IMMUNITY TO *Babesia divergens* IN THE RAT

by

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بسم الله الرحمن الرحيم

رَبِّ اشْرَحْ لِي صَدْرِي ﴿٢٥﴾ وَيَسِّرْ لِي أَمْرِي ﴿٢٦﴾ وَاحْلُلْ عُقْدَةً
مِّن لِّسَانِي ﴿٢٧﴾ يَفْقَهُوا قَوْلِي ﴿٢٨﴾

الآيات من 24 - 27 من سورة طه

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DECLARATION

The work presented in this thesis is the original work of the author and has not been submitted previously for the award of a degree at any university.

Signed:

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ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
B Cell	B lymphocyte
BCG	Bacillus Calmette Guerin
CMC	Cellular mediated cytotoxicity
Cr^{51}	Chromium 51
d	Died
DPI	Days post infection
ELISA	Enzyme Linked Immunoabsorbent Assay
FCS	Foetal calf serum
FITC	Fluorescein isothionate
HS	Hyperimmune serum
IFA	Indirect fluorescent antibody
IFAT	Indirect fluorescent antibody test
IS	Immune serum
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Intraperitoneal
IV	Intravenous
MNT	Merozoite neutralisation test
MASP	Microaerophilous stationary phase culture
NS	Normal serum
NRBC	Normal red blood cell
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

ABBREVIATIONS (Cont'd)

PRBC	Parasitised erythrocyte
RBC	Red blood cell
RES	Reticuloendothelial system
T-cell	T-lymphocyte
WBC	White blood cell
WEP	Wellcome Experimental Parasitology - numbers used to describe individual batches of stabilate
WSD	Whole serum dialysed
WSND	Whole serum not dialysed

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SUMMARY

The rat adapted strain of B. divergens was used as a model to investigate the mechanisms of immunity to this parasite. The course of infection as well as the haematological changes that accompanied the infection were followed in splenectomised rats.

The course of infection in splenectomised rats was short lasting (5-6 days) and no recrudescences were observed after the primary patent parasitaemia. The animals either died from the infection or recovered and were immune to challenge. The i.v. injection of 1.5×10^8 PRBC into splenectomised rats resulted in an infection from which most rats recover. The parasite, however, became more virulent with increasing passages and the injection of 1.5×10^8 PRBC resulted in a fatal disease. The drug diampron was used for treating infected rats. A single subcutaneous injection of diampron was enough to cure the animals.

The acute parasitaemia was accompanied by severe anaemia evident as a drop in red blood cell counts. Red cell counts started returning to normal 8-10 days after recovery. Recovery from the infection was immediately followed by a pronounced blood leukocytosis which was predominately a lymphocytosis. The possibility that lymphocytes might be accumulating in the livers of immune rats was investigated. Histological studies showed the appearance of accumulation of cells around the central veins in the livers of immune splenectomised rats. These accumulations of cells have the appearance of "pseudofollicles" as described by Weiss (1985) in splenectomised gerbils infected with P. berghei.

Preliminary histological studies have showed that some of these cells were B and T lymphocytes which might be important for the development and mediation of protective immunity in splenectomised rats.

In splenectomised immune rats, the possible removal of B. divergens PRBC by the liver was investigated using Cr^{51} labelled PRBC. It is suggested that either the parasites within the red cells were opsonised and then removed by the liver where they were phagocytosed, or the merozoites may have been destroyed by antibody as they emerged from erythrocytes and the damaged red cells were removed by the liver.

Irradiated parasites were injected into immune rats to determine whether it was intact PRBC or free merozoites which are cleared by the immune rats. It was concluded that immune splenectomised rats were able to clear PRBC from the blood stream and that immunity was not necessarily against merozoites. The effector mechanisms which were responsible for the destruction or removal of PRBC are unknown.

The possible participation of humoral factors in acquired immunity to B. divergens was investigated. Antibodies to B. divergens were detected by the IFA test in sera collected during the infection and at different times after recovery. The antibody titre as measured by this test, rose from day 5 and reached a peak on day 19 at which level it remained for at least seven weeks. The possible role of antibody in protection was investigated in vivo by passive transfer with immune and hyperimmune serum. The levels of serum protection with immune

sera rose rapidly from day 6 to reach a peak between day 7 and 13 and thereafter the levels declined rapidly. It was not possible to correlate protection with antibody levels measured by IFAT indicating that some antibodies to B. divergens were not protective. The role of antibody in protection was confirmed after fractionating immune and hyperimmune sera. The protective activity of sera collected immediately after recovery (day 7) was mainly due to IgM antibodies. The protective activity of sera collected 3-4 days after recovery (day 10) and of hyperimmune serum was mainly due to IgG antibodies. In hyperimmune sera, IgM antibodies were, however, partially protective.

In order to compare the protective activity of immune sera in vivo with its effect on parasite growth in vitro, a method for cultivation of B. divergens was established. B. divergens was cultured continuously for six months in rat erythrocytes using the candle jar technique. Parasites were cultured in rat erythrocytes in RPMI medium containing 20% FCS. Parasitaemias exceeding 35% have been observed in cultures which were initiated with 2% infected red cells. After five weeks in culture, some cultures were used to infect a splenectomised rat. The rat developed a high parasitaemia and died five days post infection, indicating that the parasite was still virulent after five weeks of culture.

Cultures with high parasitaemia contained a large number of free merozoites which were collected and either used to initiate new cultures or used for the merozoite neutralisation test, or they were stored in liquid nitrogen or at -70^{o}C for future use.

Some aspects of the mechanisms of action of antibody were tested *in vitro*. The possible role of opsonizing antibody in protection was investigated using hyperimmune serum and *B. divergens* PRBC or free parasites in the presence of peritoneal macrophages. No evidence was found for a role for opsonising antibody in protection *in vitro*.

The protective activity of immune serum *in vivo* was compared with its effect on parasite growth *in vitro*. Rat immune and normal serum inhibited parasite growth to a similar extent when tested *in vitro*, as did the immunoglobulin fractions of both sera. In passive transfer tests *in vivo*, protective activity was observed only in the unfractionated and the immunoglobulin fraction of immune serum. These results have indicated that normal serum was toxic to cultures. With immune serum, there was no evidence that antibody agglutinates merozoites and prevents their invasion *in vitro*. Evidence that antibody neutralises merozoites and prevents their invasion was, however, demonstrated only by preincubating free merozoites with immune serum, before adding them to cultures containing normal rat erythrocytes. The possibility that antibodies might only be protective in the presence of cellular elements was investigated in an ADCC assay using immune spleen cells and immune peripheral blood mononuclear cells. The results did not demonstrate specific ADCC killing by immune spleen cells or peripheral blood mononuclear cells. It is suggested that other immune mechanisms are involved in the destruction of parasites or PRBC. This might involve a non

specific type of immunity mediated by soluble factors secreted by activated T cells or macrophages or the release of toxic oxygen mechanisms which would lead to the death of the parasite within the erythrocytes and might therefore be important in B. divergens infection. It is also possible that effector cells can recognise PRBC in the peripheral circulation. The possible participation of NK cells is considered.

New isolates of B. divergens have also been adapted to long term culture. Two bovine field isolates which had been passaged into gerbils and then cryopreserved as stabilates were used. On recovery from cryopreservation, the isolates were subpassaged through two gerbils each and then either put directly into culture with gerbil or rat erythrocytes or subpassaged through a splenectomised rat for one day before going into culture without any additional red cells. No growth was observed in cultures initiated with parasitised gerbil blood in normal rat erythrocytes or those initiated with parasitised gerbil erythrocytes in normal gerbil erythrocytes. Cultures initiated with parasites in rat erythrocytes grew and the parasite was maintained continuously over a period of 75 days through 32 cultures. After five weeks of culture, some cultures were used to infect splenectomised rats successfully, while the same isolates that were injected directly from gerbils to splenectomised rats did not grow.

Some of the rat adapted cultures were initiated with human erythrocytes and the effect of diluting infected rat erythrocytes

with normal human erythrocytes was followed. The parasite was maintained for two weeks in culture and three subcultures with normal human erythrocytes. The cultures were, however, discontinued after this period, as the increase in the number of human erythrocytes in each subculture did not apparently support parasite growth and it was concluded that gerbil and human erythrocytes are not suitable for the growth of B. divergens in culture using RPMI and FCS.

These results are discussed in Chapter Nine.

B. divergens in splenectomised rats provides a useful model for investigating this parasite. Apart from the inconvenience of carrying out splenectomy, rats have advantages in that they are easy to handle, provide relatively large volumes of infected blood for immunological studies and for culturing the parasite when compared with gerbils, another laboratory host for B. divergens (Lewis and Williams, 1979). In addition, inbred rats are inexpensive and available, which would permit studies such as adoptive transfer to be carried out, not possible with cattle, the natural host of B. divergens, since cattle are expensive animals and therefore cannot be used in large numbers for experimental studies. Studies such as those carried out on the gerbil model for the screening of babesicides (Gray, 1983) can be carried out in the rat, which could provide important information relevant to the treatment of infection in humans, another abnormal host.

CHAPTER ONE

GENERAL INTRODUCTION

Chapter One

General Introduction

Babesia species are tick transmitted protozoan parasites which are found throughout the world and cause disease in wild and domestic mammals. Some of the species infect cattle (B. bovis, B. bigemina, B. divergens and B. major), sheep (B. foliata, B. motasi, B. ovis), horse (B. caballi, B. equi) goat (B. motasi, B. ovis, B.taylori), pig (B. perroncitoi, B. trautmanni), dog (B. canis, B. gibsoni, B. vogeli) and cat (B. felis). Some of these species are found in wild animals, such as B. canis in jackals, B. equi in zebra and B. divergens in reindeer (reviewed by Purnell, 1981), and some can also infect man and laboratory animals (reviewed by Callow and Dalgliesh, 1982).

