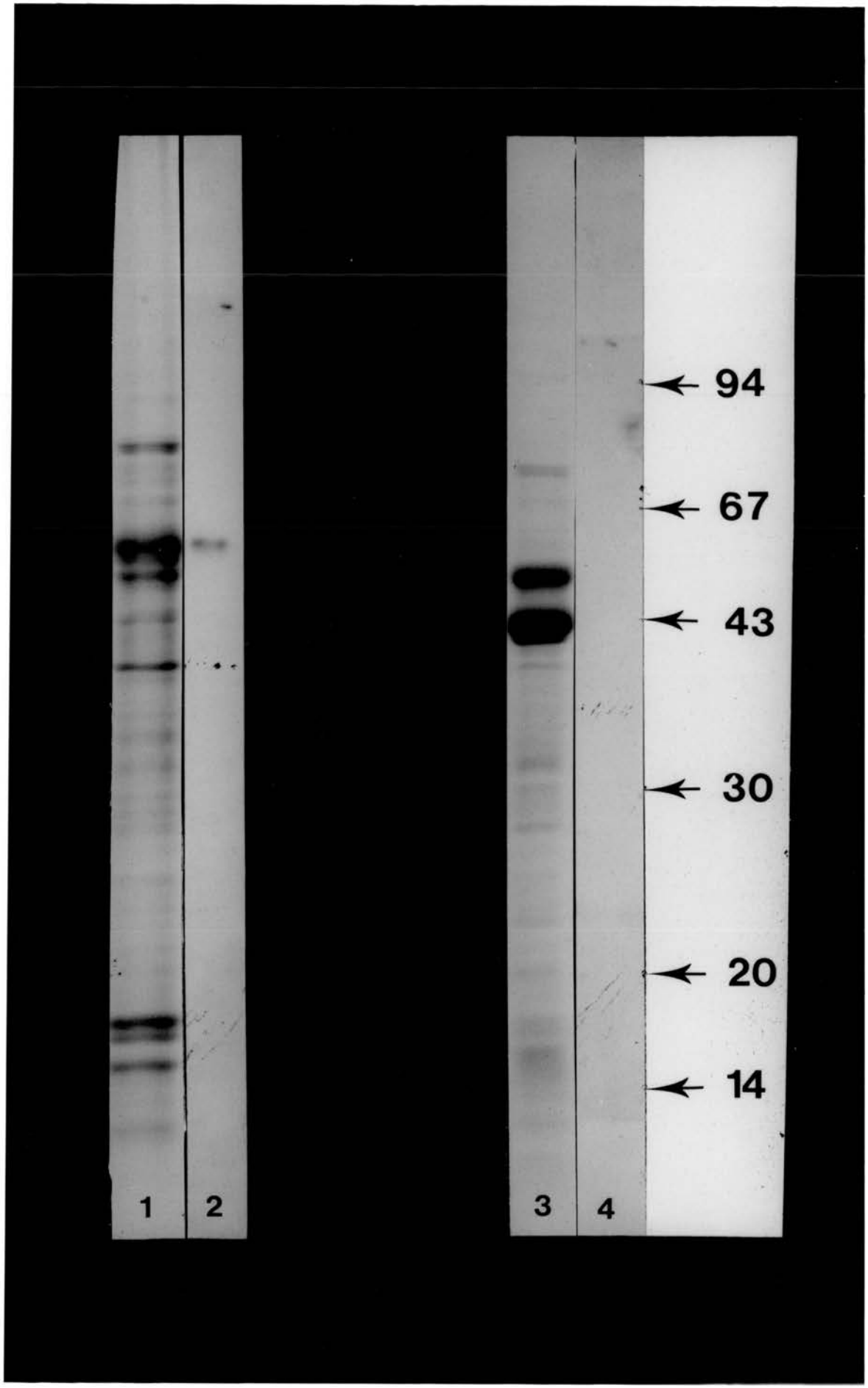
43
67
94

FIGURE 3.4

Proteins of *T. evansi* TREU 1834 recognised by anti-CRD serum.

1. Protein profile of the precipitate fraction of the lysate of TREU 1834 produced by non-detergent lysis, separated with SDS-PAGE and stained with Coomassie Blue.
2. Proteins of TREU 1834 as in 1. above probed with anti-CRD serum on "Western" blotting.
3. Protein profile of the supernate fraction of the lysate of TREU 1834 produced by non-detergent lysis, separated with SDS-PAGE and stained with Coomassie Blue.
4. Proteins of TREU 1834 as in 3. above probed with anti-CRD serum on "Western" blotting.


94
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Electron Microscopic Findings

Each *T. evansi* stock (TREU 1944, 2147 and 1834) examined by electron microscopy was seen to have a thick, compact and dense surface coat overlying the plasma membrane (Figure 3.5, Nos. 1 to 3).

Class-specific Antibodies to the ^{125}I-labelled Components of T. evansi Assayed by Coprecipitation

IgG antibodies: A single ^{125}I-labelled component with a molecular weight of 60,500 d was recognised by serum collected from both rabbits seven days after infection with TREU 2113 (Figure 3.6, Nos. 2 and 9). From 14 days after infection, a further ^{125}I-labelled component (67,000 d) was recognised. Thereafter both components were recognised throughout the post-infection and post-drug treatment periods (Figure 3.6, Nos. 3 to 14). Preinfection sera collected from both rabbits failed to react with any ^{125}I-labelled trypanosome components (Figure 3.6, Nos. 1 and 8).

IgM antibodies: A single radiolabelled component of 60,500 d, was recognised by the serum sample collected seven days after infection from one rabbit (Figure 3.7, No. 2) while the equivalent serum from the second rabbit recognised an additional component of 67,000 d (Figure 3.7, No. 9). Thereafter both bands were recognised by serum samples collected from both rabbits throughout the post-infection and post-drug treatment periods (Figure 3.7, Nos. 3 to 14).

IgA antibodies: There was considerable variation in the pattern of ^{125}I-labelled antigens of *T. evansi* recognised by IgA antibodies produced by the two rabbits. In one of the rabbits the bands, 67,000 and 60,500 d were faintly recognised by IgA in serum collected 14 days post-infection (Figure 3.8,

FIGURE 3.5

Transmission electron micrograph of section through the surface of the body of different stocks of *T. evansi*.

(Magnification x 165,000).

Number (1) TREU 1944

Number (2) TREU 2147

Number (3) TREU 1834

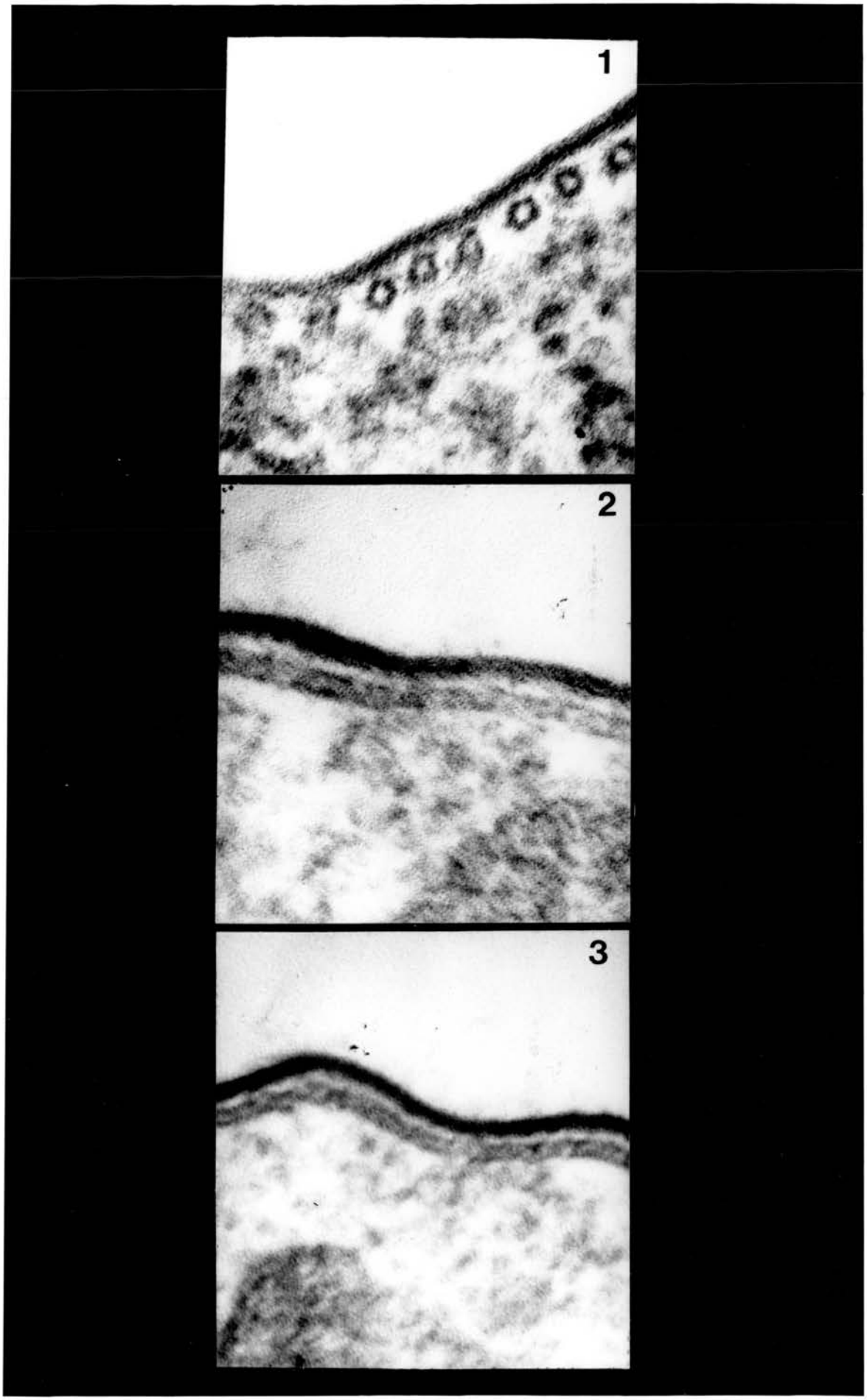
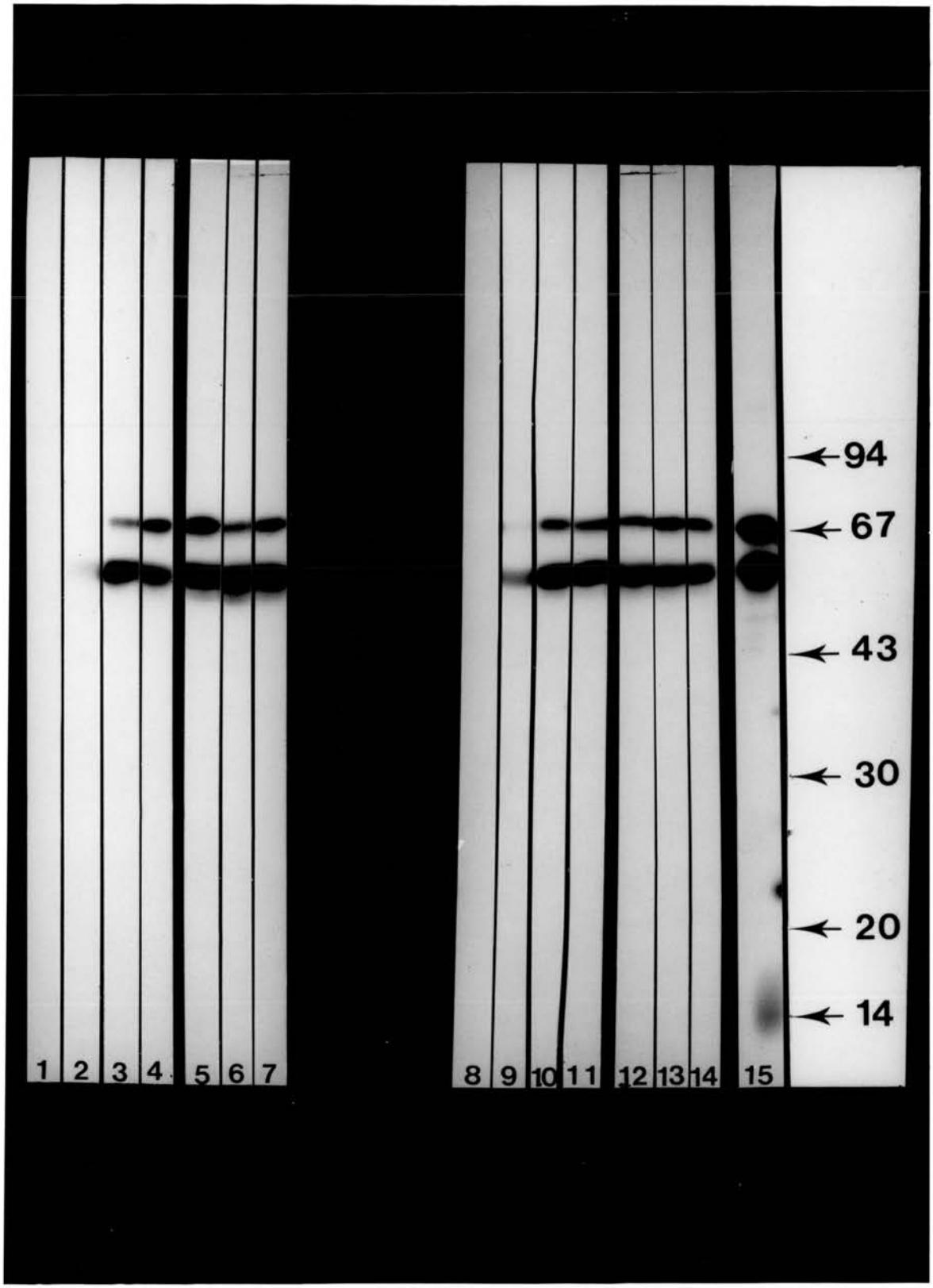

1
2
3

FIGURE 3.6

IgG antibodies produced by rabbits to the ^{125}I-labelled components of *T. evansi* during the course of infection and after drug treatment. (Numbers 1-7 and 8-14 are reactions with serum samples collected from the first and second rabbits respectively.)

1. Components identified by the normal rabbit serum.
2. Components identified by serum collected 7 days after infection.
3. Components identified by serum collected 14 days after infections.
4. Components identified by serum collected 28 days after infection.
5. Components identified by serum collected 7 days after drug treatment.
6. Components identified by serum collected 28 days after drug treatment.
7. Components identified by serum collected 39 days after drug treatment.
8. Components identified by the normal rabbit serum.
9. Components identified by serum collected 7 days after infection.
10. Components identified by serum collected 14 days after infection.
11. Components identified by serum collected 28 days after infections.
12. Components identified by serum collected 7 days after drug treatment.
13. Components identified by serum collected 28 days after drug treatment.
14. Components identified by serum collected 39 days after drug treatment.
15. ^{125}I-labelled components of *T. evansi* not reacted with any serum.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20
14
1 2 3 4 5 6 7
8 9 10 11 12 13 14 15

FIGURE 3.7

IgM antibodies produced by rabbits to the ^{125}I-labelled components of *T. evansi* during the course of infection and after drug treatment. (Numbers 1-7 and 8-14 are reactions with serum samples collected from the first and second rabbits respectively.)

1. Components identified by the normal rabbit serum.
2. Components identified by serum collected 7 days after infection.
3. Components identified by serum collected 14 days after infection.
4. Components identified by serum collected 28 days after infection.
5. Components identified by serum collected 7 days after drug treatment.
6. Components identified by serum collected 28 days after drug treatment.
7. Components identified by serum collected 39 days after drug treatment.
8. Components identified by the normal rabbit serum.
9. Components identified by serum collected 7 days after infection.
10. Components identified by serum collected 14 days after infection.
11. Components identified by serum collected 28 days after infection.
12. Components identified by serum collected 7 days after drug treatment.
13. Components identified by serum collected 28 days after drug treatment.
14. Components identified by serum collected 39 days after drug treatment.
15. ^{125}I-labelled components of *T. evansi* not reacted with any serum.

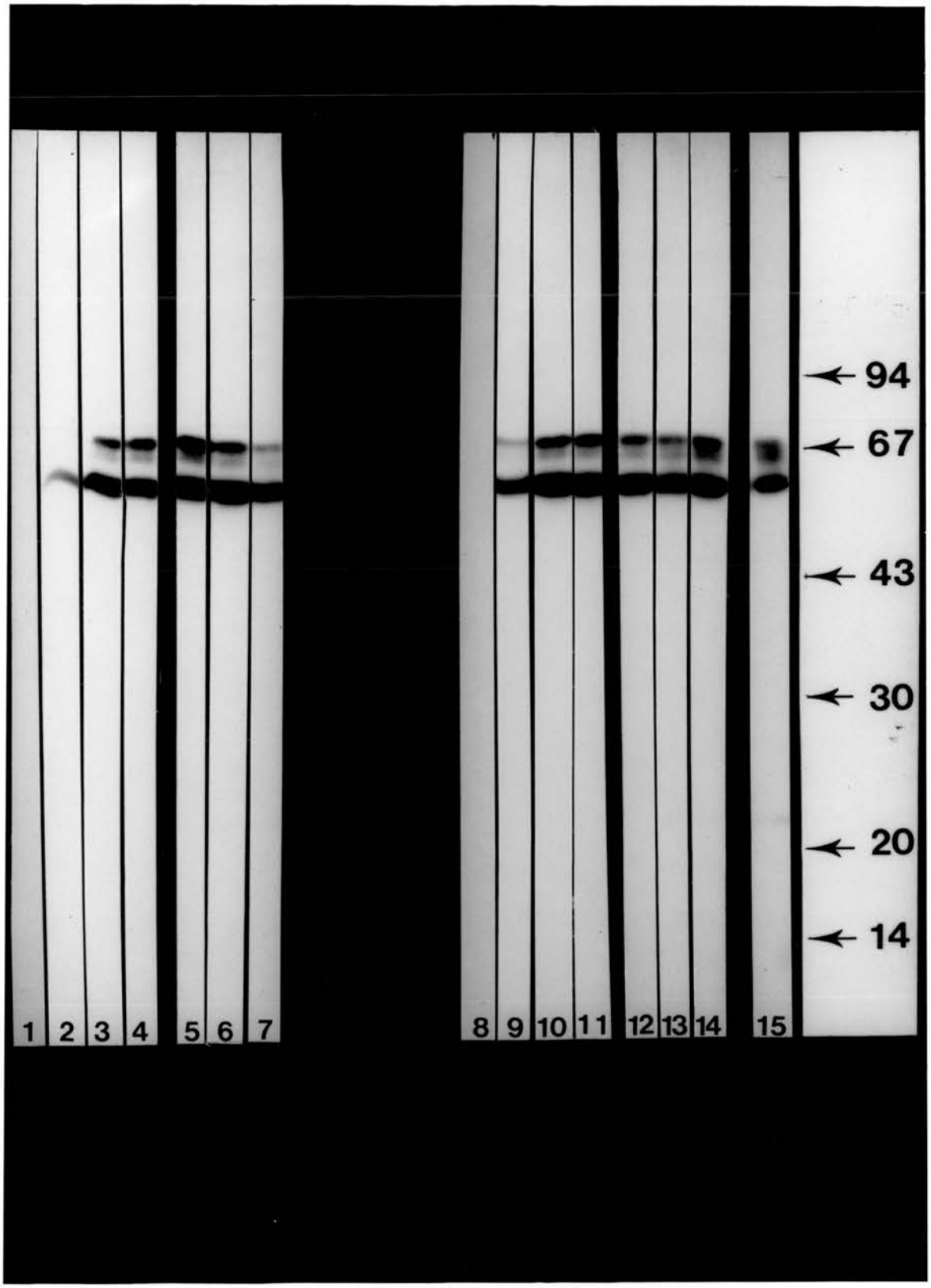
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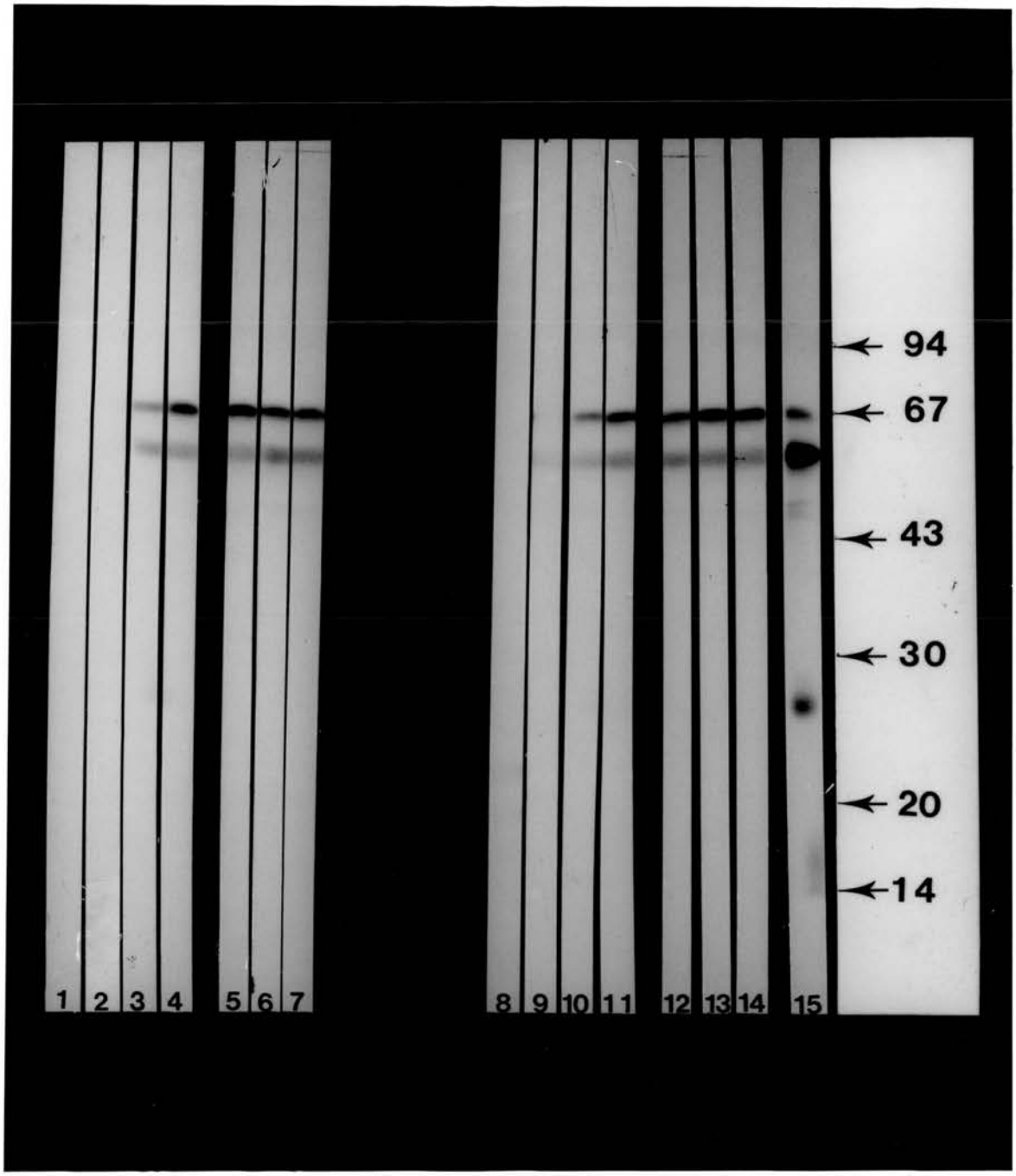
FIGURE 3.8

IgA antibodies produced by rabbits to the ^{125}I-labelled components of *T. evansi* during the course of infection and after drug treatment. (Numbers 1-7 and 8-14 are reactions with serum samples collected from the first and second rabbits respectively.)

1. Components identified by the normal rabbit serum.
2. Components identified by serum collected 7 days after infection.
3. Components identified by serum collected 14 days after infection.
4. Components identified by serum collected 28 days after infection.
5. Components identified by serum collected 7 days after drug treatment.
6. Components identified by serum collected 28 days after drug treatment.
7. Components identified by serum collected 39 days after drug treatment.
8. Components identified by the normal rabbit serum.
9. Components identified by serum collected 7 days after infection.
10. Components identified by serum collected 14 days after infection.
11. Components identified by serum collected 28 days after infection.
12. Components identified by serum collected 7 days after drug treatment.
13. Components identified by serum collected 28 days after drug treatment.
14. Components identified by serum collected 39 days after drug treatment.
15. ^{125}I-labelled components of *T. evansi* not reacted with any serum.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No. 3). Thereafter, both bands continued to be recognised by all serum samples collected from the rabbit throughout the post-infection and post-drug treatment periods (Figure 3.8, Nos. 4 to 7). IgA in serum from the second rabbit however, recognised the 60,500 d component faintly at seven days after infection (Figure 3.8, No. 9). Thereafter, both 67,000 and 60,500 d components were recognised by all the serum samples collected from the rabbit throughout both the post-infection and post-drug treatment periods (Figure 3.8, Nos. 10 to 14). The 67,000 dalton band appeared to be better recognised by the IgA antibodies than the 60,500 d band based on the intensity of staining on the blot (Figure 3.8).

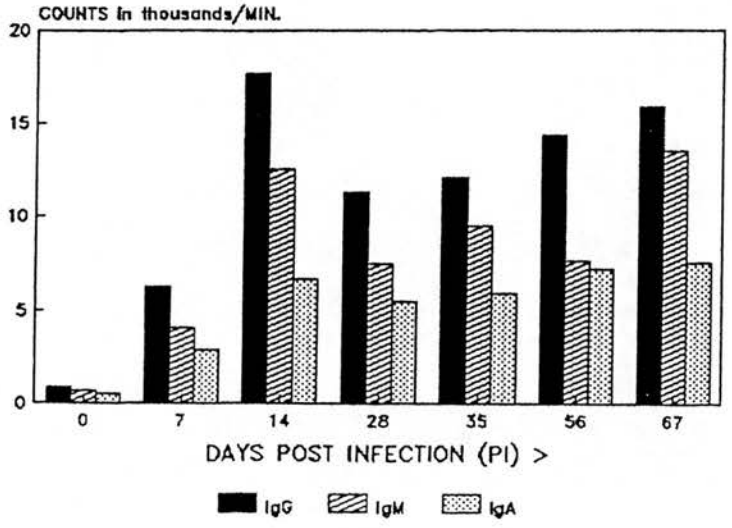
Quantification of the Class-specific Antibody Response to the Iodinated (^{125}I) Components of T. evansi

The amount of radioactivity incorporated into the complexes formed between the ^{125}I-labelled trypanosome proteins and the class-specific antibodies to rabbit IgG, IgM and IgA with the serum samples collected from the two rabbits differed at different times during the post-infection and post-drug treatment periods (Figure 3.9A and B). Least radioactive incorporation was observed in complexes formed with the serum samples collected seven days after infection, whilst the greatest amounts were recorded with serum samples collected during late infections or after drug treatment.

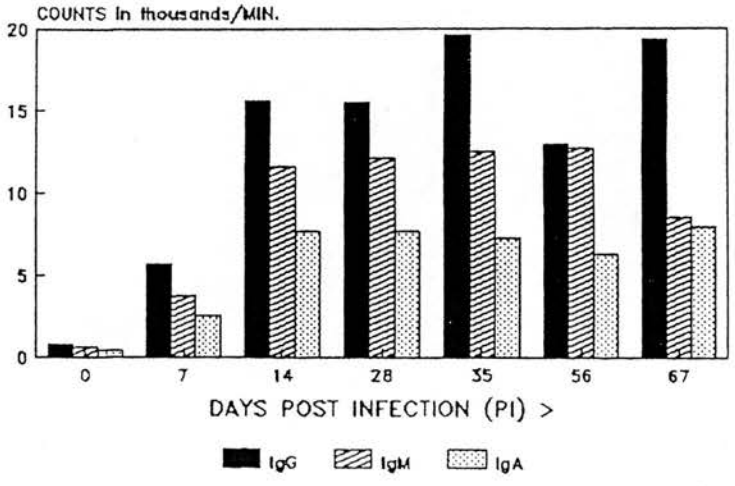
Greatest average radioactive incorporation was observed in complexes formed by reaction with IgG specification (6,500-20,000). Next in magnitude were those of IgM antiserum with 4,000-12,000 cpm and least IgA with 2,800-7,250 counts per minute per 20 μl (cpmp 20 μl). Minimal amounts of radioactivity were incorporated in the products of incubation with the normal

FIGURE 3.9

Radioactivity in the complexes precipitated by the different immunoglobulin isotypes in the serum of rabbits during the course of infection with *T. evansi* and after drug treatment. (A and B are counts from complexes precipitated by the antibodies in the serum samples of two rabbits respectively.)


COUNTS in thousands/MIN.
20
15
10
5
0
0
7
14
28
35
56
67
DAYS POST INFECTION (PI) >
IgG
IgM
IgA

(A)


COUNTS in thousands/MIN.
20
15
10
5
0
0
7
14
28
35
56
67
DAYS POST INFECTION (PI) >
IgG
IgM
IgA

(B)

rabbit serum - with 705, 450 and 317 cpmp 20 μl for IgG, IgM and IgA respectively. The amount of radioactive incorporation into the complexes formed by the three Ig isotypes, IgG, IgM and IgA is represented in Figure 3.9.

The differences between the different *T. evansi* stocks used in the present study are shown in Table 3.1.

Table 3.1 Summary of differences between the different T. evansi stocks

Stock (TREU No.)	^{125}I-labelling of trypanosomes			Anti-CRD antibody activity. (Non-detergent lysate)		EM Studies to detect the surface coat.
	Non-detergent lysate No. of bands and molecular weight.		Detergent lysate. No. of bands and Mol. Wt.	No. of bands and molecular weight		
	Supernate	Precipitate		Supernate	Precipitate	
2113	2 67000 60500	2 67000 60500	ND	1 - 60500	ND	ND
2147	2 67000 60500	2 67000 60500	ND	1 - 60500	ND	+
1733	2 67000 60500	2 67000 60500	ND	1 - 60500	ND	ND
1944	1 - 60500	1 - 60500	1 - 60500	2 67000 60500	ND	+
1380	1 - 60500	1 - 60500	ND	2 67000 60500	ND	ND
1834	0 - -	0 - -	0 - -	0 - -	1 - 60500	+

ND: Not done; -: Absent; +: Present

DISCUSSION

Radiolabelling of intact *T. evansi* organism with ^{125}I in this study resulted in the incorporation of radioactivity into two or one or no components, with radiolabelled components having a molecular weight of either 67,000 and/or 60,500 dalton bands (Figure 3.1 and Figure 3.2, No. 4a). It is likely that these labelled components represent surface components of the trypanosomes because intact organisms do not incorporate the label into other than their surface elements (Zingales, 1984). Furthermore, the radiolabelling technique employed in this study has been used extensively for surface labelling of a range of parasites (Gardiner and Dwyer, 1981; Howard *et al.*, 1982) including several trypanosome species (Gardiner *et al.*, 1983; Ross *et al.*, 1987; Camargo *et al.*, 1982a,b). The association of radiolabelling of more components with preparations containing many dead or dying trypanosomes, is a further indication that the labelling of intact trypanosomes is confined to the surface of the organism as these additional components labelled are thought to be exposed after the breakdown of the intact trypanosome.

The differences seen between the different stocks of *T. evansi* with respect to the total number and molecular weight of the individual components radiolabelled during the present study was not considered a peculiarity restricted to *T. evansi*. In *T. congolense*, Ross *et al.* (1987) iodinated a single component while Rovis *et al.* (1978) labelled more than one surface component of the same parasite. The failure to label any trypanosome component as in the case of TREU 1834 in the present study does not appear to have been reported previously. It is likely that the differences in the results of radiolabelling seen between different stocks reflect variations in the biochemical composition of the surface coat of the individual trypanosome

populations although the significance of such differences in the host/parasite relationship is unknown.

The results from electron microscopal studies (Figure 3.5, Nos. 1 to 3) clearly demonstrated no physical difference between the surface coats of the individual trypanosome populations. The failure to radiolabel any component of TREU 1834 was not, therefore, due to the lack of a surface coat and provides further evidence that failure to label TREU 1834 was probably due to the absence of specific amino-acid residues on the surface of the parasites which react with the iodination reagents or that any component bearing the appropriate amino acids is not exposed on the surface of the parasite. Furthermore, results from the Coomassie Blue staining of the total proteins of each stock of *T. evansi* evaluated in this study, indicate that both the 67,000 and 60,500 d are present in all the stocks (Figure 3.3, odd numbers). It is likely therefore that the other two factors - amino acid sequencing on the surface of the parasite or the absence of the component from the surface of the parasite could have influenced the results of radiolabelling.

Successful iodogen-mediated cell iodination (Fraker and Speck, 1978) depends on the availability of tyrosyl residues on the surface of parasites (Zingales, 1983). It is likely that in the case of some stocks such as TREU 1944 in which only the 60,500 component was identified on surface iodination (Figure 3.2, Nos. 4a and 4b) that this component was the only component exposed on the surface of the parasite having both sufficient tyrosyl residues and accessible to labelling. In other stocks such as TREU 2113 in which both components (67,000 and 60,500) were radiolabelled (Figure 3.1, Nos. 1 and 3), it is likely that both components were exposed on the surface of the parasites and with sufficient tyrosyl residues for successful labelling. In the

stock where none of the components were labelled (Figure 3.2, 3a and b), it is possible that neither component was exposed on the surface of the parasite or more likely that neither of them had sufficient exposed tyrosyl residues. The use of other iodinating techniques such as the Bolton and Hunter technique (Bolton and Hunter, 1985) that react with different amino acid residues might produce different results after surface-labelling these parasite populations.

The use of the anti-CRD serum as a means of characterising surface component of the *T. evansi* was based on the premise that the antiserum only recognises the CRD-epitope in the soluble form of VSG (sVSG) and not in the membrane bound VSG (mfVSG) where the recognition of the determinant by anti-CRD serum is impaired (Cardoso de Almeida and Turner, 1983). The CRD antibodies were found to bind to the 60,500 daltons component in all six stocks of *T. evansi* studied as a single component in the case of four stocks (Figure 3.3, Nos. 4, 6 and 8 and Figure 3.4, No. 2) and in combination with the 67,000 dalton component in two stocks (Figure 3.3, Nos. 2 and 10).

The association of the CRD with the 60,500 d component suggests that this component could represent the non-variable portion of the VSG molecule of *T. evansi* while the other surface-associated 67,000 d component might represent the variable portion. In two stocks, however the CRD epitope appeared to be distributed equally between both 60,500 and 67,000 components. This result suggests that the oligosaccharide molecule which comprises the CRD may not necessarily be restricted to the C-terminal domain of the VSG of *T. evansi* alone as has been suggested for other trypanosome species (Holder and Cross, 1981).

The surface components of trypanosomes derived from *T. evansi* TREU 1834 behaved very differently from the other stocks examined in the present

study. Of the six stocks of *T. evansi* examined, it was not possible to radiolabel any surface components of TREU 1834. Furthermore, results from the studies with the anti-CRD serum identified a single component of molecular weight 60,500 in the pellet fraction after non-detergent lysis (Figure 3.4, No. 2) that was lacking in the supernate fractions (Figure 3.4, No. 4) unlike other stocks in which the component was identified in the supernatant fraction of the lysate preparation. In the case of TREU 1834 it is possible that the glycoprotein surface coat of this stock is very tightly bound to the plasma membrane and not, therefore, released during non-detergent lysis of the cell, a process that normally releases the VSG in a water-soluble state (Auffret and Turner, 1981; Cardoso de Almeida and Turner, 1983). Although both the 67,000 and 60,500 dalton components that are associated with the surface of the other trypanosome stocks are faintly present in the total proteins of this stock, (Figure 3.3, No. 11) it is possible that they may not represent the actual surface components of TREU 1834. The two components with the molecular weight of 56,000 and 43,000 in the supernate fraction of the lysates may represent the surface components (Figure 3.3, No. 11), based on the fact that they are clearly distinct from all other components in the total protein profile of the trypanosome stock which is recognised as a common feature of VSGs on Coomassie Blue-stained gels (C.A. Ross, 1989, personal communication). These differences in the molecular weight range of the postulated surface components of TREU 1834 compared to those of the other five stocks could be a reflection of radical differences in amino acid sequences in the VSG molecules of TREU 1834 which would in turn affect the efficiency of the iodination method used for surface labelling.

The recognition of the 60,500 and 67,000 d components of *T. evansi* 2113 by serum collected early after infection provides further evidence that these

two components are surface associated, as early host antibody responses are usually directed at surface components of invading organisms. The recognition of *T. evansi* surface antigen components by the three main immunoglobulin isotypes, IgG, IgM and IgA whose production was maintained throughout the period of observation (Figures 3.6 to 3.8) confirms the importance of VSG-specific antibody response, not only, for the elimination of a particular parasite population but also for the protection of animals against reinfections with homologous trypanosomes (Emery, *et al.*, 1980; Mitchell and Pearson, 1983; Hall and Esser, 1984) as it would be difficult for parasites expressing homologous surface antigens to establish in animals having VSG-specific antibodies to the surface antigens. It is likely that the VSG-specific IgM antibodies which appeared early in animals to the surface components served to destroy the trypanosomes through lysis which could then have induced the production of IgG antibodies largely to the non-surface components (Vickerman and Barry, 1982) to remove the products of lysis or ameliorate any possible toxic consequencies of large amounts of biologically active substances produced by either dead or dying trypanosomes (Seed, 1969; Tizard and Ringleberg, 1973; Huan *et al.*, 1975) possibly by detoxification (Roitt *et al.*, 1985).

The appearance of IgA antibody to the surface antigen components of TREU 2113 is not clearly understood. IgA antibody is commonly associated with seromucous surfaces. IgA antibodies secreted during infection may be playing a supportive role in the control of parasitaemia via the activation of the alternative complement pathway or the agglutination of parasites before they are eliminated by phagocytic effector cells of the body such as neutrophils and macrophages (Roitt *et al.*, 1985; Bowry, 1984). Few studies have been undertaken to study the importance of IgA antibody response in trypanosome

infection. One such study by Herbert *et al.* (1980) reported that the total IgA level in man was unaffected while in another study Nielsen *et al.* (1978) described a reduction in total IgA level in calves infected with *T. congolense*. Although these authors were unable to explain the relevance of the findings, Nielsen *et al.* (1978) however, suggested extending the studies to IgA levels in external mucosal surfaces where IgA is at least partly responsible for protection against invading pathogens.

The identification of the two surface-associated components 67,000 and 60,500 by all classes of antibody in the serum samples collected throughout the period of observation from the rabbits indicates the continual presence of antibodies against the components throughout the period of infection and after drug treatment. The persistence of antibody to these components during the period of infection may be due to consistent re-expression of particular antigenic types such as the predominant antigen type (pVAT) particularly as TREU 2113 is known to be a predominant antigen type (T.W. Jones, personal communication). Such consistent re-expressions of a particular antigen type could act as a source of continuous antigenic stimulation leading to a build-up of specific antibodies in the serum of infected animals. The antibodies against the trypanosome surface antigens persisting in the serum after drug treatment could be those that had been produced before the institution of chemotherapy that persisted in circulation. Alternatively, it is possible that the persistence of the antibodies during the post treatment period could be associated with the elimination of the effects of the factors known to be released by living trypanosomes that block the terminal stages of differentiation of parasite-activated B lymphocytes to immunoglobulin-secreting cells or inhibits secretion of immunoglobulin by these cells (Black *et al.*, 1985). The elimination of the parasites by chemotherapy and consequently the elimination of the effects of

these factors on parasite-activated B cells are thought to contribute to the rapid production of antigen-specific antibodies after drug cure as suggested by Shapiro and Pearson (1986).

CHAPTER FOUR

CLASS-SPECIFIC ANTIBODY RESPONSE IN RABBITS EXPERIMENTALLY INFECTED WITH TRYPANOSOMA EVANSI DURING THE PERIOD OF PRIMARY INFECTION AND AFTER DRUG TREATMENT

INTRODUCTION

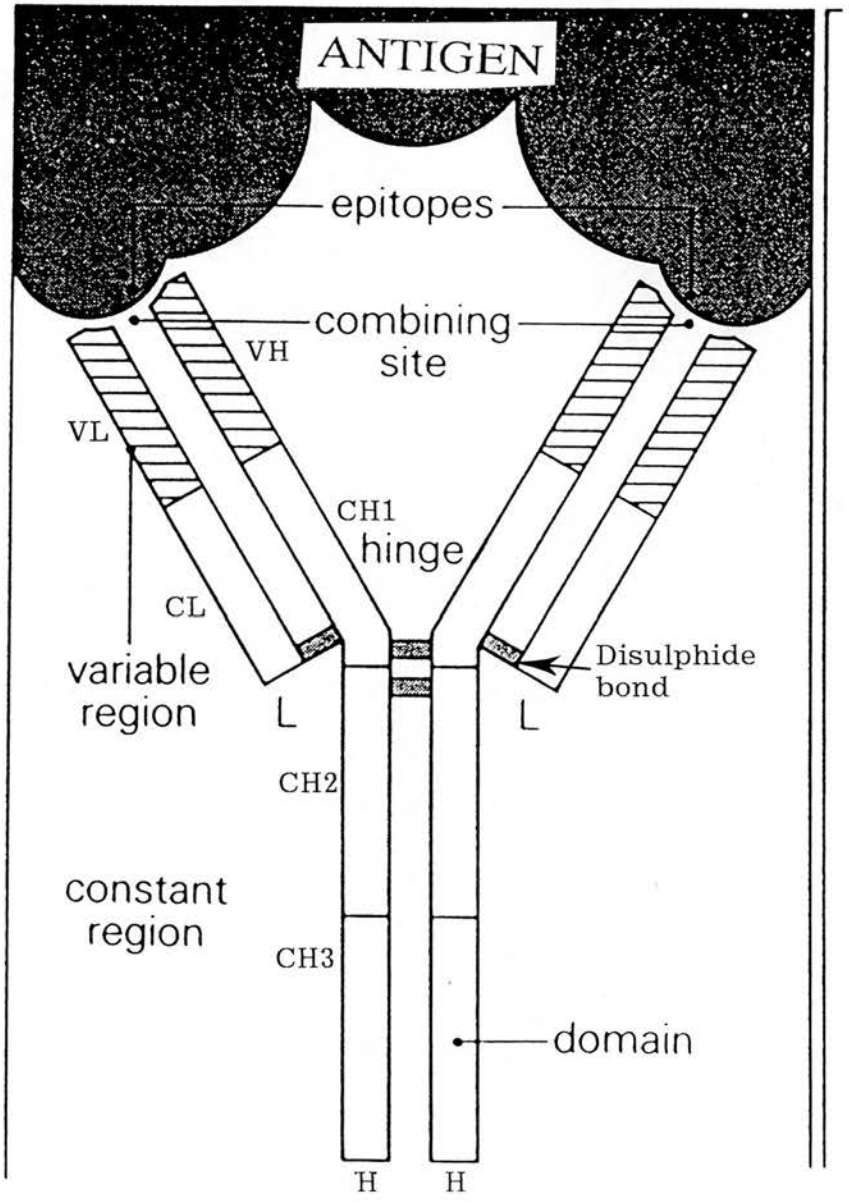
The immune system of the hosts plays a crucial role in the outcome of any parasite challenge, the success of which is influenced by the balance between the range of different immune defence mechanisms available to the host (Cabrera *et al.*, 1986). The mammalian immune system consists of two functional divisions; the innate immune system which acts as a non-specific first line of defence against infectious agents and the adaptive immune system which provides a specific reaction tailored to each infectious agent and usually results in the elimination of the agent (Roitt *et al.*, 1985). The adaptive immune system consists of two interrelated divisions, the cellular and humoral which are only mobilised when the first defences of the innate system are breached.

At the humoral level the pattern of the five main classes of immunoglobulins - IgG, IgM, IgA, IgD and IgE, elicited during the antibody response to infection is of great importance in determining the outcome of the parasite challenge (Shakib and Stanworth, 1980; Turner, 1977), as each immunoglobulin isotype can mediate a different range of biological functions such as adherence to specific defence cells or activation of the complement system (Almond and Parkhouse, 1986).

Structurally each immunoglobulin molecule consists of one or more basic units each containing four polypeptide chains - two identical high molecular weight or heavy (H) chains and two identical low molecular weight or light (L) chains. The light and heavy chains are joined by three interchain disulphide bonds into a Y-shaped molecule (Figure 4.1).

FIGURE 4.1

A schematic representation of an antibody molecule



Both light and heavy chains are further divided into a series of 12 similar subunits or domains, each domain consisting of approximately 110 amino acids. The N-terminal domain of the immunoglobulin molecule shows a marked degree of variation in its amino acid residue composition and is termed **variable** in contrast to the other more stable domains termed **constant**. Each light chain contains a single variable domain (VL) and a single constant domain (CL) while each heavy chain contains one variable region (VH) and three or four constant domains (CH_1, CH_2, CH_3 and CH_4) (Reeves, 1987). The different domains of the immunoglobulin molecule affect the biological functions of the various immunoglobulin classes and subclasses, with sites for complement system activation, and attachment of cells such as phagocytes and mast cells etc. present in the different domains (Bowry, 1984). The classification of the immunoglobulins being based on the antigenic differences between their heavy chains or Fc portion (Weir, 1983).

The ability of any antibody molecule to combine with a particular antigen is dependent on a region of the immunoglobulin molecule called the antigen binding site (Fab portion) which is formed from the variable portions of both of the heavy and light chains. The part of the immunoglobulin molecule made up of constant regions of the heavy chain (often referred to as the Fc portion from early crystallisation studies) determines the effect of such binding on the antigen. The Fc fragment of the different immunoglobulin classes differ antigenically and in their molecular weight and primary structure. After binding to an antigen has taken place via the Fab portion, the Fc fragment of the molecule binds to special Fc receptor sites present on many effector cells such as neutrophils, mononuclear phagocytes, eosinophils and B lymphocytes to activate the process that will bring about the elimination of the parasite. The exact nature of the effector process is dependent on the cell type that the

antigen-antibody complex has bound to via the Fc portion of the antibody molecule.

The most abundant immunoglobulin in the serum is IgG and it is evenly distributed between both the intra- and extravascular pools of the body. It is a monomeric protein with a molecular weight of 146,000 (Roitt *et al.*, 1985), which ensures that it can diffuse readily through the different membranes of the body, making it particularly suitable for dealing with fast-diffusing toxins and microbes localised in tissues. The affinity of the antigen-binding sites on any one IgG molecule is far greater for a particular antigen than that of other classes of immunoglobulins. Furthermore, the affinity of IgG produced in response to infection increases progressively during an immune response; in a later response it can be 10,000 times higher than in early responses (Bowry, 1984). IgG is also the main immunoglobulin class associated with secondary immune response. This probably arises from the fact that the effector cells responsible for the antibody response arising from a secondary immune response (B memory cells) are primed for producing mainly IgG (Roitt *et al.*, 1989). IgG, can be further divided into four subclasses - based on structural and antigenic differences in their heavy chains, IgG_1, IgG_2, IgG_3 and IgG_4. The differences in the heavy chains of the different subclasses are reflected in differences between their functional activities such as their ability to activate complement (Klaus *et al.*, 1979).

The other major serum immunoglobulin class is IgM. IgM is localised largely within the intravascular pool, and has a pentameric structure with a molecular weight of 970,000 (Roitt *et al.*, 1985). The pentameric structure of IgM improves upon its avidity, over that of other immunoglobulin classes. This arises from the fact that each IgM molecule has 10 antigen binding sites

in comparison with the two of each IgG molecule. The pentameric structure makes IgM especially effective in activating complement, handling of antigenically complex infectious agents and an efficient agglutinating and cytolytic agent (Steward, 1976; Roitt *et al.*, 1985).

Antibodies belonging to the other three main immunoglobulin classes are not as widespread as IgG or IgM and have a more limited and often local reactivity. The predominant immunoglobulin in seromucous secretion is IgA, although limited amounts of IgA is detectable in serum. IgA is secreted locally by plasma cells in the intestinal mucosa, the lacrimal gland, respiratory passages, salivary glands, sweat and mammary glands where it acts locally on infectious agents. IgA in external secretions is called secretory IgA (sIgA) and is a dimer joined by a J chain and contains an additional component, a protein called the secretory component - SC. This component is synthesized in the epithelial mucous membrane cells and is believed to protect dimeric IgA from proteolysis. Secretory IgA may be of either subclass of IgA (IgA1 and IgA2) and has a molecular weight of 385,000 (Roitt *et al.*, 1985). Most serum IgA in man occurs as the basic four chain monomer with a molecular weight of 150,000 daltons, while in most other mammals serum IgA is mainly polymeric, occurring mostly as a dimer.

IgA is efficient in the neutralisation of local toxins and activation of the complement system through the alternative pathway (Bowry, 1984). The Fc portion of IgA has a lower affinity than that of IgG for receptors in phagocytic cells, therefore, much of IgA's protective value may be due to its ability to combine directly with and neutralise pathogenic micro-organisms without necessarily involving any other effector system (Reeves, 1987).

Antibodies belonging to the IgD class of immunoglobulin are usually present in a low concentration constituting only about 1% of the total plasma immunoglobulin pool. They are however, present in large quantities on the membrane of circulating B lymphocytes, particularly early B cells in conjunction with IgM. Although the exact biological activity of IgD is unknown, it is believed to play an important role in antigen-triggered lymphocyte differentiation (Roitt *et al.*, 1985). IgD is not thought to mediate any of the usual effector functions attributed to immunoglobulins.

IgE, also known as 'reaginic' antibody because of its ability to fix to tissue cells so that, on reaction with antigen, histamine and other vasoactive agents are released. IgE is the least abundant of the immunoglobulins and it is usually found firmly fixed on the surfaces of mast cells and basophils via its Fc receptors. Although IgE is the least plentiful of all serum immunoglobulins, its presence is often dramatically felt when their consequential binding to specific antigens causes mast cells and basophils to release powerful inflammatory mediators such as histamines (Reeves, 1987). IgE is particularly important in the development of active immunity against helminthic parasites and the implementation of immediate hypersensitivity reaction seen in conditions such as asthma and hay-fever.

Antibodies belonging to any of the five main immunoglobulin classes could be produced by the host during the course of any infection. Antibody belonging to particular classes lacking the necessary properties to destroy parasites such as trypanosomes via complement or cell-mediated mechanisms may act as blocking antibodies which saturate antibody binding sites on the parasite thus preventing the action of protective immunoglobulins (Cabrera *et al.*, 1986). The effective control of parasite infection therefore depends on a

delicate balance between the different immunoglobulin classes during the course of infection.

There is increasing evidence that the immunoglobulin isotype produced in response to certain parasite components greatly influences the way in which the host deals with an infection (Almond and Parkhouse, 1986). This arises from a combination of the fact that some parasite components may be more antigenic than others and induce a better protective immune response from the host than other components and also that certain immunoglobulin classes perform some effector functions better than others, e.g. IgM is a more efficient complement activating antibody than IgG.

In the case of trypanosomes, the antibody response to the surface is far greater than that to the less immunogenic internal components (Le Ray, 1975). Furthermore, animals that generate an IgM antibodies response against the surface coat of the parasite would probably be better protected against the effect of the infection than those that generated a predominantly IgG response because IgM is classically considered to be more effective than IgG in the activation of complement, which is one of the principal ways by which trypanosomes are considered to be eliminated by the host (Wellde *et al.*, 1975, 1981; Murray and Urquhart, 1977; Emery *et al.*, 1980; Morrison *et al.*, 1982).

Studies on antibody response to trypanosome infection have established that infected animals produce antibodies against both the surface antigens and also the non-surface or common antigens of the parasite (Raadt, 1974; Le Ray, 1975). Antibody responses to the surface antigens play a vital role in the elimination of parasites and protection against rechallenges with homologous trypanosomes (Wellde *et al.*, 1975, 1981; Emery *et al.*, 1980; Morrison *et al.*, 1982). Differences have been reported between the immunoglobulin isotype

response in animals infected with trypanosomes by several workers, which are possibly linked to the assay systems used. Investigations based on agglutination testing reported that the primary response of rabbits to *T. b. gambiense* and rats or cattle to *T. b. brucei* infection was predominantly IgM (Seed *et al.*, 1969; Zahalsky and Weinberg, 1976). Conversely, other researchers reported that the primary response of mice and cattle to *T. b. brucei* or mice to *T. b. rhodesiense* was predominantly IgG. In these latter studies, Nantulya *et al.* (1979) used a radioimmunoassay with a purified VSG preparation as antigen and reported predominant IgG production, similar results to those of Campbell *et al.* (1978), Sacks and Askonas (1980) and Mansfield *et al.* (1981) who used VSG specific fluorescent antibody staining method. These apparently conflicting results on immunoglobulin class involvement could be due to the techniques used for the assay of antibodies. IgM is a more efficient agglutinating antibody than IgG, therefore it is likely that the agglutination test would preferentially detect IgM at the expense of IgG.

Other studies on the effect of trypanosome infection on the immune system have shown that in addition to the production of specific antibodies against parasite antigens, host B lymphocytes can be activated in a non-specific manner which results in the production of large amounts of non-specific immunoglobulins (Houba, 1969; Hudson *et al.*, 1976; Terry *et al.*, 1980) probably due to the action of trypanosome mitogens (Urquhart *et al.*, 1973; Esuruso, 1976; Assoku and Tizard, 1978). Much of these non-specific immunoglobulins produced by polyclonal activation of B cells are considered to belong to the IgM class of immunoglobulins (Hudson *et al.*, 1976; Terry *et al.*, 1980). The presence of these non-specific immunoglobulins often complicate the interpretation of results of host/parasite interaction observed in

immunological assays and can affect the overall understanding of the specific response to the parasite.

The variant surface glycoproteins of the trypanosome have attracted most attention in studies on the immunobiology of trypanosomes probably due to its high degree of immunogenicity (Le Ray, 1975) and also its role in protecting animals against reinfections (Wellde *et al.*, 1975, 1981; Emery *et al.*, 1980; Morrison *et al.*, 1982; Hall and Esser, 1984). This preoccupation of researchers with the host's response to the surface antigens of trypanosome could have been to the detriment of responses to the non-surface components of the parasites. It is likely that the technical problems of differentiating between responses to both surface and non-surface components of trypanosomes has also contributed to the fact that most studies have been restricted to the surface component. The VSG specific response by the host probably serves only to bring about the lysis of each antigenically different population of trypanosome. The way in which the host deals with the consequential release of internal antigen is largely unknown which could form an equally important part of the response to infection.

Although some studies on the range of immunoglobulin isotypes produced in response to trypanosome infection have been conducted by several workers, all of the studies have considered the parasite as a single antigenic entity, not as a collection of different antigenic components which in reality it is. Such a single antigen concept is probably only valid during the first encounter between host and parasite, particularly in the case of trypanosomes with their limited surface antigen composition. Once the host has initiated immune-mediated parasite destruction, a range of individual parasite components will become available to the host. It is possible that the type of host response to such

individual components might be important in influencing the final outcome of an infection.

Few limited studies have been undertaken on the class-specific antibody response in animals infected with *T. evansi* (Luckins *et al.*, 1978; Luckins *et al.*, 1979). These have employed techniques such as the single radial diffusion test to measure total IgM/IgG or ELISA and IFAT neither of which can associate changes in immunoglobulin levels with response to specific parasite antigens. Results obtained from these studies which evaluated only IgG and IgM levels in the infected animals showed that IgM levels were higher than IgG during the period of primary infection.

The current study is therefore designed to evaluate the nature of class-specific antibody responses in animals infected with *T. evansi*. The individual components of the parasite which have been separated by electrophoresis have been probed for their antigenicity using serum samples collected from animals at different times after infection.

MATERIALS AND METHODS

Trypanosomes

T. evansi TREU 2113 described in Chapter 2 was used for infection of all the rabbits and for the preparation of the soluble antigens used throughout this study.

Rabbit Infection

Trypanosome populations obtained from stabilate material were injected into mice. When fulminating levels of parasites were obtained, animals were exsanguinated under ether anaesthesia and about 1 ml of blood collected into

disposable syringes containing 50 units of heparin via a cardiac puncture. The parasites were then separated from the host's blood cells with anion-exchange chromatography using DEAE cellulose (DE52 Whatman Biochemicals) as described by Lanham and Godfrey (1970) and used to infect the rabbits. Eight adult New Zealand White rabbits were each infected through the ear vein with about 4 x 10^5 motile trypanosomes, suspended in ice-cold phosphate saline glucose (PSG) pH 8.0 (Appendix 2). Twenty-eight days after infection each rabbit was injected with 7 mg (active principle) of diaminazene aceturate (Berenil^R, Hoechst) intramuscularly (i/m) per kg body wt.

Collection of Serum Samples from T. evansi Infected Rabbits

Blood for the preparation of serum was collected from each rabbit before infection and on days 7, 10, 14, 17, 21, 24 and 28 after infection. Blood was also collected from each rabbit on days 7, 14, 18, 21, 28, 33 and 39 after drug treatment. Serum was prepared from these blood samples as described in Chapter 2. On each occasion that blood was collected for serum, blood samples were also collected into tubes containing EDTA (Sigma Ltd.) for the determination of packed cell volume (PCV) by the microhaematocrit method (Schalm, 1975) and for the estimation of parasitaemia initially by wet mount and subsequently by the dark ground/phase contrast buffy coat scoring system described by Paris *et al.* (1982).

Clinical Signs in Rabbits Infected with T. evansi

The body weight in kg and rectal temperature in ^oC of each rabbit was recorded immediately before each blood collection.

Separation of Soluble T. evansi Antigens by SDS-PAGE

A soluble antigen preparation of *T. evansi* TREU 2113 was prepared by freeze-thaw according to the protocol described in Chapter 2. The resulting extract was then subjected to SDS-PAGE on a 7 to 20% gradient polyacrylamide gel as described in Chapter 2. Ninety μg of the trypanosome soluble protein were loaded into each well cast in the stacking gel with one well of each gel receiving a mixture of proteins of known molecular weight (molecular weight markers, Pharmacia).

After electrophoresis, the separated trypanosome components were electrophoretically transferred to nitrocellulose membrane (Anderman and Co., U.K.) as described in the final protocol described in Chapter 2.

DETECTION OF ANTIGENIC COMPONENTS OF T. EVANSI

After the transfer, a section of the nitrocellulose membrane containing the molecular weight markers and at least one lane of the *T. evansi* soluble antigens was cut off and stained with 1% amido black (Sigma Ltd.) as described in Chapter 2. This stained portion of the blot was subsequently used for the construction of a standard curve based on the migration of the individual proteins making up the molecular weight markers. The molecular weight of individual components of *T. evansi* were calculated by interpolation from this curve.

Antigenic components in the remaining part of the nitrocellulose paper were detected using serum collected from rabbits at different times after infection and drug treatment using the final protocol described in Chapter 2 but modified to detect class-specific antibody. This modification involved the addition of an extra processing step in which a second antiserum preparation

raised in goats to an appropriate rabbit immunoglobulin isotype (Nordic^R) was used to probe any *T. evansi* antigen/antibody complex arising from the incubation of the blot with post-infection/post-treatment rabbit serum (Ag/Ab). A third antiserum raised in rabbits against goat IgG and conjugated to peroxidase (Nordic^R) followed by incubation with substrate solution was used to visualise the Ag/Ab/anti-Ig isotype complex.

In order to produce optimum antigen detection it was necessary to tailor reaction conditions according to the immunoglobulin isotype under study. Consequently a range of different serum concentrations was used. The details of the particular dilutions of the serum and antisera used in the assay are summarised in Table 4.1.

Table 4.1 Sera and antisera dilutions used for the detection of T. evansi antigens.

Immunoglobulin (Ig) class	Rabbit serum dilution (First antibody)	Goat anti-rabbit antisera dilution (Second antibody) Anti-IgG (Fc), IgM (Fc) and IgA (Fc)	Rabbit anti-goat antisera dilution (Third antibody)
IgG	1:200	1:400	1:2000
IgM	1:100	1:300	1:2000
IgA	1:100	1:300	1:2000

Incubation of the blots in the second and third antibody solutions (Nordic^{R}) were for two hours and one hour respectively.

Controls

The following controls were incorporated into every "Western" blotting procedure used for the detection of antigens of *T. evansi* in this study.

(a) Any non-specific binding of the second and third antibodies to *T. evansi* antigens was monitored by incubating of the separated *T. evansi* proteins after transfer onto nitrocellulose paper directly with the affinity purified antigen (2nd and 3rd antibodies) and substrate in the absence of any test or normal rabbit serum.

(b) Any non-specific binding of the substrate solution to the product of *T. evansi* soluble extract/rabbit serum reactions was monitored by the incubation of the separated *T. evansi* proteins bound onto nitrocellulose with a pooled post-infection rabbit serum sample and the substrate solution in the absence of the second and third antibodies respectively.

(c) Any non-specific binding of the serum protein in rabbit serum to *T. evansi* components was monitored by the incubation of the separated *T. evansi* proteins after transfer onto nitrocellulose with normal rabbit serum collected from uninfected animals, and then sequentially with the second, third antibodies and the substrate solution.

RESULTS

Total Protein Profile of T. evansi soluble Extract

At least 30 individual protein components with molecular weights ranging from 123,800 to about 13,600 daltons were identified in the supernate fraction of the trypanosome lysate used as soluble antigens after separation on SDS-PAGE and staining with Coomassie Brilliant Blue (Sigma Ltd.) (Figure 4.2).

FIGURE 4.2

Total protein profile of *T. evansi* soluble extract (supernate fraction of non-detergent lysate) separated on SDS-PAGE and stained with Coomassie Blue.

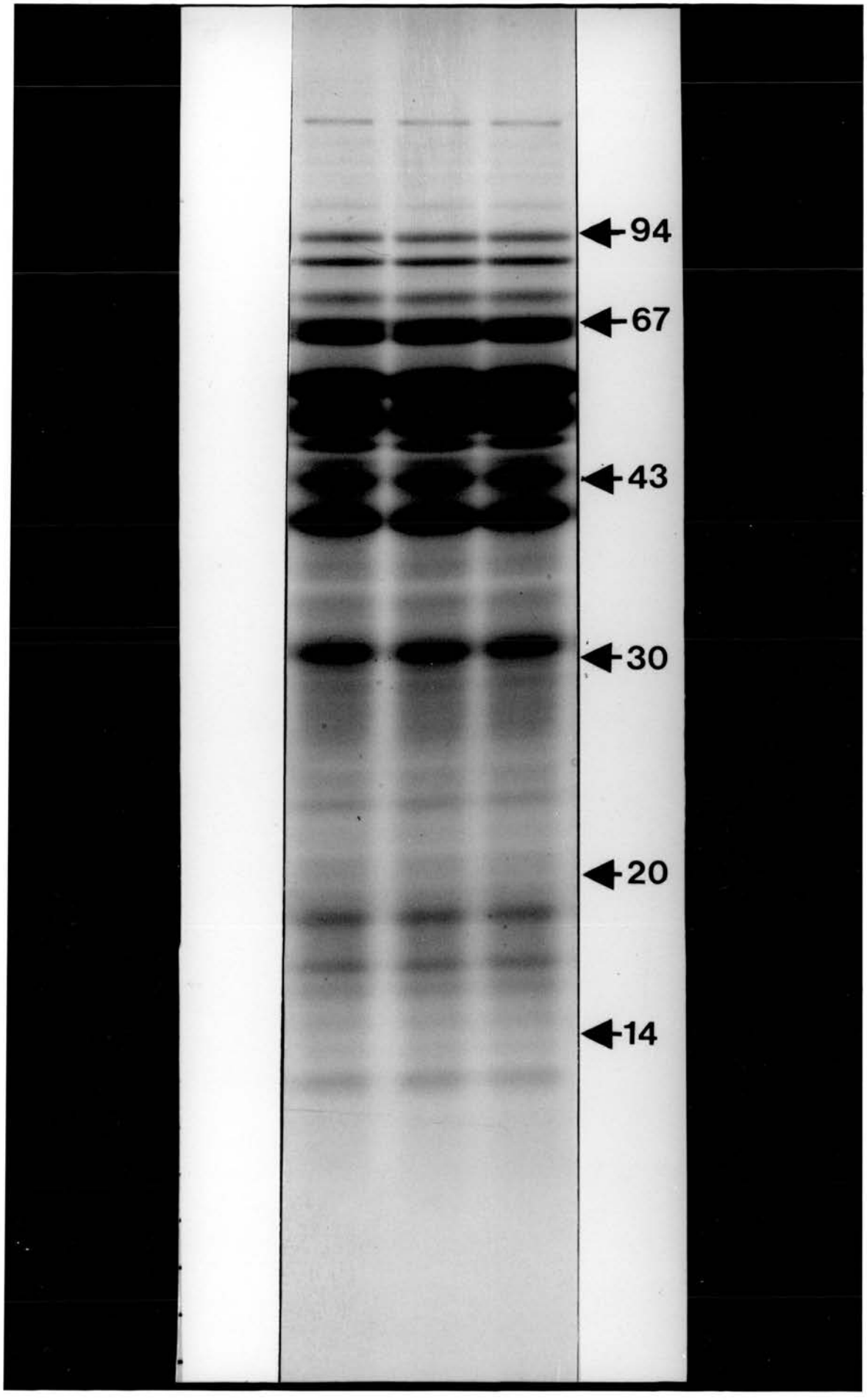

94
67
43
30
20
14

FIGURE 6.3

Skin sections prepared from tissue collected 2 hours after ANTIGEN 4 was injected intradermally into *T. evansi* infected and Berenil treated rabbit.

(A: shows clusters of polymorphonuclear leucocytes, H & E x 150; B: shows blood vessel clogged with polymorphonuclear leucocytes and erythrocytes predominantly with one to two mononuclear cells, H & E x 1,500.

T. evansi Antigens Identified by IgG Antibodies using Anti-IgG (Fc) as the Second Antibody

IgG antibodies to *T. evansi* components were first detected in the serum of rabbits seven days after infection. Although the number of antigen components labelled differed between individual rabbits, IgG antibody to two components with the molecular weight, 67,000 and 60,500 were usually labelled first either individually or together by serum collected from most of the rabbits. The 60,500 dalton component was usually labelled by serum collected early in infection (Figure 4.3, Lane 2).

Serum collected during the course of infection recognised progressively more trypanosome components, with the staining of individual bands becoming more intense as the infection progressed. Between 7 and 10 days after infection between 2 and 7 components were labelled, with serum from most rabbits recognising components with the molecular weights of 67,000, 42,000 and 32,400. By 28 days after infection the number of trypanosome components recognised increased to between 16 and 25 (Figure 4.3, Lane 8). The molecular weights of the *T. evansi* components recognised by IgG antibodies in the serum of rabbits during the period of primary infection ranged from about 94,000 to 16,500 daltons. Of these components, eight major polypeptides with molecular weights of 94,000, 85,000, 75,500, 67,000, 60,500, 43,000, 32,400 and 28,600 daltons respectively were identified by serum collected from seven of the eight rabbits up to 28 days after infection (Figure 4.3, Lanes 2 to 8). Of these eight major components, three components with molecular weights of 94,000, 67,000 and 60,500 were recognised by all eight rabbits at some time during the first 28 days of infection.

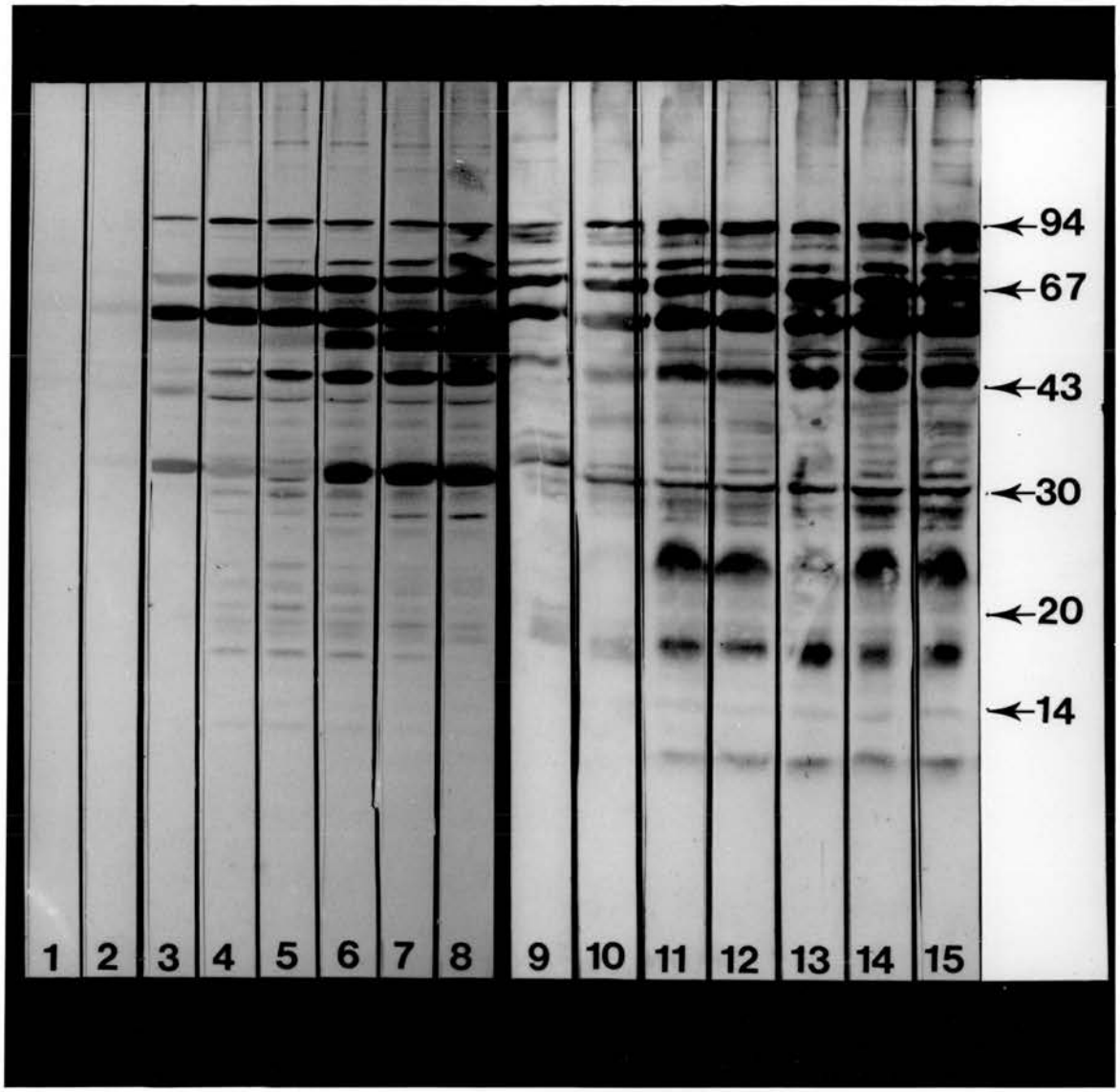
FIGURE 4.3

T. evansi antigens identified by IgG antibodies using anti-IgG (Fc) as the second antibody.

Number 1 *T. evansi* proteins probed with normal rabbit serum.

Numbers 2-8 *T. evansi* proteins probed with days 7, 10, 14, 17, 21, 24 and 28 post infection serum respectively.

Numbers 9-15 *T. evansi* protein probed with days 7, 14, 18, 21, 28, 33 and 39 post treatment serum respectively.


94
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15

The number of trypanosome components recognised by serum collected after the rabbits were treated with Berenil differed between individual animals. In general, more antigen components were identified by the IgG antibodies after drug treatment (Figure 4.3, Lanes 9 to 15) than during the post-infection period (Figure 4.3, Lanes 2 to 8).

Seven to 14 days after drug treatment there was an initial reduction in the number of antigen components recognised by the IgG antibodies (Figure 4.3, Lanes 9 and 10) when compared with the number of components recognised by serum collected 28 days after infection (Figure 4.3, Lanes 4 to 8). Serum collected seven days after drug treatment only recognised 10-12 components (Figure 4.3, Lane 9), while that collected 28 days post-infection recognised about 20 components (Figure 4.3, Lane 8) Thereafter, progressively more antigen components were labelled by serum collected after drug treatment. By 18 days after drug treatment, most of the antigen components identified during primary infection were again detected on the blot after drug treatment. Antibodies to the 32,400 dalton component disappeared while antibodies to three components of low molecular weights (25,400, 19,500 and 12,500) were detected in large amounts (Figure 4.3, Lanes 11 to 15), antibodies to which were not detected during the period of primary infection.

T. evansi Antigens Identified by IgM Antibodies using Anti-IgM (Fc) as the Second Antibody

Fewer trypanosome components were identified as antigenic by IgM antibodies in serum collected from all the animals during the post-infection period than were identified by IgG antibodies during the equivalent period. Differences, however, were seen between individual rabbits in the time taken

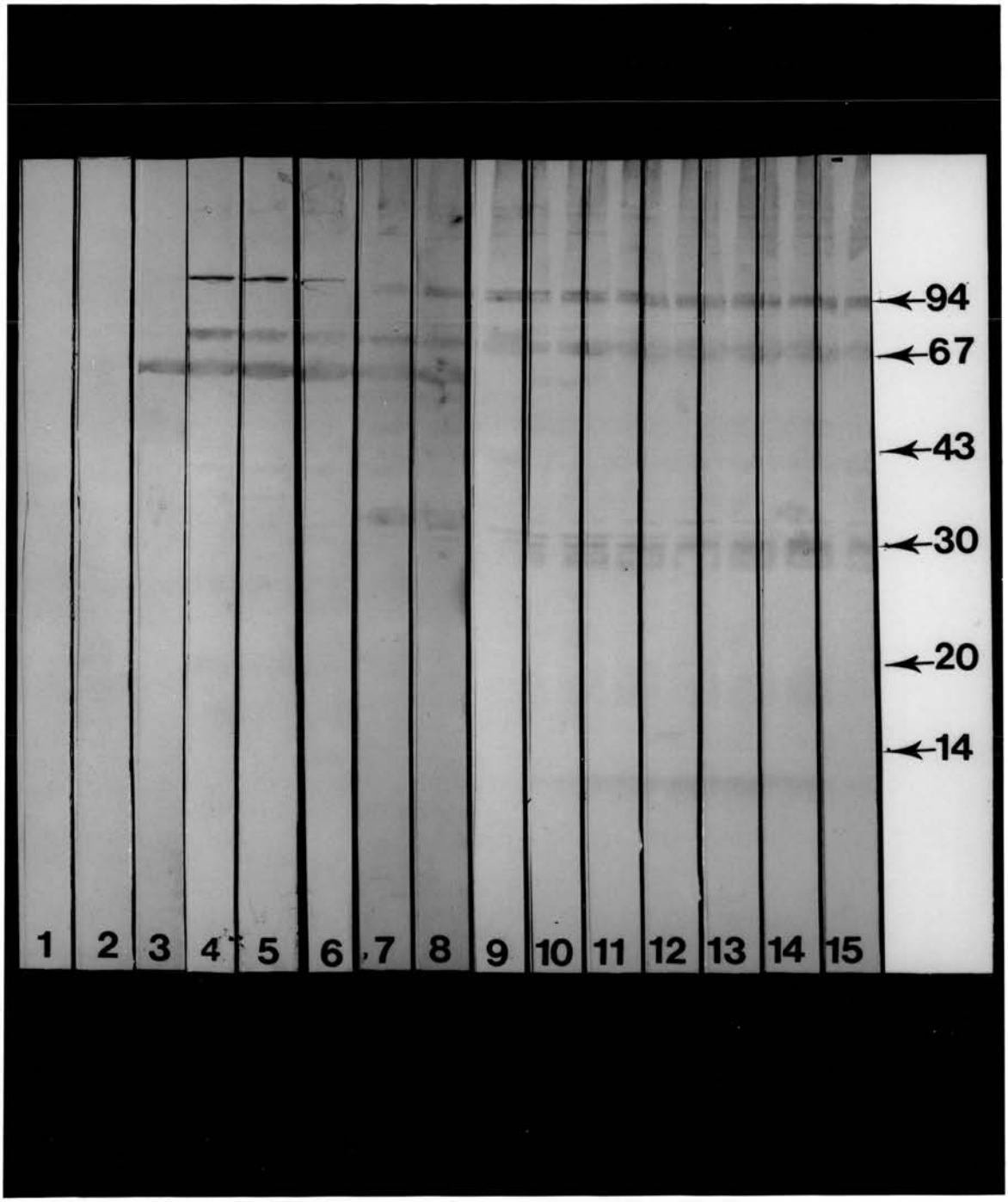
for trypanosome-specific IgM antibodies to appear. Five out of the eight rabbits recognised a single protein band 10 days after infection of either 60,500 or 67,000 daltons, while serum from three rabbits recognised the 67,000 dalton band as early as seven days after infection. By 14 days after infection however, all eight rabbits had produced IgM antibodies that recognised three major trypanosome components of molecular weight 94,000, 67,000 and 60,500 (Figure 4.4, Lanes 4 and 5). At 21 days after infection there was a reduction in both the number of antigenic components identified by the IgM antibodies and in the intensity of labelling of individual components by serum from all the rabbits (Figure 4.4, Lane 6).

Only one trypanosome components out of the three, recognised during the period of primary infection (Figure 4.4, Lanes 2 to 8) was identified by serum collected 7 days after treatment (Figure 4.4, Lanes 9 and 10) in all the rabbits. Two of the three antigen components (94,000 and 67,000) recognised by IgM antibodies during the primary infection period (Figure 4.4, Lanes 2 to 8) were labelled by the serum collected during the post-treatment period (Figure 4.4, Lanes 9 to 15). Serum from one rabbit, however recognised all the three antigen components (94,000, 67,000 and 60,500) that had been detected during the primary infection after drug treatment. Antigen components with molecular weights of 30,000, 28,600 and 12,500 were recognised by post-treatment IgM antibodies in addition to those recognised by serum collected during the post-infection period in all the rabbits.

FIGURE 4.4

T. evansi antigens identified by IgM antibodies using anti-IgM (Fc) as the second antibody.

Number 1	*T. evansi* proteins probed with normal rabbit serum.
Numbers 2-8	*T. evansi* proteins probed with days 7, 10, 14, 17, 21, 24 and 28 post infection serum respectively.
Numbers 9-15	*T. evansi* proteins probed with days 7, 14, 18, 21, 28, 33 and 39 post treatment serum respectively.


94
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T. evansi Antigens Identified by IgA Antibodies using Anti-IgA (Fc) as the Second Antibody

The pattern of *T. evansi* antigens identified by the IgA antibodies in the serum collected from the rabbits during the period of primary infection was similar to that recognised by the IgM antibodies with respect to the molecular weight range. Differences in the pattern of antigen recognition were however seen between individual rabbits. IgA antibodies in serum collected from some rabbits detected a single 67,000 dalton trypanosome component seven days after infection while others did not recognise any component until 14 days after infection. IgA from the majority of the eight rabbits however, recognised one or more trypanosome component by 10 days after infection (Figure 4.5, Lane 2). Unlike the IgM antibodies, the antigen components once identified by IgA, remained so all throughout the period of primary infection.

The major difference between the IgA antibody profile of serum collected post-infection and that collected post-treatment was the loss of antibodies to 60,500 trypanosome component in all the rabbits. Serum from all the rabbits continued to recognise the 94,000 dalton antigen component while the 67,000 component tended to be faintly and inconsistently recognised during the period. Two lower molecular weight components - 28,600 and 12,500 daltons which were not recognised by the animals during the primary infection were recognised by the IgA antibodies produced by the animals after drug treatment (Figure 4.5, Lanes 9 to 15).

The pattern of the different immunoglobulin isotypes produced by the animals and the extent of their recognition of selected *T. evansi* components during the period of primary infection and after drug treatment are summarised in Table 4.2.

FIGURE 4.5

T. evansi antigens identified by IgA antibodies using anti-IgA (Fc) as the second antibody.

Number 1	*T. evansi* proteins probed with normal rabbit serum.
Numbers 2-8	*T. evansi* proteins probed with days 7, 10, 14, 17, 21, 24 and 28 post infection serum respectively.
Numbers 9-15	*T. evansi* proteins probed with days 7, 14, 18, 21, 28, 33 and 39 post treatment serum respectively.

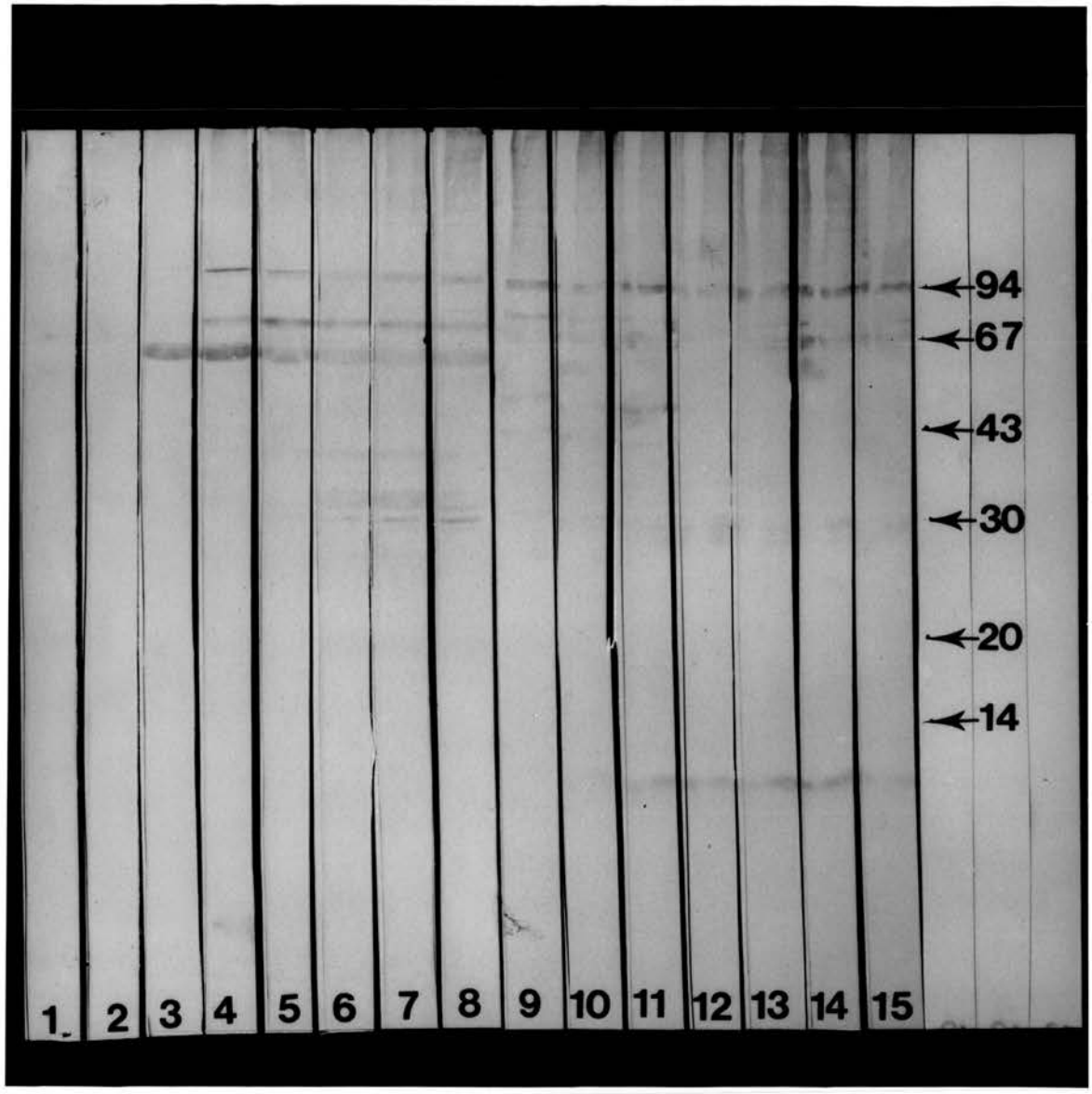
94
67
43
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Table 4.2 T. evansi major antigen components identified by the Ig isotypes produced by the rabbits during the period of primary infection and after drug treatment.

Component (KD)	Ig isotype	Days post-infection								Days post-treatment						
		0	7	10	14	17	21	24	28	7	14	18	21	28	33	39
94	IgG	-	-	+	++	++	++	++	++	+	+	+++	+++	+++	+++	+++
	IgM	-	-	-	++	++	+	+	+	+	+	++	++	++	++	++
	IgA	-	-	-	++	+	+	+	+	+	+	+	+	+	+	+
85	IgG	-	-	+	+	+	+	-	-	+	+	++	++	++	++	++
	IgM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IgA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
75.5	IgG	-	-	-	+	++	++	++	++	+	+	++	++	++	++	++
	IgM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IgA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
67	IgG	±	++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
	IgM	-	±	++	++	++	+	+	+	±	+	+	+	+	+	+
	IgA	-	±	+	++	+	+	+	+	±	+	+	+	+	+	+
56	IgG	-	-	-	-	+	+++	+++	+++	+	+	+	++	++	++	++
	IgM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IgA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	IgG	-	-	-	+	++	++	++	++	+	+	++	++	++	++	++
	IgM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IgA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32.4	IgG	-	-	++	+	+	++	++	++	-	-	-	-	-	-	-
	IgM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IgA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	IgG	-	-	+	+	++	++	++	++	+	+	++	++	++	++	++
	IgM	-	-	-	-	-	-	-	-	±	+	+	+	+	+	+
	IgA	-	-	-	-	-	-	-	-	±	±	±	+	+	+	+
28.6	IgG	-	-	+	+	+	+	+	+	±	+	+	+	+	++	++
	IgM	-	-	-	-	-	-	-	-	-	-	±	+	+	+	+
	IgA	-	-	-	-	-	-	-	-	-	-	±	+	+	+	+
25.4	IgG	-	-	+	+	+	+	+	+	±	+	+	+	+	++	++
	IgM	-	-	-	-	-	-	-	-	-	±	+	+	+	+	+
	IgA	-	-	-	-	-	-	-	-	-	-	±	+	+	+	+
19.5	IgG	-	-	-	-	-	-	-	-	-	±	+++	+++	+++	+++	+++
	IgM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IgA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.5	IgG	-	-	-	-	-	-	-	-	-	-	++	++	++	++	++
	IgM	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
	IgA	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+

-: Not stained; ±: Stained faintly or not stained by serum of some animals
+: Faintly stained; ++: Intensely stained; +++: Very intensely stained

* see P.132B

Table 4.2 T. evansi major component identified by the Ig isotypes produced by the rabbits during the period of primary infection and after drug treatment.

(Omitted from data on page 132)

Component (KD)	Ig Isotype	Days post-infection								Days post-treatment						
		0	7	10	14	17	21	24	28	7	14	18	21	28	33	39
60.5	IgG	-	+	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+++	+++	+++
	IgM	-	-	++	++	++	+	+	+	-	-	-	-	-	-	-
	IgA	-	-	++	++	++	+	+	+	-	-	-	-	-	-	-

Variations between Individual Rabbits with Respect to Antibody Production to T. evansi Antigens

The eight rabbits included in the present study were divided into two groups based on the number of antigenic components labelled by the three immunoglobulin isotypes present in their serum during the course of infection, and the time antibodies to the antigens first appeared.

One group of three rabbits recognised many trypanosome components as antigens in particular the 67,000 and 60,500 components along with more than 20 other components ranging in molecular weight from 94,000 to about 19,500 daltons for antibodies of the IgG class. Antibodies belonging to IgM and IgA classes also recognised the 67,000 and 60,500 components along with the 94,000 and 30,000 dalton components in addition to one or two other smaller components with a molecular weight of less than 30,000 which were faintly labelled. Furthermore, this group of rabbits recognised the antigen components early, at 7 and 10 days after infection and antibody to these components was maintained throughout the period of observation until the infection was terminated by chemotherapy. Figure 4.6 represents a typical series of blots from one of the rabbits in this group illustrating these points.

A second group of five rabbits recognised fewer trypanosome components as antigens than the animals in the first group. Furthermore, they did not always develop antibodies belonging to all three immunoglobulin classes recognising the two components with a molecular weight of 67,000 and 60,500 daltons. Limited recognition of these two components was most pronounced in the case of the IgM and IgA classes. IgG from the serum of the rabbits in this group did not recognise more than eight antigen components in total at any time during the period of observation. Fewer of the components also were

recognised by the IgM and IgA antibodies produced by these animals than were recognised by the animals in the first group. As well as failing to recognise many antigen components early in infection, serum from this second group lost the ability to recognise many of ithe trypanosome components as the infection progressed. This loss of antigen recognition was common to the three classes, IgG, IgM and IgA, of antibodies. Figure 4.7 represents a typical series of blots from one of the rabbits in this group illustrating these points.

Clinical Signs in Animals Infected with T. evansi

Mean packed cell volume readings of 40.33%± 3 with a range of 37 to 42% were recorded before all the animals were infected. After infection, PCV readings decreased progressively with increasing duration of infection with readings from individual animals reaching a minimum value of 22% before drug treatment. In three rabbits an increase in the PCV reading was observed seven days after infection. Thereafter, the PCV value dropped and continued to decrease until a mean pre-infection value was recorded just before and after the infection was terminated by chemotherapy.

Parasites were first detected in the blood of these rabbits 10-14 days after infection. Thereafter, parasite counts reduced until the infection was eliminated with chemotherapy (Figure 4.8A, C and E). Although there was a reduction in the body weight of all the animals between 7-10 days after infection and thereafter, fluctuations in body weight in these three rabbits were within a narrow range (Figure 4.8C). An increase in rectal temperature that coincided with the appearance of parasites in blood observed in all the animals was followed by fluctuations of rectal temperature readings within a narrow range in each of these three rabbits (Figure 4.8E).

FIGURE 4.6

T. evansi antigens identified by IgG, IgM and IgA antibodies produced by a resistant rabbit during the period of primary infection.

0 *T. evansi* proteins probed with normal rabbits serum.

1-7 *T. evansi* proteins probed with days 7, 10, 14, 17, 21, 24 and 28 post infection serum respectively for the activities of the three immunoglobulin isotypes.

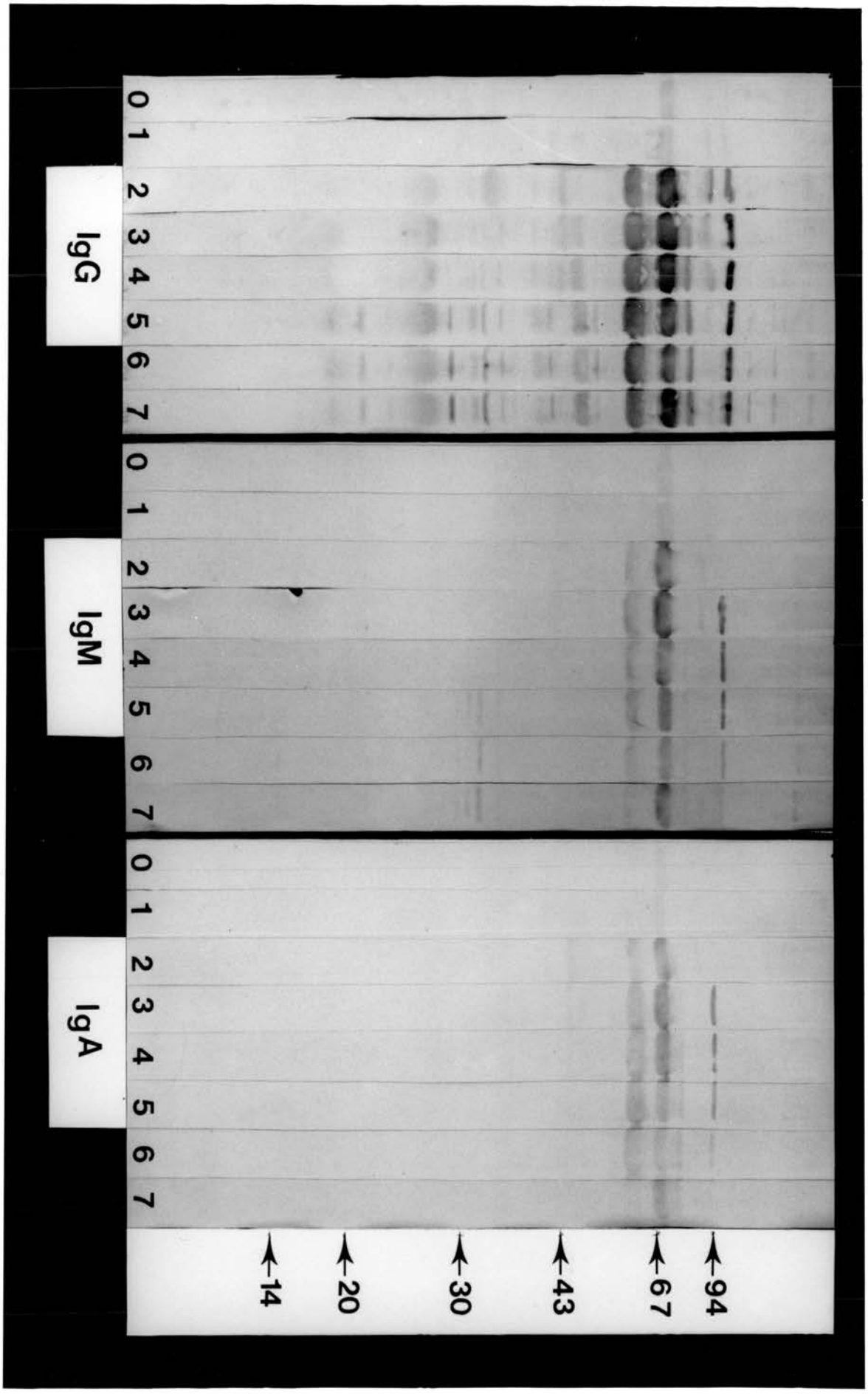

IgG
IgM
IgA
0
1
2
3
4
5
6
7
94
67
43
30
20
14

FIGURE 4.7

T. evansi antigens identified by IgG, IgM and IgA antibodies produced by a susceptible rabbit during the period of primary infections.

0 *T. evansi* proteins probed with normal rabbit serum.

1-7 *T. evansi* proteins probed with days 7, 10, 14, 17, 21, 24 and 28 post infection serum respectively for the activities of the three immunoglobulin isotypes.

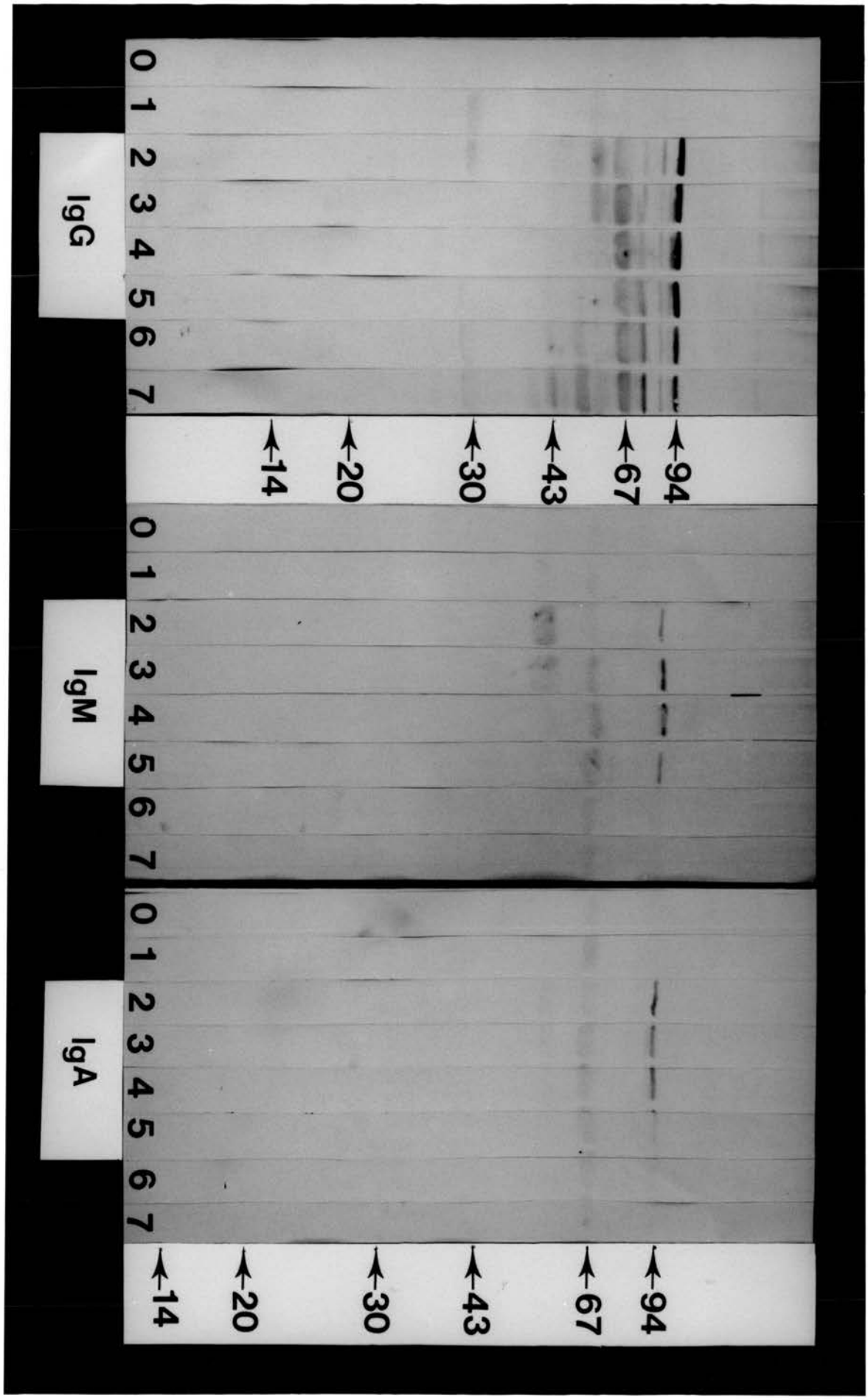

IgG
IgM
IgA
0 1 2 3 4 5 6 7
94
67
43
30
20
14

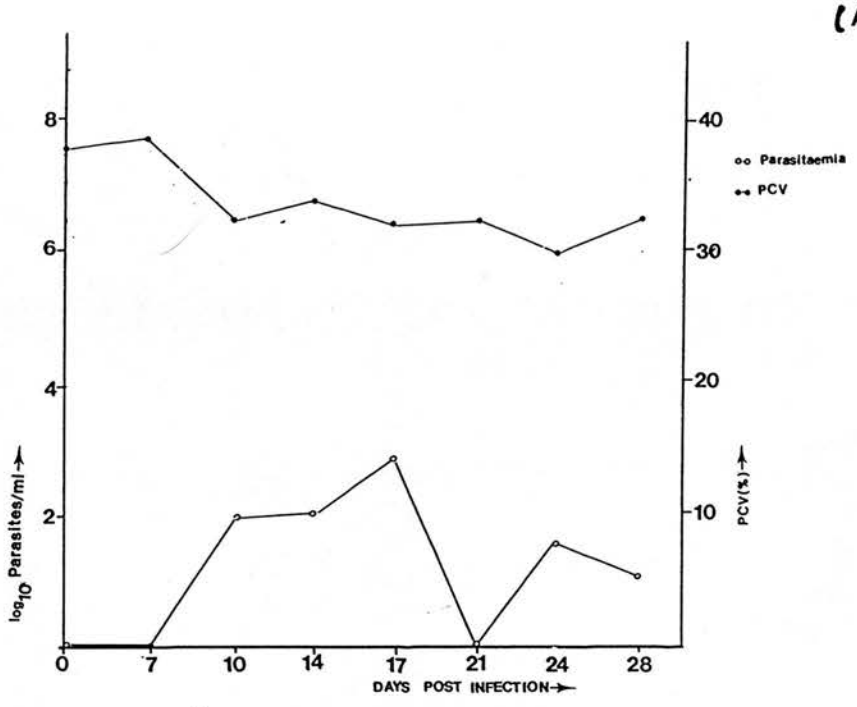
FIGURE 4.8

Clinical signs in animals infected with *T. evansi*.

A Variations of packed cell volume (PCV) with parasitaemia in a resistant rabbit.

B Variations of PCV with parasitaemia in a susceptible rabbit.

(A)


8
6
4
2
40
30
20
10
oo Parasitaemia
•• PCV
log10 Parasites/ml
PCV(%)
0
7
10
14
17
21
24
28
DAYS POST INFECTION

(B)

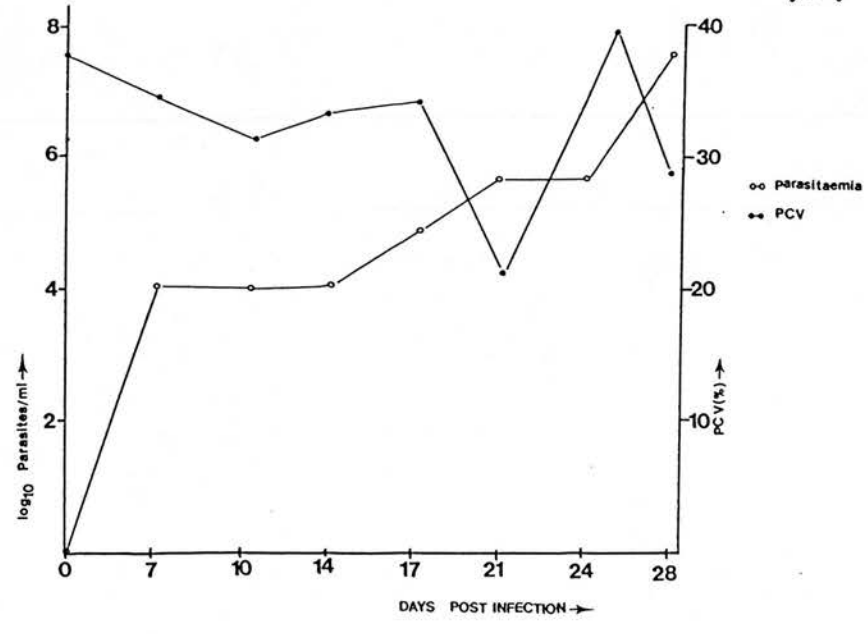

8
6
4
2
40
30
20
10
oo parasitaemia
•• PCV
log10 Parasites/ml
PCV(%)
0
7
10
14
17
21
24
28
DAYS POST INFECTION

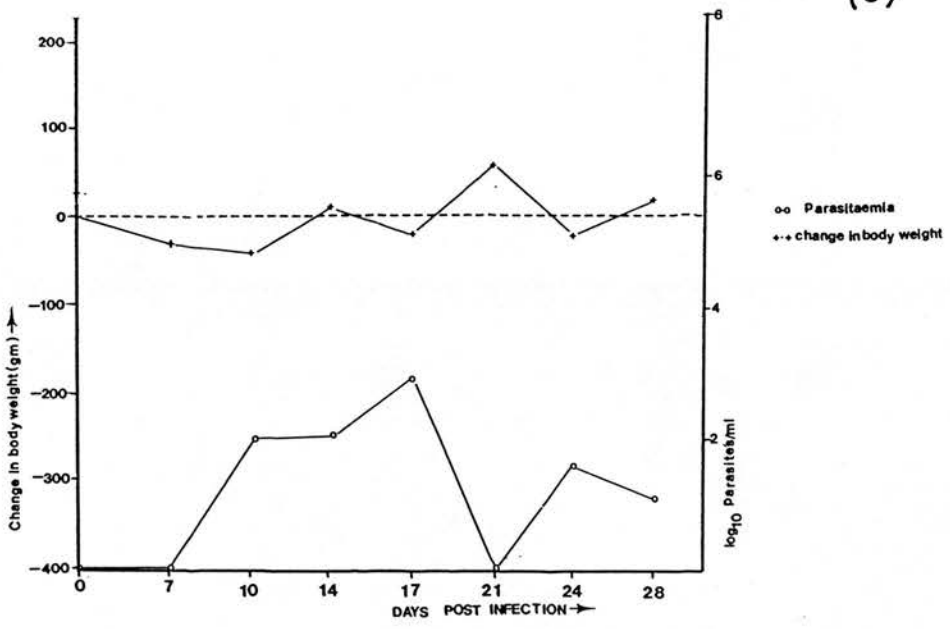
FIGURE 4.8

Clinical signs in animals infected with *T. evansi*.

C Variations in change in body weight gain with parasitaemia in a resistant rabbit.

D Variations in change in body weight gain with parasitaemia in a susceptible rabbit.

(C)


200
100
0
-100
-200
-300
-400
Change in body weight (gm)
0
7
10
14
17
21
24
28
DAYS POST INFECTION
8
6
4
2
log10 Parasites/ml
oo Parasitaemia
+-+ change in body weight

(D)

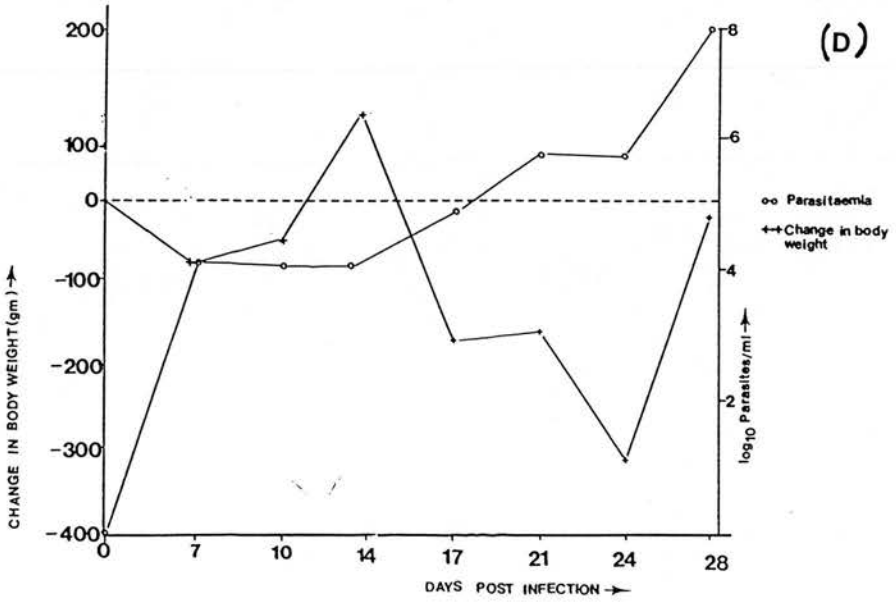
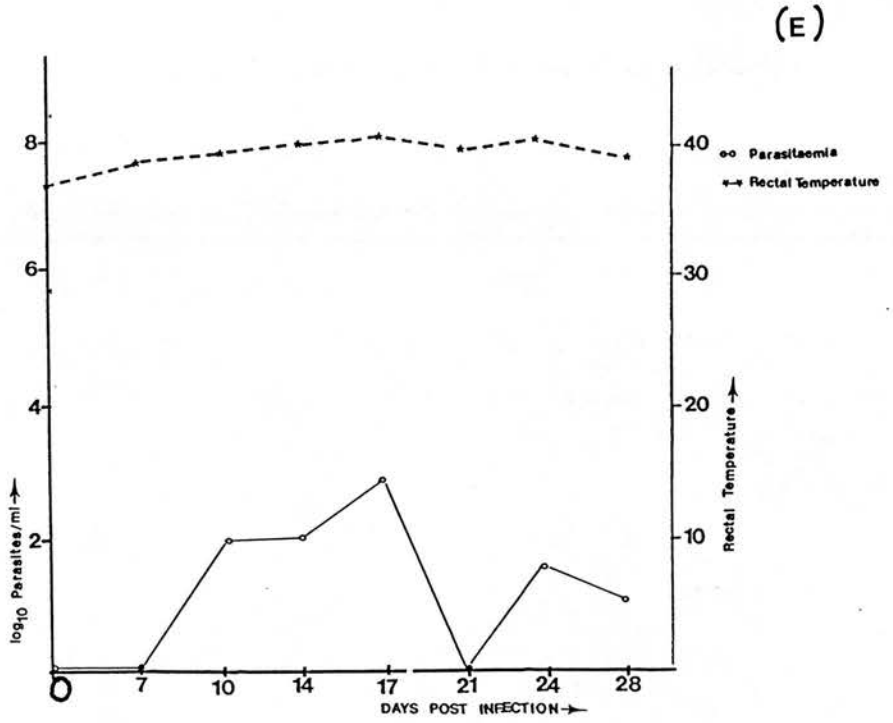

200
100
0
-100
-200
-300
-400
CHANGE IN BODY WEIGHT (gm)
0
7
10
14
17
21
24
28
DAYS POST INFECTION
8
6
4
2
log10 Parasites/ml
oo Parasitaemia
+-+ Change in body weight

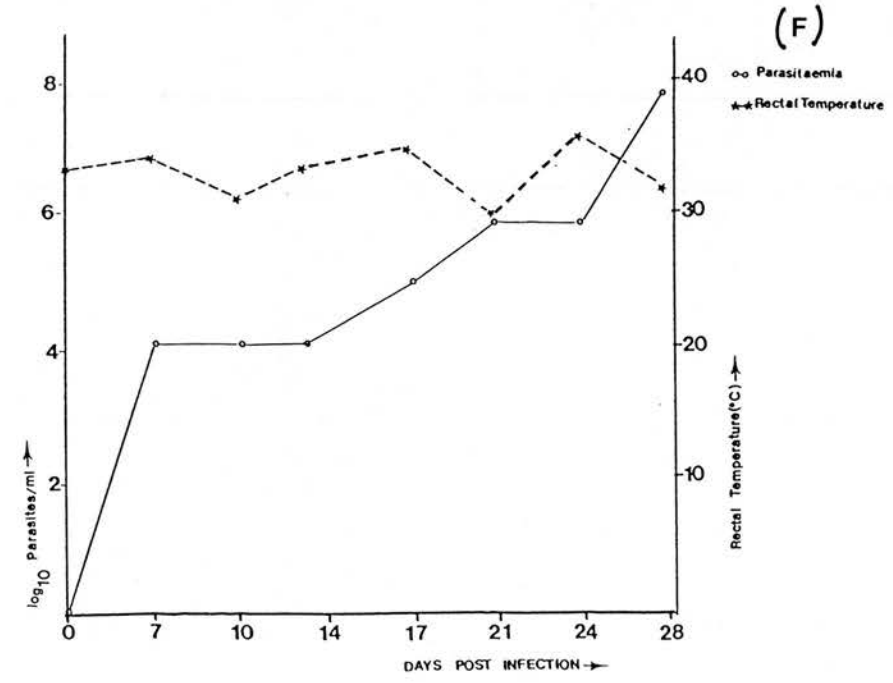
FIGURE 4.8

Clinical signs in animals infected with *T. evansi*.

E Variations in rectal temperature reading with parasitaemia in a resistant rabbit.

F Variations in rectal temperature reading with parasitaemia in a susceptible rabbit.


(E)
8
6
4
2
40
30
20
10
oo Parasitaemia
+–+ Rectal Temperature
log10 Parasites/ml
Rectal Temperature
0
7
10
14
17
21
24
28
DAYS POST INFECTION


(F)
8
6
4
2
40
30
20
10
oo Parasitaemia
+–+ Rectal Temperature
log10 Parasites/ml
Rectal Temperature(°C)
0
7
10
14
17
21
24
28
DAYS POST INFECTION

Five rabbits suffered severely from the effects of the infection showing signs of difficult breathing, marked weight loss, clogging of the eyes and nostrils by discharges and anorexia. These animals had a different PCV pattern from those in the above group of three rabbits in that, there was a progressive decrease in the PCV readings from day 7 after infection until about day 24 post-infection when marked increases in PCV values, reaching up to 47% in some rabbits, were recorded (Figure 4.8B). Haematological examination of Giemsa stained blood smears revealed the presence of few red blood cells, many of which were macrocytic and hypochromatic in the blood samples collected from these rabbits (Figure 4.9B) during the period the high PCV values were recorded. This observation contrasted markedly with the haematological picture of blood samples collected from these animals prior to infection (Figure 4.9A) in which the red blood cells appeared normal and were smaller in size with no immature cells present. During the same period, the haematological examination of blood samples collected from the other three rabbits not showing late high PCV values, did not reveal the presence of immature red blood cells. They only showed the presence of fewer red blood cells than were observed in the animals prior to infection. The erythrocytes in the blood of these rabbits were also macrocytic and hypochromatic. Parasites were detected in the blood of the five rabbits seven days after infection. Thereafter, fulminating levels of parasitaemia were observed as the infection progressed (Figure 4.8B, D and F).