Babesia was first described in Rumania in blood of sheep and cattle (Babes, 1888 - quoted by McCosker, 1981). In 1893, Smith and Kilbourne (quoted by McCosker, 1981) were the first to identify the tick transmission of and the symptoms cause by B. bigemina, which causes red water fever in cattle. This was the first demonstration of an arthropod transmitting a protozoan parasite. Since then, more than 71 different species of Babesia have been identified in both domestic and wild animals (Levine, 1971). Bovine babesiosis is economically the most important disease caused by Babesia. It has been reported that there are approximately 1.2×10^9 cattle in the world and the majority of these are potentially exposed to one or more Babesia species (McCosker, 1981).

The infective agent of Babesia (the piroplasm) multiplies within the mammalian erythrocytes. In the red cells, the parasites appear as single, oval, amoeboid, round or elongate or as pyriform bodies arranged in pairs. The size of Babesia varies depending on the species, but they are normally classified as "small" and "large" babesias.

Four species of Babesia infect cattle. B. bovis is a small babesia and is distributed all over the world. It is found in Africa, Asia, Europe, Central and South America, and in Australia where it is considered to be a major pathogen and is transmitted by Boophilus microplus. B. bigemina is a large babesia which in Australia is also transmitted by Boophilus microplus. It is found over the same geographical regions as B. bovis, although it is not highly pathogenic in Australia. It was the babesia that caused Texas fever in the United States and some reports have indicated its prevalence in the southern U.S.S.R.

B. divergens is a small babesia (1.5 x 0.4 um) which is morphologically similar to B. bovis. It is the babesia of most importance in Western and Central Europe. It is transmitted by the tick Ixodes ricinus and causes significant mortality in cattle in these areas (Barnett, 1974). B. major is a large babesia which is transmitted by the tick Haemaphysalis punctata. It is found in Europe and in the Middle East, and it is less pathogenic than any other bovine species.

Whitlock (1949) has described two states of infection in the vertebrate host, defined as "babesiosis" and "babesiasis". "Babesiosis" refers to the period where there is rapid

multiplication of the parasite, which is accompanied by clinical signs of the disease. At this stage, the host either dies or recovers from the disease. "Babesiasis" refers to the subclinical infection observed in young, passively immune animals and also those recovered from the infection. The parasites remain in the blood but their number is insufficient to cause illness.

The clinical symptoms of the disease are variable depending on the host and species of Babesia, but are generally characterised by fever, haemolytic anaemia caused by the destruction of erythrocytes as parasites multiply, haemoglobinuria (red water), followed by jaundice. Other signs may include blood stained faeces, signs of nervous disturbances, such as muscle weakness, grinding of the teeth and sunken eyes as a result of dehydration. The rectal temperature normally rises as the parasitaemia increases. Mahoney (1972), has also reported damage of the internal organs such as the liver and the kidney as a result of the infection in B. bovis. In B. bovis infection, signs of cerebral damage also appear. This cerebral damage is caused by the clumping and adherence of parasitised erythrocytes in brain capillaries causing blockage which is followed by coma and finally death of the animal. Babesia, however, may cause no significant disease in some hosts (Mahoney, 1977; Ristic and Lewis, 1977).

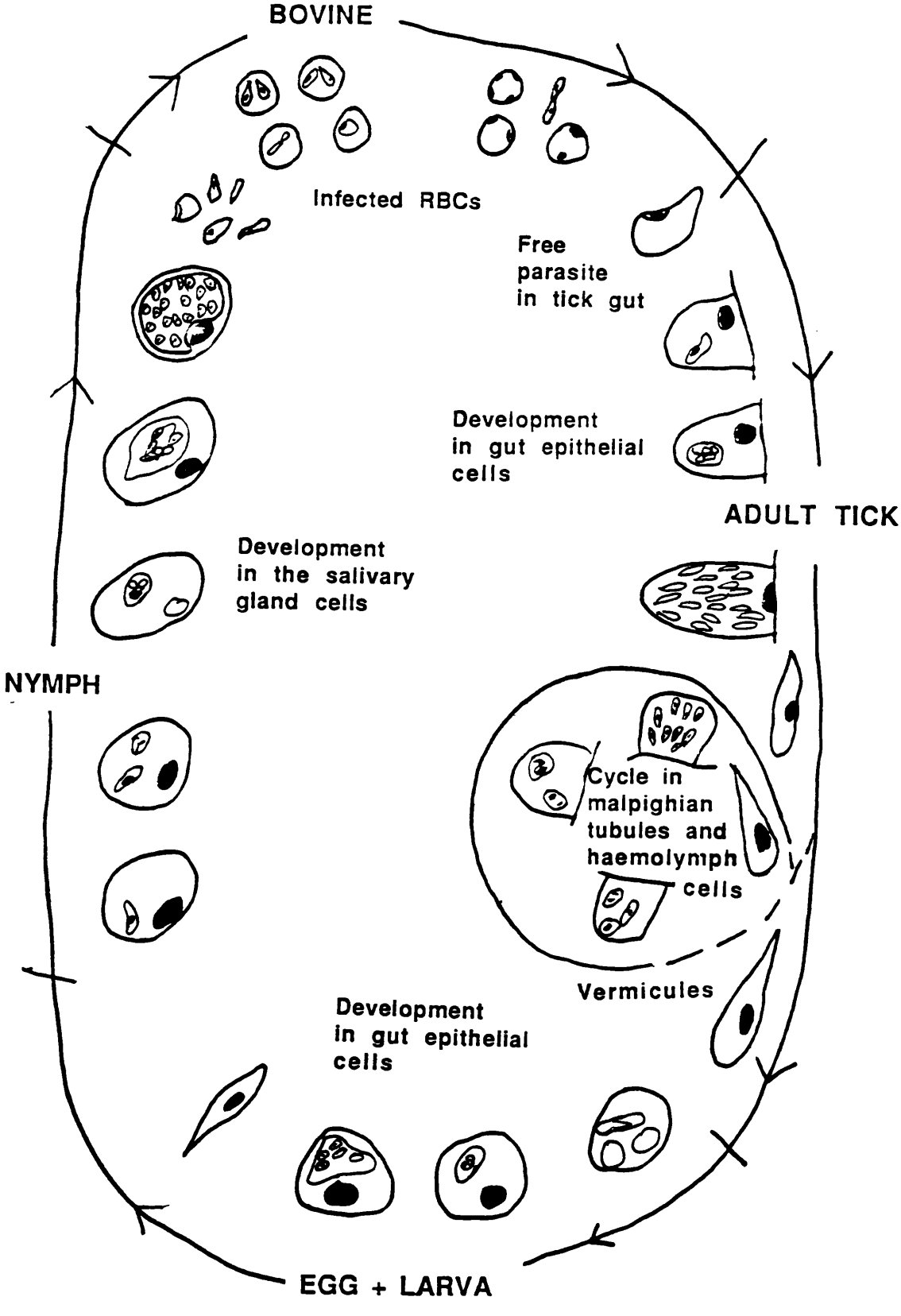
LIFE CYCLE

Babesia parasites are transmitted by hard ticks belonging to

the family Ixodidae. Although the life cycles of most Babesia species are not known or have not been studied in detail, the life cycles of the most economically important species (B. bovis and B. bigemina) have been intensively investigated by Riek (1964), who described the invertebrate phase of the life cycle of both species by following their transovarial development in the one host tick B. microplus. Similar findings were reported by Holbrook et al., 1968 for B. caballi in the tick Dermacentor nitens and by Friedhoff (1969) for B. ovis in the tick Rhipicephalus bursa. Joyner et al. (1963) have described the development of B. divergens in the tick I. ricinus, but no detailed pattern of development in the tick organs has yet been reported. Riek (1968), suggested that sexual reproduction in B. bigemina takes place in the tick in the first 24 hours after ingestion, but this has not been confirmed. Recent observations, however, on the erythrocytic stages of B. canis and B. equi in culture (reviewed by Mehlhorn and Schein, 1984) and on B. microti in Ixodes tick guts (Rudzinska et al., 1983), have thrown some light on sexual stages.

The terminology of the life cycle of Babesia is confused. Friedhoff (1981), proposed a hypothetical life cycle for Babesia in which he introduced new descriptive terms. These are currently used for the description of the known life cycles of Babesia species (Mehlhorn and Schein, 1984; Young and Morzaria, 1986). The new terms will be used for the following description of the life cycle of B. bigemina in the tick B. microplus, and in the vertebrate host (see Figure 1 - after Riek, 1964).

Figure 1:
The Life Cycle of *Babesia bigemina* in the Bovine and the One Host Tick *Boophilus microplus.* (after Riek, 1964)



In the tick

It is believed that when female Boophilus ticks feed on an infected vertebrate, most of the babesia parasites in the bovine erythrocytes die within the tick gut, but some become ray bodies, believed to be gametes (Riek, 1964). These gametes fuse to form motile kinetes (vermicules) which are then detectable in the haemolymph 3-5 days after repletion. Further multiplication and production of more kinetes occurs in the haemocytes, malpighian tubules, ovaries and muscle. After oviposition by the tick, the kinetes multiply in the ova and the daughter cells invade the gut epithelium of the developing larva, where more kinetes are produced. The final cycle takes place in the salivary gland of the nymph, in which infective sporozoites are produced and released in the salivary duct during feeding. These sporozoites infect the vertebrate host.

Most Babesia species are transmitted transovarially from infected female ticks to their progeny. B. microti which is transmitted by the tick I. dammini and B. equi which is transmitted by ticks of the genera Dermacentor, Hyalomma and Rhipicephalus are the only species to be transmitted transtadially like Theileria species. In transovarial transmission, the primary infection takes place only in the adult stage, and is then transmitted by larvae, nymphs, and adults of the following generations. For many Babesia species, vertical transmission is common. The tick can remain infective for several generations and the vertebrate host is not essential for maintaining the parasite in the tick population. Holbrook et al., 1968, working on B. caballi, reported that kinetes

(vermicules), observed in the ovaries of immature adults, will infect the next generation of ticks. Donnelly and Pierce (1975), reported that B. divergens sometimes persisted into the second generation of I. ricinus when ticks were maintained on a host not susceptible to Babesia.