After the initial reduction in body weight observed in all the rabbits after infection, fluctuations in the body weight reading within a wider range (Figure 4.8D) than the other three rabbits were observed in this group of five rabbits. Similarly the initial increase in the rectal temperature reading that coincided with the appearance of parasites in the blood observed in all the animals was

followed by fluctuations of the rectal temperature reading within a wider range in these five rabbits (Figure 4.8F) than were observed for the other three rabbits (Figure 4.8E).

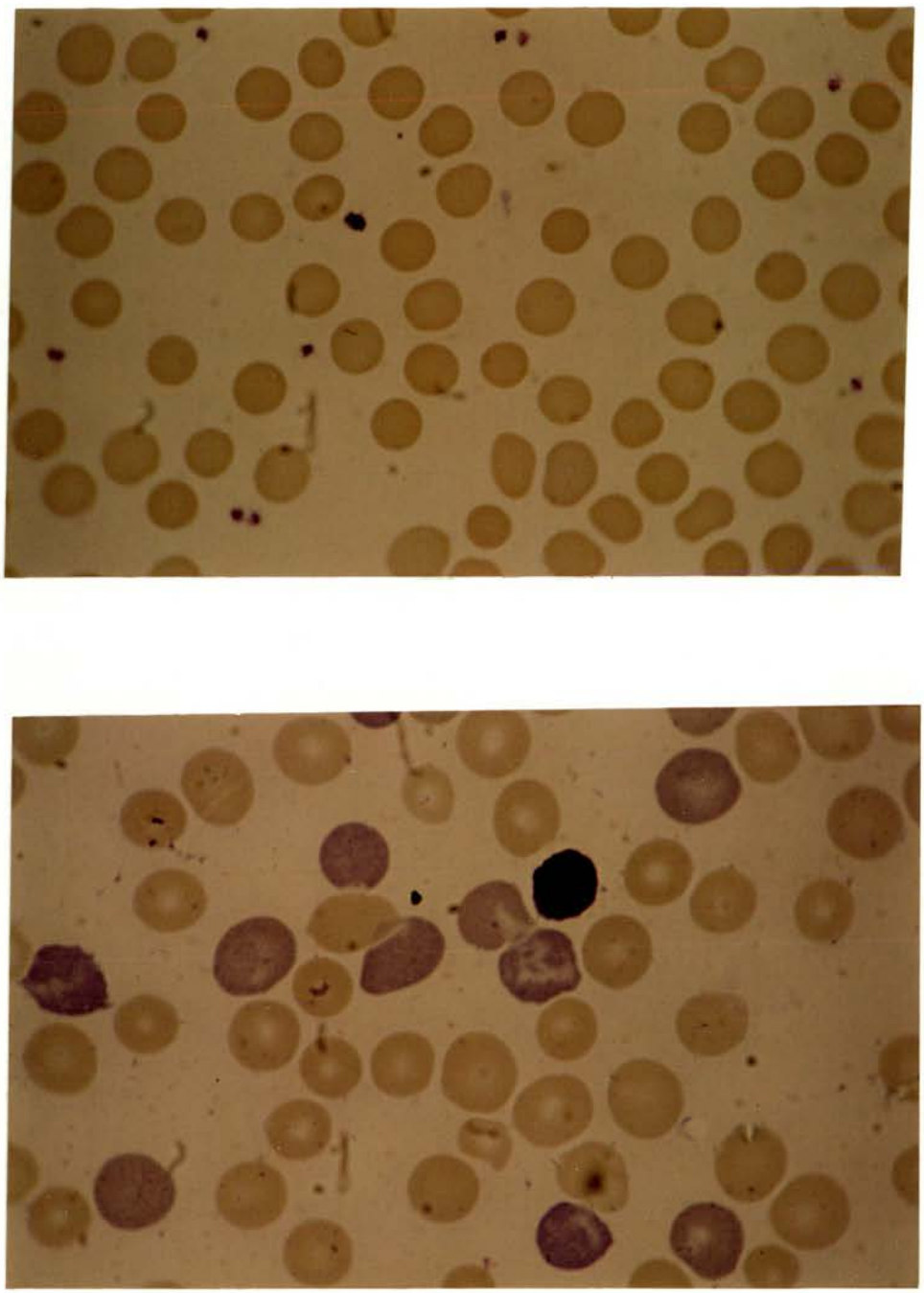
FIGURE 4.9

Giemsa's stained thin blood smears from a susceptible rabbit.

A Blood smear prepared from a blood sample collected before the rabbit was infected with *T. evansi*.

B Blood smear prepared from a blood sample collected during late period of infection when haematological examination revealed high packed cell volume (PCV) values.

Figure 4.9



DISCUSSION

A high proportion of the protein components in the trypanosome were shown to be antigenic with up to 24 of the 30 protein components being recognised by host antibodies at some point during the infection. Previous studies had clearly demonstrated that only two of the components - the 67,000 and 60,500 dalton components were associated with the surface of the trypanosome (see Chapter 3 for details of surface component characterisation). The other components are therefore, likely to be of non-surface origin and represent the internal antigens of the trypanosome.

In all the rabbits the predominant antibody response, as defined by the number of parasite components recognised and the intensity of staining of the components was IgG in nature (Figure 4.3, Lanes 1 to 8). Next in magnitude appeared to be IgM (Figure 4.4, Lanes 1 to 8) with the least response attributable to IgA (Figure 4.5, Lanes 1 to 8). The predominance of the IgG response is similar to that reported by Campbell *et al.* (1978), Nantulya *et al.* (1979), Sacks and Askonas (1980) and Mansfield *et al.* (1981) for mice and cattle infected with *T. b. brucei* or mice infected with *T. b. rhodesiense* using radioimmunoassay (RIA) or fluorescent antibody staining method (IFA). Results from the present studies differ however, from the findings of Seed *et al.* (1969), Zahalsky and Weinberg (1976) who reported a predominantly IgM response during the period of primary infection in rabbits infected with *T. b. gambiense* and rats or cattle infected with *T. b. brucei* using an agglutination assay.

It is possible that the conflicting results arise from the nature of the antigen used and type of assay employed. Assays using the agglutination test would be more likely to detect IgM principally rather than IgG due to the

efficient agglutinating property of IgM *in vitro* (Seed, 1972; 1977; Takayangi and Eriquez, 1973; Luckins, 1976; Musoke *et al.*, 1981). The agglutinating property of IgM is further enhanced with particulate antigens (Bowry, 1984) as in the case of the whole trypanosomes used as antigens in agglutination assays in which predominant IgM levels were reported. In the case of RIA, IFA and SDS-PAGE/WB equal opportunity should have been given for IgG and IgM to bind, following detection using appropriate class specific serum. The predominant IgM response recorded by the workers that used the agglutination test may therefore, be a consequence of the assay system. Workers that reported a predominantly IgG response, tended to use the antigens in a less particulate form. It is possible that these techniques were relatively less sensitive in detecting IgM (Osler, 1971) than the agglutination test or that the higher affinity of IgG over that of IgM (Bowry, 1984) enhanced the efficiency of its binding to the soluble antigens used in these assays.

The increasing IgG activity observed with the increasing duration of infection which heightened after drug treatment may be associated with the release of the internal or common antigens of the parasites which are known to elicit IgG production (De Raadt, 1974). More parasites would be destroyed by VSG-specific antibodies as the infection progresses while chemotherapy would bring about the destruction of many parasites.

Since the technique used for this assay highlights only antigen-antibody specific responses, the results obtained in this study may indicate that more trypanosome specific antibodies belong to the IgG class than IgM. This would therefore be in agreement with the views of other workers who demonstrated that most IgM produced in trypanosomiasis is probably non-trypanosome specific (Hudson *et al.*, 1976; Terry *et al.*, 1980). Alternatively, the higher

concentration of IgG in the serum in addition to its higher affinity than those of IgM may have favoured the binding of more IgG than IgM to the soluble antigens of *T. evansi* such as were used in the present study because IgM antibodies are known to bind better to particulate than soluble antigens (Bowry 1984).

The limited IgA responses observed in the rabbits in this study is in keeping with the fact that IgA antibodies are classically considered to act mainly outside the blood and are secreted at the external mucosal surfaces and therefore, are unlikely to be involved extensively in the vascular phase of an infection. IgA may, however, have a role to play in the response to trypanosomiasis infection should the parasite intrude into extravascular seromucous sites where IgA would have a role to play in the control of the parasite (Nielsen *et al.*, 1978). The ability of the *brucei* group of trypanosomes to localise in a range of extravascular sites such as testis, ear and under the skin etc. is well established (Losos and Ikede, 1970; Nagle *et al.*, 1980). Although no direct evidence for such localisation has been demonstrated for *T. evansi*, it may likely have a role to play in the increased IgA levels observed in rabbits in this study in late infections.

The greater number of antigen components detected after drug treatment compared to the number identified before treatment is probably due to the rapid destruction of the parasites by the drugs with consequential massive release of parasite components, most of which would probably be of non-surface in origin. The fact that IgM and IgA responses are largely restricted to the surface components with most of the responses to the non-surface components being predominantly IgG, probably indicates that different effector mechanisms are involved in the handling of these individual components. The

association of antibody to several low molecular weight components, 25,400, 19,500 and 12,500, (Table 4.2) with the post-drug treatment period suggests that different antigens are released following parasite destruction by the drug compared to the immune system probably suggesting different mechanisms of parasite destruction. The loss of antibodies to the 32,400 dalton component after drug treatment (Table 4.2) which happened in all the animals suggests that this component could be used as an antigen in immunoassays such as ELISA (Voller, 1977) for the diagnosis of active *T. evansi* infections, thereby overcoming the problem of distinguishing between persistent antibodies due to an ongoing infection particularly in cases of very low parasitaemia, and antibodies persisting after the infection had been eliminated by chemotherapy.

The presence of antibodies to the two surface components of *T. evansi* throughout the period of observation probably has important implications for both host and parasite as specific VSG- antibody production is considered to be important for the elimination of trypanosomes and protection against reinfections by homologous trypanosome populations (Wellde *et al.,* 1975, 1981; Emery *et al.,* 1980; Hall and Esser, 1984). Such antibody persistence might be due to the fact that the antibodies remained in circulation for the length of time of observation after they had been produced or that there was a continuous source of antigenic stimulus that maintained their production. The possible elimination of the effect of immunoglobulin-secreting prohibitive factor on antigen primed B cells due to the effect of some trypanosomes factors by chemotherapy (Black *et al.,* 1985) may have contributed in part to the maintenance of antibody levels after the elimination of the parasites by chemotherapy in this study as was suggested by Shapiro (1986).

Evaluation of the range of clinical signs shown by the animals infected with *T. evansi* during this study divided the animals into two groups. One group showed little change in PCV, low parasitaemia and no marked loss of body weight (Figure 4.8A, C and E) and were considered to be resistant to the *T. evansi* infection. The other group showed marked changes in PCV, high parasitaemia and a marked loss of body weight (Figure 4.8B, D and F) and were classed as susceptible. Similar criteria have been used by other authors to distinguish between cattle that were resistant from those that were susceptible to trypanosome infection (Roelants *et al.*, 1983).

A comparison of the nature and magnitude of the immune response of the animals classed as either resistant or susceptible on the basis of clinical signs revealed that those classed as resistant tended to produce antibodies to many trypanosome components, antibodies appeared early in infection and persisted throughout the course of the infection (Figure 4.6). Animals classed as susceptible produced antibodies to fewer trypanosome components, the antibodies were detected later in infection and were not maintained throughout the course of infection (Figure 4.7). This association, between antibody production and outcome of infection suggests that the immune system may therefore, have a role to play in determining the way in which individual animals respond to a *T. evansi* infection. The fact that the response of the immune system is under genetic control and that differences were observed between the immune response of individual animals to *T. evansi* in this study, suggests that the response of animals to *T. evansi* may be dependent on their genetic make-up. Such observation having been reported in the cases of trypanotolerance in cattle which is influenced by inheritance (Roelants, 1986). This suggests that it might be possible to produce *T. evansi*-resistant breeds of animals as has been suggested for other trypanosomes species (Roelants, 1986)

whether some factors operate for *T. evansi* as in tsetse transmitted trypanosomes is not known.

Results from the present study have indicated that an early and sustained antibody response to *T. evansi* infection is probably more capable of controlling the parasitaemia than a delayed and limited antibody response. This observation is similar to that made by Pinder *et al.* (1987) who recorded a high degree of correlation between the resistance of mice to infection with *T. congolense* and early antibody response to the epitopes exposed on live trypanosomes. These authors suggested that an early antibody response appeared to be the most important criterion for trypanosome population control and that when the antibody response is delayed trypanosome growth overwhelms the defence mechanisms of the host bringing about the death of the animal. Black *et al.* (1986) in their study with *T. brucei* also found that the resistant mice produced more antibodies than the susceptible mice. A similar association between the magnitude of the immune response and resistance to infection has also been reported with other parasites, such as helminths. Almond and Parkhouse (1986) found that the sera from mice resistant to *Trichinella spiralis* infection recognised far more parasite antigens than the susceptible ones. There is therefore mounting evidence that the ability of the resistant animals to secrete effective antibodies early in infection was an important factor to their resistance.

The association seen between resistance and response in the present study was not just limited to the surface components. The greatest differences between the resistant and susceptible animals were seen in their response to the non-surface components. The number of the non-surface components recognised by all three Ig classes, IgG, IgM and IgA in the sera of the

resistant animals (Figure 4.6) were greater in number and more intensely stained than those identified by the sera of the susceptible animals (Figure 4.7). This result suggests that the involvement of the immune response of the host to the non-surface components may be important in deciding the outcome of a trypanosome infection. This observation clearly shows that the destruction of the live trypanosomes via VSG-specific antibody might not be the only factor important in the survival of the animal. Of equal importance might be the way that the immune system deals with the products that arise from the destruction of the trypanosomes.

CHAPTER FIVE

CLASS-SPECIFIC ANTIBODY RESPONSE IN RABBITS AFTER RE-INFECTION WITH TRYPANOSOME SPECIES

INTRODUCTION

An outstanding property of trypanosomes is their ability to produce a large range of antigenically distinct parasite populations during the course of an infection, a process referred to as antigenic variation (Soltys, 1963). The effect of this is that at any one time during an infection, the trypanosome in the blood can consist of several different antigenic types. In nature therefore, it is likely that animals will be exposed to challenge with a range of antigenically distinct trypanosomes populations. Furthermore, in areas of Africa, where several species of trypanosomes are endemic, the picture would be complicated by the fact that at any one time an animal could be infected by more than one species of trypanosome.

The surface component of trypanosomes is highly immunogenic and it is well established that immunity to reinfection can easily be produced in a number of ways, such as by short-term infection followed by cure using *T. congolense* (Browning and Calver, 1943; Fulton and Lourie, 1946; Wiesenhutter, 1970) or *T. brucei* (Browning and Gulbransen, 1936). Other methods of inducing immunity include the injection of irradiated parasites, as in the case of *T. rhodesiense, T. equiperdum* and *T. equinum* into animals (Fulton and Lourie, 1946). In all cases, however, immunity was found to be strictly confined to reinoculations with homologous trypanosomes. This homologous type of protection was attributed to the specificity of the host antibodies to the particular infecting trypanosome population (Wellde *et al.*, 1975; Herbert and Wilkinson, 1977; Emery *et al.*, 1980; Nantulya *et al.*, 1980b; Wellde *et al.*, 1981; Morrison *et al.*, 1982; Hall and Esser, 1984).

Attempts to induce protective immunity experimentally in animals against an heterologous variable antigen type (VAT) challenge has been achieved by

exposing animals to infection with *T. evansi* and eliminating the infection after an extended period of time by chemotherapy. This type of immunisation process relies on maintaining the infection in the animals long enough to produce antibodies to most of the predominant VATs (pVATs) that the animal might be expected to be exposed to (T.W. Jones, personal communication). Such protective immunity is, however, limited to challenge with the pVATs that the animals had been exposed to. Animals exposed to other VAT populations succumb to the infection in the same way as animals immunized against a single VAT and then challenged with heterologous antigens (extensively reviewed in Roelants and Williams, 1982; Morrison *et al.*, 1982a).

Despite the range of antigenic types of trypanosomes circulating in nature and the extreme specificity of the host response, animals continue to survive in trypanosome endemic areas in the absence of external control measures such as chemotherapy. One factor that could account for the ability of the animals to thrive in the presence of high trypanosome challenge in the absence of chemotherapy is the tendency for stocks of trypanosomes to produce predominant VATs early in infection. In the case of tsetse transmitted trypanosomes this is further reinforced by the fact that the antigenic repertoire of metacyclic trypanosomes is limited and the existence of stock specific basic antigen types. In the case of non-tsetse-transmitted trypanosomes, although there is no basic antigen because of the lack of metacyclics, the method of pVAT development is as in tsetse-transmitted trypanosomes early in infection. Continuous exposure to basic and predominant VATs probably continues in an endemic area until most animals have produced antibodies against the range of pVATs circulating in the area. This host antibody spectrum could place a restriction on the range of VATs that can be expressed by a trypanosome

stock, making it possible for an animal to develop immunity to all the pVATs in the VAT repertoire. However, if the animal is exposed to a pVAT that is outside the range of VATs it had experienced previously then infection would result.

Should the ability of animals to control infections with trypanosomes be based solely on the production of VAT-specific antibodies (Herbert and Wilkinson, 1977; Hall and Esser, 1984) then it would be difficult to reconcile this with the absence of massive trypanosome epidemics resulting in high mortalities in many endemic areas where animals are probably exposed to a multiplicity of variant antigenic types at any one time. This paradox probably suggests that other factors could play a role in protecting animals against disease in the field, particularly in the endemic areas with high *Glossina* challenge in the absence of chemotherapy (Roelants, 1986).

Results presented in Chapter 4 on the immune response of rabbits to primary *T. evansi* infections demonstrated clearly that animals produce antibodies against both the surface and non-surface components of the parasite, with up to 20 non-surface components identified by antibody in the serum collected from animals during the first four weeks of infection. Furthermore, there appeared to be an association between such antigen recognition and susceptibility to infection, with resistant animals recognising more of the surface and non-surface antigenic components than did susceptible animals. This result implies that the response to non-surface protein components could play a role in influencing the course of infection and development of disease. The fact that some of these non-surface components are probably common not only to different stocks but also to different species of trypanosomes (Roelants and Pinder, 1984), suggests that the response to these components could play a

role in protecting animals from infection with different stocks or species of trypanosomes.

Several workers have considered the possibility that responses to the non-surface components could play a role in the protection of animals exposed to trypanosomes. This was mentioned in the review by Roelants and Pinder (1984) who concluded that past emphasis on the studies of VSG-specific antibodies produced in trypanosomiasis might have been to the detriment of identifying a possible role for the non-surface components in the protection of animals against infection. These authors however, did express some uncertainty as to the way in which such components could be involved in the development of immunity. They concluded, however, that the response probably arises following the destruction of the intact organism by VSG-specific antibodies.

The aim of the studies reported in this Chapter is to evaluate the role of antibodies produced against the individual antigen components of a stock of *T. evansi* in the protection of animals from challenge with homologous and heterologous trypanosome populations.

MATERIALS AND METHODS

Trypanosomes

T. evansi: *T. evansi* TREU 2113 described in Chapter 2 was the trypanosome stock used for all the primary infections and reinfection in the homologous *T. evansi* system.

T brucei: The stock of *T. brucei* (TREU 2049) was obtained after the initial stock EATRO 1595 acquired from Kenya was passaged once through mice. The morphology of the parasites was identical to the morphological

details of *T. brucei* listed by Hoare (1972).

T. vivax TREU: A stabilate population, TREU EO8, of a rat adapted Wellcome strain of *T. vivax* was used for the production of a further stabilate, TREU 2130, after the original parasites were passaged four times through rats. The morphology of the parasites was similar to the morphological details of *T. vivax* listed in Hoare (1972).

T. congolense: The original stock, a stabilate population - ILRAD 1180, acquired from ILRAD, Kenya from *Glossina morsitans* was passaged once through mice to produce a further stabilate - TREU 1880 which was then grown in mice before being used to infect rabbits. The parasites showed all the morphological characteristics of *T. congolense* (Hoare, 1972).

T. evansi: The stock of *T. evansi* (TREU 1834) used for the heterologous challenges in this work has been described previously in Chapter 3.

Experimental Animals

Ten adult female New Zealand White rabbits were infected with 4×10^5 motile trypanosomes of TREU 2113, from mice with fulminating infections. Trypanosomes were resuspended in 0.5 ml aliquots of ice-cold PSG (Appendix 2) and injected into the rabbits through the ear vein. Twenty-one days after infection rabbits were treated with a single intramuscular (i/m) injection of 7 mg/kg^{-1} body weight (active principle) of diaminazene aceturate, Berenil^R (Hoechst, West Germany). Thirty days after Berenil^R treatment pairs of rabbits were challenged with 4×10^5 motile trypanosomes of each trypanosome population, *T. evansi* TREU 2113, 1834, *T. brucei* TREU 2049, *T. vivax* TREU 2130 or *T. congolense* TREU 1880 grown in mice.

All animals were bled before the primary infection with *T. evansi* TREU 2113 (day 0) and on days 7, 10, 14, 17 and 21 after infection. Blood samples were also collected from the animals 7 and 14 days post-drug treatment and again on days 7, 10, 14, 17, 21 28 and 30 after challenge.

Two types of blood samples were collected on each occasion.

(a) Blood samples collected in bottles with EDTA (Sigma Ltd.) used for the estimation of parasitaemia by darkground/phase contrast buffy coat scoring system (Paris *et al.*, 1982), and packed cell volume (PCV %) estimation by the microhaematocrit technique (Schalm, 1975). Blood samples negative for trypanosomes by the darkground/phase contrast buffy coat scoring system (Paris *et al.*, 1982) were subsequently inoculated into mice. These mice were subsequently examined daily for trypanosomes for at least 30 days by examination of wet film prepared from the tail blood.

(b) Blood samples collected in bottles without anticoagulant were used for the preparation of serum, according to the method described previously in Chapter 2.

Soluble Antigens of Trypanosome Populations

Soluble antigens of each trypanosome population were prepared by freezing and thawing using the final protocol described in Chapter 2.

Antibody Responses to Infection and Challenge

The immunoglobulin isotype response of the animals to the particular trypanosome population were assessed using SDS-PAGE with 90 μg sample/well of the appropriate soluble trypanosome antigen followed by blotting of the separated components onto nitrocellulose membrane. After the

reacting of the transferred proteins with appropriate rabbit sera, the activity of the individual immunoglobulin isotypes, IgG, IgM and IgA were probed for with the appropriate immunoglobulin heavy chain (Fc) specific antisera (Nordic^{R}) as described in Chapter 4.

Soluble antigen preparation of *T. evansi* TREU 2113 was tested against serum samples collected from all the 10 rabbits, 0, 7, 14, 17 and 21 days after infection, 7 and 14 days after drug treatment and 7, 14, 17 and 30 days after challenge.

Soluble antigen preparation from the other trypanosome populations were tested against serum samples collected from each of the 10 rabbits 14 days after infection with *T. evansi* TREU 2113, 14 days after drug treatment and 21 days after rechallenge with the respective trypanosome population used as antigen.

Ten adult female New Zealand White rabbits were infected in pairs with 4 x 10^{5} motile parasites of each of the trypanosome populations used in the study in order to establish the normal prepatent periods of these parasites in the absence of any priming with *T. evansi* stock (TREU 2113) used for all the primary infections.

RESULTS

Parasitaemia Profiles during Primary Infection with T. evansi TREU 2113

Trypanosomes were first detected in the blood of nine of the 10 rabbits seven days after infection with *T. evansi* TREU 2113. In the remaining rabbit, parasites were not detected until 10 days after infection (Figure 5.1E). Thereafter, fluctuations in levels of parasitaemia were observed in all the rabbits until day 21 post-infection when they were treated with Berenil^{R} (Hoechst, West Germany) (Figure 5.1A to E). Up to 30 days after treatment

no parasites were seen in the blood of 10 of the rabbits used. Two of the original rabbits included in the study relapsed during this time and were not used for the challenge studies.

Parasitaemia during challenge period: Differences were seen in both the prepatent period and pattern of parasitaemia in the animals after the challenge period when compared to the control animals and were linked to the trypanosome population used for challenge. The only rabbits in which trypanosomes were not detected after challenge were those reinfected with the homologous stock, TREU 2113. Trypanosomes were not detected in the blood of the infected rabbits after drug treatment and up to 30 days after challenge (Figure 5.1A), while trypanosomes appeared in the blood of naive animals infected with TREU 2113, 7 days after infection. In the case of heterologous *T. evansi* challenge (TREU 1834) parasites were first seen on day 21 after rechallenge in both rabbits compared to 7 days after infection in the naive animals. A slight increase over the initial level of parasitaemia recorded 21 days after reinfection was seen in both rabbits by the last parasitaemia estimation at 30 days after reinfection (Figure 5.1B).

Trypanosomes were detected in the blood samples collected from both rabbits challenged with *T. brucei* TREU 2049 for the first time 17 days after rechallenge compared to 7 days after infection with the same population in naive rabbits. Blood parasite counts remained stable in one infection/cure rabbit up to day 21 post-challenge after which parasites were not detected again by buffy coat microscopal examination or mouse inoculation for the remainder of the observation period (Figure 5.1C). In the case of the second infection/cure rabbit, after the initial appearance of trypanosomes on day 17 after challenge, parasites were not detected in the blood of the rabbit up to the

point when the last blood examination was conducted at 30 days after challenge (Figure 5.1C).

Parasites were detected for the first time in the blood of one of the rabbits challenged with *T. vivax* seven days after challenge. Thereafter, increases in the level of parasitaemia with a slight fluctuation were observed throughout the rest of the period of observation (Figure 5.1D). Parasites appeared in the blood of the second rabbit for the first time 10 days after reinfection. This was followed by a fall in the level of parasitaemia starting from 14 days after reinfection. From 28 days after reinfection, an increase was seen in the level of parasitaemia which was maintained for the rest of the period (Figure 5.1D). Parasites appeared in the blood of naive rabbits infected with this stock of *T. vivax* 10 to 11 days after infection.

Trypanosomes were detected in the blood samples of the two rabbits challenged with *T. congolense* for the first time on day 10 after reinfection compared to 11 to 12 days in naive rabbits. After this period fluctuations in parasitaemia were recorded with higher parasite counts obtained from one rabbit until day 30 post-infection (Figure 5.1E).

Antibody Responses to TREU 2113 during the Primary Infection Period

Reaction with TREU 2113 as soluble antigen: All the rabbits produced antibodies belonging to each of the three classes of immunoglobulins, IgG, IgM and IgA, to a range of components of *T. evansi* TREU 2113 during the period of primary infection. IgG activity was predominant in that antibody belonging to this class recognised more components (Figure 5.2A, 3A, 4A, 5A and 6A, Nos. 2 to 5) than did those belonging to the IgM class (Figure 5.2B, 3B, 4B, 5B and 6B, Nos. 2 to 5). Antibodies belonging to IgA class recognised the

least number of components (Figure 5.2C, 3C, 4C, 5C and 6C, Nos. 2 to 5).

Reactions with TREU 1834, TREU 2049, TREU 2130 and TREU 1880 as soluble antigens: Differences were seen in the number of components of each trypanosome population recognised as antigen by the serum collected 14 days after infection with *T. evansi* TREU 2113.

In the case of the heterologous *T. evansi* population (TREU 1834) two antigenic components with molecular weights of 67,000 and 41,800 daltons were recognised by the IgG antibodies in the 14 day serum (Figure 5.3A, No. 12). None of the proteins of TREU 1834 were, however, recognised as antigens by antibodies belonging to either the IgM (Figure 5.3B, No. 12) or IgA class (Figure 5.3C, No. 12) of immunoglobulin. The soluble antigen preparation of *T. brucei* (TREU 2049) contained at least 30 clearly recognisable antigenic components with molecular weights between 125,000 and 20,000 daltons which reacted with the IgG antibodies in the 14 day serum (Figure 5.4A, No. 13). Between four and five of the *T. brucei* components were recognised as antigens by antibodies belonging to the IgM and IgA class of immunoglobulins (Figure 5.4B and 4C, No.13). The IgG from the 14 day serum recognised two *T. vivax* components but only faintly (Figure 5.5A, No. 13). IgM from the same serum recognised one of these components (Figure 5.5B, No. 13) while IgA in addition to recognising the two components identified by IgG, recognised three other components, bringing the number of *T. vivax* components recognised by IgA antibodies to five in total (Figure 5.5C, No. 13). None of the *T. congolense* components were recognised as antigens by either the IgG (Figure 5.6A, No. 13), IgM (Figure 5.6B, No. 12) or IgA (Figure 5.6C, No. 13) antibodies in the serum collected 14 days after infection with TREU 2113.

Antibody Response during the Post-drug Treatment Period

Reactions with T. evansi TREU 2113 soluble antigens: Antibodies belonging to the three immunoglobulin classes in the serum collected from animals after drug treatment reacted with soluble antigens of *T. evansi* TREU 2113. More antigenic components of *T. evansi* were recognised during the post-treatment period than were recognised during the post-infection period. IgG antibody activity predominated in that this class of antibody identified more *T. evansi* components (Figure 5.2A, 3A, 4A, 5A and 6A, Nos. 6 and 7) than IgM (Figure 5.2B, 3B, 4B, 5B and 6B, Nos. 6 and 7) or IgA which recognised the least number of components (Figure 5.2C, 3C, 4C, 5C and 6C, Nos. 6 and 7).

Reactions with soluble antigens of TREU 1834: Four antigenic components of *T. evansi* TREU 1834, which were additional to the two 67,000d and 41,800d components, recognised by serum collected from the animals during the post-infection period with *T. evansi* TREU 2113, were recognised by serum collected from the two animals 14 days after drug treatment. One of the new components had a molecular weight of 78,000 daltons, while the remaining three components had molecular weights ranging from 36,000 to 48,000 daltons (Figure 5.3A, No. 13). None of the components of *T. evansi* TREU 1834 were recognised by the IgM (Figure 5.3B, No. 13) or IgA (Figure 5.3C, No. 13) antibodies in the serum collected from the animals 14 days after drug treatment.

The *T. brucei* components recognised as antigens by IgG antibodies in serum collected from the rabbits 14 days after drug treatment (Figure 5.4A, No. 14) were similar in molecular weight range with those recognised by IgG antibodies in serum collected 14 days after infection with TREU 2113 (Figure

5.4A, No. 13), apart from the fact that components with molecular weight less than 31,000 daltons were more faintly stained by serum collected after drug treatment than that collected during the post-infection period. Fewer *T. brucei* components were recognised as antigens by IgM antibodies in serum collected from the rabbits after drug treatment (Figure 5.4B, No. 14) than were recognised by IgM in serum collected after infection with *T. evansi* TREU 2113 (Figure 5.4B, No. 13). More *T. brucei* components were recognised as antigens by IgA in 14 day post-treatment serum (Figure 5.4C, No. 14) than were identified by IgA antibodies in serum collected 14 days after infection with *T. evansi* TREU 2113 (Figure 5.4C, No. 14). More *T. vivax* components were recognised as antigenic by IgG (Figure 5.5A, No. 14), IgM (Figure 5.5B, No. 14) and IgA (Figure 5.5C, No. 14) antibodies respectively in the sera collected from the rabbits 14 days after drug treatment in comparison with the number of *T. vivax* components identified as antigenic by the three antibody classes in the serum collected from the rabbits 14 days after infection with *T. evansi* TREU 2113 (Figures 5.5A, B and C, No. 13). A group of two to three *T. congolense* components with molecular weights above 94,000 daltons were recognised by IgG (Figure 5.6A, No. 14) and none by IgM (Figure 5.6B, No. 14) and IgA (Figure 5.6C, No. 14) antibodies present in the serum collected 14 days after drug treatment.

Antibody Response during the Challenge Period

Response to challenge with T. evansi TREU 2113: Serum samples collected from rabbits after challenge with *T. evansi* TREU 2113 recognised more components of *T. evansi* TREU 2113 than any of the serum samples collected after primary infection with TREU 2113 or after Berenil treatment. IgG antibody activity (Figure 5.2A, No. 8 to 11) was highest during challenge

as judged by the number of *T. evansi* antigen components recognised and their intensity of labelling. Next to this was the number of components recognised by IgM (Figure 5.2B, Nos. 8 to 11) and lastly IgA (Figure 5.2C, Nos. 8 to 11) antibodies in serum samples collected 21 days after challenge with *T. evansi* TREU 2113. The IgG antibodies, apart from recognising two *T. evansi* TREU 2113 components, with molecular weights - 67,000 and 60,500 daltons, more intensely during the challenge period than after primary infection and drug treatment, also recognised several additional components within the molecular weight range of 43,000 and 12,500 daltons (Figure 5.2A, Nos. 8 to 11) that were not recognised by any serum sample collected before challenge (Figure 5.2A, Nos. 1 to 7). IgM antibodies in the serum samples collected from animals after challenge with *T.. evansi* TREU 2113 recognised two components with molecular weight of 85,000 and 75,500 daltons and some other components with molecular weights around 30,000 daltons (Figure 5.2B, Nos. 8 to 11) that were not recognised before the challenge (Figure 5.2B, Nos. 1 to 7). No new components were recognised by IgA antibodies in the serum collected after challenge while antibodies to the 60,500 dalton component identified during primary infection (Figure 5.2C, Nos. 1 to 4) were absent in the serum samples collected after challenge (Figure 5.2C, Nos. 8 to 11).

Response to heterologous challenge: Serum collected from animals 21 days after challenge with *T. evansi* 1834 were tested against separated proteins of TREU 1834 to find out the antibodies whose production was induced by the reinfections. The IgG in the serum recognised about seven components of the trypanosome (Figure 5.3A, No. 14). One component with a molecular weight of 67,000, and a group of low molecular weight components with a molecular weight of around 30,000 daltons were common to both *T. evansi* TREU 2113 and 1834. None of the components of TREU 1834 were recognised by IgM

(Figure 5.3B, No. 14) or IgA (Figure 5.3C, No. 14) antibodies in the serum collected 21 days after challenge.

The pattern of antigen recognition in rabbits challenged with *T. brucei* (Figure 5.4A, 4B and 4C, No. 15) and *T. vivax* (Figure 5.5A, 5B and 5C, No. 15) was similar to those observed in animals reinfected with TREU 1834 in that increasing numbers of the components of the challenge trypanosome stock were identified by serum samples collected from animals 21 days after rechallenge. In all cases, the 67,000 dalton component and a group of lower molecular weight components around 30,000 and 43,000 daltons were common to the *T. evansi* TREU 2113 stock used for the initial infection and the rechallenge trypanosome species. IgG activity expressed in terms of the number of components identified and the degree of labelling was, however, greater than that of IgM with IgA activity least during the rechallenge period. Of the five components of TREU 1834 faintly recognised by the IgG antibodies collected from the rabbits 21 days after reinfection with TREU 1834 only a few components with molecular weights from 94,000 daltons and above appeared to be common between TREU 2113 and *T. congolense*. No components of *T. congolense* were recognised by IgM (Figure 5.6B, No. 14) or IgA (Figure 5.6C, No. 14) in sera collected from the rabbits during the post-challenge period.

FIGURE 5.1

Parasitaemia in rabbits during primary infection with *T. evansi* and challenge with different stocks and species of trypanosomes.

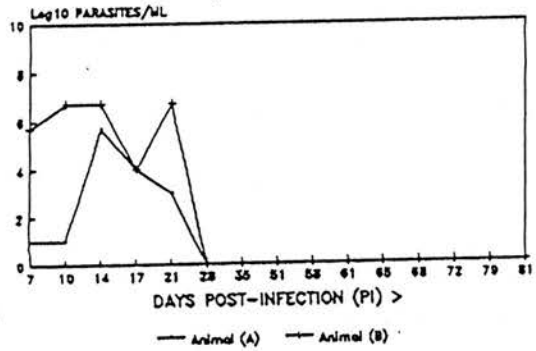
A Rabbits infected with *T. evansi* TREU 2113 and challenged with the same stock.

B Rabbits infected with *T. evansi* TREU 2113 and challenged with *T. evansi* TREU 1834.

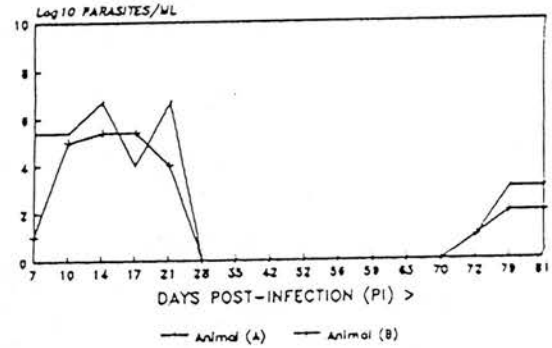
C Rabbits infected with *T. evansi* TREU 2113 and challenged with *T. brucei*.

D Rabbits infected with *T. evansi* TREU 2113 and challenged with *T. vivax*.

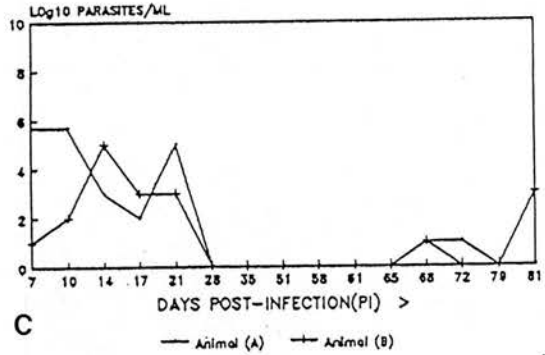
E Rabbits infected with *T. evansi* TREU 2113 and challenged with *T. congolense*.



A



B



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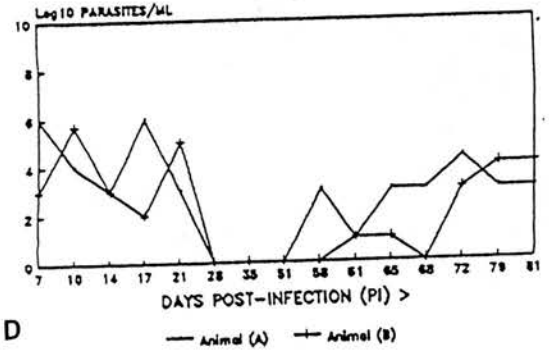

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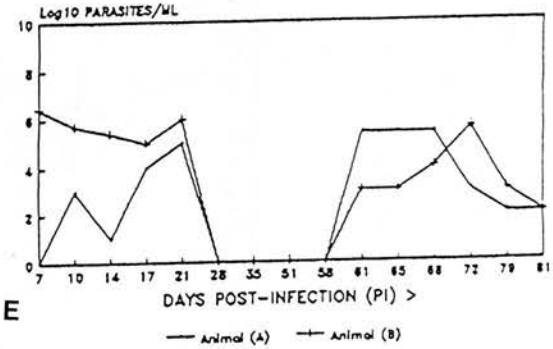

E

21: drug treatment
51: day of challenge

FIGURE 5.2A

T. evansi TREU 2113 antigens identified by IgG antibodies produced by a rabbit during the period of primary infection, after drug treatment and rechallenge with the same stock.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-11	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17 and 30 after rechallenge with the same stock.

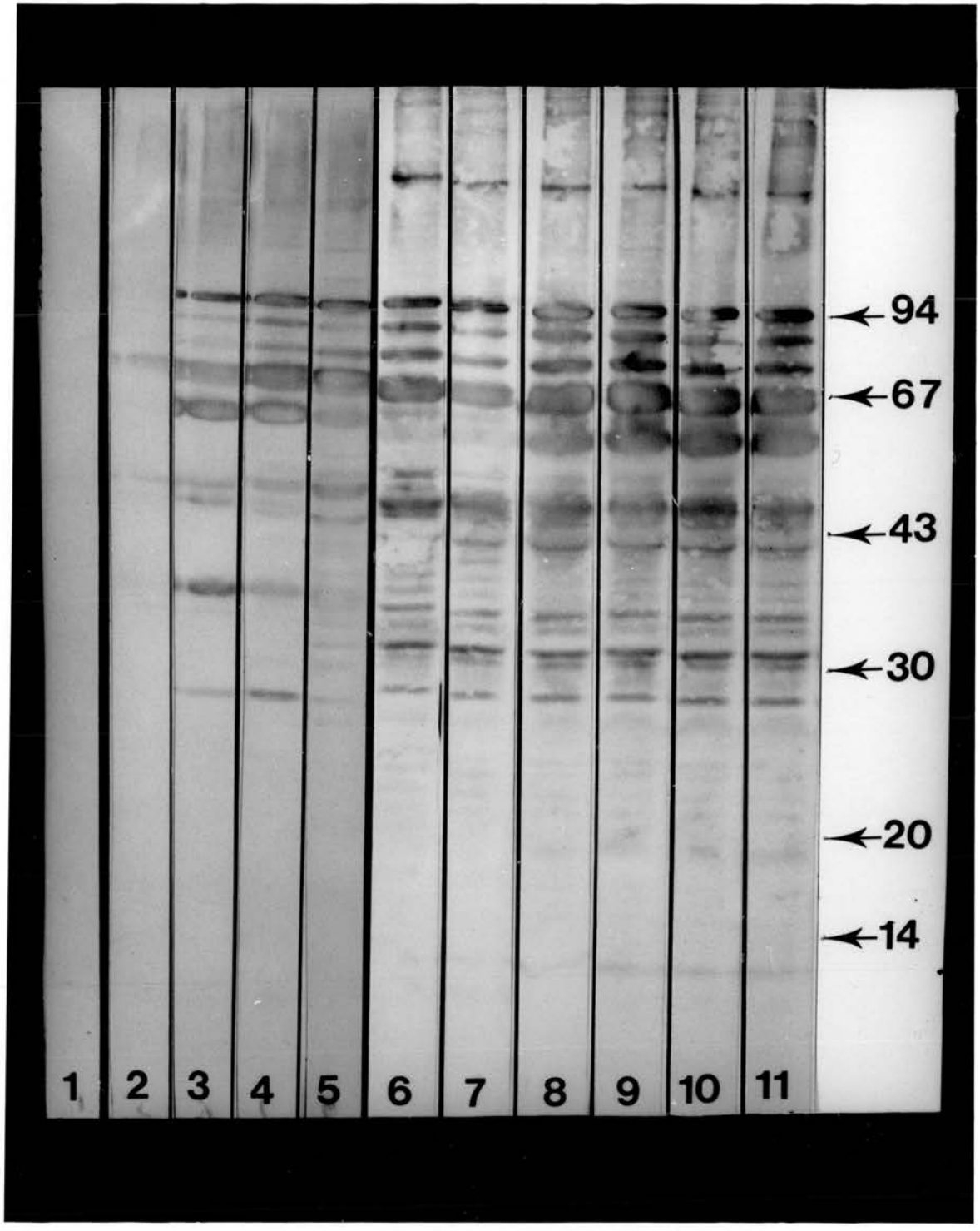
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FIGURE 5.2B

T. evansi TREU 2113 antigens identified by IgM antibodies produced by a rabbit during the period of primary infection, after drug treatment and rechallenge with the same stock.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-11	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17 and 30 after rechallenge with the same stock.

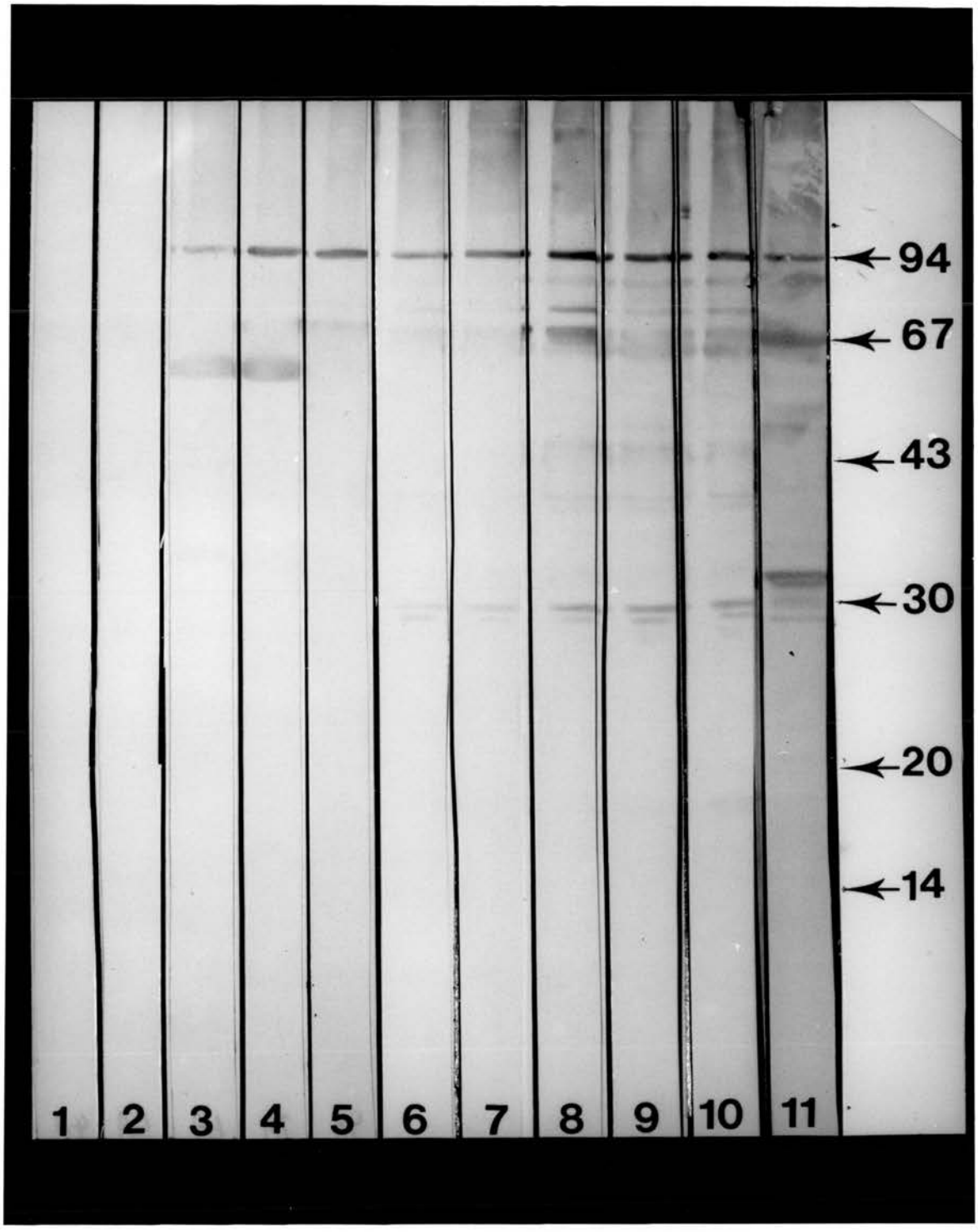
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FIGURE 5.2C

T. evansi TREU 2113 antigens identified by IgA antibodies produced by a rabbit during the period of primary infection, after drug treatment and rechallenge with the same stock.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post-drug treatment respectively.
Numbers 8-11	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17 and 30 after rechallenge with the same stock.

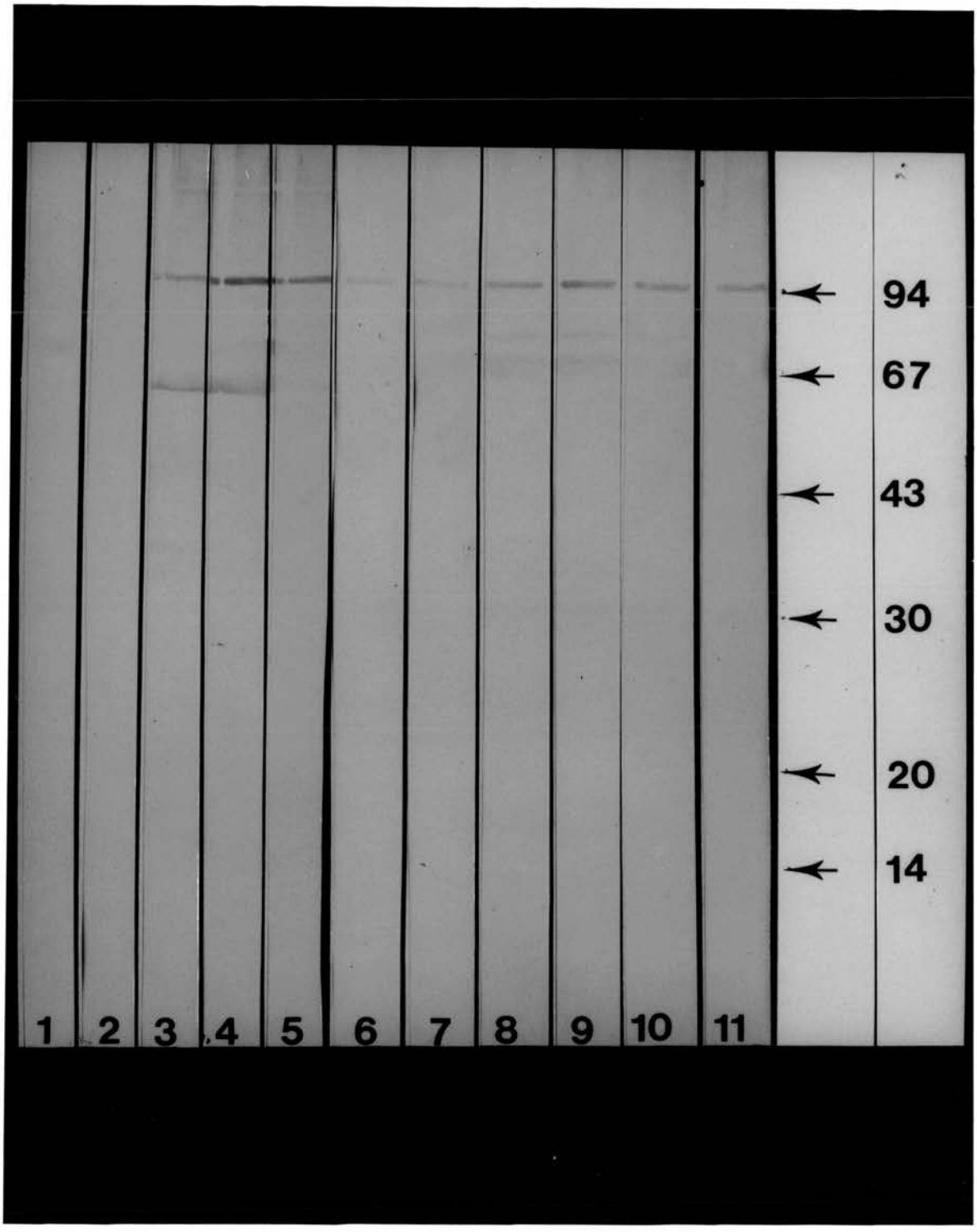
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FIGURE 5.3A

Antigens of *T. evansi* TREU 2113 and 1834 identified by IgG antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with TREU 1834.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-11	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17 and 30 after challenge with *T. evansi* TREU 1834.
Number 12	Proteins of *T. evansi* TREU 1834 probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 13	Proteins of *T. evansi* TREU 1834 probed with serum collected 14 days after drug treatment.
Number 14	Proteins of *T. evansi* TREU 1834 probed with serum collected 21 days after challenge with the same stock.

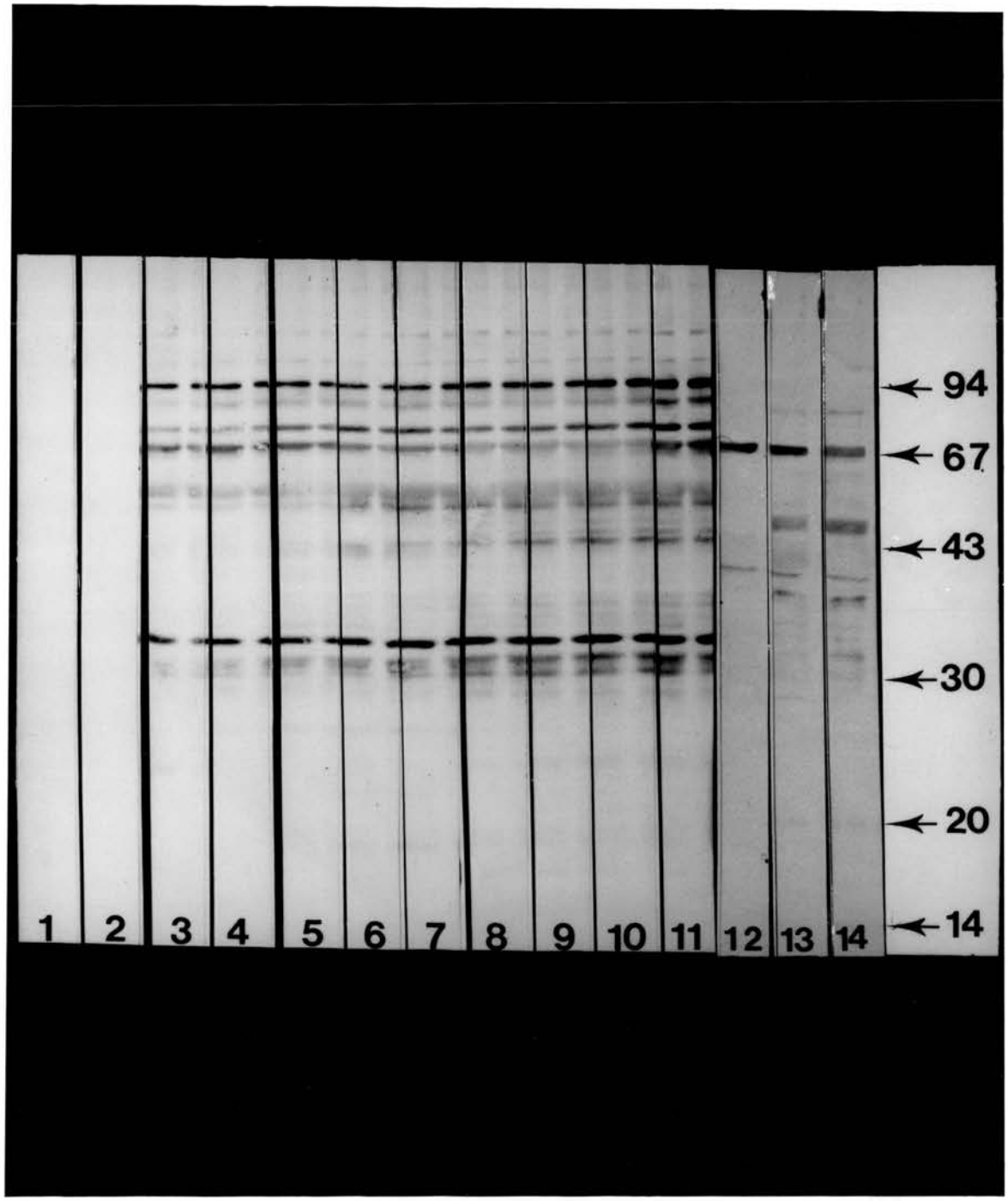
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FIGURE 5.3B

Antigens of *T. evansi* TREU 2113 and 1834 identified by IgM antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with TREU 1834.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-11	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17 and 30 after challenge with *T. evansi* TREU 1834.
Number 12	Proteins of *T. evansi* TREU 1834 probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 13	Proteins of *T. evansi* TREU 1834 probed with serum collected 14 days after drug treatment.
Number 14	Proteins of *T. evansi* TREU 1834 probed with serum collected 21 days after challenge with the same stock.