Transmission occurs at different times in the parasitic life of the one host tick. For example, transmission occurs from larvae in B. bovis and from nymphs in B. bigemina and B. caballi (Riek, 1964; 1966; Holbrook, 1968). In the two host tick as in B. ovis, infection occurs only through adult stages of the tick and transmission happens through the subsequent nymphs or adults. B. divergens have a three host tick as vector. Here infections can only be initiated in the adult stage of the I. ricinus tick, subsequently larvae, nymphs and adults are all infective, but the larvae and nymphs of the first generation cannot acquire the infection (Donnelly and Pierce, 1975). When adult female ticks feed on infected vertebrate hosts, they are able to transmit the infection to other hosts. Lewis and Young (1980) found that adult ticks which were fed on calves infected with a human strain of B. divergens were able to transmit the parasite to other splenectomised calves and to Meriones unguiculatus gerbils. Other babesias which have a three host tick as vector are B. canis and B. major, which are transmitted by R. sanguineus and H. punctata respectively.

Erythrocytic parasites are believed to be the main source of infection for ticks. Infectivity for ticks may, however, be lost as a result of continuous blood passaging of the parasite as was

demonstrated by Stewart (1978) for B. bovis.

In the vertebrate

During tick feeding, the sporozoites are inoculated into the vertebrate host, where they attach and penetrate the erythrocytes (Hoyte, 1961). The mechanisms of entry of sporozoites into erythrocytes are not known. The development of most Babesia species in the vertebrate hosts occurs in the erythrocytes (Friedhoff, 1981). B. microti and B. equi are the only species in which sporozoites invade the lymphocytes of their hosts (reviewed by Mehlhorn and Schein, 1984). The sporozoites within the erythrocytes then become trophozoites, which undergo asexual division (merogony) to form merozoites. In most Babesia species, two merozoites are produced, while B. microti and B. equi produce four to form what is called a "Maltese cross". The merozoites are covered by a pellicle consisting of three membranes. They have an apical and a posterior polar ring, micronemes, rhoptries and sub-pellicular microtubules. Only B. equi merozoites possess a micropore, which is a typical feature of malaria parasites. Some Babesia species (e.g. B. bovis, B. bigemina, B. caballi), have, in addition, the so-called spheroid bodies near the nucleus. The function of these is not known.

The merozoites then escape the erythrocytes, after destroying them to invade more red blood cells. This asexual division continues until the host either dies or recovers from the infection. The invasion of erythrocytes by merozoites was well studied in some Babesia species (Mehlhorn and Schein, 1984; Rudzinska, 1981). Some reports have indicated the role of

complement in the invasion of erythrocytes by merozoites (Jack and Ward, 1980). Some of the parasites inside the erythrocytes do not divide but acquire unusual shapes. These are considered to be gametocytes which are transformed to what are called gamonts in either the erythrocytes as in B. canis (Mehlhorn et al., 1980) or in the gut lumen of the tick as in B. microti (Rudzinska et al., 1979).

IMMUNITY TO Babesia

Both non specific and specific immune mechanisms are involved in the host's resistance to Babesia infection. Non specific immune mechanisms are normally possessed by all animals, while specific mechanisms are acquired as a result of an active infection, immunisation, or passive transfer of protective elements from an immune donor. These specific immune mechanisms are directed against the parasite or its products.

Non specific immune mechanisms

a. Host specificity

The various Babesia species were assumed to be restricted to the host in which they were discovered (Levine, 1971). The detection of Babesia in man, however, and the search for suitable laboratory models for research studies, stimulated a new interest in experiments examining the host specificity of these parasites. Some domestic and laboratory animals were not susceptible to infection with species of Babesia other than their own natural parasites, for example B. caballi and B. equi (Crowe and Pullen, 1968; Frerichs et al., 1969), will only infect horses and donkeys and the two parasites, B. bigemina and B. bovis will only infect

bovines, although Callow (1965) has reported that B. bigemina would infect sheep and horse, and Enigk and Friedhoff (1963) have suggested that the same parasite would also infect splenectomised gazelle, but these reports were not confirmed. B. hylomysci which was isolated from an African tree rat (Hylomyscus), was adapted to laboratory mice and also produced infections in a squirrel and a splenectomised rhesus monkey (Bafort et al.,1970). In mice the infection was virulent after several passages (Hussein, 1977a). B. rodhaini was also isolated from the African tree rat host (Thamnomys) into laboratory mice (Van den Berghe et al., 1950) and became virulent later in this host (Beveridge, 1953), and produced subpatent infections in several primates (Hawking, 1973). These last two species of Babesia have not been reported in domestic animals or man.

Removal of the spleen seems to be an important factor in breaking down natural resistance to infection with Babesia. Skrabalo and Deanovic (1957), reported the first acute case of B. divergens in a splenectomised man. Since then, more than 22 cases of babesiosis in splenectomised humans have been reported (reviewed by Rosner et al., 1984). Splenectomy of deer and goats also rendered them susceptible to this parasite (Enigk and Friedhoff, 1962). Injection of bovine blood infected with B. divergens into splenectomised chimpanzees and rhesus monkeys initiated acute parasitaemias (Garnham and Bray, 1959; Garnham and Voller, 1965). B. divergens was also adapted to splenectomised laboratory rats (Phillips, 1984). This parasite, however, infected intact reindeer (Nillson et al., 1965) and mongolian gerbils (Lewis and Williams, 1979; Lewis et al., 1981b;

Liddel et al., 1980). B. microti naturally infects field mice (Microtus pennsylvanicus) and white footed or deer mice (Peromyscus leucopus) and man (Healy et al., 1976) in the United States, and in the laboratory will also infect intact rats, hamsters, gerbils and mice (Nowell, 1970; Gleason et al., 1970; Ruebush and Hanson, 1979) and rhesus monkeys (Ruebush et al., 1979). Ristic et al. (1971) were, however, unable to infect splenectomised dogs with this parasite.

b. Age and Sex

Susceptibility of some hosts to Babesia infection can depend on the age of the host. In some studies older cattle were found to be more susceptible to babesiosis than young calves (Riek, 1963; Smith et al., 1978). With B. rodhaini in rats, however, where neonates exhibited resistance to Babesia, this was thought to be in some cases at least, the result of immunity passively acquired from the mother (Beveridge, 1953; Phillips, 1968). Other workers, did not however, observe an age effect in the hosts. For example Brocklesby et al. (1971) reported that young and old cattle responded in a similar way to B. divergens infection.

Little is known about the effect of sex on the susceptibility of the host of Babesia infections. Male mice were found to be more susceptible than females to B. rodhaini (Goble, 1966) and B. microti infections (Rosenberg and Evans, 1979; Cox, 1980; Irvin et al., 1981).

c. Cross infections

Concurrent infections with Babesia may induce specific resistance to other parasites, for example Cox (1970), Cox and Young (1969) and Cox and Turner (1970b) demonstrated that mice that recovered from B. microti and B. rodhaini infections were not only protected against reinfection but were also partially protected against Plasmodium vinckei and P. chabaudi. These authors thought that this cross protection was a result of shared common antigens between the two species. Mahoney (1972) reported that splenectomised calves infected with T. mutans were more resistant to B. bovis than calves free from Theileria. Mahoney (1972) suggested that this cross resistance was due to the fact that the reticuloendothelial system was being stimulated by Theileria infection or possibly due to the alteration of the erythrocytes in the doubly infected animals making them less suitable for B. bovis.

Immunostimulation

Non specific stimulation by a number of agents has been intensively studied particularly in the rodent parasite B. microti and B. rodhaini (Cox, 1980). Pretreating mice with agents such as BCG (Clark et al., 1976) and Corynebacterium parvum (Clark et al., 1977a; Corrier and Wagner, 1984), protected them against subsequent challenge. Protection against B. rodhaini in rats can be achieved by pretreating them with C. parvum (Cox and Saleh, 1983). Corrier and Wagner (1984) found that C. parvum has a slight protective effect against B. bigemina. Brocklesby and Purnell (1977) could not, however, protect cattle against

B. divergens after injecting them with BCG.

The appearance of intraerythrocytic damaged parasites (crisis forms) in mice previously infected with BCG or other agents is thought to be due to soluble non antibody factors which mediate the killing of Babesia parasites in the red cells (Clark et al., 1977a, b, c). These soluble factors are associated with macrophage activation (Clark, 1979a), interferon tumour necrosis factor (Clark, 1979a) and natural killer (NK) cells (Allison et al., 1978; Clark, 1979b; Irvin et al., 1981). Such soluble factors may also damage malaria parasites and could explain the cross immunity existing between rodent babesias and rodent malaria parasites as suggested by Cox (1978). Clark (1978) speculated that these soluble non antibody factors are released as the result of endotoxin shock, which is produced as a result of the presence of E. coli liposaccharides during the rising parasitaemia in mice infected with Babesia and Plasmodium parasites.

Specific immune mechanisms

a. Acquired immunity

The course of Babesia infection depends on the host and the species involved. In some hosts the infection is fatal and rapidly kills the host. In others, the host can recover and is strongly immune to reinfection. Sergent et al., (1924) introduced the term "premunition" to describe the situation where the persistence of subclinical infection in recovered animals is necessary to maintain immunity. In recent years, however, work done on a number of Babesia species, indicated that immunity

remained for several years in animals after completely recovering from the infection i.e. sterile immunity, such as in B. divergens (Davies et al., 1958; Joyner and Davies, 1967), B. bovis (Mahoney et al., 1973b) and B. bigemina (Callow, 1967) in cattle, B. caballi in horses (Frerichs et al., 1969), and B. rodhaini in rats (Phillips, 1969a). In Australia, immunity in cattle infected with B. bovis and treated with drugs, persisted at least for six months after chemotherapy (Callow et al., 1974). The persistence of immunity was demonstrated after challenge with both homologous and heterolgous parasite strains (Johnston et al., 1978).

b. Role of antibody

The role of antibody in protection has been demonstrated by passive transfer experiments from immune animals. Mahoney (1967a), Mahoney et al. (1979) demonstrated that antibodies play an important role in B. bovis infection in cattle, especially when hyperimmune serum was used. The protective activity of hyperimmune serum was confined to the IgG fraction of the serum. Antiserum was also effective against B. rodhaini in mice (Roberts et al., 1972; Roberts and Tracey Patte, 1974) and in rats (Phillips, 1969b; Rogers, 1974). Protection was achieved as a delay in the onset of parasitaemia when antiserum was injected at the time of the infection. Some other workers, however, failed to show any protection with immune serum and suggested therefore that antibody has a minor role in protection as was reported for P. berghei and B. rodhaini (Mitchell et al., 1978) and B. microti (Allison et al., 1978) infections. Work done on rodent malaria,

however, has shown that the protective activity of serum is short lasting and that serum collected 5-6 days after peak activity is not protective (Phillips and Jones, 1972; McLean and Phillips, 1979). It is possible therefore that workers who failed to show protection with immune serum, were collecting serum at the wrong time.