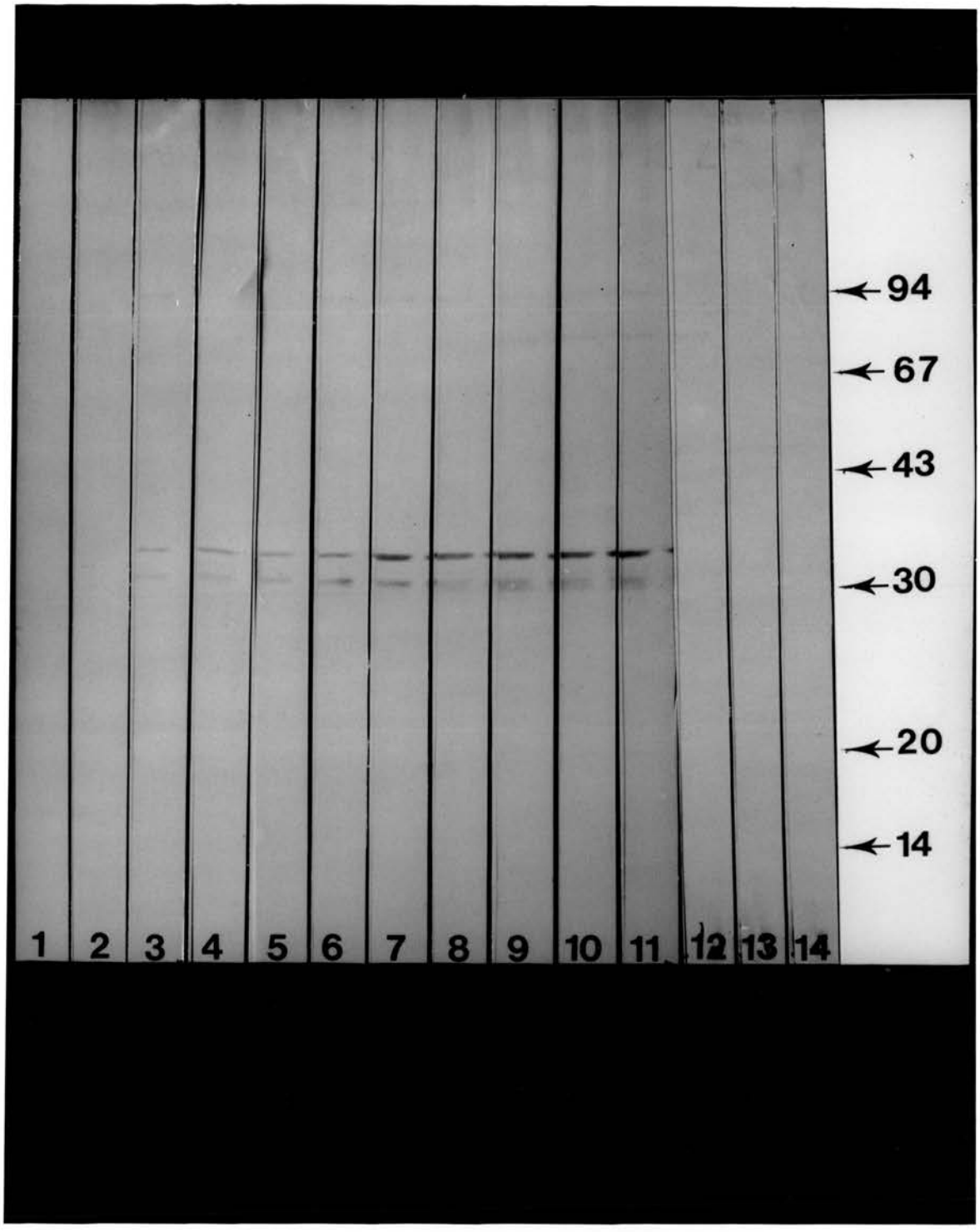
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FIGURE 5.3C

Antigens of *T. evansi* TREU 2113 and TREU 1834 identified by IgA antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with TREU 1834.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-11	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17 and 30 after challenge with *T. evansi* TREU 1834.
Number 12	Proteins of *T. evansi* TREU 1834 probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 13	Proteins of *T. evansi* TREU 1834 probed with serum collected 14 days after drug treatment.
Number 14	Proteins of *T. evansi* TREU 1834 probed with serum collected 21 days after challenge with the same stock.

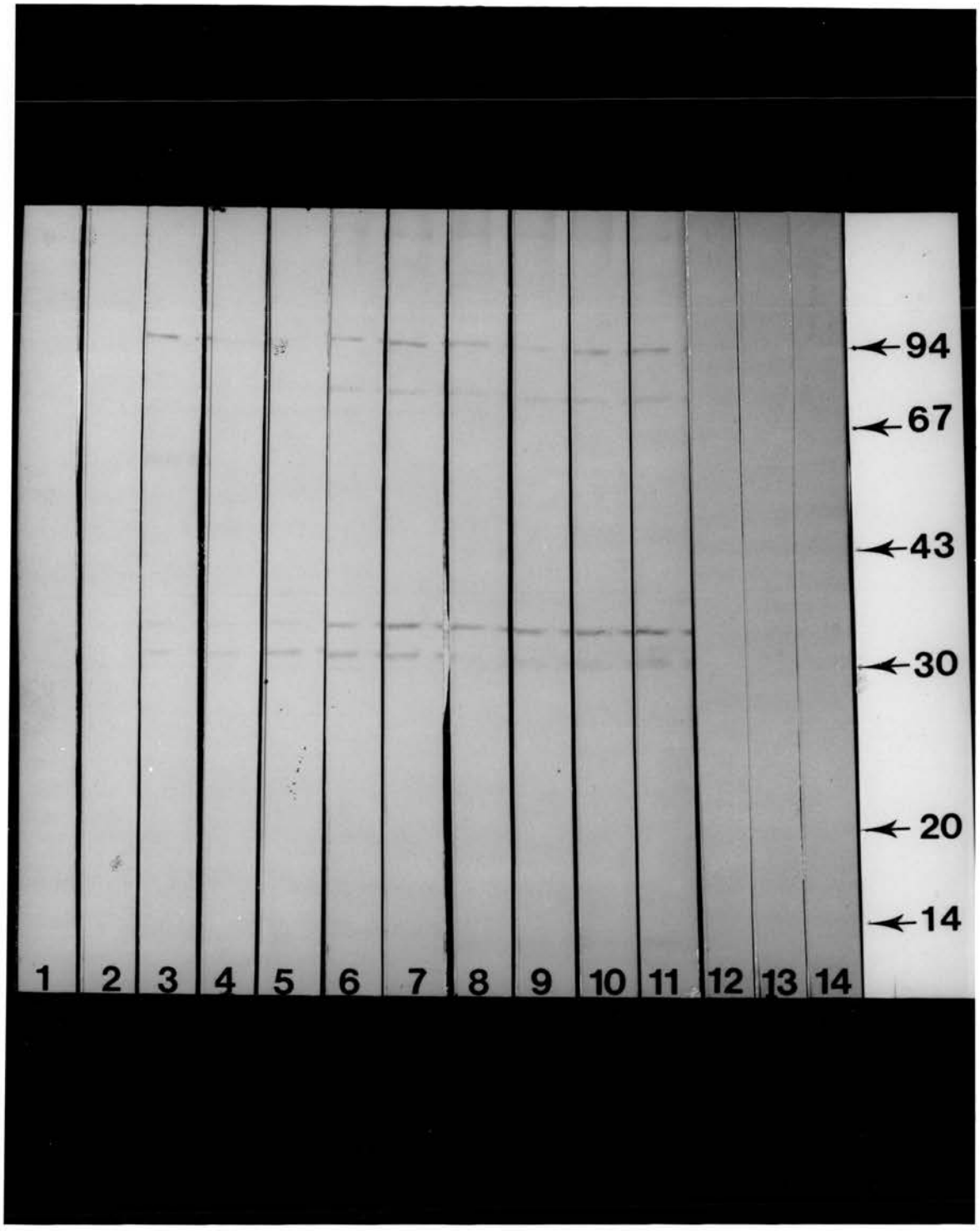
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FIGURE 5.4A

Antigens of *T. evansi* TREU 2113 and *T. brucei* identified by IgG antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. brucei*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17, 21 and 30 after challenge with *T. brucei*.
Number 13	Proteins of *T. brucei* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. brucei* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. brucei* probed with serum collected 21 days after challenge with the same stock.

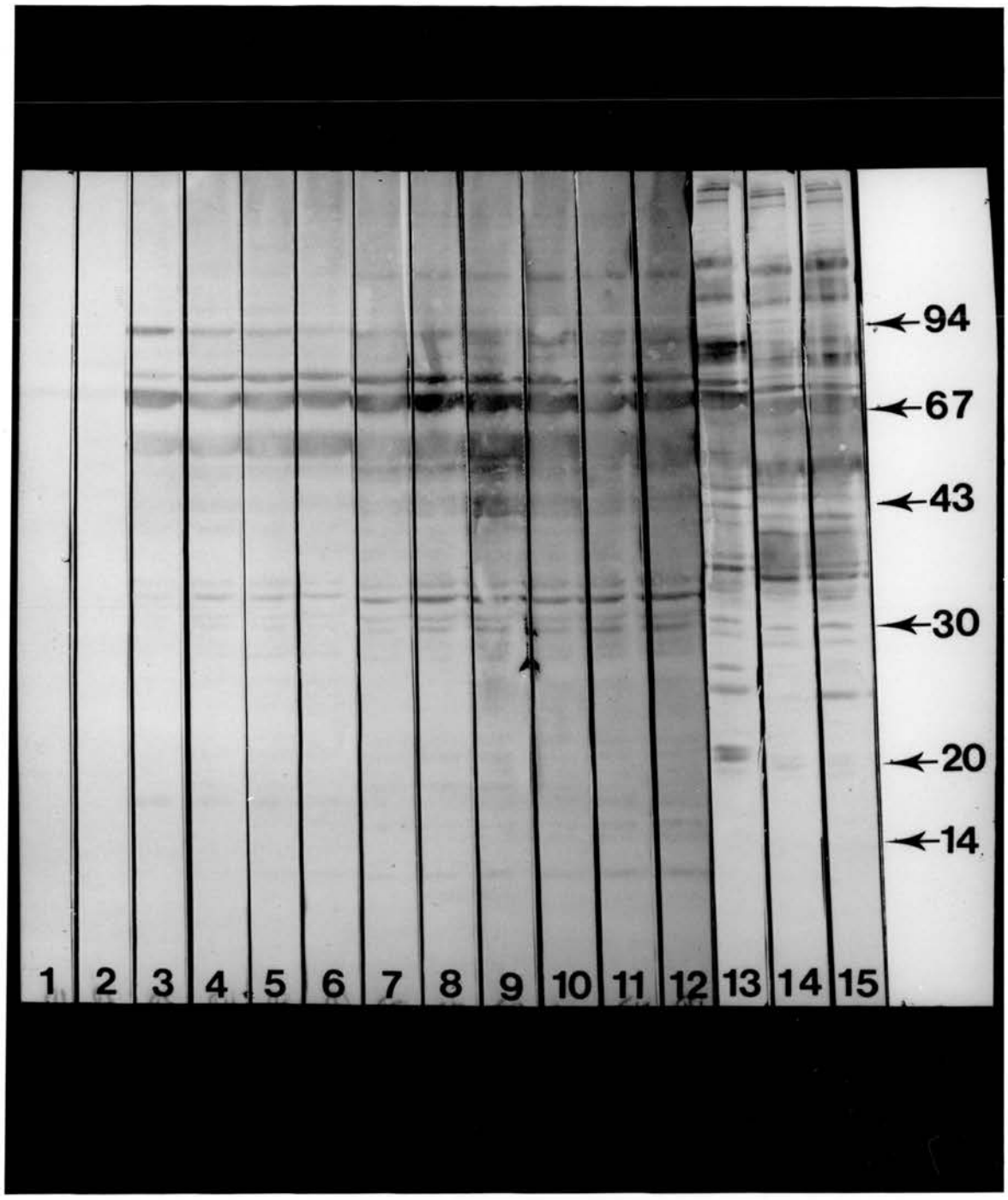
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FIGURE 5.4B

Antigens of *T. evansi* TREU 2113 and *T. brucei* identified by IgM antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. brucei*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17, 21 and 30 after challenge with *T. brucei*.
Number 13	Proteins of *T. brucei* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. brucei* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. brucei* probed with serum collected 21 days after challenge with the same stock.

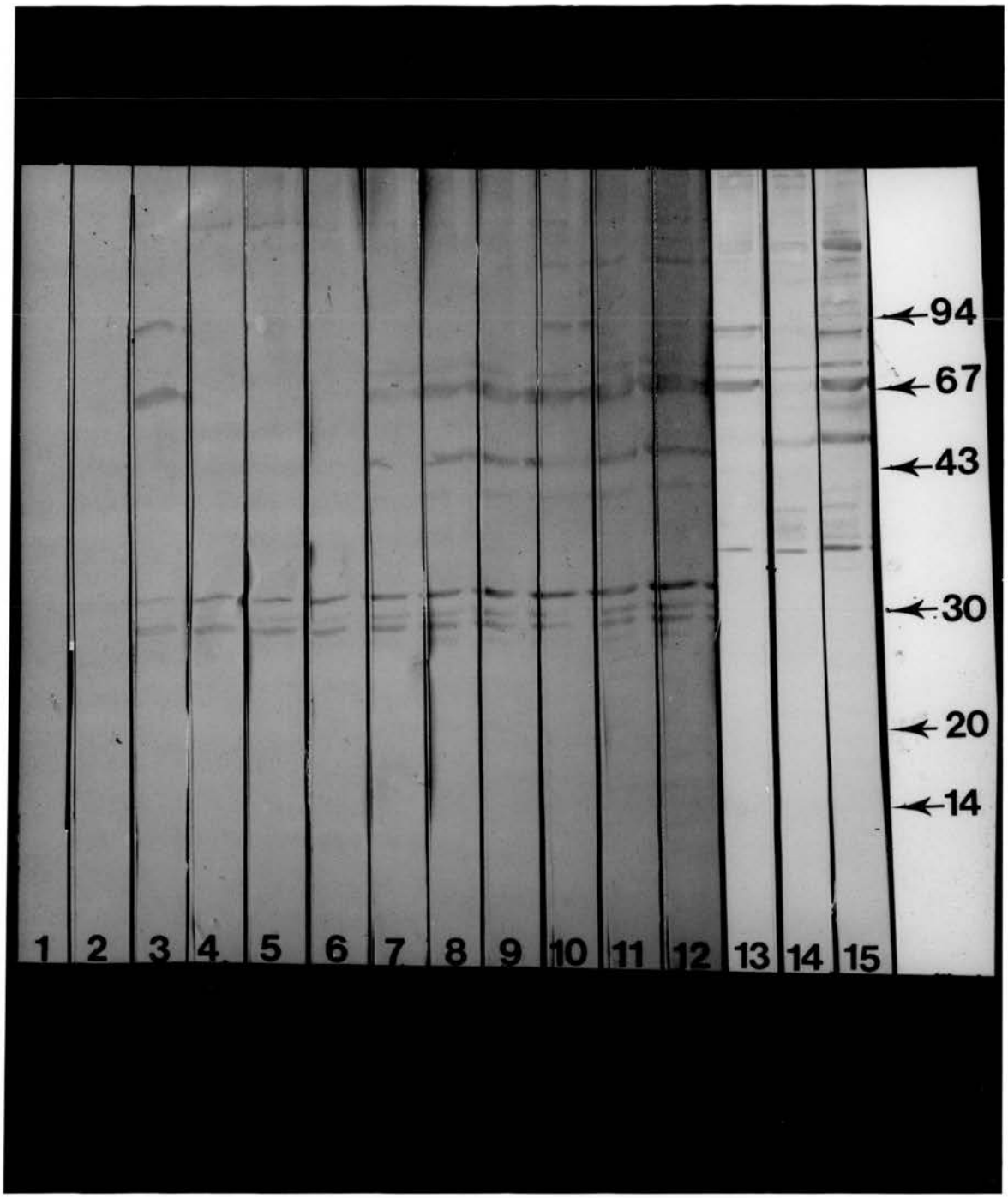
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FIGURE 5.4C

Antigens of *T. evansi* TREU 2113 and *T. brucei* identified by IgA antibodies produced by a rabbit during the period of primary infection with TREU 2113, after drug treatment and challenge with *T. brucei*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post-infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17, 21 and 30 after challenge with *T. brucei*.
Number 13	Proteins of *T. brucei* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. brucei* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. brucei* probed with serum collected 21 days after challenge with the same stock.

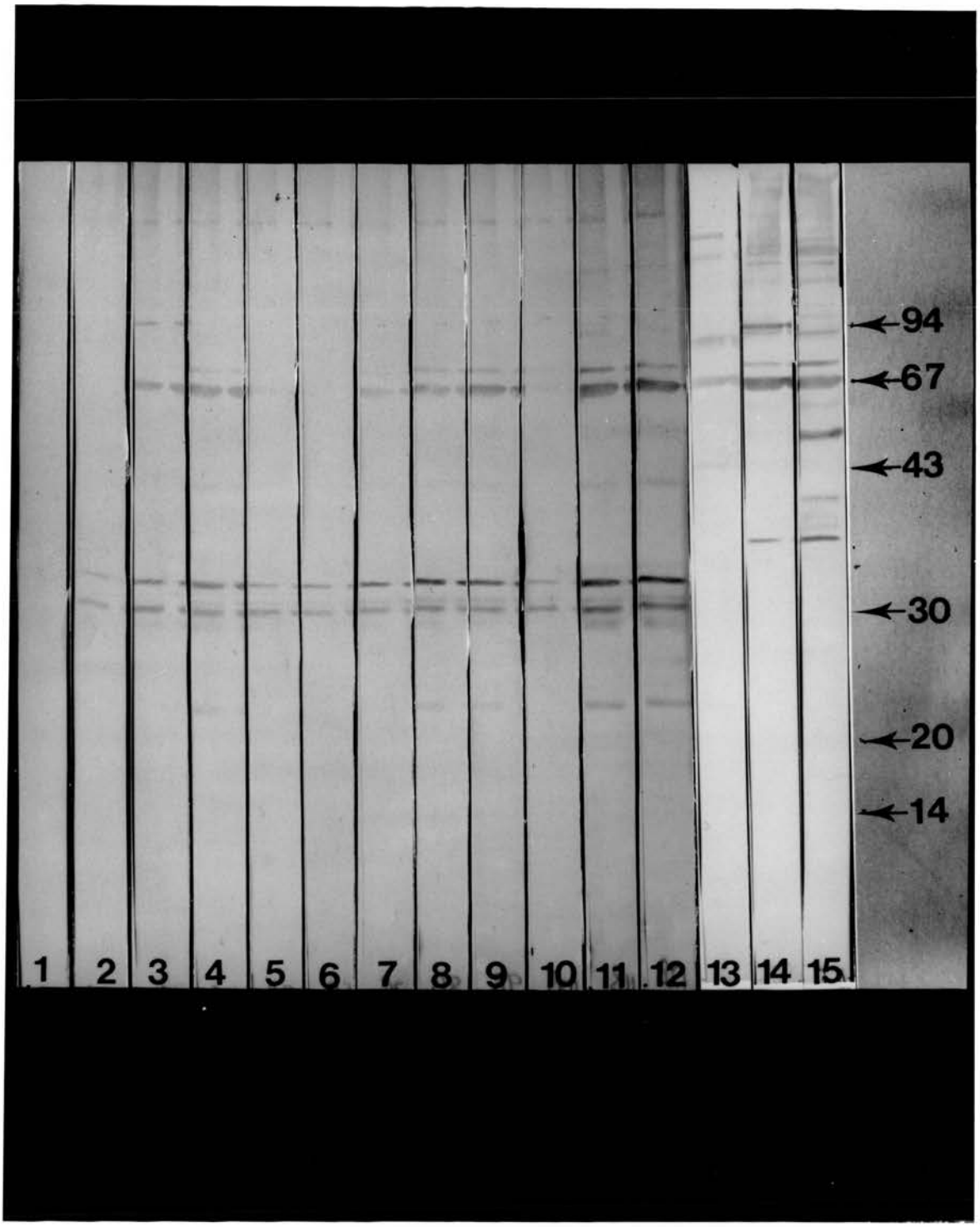
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FIGURE 5.5A

Antigens of *T. evansi* TREU 2113 and *T. vivax* identified by IgG antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. vivax*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17, 21 and 30 after challenge with *T. vivax*.
Number 13	Proteins of *T. vivax* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. vivax* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. vivax* probed with serum collected 21 days after challenge with the same stock.

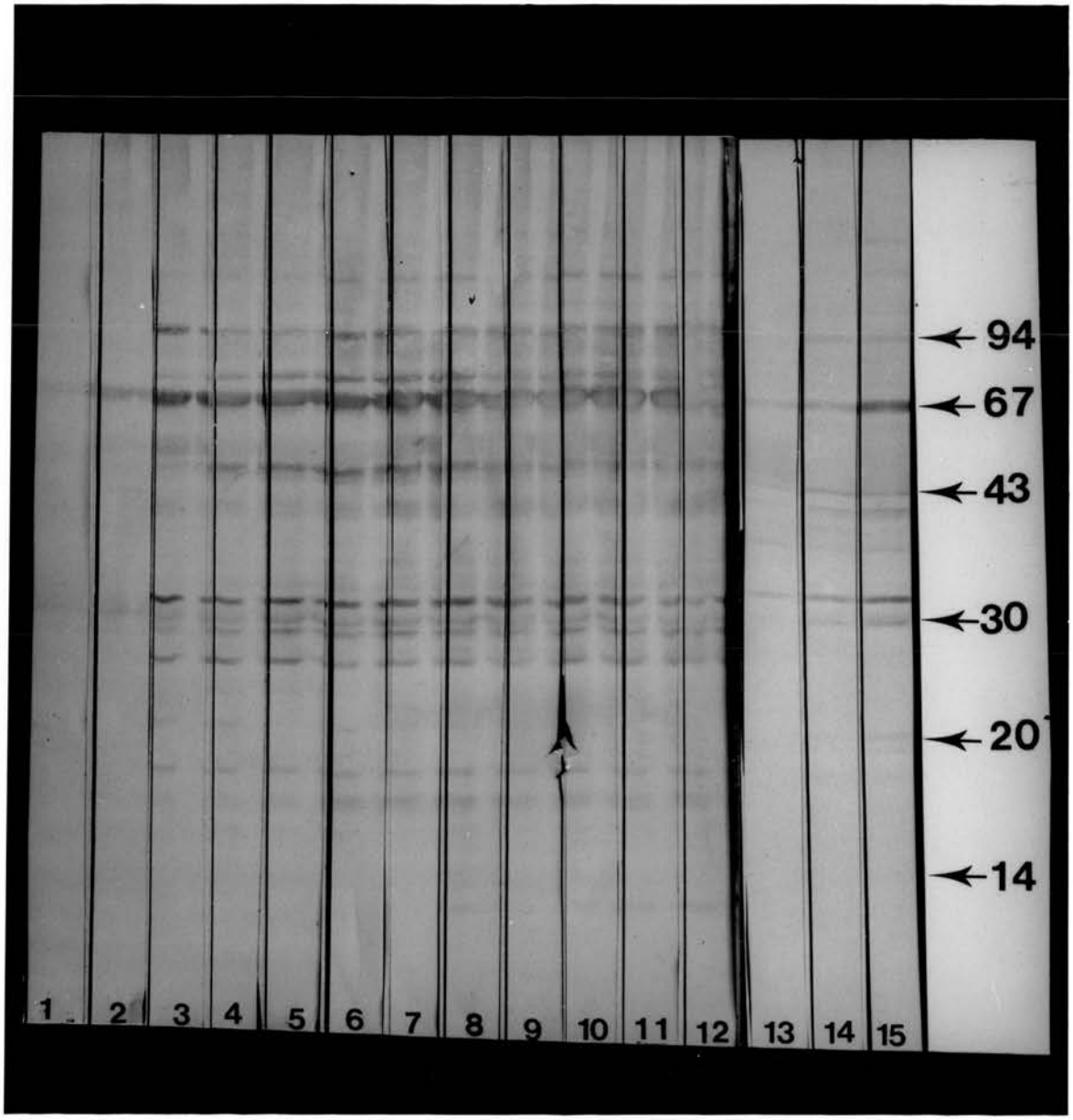
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FIGURE 5.5B

Antigens of *T. evansi* TREU 2113 and *T. vivax* identified by IgM antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. vivax*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17, 21 and 30 after challenge with *T. vivax*.
Number 13	Proteins of *T. vivax* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. vivax* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. vivax* probed with serum collected 21 days after challenge with the same stock.

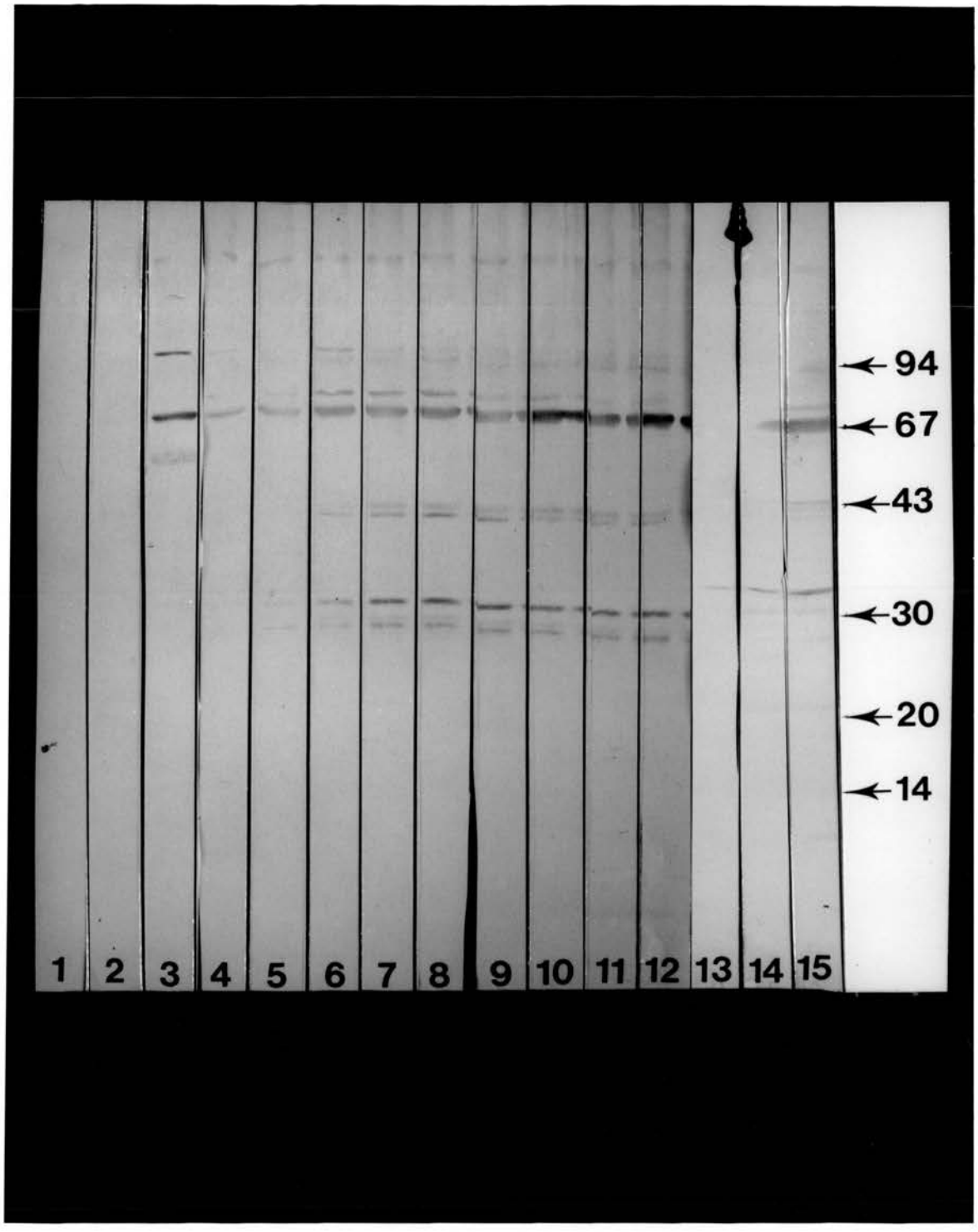
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FIGURE 5.5C

Antigens of *T. evansi* TREU 2113 and *T. vivax* identified by IgA antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. vivax*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17, 21 and 30 after challenge with *T. vivax*.
Number 13	Proteins of *T. vivax* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. vivax* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. vivax* probed with serum collected 21 days after challenge with the same stock.

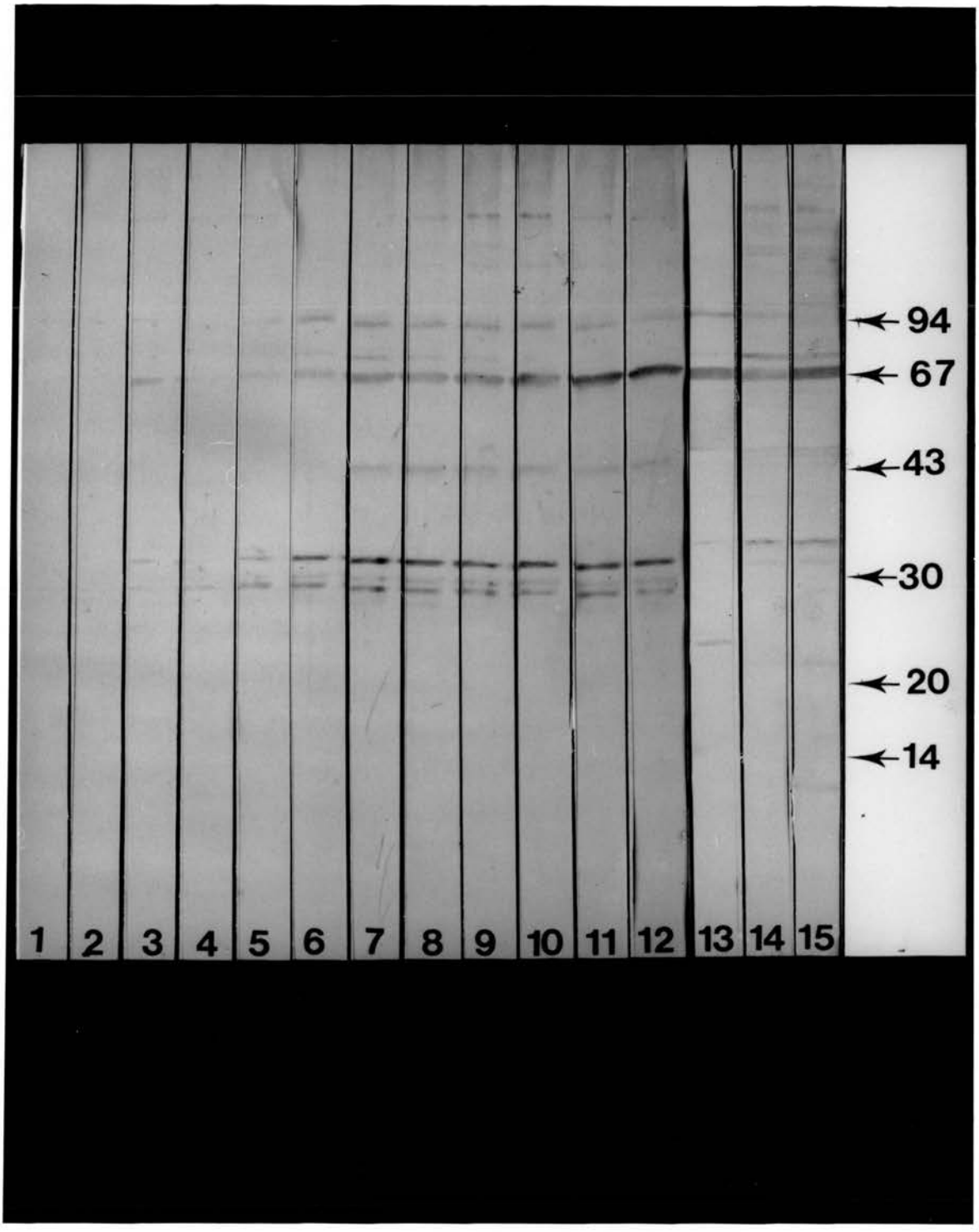
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FIGURE 5.6A

Antigens of *T. evansi* TREU 2113 and *T. congolense* identified by IgG antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. congolense*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17, 21 and 30 after challenge with *T. congolense*.
Number 13	Proteins of *T. congolense* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. congolense* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. congolense* probed with serum collected 21 days after challenge with the same stock.

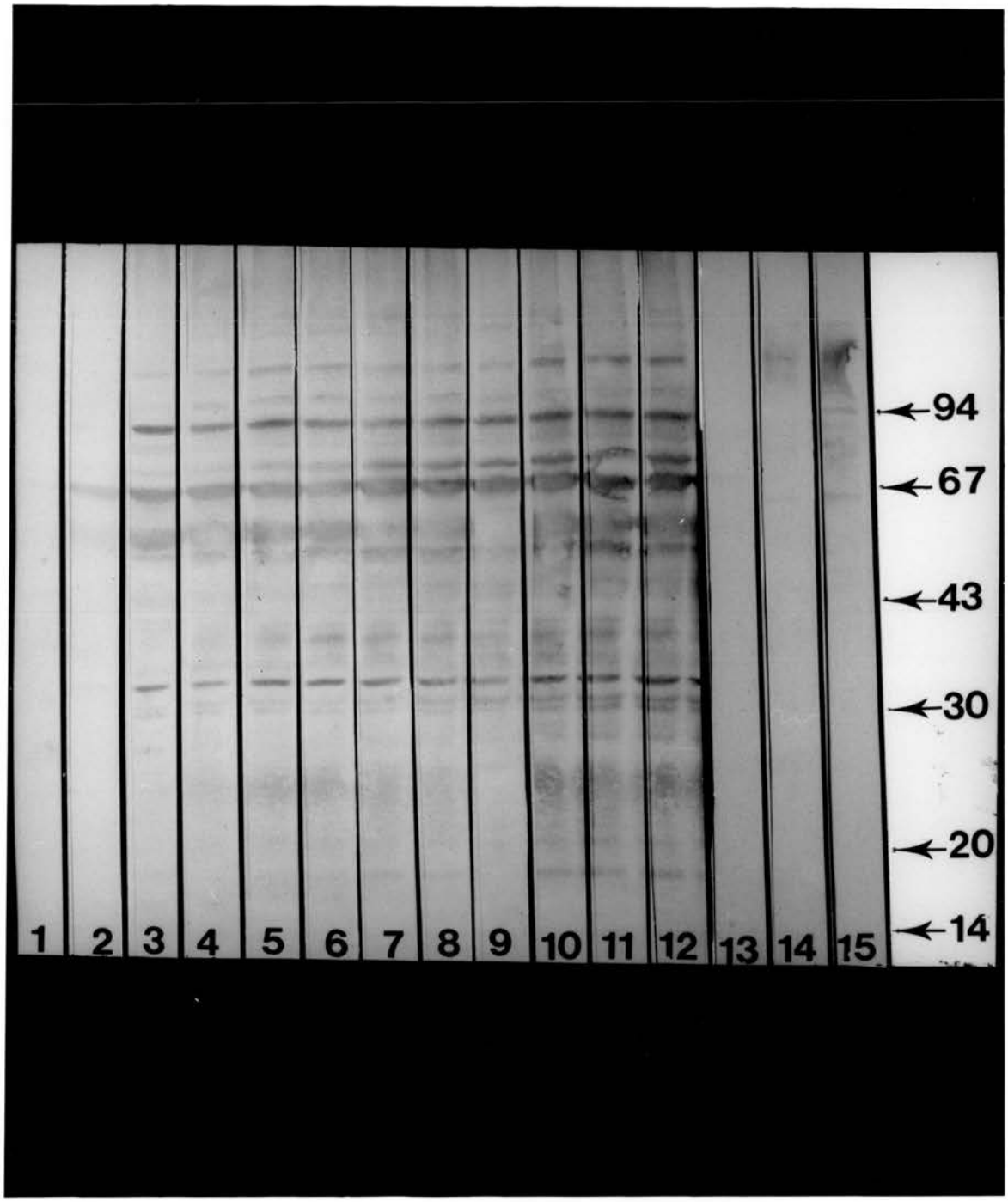
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FIGURE 5.6B

Antigens of *T. evansi* TREU 2113 and *T. congolense* identified by IgM antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. congolense*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-11	Proteins of *T. evansi* TREU 2112 probed with serum samples collected on days 7, 14, 17 and 30 after challenge with *T. congolense*.
Number 12	Proteins of *T. congolense* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 13	Proteins of *T. congolense* probed with serum collected 14 days after drug treatment.
Number 14	Proteins of *T. congolense* probed with serum collected 21 days after challenge with the same stock.

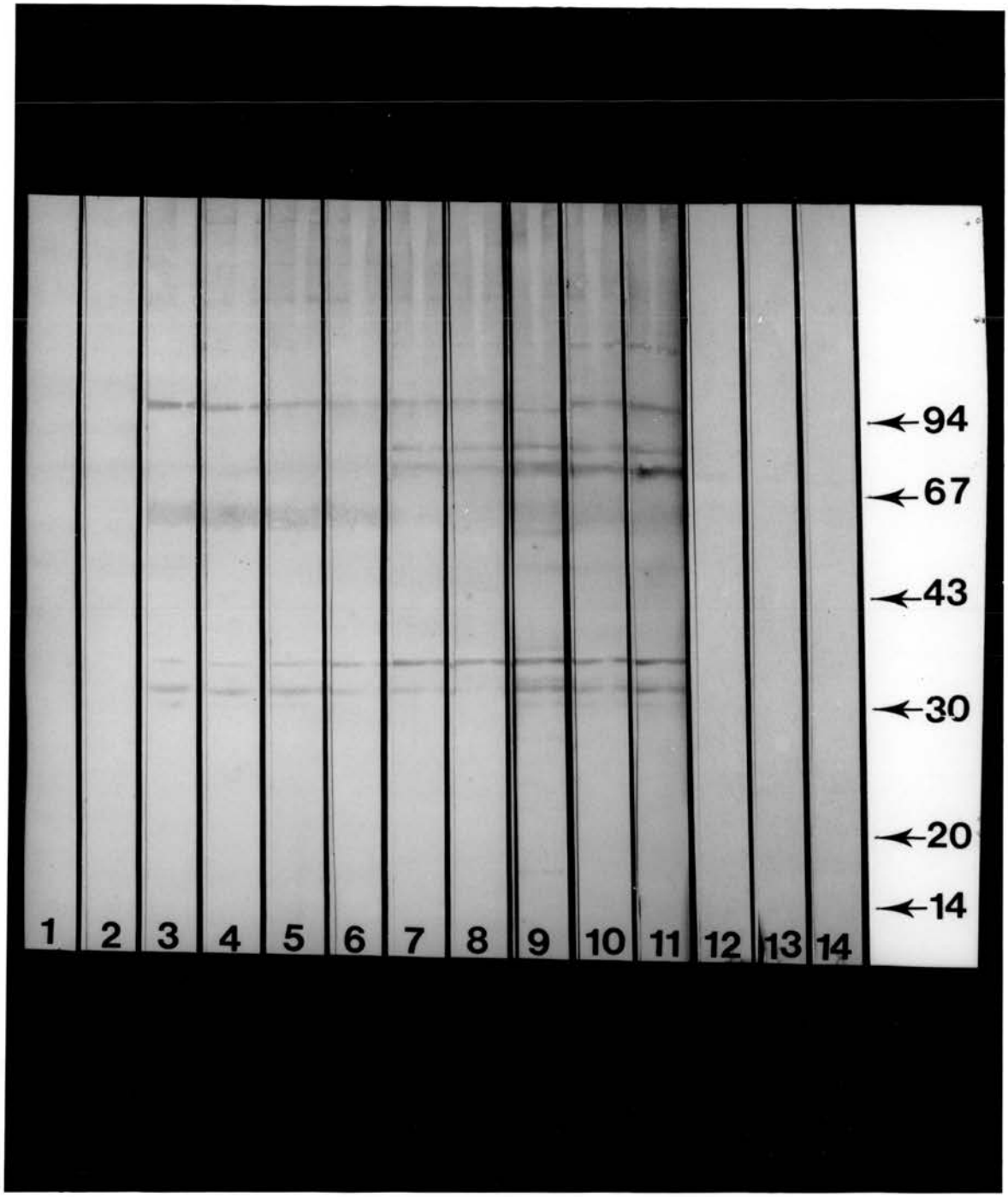
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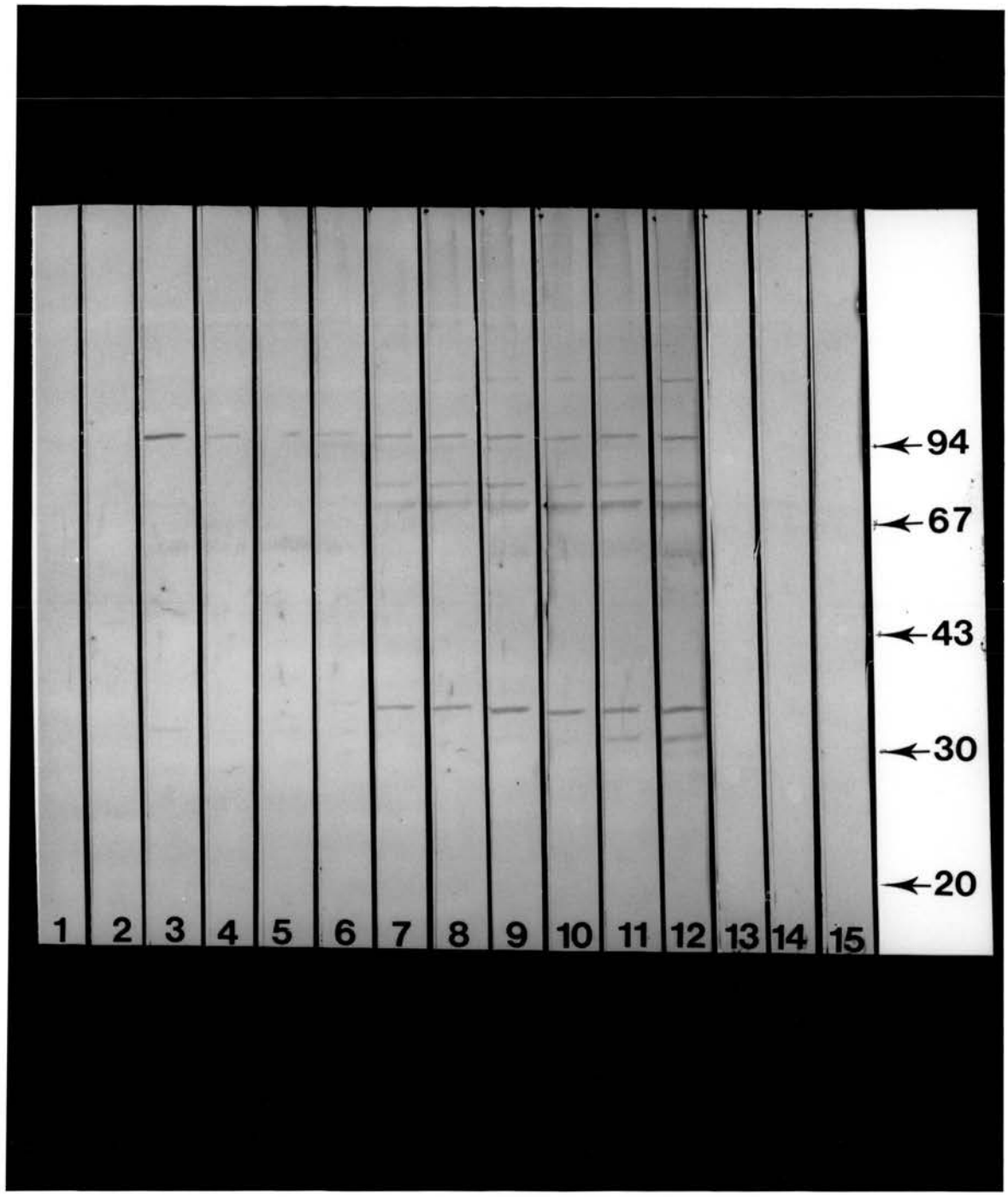
FIGURE 5.6C

Antigens of *T. evansi* TREU 2113 and *T. congolense* identified by IgA antibodies produced by a rabbit during the periods of primary infection with TREU 2113, after drug treatment and challenge with *T. congolense*.

Number 1	Proteins of *T. evansi* TREU 2113 probed with normal rabbit serum.
Numbers 2-5	Proteins of *T. evansi* TREU 2113 probed with days 7, 14, 17 and 21 post infection serum respectively.
Numbers 6-7	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7 and 14 post drug treatment respectively.
Numbers 8-12	Proteins of *T. evansi* TREU 2113 probed with serum samples collected on days 7, 14, 17 and 30 after challenge with *T. congolense*.
Number 13	Proteins of *T. congolense* probed with serum collected 14 days after infection with *T. evansi* TREU 2113.
Number 14	Proteins of *T. congolense* probed with serum collected 14 days after drug treatment.
Number 15	Proteins of *T. congolense* probed with serum collected 21 days after challenge with the same stock.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DISCUSSION

In a study concerned with immunization of animals, the protocol used will influence the results obtained. In the current study the protocol adopted was designed to ensure that the animal had produced antibodies to the maximum number of *T. evansi* antigens before challenge. Previous studies in Chapter 4 had established that these conditions could be met by infection followed by cure from 21 days after infection. In the present study challenges after the primary infection were carried out 30 days after drug treatment to minimise the possible residual prophylactic effect of Berenil^{R} which has been recorded to remain for up to three weeks in cattle (Van Hoeve and Cunningham, 1964). Trypanosomal activity of Berenil in rabbits treated at the dose rate used in the present study has been shown to be absent by 30 days after treatment (T.W. Jones, personal communication). The previously reported phenomenon of parasite relapse following late drug treatment was only observed in two rabbits after drug treatment possibly due to the high dose rate of Berenil used.

The only absolute measure of assessing protection in the case of trypanosomiasis is the absence of parasites from the blood after challenge. This was only achieved in the case of homologous challenge with TREU 2113 (Figure 5.1A). This observation is in agreement with the findings of many workers who clearly demonstrated that both domestic animals and laboratory rodents are rendered resistant to the homologous trypanosome population after infections are followed by drug cure (Lavern and Mesnil, 1902; Ehrlich *et al.*, 1902; Hornby, 1941; Wiesenhutter, 1970; Nantulya *et al.*, 1980b).

The absolute protection seen in animals infected with homologus trypanosomes is likely to be due to the presence of specific antibody production

in particular against the parasite surface antigens because these components were better recognised in these rabbits during the challenge period (Figure 5.2A, Nos. 8-11) than were recognised by the animals challenged with the rest of the trypanosome populations used in the present study in which absolute protection was not recorded. Responses to the internal components of the trypanosomes may have also contributed to the absolute protection observed in the animals challenged with the homologous trypanosome stock as more of the internal components of the parasite were identified by sera collected from these animals than were recognised by sera collected from the other rabbits challenged with the heterologous trypanosome populations. This specific response to the homologous parasite antigens is considered to be anamnestic in nature as would be seen from the increased intensity of labelling and the increased number of parasite components recognised by the antibodies particularly belonging to the IgG class, produced by the animals during the reinfection period (Figure 5.2A, Nos. 8-11). Similar anamnestic responses to antigens of a particular parasite population have been reported for some IgG subclasses by Musoke *et al.* (1981) in cattle infected with *T. brucei*. The role of such an IgG response to protection against *T. evansi* infection, in relation to the activities of IgM and IgA was however, not investigated in the present study but there is ample evidence that it may be playing an important role.

In the case of heterologous challenge, with a different stock of the same parasite species *(T. evansi* TREU 1834; Figure 5.1B) and *T. brucei* (Figure 5.1C) partial protection of animals as indicated by extended prepatent periods and lower parasite numbers during reinfection compared with the respective challenge populations in naive animals was observed. It was considered possible that the partial protection observed in animals challenged with *T. brucei* could be associated with the fact that many antigens of *T. brucei*, one of

which corresponded in molecular weight (67,000 daltons) to one of the surface components of *T. evansi* TREU 2113, were recognised particularly by IgG antibodies (Figure 5.4A, No. 13) some by IgM (Figure 5.4B, No. 13) and IgA (Figure 5.4C, No. 13) in the serum collected from animals after the primary infection with *T. evansi* TREU 2113. Responses in animals challenged with *T. evansi* TREU 1834 were peculiar, however, in that only two components of TREU 1834, one of them again corresponding to the surface component of *T. evansi* TREU 2113 with the molecular weight of 67,000 daltons, were recognised by the IgG antibodies (Figure 5.3A, No. 12) and none by IgM (Figure 5.3B, No. 12) and IgA (Figure 5.3C, No. 12) in the serum collected from animals after infection with *T. evansi* TREU 2113. It is possible that the recognition of the 67,000 dalton component may have contributed to the partial protection observed in these animals in the absence of specific antibodies against other components of TREU 1834 because the 67,000 dalton component was always recognised in all cases of absolute protection in the present study. It is also possible that non-antibody dependent factors in the absence of specific antibodies to the antigens of *T. evansi* TREU 1834 could be involved in the inhibition of the early establishment of the challenge parasite populations and low levels of parasitaemia observed in animals challenged with TREU 1834 during the post-reinfection period (Figure 5.1B). Such a possibility has been advanced by Lloyd *et al.* (1979) to explain the suppressed response they observed in the absence of any evidence of cross-immunity to an antigenically distinct stock of *T. congolense* used in their reinfection studies. These authors considered that this factor might have interfered at some point with parasite replication. The role of non-antibody dependent factors on the development of other species of trypanosomes such as *T. lewisi* and *T. musculi* have been observed by some workers (Targettand Veins, 1979) and it may be

possible that these factors affecting other Stercorarian species of trypanosomes may also be important in the development of some salivarian species of trypanosomes.

Animals reinfected with *T. congolense* and *T. vivax* were not protected against the reinfecting parasite populations in that the prepatent period of the parasites in these test animals were similar to those observed in naive rabbits and also the other fact that the animals had fulminating parasitaemias all through the challenge period (Figure 5.1D and E). The lack of protection observed in these animals is thought to be due to the very limited recognition of *T. vivax* antigens (Figure 5.5A,B and C, No. 13) and none of the *T. congolense* antigens (Figure 5.6A, B and C, No. 13) by the three classes of antibodies under study, present in the serum collected from the rabbits after infection with *T. evansi* TREU 2113.

The most important aspect of the host response in this study appears to be the pattern of antibody response to the different components of the parasite. Results from this study show that when specific antibody response against the surface component is pronounced, as was observed with the rechallenge with homologous species (TREU 2113), where the 67,000 and 60,500 d components (parasite surface-associated components - See Chapter 3 for details) were very prominently identified by antibodies in serum collected when the animals were infected with *T. evansi* TREU 2113, absolute protection occurred. Partial protection was observed in cases such as in the animals challenged with *T. brucei*, where mostly the components corresponding to the non-surface and only one, of molecular weight 67,000, of the surface components of *T. evansi* TREU 2113, used for the primary infection, were identified by antibodies collected from the serum of rabbits after infection with *T. evansi* TREU 2113

(Figure 5.4A, B and C, No. 13). In cases where the antibodies recognised very limited numbers or none at all of both the components corresponding to the surface and non-surface components of *T. evansi* TREU 2113 as was observed in animals challenged with *T. vivax* (Figure 5.5A, B and C, No. 13) and *T. congolense* (Figure 5.6A, and C, No. 13 and 5.6B, No. 12) no protection against the challenge parasite population was observed. These observations are in agreement with the findings of several workers (Wellde *et al.*, 1975; Emery *et al.*, 1980; Morrison *et al.*, 1982; Hall and Esser, 1984) who associated absolute protection to homologous parasite challenge with the production of VSG-specific antibodies.

The close antigenic relationship observed between the stock of *T. evansi* used for all the primary infections, TREU 2113, and *T. brucei* (TREU 1880) is thought to be due to the fact that they belong to the same subgenus - *Trypanozoon*. It is also interesting to observe that most of the cross- reactions between these two species were with the components that correspond to the non-surface component of TREU 2113 which should be expected (Roelants and Pinder, 1984), as the non-surface components are known to be common not only to different stocks but species of trypanosomes. It is difficult to explain why no such relationship was observed between the two stocks of *T. evansi* used in this study. This may indicate that TREU 1834 is not a typical *T. evansi* population in that the same stock of parasites showed peculiar reactions in studies involving several other species of *T. evansi* used in Chapter 3 for studies involving the surface coat in which using the same technique the surface coat of TREU 1834 could not be labelled. The closer antigenic relationship observed between *T. evansi* and *T. vivax* but not with *T. congolense* in this study is interesting. These relationships, if found to apply generally and not just to a few stocks of the different trypanosome species,

could be useful in selecting animals for rearing in areas with a different history of endemicity for the different species of trypanosomes, as results from this study indicate that animals previously exposed to *T. evansi* are likely to suffer a less severe form of *T. brucei* infection than *T. congolense* infection.

Results from the studies in this chapter have therefore, confirmed the importance of antibodies produced against the surface components of a particular trypanosome population in protecting animals from reinfection with the homologous parasites. The observations concerning the possible role of antibody responses to the non-surface component of the parasite and the possibility of other non-antibody dependent factors influencing the outcome of infections in primed animals are some of the important findings of the present study which may be important for the development of alternative control strategies that should be further investigated.

CHAPTER SIX

EARLY EVENTS FOLLOWING CHALLENGE OF RABBITS WITH TRYPANOSOMA EVANSI AND T. EVANSI COMPONENTS

INTRODUCTION

Trypanosomes have to be introduced through the skin of the animal or human host by an arthropod vector before the trypanosome infection can establish. The first reaction to the parasite at the skin barrier, can therefore be important in influencing the outcome of the infection. The most obvious and well documented host's reaction to the introduction of trypanosomes into the skin is the development of the chancre (Burtt and Fairbairn; 1945; De Raadt *et al.,* 1966) a localised skin reaction at the bite site within 7-11 days after infection which then gradually subsides up to one month after infection. Chancres have been observed in a range of hosts such as man and rabbits subjected to infection with *T. rhodesiense* (Fairbairn and Godfrey, 1957; Willet and Gordon, 1957; Emery and Moloo, 1980), and cattle and goats infected through tsetse bites with *T. brucei, T. vivax* and *T. congolense* (Luckins and Gray, 1978; Gray and Luckins, 1980; Emery *et al.,* 1980a; Emery and Moloo, 1981; Luckins, 1981; Dwinger *et al.,* 1987; Dwinger, 1988).

While chancres can be induced by all three species of tsetse- transmitted trypanosomes that infect domestic livestock, the size of the reaction differs between trypanosome species. For any unit parasite inoculation the lesions caused by *T. brucei* are larger and the reaction more intense than those produced by *T. congolense* or *T. vivax* (Emery *et al.,* 1980; Akol and Murray, 1983). Furthermore, certain stocks of *T. congolense* and *T. vivax* do not appear to induce any detectable skin reactions (Luckins and Gray, 1979; Dwinger, 1985).

Results from detailed studies on the properties of the initial host response at the skin level to tsetse-transmitted infections suggest that these reactions are mostly specific immune reactions directed against the parasites. Such reactions

are antibody-mediated and are effected by trypanolysis as has been experimentally demonstrated by several authors (reviewed in Vickerman and Barry, 1982) who clearly linked the development of chancres only with trypanosomes and not with uninfected fly bites or trypanosome extracts.

At the early stages the cell types in the chancre are predominantly neutrophils. This characteristically rapid increase in the number of neutrophils in the early stages of chancre development (Emery *et al.*, 1980) suggests that the development of chancre is not a classical cell-mediated immune (delayed type hypersensitivity) response (Vickerman and Barry, 1982). Furthermore, the chancre does not exhibit the clinical and histological characteristics of the four types of classical delayed type hypersensitivity reaction (Roitt *et al.*, 1989). The fact that the chancre develops in naive animals also rules out the reaction as a hypersensitivity type of reaction. These features along with infiltration by lymphocytes, lymphoblasts, plasma cells, macrophages and eosinophils usually observed in chancre reactions (Emery and Moloo, 1980) suggest that the chancre represents a complex and dynamic combination of an acute inflammatory response coupled with some kind of an immunological reaction evoked by the presence of the trypanosome.

Functionally, the chancre probably plays a dual role in host/parasite relations. The first role which is of advantage to the parasite, is that the chancre could provide focus for the generation of a range of antigenically different parasites prior to the appearance of initial parasitaemia in the host. The second role being the provision of antigens which serve to prime the draining lymph nodes (Emery and Moloo, 1980). This action would be of advantage to the host, as such a priming would set into motion some immunological defence reactions to infection. Results from studies on *T.*

congolense conducted by Luckins and Gray (1979) indicated that the initial antigenic diversification of trypanosomes occurs within the chancre.

No reports of chancre development studies have been made in the case of *T. evansi* infection, possibly as chancres have always been associated with the bites of tsetse flies infected with trypanosomes. Furthermore, the intradermal route is very rarely used for infecting animals experimentally with *T. evansi,* unlike infection induced with the tsetse-transmitted trypanosome species via tsetse flies.

Results from earlier studies on host antibody response to *T. evansi* infections presented in Chapters 3 and 5 have clearly demonstrated the involvement of the surface and non-surface components of the trypanosome in the immune response generated by the host when the trypanosomes were introduced intravenously into the hosts. More importantly, an association was made between the number of the components of the parasites identified by the antibodies produced by the host and the degree of resistance or susceptibility of infected animals. Animals that recognised both the surface and a large number of the non-surface components were found to be more resistant than those that failed to recognise either components well. However, it is unknown whether such responses have any relevance in the very early stages of natural infections introduced through the skin.

The present study was therefore designed to study the nature of reactions developing at the site of intradermal inoculation of *T. evansi* using intact parasites and extracts of trypanosomes in an attempt to understand the host/parasite relation in *T. evansi* infections at the point of first contact between the parasite and the host better.

MATERIALS AND METHODS

TRYPANOSOMES

T. evansi TREU 2147, an immediate derivative of TREU 2113 used in previous studies was used for the infections, superinfections and the preparation of the antigens used for the studies undertaken in this chapter.

ANTIGENS

(a) Whole Trypanosomes

Response to infection with intact trypanosomes was undertaken by inoculation of whole trypanosomes resuspended in 0.1 ml ice-cold PSG (Appendix 2) intradermally into the rabbits at two different doses - 4 x 10^5 (ANTIGEN 1) and 1 x 10^5 (ANTIGEN 2). The trypanosomes were obtained from mice with fulminating infections and separated from the host's blood cells using DEAE cellulose (DE52, Whatman Biochemicals) as described previously before being inoculated into rabbits.

(b) T. evansi Soluble Extract

Host response to the injection of whole trypanosome extract, and isolated surface and non- surface components were based on reactions to appropriate parasite materials obtained from soluble antigen preparations prepared by freeze-thawing as described in Chapter 2. The supernate fraction of such parasite lysate after centrifugation at 10,000 g for 45 minutes at 4^{o}C was made up to 0.1 ml with distilled water and used as ANTIGEN 3.

Fractionation of T. evansi soluble antigens into surface and non-surface components: A soluble extract of *T. evansi* TREU 2147 as prepared above were separated into individual protein components by SDS-PAGE as described

in the final protocol in Chapter 2. The separated protein components were isolated from two sections of the gel by the method described by Gill *et al.* (1986). One section contained components with molecular weights of 67,000 and 60,500 daltons known to be the surface components of the parasite, while the other section contained components known to be non-surface in origin from the studies conducted on the identification of the surface components of the parasite in Chapter 3.

Briefly, 1350 μg total proteins of the soluble extract of *T. evansi* contained in 0.5 ml of SDS sample buffer (Appendix 14) was distributed amongst 13 sample wells and subjected to SDS-PAGE on 7-20% acrylamide gel slabs. After electrophoresis a longitudinal section of the gel containing one track of the *T. evansi* separated proteins and another track containing the standard molecular weight proteins (Pharmacia) was cut out from the gel and stained with Coomassie Blue (Sigma) to visualise the proteins. Using this stained part of the gel as a template, a horizontal strip of the unstained portion of the gel corresponding to the two surface associated components of molecular weights 67,000 and 60,500 on the stained gel section was carefully cut out from the unstained portion of the gel. The gel section containing the two protein components was then transferred into a 10 ml centrifuge tube containing 1 ml ice-cold PBS, macerated with a glass rod and the proteins contained in the gel left to elute for 90 minutes at $4^{O}C$. After centrifuging at 10,000 g for 45 minutes at $4^{O}C$, the supernatant was aspirated, dialysed against distilled water for 24 hours and freeze-dried. Before use the freeze-dried sample was resuspended in 0.1 ml of distilled water and used as ANTIGEN 4. The remaining portion of the unstained gel containing the non-surface components of TREU 2147 was treated in a similar fashion to the portion as for ANTIGEN 4 except that an extraction volume of 2 ml was used. After

freeze-drying the sample was reconstituted in 0.1 ml distilled water and used as ANTIGEN 5.

Control (non-trypanosome) solution: A section of a polymerised gel that had SDS sample buffer and no trypanosome proteins in the wells of the stacking gel during electrophoresis, was cut out and put into a 10 ml centrifuge tube containing 2 ml ice-cold PBS. The gel was treated as in ANTIGEN 5. After dialysis and freeze-drying the sample, the resulting material was reconstituted in 1 ml of distilled water. 0.1 ml of this preparation was used as SDS-PAGE control solution.

The protein concentration of all the trypanosome fractions were determined photometrically using BCA^R (Pierce Chemical Company, USA).

EXPERIMENTAL ANIMALS

(1) Infected Animals

Two adult female New Zealand White rabbits were infected with 4 x 10^5 motile trypanosomes through the ear veins. Each animal was allowed to carry the infection for 21 days before they were each injected intradermally with 0.1 ml PSG containing 4 x 10^5 motile trypanosomes (ANTIGEN 1), 1 x 10^5 motile trypanosomes (ANTIGEN 2), 33 μg protein of ANTIGENS 3, 4 and 5 and the control solution respectively. Each antigen administration was duplicated on each side of the back giving rise to four tracks of antigens, running longitudinally from the shoulders to the hindquarters as shown in Figure 6.1. The preparation of the animals prior to the inoculation of the antigens and control solution involved the shaving of both sides of the back 24 hours before the samples were injected. One hour before the samples were inoculated the shaved portions of the back were sterilised with 70% alcohol.

(2) Infected and Drug Treated Animals

Two adult female New Zealand White rabbits were infected through the ear vein with 4 x 10^5 motile trypanosomes as above. The animals were allowed to carry the infection for 21 days before they were treated with 7 mg (active principle) of diaminazene aceturate (Berenil^R, Hoechst, W. Germany) injected intramuscularly (i/m) deep into the thigh muscles per kg body wt. Twenty five days after drug treatment the animals were each inoculated intradermally with ANTIGENS 1 to 5 and the control solution as in the case of the infected animals.

Infected animals were examined for the presence of trypanosomes in the blood 7, 14 and 21 days after infection and on days 7, 14 and 25 days post drug treatment in the animals that were drug treated, by the buffy coat microhaematocrit method (Woo, 1970).

(3) Uninfected Control Animals

Two adult female New Zealand White rabbits, without previous exposure to *T. evansi* or *T. evansi* extracts were used as control animals in this study. One rabbit served as a control for the infected rabbits and the other for the drug treated rabbits. The animals were injected with ANTIGENS 1 to 5 and the control solution on equivalent days as the infected and the infected and drug treated rabbits.

RESPONSE TO ANTIGEN ADMINISTRATION

Skin Thickness

A double skin thickness was formed by pinching up the skin at the site of the antigen inoculation with the fingers before measurements were taken from

each site with vernier callipers in mm. These measurements were taken before the antigens were inoculated and subsequently at 2, 24 and 48 hours after the administration of the antigens. The 2, 24 and 48 hour skin measurements were taken from all the sites of antigen inoculation along three longitudinal tracks, one (Lane B) and two (Lanes C and D) tracks on each side of the back respectively (Figure 6.1).

Cellular Infiltration

Biopsies were taken after the animals had been sedated by infiltrating Xylocaine-HCl locally through the subcutaneous (s/c) route in such a way that the parts of the body from which samples were obtained were completely anaethetised in that animals did not react to pin pricks on those parts.

Two hour post antigen inoculation skin biopsies were collected from each antigen inoculation site along one track (Lane A) on one side of the body, with the 3 mm biopsy punch (Baker Laboratories Ltd.). The 48 hour skin biopsies were collected from each site of antigen inoculation from two (Lanes B and C) of the three remaining tracks, one track from each side of the back.

Each skin biopsy was divided into two equal parts, one for light and the other for electron microscopic studies. Tissues for light microscopic studies were fixed in 10% buffered formal saline. Skin sections 5 μm thick were cut from paraffin-embedded blocks prepared by conventional methods and stained with Giemsa or Mayers haematoxylin and eosin (Cook, 1974). Tissue samples destined for electron microscopy were fixed in 2.5% glutaraldehyde in cacodylate buffer pH 7.2 (Appendix 12) and processed for electron microscopic studies using the methods described in Chapter 3 with the only modification being that tissues were fixed until required for processing.

RESULTS

PARASITAEMIA

Results of microscopal examination of the buffy coat of blood samples, revealed parasites in the blood of the infected rabbits on day 21 post infection in all the infected rabbits before the antigens were administered. Parasites were not seen in the blood of the drug treated animals on 7, 14 and 25 days after drug treatment.

LOCAL SKIN REACTIONS IN RABBITS AFTER INTRADERMAL INOCULATION OF T. EVANSI AND T. EVANSI COMPONENTS

(a) Changes in skin thickness

Each of the five antigens (ANTIGENS 1-5) and the control solution caused some changes in skin thickness at the sites of inoculation in all animals. The greatest increases being observed in the infected and drug treated rabbits and the least in the uninfected naive rabbits used as control animals.

Inoculation of live trypanosomes (ANTIGENS 1 and 2) into naive rabbits resulted in a mean increase in skin thickness in the two rabbits of 0.9 mm (ANTIGEN 1) and 0.57 mm (ANTIGEN 2) at 48 hours post-antigen inoculation skin compared to pre-antigen inoculation measurements. Inoculation of live trypanosomes resulted in a continuous increase in skin thickness that took place from 2 to 48 hours post antigen inoculation (Figure 6.2A). The greatest increase in the skin thickness in naive rabbits, with both antigens, was seen 48 hours post inoculation (Table 6.1).

Inoculation of trypanosome extracts (ANTIGENS 3 to 5) into naive rabbits resulted in a mean increase in the skin thickness of the two rabbits of 0.17 mm with ANTIGEN 3, 1.00 mm with ANTIGEN 4 and 0.13 mm with

ANTIGEN 5 by 48 hours after inoculation. Skin thickness was observed to decrease by 48 hours after the administration of each of these antigen (Figure 6.2A). The greatest increase in skin thickness, for this group of antigens, was obtained 24 hours after antigen inoculation (Table 6.1).

Inoculation of live trypanosomes (ANTIGENS 1 and 2) into the infected rabbits resulted in a mean increase in the skin thickness of the two rabbits of 1.3 mm (ANTIGEN 1) and 1.02 mm (ANTIGEN 2) at 48 hours post-antigen inoculation compared to the pre-antigen inoculation skin measurements. The skin thickness decreased in these rabbits 48 hours after the administration of the antigen (Figure 6.2B), with the greatest increase in the skin thickness seen 24 hours post inoculation (Table 6.1).

Inoculation of trypanosome extracts (ANTIGENS 3 to 5) resulted in a mean increase in the skin thickness of 0.85 mm with ANTIGEN 3, 1.98 mm with ANTIGEN 4 and 0.96 mm with ANTIGEN 5 48 hours post-antigen inoculation. By 48 hours after antigen inoculation decreases in skin measurements were observed (Figure 6.2B). The greatest increase in skin measurements were obtained 24 hours after inoculation (Table 6.1).

Inoculation of live trypanosomes (ANTIGENS 1 and 2) into rabbits infected with *T. evansi* and treated with Berenil resulted in a mean increase in the skin thickness of 1.50 mm (ANTIGEN 1) and 1.25 (ANTIGEN 2) by 48 hours post-antigen inoculation. The skin thickness decreased in both these rabbits at 48 hours after the administration of the antigen (Figure 6.2C) with greatest mean increase in skin thickness obtained 24 hours after antigen inoculation (Table 6.1). Inoculation of trypanosome extracts (ANTIGENS 3 to 5) resulted in a mean increase in the skin thickness of the two rabbits of 1.05 mm (ANTIGEN 3), 3.00 mm (ANTIGEN 4) and 1.25 mm (ANTIGEN

5) at 48 hours post-antigen inoculation. The skin thickness measurements were observed to decrease at 48 hours after antigen inoculation (Figure 6.1C) with greatest increases in skin measurements obtained 24 hours after antigen inoculation (Table 6.1).

Inoculation of the control solution resulted in a slight increase in the skin thickness in all the three groups of rabbits. This ranged from an average value of 0.02 in naive rabbits, 0.42 mm in the infected animals to 0.46 mm in the infected and drug treated animals. The skin thickness measurements were observed to decrease in all the animals 48 hours after sample administration (Figure 6.1C) with the greatest increase obtained 24 hours post sample inoculation (Table 6.1).

FIGURE 6.1

(a) Numbers 1-5 indicate ANTIGENS and sites of inoculation. No. 6 = control solution.

(b) Skin measurements for the 2, 24 and 48 hour post antigen inoculations repsectively were obtained from all the antigen inoculation sites along LANES B, C and D.

(c) Tissues for 2 hour post antigen inoculation biopsies were obtained from all antigen inoculation sites in LANE A while samples were obtained from LANES B and C for the 48 hour post antigen inoculation biopsies.

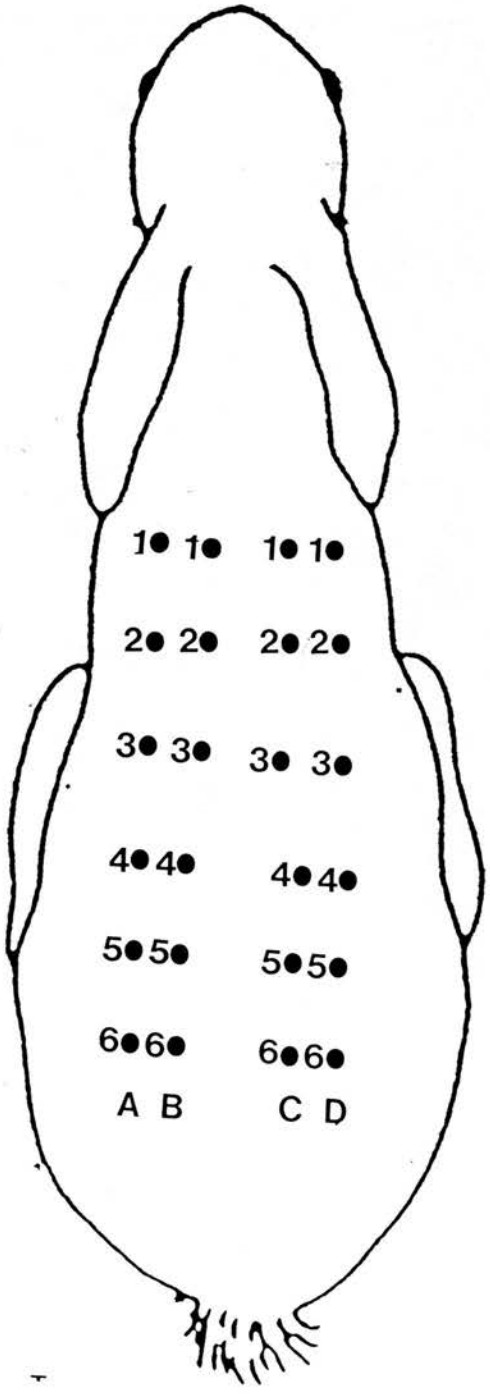

1 1 1 1
2 2 2 2
3 3 3 3
4 4 4 4
5 5 5 5
6 6 6 6
A B C D

FIGURE 6.2

Increase in skin thickness of rabbits challenged with *T. evansi* and *T. evansi* components.

A Uninfected rabbits.

B Rabbits infected with *T. evansi* before challenge.

C Rabbits infected with *T. evansi* and treated with Berenil before challenge.

FIGURE 8.1

Class-specific antibody production in primed rabbits challenged with *T. evansi* soluble antigens of immune complexes.

A and B Rabbits challenged with *T. evansi* soluble antigens.

C and D Rabbits challenged with immune complexes formed with *T. evansi* soluble antigens and post infection serum.

Table 6.1 Average increase in skin thickness (mm) in the three groups of rabbits between skin measurements at 0 hours and at any time of maximum skin thickness

Antigens	1	2	3	4	5	6
Infected rabbits	1.50	1.15	0.95	2.02	0.98	0.70
Infected and drug treated rabbits	1.70	1.35	1.15	3.53	1.25	0.68
Naive rabbits	+ 0.8	+ 0.57	0.09	1.02	0.17	0.06

(1) +: Highest mean skin measurement obtained after 48 hours and the rest after 24 hours post antigen inoculation.

(2) Mean measurement of normal skin thickness (mm): 1.45

HISTOLOGICAL EXAMINATION OF SKIN BIOPSIES

Microscopal examination of sections prepared from tissue samples collected from the sites of antigen inoculation, in the three groups of animals, revealed similar types of cellular infiltration with differences restricted to the cell types seen at the different sampling times of 2 and 48 hours post-antigen inoculation. The differences seen were in the magnitude of cellular infiltration at the site of antigen inoculation with the highest level of infiltration in each case occurring in the infected and drug treated animals. Minimal cellular infiltration was observed in sections prepared from tissues obtained from sites where the control solution was inoculated.

Cellular Reactions in Skin Biopsies Collected Two Hours after Antigen Inoculation

Animals showed severe signs of inflammation at the sites of inoculation two hours after ANTIGENS 1-5 were injected into the skin of animals. The intensity of inflammation appeared greatest at sites where ANTIGEN 4 was injected in all groups of rabbits. Microscopal examination of biopsy sections prepared from tissues collected from the animals two hours after the injection of the antigens revealed marked cellular infiltration, congestion and oedema. The cell types present were predominantly neutrophils and eosinophils with one or two mononuclear cells in each microscopic field. These mononuclear cells were mainly lymphocytes. Blood vessels were often clogged by these cells and large numbers of red blood cells. Cellular aggregation was greatest in the dermis with cellular infiltration most pronounced at sites where ANTIGEN 4 was injected in all the groups of animals (Figure 6.3A and B) In general, fewer numbers of polymorphonuclear leucocytes, neutrophils and eosinophils, were

seen at sites where live trypanosomes were injected (ANTIGENS 1 and 2) than where the trypanosome extracts (ANTIGENS 3, 4 and 5) were inoculated. Very few cells were seen in the sections prepared from tissues obtained from the normal skin of rabbits where no antigens were injected (Figure 6.5B) and from sites where the control solution was injected (Figure 6.6A). Trypanosomes were not observed in sections prepared from tissues collected two hours after antigen inoculation even in the sites where the highest numbers of trypanosomes (ANTIGEN 1) were inoculated.

Cellular Reactions in Skin Biopsies Collected 48 Hours after Antigen Inoculation

Signs of acute inflammation were reduced in all animals 48 hours after the inoculation of all the antigens. Animals were however, still sensitive to touch at the sites of antigen inoculation because of inflammation particularly when the teeth of the vernier callipers were closed up on the skin.

Cellular infiltration seen in sections prepared from all tissues 48 hours after antigen inoculation were dominated by mononuclear cells with perivascular cuffs formed around blood vessels (Figure 6.5A). The cell types identified were predominantly lymphocytes, some monocytes and plasma cells and a few macrophages. Most of the cellular changes were observed in the dermis. Few trypanosomes were seen in the section appearing as single organisms in some of the tissue sections (Figure 6.5B). Neutrophils and eosinophils were observed in sections from sites where the trypanosome components (ANTIGENS 3 to 5) were inoculated, unlike those sites where live trypanosomes (ANTIGENS 1 and 2) were injected in which mostly mononuclear cells were seen.

Control Biopsies

Minimal reaction to the control solution was seen in all cases with a few neutrophils and eosinophils seen scattered throughout sections obtained two hours after inoculation. A few mononuclear cells were seen in sections prepared from tissues collected 48 hours after the inoculation of the control solution (Figure 6.6A).

Microscopal examination of sections obtained from tissue collected from sites where no antigens were injected revealed the presence of few mononuclear cells. The expected structural components of the normal skin, such as fibroblasts and reticulum cells were present (Figure 6.6B).

Electronmicroscopal Examination

Electron microscopal examination of biopsy sections revealed that the neutrophils were activated at sites where ANTIGEN 4 was injected as they were seen engulfing many particulate materials, some of which appeared to be collagen fibres (Figure 6.4A to C). No such activation was seen in sections prepared from tissues where the other antigens and the control solution were injected (Figure 6.3D).

FIGURE 6.3

Skin sections prepared from tissue collected 2 hours after ANTIGEN 4 was injected intradermally into *T. evansi* infected and Berenil treated rabbit.

(A: shows clusters of polymorphonuclear leucocytes, H & E x 150; B: shows blood vessel clogged with polymorphonuclear leucocytes and erythrocytes predominantly with one to two mononuclear cells, H & E x 1,500.

Figure 6.3

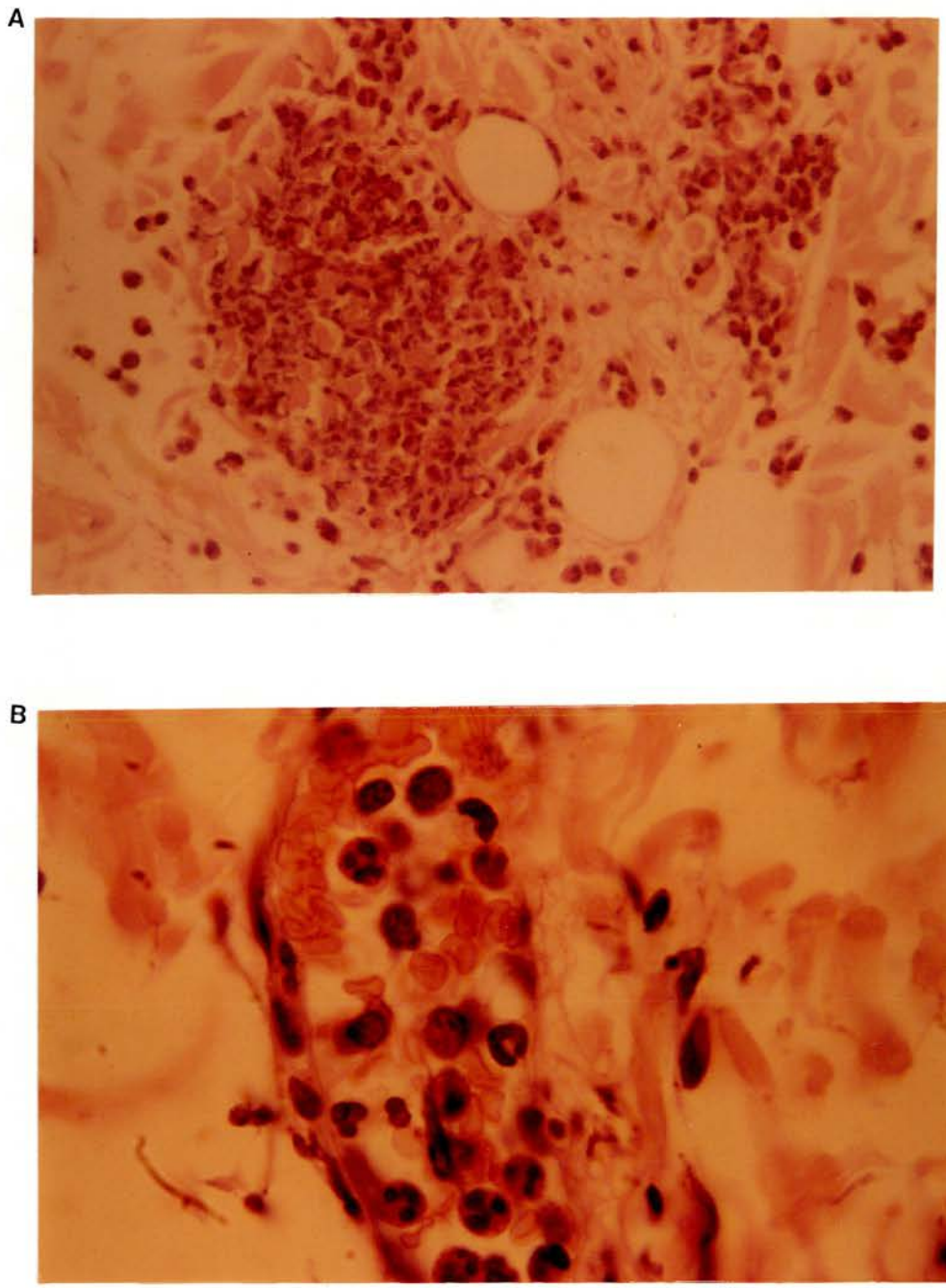


FIGURE 6.4

Cellular activity in sections from tissues obtained from sites of ANTIGEN inoculation 2 hours post-antigen administration.

(A: x 4,600; B: x 7,700; C: x 10,000 show activated neutrophils at different stages of phagocytosis; D: x 2,500 shows activities at a site where the control solution was injected.

All tissues were obtained from infected and Berenil treated animals.)

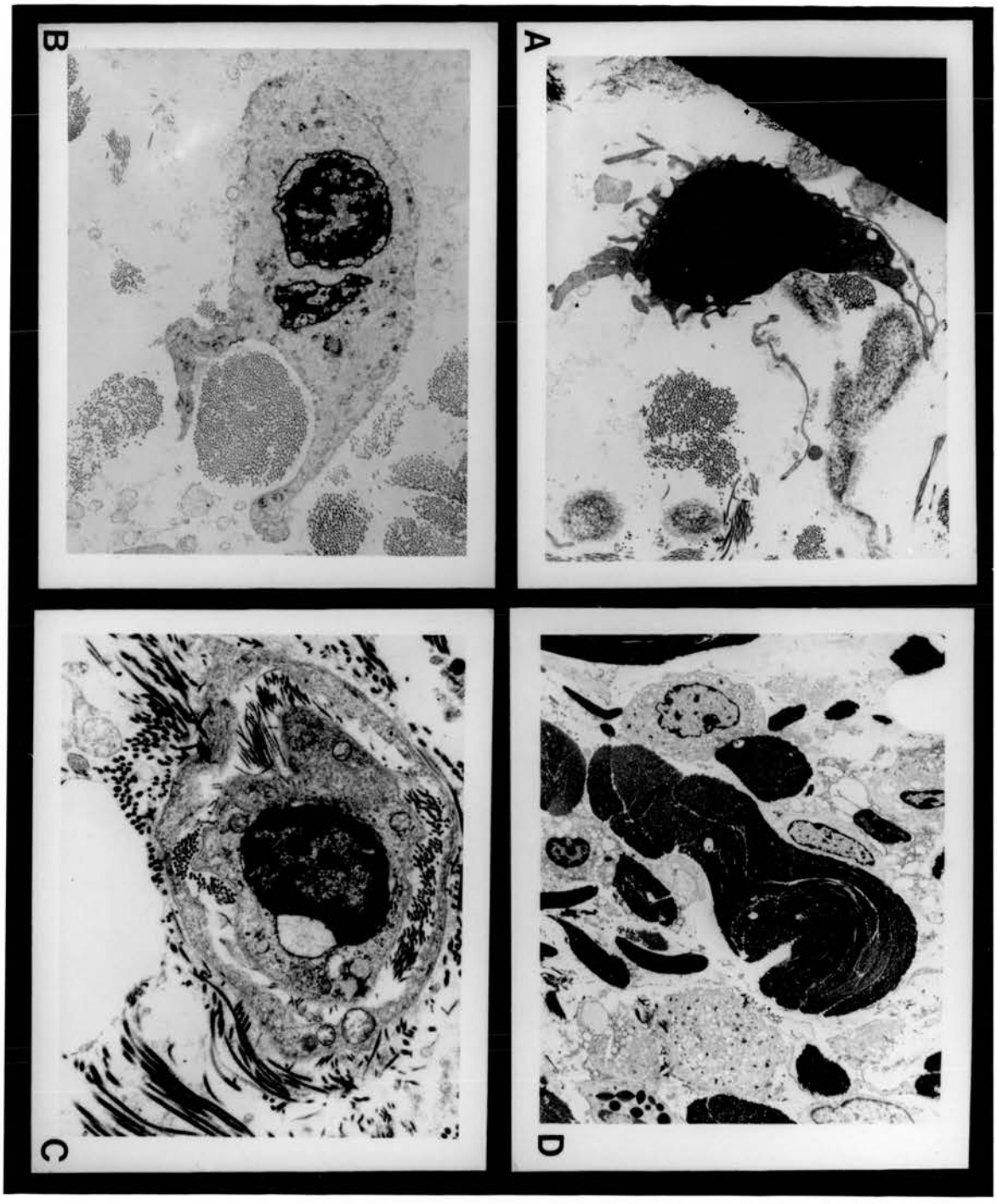

A
B
C
D

FIGURE 6.5

Giemsa'a stained section prepared from tissue collected 48 hours after live trypanosomes (ANTIGEN 1) were intradermally injected into *T. evansi* infected and Berenil treated rabbits.

A shows clusters of mononuclear cells with many of them forming distinct cuffs around blood vessels. x 500.

B shows a single trypanosome (T) and some mononuclear cells identified in the skin section. x 1,500

Figure 6.5

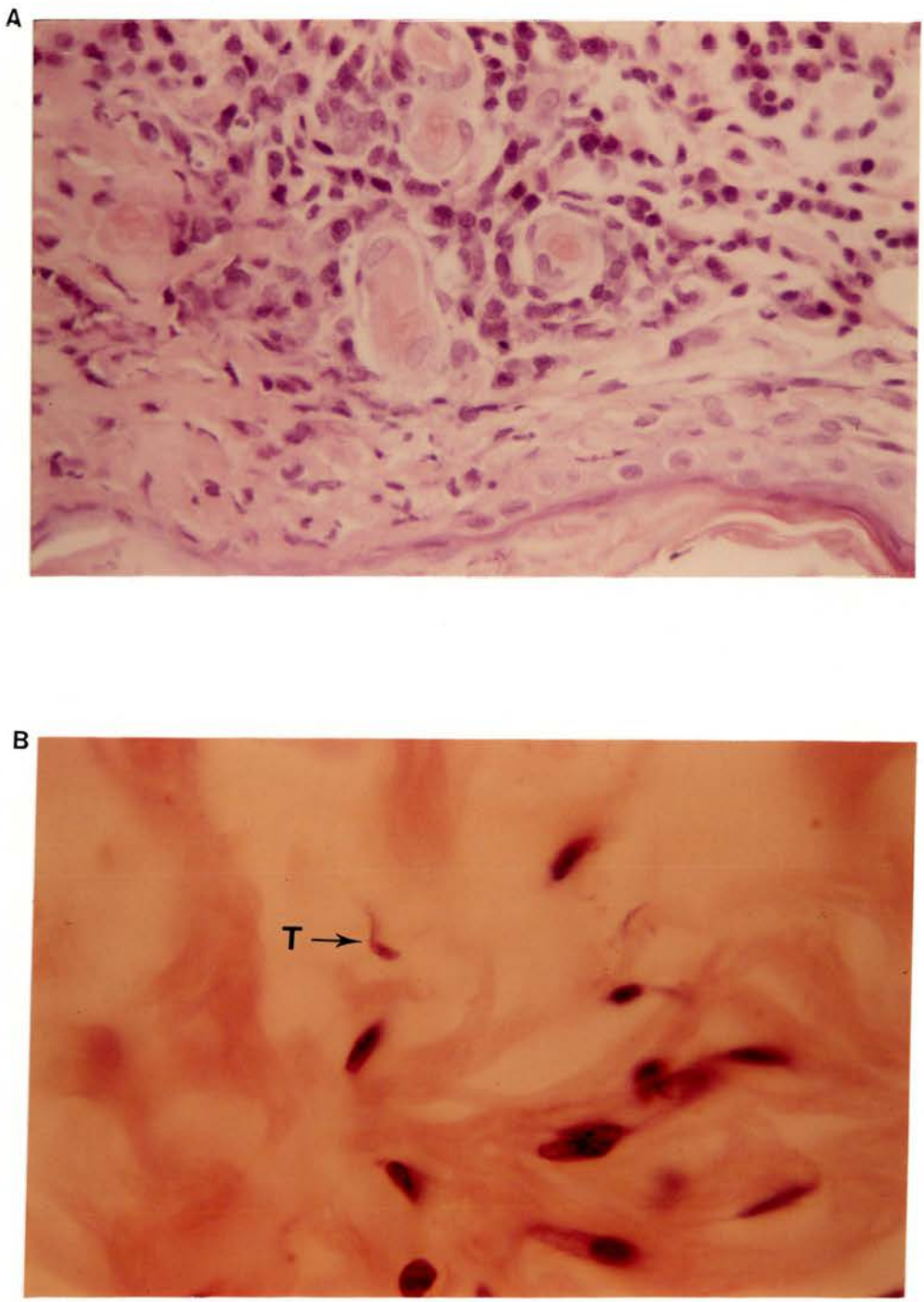


FIGURE 6.6A

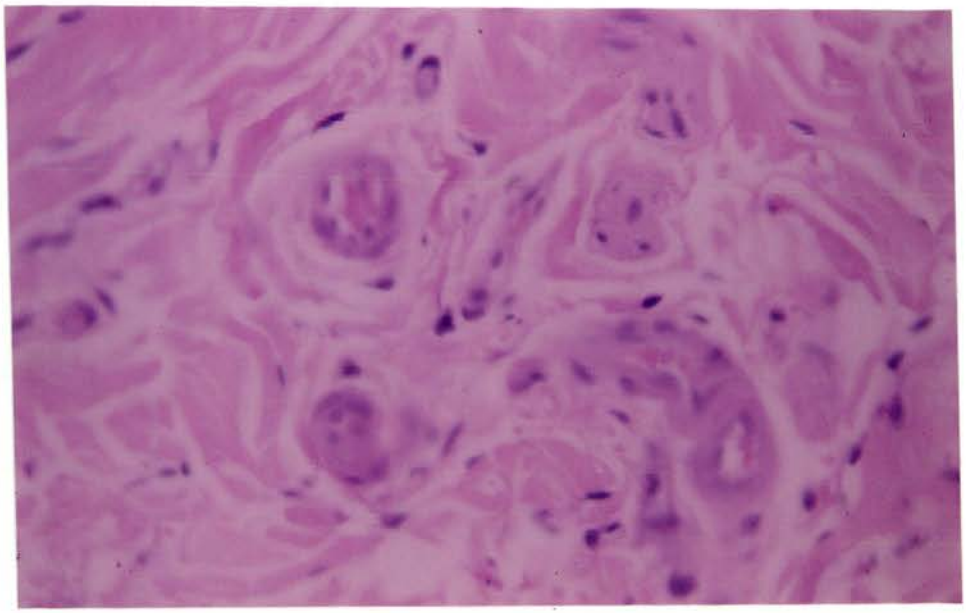
Section from tissue collected 48 hours after the control solution was injected intradermally into *T. evansi* infected and Berenil treated rabbit, showing a few mononuclear cells. x 500.

FIGURE 6.6B

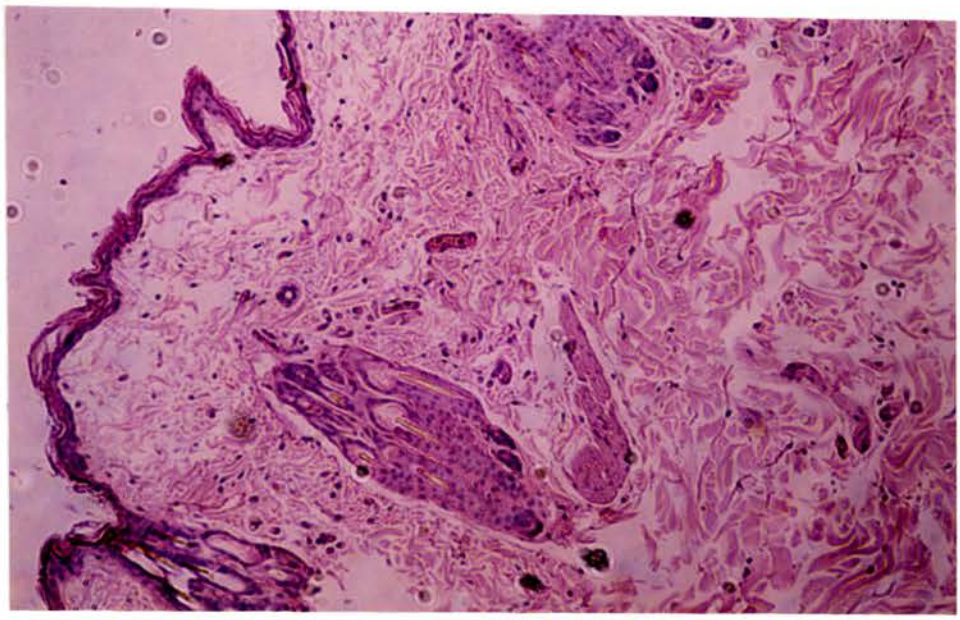
Giemsa's stained section from tissue collected from the skin of an uninfected rabbit that did not receive any intradermal ANTIGEN inoculation, showing a few mononuclear cells scattered in the dermis of the skin. x 150.

Figure 6.6

A



B



DISCUSSION

Some form of reaction was observed at sites where each of the antigens were inoculated. Differences were seen however, in the magnitude of the response as indicated by changes in skin thickness and also in the degree of cellular infiltration at each antigen inoculation sites. Most marked changes were observed in animals that had experienced an infection previously when compared with naive animals.

The reaction of naive animals used in this study would be comparable to animals being exposed to trypanosomiasis in the field for the first time. They all responded to the intradermal inoculation of the parasites and parasite components by developing a palpable swelling at the site of antigen inoculation (ANTIGENS 1-5). Swellings of a lesser magnitude were observed at sites where the control solution was injected indicating that the bulk of increased skin thickness observed at the sites of antigen inoculation was due to the trypanosome or trypanosome components injected into those areas. A similar observation was made by Dwinger (1985) who considered that these increases in skin thickness and cellular activity at sites of antigen inoculation were associated with trypanosomal factors. The swellings observed in the present study showed all signs of acute inflammation - hotness, red colouration and pain 2 and 24 hours after antigen inoculation. By 48 hours after the antigen inoculation, reactions at the sites of inoculation were less intense. While the swellings at the site of inoculation of live trypanosomes in the naive animals increased throughout the period of observation, swellings at the sites where the rest of the antigens (ANTIGENS 3 to 5) and the control solution were injected were observed to reduce 48 hours after inoculation.

The continuous increase in skin thickness observed at sites where live trypanosomes were injected in the naive animals (Figure 6.2A) compared with that obtained with infected (Figure 6.2B) and the infected and drug treated rabbits (Figure 6.2C) which showed a reduction in the skin thickness by 48 hours after the inoculation of the trypanosomes, is probably the result of the fact that absence of trypanosome-specific antibodies in the naive animals, allowed more parasite multiplication to take place in the skin of naive animals before the invasion of the circulatory system by the parasites (Emery and Moloo, 1980). Specific antibodies produced by trypanosome-infected animals are known to destroy the parasites in the skin by trypanolysis (reviewed in Vickerman and Barry, 1982).

The general reduction in skin thickness when the other antigens and control solution were injected in all groups of animals is probably due to the fact that the preparations were cleared rapidly from the site of inoculation by the host immune system.

All the animals showed varying degrees of response to both the surface and the non-surface components of the parasites, a probable indication that the host immune response to the two components of the parasite, demonstrated in the previous studies reported in Chapter 3 and 4 is effective at the first point of contact between the host and the parasite at the skin level. The fact that the host response was strong against the surface (ANTIGEN 4) than the internal (ANTIGEN 5) components in all the animals is in agreement with previous findings of this study reported in Chapter 4 and the findings of Le Ray (1975). The fact that these responses and in particular those against the surface components were most marked in the animals that had previously experienced an infection by the same parasites (Figure 6.2C and Figure 6.2B)

when compared with those of the naive animals (Figure 6.2A) may be an indication that absolute protection against reinfection by a homologous trypanosome population due to the activities of VSG-specific antibodies produced by the host (Herbert and Wilkinson, 1977; Morrison *et al.*, 1982; Hall and Esser, 1984) may be equally effective at the skin level, as in the bloodstream.

Apart from the role of antibodies in destroying the parasites, it is possible that effector cells sensitised as a result of the previous exposure to the antigen could play an important role in the generation of immune response on second exposure of the animal to the antigen at the skin level. This is likely as a greater amount of cellular reaction was observed in the infected animals than in the naive animals. The reduced level of reactions observed in animals still carrying patent infections by the time they were challenged (Figure 6.2B) in comparison with the drug treated animals (Figure 6.2C) could be associated with an inhibited immunological response reported in trypanosome infected animals (Murray *et al.*, 1974c).

The cells types identified in the cellular infiltrate at the sites where the antigens were injected two hours after administration (Figure 6.3A and B) and particularly the greater magnitude of response seen in animals previously exposed to infections by homologous parasites are characteristic of immediate type hypersensitivity reaction. Furthermore, the cell types in the infiltrate and also the magnitude of response in sensitised animals 48 hours after the administration of the antigens (Figure 6.5A and B) are characteristic of delayed type hypersensitivity reactions (Roitt *et al.*, 1989). Similar reactions have been reported by Dwinger (1985) in sensitised goats exposed to bites from *T. congolense* infected tsetse flies which he thought were normal reactions that

precede the establishment of the chancre.

The presence of activated neutrophils at sites where the surface components (ANTIGEN 4) were injected (Figure 6.4A to C) which were absent from sites where the rest of other antigens (ANTIGENS 1, 2, 3 and 5) and the control solution (Figure 6.4D) were injected, probably suggests that the surface component of the trypanosome might be an important factor in the induction of intense inflammatory responses routinely observed at sites where trypanosomes or trypanosome components are injected into the skin of an animal such as those of Seed (1969) and Dwinger (1985).

In conclusion, the results of the experiments conducted in this chapter have demonstrated that both the surface and non-surface components of the parasite induce a response at the skin level when introduced into both naive and sensitised hosts, with the surface components eliciting more intense reactions than the non-surface components. Furthermore, there were indications that the development of a state of immediate type hypersensitivty reaction, which preceded the development of a delayed type reaction, may also be important in influencing the outcome of challenge of previously infected animals with *T. evansi*. The overall effect of such conclusions is that the reactions at the skin level could be important in the development of *T. evansi* infections and probably operating in a similar way to those described for the tsetse transmitted species of trypanosomes (Luckins and Gray, 1979; Emery and Moloo, 1980).

CHAPTER SEVEN

COMPLEMENT (C3) LEVELS AND ACTIVATION IN RABBITS EXPERIMENTALLY INFECTED WITH TRYPANOSOMA EVANSI

INTRODUCTION

Complement has long been known to have important effector functions in the immune response, such as the opsonization of particles for ingestion by phagocytic cells (Staines *et al.*, 1985; Klaus and Ursul, 1986), promotion of lysis of microorganisms (Murray and Urquhart, 1977; Shirazi *et al.*, 1980) and the development of inflammatory responses (Frank, 1975; Staines *et al.*, 1985).

A fundamental property of the complement system is its component nature which involves the breakdown or activation of certain of the complement components which in turn gives rise to new products which then act directly or indirectly to trigger off a series of effector reactions ultimately leading to the elimination of an infective agent. Some of these reactions affect the host itself such as mediating changes in vascular permeability with consequent attraction of polymorphonuclear and mononuclear leucocytes into an area where foreign bodies or infectious agents are present (Frank, 1975). The products of complement activation are also known to bring about directly T-cell independent B cell activation which leads to the differentiation of B cells into plasma or antibody producing cells (Pryjma *et al.*, 1974). The activities of the complement system act in concert with other arms of the immune system in the elimination or control of invading microorganisms in an infected animal.

A total of 30 individual proteins are recognised as part of the complement system (Lambris, 1988). Nine of which (C1 to C9) are the major ones which are directly involved in what is commonly referred to as the complement cascade whose activation leads to the elimination of the intruding pathogen. The activation of the complement cascade is brought about through two interrelated routes referred to as the classical and alternative pathways. The ultimate result of both pathways is the elimination of the offending pathogen

through cell lysis, phagocytosis, opsonization or other chemotactic processes that lead to the development of inflammatory responses. Such responses serve to localise the foreign particles before they are destroyed by host defensive cells (Staines *et al.*, 1985; Roitt *et al.*, 1985).

The most thoroughly studied of the two pathways of complement activation, is the classical pathway. This is initiated by the presence of antigen-antibody complexes and requires the binding of antibodies to the antigen before it can be activated. The presence of antibody belonging to the immunoglobulin classes IgG and IgM in the complexes are considered to be the only ones that can activate the classical complement pathway (Bowry, 1984). The alternative pathway can be activated in the absence of antibodies with some proteins such as bacterial cell walls and trypanosome components bringing about the direct activation of the pathway (Table, 1982; Staines *et al.*, 1985). Of the five classes of antibodies only IgA and IgE are known to bring about the activation of the alternative pathway (Frank, 1975). In practice both pathways act together to bring about the destruction and removal of the foreign particles or infectious agents.

Of the many individual elements identified as part of the complement systems, a single component - C3 - has been the focus of intensive studies mainly due to its functional versatility and the fundamental role it plays in the activiation of both the classical and alternative pathways (Lambris, 1988). C3 can be activated via the alternative pathway by the direct action of parasites or their fragments or indirectly via the classical pathway by an enzyme known as C3 convertase. This enzyme is only formed after the antigen/antibody complex formed as a result of the host's response to the presence of the parasite has activated a single complement component - C1 which subsequently cleaves two

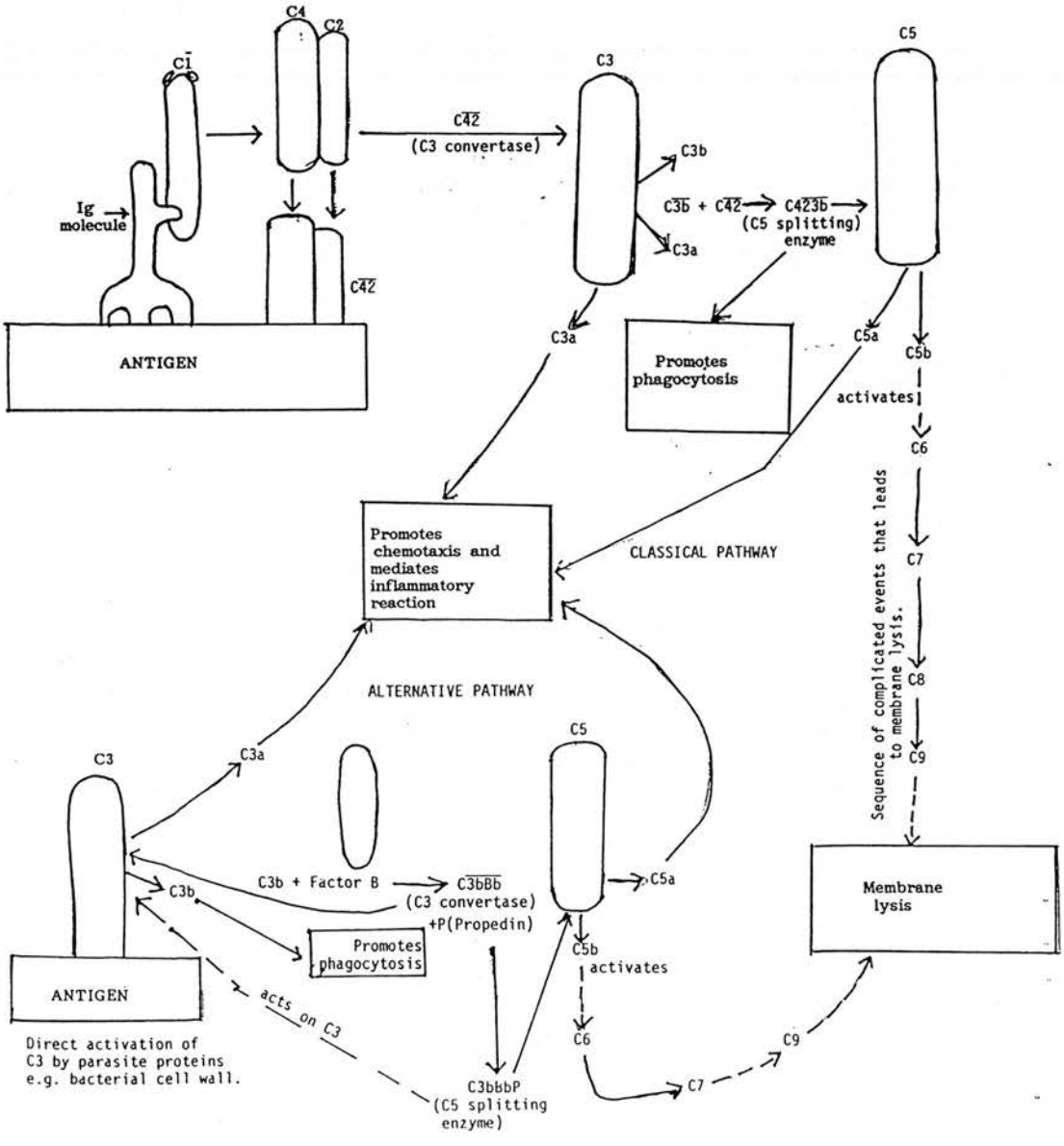
further components - C4 and C2. The activation of C3 by both pathways leads to the activation of the complement cascade, a chain of complicated events that serves to eliminate the pathogen. The range of activities that result in the activation and development of the two pathways - classical and alternative, are shown in Figure 7.1.

The cleavage of C3 results in the production of two new complement components - a smaller C3a, and a larger fragment, C3b. These fragments either react with other complement components resulting in enzymes that play an important role in the development of the appropriate pathways or act directly mediating the development of other host effector functions such as opsonization. C3b will combine with the existing enzyme C3 convertase to form another enzyme which cleaves another complement component - C5. The cleavage of C5 ensures the subsequent activation of the rest of the complement components (C6 to C9) to the point that lysis of the microorganism is achieved. C3b also plays a major role in the activities of the alternative pathway by combining with factor B to produce an enzyme complex with C3-splitting activity and also in the opsonization of parasites (Gigli and Nelson, 1968). Furthermore, it has been shown that a number of defence cells in the body such as B lymphocytes, polymorphonuclear neutrophils and macrophages have surface receptors for C3b (Lay and Nussenzweig, 1968). Subsequently, host cells or pathogens with C3b on their surface will adhere to any of these cells with C3b receptors. The consequence of this adherence will depend upon the particular cell type, in the case of phagocytic cells, such adherence will lead to the ingestion of opsonized particles etc.

C3a can act directly and mediate the formation of inflammatory responses through its chemotactic activities and effect on mast cells which cause them to

FIGURE 7.1

Schematic representation of the complement activation through classical and alternative pathways.



release histamine (Dias *et al.*, 1967; Staines *et al.*, 1985).

Apart from the role of C3 in the activation of the complement cascade and subsequent destruction of parasites, C3 has also been demonstrated to play a major role in the generation of B memory cells (Klaus and Humphrey, 1977). These cells are a specialised subgroup of B lymphocytes, which represent a small percentage of the total B cell population in circulation when the immune system encountered a particular antigen the first time. The memory cells bear the memory of the initial exposure and are expanded in number when the host immune system encounters the same antigen again. This response boosts the immune response to the particular antigen at the time of a secondary immune response (Irvine, 1979). C3 has also been demonstrated to enhance cellular cooperation possibly by facilitating antigen processing and presentation (Pepys, 1972; Pepys *et al.*, 1976). Furthermore it has been suggested that the binding of activated C3 to C3 receptors on B cells (Bianco *et al.*, 1970) might provide a necessary "second signal" for T-cell-independent B-cell activation (Dukor and Hartmann, 1973) leading to the production of antibodies by B cells without the influence of T cells.

Studies conducted on the role of complement in trypanosome infection particularly C3, are limited and the results obtained often equivocal. For instance, in mice depleted of complement and infected with *T. cruzi,* significant increases in parasitaemia and mortality were recorded when compared with non-complement depleted *T. cruzi* infected mice (Budzko *et al.*, 1975; Keirzenbaun, 1976). This demonstrated that complement is important for *in vivo* control of parasitaemia in trypanosomiasis. This finding was also confirmed by Cunningham *et al.* (1978) who conducted similar studies on mice infected with *T. cruzi.* Flemming and Diggs (1978) showed that

antibody-mediated cytotoxicity in trypanosomiasis was dependent upon complement activation while Balber *et al.* (1979) demonstrated the absolute complement requirement for the lysis of antibody treated *T. brucei* and *T. congolense* populations. Other studies that demonstrated the importance of the complement system in the elimination of trypanosomes were reviewed in Nielsen (1985). The principal observation in these studies is that trypanosomiasis affects the level of complement in infected individuals with a consequent worsening of the clinical state of the animals if parasites were not eliminated by chemotherapy.

A reduction in the level of circulating complement components has been reported in infections with salivarian trypanosomes of man (Greenwood and Whittle, 1976), rhesus monkeys (Nagle *et al.*, 1974) and cattle (Kobayashi and Tizard, 1976; Nielsen *et al.*, 1978; Rurangirwa *et al.*, 1980; Table *et al.*, 1980). These authors established that the reduced level of complement observed in these infections affected the course of the disease by worsening the clinical state of the infected animals. Other studies in which reduced levels of complement components have been observed include those of Basson *et al.*, (1977) in which the reduction in the levels of C4 and factor B were indications that the activation of both the classical and alternative pathways occur in trypanosomiasis.

Although the importance of complement in the control of parasitaemia in trypanosomiasis has been demonstrated by several workers, some other workers such as Shirazi *et al.* (1980) did not find a significant difference in the level of parasitaemia between normal and C3-depleted mice. Dalmasso and Jarvinen (1980) while studying the infection of mice and guinea-pigs with *T. cruzi* did not find any difference in the level of parasitaemia in mice genetically deficient

in C5 and normal mice. Similarly, studies with guinea-pigs genetically deficient in C4 and normal guinea-pigs gave no indication of differences in the course of the infection. This result according to the authors suggests a minor role for the classical if at all, but not the alternative pathway in the control of parasitaemia. Other studies by the same authors (Jarvinen and Dalmasso, 1976) with *T. lewisi*-infected rats however, showed a tremendous decrease in serum complement C3 and C4 levels with C3 levels highly correlated to parasitaemia. Another complement component - C6 was not decreased as measured by haemolytic assay, and the parasitaemia in C4-deficient rats were not significantly different from those of normal rats. Nielsen *et al.* (1978a) also found that while the levels of some early complement components such as C1 and C3 were depleted in calves infected with *T. congolense*, that the levels of some late complement components such as C8 were not reduced. These results led these authors to doubt the importance of the classical complement pathway in the elimination of trypanosomes since without the utilization of the late complement components the effector process of the system will not be achieved.

One mechanism responsible for the reported hypocomplementaemia in trypanosomiasis could be extensive activation of complement by antigen-antibody complexes (Musoke and Barbet, 1977). Such a process would reduce the level of complement in circulation as complement fragments would be utilised in the process of parasite elimination, probably by both the classical and alternative pathways. The complex nature of complement in the host, makes it much easier to study the complement system using *in vitro* systems that are much easier to control. One of such *in vitro* systems is that used by Klaus *et al.* (1979) to study the activation of the classical and alternative complement pathways.

In trypanosomiasis few *in vitro* studies have been conducted on the activation of complement (Musoke and Barbet, 1977; Cunningham *et al.*, 1978). Each of these workers adopted the technique developed by Clark and Freeman (1968) based on two-dimensional immunoelectrophoresis which distinguishes between native C3 and its activated products which other techniques used for assessing complement activation such as lytic assays (Ferrante and Allison, 1983) are incapable of doing. The electrophoretic technique exploits differences in electrophoretic mobility of the native complement component and its activated products. With this technique Musoke and Barbet (1977) demonstrated the activation of human complement by an isolated variant-specific surface antigen of *T. brucei* through the classical pathway while Cunningham *et al.* (1978) showed that viable trypanosomes and trypanosome culture supernates activate complement primarily also through the classical pathway.