The antibody levels in the serum can be detected by various serological tests (Todorovic, 1975; Bidwell et al., 1978), but levels measured by these tests do not relate to protective levels, indicating that some antibodies are not protective (Callow et al., 1974). The mode of action of antibody in vivo is not clear. Some reports have indicated a role for monocytes and neutrophils in the destruction of Babesia, as was reported for B. canis (Neitz, 1938), where phagocytosis of free parasites, parasitised red cells and non infected red cells has been promoted by specific antibodies that combine with free parasites, parasite antigen on or in the red cell membrane. Rogers (1974) observed that the addition of immune serum in vitro increased the phagocytosis of B. rodhaini by peritoneal macrophages and suggested that opsonising antibody plays an important role in protection when the opsonising activity was correlated with the protective activity of immune sera in passive transfer experiments. Mahoney et al,1979 passively protected cattle against B. bovis and suggested that merozoites may be destroyed as they leave the red blood cells but that parasites within the red cells were not affected by antibody. Mathematical analysis of the rate of removal of B. bovis, however indicated that some infected red

cells must have been opsonised and engulfed by phagocytes.

The recent advances in the *in vitro* cultivation of *Babesia* permits more detailed examination of the effect of antibody and/or cells on parasite growth in culture. Erp *et al.* (1978) were the first to develop a method for short term cultivation of *B. bovis*. Later, Bautista and Kreier (1979) cultivated *B. microti* in short term cultures and tested the effect of addition of immune hamster serum on parasite growth *in vitro*. They found that the addition of immune hamster serum inhibited parasite growth and suggested that immune serum prevented the invasion of red cells by the parasite. In further experiments, the same workers cultured *B. microti* from hamsters over peritoneal macrophages with immune serum, and suggested that opsonisation and phagocytosis was not the major cause of parasite death in culture, but immune macrophages produced a soluble factor which inhibited growth of *B. microti* in the presence of immune serum (Bautista and Kreier, 1980).

The development of the microaerophilous stationary phase cultures (MASP) for *B. bovis* (Levy and Ristic, 1980), where a large quantity of cell free merozoites and soluble *Babesia* antigens were released in the culture supernatant, has provided a tool to examine the protective role of anti-*Babesia* antibodies. It was found that when free merozoites of *B. bovis* were preincubated with sera of recovered or immunised cattle and then introduced to *in vitro* culture, they failed to invade erythrocytes (reviewed by Ristic and Levy, 1981).

It has been reported for *P. berghei* (Miller *et al.*, 1975) and *B. bovis* (Gravely *et al.*, 1979), that the merozoite surface

coat elicits the production of antibodies that prevent erythrocyte invasion by parasites and facilitates their immune destruction by phagocytic cells. Ristic and Levy (1981) were able to recover a large quantity of B. bovis soluble antigens in a MASP culture and found that if they were preincubated with immune serum, antibody lost its ability to prevent in vitro infection of erythrocytes by B. bovis merozoites because it was neutralised by the soluble antigens. Smith et al. (1981) observed that antisera to soluble antigens released into the supernatant of B. bovis cultures reacted with the merozoite surface coat causing its lysis. Kuttler et al. (1982, 1983), vaccinated cattle with culture derived soluble B. bovis antigens, and found that cattle responded with a strong humoral response to B. bovis challenge as measured by the indirect fluorescence antibody test (IFAT), and that immunity induced by vaccination persists for at least six month periods. They suggested, however, the participation of T-helper cells in immunity since it was observed that vaccinated cattle were still immune to babesiosis when circulating antibody titre was below detectable levels as determined by the IFA test.

c. Cellular immunity

Many reports on Babesia infections indicate that T cells play an important part in acquired immunity to this parasite. Roberts (1968) compared immunity to B. rodhaini in rats in transfer experiments using immune spleen cells and immune serum. He concluded that adoptive transfer of immunity is effective, whereas passive transfer of serum led to the enhancement of

Babesia infections. Clark and Allison (1974) reported that parasitaemias in neonatally thymectomised mice infected with B. microti (King's strain) were more severe than in normal mice. Mitchell (1977) reported the same observations in thymectomised mice infected with B. rodhaini. Ruebush and Hanson (1980) working on B. microti (Human strain), also using thymectomised mice, concluded T cells were the major cells required for the development of acquired immunity to this parasite in mice. In further experiments, the same authors observed that spleen, or nymph node cells of recovered mice could adoptively transfer immunity to recipients and in the spleen cells it was T cells and not B cells which carried immunological memory of B. microti. They suggested that these T cells stimulated macrophages to produce a soluble factor toxic to the parasite. Evidence that cell mediated immunity plays a part in immunity to Babesia came also from the results of leukocyte migration inhibition tests in hamsters infected with B. microti (Perez et al., 1977) and in delayed skin tests in B. equi infection in donkeys (Banarjee et al., 1977; Singh et al., 1979). The role of T cells in protection was also reported for malaria infections in thymectomised rats (Brown et al., 1968 ; Stechschulte, 1969), hamsters (Chapman and Hanson, 1971), mice (McDonald and Phillips, 1978) and athymic nude mice (Clark and Allison, 1974) were more susceptible to P. berghei, P. chabaudi or P. yoelii than intact controls.

T cells may act as helper cells in protective antibody production or as mediators of various cell mediated immune

mechanisms. Wolf (1974), studied the effect of antilymphocyte serum and splenectomy on resistance to B. microti infection in hamsters. He suggested that although cellular immunity is a major factor in host resistance, humoral antibodies may contribute to controlling the level of parasitaemia. Previously Phillips (1970) reported that adoptively transferred cells may promote antibody production in the adoptive transfer of immunity to P. berghei in the rat. Meeusen et al. (1984a), working on B. microti in mice suggested that antibodies produced by sensitised B cells were responsible for the adoptive transfer of immunity. In further experiments, the same workers suggested the possible role of subpopulations of B cells, namely antibody forming cells in the adoptive transfer of immunity with immune spleen cells (Meeusen et al., 1984b). Antibody forming cells were also responsible for the adoptive transfer of immunity in P. berghei infection (Gravely and Kreier, 1976). Allison et al. (1978) examined the role of B, T and other unidentified cells in acquired immunity to Babesia. They transferred resistance to mice infected with B. microti, using spleen cells and found that the protective activity of the spleen cells was not reduced by treatment with anti-thyserum (which removes T cells) or by nylon wool separation (which decreases the number of B cells).

Natural killer cells

The role of natural killer cells in cellular immune responses to babesial infections has been studied by several workers. Eugui and Allison (1980), reported that differences in the susceptibility to B. microti infection among various mouse

strains could be correlated with natural killer cell activity. They found that in CBA, C57/Bl and B10.A mice, B. microti infection is rapidly controlled, whereas in A strain mice the parasitaemia persists for a long time, and they correlated the greater resistance of CBA, C57/Bl and B10.A mice with the rise in the natural killer cell activity in the spleen which is measured by the capacity of spleen cells to kill sensitive tumour target cells in vitro. In contrast, A strain mice had a much lower NK activity in their spleens. It was suggested that the increase in NK activity results from sensitised T cells and macrophages activated by T cell products which stimulate natural killer cells through the release of interferon which in turn releases factors that inhibit the development of the parasite. Irvin et al. (1981) also working on B. microti found that congenitally asplenic mice (DH/+mice) were less susceptible to the infection than surgically splenectomised mice, and suggested that the functional splenic activity in congenitally asplenic mice is taken over by other tissues which may be mediated by natural killer cells. Wood and Clark (1982) have shown, however, that increased natural killer cell activity in the spleens of B. microti and P. vinkei peteri infected mice was not associated with the host's response against these parasites. They found that parasitaemia in mice (CBA/Ca mice) infected with B. microti or P. v. peteri increased normally after maximal NK activity had been achieved. They also observed that the course of infection with B. microti of P. v. peteri was normal in mice pretreated with strontium^{89} or oestradial which both reduce NK activity, and the course of infection with B. microti was also unaltered in

beige mice which are genetically deficient in NK cells. After all the above observations they concluded that it is unlikely that NK cell are important in the resolution of B. microti and P. v. peteri infections in mice.

Role of the spleen

The spleen plays an important role in the development of acquired immunity to Babesia infections (reviewed by Carson and Phillips, 1981). It has been reported that removal of the spleen in rats infected with B. rodhaini results in their death (Matson, 1964; Phillips, 1969c). Todorovic et al. (1967), working on the same parasites in the same hosts as above, reported, however, that rats do not always die from B. rodhaini infection when splenectomised. Removal of the spleen also had no effect on cattle infected with B. bovis (Legg, 1935). Splenectomy also allows the establishment of Babesia in animals and man to which these hosts are usually completely immune. For example, Garnham and Voller (1965) reported that intact chimpanzees were completely resistant to B. divergens, but after splenectomy, they become highly susceptible. In man, severe cases of babesiosis occurred in splenectomised individuals (reviewed by Rosner et al., 1984).