In view of the central role that C3 plays in the activation of the classical and alternative complement pathways, and its established importance in the control of parasitaemia in trypanosomiasis, the series of experiments reported in this chapter were designed to evaluate:

(1) The changes of C3 levels in rabbits infected with *T. evansi* during the period of primary infection and after drug treatment.

(2) The extent of C3 activation *in vivo* in these animals.

(3) Complement activation using *in vitro* systems specific for the classical and alternative pathways; and

(4) The efficiency of different immunoglobulin isotypes in activating C3.

MATERIALS AND METHODS

Trypanosomes

T. evansi TREU 2147 described previously in Chapter 3 was used for all the infections and preparation of soluble antigens used for the production of complexes for the C3-cleavage assays. It was also the source of parasites used for the C3-cleavage assays involving the use of whole trypanosomes.

Animals

Four adult female New Zealand White rabbits were used for the experiment, each infected with 4 x 10^5 motile trypanosomes through the ear vein. The trypanosomes had been separated from the blood of mice with fulminating infections with DEAE cellulose (DE52 Whatman Biochemicals) as described previously. Twenty-four days after infection each infected rabbit was treated with 7 mg (active principle) kg^{-1} body weight of diaminazene aceturate (Berenil^R, Hoechst, W. Germany). Rabbits were bled into bottles containing EDTA (Sigma Ltd.) before infection, days 7, 11, 18, 21 and 24 post infection and days 8 and 14 post drug treatment, for the preparation of plasma and parasite estimation. Plasma samples were aliquoted into small volumes and stored at -79°C until needed.

Blood for serum preparation was collected on equivalent days. These sera samples were used for the determination of C3 activation and extraction of immunoglobulin classes. Sera for immunoglobulin extraction were prepared as previously described in Chapter 2 and stored at -20^{O}C until needed, while blood from which sera used for the determination of C3 activation were obtained, was kept on ice for 20 minutes before the sera were aspirated and processed as described in Chapter 2. Resulting sera samples were aliquoted in

small volumes and stored at -79°C until needed.

Estimation of Relative C3 Levels

C3 levels in the plasma samples were measured by rocket immunoelectrophoresis (Laurell, 1966) in a 1% agarose gel, pH 8.6, in barbitone acetate buffer (Appendix 15) containing 2.5% sheep anti-rabbit C3 (ICN Immunobiologicals, UK) and 3% PEG 6000 (Sigma Ltd.).

Ten μl of plasma was placed into each well with a diameter of 5 mm cut out of the gel with a metal punch. Current was applied at a constant voltage of 150V and the proteins allowed to run across the agarose gel containing C3 antisera for about 18 hours. The gels were subsequently washed in PBS (Appendix 1) for 24 hours and in destain solution for an hour to fix the protein before being dried in a stream of hot air from a hair-drier. The dried gels were stained with Coomassie Blue as described previously in Chapter 2 to enhance contrast and expose the components bound to the gel.

In vivo C3 Activation Assay

The presence of cleavage products of C3 in serum of rabbits infected with *T. evansi* was assayed using a two-dimensional immunoelectrophoretic technique (counter-electrophoresis) of samples as described by Hudson and Hay, (1976) and Johnstone and Thorpe (1987).

This was conducted as described previously for the estimation of the relative C3 levels in the plasma samples. The only modifications were that 250 volts were used and electrophoresis was allowed to run for about seven hours. No C3 antisera was included in the agarose gel. Tracks of separated proteins from the first dimensional electrophoresis were cut off from the agarose gel

and laid on one edge of a Gelbond^{R} film (Pharmacia). Fifteen ml of warm 1% agarose in barbitone acetate buffer pH 8.6 (Appendix 15) containing 2.5% sheep anti-rabbit C3 (ICN Immunobiologicals, UK) and 3% PEG 6000 (Sigma Ltd.) was then poured onto the Gelbond in such a way that the liquid agarose merged smoothly with the solidified track of agarose containing the separated proteins. Electrophoresis was then carried out for about 16 hours with a constant voltage of 150V in such a way that the track of agarose containing the separated serum proteins was on the cathodic side of the electrophoresis plate so that when the current was applied the separated proteins migrated across the agarose gel bed containing the C3 antisera. All electrophoresis was carried out with the barbitone acetate buffer (Appendix 15). Gels were subsequently processed and stained with Coomassie Blue as described previously in the section dealing with the estimation of relative C3 levels in the plasma.

Fractionation of Immunoglobulins in Sera from T. evansi-infected rabbits

The immunoglobulins from *T. evansi* infected and uninfected rabbits for the assessment of C3 activation were fractionated by salt precipitation, gel filtration and ion-exchange chromatography.

(a) Isolation of IgG by anion exchange chromatography: DEAE-Affi-Gel Blue was used according to the manufacturer's instructions for the isolation of serum IgG.

Briefly, a column of DEAE Affi-Gel Blue with a total bed volume of 5 ml gel was used for each 1 ml of the serum processed. After packing the column, the gel was washed free of residual dye with at least five bed volumes of 0.1M acetic acid, adjusted to pH 3.0 containing 1.4M NaCl and 40% (v/v) isopropanol. After washing, the gel was equilibrated with at least 12 bed

volumes of buffer consisting of 0.02M Tris-HCl, pH 8.0, 0.028M NaCl, and 0.02% NaN3.

Serum from infected and non-infected rabbits was dialysed against 0.02M Tris-buffer pH 8.0 (Appendix 16) respectively overnight. The samples from the infected animals were made up of a mixture of equal volumes of sera collected 14 and 18 days after infection from rabbits infected with *T. evansi* TREU 2147. The dialysed serum samples were then applied to columns of DEAE-Affi Gel Blue at the rate of 2 ml per 10 ml of equilibrated gel bed. Unbound materials were then eluted with three bed volumes of the recommended buffer (Appendix 16) and 3 ml fractions of the eluate were collected. The absorbence of each fraction at 280 nm was determined with a spectrophotometer and the fractions showing the highest absorbences were pooled together. These pooled fractions were dialysed against distilled water for 24 hours at 4°C, freeze-dried, reconstituted to a volume of 1 ml with distilled water and stored at -20°C until required.

(b) Isolation of IgG by DEAE cellulose method (DE52): The DE52 method for the extraction of IgG of Shubbar (1978) was used. The practical steps for the extraction adopted were those described in detail by Woldehiwet (1981).

Briefly, DE52 (Whatman Biochemicals, U.K.) swollen in phosphate buffer (KH_2PO_4, 0.5M) for 24 hours was transferred into a stoppered Buchner flask and degassed under pressure. The pH of the slurry was then adjusted to 7.6 by the addition of Na_2HPO_4, 0.5M. The slurry was then washed by sedimentation and filtered twice with 0.1M phosphate buffer pH 7.6 through Whatman 54 filter paper under reduced pressure. Washing was continued until the pH of the filtrate was the same as that of the buffer, after

which the slurry was then used to pack a 40 cm by 2.5 cm column.

Fifteen ml of a mixture of serum consisting of equal volumes of serum collected 14 and 18 days post infection were dialysed against tap water adjusted to pH 6.1 at 4^{O}C for 72 hours. The precipitate was removed by centrifugation at 2000 g for 30 minutes at 4^{O}C. The resulting supernatant was used as the starting sample for IgG extraction. Fifteen ml of serum from uninfected rabbits was treated similarly.

Fifteen to 20 ml of a 50% saturated solution of ammonium sulphate was added to the supernatant. The precipitate formed was centrifuged at 2000 g for 30 minutes at 4^{O}C and the resulting supernatant discarded. The precipitate was redissolved in distilled water to a final volume of 15 ml. The ammonium sulphate precipitation and redissolution in distilled water were repeated twice. The final precipitate was redissolved in 5 ml of distilled water, loaded in dialysis tubes and dialysed against 0.02M phosphate buffer pH 7.6 for 24 hours. The dialysed material was then loaded onto the DE52 ion-exchange column. Unbound materials were eluted in 10 ml volumes sequentially with 0.01M, 0.02M and 0.05M phosphate buffer pH 7.6 (Reid *et al.*, 1971). The second, third and fourth fraction had the highest absorbences at 280 nm wavelength. These fractions were pooled together, dialysed against distilled water for 24 hours at 4°C, freeze-dried and reconstituted in 5 ml of distilled water and stored at -20°C in 200 μl volumes until required.

Isolation of IgM by Gel Chromatography on Sephadex G200

Thirty g of Sephadex-G200 (Pharmacia Ltd., London) was allowed to swell at 4^{O}C for three days in two litres of 0.1M Tris-HCl buffer + 1M NaCl, pH 8.0, containing 0.02 per cent sodium azide (Jonas, 1969; Shubbar, 1978).

The slurry was put into a stoppered Buchner flask and degassed under pressure.

The slurry was then used to fill a 90 cm by 2.5 cm glass column and the gel bed allowed to pack under a flow rate of 24 ml per hour for three days with 0.1M Tris-HCl buffer plus 1M NaCl, pH 8.0. The homogeneity of the gel bed was determined by running a volume of a 2 mg per ml solution of blue dextran 2000 (Pharmacia Fine Chemicals) through the column. After this the gel bed was again washed clean with excess buffer.

Thirty ml of a mixture of equal volumes of serum collected 14 and 18 days post infection were dialysed against tap-water adjusted to pH 6.1 at $4^{o}C$ for 72 hours. The precipitate was then separated from the supernatant by centrifugation at 2000 g for 30 minutes at 4°C. The resulting precipitate was redissolved in 0.1M Tris-HCl buffer pH 8.0 to a final volume of 5 ml. This was then applied to the Sephadex G-200 column and left to fractionate at a flow rate of 24 ml per hour with Tris-HCl buffer plus 1M NaCl pH 8.0. Five ml fractions representing the unbound material were collected and the absorbences monitored by the column monitor at 280 nm. The fractions having the highest absorbance were pooled together. The pooled fractions were dialysed against distilled water for 24 hours at 4°C, freeze-dried and redissolved in distilled water to make a volume of 5 ml. This was then aliquoted into 200 μl volumes and stored at -20°C until required. The same IgM extraction process was repeated with 15 ml of serum from uninfected rabbits.

Evaluation of the Purity of Immunoglobulin Fractions by Double Immunodiffusion Test

In order to test the purity of the fractionated immunoglobulins, the double immunodiffusion test described by Johnson and Thorpe (1987) was used. Briefly, 15 μl of undiluted samples of the individual pooled fractions from chromatography were placed into one of the three 5 mm diameter centrally located wells cut in a 1% agarose gel, pH 8.6, in barbitone acetate buffer (Appendix 15) containing 3% PEG 6000 (Sigma Ltd.). Ten μl of affinity purified antisera (Nordic^{R}) raised in goats to rabbit IgG, IgM and IgA were then placed into three 5 mm diameter satellite wells which formed an arc around the centrally located wells containing the extracted fraction. The gels were then placed in a moistened lunch box and kept at 4°C until precipitation lines were formed. Unreacted materials were removed from the gels by washing in PBS for 24 hours; transferred to destain for an hour to fix the proteins before being dried in a hot air stream from a hair drier. The dried gels were then stained in Coomassie Blue as described previously in Chapter 2.

Immune Complex Formation for Studies on In vitro C3 Activation

(a) Complex formation with whole trypanosomes and trypanosome soluble antigens: One x 10^8 motile trypanosomes separated from the host blood cells with DE52 (Whatman Biochemicals, U.K.) as described previously in Chapter 2 were mixed with 200 μl of a mixture of equal volumes of serum collected 14 and 18 days post infection or serum collected from uninfected rabbits respectively. *T. evansi* soluble antigens obtained by freeze-thaw and centrifugation from 1 x 10^8 trypanosomes were similarly mixed with 200 μl of the two sera samples as described above. These mixtures were incubated at

37°C for 30 minutes and at 4°C for 24 hours. The resulting precipitates were separated and washed twice with veronal buffer (Appendix 17) by centrifuging at 1,200 g for five minutes before use.

(b) Immune complexes formed with fractionated immunoglobulins and whole trypanosomes

(i) Complexes formed with IgG: 1 x 10^8 motile trypanosomes separated from host blood cells with DE52 (Whatman Biochemicals, U.K,) were mixed with 200 μl of IgG, containing 1000 μg of proteins, obtained by fractionation of post infection and normal rabbit serum. These mixtures were incubated at 37°C for 30 minutes and 4°C for 24 hours. The resulting precipitates were separated and washed twice with veronal buffer (Appendix 17) by centrifuging at 1,200 g for five minutes before use.

Complexes formed with IgM: 1 x 10^8 motile trypanosomes separated from the host blood cells with DE52 (Whatman Biochemicals) were mixed with 200 μl of IgM, fractionated from post infection serum and serum obtained from uninfected rabbits containing 1,000 μg of proteins. These mixtures were incubated at 37°C for 30 minutes and 4°C for 24 hours. The resulting precipitates were separated and washed twice with veronal buffer (Appendix 17) by centrifuging at 1,200 g for five minutes before use.

In vitro C3 Activation Assay

The ability of the products of reaction between the parasites or *T. evansi* soluble antigens and post infection or normal rabbit serum, to activate C3 *in vitro* was assayed using the two dimensional electrophoretic technique described by Klaus *et al.* (1979).

The technique involved the analysis of rabbit complement (C^{Rab}) (fresh rabbit serum stored at -79°C) with first and second-dimensional immunoelectrophoresis (Hudson and Hay, 1976; Johnstone and Thorpe, 1987) after treatment with the complexes. The ability of the complexes formed with sera collected from infected rabbits to activate C3 through the classical and alternative pathways was investigated. The ability of the complexes formed with IgG or IgM to activate C3 through the classical pathway alone was also investigated. For each of the systems a control was incorporated and these were the products of incubation of the normal rabbit serum or immunoglobulins (IgG and IgM) fractionated from normal rabbit serum with either whole trypanosomes or their soluble antigens.

C3 Activation through the Classical Pathway

All the complexes mentioned above were individually resuspended in 50 µl veronal buffered saline (Appendix 17) containing 1.0 mM $MgCl_2$ and 0.15 mM $CaCl_2$. Fifty µl of fresh rabbit serum (C^{Rab}) was added to each tube before the tubes were incubated at 37°C for 45 minutes. For each of the systems a control was incorporated. After the incubation antigen-antibody crossed electrophoresis (Laurell, 1965) was performed as described previously for the one dimensional system in agarose gels containing 0.01M EDTA. The second dimension was run as described previously in the presence of 2.5% sheep anti-rabbit C3 (ICN Immunobiologicals, UK) (Pryjma *et al.*, 1974), after which the gels were processed as described previously and stained with Coomassie Blue.

C3 Activation through the Alternative Pathway

Complexes formed between whole trypanosomes or *T. evansi* soluble antigens and trypanosome specific sera with their controls were individually resuspended in 50 μl of veronal buffered saline (Appendix 17) containing 25 mM $MgCl_2$ and 25 mM ethylene-glycol tris-(aminoethyl)-tetra- acetic acid (EGTA, Sigma Ltd.). This buffer provides conditions for activation of the alternative complement pathway only (Sandberg and Osler, 1971). Fifty μl of fresh rabbit serum (C^{Rab}) was added and the tubes incubated at 37°C for 45 minutes. Antigen-antibody crossed electrophoresis (Laurell, 1965) was performed as described previously for the one dimensional system in agarose gels containing 0.01M EDTA, the second dimension being run as described previously in the presence of 2.5% sheep anti-rabbit C3 (ICN Immunobiologicals, UK) (Pryjma *et al.*, 1974). Gels were subsequently processed and stained with Coomassie Blue as described previously.

RESULTS

Relative C3 Levels during T. evansi Infection

There was an initial increase between 5 and 7% in circulating C3 levels in all the animals by seven days after infection when compared with preinfection levels. This was followed by a gradual decrease up to 11 days after infection in all the rabbits. Thereafter, a rapid fall in the levels of circulating C3 was seen which continued up to around 21 days after infection. A reduction of 70% of the preinfection C3 level was attained in one of the four rabbits (Figure 7.2A, No. 21) at this time. A rise in the C3 level immediately before drug treatment occurred in the same rabbit 24 days after infection (Figure 7.2A, No. 24). After drug treatment, an increase in the level of circulating C3 was recorded in

all the rabbits (Figure 7.2A to C: Nos. 32 and 38). The day 8 post drug treatment serum sample of one of the rabbits (Figure 7.2B, No. 32) was misplaced.

Parasitaemia Levels in T. evansi Infected rabbits

Parasites were first detected in the blood of each rabbit seven days after infection. This was followed by a general increase in parasite counts until 11 days post infection. Thereafter, the level of parasitaemia fluctuated in individual rabbits before they were drug treated 24 days after infection (Figure 7.3A to D). No parasites were detected in the blood of all the rabbits up to 14 days after drug treatment when the last samples were collected.

C3 Activation in T. evansi Infected Rabbits

A single precipitation peak was observed in the gel after the analysis of serum samples collected before infection, seven days after infection and 8 days after drug treatment (Figure 7.4, Nos. 9, 10 and 13). Two precipitation peaks were observed when serum samples collected 21 and 24 days after infection were analysed (Figure 7.4, Nos. 11 and 12). The height of the faster moving precipitation peak was greater in the serum sample collected 21 than 24 days post infection.

Assessment of the Purity of Ig Isotype (IgG and IgM) Preparation

(1) A single broad precipitation line was formed when the Affigel-Blue extract (Figure 7.5, No. 1) was tested against antisera to rabbit IgG (Nordic) (Figure 7.5, 1a). No precipitation lines were seen when the extract was probed with antisera against IgM (Figure 7.5, No. 1b) or IgA (Figure 7.5, No.

1c).

(2) A single fine precipitation line was formed when the Sephadex G-200 extract (Figure 7.5, No. 2) was probed with antisera against IgM (Figure 7.5, No. 2b). No precipitation lines were formed when the same extract was probed with rabbit IgG (Figure 7.5, No. 2a) and IgA (Figure 7.5, No. 2c) antisera respectively.

(3) A single fine precipitation line was formed when the DE52 extract (Figure 7.5, No.3) was probed with antisera against rabbit IgG (Figure 7.5, No. 3a). No precipitation line was formed when the same extract was probed with antisera against rabbit IgM (Figure 7.5, No. 3b) and IgA (Figure 7.5, No. 3c) respectively.

In vitro C3 Activation by Immune Complexes

Complexes formed with whole trypanosomes: Two precipitation peaks were seen in the gels after the electrophoresis of the product of reactions between the complexes and rabbit complement (C^{Rab}) under the conditions for activation by both the classical (Figure 7.6, No. 5) and alternative pathways (Figure 7.6, No. 7). Differences were seen in the height of the cleavage products, with the precipitation peak to the right side of the cathode, higher in the classical than the alternative pathway. In both the classical (Figure 7.6, No. 6) and the alternative (Figure 7.6, No. 8) pathways, incubation with products of trypanosomes and normal rabbit serum yielded a single precipitation peak.

Complexes formed with T. evansi soluble antigens: Five precipitation peaks were observed after immunoelectrophoresis of the products of reactions between the complexes formed with the soluble antigens of *T. evansi* and the

post infection serum with rabbit complement (C^{Rab}) under the conditions of activation of complement through the classical (Figure 7.6, No. 1) and three precipitation peaks by the alternative (Figure 7.6, No. 3) pathways. In both the classical (Figure 7.6, No. 2) and alternative (Figure 7.6, No. 4) pathways a single precipitation peak was seen with the control system in which the soluble antigens had been incubated with normal rabbit serum and rabbit complement (C^{Rab}).

Complexes formed with whole trypanosomes and immunoglobulin isotypes: Two over-lapping precipitation peaks were observed after immunoelectrophoresis of the products of reactions between the complexes formed with whole trypanosomes and IgG with rabbit complement (C^{Rab}) (Figure 7.7, No. 1) and two small precipitation peaks with the products formed with IgM (Figure 7.7, No. 4) under the conditions of activation of complement through the classical pathway. With both preparations only a single precipitation peak was observed with the control system in which the complexes were formed between the whole trypanosomes and IgG (Figure 7.7, No. 3) and IgM (Figure 7.7, No. 6) fractionated from normal rabbit serum and rabbit complement (C^{Rab}). Figures 7.7, Nos. 2 and 5 are sketches for Figures 7.7, Nos. 1 and 4 respectively.

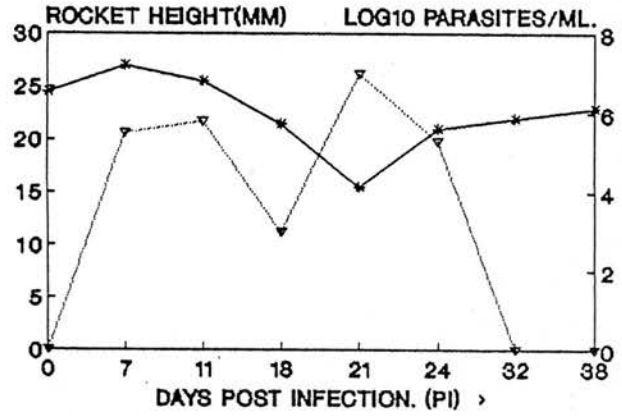
FIGURE 7.2

C3 complement levels and parasitaemia in four rabbits experimentally infected with *T. evansi*.

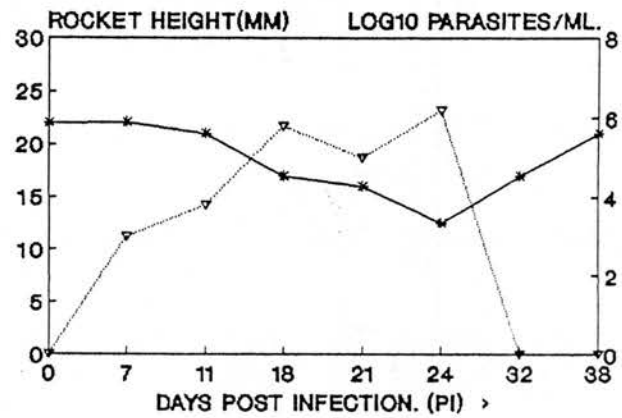
24 Day of drug treatment

- Rocket Height

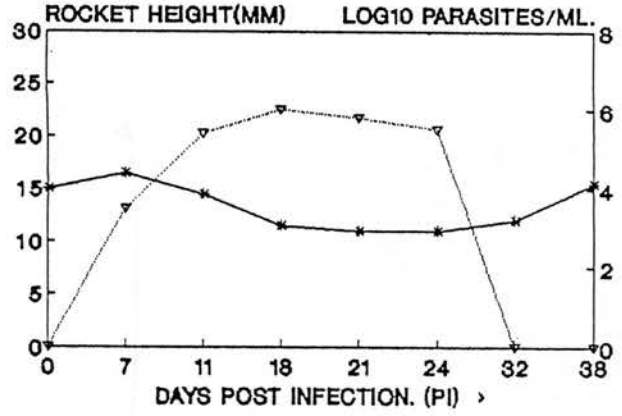
∇-∇ Parasitaemia.



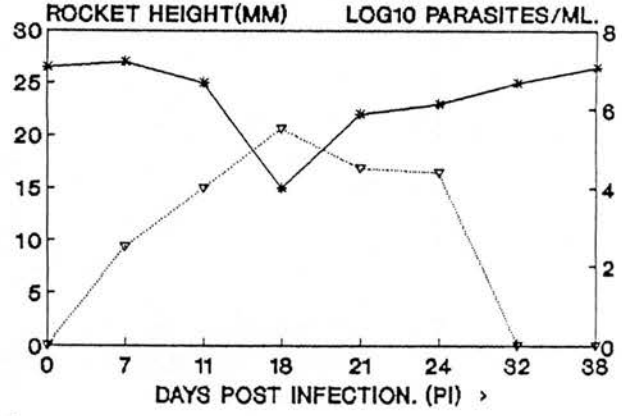
(A)



(B)



(C): 24-day of drug treatment.



(D)

FIGURE 7.3

Levels of C3 in plasma of rabbits infected with *T. evansi*.

0	Normal rabbit plasma.
7-24	Days 7, 11, 18, 21 and 24 post infection plasma respectively.
32 and 38	Days 8 and 14 post treatment plasma respectively.

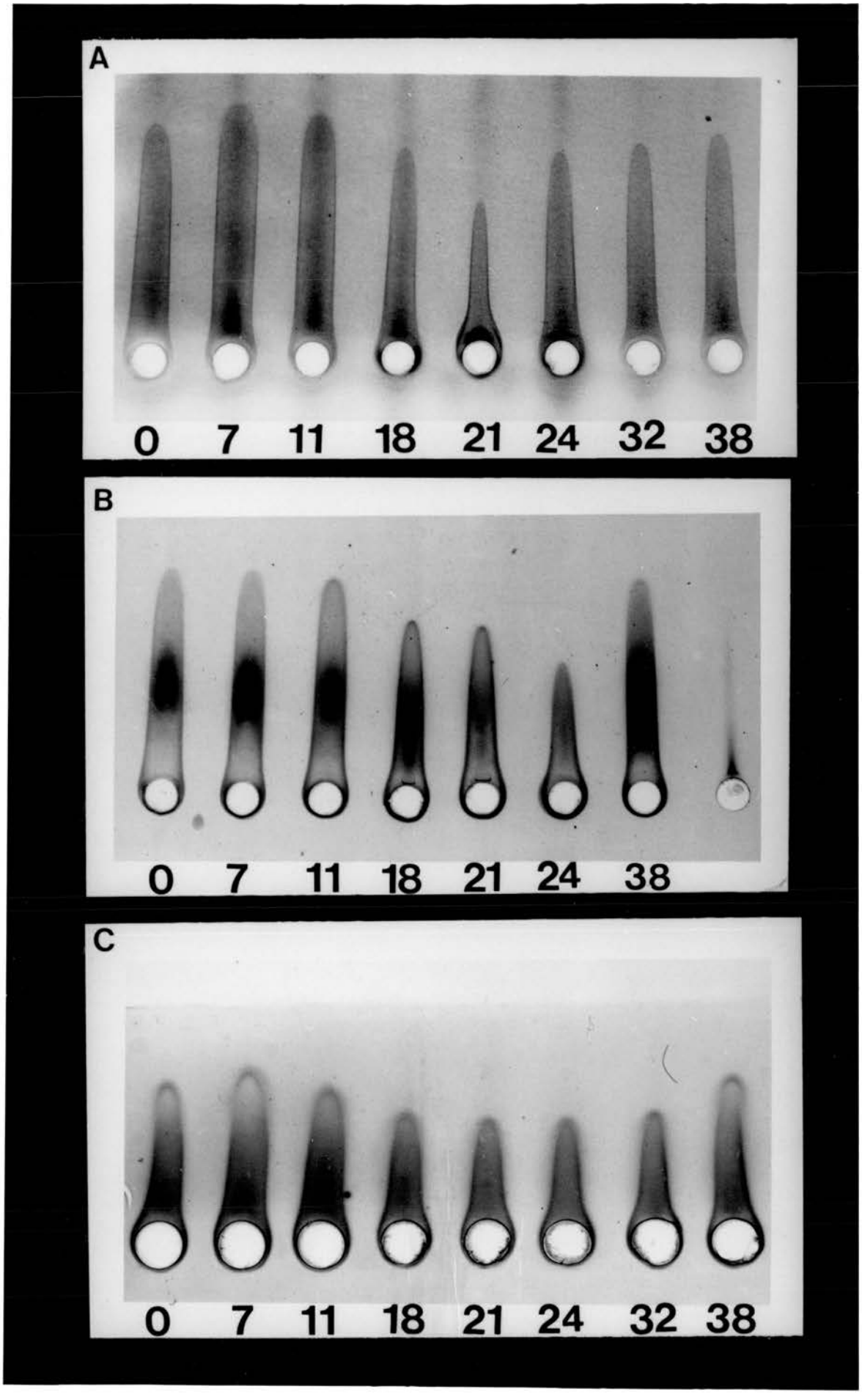

A
0 7 11 18 21 24 32 38
B
0 7 11 18 21 24 38
C
0 7 11 18 21 24 32 38

FIGURE 7.4

Activation of C3 in serum of rabbits infected with *T. evansi*.

Numbers 0-38 C3 complement levels in days 0, 7, 11, 18, 21, 24 post infection and days 8 and 14 post treatment plasma respectively.

Numbers 9-13 C3 activation in days 0, 7, 21, 24 post infection and day 8 post treatment serum respectively.

Number 14 Schematic representation of No. 11.

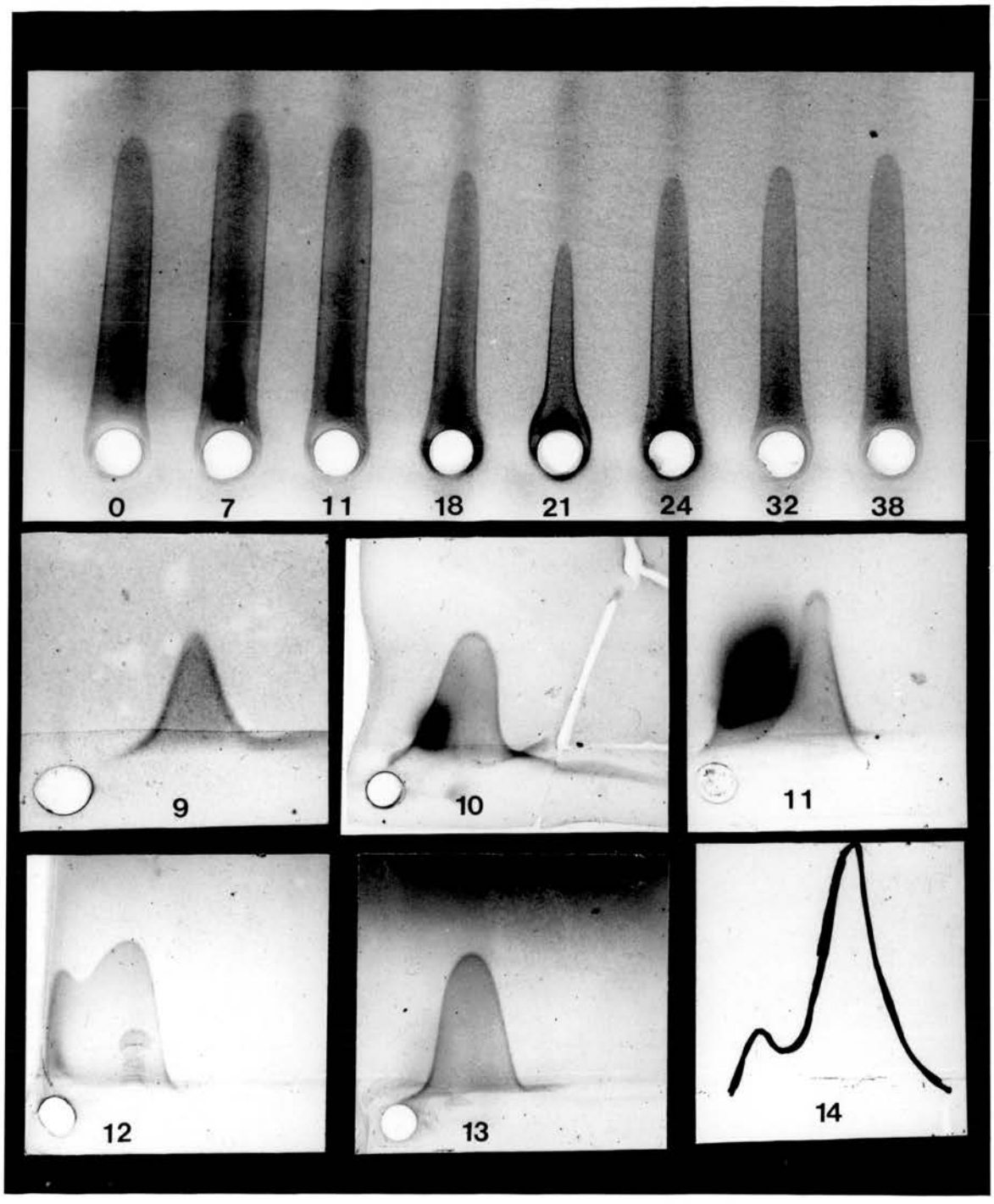

0
7
11
18
21
24
32
38
9
10
11
12
13
14

FIGURE 7.5

Double immunodiffusion test for the verification of the purity of the immunoglobulin extracts.

Number

1. Extract from Affigel
    (a) Antiserum against rabbit IgG
    (b) Antiserum against rabbit IgM
    (c) Antiserum against rabbit IgA.
2. Extract from Sephadex G200
    (a) Antiserum against rabbit IgG
    (b) Antiserum against rabbit IgM
    (c) Antiserum against rabbit IgA.
3. Extract from DEAE cellulose (DE52)
    (a) Antiserum against rabbit IgG
    (b) Antiserum against rabbit IgM
    (c) Antiserum against rabbit IgA.

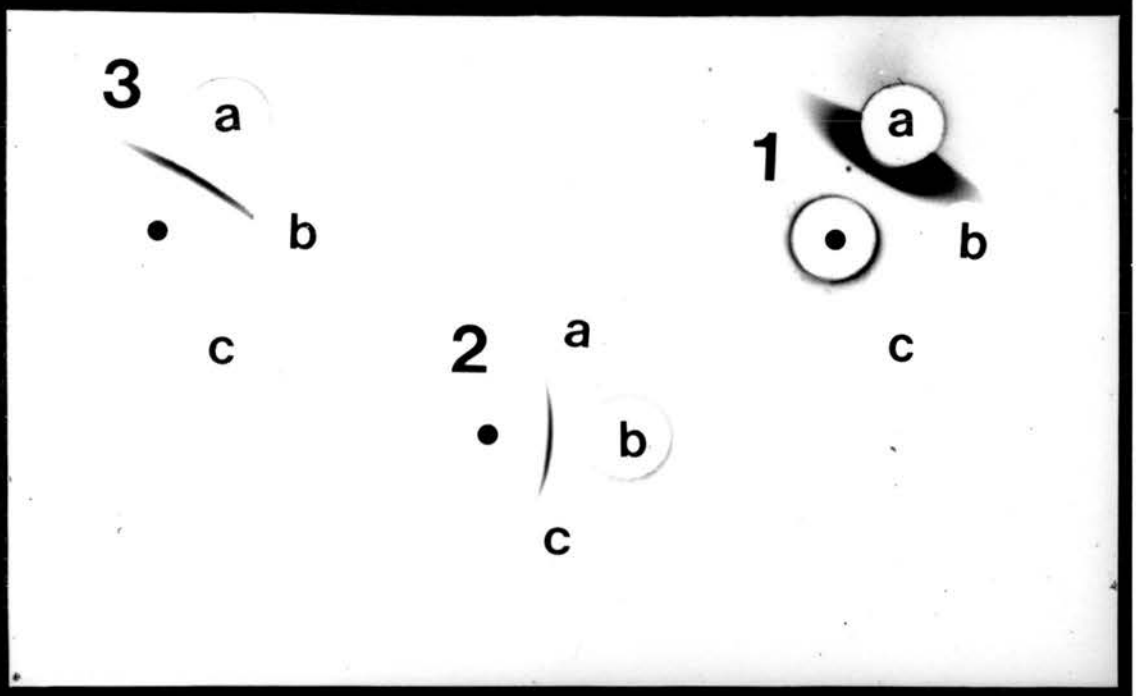

3
a
b
c
1
a
b
c
2
a
b
c

FIGURE 7.6

In vitro C3 activation through the classical and alternative pathways.

1. Activation of C3 through the classical pathway by complexes formed with *T. evansi* soluble antigens and post infection serum.
2. Activation of C3 through the classical pathway by products of normal rabbit serum and *T. evansi* soluble antigen incubation.
3. Activation of C3 through the alternative pathway by complexes formed with *T. evansi* soluble antigens and post infection serum.
4. Activation of C3 through the alternative pathway by products of normal rabbit serum and *T. evansi* soluble antigen incubation.
5. Activation of C3 through the classical pathway by complexes formed with live trypanosomes and post infection serum.
6. Activation of C3 through the classical pathway by products of normal rabbit serum and live trypanosome incubation.
7. Activation of C3 through the alternative pathway by complexes formed with live trypanosomes and post infection serum.
8. Activation of C3 through the alternative pathway by products of normal rabbit serum and live trypanosome incubation.

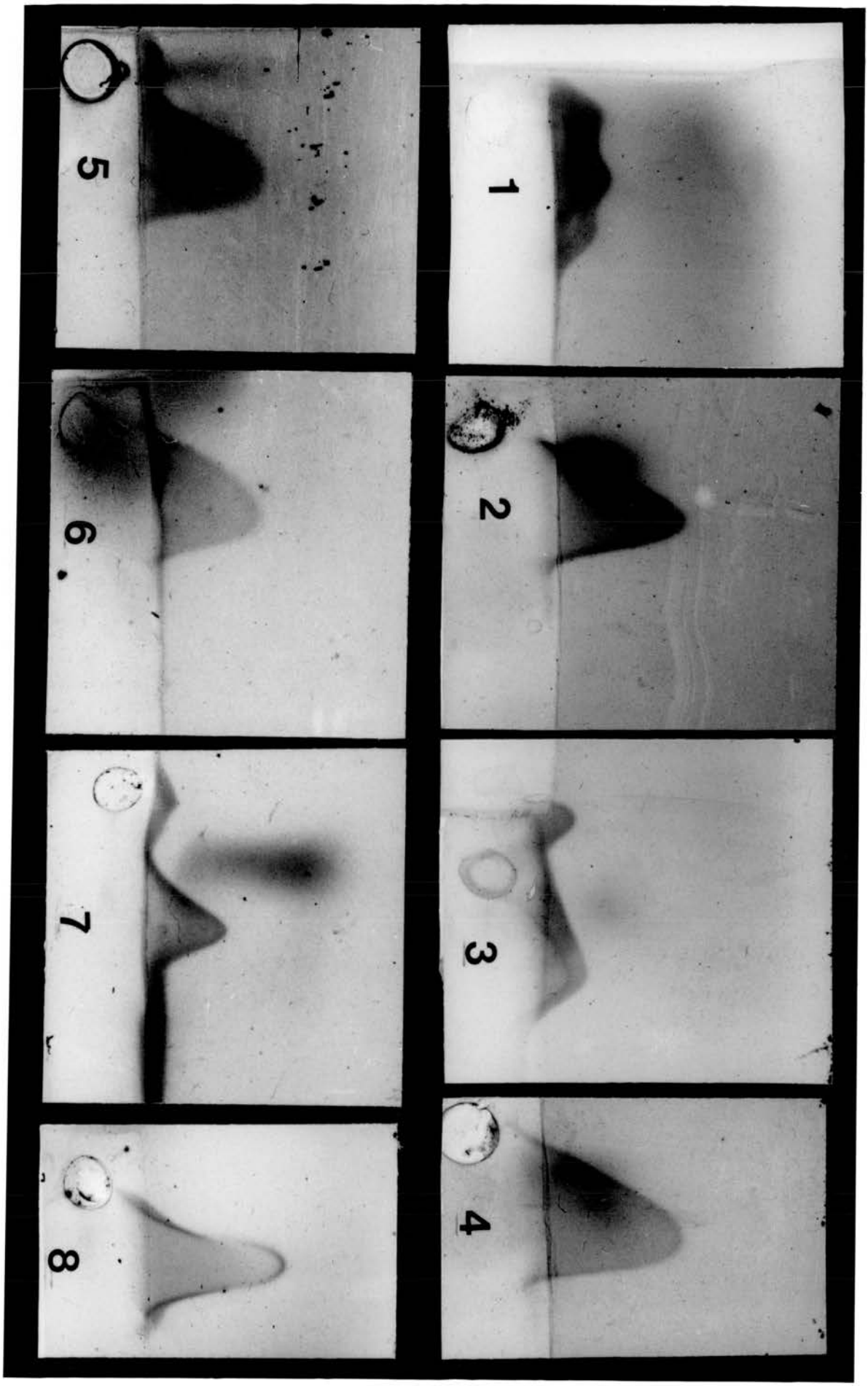
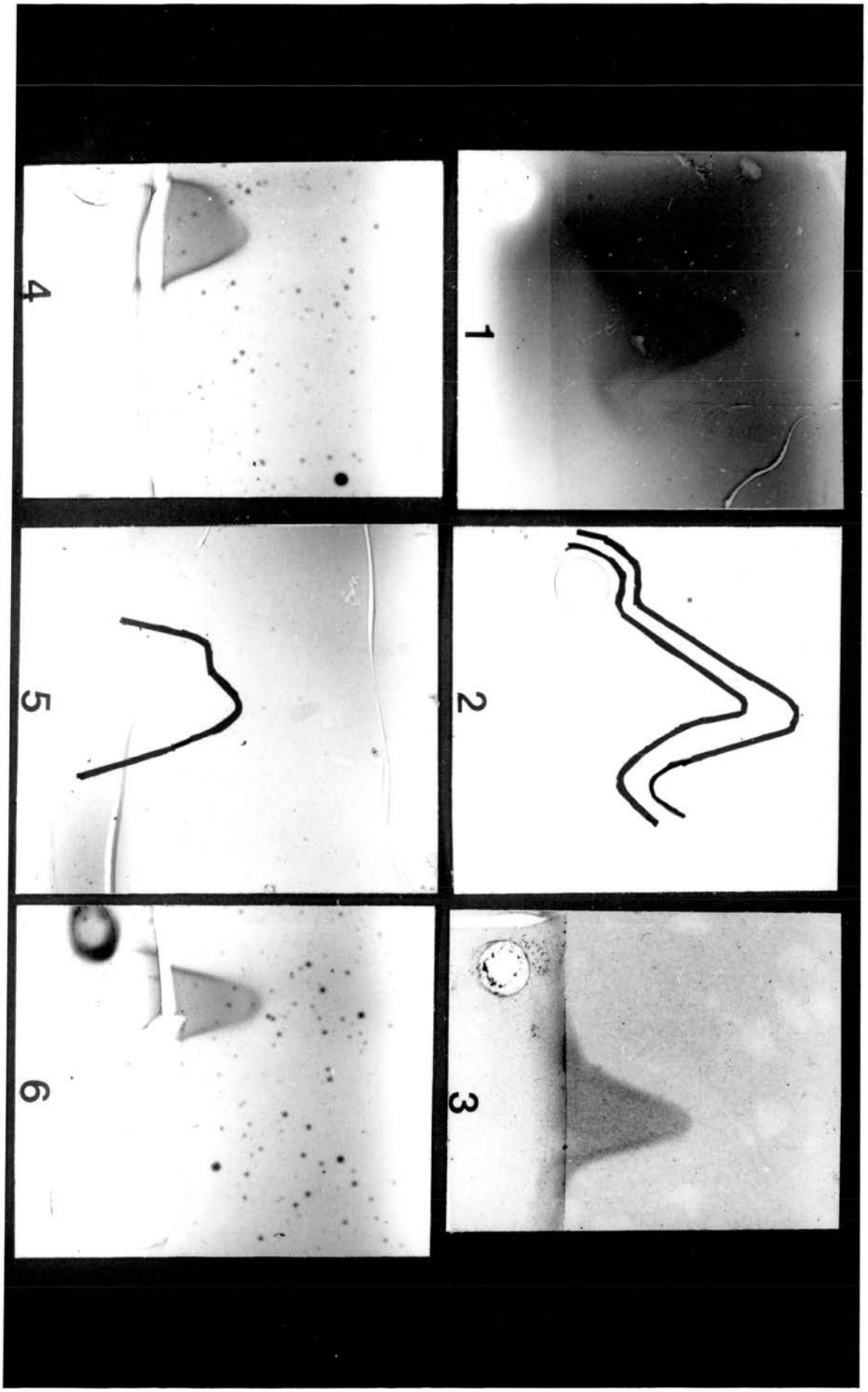

1
2
3
4
5
6
7
8

FIGURE 7.7

In vitro C3 activation through the classical pathway by complexes formed with immunoglobulin isotypes.

1. Activation of C3 by complexes formed with live trypanosomes and IgG fractionated from post infection serum.
2. Schematic representation of 1.
3. Activation of C3 by products of reactions between live trypanosomes and IgG fractionated from normal rabbit serum.
4. Activation of C3 by complexes formed with live trypanosomes and IgM fractionated from post infection serum.
5. Schematic representation of 4.
6. Activation of C3 by products of reactions between live trypanosomes and IgM fractionated from normal rabbit serum.


1
2
3
4
5
6

DISCUSSION

A consistent feature observed in all the rabbits infected with *T. evansi* was a fall in the C3 levels as the infection progressed. Similar observations have been reported by other workers for cattle infected with *T. congolense* and *T. vivax* (Kobayashi and Tizard, 1976; Nielsen *et al.*, 1978a; Rurangirwa *et al.*, 1980) and in rats infected with *T. lewisi* (Jarvinen and Dalmasso, 1976). One possible cause of this drop in C3 levels could be C3 activation by antigen-antibody complexes (Musoke and Barbet, 1977) that occurs during the elimination of the parasites by the host immune system through complement dependent effector mechanisms (Murray and Urquhart, 1977; Greenblatt *et al.*, 1983; Ngaira *et al.*, 1983). The observations made in the current study that the lowest levels of C3 were seen in rabbits during periods of highest parasitaemia (Figure 7.3a to d) and in one rabbit, an increase in C3 level with a fall in the level of parasitaemia (Figure 7.2A, No. 24 and Figure 7.3a) and a rapid increase in C3 levels after chemotherapy in all the rabbits (Figure 7.2A to C, Nos. 32 to 38) suggest a possible relationship between C3 levels and the parasite burden. Increasing number of parasites would probably require more C3 for their removal compared to lower parasites numbers. The number of parasites in circulation could also have a direct effect on the level of C3 in circulation as there is evidence that live trypanosomes release large quantities of complement activating factor (Cunningham *et al.*, 1978) which would again link parasite number with complement reduction.

During the early part of the infection an initial rise in C3 levels was observed in all the rabbits used in the present study (Figure 7.2A to C). A similar observation was made by Shirazi *et al.* (1980) although of a greater magnitude. In the present study an initial rise of 5 to 7% of the preinfection

C3 level was recorded, while Shirazi and his colleagues observed a two to three-fold increase. The reason for this initial rise is probably associated with the fact that C3 is produced in large amounts during the acute phase of infections (Horning and Arquembourg, 1965) and also that mononuclear phagocytes, which are known to secrete C3, are highly active in the initial stages of trypanosome infections (Longstaffe, 1974). Reported increases in numbers and activity of the cells of the mononuclear phagocytic system (MPS) of the liver a few days after trypanosome infection (Murray *et al.*, 1974b) may probably play a role in the initial rise in C3 levels as the liver is known to secrete about 90% of C3 (Lambris, 1988). The activation of C3 results in the splitting of C3 into subunits, which migrate with different speeds in the electric field and the principle of complement activation assay relies on the property of the activated products to move faster across the electrophoresis field than the native component which usually appear as a precipitation peak behind the faster moving precipitation peak(s) on the right side of the cathode, the cleavage products (Clark and Freeman, 1968; Pryjma *et al.*, 1974).

The results of the experiments on C3 activation in serum of rabbits infected with *T. evansi* showed that with the normal rabbit serum, serum collected seven days after infection and eight days after drug treatment only one precipitation peak was recorded respectively (Figure 7.4, Nos. 9, 10 and 13). With serum samples collected 21 and 24 days after infection two peaks were observed (Figure 7.4, Nos. 11 and 12). The presence of more than one precipitation peak is indicative of C3 activation while a single peak depicts non-activation of C3 (Pryjma *et al.*, 1974). The activation products of C3 namely C3a and C3b, move faster than the native C3 over the electrophoretic field and appear on the right side of the cathode whilst the native C3 appears behind the activation products. The two cleavage products of C3 appear as a

single peak because they have shared antigenic determinants (Hudson and Hay, 1976). The identification of these cleavage products by antibodies against the native C3 indicates that the breakdown products have some antigenic determinants in common with the native C3. The observation of activation of C3 during infection probably confirms our earlier suggestion that C3 hypocomplementaemia may be due to activation of C3. The fact that the highest degree of C3 activation occurred at a period of highest parasitaemia (Figure 7.4, No. 11 and Figure 7.2a) in comparison with when the parasitaemia was low (Figure 7.4, No. 12 and Figure 7.2a) probably shows that the demand for C3 activation was linked to the level of parasitaemia.

When whole trypanosomes and *T. evansi* soluble extracts were used for the formation of immune complexes with the post infection serum, all the complexes caused the activation of C3 to some degree. Complexes formed with soluble antigen complexes differed in their activation in that more cleavage products were produced as seen by a large number of precipitation peaks obtained (Figure 7.6, Nos. 1 and 3) than when complexes were formed with live trypanosomes which resulted in the formation of a single cleavage product was observed (Figure 7.6, Nos. 5 and 7) were used. The presence of multiple peaks is thought to be due to a further breakdown of C3 cleavage products. Factors such as C3b inactivator present in the serum are known to break down the cleavage products, C3b, further into C3c and C3d which could result in the appearance of multiple peaks on the gel, particularly when C3b binds to a cell membrane (Frank, 1975). The fact that multiple component formation was not seen with complexes formed with whole trypanosomes suggests that other, internal, components of the parasite present in the soluble antigen preparation could have the ability to break down C3 cleavage products further or stimulate C3b inactivation. The absence of multiple peaks from the

in vivo assay for C3 activation (Figure 7.4, Nos. 11 and 12) may be an indication that multiple component formation could also be an artifact arising from the effects of chemicals or immunochemical processes (Nielsen and Duncan, 1988). The absence of such a finding in the serum from infected rabbits could suggest that a situation that can give rise to such extensive parasite lysis and soluble antigen release is unlikely in natural situations.

IgG was observed to be more efficient than IgM in activating C3 based on the height of the activated C3 products formed that was higher with IgG than IgM (Figure 7.7, Nos. 1 and 4). A similar observation was made by Ishizaka *et al.* (1968), Scott and Russell (1972), Klaus *et al.* (1979) and Van der Zee *et al.* (1986). Ishizaka *et al.* (1986) showed that rabbit IgM is more effective in activating complement when it reacts with a particulate antigen than when the same antigen is used in a soluble form. One possible explanation for the observation made in the present study could be that the IgM antibodies produced in *T. evansi* are not as antigen specific as the IgG and therefore may not bind very well to the trypanosome antigens to form complexes that would bring about complement activation. This view is supported by the findings of other workers (Hudson *et al.*, 1976; Terry *et al.*, 1980) that most IgM produced in trypansomiasis are non-specific to the antigen, probably produced as a result of polyclonal B cell stimulation.

The observation of larger and greater numbers of peaks in the classical pathway assay (Figure 7.6, Nos. 1 and 5) than the alternative pathway (Figure 7.6, Nos. 3 and 7) probably indicates that C3 activation in *T. evansi* is more effectively brought about through the classical than the alternative pathway, as the two immunoglobulins, IgG and IgM, were also found to cause varying degrees of C3 activation through the classical pathway (Figure 7.7, Nos. 1 and

4). This observation is in agreement with those of Jarvinen and Dalmasso (1976) who demonstrated that the classical pathway is superior to the alternative in activating C3 in *T. lewisi* infections. The fact that *T. evansi* infections had an effect on C3 is important because this could have far reaching effects on how the host not only reacts to an ongoing infection, but could also compromise the host's ability to respond to subsequent infections as C3 has been shown to play an important role in the development of immunological memory (Klaus and Humphrey, 1977).

CHAPTER EIGHT

THE ROLE OF COMPLEMENT (C3) IN THE GENERATION OF B MEMORY CELL FUNCTION IN RABBITS PRIMED WITH ANTIGENS OF TRYPANOSOMA EVANSI

INTRODUCTION

Although a major function of the immune system is to overcome current infection, it also has an equally important role in protecting animals from subsequent infections (Bowry, 1984; Roitt *et al.*, 1985). Such secondary responses of the host's immune system to an antigen differ from the primary response in both the magnitude and rapidity with which secondary responses are brought about. The ability of a host to mount a secondary response is dependent on "immunological" memory, a system by which the host immune system retains a record of an antigen it had experienced previously which it uses to react very rapidly and in a highly specific manner to the antigen on subsequent exposure. The concept of immunological memory is fundamental to the control of pathogens by vaccination. At the cellular level expression of immunological memory is linked to the expansion of populations of lymphocytes which were originally generated during the primary response but have persisted after recovery.

During maturation a proportion of the host lymphocytes after formation in the bone marrow migrate to the thymus for maturation into T-cells from where they eventually migrate to, and settle in the spleen and lymph nodes. During their passage through the thymus they become subtly altered in that the cells acquire functional specificity in the range of antigens they could recognise. Other lymphocytes do not pass through the thymus but settle directly in the lymph nodes and spleen. There they also mature, differentiate and develop functional specificity in the type of antibodies they can secrete. This second group of lymphocytes are called the B lymphocytes (Staines *et al.*, 1985). Each B cell carries on its surface an antibody molecule which acts as a receptor for antigens. In effect, each B cell is programmed to make just one

antibody, which is placed on its surface as an antigen receptor. Although these individual effector cells are somewhat restricted in their numbers and ability to recognise or react with antigens an effective immune response is brought about by the process of selection and clonal expansion of these effector cells. A B cell when exposed to an antigen that combines with its antigen-specific receptors becomes activated and divides several times to form a clone of daughter cells which start secreting antibody of the same type as that expressed originally on the surface of the parent cell, which in turn initiates a chain of events that culminates in the probable destruction of the antigen. Similar expansion of the parent cells on antigenic stimulation occurs with T-cells with the result that many daughter T-cells with the same range of antigenic specificity are produced.

After recovery from the infection, most of the T and B cells disappear but a small population of the lymphocytes persist and remain in circulation for many years charged with maintaining the memory of exposure to the antigen. These cells are referred to as T-memory (Tm) or B-memory (Bm) cells and are responsible for initiating the rapid and highly specific responses generated when the immune system of the host is exposed to the same antigen again.

Although the direct binding of an antigen to the appropriate surface receptor of the B memory cells is known to stimulate its proliferation and maturation into antibody-producing cells, other factors have been implicated in the process. The role of non-immunoglobulin receptors on B lymphocytes in the initiation of cell triggering by antigen has received particular attention in recent years (Klaus and Humphrey, 1977). Results from these studies suggest that the binding of activated C3 to the C3 receptors on B cells (Bianco *et al.*, 1970) may provide a necessary "second signal" for T-cell-independent B-cell

activation (Dukor and Hartmann, 1973). To investigate this possibility, Klaus and Humphrey (1977) evaluated the role of C3 complement component in the generation of B memory cells. Their observations led them to suggest that the stimulation of B-memory cells is greatly enhanced by the formation of antigen-antibody-C3 complexes on dendritic cells in lymphoid follicles. In effect they proposed that C3 stabilises and enhances the presentation of the antigen by the dendritic cells to the Bm cells, a sequence of events that would lead to the rapid proliferation and maturation of the B memory cells into antibody producing daughter B cells. Results from a later study (Klaus, 1979) indicated that the localisation of antigen-antibody (Ag-Ab) complexes in lymphoid follicles is abrogated by the depletion of C3 levels which could have an adverse effect on secondary immune response. These observations, together with evidence from other studies (reviewed by Thorbecke and Lerman, 1976) strongly suggests that the activation of Bm cells requires the deposition of Ag-Ab-C3 complexes in lymphoid follicles as an important first step. These complexes are envisaged as providing an antigen-specific B-cell trap within the developing germinal centre (Ponzio *et al.*, 1977). Further support for this concept was provided from studies that showed that preformed Ag-Ab complexes induce Bm cell generation more effectively and more rapidly than soluble antigens (Klaus, 1978).

It is likely, therefore, that C3 plays an important role in the response of an animal during secondary exposure to an antigen, through the activation of Bm cells (Klaus and Humphrey, 1977), although there is no direct evidence of its importance in responses to infections with pathogens such as trypanosomes. Most of the studies on the importance of C3 levels in trypanosomiasis had concentrated on the relationship of C3 with parasitaemia in infected animals (reviewed in Nielsen, 1985). Information on the ways in which C3 could

affect the ability of an animal to mount an immune response particularly during secondary infections is unavailable.