The spleen is important in that it is the major site of phagocytosis. Free parasites and infected and uninfected erythrocytes were reported to be phagocytosed in this organ in B. rodhaini infection in rats (Phillips, 1969c) and mice (Roberts et al., 1972). The spleen is also important in the production of antibabesial antibodies and in cellular immunity (Roberts, 1968;

Phillips, 1970b), and splenectomy reduces the level of antibodies against various antigens present in the blood circulation (Winebright and Fitch, 1962; DeCarvalho et al., 1967). The spleen may also remove parasites from circulation by the process of pitting, as it was reported for P. knowlesi infection (Schnitzer et al., 1972), and the parasite numbers are therefore reduced before any specific immune response. Roberts and Tracey Patte (1974), suggested however the "pitting" is not important in the control of B. rodhaini infection in mice. The spleen seems also to be important in dealing with antigenic variation. Phillips (1969c) reported that in normal rats, the spleen plays an important role in the control of the first wave of B. rodhaini parasites. When the rats survived the primary attack, the spleen was primed for the secondary wave of antigenically different B. rodhaini and was much more efficient in controlling this. When the rats were splenectomised after the first parasitaemia, the relapse was fatal. Phillips (1969c) suggested that after splenectomy the memory of the previous attack had been lost and the relapse strain developed as if it were a primary infection. Antigenic variation has also been reported for B. rodhaini in mice (Roberts et al., 1972) and B. bovis in cattle (Curnow, 1973a, b).

IMMUNISATION

Bovine babesiosis is considered to be a serious problem which threatens the health of cattle in many regions of the world. Although the disease has been eliminated in countries such as the United States (Bram and Gray, 1983), it is still

persisting and spreading due to the transport of infected domestic animals across the world (Young and Morzaria, 1986). This, and the cost of eradicating ticks, makes establishment of effective control programmes difficult. Control of bovine babesiosis is therefore mainly directed at the development of methods for controlling the disease rather than eradicating the tick vector. Various vaccination methods have been developed for disease control, and three principle methods of vaccination are being followed:-

1. The use of parasite antigens derived from infected hosts.
2. The use of culture derived antigens.
3. DNA technology.

1. The use of parasite antigens derived from infected hosts

A. Live vaccines

The oldest form of immunisation against babesiosis consisted of inoculating infected blood into susceptible animals (Hunt, 1897 - quoted by Mahoney, 1972). In Australia, immunisation of cattle against babesiosis caused by B. bovis was achieved by using live vaccines which have been attenuated or made avirulent by rapid passage of infected blood through splenectomised calves (Callow, 1977). This method has been extensively used for vaccinating cattle against babesiosis in the state of Queensland, Australia (FAO, 1984). In Ireland, it was not, however, possible to attenuate B. divergens by such means (Taylor et al., 1983; Murphy et al., 1986).

There are risks of using live vaccines, and these include; first, the accidental transmission of unrelated diseases such as

bovine leukosis (Hugosan et al., 1968); secondly, repeated vaccination of animals results in the production of antibodies to blood group antigens which in turn cause haemolytic disease in the newborn (Langford et al., 1971) and thirdly, the live vaccines might occasionally cause severe babesiosis and transmission of the attenuated parasite from vaccinated animals can cause reversion to virulence (Young and Morzaria, 1986).

b. Irradiated parasites

Other attenuated vaccines for the control of bovine babesiosis have been in the form of irradiated infected blood (reviewed by Wright, 1984). Previous reports have shown that rats and mice can be protected against challenge with B. rodhaini (Phillips, 1970a; 1971) and cattle against B. bovis (Mahoney et al., 1973a), B. major (Brocklesby et al., 1972; Purnell et al., 1978; 1979a), B. bigemina (Bishop and Adams, 1974) and B. divergens (Lewis et al., 1979a; 1980a; Taylor et al., 1980) by prior inoculation of irradiated parasites. Phillips (1971) suggested that the use of irradiated infected blood to protect mice and rats against challenge with B. rodhaini might provide a method of vaccination against Babesia of domestic animals. Lewis et al. (1979a) speculated that protection resulted from the combined inoculation of a large number of parasites which have been irradiated at a dose to kill them, and a small number of live parasites which had survived irradiation. This was later confirmed by Purnell and Lewis (1981). Taylor et al. (1983), however, concluded that this method of producing attenuated vaccines (i.e. irradiation of infective blood) is not reliable to

protect cattle against B. divergens infection, since the irradiation of infected blood results in the destruction of most of the parasites in the infective dose rather than the reduction of viability of individual parasites, and some parasites may survive to produce a clinical infection.

c. Inactivated vaccines

The early work of immunisation against babesiosis showed that immunisation can be achieved with either antigens derived from parasitised erythrocytes or the plasma of infected blood (Mahoney, 1981). Antigens that were contained in the infected blood were purified, lyophilised or used fresh and inoculated with an adjuvant. Mahoney (1967b) and Phillips (1967), induced protection against B. bovis and B. rodhaini respectively by the inoculation of washed parasite suspension in Freund's complete adjuvant into susceptible hosts.

Mahoney and Wright (1976) found that B. bovis infected erythrocytes protected susceptible non-splenectomised adult cattle even against a heterologous strain of B. bovis when injected subcutaneously or intramuscularly in Freund's complete adjuvant. Some protection against B. divergens was conferred by using fractions of parasitised erythrocytes separated by isolectric focussing (Taylor et al., 1984). Goodger et al. (1983), also found that a simple distilled water lysate of infected erythrocytes, was effective against homologous challenge to B. bovis when used as a vaccine. Increasingly more efficient methods have been developed for purifying such antigens by the Australian workers (reviewed by Irvin, 1987). The antigens

released into the plasma of B. bovis infected cattle (Mahoney, 1971; Mahoney and Goodger, 1972) and B. divergens (Purnell and Brocklesby, 1977) were, however, less protective than the antigens contained in the infected erythrocytes. Goodger et al. (1987) reported that although an immune complex detected in the plasma of animals recovering from acute B. bovis infection contained babesial antigens as well as the major proteins, it had little or no role in protective immunity. Banarjee and Prasad (1985) however, reported that a vaccine which consists of plasma separated from B. bigemina infected blood, gave better protection in calves even when challenged 264 days after vaccination. On the other hand, a vaccine which is composed of the lysate of infected erythrocytes containing B. bigemina parasites and some erythrocyte stromata was less protective. The plasma antigens of two other non-bovine babesias, B. rodhaini and B. canis were also protective, not only in homologous but also in the heterologous situations (Sibnovic et al., 1967; 1969).

2. **The use of culture derived antigens** (attenuated vaccines)

Methods for in vitro culture of Babesia species have facilitated the collection and preparation of antigens for immunisation trials. The culture approach has been extensively studied by the American workers and is based on the development of culture methods for B. bovis (Erp et al., 1978; Levy and Ristic, 1980). Smith et al. (1979; 1981), reported that cattle could be protected against homologous challenge with cell free soluble antigens prepared from culture supernatants. The development of a MASP culture system (Levy and Ristic, 1980),

resulted in a series of studies where a high yield of cell culture derived antigens were produced. These were purified, mixed with a Quil-A saponin adjuvant and used to immunise cattle. Immunised cattle had milder infections than controls when both groups were given homologous (Kuttler et al., 1982; 1983) or heterologous challenge (Montenegro-James et al., 1985; 1987). Other studies have shown however, that culture derived antigens were less protective against a heterologous challenge than live culture derived parasites (Timms et al., 1983; 1984). The application of new techniques in immunochemistry has led to the identification and characterisation of antigens on the surface of Babesia merozoites that might be associated with protection. Montenegro-James et al. (1983) found that the principle antigens associated with protection are located on the surface coat of merozoites. James et al. (1981) found that protection of the merozoite surface coat is associated with proteins of 30-40 KDa. Wright et al. (1985) found that vaccination against B. bovis could be achieved with a low molecular weight antigen which appeared to be 29 KDa. Winger and Canning (1985) and Winger et al. (1987a) have raised monoclonal antibodies against merozoites of B. divergens. Winger et al. (1987a or b) were able to induce partial protection in gerbils to homologous challenge by immunisation, with an affinity purified 50-60 KD B. divergens merozoite antigen.

Chemotherapy

A large number of chemical compounds have been used in the treatment of babesiosis. Compounds such as amicarbalide,

imidocarb, quinuronium sulphate and oxytetracyclines have been extensively used especially in the treatment of bovine babesiosis (reviewed by Kuttler, 1981). Amicarbalide has been successfully used for the treatment of B. divergens and B. bigemina in cattle when it was administered intramuscularly (Adams et al., 1978; Barnett, 1965; Shone et al., 1961). Another compound, imidocarb, in addition to its therapeutic properties, can also act prophylactically and protection of up to 15 weeks has been recorded against B. bovis (Todorovic et al., 1973). The prophylactic activity of the drug was found, however, to be limited since it did prevent the exposure of cattle infected with B. bovis (Callow and McGregor, 1970; Dalgliesh and Stewart, 1977) and with B. divergens (Lewis et al., 1981a) to new infections. De Vos et al. (1986) reported that the prophylactic activity of imidocarb in B. bovis infection can be disadvantageous since it can suppress the establishment of immunity in cattle immunised with live vaccine. Another aspect of imidocarb is that infection of ticks which feed on imidocarb treated cattle can be reduced or eliminated as reported for B. bigemina and B. microplus ticks (Kuttler et al., 1975; De vos et al., 1984). This drug was also used for the treatment of canine babesiosis (Adeyanju and Aliu, 1982; Guelfi, 1982) and it was found to be effective in in vitro short term cultures of B. bovis (Akinboade, 1984). Another compound, quinuronium sulphate, has been used for treating splenectomised calves infected with B. divergens (Purnell, 1981). Gray (1983) made a comparative study on the chemotherapy of the gerbil Meriones unguiculatus infected with B. divergens and found

that four drugs, namely quinuronium sulphate, diminazene aceturate, amicarbalide, and imidocarb, were all effective, leaving the gerbils with a sterile immunity. Tetracycline is another drug which was used to ameliorate the severity of post immunisation reactions in highly susceptible cattle given live B. bovis vaccine (Pipano et al., 1985) and was recently used for the treatment of cattle infected with B. divergens (Taylor et al., 1986).

2. DNA technology

The various approaches to vaccination against Babesia have concentrated on the use of live, attenuated or irradiated parasites derived from infected hosts as described above. There are, however, risks in using these vaccines as they might be a source of transmitting unrelated diseases to the vaccinated hosts, and although irradiated parasites have been extensively used against challenge with B. bovis (Mahoney et al., 1973a), this method was found to be unreliable since the radiating dose was found to be important, for example, strong protection was achieved when low doses of irradiation were used, which inactivated the majority but not all of the parasites. On the other hand partial protection was achieved when high doses of irradiation were used. Similar results have been obtained for B. major (Purnell et al., 1978) and with B. divergens (Lewis et al., 1979; Taylor et al., 1980). The difficulty in producing large amounts of parasite material from infected hosts has also presented a problem and led to the need for finding an alternative source for producing parasite antigens. The

development of methods for the *in vitro* cultivation of *P. falciparum* (Trager and Jensen, 1976) and *B. bovis* (Levy and Ristic, 1980) in recent years has presented a partial solution for producing a large number of these antigens. Sufficient amounts for the synthesis of parasite antigens was achieved by the use of recombinant DNA technology which was based on gene cloning and allowed the expression of eukaryote genetic material in organisms such as *Escherichia coli* (Wakelin, 1984). Although there are no reports on applying recombinant DNA technology for producing vaccines against *Babesia*, this method has been extensively tested for producing vaccines against malaria. This was achieved by first identifying antigens responsible for protection using monoclonal antibody technology, which in turn made it possible to understand their molecular structure. A number of protective antigens have been identified in the merozoite and schizont stages of both *P. yoelii* in mice, *P. knowlesi* in primates, and for *P. falciparum* in humans (Wakelin, 1984). Wright *et al.* (1983) have isolated an antigen, using a monoclonal antibody raised against a soluble *B. bovis* antigen. This antigen has been shown to provide protection against challenge in immunisation trials on cattle (Wright *et al.*, 1985). Wright *et al.* (1983) speculated that since this antigen was small and a single polypeptide chain it could be an ideal candidate for producing large amounts of vaccines using recombinant DNA technology. Using DNA technology, it was possible to extract total RNA from thoraxes of *P. knowlesi* infected mosquitoes, and it was possible to prepare double stranded cDNA, which was inserted into plasmids and cloned in

E. coli. Subsequently it was possible to screen recombinant organisms for the sporozoite antigen using monoclonal antibody technology and successful recombinants have been produced (Wakelin, 1984).

EXPERIMENTAL RATIONALE

Babesia divergens is the babesia of major importance in the United Kingdom and Ireland where it causes losses in cattle. Little is known about the mechanisms of immunity to this parasite in the bovine. Phillips (1984) has adapted the parasite to laboratory splenectomised rats using infected bovine blood. It was of interest, therefore, to study the mechanisms of immunity to this parasite using the rat adapted strain as a model although it is appreciated that the immune mechanisms in the bovine might be different from those in the rat.

Although the spleen plays a major part in the development of acquired immunity in splenectomised animals, extra splenic sources may take over the function of the spleen and immune mechanisms may develop in other sites (Allison et al., 1978; Carson and Phillips, 1981). The course of infection of B. divergens in splenectomised rats was short lasting and no recrudescences were observed after the primary patent parasitaemia. The rats either died or recovered and were immune to reinfection. The persistence of acquired immunity was apparently not dependant on the spleen. Reports on murine malaria have shown that the liver is a major site for parasite destruction and for development of protective immunity. Increased cell migration and leukocyte accumulation in the liver

have been reported in P. berghei (Playfair and de Souza, 1982; Weiss, 1985), and in P. chabaudi (Kumararatne et al., 1987) infections. Histological studies on livers collected from recovered rats were carried out to investigate the possible role of this organ in the destruction of parasites and in protective immunity. The haematological changes that accompanied the infection were also followed.

There is good evidence that antibody is involved in the acquired immunity to Babesia. Passive transfer of immunity with serum from immune animals has been reported for B. canis in the dog (Nocard and Motas, 1902), B. rodhaini in rats (Matson, 1964; Phillips, 1969a and b; Abdalla et al., 1978) and B. bovis in cattle (Mahoney et al, 1979), Using the same parasite as in the present study but in mongolian gerbils, Liddel et al. (1982), reported however, a minor role of antibody in protection. The role of antibody in protection against B. divergens was studied in passive transfer experiments with sera collected during the infection at different times after recovery and with hyperimmune serum. The different immunoglobulin classes were determined and the antibody levels in the sera were measured by the indirect fluorescence antibody test (IFAT). Immunological studies with B. divergens in the rat may therefore provide a suitable model system, the results of which could be extrapolated to Babesia in the bovine.

The development of methods for in vitro cultivation of malaria and Babesia has provided a laboratory tool for investigations of various aspects of human malaria and bovine

babesiosis that were not possible before. Trager and Jensen (1976), were the first to develop a method for long term cultivation of P. falciparum using the candle jar technique. Levy and Ristic (1980) have developed a method for continuous cultivation of B. bovis in a microaerophilous stationary phase culture (MASP) in which the isolation of infectious merozoites and soluble antigens was possible and provided an opportunity for various in vitro studies on babesiosis without the need of use of animals. Studies such as the properties of protective anti-B. bovis antibody drug sensitivity and the development of vaccines were carried out in vitro.

In order to compare the protective activity of immune serum in vivo with its effect on parasite growth in vitro, a method for cultivation of B. divergens was developed. Three isolates of B. divergens were adapted to long term cultures in rat erythrocytes using the candle jar technique. Apart from producing a large number of infected erythrocytes, the cultures produced extracellular Babesia merozoites. Some aspects of the mechanisms of action of antibody, the effect of adding immune or normal serum to culture on parasite growth and some aspects of cellular immunity were studied in vitro. The development of a method for the long term cultivation of B. divergens has also provided an opportunity to adapt new Babesia isolates for long term cultivation and to test suitability of erythrocytes from non rat species to support parasite growth in culture.

It has been reported that immunogens consisting of soluble exoantigens collected from culture supernatants are prime candidates for babesiosis vaccines (Levy and Ristic, 1980). The

in vitro cultivation of *B. divergens* therefore provides a good tool for further studies on protective anti-*B. divergens* antibodies, for drug sensitivity assays, and for studies utilising cell culture derived antigens which are released in the culture supernatant. This might provide valuable knowledge for producing a suitable vaccine for bovine babesiosis.

CHAPTER TWO

MATERIALS AND METHODS

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Materials and Methods

Host animals

Wistar rats and gerbils were bred in the Wellcome Laboratories for Experimental Parasitology, University of Glasgow. The sex and the age of the animals at the start of each experiment are given in the text.

Splenectomy

Splenectomy was carried out under ether anaesthesia (Phillips, 1969c; 1984). Rats were not infected until at least seven days after splenectomy.

Parasite

B. divergens was originally cryopreserved in liquid nitrogen as infected bovine blood (Phillips and Wilson, 1978). These parasites were subsequently adapted to splenectomised rats and cryopreserved (Phillips, 1984) until used in the present project. The blood was recovered from liquid nitrogen using the method of Gray and Phillips (1981). Parasites were recovered from stabilate by thawing under running water and diluted 50 : 50 with 17.5% sorbitol (see Appendix C) and immediately injected into a splenectomised rat, intravenously (i.v.) into the tail vein or intraperitoneally (i.p.). The parasite was subpassaged through splenectomised rats every 2-3 days, or kept at 4°C for 3-4 days before it was subpassaged into splenectomised rats.

Parasitaemia and course of infection

Babesia infections were evaluated by counting the percentage of infected red cells in thin blood smears taken daily from the tails of infected rats. The blood smears were air dried, fixed in 100% methanol (Analar, BDH Ltd.) and stained in 10% Giemsa's stain (BDH) in phosphate buffer pH 7.2 (see Appendix A). The blood smears were examined under oil immersion using a 10 x eye piece and a x 100 objective on a Leitz SM Lux binocular microscope. Low parasitaemias were counted by counting the number of parasites observed in a total of 30 field (i.e. approximately 10,000 cells). Parasitaemias were considered to be subpatent when no parasites were observed in 30 fields. If more than 3-4 parasites were observed per field, the number of parasites and erythrocytes were counted in 1-3 fields (300-500 erythrocytes).

The course of infection of a group of rats is presented in a graphical form by plotting the geometric mean of the parasitaemia (Mean Log^{10} of the number of PRBC/10^5 RBC) against time (expressed as days post infection). Vertical bars showing one standard deviation are included where necessary.

Infection of rats

Rats were infected with parasitised red blood cells (PRBC) collected in heparin (10 i.u./ml). The blood was diluted where necessary using RPMI 1640 (Gibco) (see Appendix B). Parasites were kept on ice until they were inoculated into the rats.

Cryopreservation of parasites

Parasites were cryopreserved using the method of Phillips and Wilson (1978). Infected blood was collected into heparin (10 i.u./ml) and diluted 50 : 50 with a solution of sorbitol/glycerol (Appendix C). The sorbitol glycerol was added to the blood, slowly dropwise with frequent mixing 0.5 - 1ml aliquots were snap frozen and stored in liquid nitrogen as stabilates.

Haematology

Blood was collected from the tail of the rats into heparinised glass capillary tubes. Red blood cell counts and white blood cell counts were made with a haemocytometer after dilution of red blood cells in PBS (pH 7.2) (see Appendix A) and white blood cells in white cell diluting fluid (Appendix D). Differential white blood cell counts were made by examining Giemsa's stained blood smears and counting the different types of white blood cells present.

Raising and collection of sera

Large volumes of immune, hyperimmune and normal sera were collected by exsanguination. The different sera were raised in rats as follows:

a. Immune serum

Immune serum was obtained from donors which were inoculated with 1×10^4 PRBC and recovered. Immune sera were collected at different times after recovery as will be described at the start of each experiment.

b. Hyperimmune serum

Hyperimmune serum was obtained from donors which were inoculated with 1 x 10^4 PRBC and challenged at least twice at one monthly intervals. The rats were bled one week after the last challenge.

c. Normal serum

Normal serum was collected from normal non infected rats.