One well established consequence of trypanosome infection, is C3 depletion which results in lowered C3 levels throughout infection (Jarvinen and Dalmasso, 1976; Nielsen *et al.*, 1978a; Nielsen *et al.*, 1978b). In consideration of the fact that C3 is probably involved in the sequence of immunological activities that lead to the generation of secondary immune response and that C3 levels are depressed thoughout infection with *T. evansi*, studies in this chapter were designed to evaluate the influence of C3 depletion (Chocrane *et al.*, 1970; Klaus, 1978) on the ability of rabbits primed with *T. evansi* antigens to respond to the same antigen on subsequent exposure. The changes in antibody production by the primed animals during the challenge period were used as an indirect measure of Bm cell function.

Two experiments were carried out, one involved the evaluation of the ability of rabbits primed with *T. evansi* soluble antigens to produce antibodies when exposed to the same soluble antigens alone or to complexes formed between the soluble antigens and *T. evansi* specific sera from rabbits. The second study involved the evaluation of the effect of C3 depletion on the ability of rabbits primed with *T. evansi* soluble antigens to produce antibodies when exposed to complexes formed between the soluble antigens and *T. evansi* specific sera from rabbits.

MATERIALS AND METHODS

1. ANTIBODY PRODUCTION IN RABBITS PRIMED WITH T. EVANSI SOLUBLE ANTIGENS ON CHALLENGE WITH HOMOLOGOUS ANTIGENS OR IMMUNE COMPLEXES FORMED WITH T. EVANSI SPECIFIC SERA

Trypanosomes

T. evansi TREU 2147 described previously in Chapter 3 was used in this study for the preparation of soluble antigens (Ag) and soluble antigen-*T. evansi* specific serum (Ag-Ab) complexes. Separation of the trypanosomes from host blood cells was achieved with DEAE cellulose (DE52, Whatman Biochemicals) as described in Chapter 2. *T. evansi* soluble antigens used for the priming and challenge of rabbits and making of immune complexes were prepared as described in Chapter 2 by lysing the trypanosomes with three cycles of freezing and thawing with solid carbon dioxide and separation of the supernatant fraction, the soluble antigens, by centrifugation.

Animals

Four female adult New Zealand White rabbits were used. Blood samples were collected from each rabbit before they were inoculated with *T. evansi* soluble antigens (day 0) and on days 1, 10, 14, 17, 21, 24, 32 and 38 post-antigen inoculation. A second batch of blood samples was collected from these animals on days 2, 7, 14 and 21 after challenge with soluble antigens or the immune complexes. Serum was prepared from these blood samples as described in Chapter 2.

Protein Estimation

The protein concentration in all samples of both antigen and immune complexes were estimated photometrically by the BCA^{R} technique as described in Chapter 2.

Formation of Antigen-Antibody (Ag-Ab) Complexes

Immune complexes were formed by incubating 2 mg of the soluble antigens with 500 µl of a pooled serum sample consisting of equal volumes of sera collected on days 14, 17 and 18 from two rabbits infected with TREU 2147. The mixture was incubated at 37^{o}C for 30 minutes and then at 4^{o}C overnight. Any complexes formed were centrifuged at 1,200 g for 10 minutes and washed once with PBS before use.

Response of Rabbits to Challenge with Soluble Antigens or Complexes

Each of four rabbits was inoculated through the ear vein with 1 mg of the soluble antigen of *T. evansi* TREU 2147 made up to 1 ml in PBS. Sixty-three days after the first administration of the soluble antigens, two rabbits were then inoculated through the ear vein with 0.5 mg of the soluble antigen made up to 1 ml in PBS, while the two other rabbits were inoculated through the same route with 0.5 mg of Ag-Ab complex made up to 1 ml in PBS.

Enzyme-linked Immunosorbent Assay (ELISA) for the Estimation of Antibody Levels to T. evansi Soluble Antigens

To quantify the level of class-specific antibodies, IgG, IgM and IgA, produced by the primed animals on secondary challenge with soluble antigens alone or with immune complexes, the double "sandwich" ELISA technique was

used. For the detection of the class-specific antibodies a modification of the practical steps of ELISA described by Luckins (1977) was used.

Briefly, 100 µl of 1µg/ml of *T. evansi* TREU 2147 soluble antigen in coating buffer (Appendix 18) was used to coat the micro-ELISA plates overnight. After coating, the plates were washed with PBS/Tween 20 three times at intervals of three minutes per wash and the parts of the plate not coated with the antigens were blocked with 1% bovine serum albumin in PBS (w/v) (BSA, Sigma Ltd.) to prevent the non-specific binding of reagents to the plate. After blocking for 60 minutes at 37°C the plates were again washed as described above. Thereafter, 100 µl of the sera samples diluted 1:200 for IgG and 1:100 for IgA and IgM were added to the wells and incubated for 30 minutes at 37°C. The plates were again washed as before. After washing, 100 µl of affinity purified antisera (Nordic^{R}) raised in goats to the heavy chains (Fc) of rabbit IgG, IgM or IgA at a dilution of 1:6000 for IgG and 1:1000 for IgM and IgA respectively were added into the wells and incubated for 30 minutes at 37°C. After another cycle of three washes, 100 µl of affinity purified antisera (Nordic^{R}) raised in rabbits to the heavy and light chains of goat IgG conjugated to peroxidase were added to each well of the micro-ELISA plate at a dilution of 1:4000. After an incubation period of 30 minutes at 37°C, reactions were visualised by the addition of a substrate solution. The substrate used was 3,3',5,5'-tetramethyl-benzidine (TMB, Sigma Ltd.), 10 mg of which was dissolved in 1 ml dimethyl sulphoxide and then added to 99 ml citrate acetate buffer (0.1M, pH 6.0), followed by 15 µl of 30% hydrogen peroxide immediately before use. The reaction was stopped after 15 minutes by the addition of 50 µl 1M sulphuric acid and the optical density (OD) of each test well was read at 450 nm wavelength using a spectrophotometer.

2. THE EFFECT OF C3 DEPLETION ON THE ABILITY OF RABBITS TO PRODUCE A SECONDARY RESPONSE TO T. EVANSI ANTIGENS

Trypanosomes, soluble antigen administration and serum preparation were as described for the previous group of animals referred to in the first part of this chapter. In the present study the complement of some rabbits in this experiment was depleted by the administration of cobra venom factor (Chocrane *et al.*, 1970; Klaus, 1978) after which the animals were challenged with immune complexes only.

C3 Depletion

Fifty μg of pure cobra venom factor, CVF (Sigma Ltd.) made up to 1 ml in PBS was injected into two rabbits 46 days after the administration of the soluble antigens followed by a second injection three days later. Two control rabbits were also injected with the soluble antigens on the corresponding days which did not receive the CVF injection.

Secondary Exposure to T. evansi Antigens

Each of the four rabbits were inoculated through the ear vein with 0.5 mg of *T. evansi* (TREU 2147) Ag-Ab complex prepared as described in the first part of this chapter one day after the first administration of the first CVF injection.

Serum and Plasma Collection

Blood for the preparation of serum was collected from each of the rabbits before they were injected with the soluble antigens (day 0) and on days 7, 14 and 21 after the soluble antigen injection. Another batch of blood samples were collected from the animals on 1, 3, 7, 14 and 21 days after the administration of the first CVF injection.

On each of the days blood samples were collected for serum, separate blood samples were collected in bottles containing EDTA (Sigma Ltd.) for the preparation of plasma for the estimation of C3 levels in circulation by rocket immunoelectrophoresis (Laurell, 1966) as described in Chapter 7. Plasma samples were aliquoted into smaller volumes and stored at -79^{O}C until needed.

RESULTS

1. ANTIBODY PRODUCTION IN RABBITS PRIMED WITH T. EVANSI SOLUBLE ANTIGENS ON CHALLENGE WITH HOMOLOGOUS ANTIGENS OR IMMUNE COMPLEXES FORMED WITH T. EVANSI SPECIFIC SERA

Each rabbit produced the three classes of immunoglobulins, IgG, IgM and IgA, that recognised the soluble antigens of *T. evansi* as indicated from ELISA results (Figure 8.1A to D). The pattern of immunoglobulin isotype production was similar in all the rabbits during the period following the administration of the soluble *T. evansi* antigens. Basically, a rise in the level of IgM was recorded in the four rabbits between 10 and 14 days after the administration of the soluble antigens until about 21 days after the soluble antigen administration when the level of IgM was observed to wane in all the rabbits. IgG levels increased in all the animals from 10 days after the administration of the soluble

antigen and remained fairly stable, although with some decrease in levels in individual animals, until 63 days after the administration of the soluble antigens when the animals were either challenged with soluble antigens or Ag-Ab complexes. IgA levels although lower than IgG and IgM produced by the animals during the period, remained fairly stable all through the period following the administration of the soluble antigens in three of the animals. The IgA level was found to be consistently low in one rabbit (Figure 8.1D).

Response to Challenge

In the case of both rabbits challenged with soluble antigen there was an increase in the level of antibodies (Figure 8.1A and B, Nos. 63 to 84). A slight increase in IgG antibody production was followed by a gradual increase in the IgM and lastly IgA antibodies which showed a slight increase in level. Differences were, however, seen between the level of antibody produced by individual rabbits based on the ELISA readings (Figure 8.1A and B, Nos. 63 to 84). One of the rabbits responded earlier to the rechallenge by producing higher levels of antibodies two days after the administration of the soluble *T. evansi* antigens (Figure 8.1B, Nos. 65 to 84) compared to the second rabbit that showed a comparable increase in antibody level seven days after the administration of the soluble antigens (Figure 8.1A, Nos. 70 to 84).

There was also a rapid and marked increase in the level of antibodies produced by the two rabbits challenged with the Ag-Ab complexes. In both rabbits there was a clear increase in the level of IgG and IgM produced seven days after the administration of the complexes (Figure 8.1C and D, Nos. 70 to 84). About a three-fold increase in the level of IgG produced by the animals after the injection of the soluble antigens (Figure 8.1C and D, Nos. 1 to 38)

were observed from seven days after the administration of the immune complexes (Figure 8.1C and D, Nos. 70 to 84). After the initial increase, IgG levels remained stable with minor fluctuations until the observation was discontinued. About a one-and-a-half to two-fold increase of the IgM produced by the animals after being injected with soluble antigens were observed after they were challenged with the immune complexes. Thereafter a gradual decrease in the level of IgM was observed until the observation was discontinued (Figure 8.1C and D, Nos. 70 to 84). Slight increases in IgA levels were observed in the two rabbits within the period of observation.

2. EFFECT OF C3 DEPLETION ON SECONDARY RESPONSE TO T. EVANSI ANTIGEN

Rocket electrophoresis of the plasma samples collected from the two control rabbits primed with the soluble antigens of *T. evansi* which did not receive the CVF injection showed that C3 levels remained stable in the two rabbits throughout the period of observation (Figure 8.2A and B).

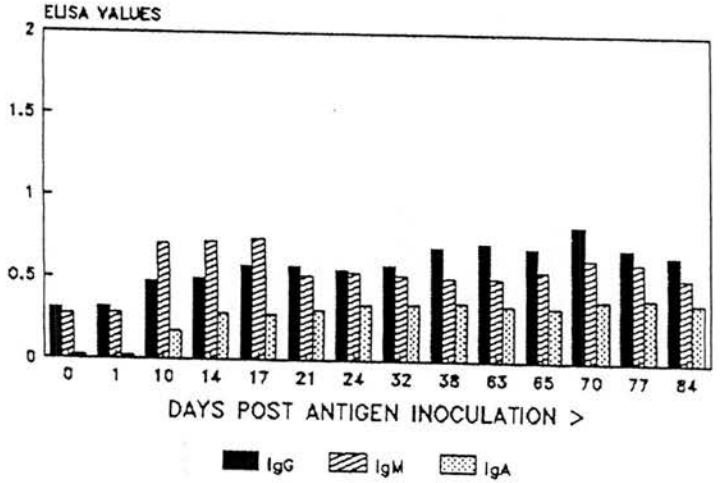
Rocket electrophoresis of the plasma samples collected after the rabbits were injected with CVF however, showed that C3 was completely removed from the plasma samples collected one and three days after the animals were injected with CVF (Figure 8.2B and D, Nos. 47 and 49). Analysis of plasma samples collected on 7, 14 and 21 days after the first administration of CVF showed a gradual restoration of plasma C3 levels in both rabbits (Figure 8.2C and D, Nos. 53 to 67).

FIGURE 8.1

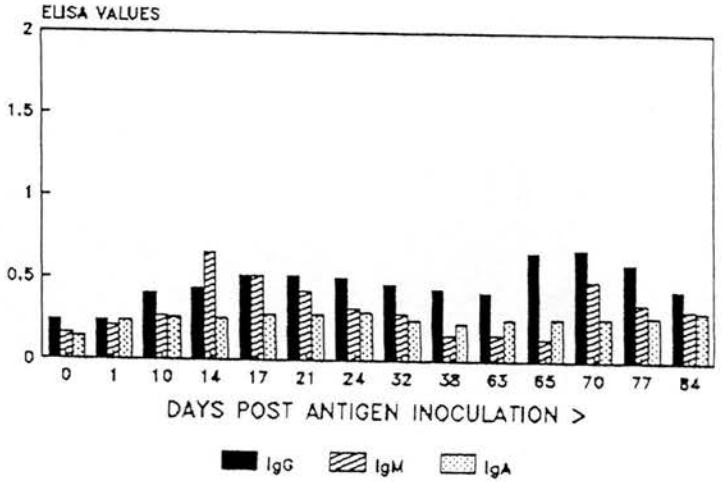
Class-specific antibody production in primed rabbits challenged with *T. evansi* soluble antigens of immune complexes.

A and B Rabbits challenged with *T. evansi* soluble antigens.

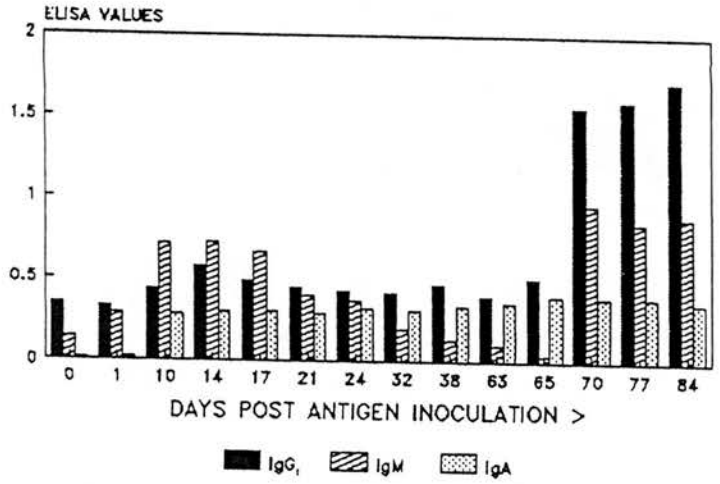
C and D Rabbits challenged with immune complexes formed with *T. evansi* soluble antigens and post infection serum.



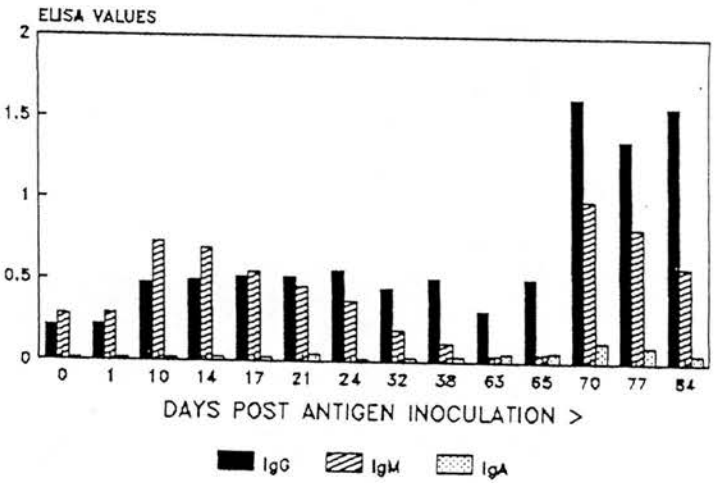
(A)



(B)



(C) : 63 = DAY OF CHALLANGE .



(D)

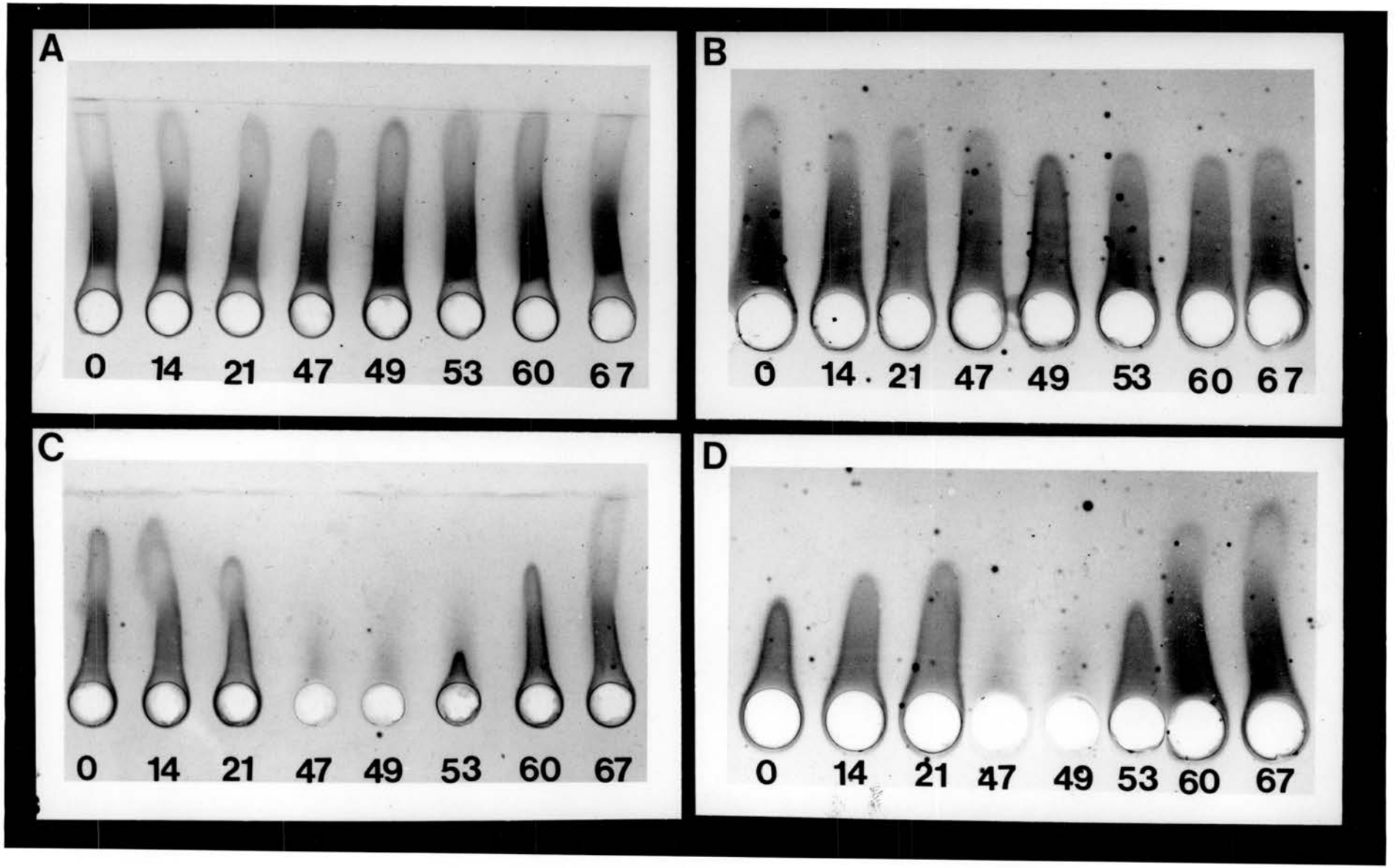
FIGURE 8.2

C3 levels in plasma of cobra venom treated and non-treated rabbits.

A and B: C3 levels in rabbits not treated with cobra venom factor.

C and D: C3 levels in rabbits treated with cobra venom factor.

(Cobra venom factor was administered on day 46 after the rabbits were injected with *T. evansi* soluble antigens and a second injection given three days later.)


A
0 14 21 47 49 53 60 67
B
0 14 21 47 49 53 60 67
C
0 14 21 47 49 53 60 67
D
0 14 21 47 49 53 60 67

Response of of Rabbits to T. evansi Soluble Antigens Prior to the Administration of the CVF Injection

The pattern of antibody production as revealed by ELISA readings were similar to those of the four rabbits in the first part of the experiment conducted in this chapter, where *T. evansi* primed rabbits were challenged with either soluble *T. evansi* antigens or with immune complexes. All the animals in the present part of the study produced the three classes of antibodies, IgG, IgM and IgA against the *T. evansi* soluble antigens (Figure 8.3A to D, Nos. 14 and 21). Variations in the antibody level produced by individual rabbits were also similar to those in the first part of the experiment.

ANTIBODY RESPONSE BY RABBITS CHALLENGED WITH IMMUNE COMPLEXES

The two *T. evansi* antigen-primed control rabbits produced the three classes of antibodies, IgG, IgM and IgA to *T. evansi* soluble antigens as shown by the ELISA readings after they were challenged with the immune complexes (Figure 8.3C and D, Nos. 47 to 67). Two- to three-fold increases in levels of IgG and IgM were seen in both animals after challenge compared to pre-challenge levels (Figure 8.3C and D, Nos. 53 to 67). The IgG level remained stable after the initial rapid increase, but gradual reductions in the IgM levels were seen in both rabbits in the remaining period of observation. IgA levels in both rabbits appeared unaffected during the post-immune complex administration period.

In the case of the two rabbits primed with *T. evansi* soluble antigen but treated with CVF, the administration of complexes produced a response consisting of the three classes of antibodies under consideration, IgG, IgM and

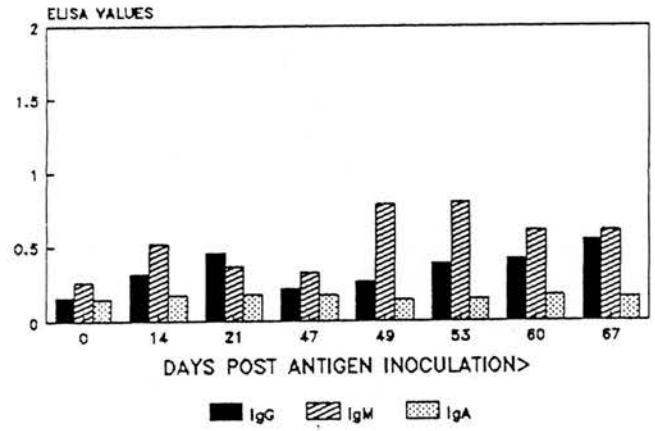
IgA. During this period the two rabbits produced more IgM than IgG antibodies with IgA ranking last in the amount produced based on the ELISA readings. Two- to three-fold increases in the IgM level produced before the animals were C3 decomplemented was produced from three days after the rabbits were injected with the CVF injection until day 21 post-CVF administration when the observation was terminated (Figure 8.3A and B, Nos. 49 to 67). About one-and-a-half times the IgG levels produced before the animals were injected with CVF was produced seven days after CVF injection and the immune complex administration until day 21 post-CVF administration when the observation was discontinued (Figure 8.3A and B, Nos. 53 to 67). The IgA level in one rabbit remained stable (Figure 8.3A, Nos. 47 to 67) while slight increases in IgA levels were observed in one rabbit from days 14 to 21 post-CVF administration.

FIGURE 8.3

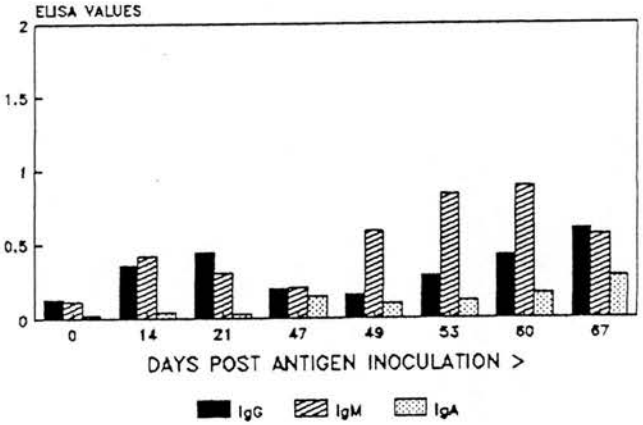
Class-specific antibody production by cobra venom treated and non-treated rabbits.

A and B Rabbits injected with cobra venom factor.

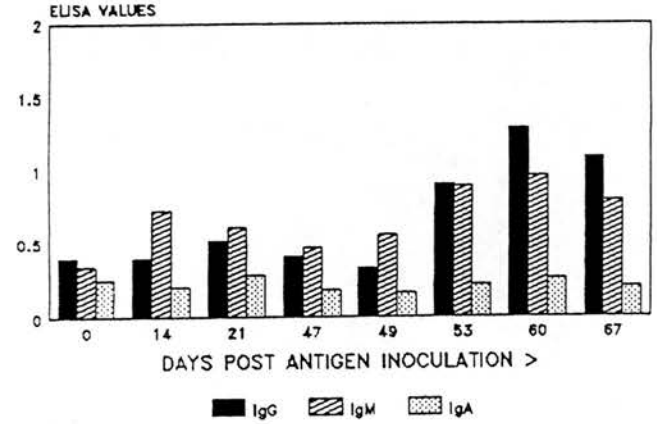
C and D Rabbits not injected with cobra venom factor.



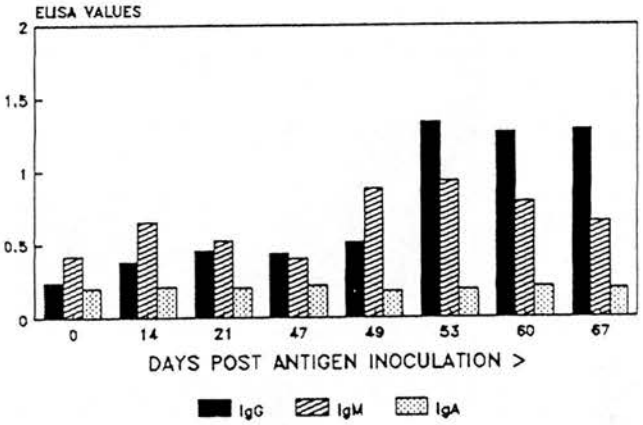
(A)



(B)



(C) : 46 & 48 DAYS OF CVF INJECTION .



(D) : 47 = DAY OF CHALLANGE .

DISCUSSION

It is impossible to measure directly the numbers of Bm cells or their direct response to antigens without reverting to the use of complex procedures, such as labelling the cells with radioactive reagents. To overcome this, an indicator of Bm cell function was based on an estimation of antibody levels produced by the animals during the period of secondary exposure. The suitability of such an index of Bm cell function stems from the fact that Bm cells are responsible for the antibody production during the period of secondary immune response via the activities of their antibody-secreting progeny (Irvine, 1979; Roitt *et al.*, 1989). The greater the Bm cell response then the greater the antibody response.

Results from the present study indicated that Ag-Ab complexes were more potent than soluble antigens alone in the activation of Bm cell function as higher levels of antibodies were produced by the animals challenged with the immune complexes (Figure 8.1C and D, Nos. 70 to 84) than those challenged with soluble antigens alone (Figure 8.1A and B, Nos. 70 to 84). The predominant IgG production by the animals during this period is thought to support the fact that the antibodies produced reflect the Bm cell function because Bm cells are more potent than unprimed B cells in the production of IgG (Roitt *et al.*, 1989). This observation is in agreement with the findings of Klaus (1978) who found that Ag-Ab complexes used in his study elicited stronger antibody responses than soluble antigens alone.

The complete absence of detectable levels of C3 in the plasma samples collected from rabbits soon after the injection with CVF (Figure 8.2C and D, Nos. 47 and 49) confirms the pharmacological property of CVF as a potent C3 decomplementing agent (Cochrane *et al.*, 1970) and that both rabbits could be

considered to be decomplemented at the time of challenge with the immune complexes.

There was a difference in the nature and magnitude of antibody production between the C3 intact (Figure 8.3C and D) and C3 depleted animals (Figure 8.3A and B). Higher levels of antibodies were produced during the challenge period by the C3 intact rabbits which were predominantly IgG (Figure 8.3C and D, Nos. 49 to 67) while the C3-depleted rabbits produced lower levels of IgG with high levels of IgM within the same period of observation (Figure 8.3A and B, Nos. 49 to 67). This result indicates that C3 has a role to play in the response of these primed animals to re-exposure to the homologous antigen, probably by stimulating the activation of Bm cells (Klaus and Humphrey, 1977). The reduced immune response observed in the C3-depleted animals compared to the complete absence of response observed by Klaus and Humphrey (1977) in thymectomized mice whose C3 levels were depleted before challenge, probably indicates that some C3 independent factors may be affecting the secondary immune response in the C3-depleted animals in our study. This might be linked to T-cell dependent secondary responses since we used thymus-intact rabbits for our studies.

The enhanced IgM production observed in the C3-depleted animals in the present study could be due to the fact that a factor that sustained the antibody production in animals in the absence of C3, may be more specific in stimulating IgM producing Bm cells than IgG or IgA producing Bm cells. Alternatively, it could indicate a state of non-stimulation of Bm cell function in these animals because of the absence of C3. In the absence of C3 which completes the bridge between the dendritic cells and the Bm cells (Klaus and Humphrey, 1977) that brings about Bm cell activation, it is likely that the

immune system of these animals reacted to the immune complexes as if they were being introduced into the animal for the first time and therefore, produced more IgM antibodies, which are characteristic of primary infections (Irvine, 1979; Roitt *et al.*, 1985).

The observations made in the present study could be of practical significance in the control of trypanosome infections due to the well documented reduction in C3 levels in trypanosomiasis (reviewed in Nielsen, 1985) which was also observed in the present study reported in Chapter 7. These results provide evidence that apart from the depletion of C3 compromising the ability of the immune system of the infected animals from eliminating the parasites via complement mediated effector mechanisms (Murray and Urquhart, 1977), it may also be important in determining the way in which an animal responds to a secondary exposure to trypanosome infections probably by breaching the sequence of events that leads to the activation of "immunological" memory.

CHAPTER NINE

PATHOLOGICAL CHANGES IN SELECTED ORGANS OF RABBITS EXPERIMENTALLY INFECTED WITH TRYPANOSOMA EVANSI

INTRODUCTION

After infection with African trypanosomiasis the mammalian host mounts an antibody response against the parasite. This response appears to be directed strongly against the variable surface antigens of the parasite, and feebly against the non-surface components (Le Ray, 1975). The antigen-specific antibodies enable the host to eliminate the parasites in the bloodstream by a combination of antibody-dependent phagocytosis (Greenblatt *et al.*, 1983; Ngaira *et al.*, 1983) and/or complement-mediated lysis (Murray and Urquhart, 1977). Extravascular localisation has been demonstrated for *T. brucei* (Losos and Ikede, 1970) and *T. rhodesiense* (Nagle *et al.*, 1980) with the suggestion that such parasite populations serve as the source of parasites that give rise to new antigenic variants that re-invade the circulatory system. A variety of tissue lesions arising from the effects of these localised parasite populations may, complicate the trypanosome disease syndrome subsequently leading to the death of the host (Koten and Raadt, 1969; Omerod, 1970; Hutt and Wells, 1971; Losos and Ikede, 1972; Sadun *et al.*, 1973; Murray *et al.*, 1974a; Poltera *et al.*, 1976). Although some of these findings have been well documented, the exact nature of the changes undergone by these organs during trypanosome infection and the sequence of events that lead to the death of an infected host, particularly in animals such as rabbits that die without experiencing fulminating infections is still unresolved (Seed, 1969). Results of some studies indicate that immune responses of the host to the parasites while providing a means of eliminating the parasite through the activities of the antibodies (Greenblatt *et al.*, 1983; Ngaira *et al.*, 1983) can contribute to the development of pathological events such as the deposition of immune complexes in organs such as the kidney (Nagle *et al.*, 1974; Van Marck Vervoort, 1980), leading to kidney damage and subsequently the death of the host. Immune complex-

mediated damage akin to type III allergic reactions has also been suggested to occur and also constitute another way through which the immune response of the host animal aimed at alleviating the plight of the infected animal could lead to the development of some undesirable consequences. Such reactions are thought to arise following the repeated destruction of trypanosome populations by the host antibody and the continuous shedding of antigens by live trypanosomes into the antibody-laden surrounding (Vickerman and Barry, 1982). The development of autoimmune reactions against host components such as immunoglobulins (Klein *et al.*, 1970), liver, brain, kidney and heart tissues (Mackenzie and Boreham, 1971; Mansfield and Kreier, 1972), spleen cells (Mitry-Martin *et al.*, 1979), thymocytes (Kobayakawa *et al.*, 1979), and erythrocytes (Kobayakawa *et al.*, 1979; Rickman *et al.*, 1981) have been demonstrated in trypanosomiasis and is an undesirable consequence of the host immune response against the parasites.

The third complement component (C3), which plays an important role in both the generation of B memory (Bm) cells, (Klaus and Humphrey, 1977) and lysis of trypanosomiasis is affected by trypanosome infections. Studies conducted by several workers reviewed by Neilsen (1985) have amply demonstrated that marked C3 decomplementaemia occurs in trypanosomiasis, which suggests that apart from trypanosome infection having an immediate effect on the animal as a result of the activities of the parasites and possibly other consequences of the disease, it is likely, therefore that the infection may also compromise the ability of an infected animal to deal with a current infection and mount a significant secondary response. The pathological consequences of trypanosomiasis on some organs, e.g. glomerular hypercellularity which subsequently leads to the development of proteinuria and death of infected animals have been associated with the deposition of C3

and some antibody classes particularly IgG and IgM in the renal glomeruli. Furthermore, the liver which produces more than 90% of C3 (Lambris, 1988), is also known to be damaged in infections caused by trypanosome species transmitted by tsetse flies such as *T. congolense* (Morrisen *et al.*, 1982b).

The implication of these observations is that while some changes taking place in the organs of trypanosome infected animals may be associated with the direct activities of the parasites, some other changes may be the consequences of the host's defence response which could be both to the advantage and disadvantage of the host. Based on this premise, the studies in this chapter were designed to evaluate the changes occurring in some selected organs considered to be associated with the immunobiology and/or pathology of trypanosomes with a view to distinguishing between changes that are pathological and consequently destructive from those that are physiological and protective. The organs selected for evaluation included the kidneys, lymph nodes, liver and the spleen. The selection of other organs was based on the routine observation that they are usually inflamed in rabbits infected with *T. evansi* and included the vulva, the ears and the eyelids.

MATERIALS AND METHODS

Six adult female New Zealand White rabbits were used for the study. Four of the rabbits were infected with 4 x 10^5 motile trypanosomes, TREU 2147, from mice with fulminating infections. The trypanosomes were separated from the host blood cells using anion-exchange chromatography with DEAE cellulose (DE52, Whatman Biochemicals) as described previously in Chapter 2, resuspended in 0.5 ml aliquots in ice-cold PSG (Appendix 2) and injected into rabbits through the ear vein. The two other rabbits were

sacrificed as uninfected control animals.

Two of the rabbits infected with *T. evansi* were treated with the trypanocidal drug, diaminazene aceturate (Berenil^{R}, Hoechst, W. Germany) (7 mg active principal per kg body weight) 28 days after infection. The two remaining infected rabbits were sacrificed 28 days after infection. The two uninfected rabbits were also sacrificed at the same time.

HISTOLOGY

Control Animals

Animals were killed with Pentobarbitone sodium, Euthatal^{R} (May and Baker (U.K.) Ltd.) by intravenous injection. Tissues were collected from the prefemoral and prescapular lymph nodes, the spleen, liver, kidneys and the vulva, from the tip of the ear and the eyelid from each animal.

Infected Animals

Tissues were obtained with a 3 mm diameter tissue punch (Baker Laboratories, USA) under Xylocaine-HCl local anaesthesia from the vulva, through the outer skin 7 and 17 days after infection from two rabbits. Twenty-eight days after infection the two rabbits were killed with Euthetal^{R} (May and Baker (U.K.) Ltd.) and tissues also collected from all the organs under study in this experiment. Tissue preparation and staining were carried out as described for the tissue samples collected from the control rabbits.

Infected and Drug Treated Animals

Tissues were obtained with the 3 mm diameter tissue punch from the vulva of the two rabbits 7, 17 and 28 days after infection.

The two rabbits were killed 3 and 20 days after drug treatment with Euthetal respectively. On each of these days, tissues were collected from all the organs under study and sections prepared and stained as described previously. All tissues were then fixed in 10% buffered formal saline before sections 5 μm thick were cut from paraffin-embedded blocks and stained with Mayer's haematoxylin and eosin or Giemsa (Cook, 1974).

RESULTS

The histopathological examination of stained tissue sections revealed the presence of some changes in the organs of the infected animals which were not present in the sections collected from tissues of *T. evansi* uninfected animals as described below.

The Liver

The livers of the non-infected animals at necropsy were firm, had sharp edges and were not swollen. Sections of tissues collected from the livers of the non-infected animals showed radiating hepatic cords, well defined sinusoids and no cellular infiltration (Figure 9.1A) indicative of normal hepatic architecture (Wheater *et al.*, 1987). The livers of the infected rabbits were, however, grossly swollen had rounded edges, were tarry red in colour and friable. Marked cellular infiltration mainly in the portal tracts, and fatty degeneration of the hepatocytes, were the main features observed in the tissue sections prepared from tissues collected from the livers of rabbits killed 28 days

after infection (Figure 9.1B). Cell types in the filtrate were mainly small and large lymphocytes, plasma cells, macrophages and a few neutrophils in the blood vessels (Figure 9.1C). In many sections the hepatic cords were disorganised with small foci of centrilobular hepatocyte degeneration. There was an increase in the number of Kupffer cells lining the hepatic sinusoids. Vacuolations of variable sizes, disruption of the sinusoids, some evidence of nuclear fragmentation and periportal haemorrhages were some of the other findings seen in the stained tissue sections. No trypanosomes were identified in any of the liver sections examined (Figure 9.1B). Sections prepared from tissues obtained from the liver after drug treatment showed a marked reduction in cellular infiltration and this was more evident in the sections from the tissues collected 20 days after than three days after drug treatment.

The Vulva

Grossly, the vulva of the non-infected animal was not swollen. Histological sections revealed a well defined epidermis, dermis and a thick fatty layer with a few mononuclear cells scattered around the dermis (Figure 9.2A). Grossly the vulva of the infected rabbits were swollen from around 17 days after infection and remained so until day 28 after infection. Microscopal examination of stained sections of tissues obtained from the vulva seven days after infection revealed no obvious histopathological changes other than signs of cellular infiltration. Sections from tissues collected 17 and 28 days after infection revealed the presence of extensive oedema, massive cellular infiltration into the dermis of the vulva skin and vulva parenchyma (Figure 9.2B). The cell types in the infiltrate include numerous small and large lymphocytes, plasma cells and macrophages. The most striking observation made was the presence of a large population of trypanosomes throughout the

vulval dermis. Trypanosomes were seen in the sections prepared from tissues collected 17 days after infection with the highest parasite numbers observed from sections obtained from tissues collected 28 days after infection (Figure 9.2C). Parasite counts from the blood at this time, 28 days after infection, were found to be lower than counts obtained 17 days after infection. In the case of drug treated animals much trypanosome nuclear debris was observed in the tissue section obtained from the vulva (Figure 9.2D) which appeared to be smaller in size, in sections obtained 20 days after than three days after drug treatment. Cellular infiltration was also reduced in the sections from tissues collected after drug treatment than in sections from tissues obtained 28 days after infection. Many plasma cells and some macrophages were however, seen in the sections after the animals had been drug treated.

The Spleen

Grossly the spleens of the infected animals were about three to five times larger than those of the non-infected animals. Sections from tissues obtained from the spleens of the uninfected rabbits showed well defined splenic cords lined by macrophages with about one neutrophil seen in about three microscopic fields. Many erythrocytes with well defined outlines were identified in the venous sinuses (Figure 9.3A). The most extensive change following *T. evansi* infection in the spleen was observed in the red pulp which was found to be more extensive, engorged and hyperplastic. The sinuses were distended by erythrocytes and macrophages, with some of the macrophages containing trypanosomal nuclei. Trypanosomal nuclei were also observed scattered throughout the red pulp. Extensive vacuolar spaces were observed in the pulp cords with one to two neutrophils identified per microscopic field. Some plasma cells were also identified in the red pulp parenchyma (Figure

9.3B). After drug treatment lesser amounts of cellular infiltration and engorgement were observed in stained sections. Few trypanosomal nuclear materials were still evident at this time in the red pulp which appeared to be smaller in size than in sections of tissues obtained 28 days after infection.

The White Pulp: The white pulp of the spleens of the control animals had defined boundaries and were clearly demarcated from the adjoining red pulp (Figure 9.4A). Microscopal examination of stained sections through the white pulp of the control rabbits revealed the presence of many small and large lymphocytes and macrophages. Plasma cells and erythrocytes were not evident in the sections. In comparison with the control animals, the lymphoid nodules of the white pulp of spleen tissue collected 28 days after infection were hyperplastic and diffuse in nature with the lymphoid nodules merging into the extensive red pulp in many places (Figure 9.4B). Microscopal examination of the stained sections of the white pulp from the spleen of the infected animals revealed the presence of predominantly large and some small lymphocytes, many macrophages within the periarteriolar sheath and nodules. Some red blood cells were also present in the sections. No trypanosomes were detected in the white pulp of the infected animals. After drug treatment, there was a reduction in the number of cells identified in the white pulp. Plasma cells, however, were still evident in the sections by this time.

The Lymph Nodes

The lymph nodes of the infected animals in comparison with those of the controls were swollen. Stained sections of tissues obtained from the lymph node of uninfected control animals had well defined follicular areas in the cortex. The paracortical and medullary zones appeared normal (Wheater *et*

al., 1978). There was no evidence of cellular proliferation into the different zones of the lymph node (Figure 9.5A).

Similar histological changes were observed in the stained sections of the prefemoral and prescapula lymph nodes obtained from the rabbits 28 days after infection. The cortical follicles were poorly defined due to very active hyperplasia with many large lymphocytes, plasma and macrophages evident in the sections. Active proliferation of macrophages, indicated by the presence of the "starry-sky" appearance of such areas was evident in some sections of the paracortex and the medulla. Marked cellular infiltration into the substance of the lymph node was generally evident (Figure 9.5B). The medulla of the lymph nodes from infected animals was extensive and the medullary cords were filled with many plasma cells, large and small lymphocytes. Tingible body macrophages were seen in large numbers. No trypanosomes were seen in any of the sections. After drug treatment, there was a reduction in the amount of cellular infiltration in both the follicular and paracortical areas of the lymph nodes. The follicular areas were, however, still diffuse with most areas of the paracortex still retaining the starry-sky appearance.

The Kidneys

The microscopal appearance of the stained sections of kidney tissues collected from uninfected animals (Figure 9.6A) was as described for the normal kidney (Wheater *et al.*, 1987). Grossly, kidneys from the infected animals were slightly swollen and the microscopal examination of stained sections of tissues collected 28 days after infection revealed evidence of glomerulitis and cloudy swelling. There was an increase in cellularity and cellular infiltration in all the sections of the kidney and in particular around

the renal pelvis (Figure 9.6B). Marked mononuclear cellular infiltration into the renal corpuscles and papillae with most of the cells appearing as clusters in the collecting tubules was a common feature in the sections of the kidneys from the infected animals (Figure 9.6C). The cell types in the infiltrate were mainly plasma cells, small and large lymphocytes and macrophages. Some scattered haemorrhagic foci were seen all over the cortex and in the renal corpuscles (Figure 9.6B). No trypanosomes were observed in any of the sections of the kidneys examined. After drug treatment there was a reduction in the magnitude of cellular infiltration in the renal papilla but not in the corpuscles.

The Ear and the Eyelids

The stained sections of the tissues obtained from the ear and eyelids of the uninfected animals were clearly divided into the epidermal and dermal sections with no evidence of cellular infiltration. Grossly, the ear and eyelids of infected animals were swollen. Microscopal examination revealed the presence of many macrophages and large lymphocytes with some small lymphocytes, plasma cells and few neutrophils. Many trypanosomal nuclei were evident in the sections with many of them detected in the macrophages (Figure 9.6D). After drug treatment, there was a reduction in the magnitude of cellular infiltration in the stained sections of the ear and eyelid.

FIGURE 9.1A

Giemsa's stained section of the liver of a non-infected rabbit showing radiating hepatic cords and well defined sinusoids. x 150.

FIGURE 9.1B

Giemsa's stained section of the liver of a rabbit 28 days after infection showing marked cellular infiltration in the portal tracks and fatty degeneration of the hepatocytes. x 150.

Figure 9.1

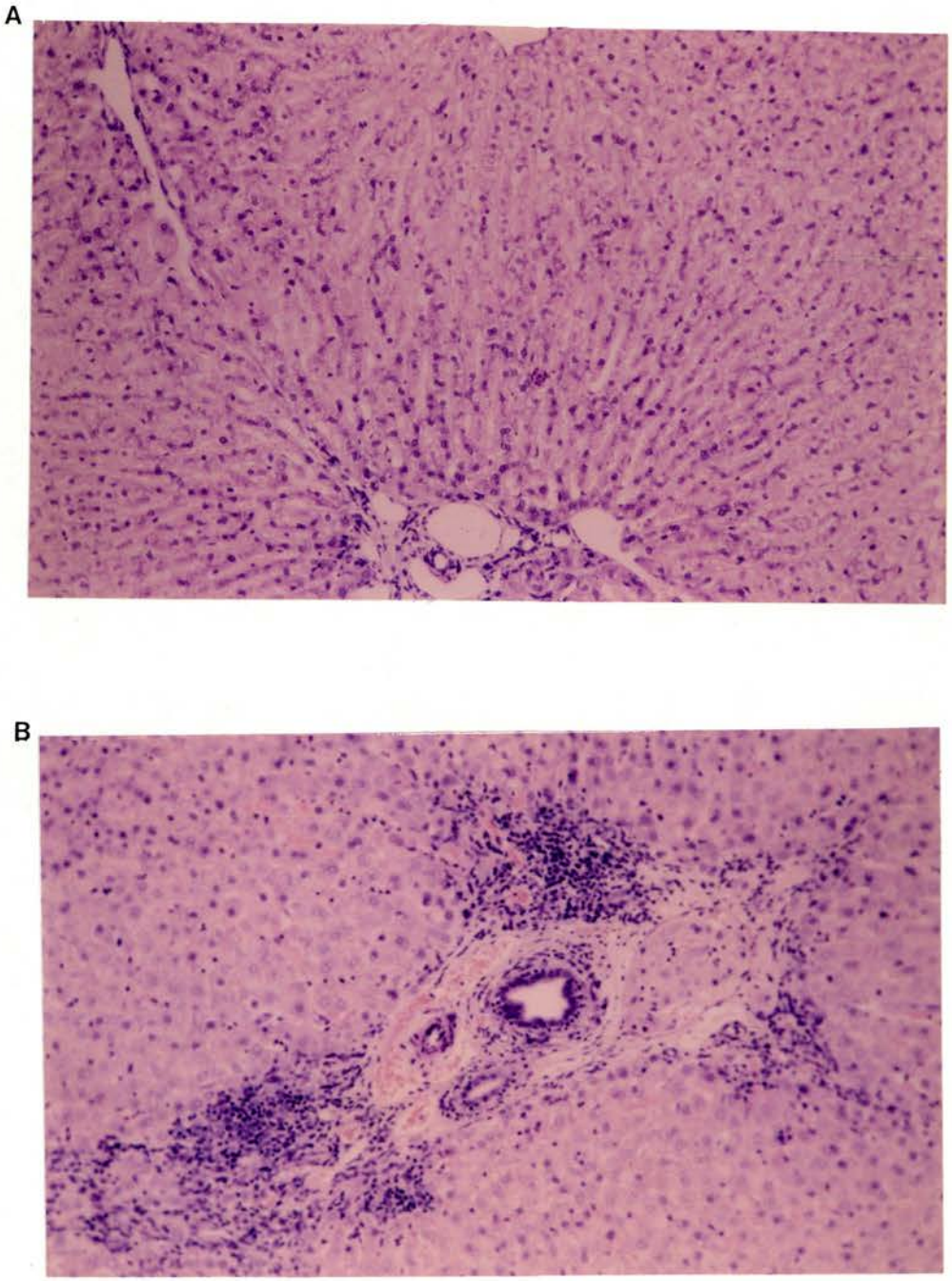


FIGURE 9.1C

Giemsa's stained section of the liver of a rabbit 28 days after infection showing marked mononuclear cellular infiltration and the presence of some polymorphonuclear leucocytes. x 500.

Figure 9.1C

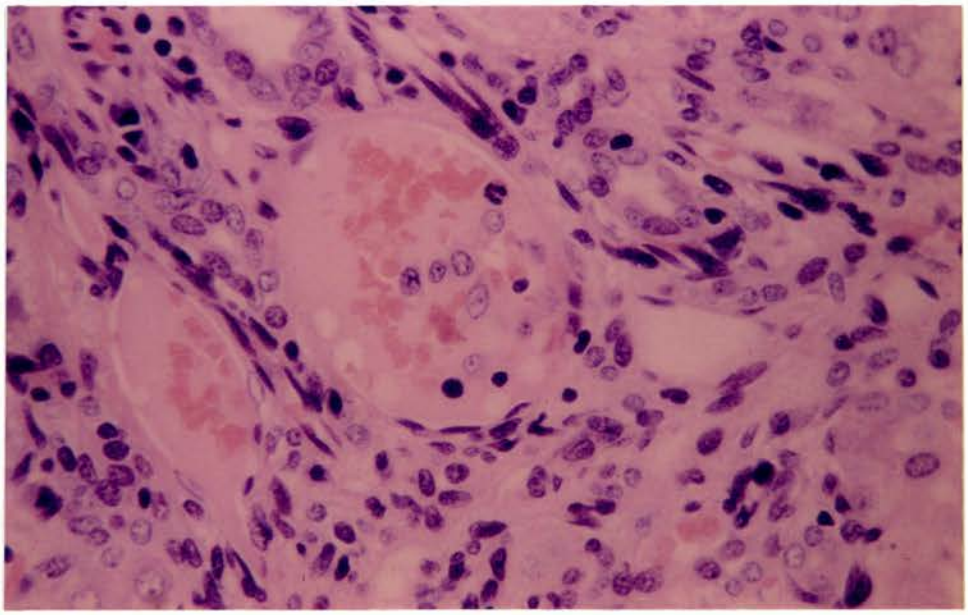


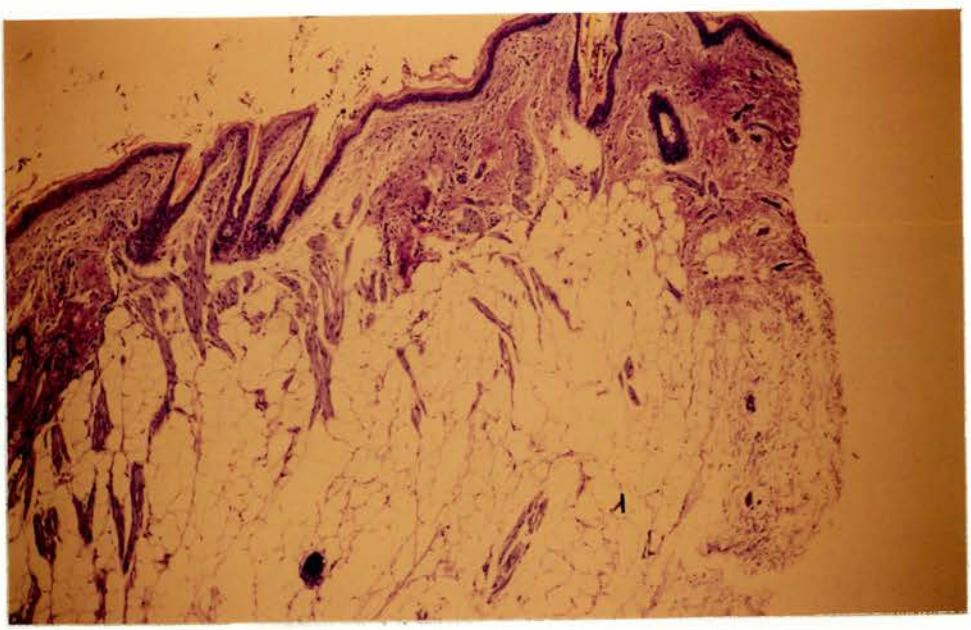
FIGURE 9.2A

Giemsa's stained section of the vulva of a non-infected rabbit showing well defined epidermis, dermis and thick fatty layer with some mononuclear cells scattered in the dermis. x 50.

FIGURE 9.2B

Giemsa's stained section of the vulva of a rabbit 17 days post infection showing oedema and extensive cellular infiltration. x150.

A



B

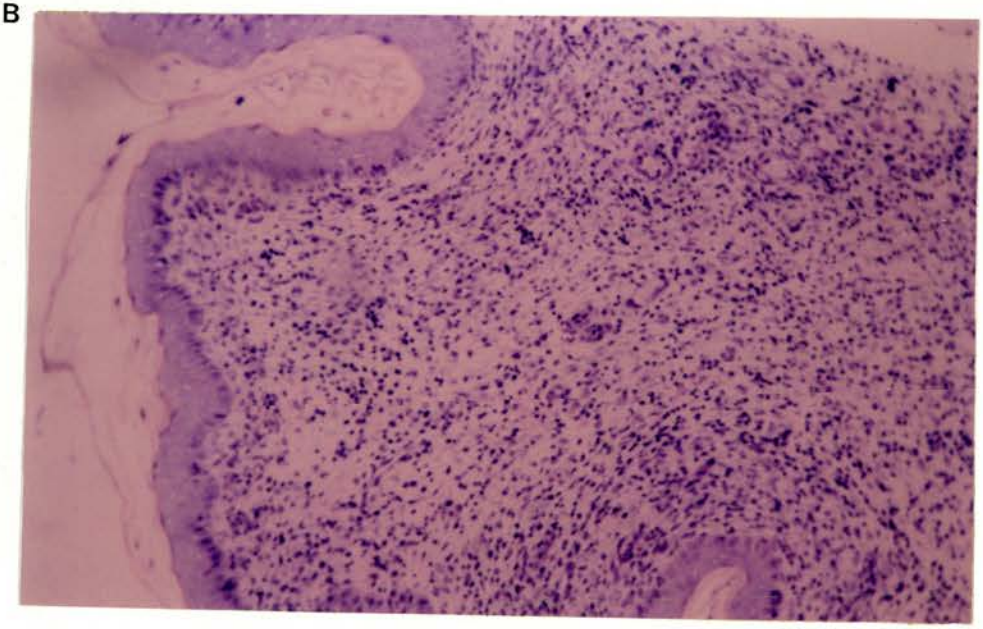


FIGURE 9.2C

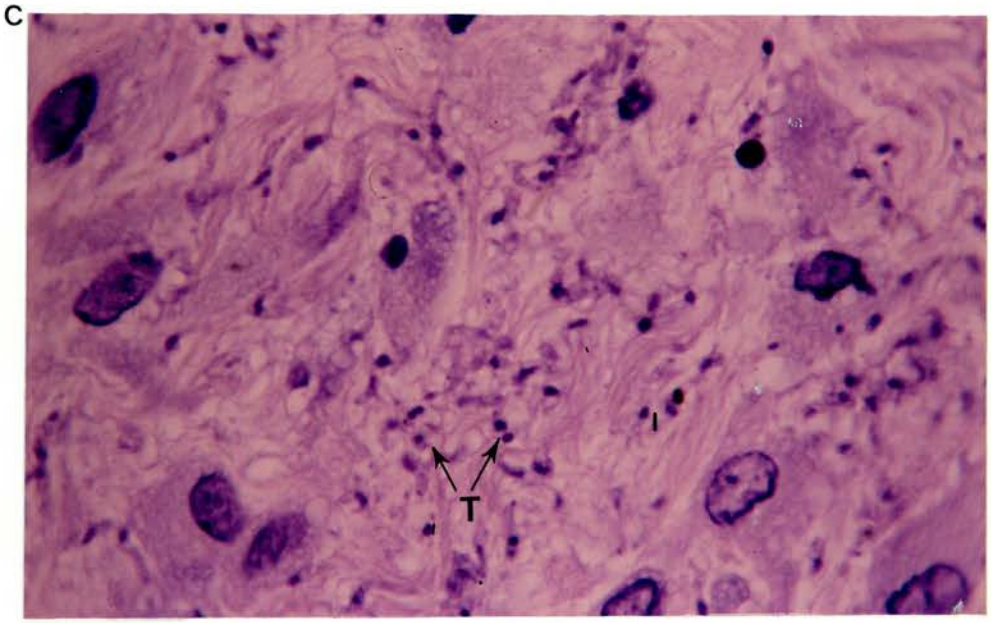
Giemsa's stained section of the vulva of a rabbit 28 days after infection showing many trypanosomes (T) x 1,500.

FIGURE 9.2D

Giemsa's stained section of the vulva of a rabbit after drug treatment showing debris of trypanosome nuclei (N).

x 1,500.

Figure 9.2



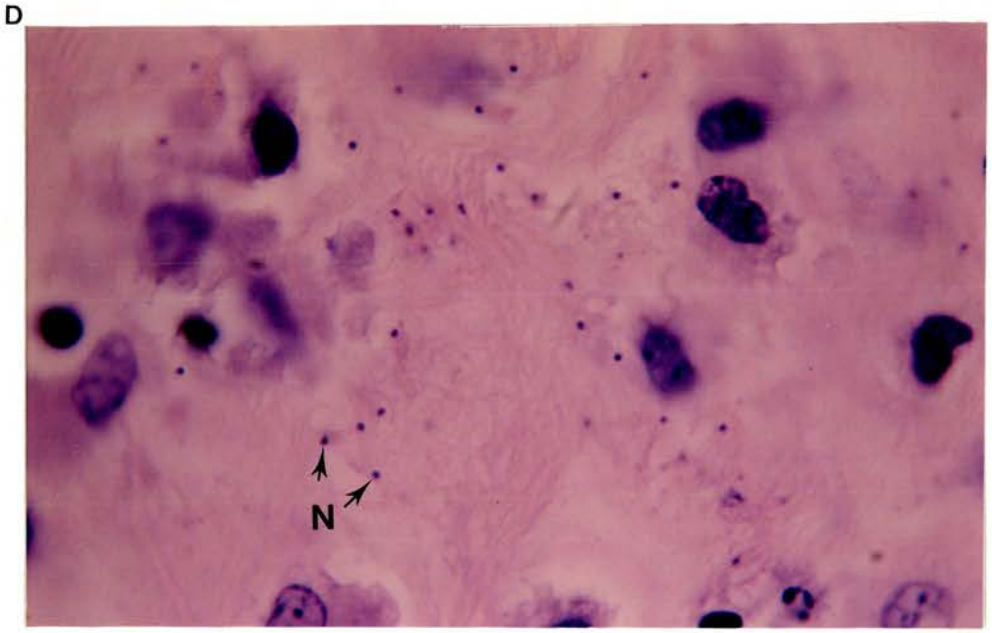


FIGURE 9.3A

Section of the spleen of an uninfected rabbit through the red pulp showing well defined splenic cords, lined by macrophages and engorged with erythrocytes (H & E x 1,500).

FIGURE 9.3B

Section of the spleen of a rabbit through the red pulp from tissues collected 28 days after infection showing extensive vacuolar spaces with cellular infiltration of plasma cells, lymphocytes and macrophages (M) with some of the trypanosomal nuclei (T) phagocytosed by macrophages (M) (H & E x 1,500).

Figure 9.3

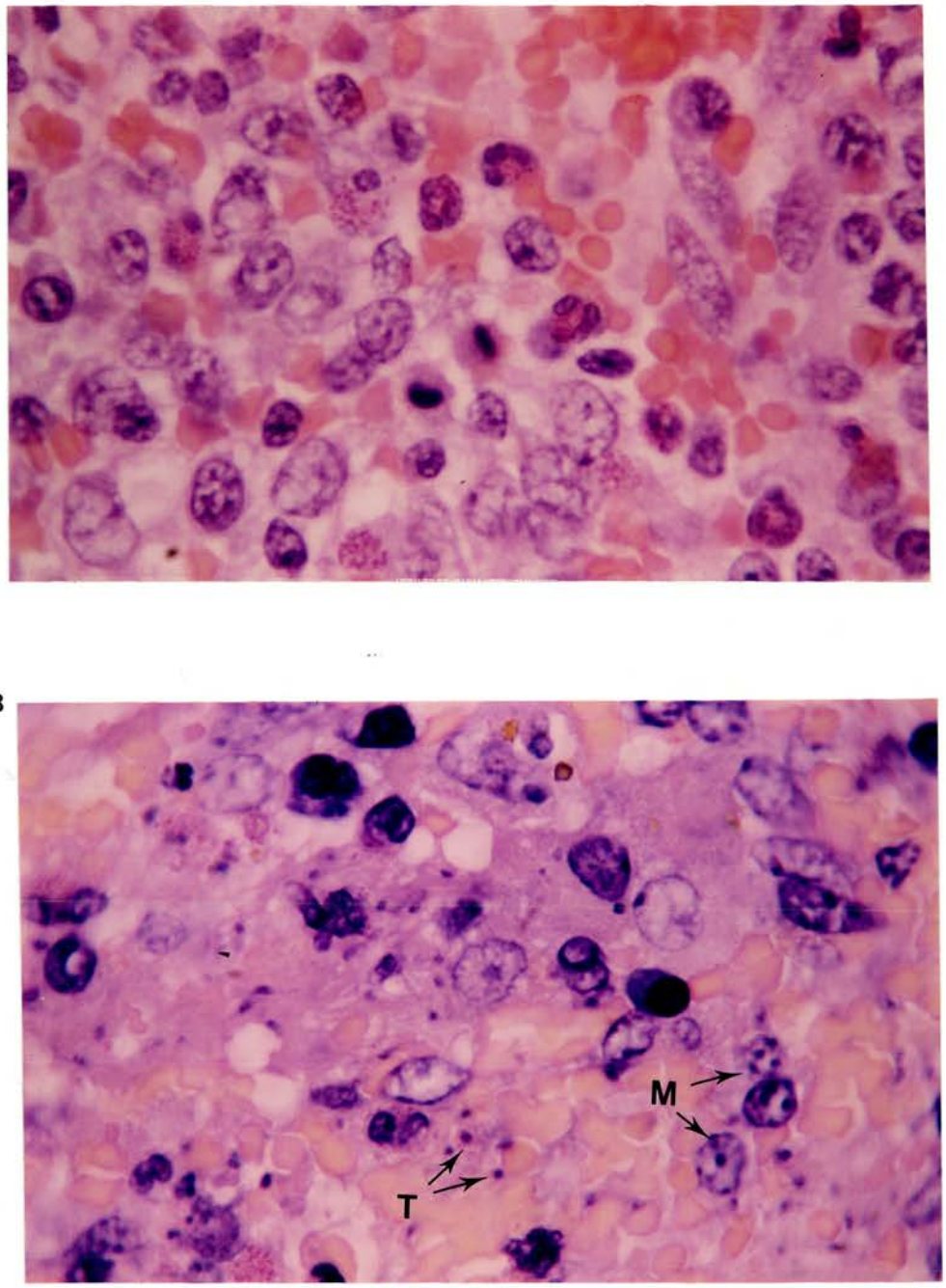


FIGURE 9.4A

Giemsa's stained section through the spleen of a non-infected rabbits showing non-proliferating lymphoid nodules (L) clearly demarcated from the adjoining red pulp. x 50.

FIGURE 9.4B

Giemsa's stained section through the spleen of an infected rabbit collected 28 days post infection showing hyperplastic and diffuse lymphoid nodules (L) merging into the extensive red pulp. x 50.

Figure 9.4

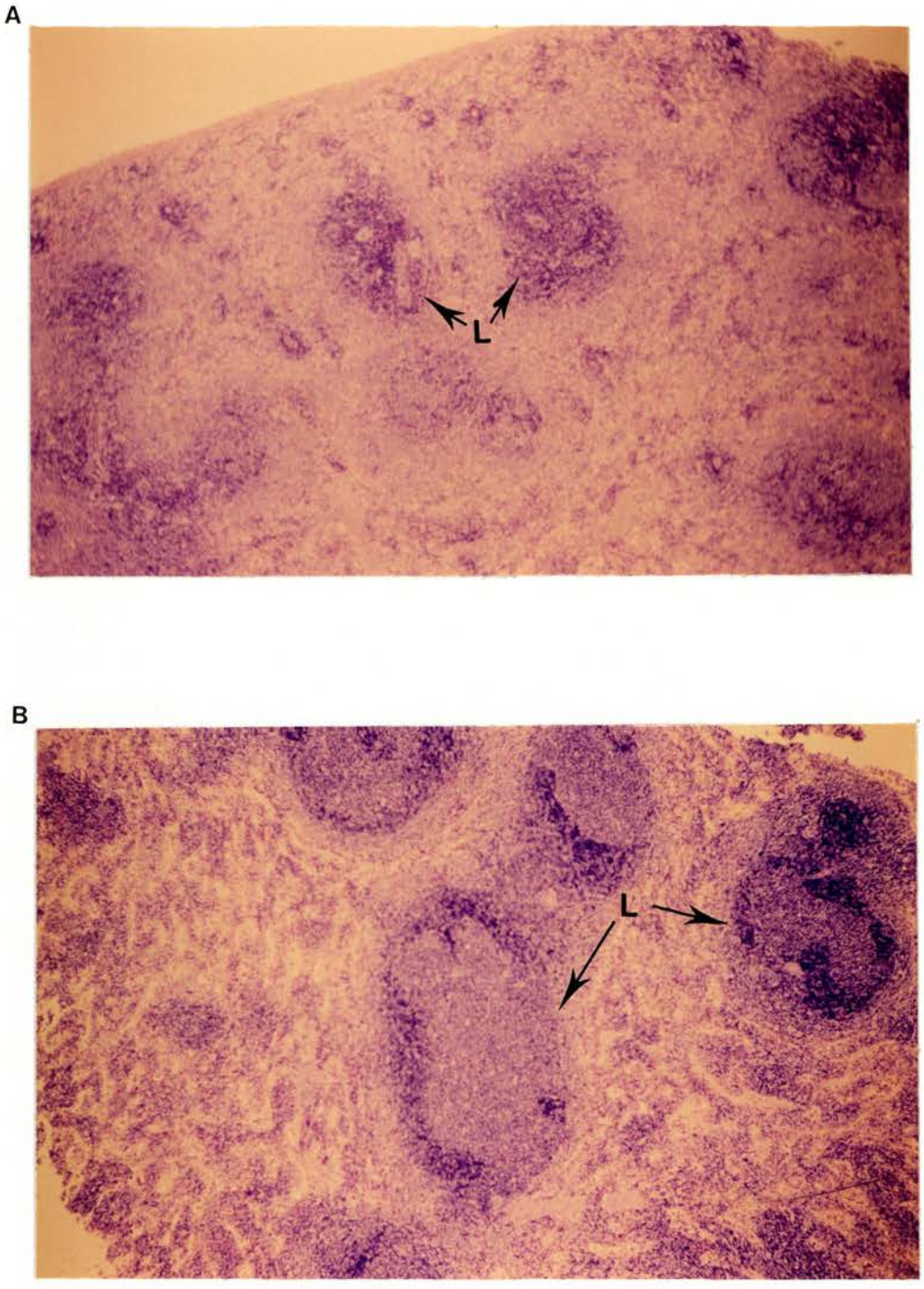


FIGURE 9.5A

Giemsa's stained section through the lymph nodes of a non-infected rabbit showing well defined follicular areas (F) in the cortex, paracortical and medullary zones. x 50.

FIGURE 9.5B

Giemsa's stained section through the lymph node collected 28 days after infection showing poorly defined and hyperplastic follicular areas (F) in the cortex and starry-sky appearance (S) of some areas in the paracortex and the medulla. x 50.

Figure 9.5

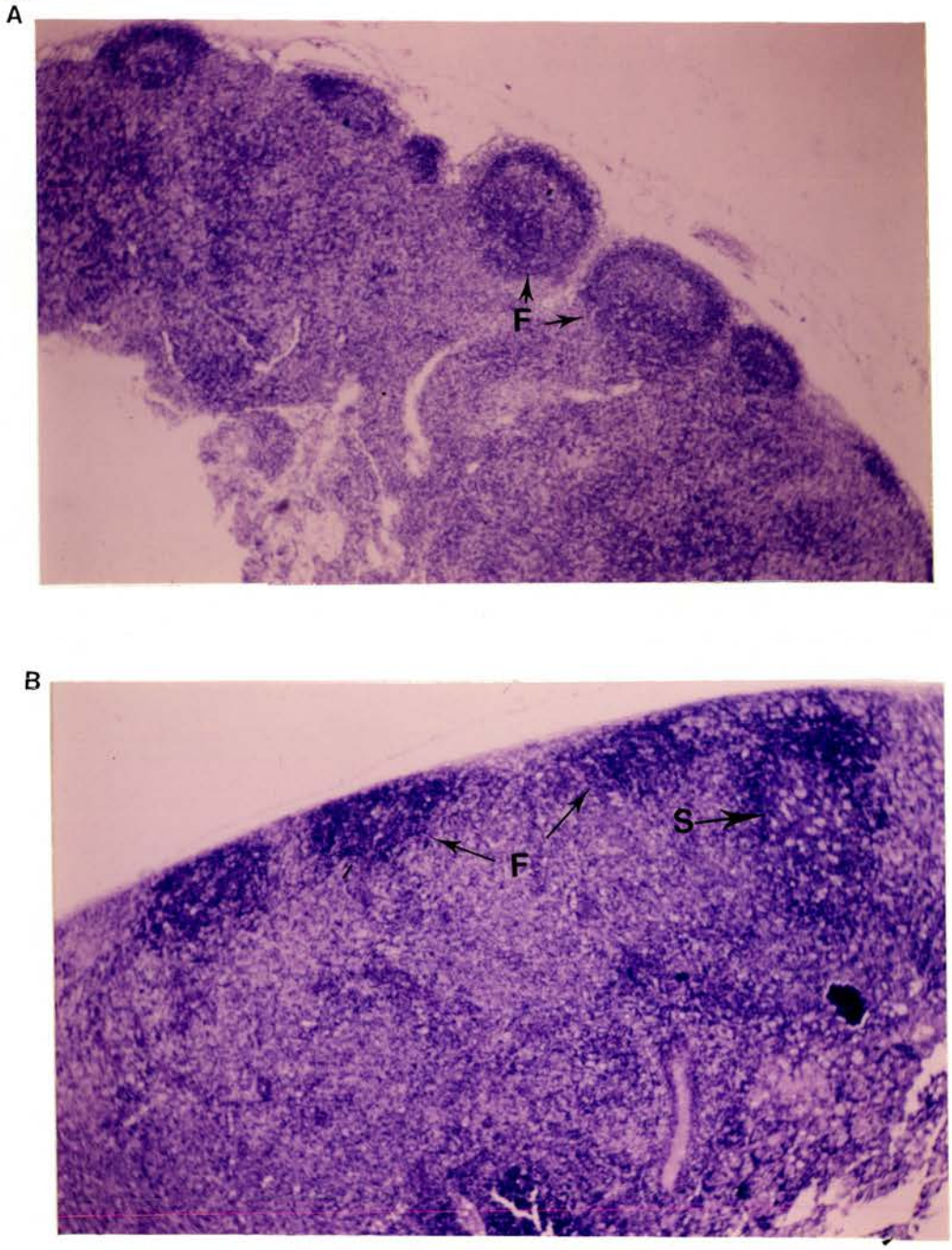


FIGURE 9.6A

Giemsa's stained section of the kidney of the non-infected rabbit showing collecting tubules devoid of any cellular infiltration. x 150.

FIGURE 9.6B

Giemsa's stained section of the kidney collected 28 days post infection showing signs of marked glomerulitis, cloudy swelling and marked cellular infiltration. x 150.

Figure 9.6

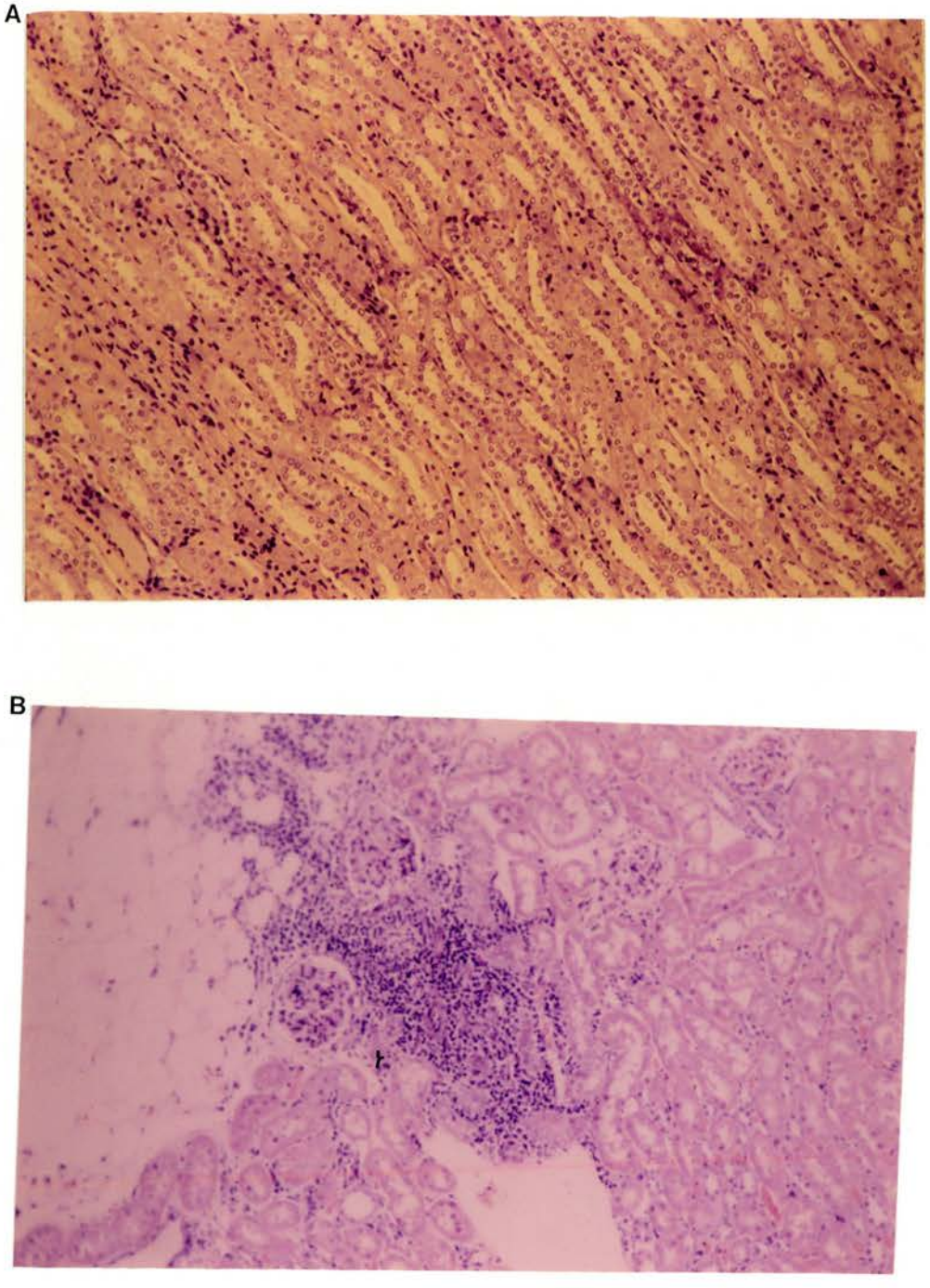


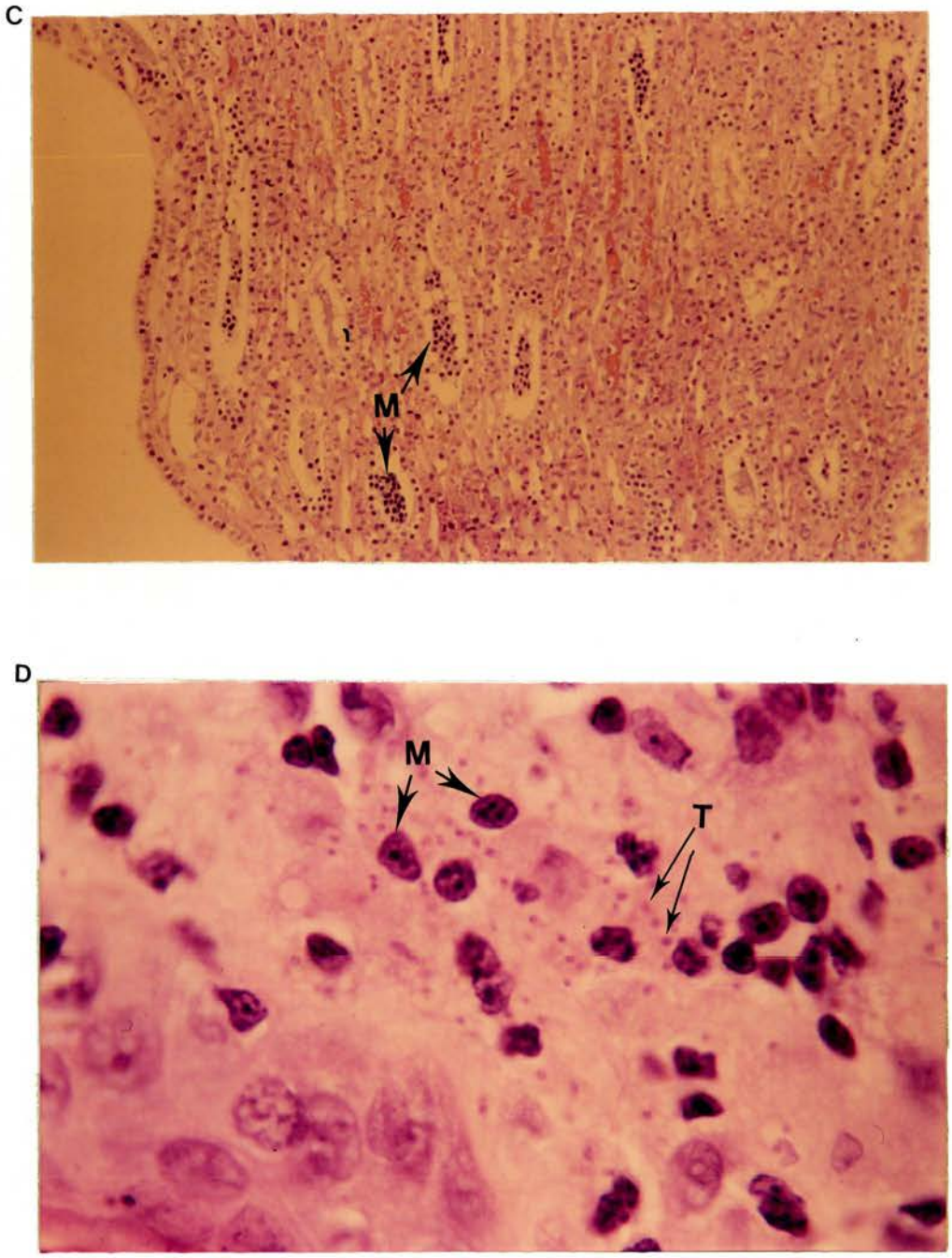
FIGURE 9.6C

Giemsa's stained sections of the kidney collected 28 days post infection showing clusters of mononuclear cells (M) in the collecting tubules. x 150.

FIGURE 9.6D

Giemsa's stained section of tissues from the eyelid collected 28 days after infection showing marked mononuclear cellular infiltration and the presence of many trypanosome nuclei (T). Many trypanosome nuclei were phagocytosed by the macrophages (M). x 1,500.

Figure 9.6



DISCUSSION

The findings in this study show that pathological changes occurred in the organs of rabbits infected with *T. evansi* which were not seen in organs of non-infected rabbits. These changes were thought to be due to the infection except in the case of the spleen where the effect of the anaesthetic agent was thought to have contributed to the engorgement observed because such agents are known to cause the pooling of blood in the spleen of animals.

The changes in the liver are thought to be a direct consequence of the activity of the parasites or the effects of the products of the infection. Apart from the direct involvement of the liver in nutrition, it has the other role of producing more than 90% of the third complement component (C3) (Lambris, 1988) which plays a central role in the activation of both the classical and alternative pathways, the routes through which the effector functions of the complement system during an infection is brought about (Roitt *et al.*, 1985). Antibody-dependent effector mechanisms such as cell lysis, one of the ways trypanosomes are known to be eliminated by the immune system of the host (Greenblatt *et al.*, 1983; Ngaira *et al.*, 1989) are also known to be dependent on the complement system (Staines *et al.*, 1985). Furthermore, the ability of the immune system of animals previously exposed to an antigen to mount an adequate and specific secondary immune response has been linked with the role of C3 in the generation of B memory cells (Klaus and Humphrey, 1977). The liver damage due to *T. evansi* infection would therefore, affect some of these important hepatic functions and may probably explain the decreasing levels of C3 with increasing levels of parasitaemia and duration of infection in trypanosomiasis observed by several workers (reviewed by Nielsen, 1985).

The initial increase in C3 levels during the early stages of infection reported in Chapter 7, is in agreement with the findings of Shirazi *et al.* (1980) and is thought to be associated possibly with an initial attempt of the liver to cope with the increasing C3 requirement of the immune system for the elimination of the parasites by producing more C3, which possibly failed because of the pathological effects of the infection on the liver. Thereafter, the level of C3 was thought to have consistently dropped with the increasing duration of infection because of the imbalance between C3 production by the apparently damaged liver and use by the immune system with increasing parasitaemia.

The observation of restorative changes such as reduced inflammatory changes in the liver after drug treatment may possibly explain in part the increased C3 levels observed in infected animals after drug treatment reported in Chapter 7. The elimination of the parasites by chemotherapy and the return to normality of the liver and consequently its functions, such as C3 production, would therefore, boost the level of C3 in the blood as limited amounts of C3 would be consumed by the immune system in the absence of parasites.

The marked increase in the number of these mononuclear cells seen in sections of vulval tissues collected 28 days after infection is thought to be associated with the defence response of the host to the trypanosome infection and the presence of considerable numbers of parasites in the vulval connective tissue. The cells present were largely lymphocytes, plasma cells and macrophages indicating that these changes are protective, in response to the presence of the infection (Bowry, 1984). Of particular interest is the antibody class most likely to be secreted by the cells in the vulval dermis which are likely to be predominantly IgA considering the seromucosal nature of the cells

beneath the vulva (Roitt, *et al.*, 1985). The increased serum IgA levels observed during the late infection periods in animals reported in Chapter 5, is thought to be in part attributable to the antibody response of the host to the numerous parasites localised in extravascular sites such as the vulva. The increase in the lymphoid cells and macrophages in the vulval dermis in this study is therefore thought to have been engendered by the localisation of many trypanosomes in the vulva during the late infection periods (Figure 9.2C). Similar localisations of other trypanosome species belonging to the *brucei* group such as *T. b. brucei* (French and Hornby, 1934-1935; Losos and Ikede, 1970) and *T. b. rhodesiense* (Nagle *et al.*, 1980) in different extravascular sites have been reported. These extravascular sites in *T. evansi* infections may be playing the role of providing sites for trypanosome antigenic diversification and escape of parasites from the hostile immunological climate in the blood and the provision of parasites for relapsing infections that has been suggested for the tsetse transmitted species of trypanosomes (Jennings *et al.*, 1978; Nagle *et al.*, 1980).

Although *T. evansi* is naturally transmitted by biting flies, it is closely related to *T. equiperdum* which is transmitted sexually (Hoare, 1972). Consideration should therefore be given to the possibility of sexual transmission of *T. evansi* at least in occasional circumstances considering the localisation of large parasite populations in the vulval dermis observed in this study. The difference in the appearance of the parasites in the vulva before and after drug treatment respectively, show that while the parasites still retained their morphology before drug treatment (Figure 9.2C), after chemotherapy all that was left were fragments of the nuclear material (Figure 9.2D). The ability of the drug to enter tissue fluid is important, because once the disease is diagnosed and chemotherapy appropriately administered whatever role the

extravascular parasite pool may be playing would be compromised if not completely abrogated.

The changes observed in the spleen (red and white pulp respectively) and the lymph nodes are also considered to be mainly protective in nature, in response to the infection. The extensive distension of the sinus lumina of the red pulp with erythrocytes and macrophages, with some of the latter observed to contain trypanosomal nuclei (Figure 9.3B) are similar to the observation made by Brown and Losos (1977). While it is suspected that the anaesthetic agent, Euthatal^{R} used for killing the animals may have contributed to the pooling of blood in the spleen, the presence of large numbers of macrophages is thought to be a direct response of the host immune system to the presence of the parasites. Additionally the presence of many lymphocytes and plasma cells in the red pulp is suggestive of an increased immunological activity of the spleen during *T. evansi* infection. These findings support the view that trypanosomes are destroyed in the red pulp in great numbers which is in agreement with the findings of several workers (Wallhausen and Mansfield, 1979; Albright *et al.,* 1977).

The presence of large numbers of trypanosomal nuclei in the splenic sinuses (Figure 9.3B) may indicate vigorous splenic phagocytosis of parasites. The increased number of lymphocytes, plasma cells and macrophages in the white pulp of the spleen of rabbits infected with *T. evansi* (Figure 9.4B) in comparison with those of the control animals (Figure 9.4A) is considered further evidence of increased cellular activation probably linked to increased immunological activity of the white pulp which is thought to be associated with the infective process. Similar changes were also observed in the lymph nodes. The proliferative activity and diffuse nature of the follicular areas in the cortex

of the lymph nodes of rabbits infected with *T. evansi* (Figure 9.5B) in comparison with those of the control animals which had a clearly defined and well demarcated follicular area from the adjoining paracortical zone (Figure 9.5A) is similar to the observations made in *T. congolense* infected mice (Morrison *et al.*, 1982b) and rats infected with *T. congolense* and *T. brucei* respectively (Brown and Losos, 1977). The follicular area was expanded due to the active proliferation of the lymphocytes and plasma cells. The paracortical areas in our studies, as was the case in those of Morrison *et al.* (1982b), Brown and Losos (1977) were not very active. In some areas of the paracortex however, proliferative activity was observed in the present study. These areas in the paracortex were found to contain many macrophages which probably explains the starry-sky appearance of such areas, a phenomenon that is associated with the presence of large numbers of activated macrophages. The marked proliferative activity observed in the follicular (B cell) areas in comparison with the paracortical (T cell) areas (Roitt *et al.*, 1985; Wheater *et al.*, 1987) probably signifies that antibody dependent effector mechanisms (Murray and Urquhart, 1977; Greenblatt *et al.*, 1983; Ngaira *et al.*, 1983) rather than the cell-mediated process are more important in the elimination of trypanosomes. Our observations however, differed from those of Brown and Losos (1977) and Morrison *et al.* (1982b) in one respect, in that we could not demonstrate any trypanosomes in the lymph nodes. It is possible that the different host/parasite systems used influenced the findings or that the rate of destruction and elimination of *T. evansi* by the phagocytic system of the lymph node is both efficient and rapid so that little if any components of the parasites are left. Alternatively, it may be that for some reason *T. evansi* does not become established in the lymph node. Our findings however, are in agreement with those of Seiler *et al.* (1981) who failed to find any parasites in

the lymph nodes of horses that died of *T. evansi* infections. The major difference however, between our findings and those of Seiler *et al.* (1981) is that we found trypanosomes in some organs such as the spleen (the red pulp) (Figure 9.3B), the vulva (Figure 9.2C), the ear and eyelid (Figure 9.6D) which these authors did not report.

The changes observed in the kidneys of rabbits infected with *T. evansi* in this study are probably a direct effect of the infection on the kidneys and/or an undesirable consequence of the immunological response of the host. The extensive mononuclear cellular infiltration in which some of them appear in clusters in the tubules (Figure 9.6C) marked tubular degeneration with nuclear fragmentation and glomerulitis with scattered haemorrhagic foci (Figure 9.6B) are all indicative of severe pathological processes possibly brought about by the *T. evansi* infections. The presence of many plasma cells in the renal corpuscles, collecting tubules, in addition to numerous lymphocytes and macrophages are thought to be developments that could lead to the formation and accumulation of immune complexes that may build up to block glomerular filtration and cause kidney damage and possibly renal failure. This observation has been made with different trypanosome species infecting different hosts by several workers (Nagle *et al.*, 1974; Van Marck and Vervoort, 1980).

The results of the present studies show that *T. evansi* infected animals mount a vigorous conventional defence response both locally and systemically to the presence of the parasite. The studies have further shown extensive pathological changes directly related to the presence of the parasites in the tissues, liver damage which could lead to depression of defence responses and extensive kidney damage that could lead to the development of toxic changes due to retained metabolites and acid/base imbalance. The possibility that

vulvo-vaginal localisation of the parasites may be a factor in the transmission of *T. evansi* deserves further investigation.

CHAPTER TEN

GENERAL DISCUSSION

GENERAL DISCUSSION

The interaction of the parasite and the host immune system has attracted a lot of attention over the years, principally from the point of view of evolving efficient control measures such as the development of vaccines. The interpretation of results from such studies have often been complicated by the multicomponent nature of the parasite antigens overlaid by a consequently complex immune response by the host. In the case of trypanosome infection this is further complicated by the process of antigenic variation and antigenic diversity of different stocks of the same species of parasite. The host can produce antibodies belonging to one or more of the five main classes of immunoglobulins, IgG, IgM, IgA, IgD and IgE which can be directed against one or all of the many parasite components. Furthermore, in the case of trypanosome infections, the host immune system can produce immunoglobulins which are not specific to the invading organism (Hudson *et al.*, 1976; Terry *et al.*, 1980) which adds to the complexity of the host response to the parasite.

Of principal importance in the study of the role of the immune system in host/parasite interactions is the assay system used. An assay system of choice should not influence the nature of results obtained, in order that the interpretation of the observations should truly reflect changes in the host/parasite relationship. Most assays have tended to use crude extracts of trypanosomes as antigens, which makes it impossible for the host's response to individual antigens of the parasite to be evaluated. To obtain the maximum amount of information on host/parasite relationship, however, an assay system should be capable of dissecting the host response to individual antigens of the parasite.

In the present study SDS-PAGE was used to separate the components of *T. evansi* on the basis of molecular weight which was then probed for antigenicity with "Western" blotting (Towbin *et al.*, 1979) using sera samples collected from rabbits infected with *T. evansi*. The system was also adapted so as to provide information on the immunoglobulin class response to the separated components. Of importance in an assay system involving extensive comparative studies such as the present study is reproducibility of results and avoidance of artifacts. This often involves the development of assay systems specifically tailored for the identification of host response to the individual parasite components. An integral part of this process, therefore, was the identification of factors having the greatest influence on reproducibility of results with special reference to the rabbit/*T. evansi* system under study.

The factor having the greatest influence on results was identified as the blocking condition which controlled the effect of background reactions on specific host/parasite interactions and ensured that labelled components could be clearly identified. A 4% goat serum preparation in blocking buffer (v/v) provided maximum reduction of background staining.

In the case of trypanosome infection, host antibody response to the surface antigens is considered to play a principal role in the host/parasite interaction as the surface coat is the first point of contact between the host and the parasite and probably the first point of activation of the effector process that could lead to the elimination of the parasite. The host response to the surface of trypanosomes is important because VSG-specific antibodies are possibly the main resource which the host uses to eliminate the parasites and also get protected against reinfection with the same parasite population (Wellde *et al.*, 1975, 1981; Emery *et al.*, 1980; Morrison *et al.*, 1982b; Hall and Esser,

1984). The host reaction to the surface of the trypanosome however, is complicated by the changing nature of the surface coat of the parasites during the course of infection brought about by the process of antigenic variation (Soltys, 1963) which helps the parasite to evade the host immune system. Antigenic variation also renders improbable the use of immunoprophylaxis for the control of trypanosomiasis (Cardoso de Almeida and Turner, 1983).

The identification of the surface component of trypanosomes and the evaluation of the host's response to it during the course of infection is important for a better understanding of the host/parasite relationship. It is also important that the surface components can be identified in crude extracts so that they could be excluded as reagents for the development of diagnostic probes as their variable nature limits their usefulness in this respect. The SDS-PAGE/"Western" blotting system used in the present study separates the components of the parasite into individual elements but clearly cannot distinguish the components that are surface related from those that are non-surface in origin. For the analysis of responses to the individual antigenic components of the parasite, it is important to identify the origin of the components in the intact trypanosome.

The identification of surface components of parasites is usually undertaken by labelling the surface of organism and the most commonly used label for this is radioactive iodine (^{125}I) which can be attached to the surface of the parasite by a variety of methods such as the IODO-GEN-mediated technique (Fraker and Speck, 1978). After such surface iodination of *T. evansi* stocks in the present study two components with molecular weights of 67,000 and 60,500 daltons were identified as being associated with the surface of the organism. Differences were, however, seen between the number of these components

exposed on the surface of the different stocks of *T. evansi* studied. In some stocks, both components were labelled while in other stocks, only one of the components was visualised. In one stock the particular iodination technique used in the present study failed to label any component, despite the fact that both of these components were present in the total protein profile of the stock and also that electron microscopy revealed that the particular stock of *T. evansi* appeared to have a normal surface coat. Further studies on the characteristics of the surface elements of the *T. evansi* stocks studied using reagents directed at specific parts of the VSG molecule suggested that the number of surface-associated components identified in the different stocks by the IODO-GEN labelling technique is probably a function of the types of amino acid molecules exposed on the surface glycoprotein coat of individual parasite stocks.

Results from studies on the various aspects of *T. evansi* infection carried out during the present study, clearly demonstrated that the host produces antibodies to a large number of *T. evansi* antigens during the course of the infection. From these results it is clear that the immune response to infection was not limited to the surface of the parasite as antibodies appeared to a range of non-surface components as well. Apart from being able to identify individual antigenic components of *T. evansi* the assay system was also used to follow the host's immune response to the surface and non-surface components during the course of the infection.

The host's response to the surface components of *T. evansi* involved the production of antibodies belonging to each of the three main immunoglobulin isotypes studied - IgG, IgM and IgA, while responses to the non-surface components were predominantly IgG in nature. The difference in the pattern of response is likely to be related either to the different effector roles of the

individual antibody classes or differences in antigen processing of the surface and non-surface components of the parasites. It is likely that while the surface-specific IgM response serves to destroy the intact trypanosomes by lysis (Mitchel and Pearson, 1983), the IgG response may be important in handling the consequences of the parasite lysis and dealing with less particulate antigens released after the lysis of the trypanosomes.

Naive animals were observed to mount a response to the intact parasites and parasite extracts when introduced directly into the skin as in the case of a natural infection. The isolated surface components appeared to induce the most severe form of skin reaction in the animals in comparison with the non-surface components. In the case of previously infected animals two types of reactions, classed as immediate and delayed type hypersensitivity reactions based on their time of development and cell types present in sections prepared from tissues collected from sites of antigen inoculation, were observed. These reactions were thought to constitute the early events leading to the development of the chancre (Dwinger, 1985). It is likely that if these types of hypersensitivity reactions occur in animals in the field, they may be important, as suggested by Dwinger (1985) in hampering the multiplication of the parasites in the skin and possibly delay their subsequent escape to the bloodstream.

An outstanding feature of the host response observed in this study was the persistence of trypanosome-specific antibodies with antibodies recognising particular antigens of the parasite remaining in circulation for up to 28 days after infection and for up to 39 days after drug treatment, despite the fact that the parasites had been eliminated by the drug. This persistence was a feature of the antibodies to both the surface and non-surface components of the

parasite. Persistence of antibodies to surface components of the parasite during infection might have been engendered by the re-expression of particular VATs such as predominant VATs (pVATS). Such persistence would be of advantage to both host and parasite as the presence of antibodies to the pVATS could serve to limit variable antigen development, thereby acting as a selection pressure against the emergence of the pVATs which are often more pathogenic than the later emerging non-predominant VATs. The persistence of non-surface antibodies may be due to the continuous destruction of emerging new parasite populations by VSG-specific antibodies and the consequent release of internal components, antibodies to which would be continuously produced by the infected animal. The persistence of antibodies after drug treatment may be as a result of the persistence in circulation of antibodies produced by the host before the parasites were eliminated by chemotherapy. The persistence of antibodies to non-surface components constitutes one of the major problems facing serological diagnosis of trypanosomes in that the majority of assays cannot distinguish between currently infected animals and those in which the infection has been eliminated by drug treatment.

The predominance of IgG antibodies as opposed to IgM antibodies to *T. evansi* antigens during the course of the infection is another important finding of this study because the observation is not in agreement with those of several authors that recorded predominant IgM production in trypansomiasis. The analysis of our finding suggests that the high IgM levels often recorded in trypanosomiasis by other workers (Seed *et al.*, 1969; Zahalsky and Weinberg, 1976) might be related to the assay system used which biased the results obtained towards a particular antibody class such as the propensity of agglutination tests for IgM antibodies or are the products of polyclonal B

lymphocyte stimulation which occurs with the IgM class of immunoglobulins in trypanosomiasis (Hudson *et al.*, 1976; Terry *et al.*, 1980).

The change in IgA levels observed in rabbits in this study during late infection periods and after drug treatment is considered to be associated with the intrusion of the parasites into seromucosal extravascular surfaces. Such sites are associated with IgA secretion (Roitt *et al.*, 1985) and the high levels of IgA recorded in the serum in this study was thought to be due to a spill-over of IgA produced in such places as the vulva where many parasites were identified during the late periods of infection. Studies of changes taking place in some selected organs of rabbits infected with *T. evansi* showed that while the changes in the kidney and the liver are pathological and more likely to the disadvantage the host, changes in other organs like the lymph node and spleen appeared to be more of an enhancement of their physiological functions against the parasite which are linked to the immunological responses of the infected animals.

Based on the observation of clinical signs, animals in this study could be divided into two broad groups: susceptible and resistant. Results obtained from evaluating the immune responses of these two groups to the antigens of the parasites showed that the extent the animals were able to mobilise their immune system against the presence of parasites was directly linked to their status of resistant or susceptible. However, once an animal has experienced infection with *T. evansi* other factors appeared to influence the outcome of infection, the major one being whether or not the animal had experience of the particular antigenic type in the challenge population. If it had experienced the particular parasite population or VAT previously and still had antibodies to it, no infection occurred, as the animal was protected against the homologous

parasite population a clearly established phenomenon in trypanosomiasis (Fulton and Lourie, 1946; Wiesenhutter, 1970; Wellde *et al.*, 1975; Nantulya *et al.*, 1980b). If the animal had not however, experienced the VAT previously, some other factors, possibly non-humoral, appeared to influence the response of previously infected animals to challenges. Such a factor which appeared to hinder the establishment of some challenge populations of *T. evansi* did not appear to be linked to the presence of trypanosome specific antibodies. In the case of *T. brucei*, the late establishment and low level of challenge parasitaemia is considered to be associated with the large number of antigens of this stock of *T. brucei* that reacted with the antibodies produced against *T. evansi*. In the case of *T. vivax* and *T. congolense* there was no apparent protection of animals previously infected with *T. evansi* probably due to the fact that few, if any, antigens of these two parasites were common to *T. evansi*.

Accessory systems such as the complement system that are known to play an important role in the elimination of parasites by the host immune system were seen to be affected in the present study. Studies on the principle component of the complement system, C3, (Lambris, 1988) showed that there was a general reduction in C3 levels during infection with *T. evansi*. Such a reduction in the level of C3 can compromise the ability of the animal to eliminate parasites via complement mediated lysis (Murray and Urquhart, 1977). The reduction in complement (C3) levels probably arose from a disturbance in the balance between C3 production, and consumption by the host immune system. Results from the present study provided evidence that the reduction may have been as a result of changes on both sides of the production/consumption equation. The damage of the liver which produces more than 90% of C3 (Lambris, 1988) and the continuous cleavage of C3 as were observed in the present study were thought to have contributed largely to

the reduction of C3 levels in circulation with the progress of the infection. Evidence was obtained from the present study which indicated that reduced C3 level could be detrimental in the way in which an animal reacts to a second exposure to the same antigens of *T. evansi* probably by influencing the functions of the special B cell population (B memory cells) responsible for the retention of memory (Klaus and Humphrey, 1977).

Results from the present study indicate the importance of broadening the baseline of trypanosome research with the adaption of a more integrated approach taking into consideration the fact that no parts of the trypanosomes or their mammalian hosts act in isolation both in health and in disease. In this respect emphasis in trypanosome research should go beyond the antibody interaction with the surface coat of the parasite to include other areas such as the humoral and non-humoral host response to the non-surface component of the parasite, better appreciation of the role of accessory systems such as the complement system and changes taking place in other organs of the host during infection that may not be completely pathological but protective in nature. Such an integrated approach may yield better and long-term solutions to the problems of trypanosomes.

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Appendix 1
Phosphate buffered saline (PBS)

Sodium chloride (Analar, BDH)	8.0 g
Potassium chloride (BDH)	0.2 g
Disodium hydrogen phosphate, anhydrous (BDH) (Disodium hydrogen orthophosphate, anhydrous)	1.15 g
Potassium dihydrogen phosphate (BDH) (Potassium dihydrogen orthophosphate)	0.2g

Made up to one litre with distilled water
pH 7.3

Appendix 2
Phosphate saline glucose (PSG)

Disodium hydrogen phosphate, anhydrous (BDH) (Disodium hydrogen orthophosphate, anhydrous)	13.48 g
Sodium dihydrogen phosphate (BDH) (Sodium dihydrogen orthophosphate)	0.78 g
Sodium chloride (Analar, BDH)	4.25 g

Made up to one litre with distilled water

For use, dilute solution prepared above in the ratio of 6:4 with distilled water and add 10 gm of glucose (BDH) to give a 1% solution, adjusted to pH 8.0 with 5% phosphoric acid (BDH).

Appendix 3
Enzyme protectant solution

2 mM 6-Amino-n-hexanoic acid
(E-Amino-caproic acid) (BDH)
2 mM DL-Dithiothreitol (Sigma Ltd.)
2 mM Ethylenediamine tetraacetic acid (EDTA) (Sigma Ltd.)

Appendix 4

(a) Stock 30% acrylamide solution

Acrylamide (Sigma)	154g
N,N methylene bisacrylamide (Sigma)	4 g
Mixed bed ion exchanger (Duolite MB6113, BDH)	10g

Made up to 500 ml with distilled water by heating slowly until dissolved. The mixture is finally filtered to remove the mixed resins.

(b) Lower Tris x4

*Tris (Sigma)	36.4 g
**SDS (Sigma)	0.8 g
Distilled water to	200 ml

pH changed to 8.8 using concentrated HCl (BDH)

*Tris = trishydroxymethylamine
**SDS = sodium dodecyl sulphate

(c) Upper Tris x4

Tris	12.12 g
SDS	0.8 g
Distilled water to	200 ml

pH changed to 6.8 using concentrated HCl

(d) Ammonium persulphate solution

Ammonium persulphate (BDH)	1.0 g
Distilled water to	10 ml

Stored at 4°C for one week.

(e) Stacking gel (4.5% acrylamide)

Upper Tris x4	50 ml
30% acrylamide	30 ml
Distilled water to	200 ml

(f) Separating gels

	% acrylamide	
	7%	20%
Lower Tris x4	50	50 ml
30% acrylamide	46	132 ml
Sucrose (Analar, BDH)	-	30 g

Distilled water to 200 ml

Appendix 5
Electrophoresis tank buffer (pH 8.3)

Tris	3.03 g	25 mM
Glycine (BDH)	14.14g	0.192M
SDS	1.0 g	0.1%

Distilled water to 1,000 ml

Appendix 6
Destain solution

Methanol (BDH)	2,500 ml (25%)
Glacial acetic acid (BDH)	1,000 ml (10%)

Distilled water to 10,000 ml

Appendix 7
Silver stain solution

(i) Solution 1

Methanol	50%
Glacial acetic acid	10%

in distilled water

(ii) Solution 2

Methanol	5%
Glacial acetic acid	7%

in distilled water

(iii) Solution 3

Gluteraldehyde (Taab Labs)	5.0%
Sodium tetraborate (BDH)	1.9%

in distilled water

Appendix 8
Developer

Sodium carbonate (BDH)	3 g
37% formaldehyde (Analar, BDH)	50 μl

Distilled water to 100 ml

Appendix 9
Blocking buffer x10

Tris	60.5 g	50 mM
Sodium chloride (BDH)	87.0 g	150 mM
EDTA	3.72 g	1 mM
Nonidet P40 (NP40) (Sigma)	5.0 ml	0.005%
Gelatin (BDH)	25.0 g	2.5%
Thiomersal	2.0 g	0.2%
Distilled water to 1,000 ml		

pH changed to 7.4 using concentrated HCl. For use a x1 buffer strength was made by diluting the x10 buffer 1:10 with distilled water and 4% fresh goat serum added.

Appendix 10
Substrate solution

(i) Tris buffered saline (TBS)

Tris	4.85 g	20 mM
Sodium chloride	58.48 g	500 mM

Distilled water to 2,000 ml
pH changed to 7.5 with concentrated HCl.

(ii) 60 mg 4-chloro-1-naphthol (Sigma) was dissolved in 20 ml ice-cold technical methanol (Solution 1).
Solution 1 was added to 100 ml of TBS. Following this 60 μl of ice-cold 30% hydrogen peroxide (Analar, BDH) was added immediately before use.

Appendix 11
Immunocoprecipitation 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
NP40 10-0%		20 ml	0.2%

Make up to slightly less than 1,000 ml. Check pH 8.0 and finally make up to 1,000 ml.

Appendix 12
0.2M sodium cacodylate buffer
(Double working strength)

Sodium cacodylate (BDH) 2.14 g

Made up to 500 ml with distilled water. pH adjusted to 7.3 with HCl and stored at 4°C. Before use the buffer is double diluted with distilled water to make a single strength buffer. 12 ml of 25% EM grade gluteraldehyde is then added to 88 ml of the single strength (0.1 M) cacodylate buffer, pH 7.3.

Appendix 13

(i) Araldite mix

Araldite CY212 (Agar Scientific Ltd.)	50 ml
Dodecyl succinic anhydride (" ")	50 ml

(ii) Accelerator

2,4,6-tri(dimethylaminomethyl) phenol (DMP-30) (Agar Scientific Ltd.) - 1 volume.

Dibutylphthalate (Agar Scientific Ltd.) - 2 volumes

Appendix 14
Sample buffer (Reducing type)

Tris	45 mg (18.5 mM)
EDTA (disodium salt, Sigma Ltd.)	223 mg (30 mM)
SDS	1.0 g (5% w/v)
2-mercaptoethanol (Sigma Ltd.)	0.45 ml
Glycerol (Analar, BDH)	5.0 ml (25% w/v)
Bromophenol blue (Sigma Ltd.)	Spatula tip
Distilled water to	20 ml

Appendix 15
Barbitone acetate buffer

Barbitone acetate buffer was prepared as instructed by the manufacturers (Shandon, U.K.), by dissolving 16.5 g of barbitone acetate (Electrafor®) in 500 ml of warm distilled water before being made up to 1,000 ml with cold distilled water.

Appendix 16

0.02M Tris - HCl, pH 8.0
0.028M NaCl, 0.02% NaN_3 (BDH)

Appendix 17
Veronal buffer

Sodium diethylbarbiturate (BDH)	5.15 g
Diethylbarbituric acid (BDH)	0.92 g
Distilled water to	1,000 ml
pH 8.6	

Appendix 18
Carbonate bicarbonate buffer, pH 9.6

Sodium carbonate	1.59 g
Sodium bicarbonate	2.93 g
Distilled water to	1,000 ml

Appendix 19
Blotting buffer

For use with semi-dry Ancos blotter (Dako Ltd.)

Electrophoresis tank buffer	100 ml
Technical methanol (BDH)	100 ml
Distilled water	300 ml

Appendix 20
Molecular weight markers

A commercial mixture of six molecular weight protein standards were used covering a low molecular weight range (Pharmacia). The contents of one vial were reconstituted in 1.0 ml x 1 SDS sample buffer and heated to 100°C for three 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 anhydrous (30 kdal), soyabean trypsin inhibitor (20 kdal) and lactalbumin (14 kdal).

Appendix 21

Manufacturer' and Suppliers' Names and Address

Agfa Professional Photographic Division, 27, The Great West Road, Brentford, Middlesex, T88 9AX, 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.

BioRad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Hertfordshire, England.

Dako Ltd., 27, The Arcade, The Octagon, High Wycombe, Buckinghamshire, England.

Glaxovet Ltd., Greenford, Middlesex, UB6 OHE, England.

Ilford Ltd., Mobberley, Cheshire, England.

Kodak Ltd., Hemel Hempstead, 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.

Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, England.

Pierce Chemicals - Suppliers: Life Science Laboratories Ltd., Sedgewick Road, Luton, LU4 9DT, England.

Shandon Southern Products Ltd., 93 Chadwick Road, Astmoor, Runcorn, Cheshire, England.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7NH, 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.

Appendix 22

Papers arising from the thesis.

Two posters were presented from the work arising from this thesis. (Abstracts attached)

(a) U.E. Uche, Jones, T.W. and Boid, R. (1988).
Class-specific antibody response and variations between susceptible and resistant rabbits experimentally infected with *Trypansoma evansi* (Presented at the 12th International Congress for Tropical Medicine held at the Institute of Tropical Medicine, Antwerp, Belgium, 15th to 16th September 1988.)

(b) Uche, U.E., Jones, T.W. and Boid, R. (1989).
Complement levels in rabbits infected with *Trypanosoma evansi*. (Presented at the Meeting of the Royal Society for Tropical Medicine and Hygiene at Glasgow University 24th May, 1989.)

CLASS-SPECIFIC ANTIBODY RESPONSE AND VARIATIONS BETWEEN SUSCEPTIBLE AND RESISTANT RABBITS EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA EVANSI*

UCHE, U.E., JONES, T.W. & BOID, R.

Centre for Tropical Veterinary Medicine,
Easter Bush, Roslin, Midlothian EH25 9RG,
University of Edinburgh, Scotland.

ABSTRACT

Using SDS-PAGE and Western blotting, IgG antibody response was found to predominate in rabbits experimentally infected with *Trypanosoma evansi* in terms of the number of homologous *T. evansi* antigenic bands recognised and the degree to which these bands were labelled during the course of the infection. Animals that developed parasitaemia later or had low levels of parasitaemia, as the infection progressed were considered to be more resistant to the infection. These resistant animals in addition to recognising the *T. evansi* antigens earlier, identified more of the antigens than their susceptible counterparts as the infection progressed. The susceptible animals as well as developing patent parasitaemia earlier, with higher parasite counts being attained as the infection progressed were found to recognise the *T. evansi* antigens later with fewer bands labelled during the course of the infection.

Royal Society of Tropical Medicine and Hygiene

Report of Demonstration/Poster at Laboratory Meeting

Author(s)

U.E. UCHE, T.W. JONES & R. BOYD

Title

Complement levels in rabbits infected with T. evansi

Author(s) Address(es)

Centre for Tropical Veterinary Medicine, University of Edinburgh, ROSLIN, Midlothian, Scotland.

(See reverse for instructions)

C3 levels in rabbits infected with T. evansi were measured by rocket immunoelectrophoresis in 1% agarose gel containing 2.5% sheep anti-rabbit C3 serum and 3% PEG6000. Plasma samples were collected at regular intervals over the first 24 days of infection at which point the rabbits were treated with Berenil^{R} (Hoecht) at 7 mg/kg active principle. Further samples were collected at 8 and 14 days after treatment.

A slight rise in C3 levels was seen early in infection in most animals probably due to increased mononuclear phagocyte activity during this stage of the infection. Thereafter, a progressive fall in C3 levels occurred up to the point of treatment. This fall in C3 levels could be due to C3 consumption by the immune system undertaking parasite destruction and/or altered activity of certain mononuclear phagocytic cells due to trypanosome infection leading to reduced C3 production. After treatment C3 levels showed a progressive rise usually returning to pre-infection levels by 14 days after treatment.