In each experiment, the blood from each day was pooled and allowed to clot. The clot was loosened from the edges of the container and left for 2-3 hours at room temperature. The serum was collected and contaminating erythrocytes removed by centrifugation (300g for five minutes). The serum was then aliquoted, frozen and stored at -20°C. Small volumes of sera were collected from the tail blood by the method of Gray (1979) using hard glass capillary tubes.

Indirect fluorescent antibody test

Infected red blood cells were collected from infected rats and used as antigen. The parasitised red blood cells were washed three times in 20ml PBS (pH 7.2) by centrifugation (300g for five minutes) and resuspension. After the last wash, the cells were resuspended in PBS to approximately 50% haematocrit and used to make blood smears covering the microscope slide. Parasitaemias of 8-20% were used for antigen slide preparation. The slides were dried over silica gel, packed with silica gel and stored at -20°C. Before use the slides were brought up to room temperature in a dessicator.

The IFAT used was basically the method of Van Meirvenne et al. (1975). Reaction zones were marked on antigen slides using an H series texpen (Deacon Laboratories). The slides were washed twice in PBS, drained and rehydrated for 15 minutes in PBS. The area around reaction zones was dried and serial dilutions of test and control sera were added to the reaction zones. The slides were incubated for 15 minutes then washed and rehydrated as above. FITC Conjugated rabbit antiserum to rat Ig (Sera-Lab) diluted 1 in 400 in PBS containing Evans blue (1 in 10,000 w/v) was applied to the slides and incubated for a further 15 minutes. The optimal working concentration of the rabbit anti rat Ig was determined against a known positive rat anti-B. divergens serum. The slides were then washed again and rehydrated, mounted 1 : 1 PBS/glycerol. Fluorescence was observed using a Leitz Ortholux microscope. The overhead ultraviolet source was a Wotan HBO-50 mercury lamp with 2 x KP 490 exciting filters and a TK 510 dichroic beam splitting mirror and a K 515 suppression filter. The slides were examined using a x 50 water objective and x 12 eye pieces. The titre of the serum was considered to be the last dilution of serum at which specific parasite fluorescence was observed.

Passive transfer of sera

Rats were injected with the different sera i.v. or i.p. (as will be described at the start of each experiment) within one hour of infection. Their subsequent course of infection was followed in blood smears (see above). Results of passive transfer tests were usually presented in a graphical form as

described for the course of infection.

FRACTIONATION OF ANTISERA

Isolation of IgG from serum using DEAE cellulose

The IgG fraction of pools of hyperimmune sera, immune sera and normal sera was isolated using a variation of the method described by Johnstone and Thorpe (1982). First the Ig fraction of the serum was collected as follows: Twenty mls of serum was thawed and warmed to 25°C. Anhydrous sodium sulphate (Analar) was added to 18% (w/v) and the solution incubated for 30 minutes at 25°C. It was then centrifuged at 3000g for 30 minutes at room temperature. The supernatant was discarded and approximately 3-5mls of the precipitate redissolved in 10mls distilled water and anhydrous sodium sulphate added to make a 14% solution. The solution was incubated for 30 minutes at 25°C and centrifuged as above. The precipitate was again dissolved in water and dialysed overnight against 0.07 M phosphate buffer pH 7 (see Appendix A). The IgG was isolated from the Ig fraction using ion exchange chromatography. DEAE cellulose (Whatman) was used as an ion exchanger. This was equilibrated with the 0.07 M phosphate buffer pH 7 (Appendix A) and packed into a column 1.6 x 30 cm (Pharmacia) and washed with the same buffer at room temperature overnight. When both exchanger and sample were fully equilibrated, the serum sample was applied to the column and eluted with the starting buffer. 6-9ml/tube fractions were collected in a fraction collector (LKB).

Isolation of IgM on sephadex G200

Pools of hyperimmune, immune and normal sera were fractionated using the method described by Hudson and Hay (1976), except that borate buffer (pH 8) (see Appendix A) was used for elution instead of phosphate buffer saline, as follow:-

Seventeen grams of sephadex G-200 powder (Pharmacia) were heated in about 750ml of phosphate buffered saline (pH 7.2) in a boiling bath for five hours. The gel was then poured into a column 100 x 2.5cm (Pharmacia) along a glass rod to avoid air bubbles. The column outlet was left open during packing. The gel was equilibrated with borate buffer (pH 8) overnight. After the gel was equilibrated, 1ml of blue dextran solution (see Appendix E) was added to the column. This is to check on the homogeneity of the column and on the void volume. After the blue dextran was washed into the gel, the buffer reservoir was attached to the column. As soon as the blue dextran had entered the gel, the eluate was collected in a graduate cylinder. The volume needed for the blue dextran to reach the outlet was approximately 100ml of buffer. The serum sample (20ml of serum) was then centrifuged for five minutes at 300g, applied to the column, and eluted with approximately 100ml of borate buffer. Fractions of 5-10ml were collected in a fraction collector (LKD).

Isolation of IgM on sephacryl S-300

Pools of immune serum (day 7 = serum collected immediately after recovery) were eluted from a column of sephacryl S-300 (Pharmacia), with either borate buffer (pH 8) (see Appendix A) or 0.1M Tris Nacl (pH 8) (see Appendix A). Sephacryl S-300 was

already packed in a column 2 x 100 cm (Pharmacia) in the Veterinary Parasitology Department, Glasgow University. The serum was dialysed against either borate buffer or Tris NaCl overnight. The gel was equilibrated with either borate buffer or Tris NaCl overnight, and the serum sample was applied. Fractions of 5-10mls were collected as above.

Estimation of rat immunoglobulin IgG and IgM

The relative concentration of IgG and IgM in the eluted samples was measured by a spectrophotometer (LKB) at 280nm. Fractions of IgG and IgM were pooled separately and placed in dialysing tubes and dialysed overnight against phosphate buffered saline (pH 7.2). The samples were concentrated up to half their volumes by sprinkling polyethylene glycol 6000 (BDH) over the dialysing sac. Rat IgG and IgM were demonstrated by either agar gel immunoelectrophoresis (the Shandon System) (see below) or by double diffusion test using the rat monoclonal typing kit (Serotec, Oxford) against anti rat whole serum (Sigma), sheep anti rat IgG (heavy chains) (Serotec) or sheep anti rat IgM (Serotec). IgG fractions were kept at -20^{o}C until used. IgM fractions were normally kept at 4^{o}C.

Immunoelectrophoresis on IgG and IgM fractions

Immunoelectrophoresis was carried out using the Shandon System according to the manufacturer's instructions with some modifications. Briefly 3 - 3.5ml of 1% Agarose 15 (Electron - BDH) in barbitone buffer (pH 8.6 - Appendix A) were poured onto microscopic slides which had previously been covered with gel bond (Pharmacia). The slides were kept in a humid chamber.

Wells were cut in each slide using a gel cutter (Shandon), and the agar plugs were removed using a pasteur pipette attached to a vaccum line. The wells were filled with 2 - 3 ul of the fractions tested on whole normal serum (control) to which 2 ul of bromophenol blue (BDH) were added (bromophenol blue is used as a marker throughout the electrophoresis). The electrophoresis tank (Model 600 electrophoresis chamber - Shandon) was filled with barbitone buffer (Appendix A). The slides were placed in the tank and each end of the slide was connected to the buffer chamber using a filter paper. Immunoelectrophoresis was applied at a current of approximately 2mA/cm width of slide at a total voltage drop of 150-200 volts for 1 1/2 - 2 hours. The slides were taken out of the electrophoresis chamber and antiserum troughs were cut. Approximately 90-100 uls of anti rat whole serum (Sigma) or anti rat IgG or IgM (Serotec) (neat or diluted 1 in 10) was added to each trough. The slides were kept in a humid chamber at $4^{o}C$ for 24-48 hours. At the end of this period, the slides were washed x 3 with PBS (Appendix A) for 20 minutes, followed by two ten minute washes in distilled water. The gels were then covered either by leaving them at room temperature for 24-48 hours after they were covered with a thick absorbent tissue (Whatman no.4), or they were dried using a current of warm air. The gels were stained with coomasie blue (Appendix E), then washed in a destain solution (Appendix E) until the background was clear. The gels were dried using a current of warm air after which they were ready for inspection.

Quantitative determination of protein concentration

The quantitative concentration of IgG and IgM was preliminarily determined using the protein assay reagent (Pierce).

Chromium-51 labelling of infected and non infected RBC

Infected blood was obtained by bleeding splenectomised infected rats with a parasitaemia of approximately 45% using heparin as anticoagulant (10 iu heparin/ml blood). The blood was pooled and spun at 700g for five minutes. The supernatant was discarded and 200uci of sodium chromate $Na_2Cr^{51}0_4$ (Western Infirmary, Glasgow) was added to each ml of packed cells. The blood was incubated at 37°C for 30 minutes. Labelled cells were washed three times with PBS (pH 7.2) and resuspended in PBS to give a concentration of 5 x 10^8 PRBC/ml. Normal uninfected blood from normal rat donors was labelled in a similar manner and used as a control.

Clearance and organ uptake of chromium-51 labelled erythrocytes

Groups of immune and non-immune splenectomised rats were injected intravenously with 5 x 10^8 chromium^{51} (Cr^{51}) labelled parasitised red blood cells (PRBC) or Cr^{51} labelled normal red blood cells (NRBC) which were equivalent to the total number of erythrocytes injected into the group receiving infected cells. Two rats from each group were sacrificed and bled at intervals as will be described in the experiment and the volume of blood collected was noted. The radioactivity of the blood injected and the blood collected from the rats was measured in a 1280 ultra gammacounter (LKB). The radiolabel present in circulation was

expressed as percentages of the counts which were present in the blood injected into the rats. Blood smears were taken from the tail vein of infected rats every 30 minutes. To determine organ uptake of chromium^{51} labelled erythrocytes, the livers and lungs of immune and non immune rats that received either Cr^{51} labelled PRBC or Cr^{51} labelled NRBC were collected at the same intervals when the rats were sacrificed for blood collection. Radioactivity was measured in weighed samples of tissue and total organ uptake was calculated based upon the determined wet weight of the organ.

Organ uptake was expressed as the percentage of total organ

$$\text{uptake} = \frac{\text{Counts per 20 seconds organ}}{\text{Counts per 20 seconds injected}} \times 100$$

Irradiation of parasites

Whole infected blood was divided into two aliquots. One aliquot was irradiated with 40k rad using a cobalt 60 source (Veterinary Physiology, Glasgow University). The other aliquot was not irradiated and received the same handling as the irradiated blood before it was injected into the rats.

Liver histology

Livers from rats that recovered from a primary infection or from immune rats and naive non-infected rats, were collected, cut into small pieces, fixed in formal saline (see Appendix A) and processed by standard paraffin wax embedding for histology. The rest of the tissue was fixed in isopentane (Analar) then snap frozen in liquid nitrogen. The tissues were then kept in small

plastic tubes and stored at -70°C, until used (see below).

Immunofluorescence staining for determination of B and T lymphocytes in rat liver

The immunofluorescence technique as described by Poulter et al. (1983) was used to demonstrate B and T lymphocytes in rat livers. Rabbit anti rat IgG-FITC conjugate (Sigma) was used to demonstrate B-cells. T cells were demonstrated by monoclonal anti rat T lymphocytes clone W_3/13HLK (Sera-Lab) in combination with fluorescein conjugated (FITC) sheep anti mouse gamma globulin (Scottish antibody production unit). Briefly 5 um of frozen liver sections which were embedded in O.C.T. compound (Tissue TEK-II) were cut on a cryostat Mod.2700 Frigocut, Reichert-Jung, at -25°C, and air dried for one hour at room temperature. The sections on slides were then fixed with acetone (Analar) for five minutes at room temperature. The slides were washed for ten minutes in PBS at room temperature and placed horizontally in humid chambers and 5-10ul of appropriate dilutions of either rabbit anti rat IgG-FITC conjugate or monoclonal anti rat T lymphocytes was added. The slides were incubated at room temperature for 30 minutes and washed for another 30 minutes in PBS. 5-10ml of appropriate dilutions of FITC conjugate sheep anti mouse gamma globulin was applied to the slides which had the monoclonal anti rat T lymphocytes and incubated for 30 minutes. These slides were washed in PBS for 30 minutes and finally all slides were mounted in 1 : 1 glycerol/PBS solution. Fluorescence was observed using a Leitz Ortholux microscope.

CULTURE OF PARASITES

Infected Blood

Rat adapted strain of B. divergens

The cultures were normally initiated with a starting parasitaemia of 2%. The infected blood was either obtained from infected splenectomised rats with a parasitaemia of 2% or from infected rats with higher parasitaemias of 4-10% which was diluted with normal uninfected blood to a starting parasitaemia of approximately 2%. A blood smear was normally prepared from this mixture. The rats were bled aseptically by cardiac puncture and blood was defibrinated by dispensing it into a flask containing sterile glass beads. The defibrinated blood was transferred to sterile universals and centrifuged for ten minutes at 800g. The plasma and buffy coat were removed and the cells resuspended in incomplete medium (see Appendix B) and again centrifuged. The blood was given two more washes in incomplete medium as above. The infected blood was then resuspended in a small volume of incomplete medium and kept at $4^{o}C$ until used.

Bovine isolates of B. divergens

Two bovine isolates which had been passaged into gerbils and then cryopreserved as stabilates were recovered from cryopreservation and subpassaged to gerbils and then either put into culture with gerbil or rat erythrocytes (see below), with a starting parasitaemia of 2%, or subpassaged through splenectomised rats before going into culture. Infected rats and gerbils were bled aseptically by cardiac puncture and the blood

was defibrinated and washed as above before diluting it with normal uninfected rat or gerbil erythrocytes.

Uninfected erythrocytes

Rat and gerbil erythrocytes

Uninfected blood was obtained from normal intact male or female adult gerbils or rats. The gerbils or rats were bled aseptically by cardiac puncture, the blood was defibrinated and washed as bove. The uninfected gerbil or rat blood was resuspended in incomplete medium (see Appendix B) and kept at 4°C until used.

Human erythrocytes

Outdated (nine weeks after donation) normal human blood group O rhesus positive red blood cells were kindly provided by the West of Scotland Blood Transfusion Service, which were received in 450ml packs of whole blood collected into citrate phosphate dextrose adenine (CPDA). Aliquots were removed aseptically and washed as above. The blood was then resuspended in incomplete medium (see Appendix B) before being stored at 4°C for up to four weeks before use in cultures.

Foetal calf serum and heat inactivated foetal calf serum

Foetal calf serum (FCS) was provided by Gibco Ltd. This was received deep frozen and was thawed on arrival. It was then dispersed in 20ml aliquots or heat inactivated at 56°C for 30 minutes, before aliquoting. All aliquots were frozen and stored at -20°C until used.

Cultivation procedure and routine maintenance of *B. divergens* in vitro

The candle jar technique (Trager and Jensen, 1976) was used for culturing either the rat adapted strain or the bovine isolates of *B. divergens*. Packed cells containing 2% infected rat or gerbil erythrocytes were diluted with complete medium (see Appendix B) to make a 10% haematocrit suspension. This was dispensed in 35mm plastic petri dishes (Cel-Cult) with 1.5mls in each. The dishes were placed in a humidified glass dessicator with a candle. The candle was lit and the cover put on with the stop cock open. When the candle flame went out the stop cock was closed. This provides an atmosphere low in oxygen and high in carbon dioxide. The jar was put into a 37^{o}C incubator. Each day the cultures were removed from the candle jar and the medium replaced with approximately 1.5ml fresh complete medium. The dishes were then returned to the candle jar and to the incubator. Blood smears were taken daily from the cultures, fixed and stained with Giemsa's stain (Appendix A). The parasites were counted in relation to the red blood cells to determine the extent of multiplication. Subcultures were prepared by diluting the cultures every 48-72 hours depending on the parasitaemia. Cultures were normally diluted when they reached a 10-12% infected red cells. These were diluted 1 in 5 or 1 in 6 with normal red blood cells and a 10% haematocrit suspension was made with complete medium. The cultures were dispensed in fresh petri dishes (1.5ml each) and reincubated as described above.

Recovery of cryopreserved cultures

Cryopreserved cultures were recovered from stabilates by thawing in a 37°C water bath. The contents in one ampoule were transferred to a sterile universal tube and diluted with 0.5ml of 4.5% saline followed by 4.5mls of 3.5% saline (see Appendix A). This was added dropwise with frequent mixing. The PRBC were washed twice in incompleted medium. This method proved to be unsuitable for recovering B. divergens cultures as will be described later in the text.

Collection of free merozoites

Extracellular merozoites were collected from cultures with 25-30% infected red cells. The cultures were pooled and centrifuged at 200g for ten minutes to sediment erythrocytes. The supernatant fluid was retained and spun again at 400g for ten minutes, to bring down the remaining erythrocytes. The supernatant fluid was retained and spun at 2000g for 20 minutes to pellet the merozoites. The pellet was resuspended in 3mls of incomplete medium (Appendix B) and spun at 2000g for 20 minutes. The pellet was resuspended in 200ml of incomplete medium. Some of the merozoite suspension was further diluted 1 in 200 with PBS and a count was made with a haemocytometer. The pellet contained a large number of merozoites, red cell ghosts but no intact erythrocytes. All procedures were carried out at 4°C.

Cryopreservation of free merozoites

Free merozoites were cryopreserved using the method of Canning (personal communication). Merozoites were resuspended in a cryoprotectant which is composed of 10% dimethylsulphoxide

(DMSO) in Puck's saline (Gibco) containing 20g/L glucose (see Appendix C). 0.5ml aliquots in sterile ampoules were made and placed in an expanded polystyrene box packed with insulating clips and was placed in -70°C for 16-24 hours. The vials were then transferred to liquid nitrogen. Some merozoites were also frozen in incomplete medium at -20°C for future use.

Precipitating of the Ig fraction from normal and immune serum

The Ig fraction of immune and normal serum was precipitated using anhydrous sodium sulphate as described before, except that 2 x 5mls aliquots of either immune or normal serum were used. To one aliquot sodium sulphate was added to give 18% w/v solution. This was kept at 4°C until used. To the other aliquot sodium sulphate was added also to give 18% w/v solution, centrifuged as described before, redissolved in distilled water and sodium sulphate was added to give a 14% w/v solution. Both the 18% and the 14% w/v Ig solutions were aliquoted and either dialysed with PBS overnight or kept at 4°C until used. All aliquots were filtered aseptically using sterile 0.22um filters (Millex - GV), before they were added into culture.

Heat inactivating immune and normal sera

Immune and normal sera were heat inactivated in a 56°C water bath for 30 minutes.

Collection of free parasites

Free parasites were collected by lysing infected red blood cells obtained from infected rats with 0.2% saline (see

Appendix A). Infected blood was washed three times with PBS (pH 7.2). After washing the supernatant was removed and six volumes of prewarmed 0.2% saline was added to the pellet. The mixture was stirred for 20 minutes and then spun at 900g for ten minutes. The supernatant was aliquoted in small eppendorfs and the aliquots were spun for 7-10 minutes using a microcentaur (MSE) at 11,600g. The pellet contained free parasites.

Opsonisation test using hyperimmune serum and free parasites or parasited red blood cells

Peritoneal macrophages were collected as follows:- Normal uninfected rats were killed and about 20ml of medium 199 (Flow) (see Appendix B) were injected into the peritoneal cavity. The medium containing macrophages was flushed out and spun at 700g for five minutes. The cells were resuspended in medium 199 containing 5% foetal calf serum and approximately 1.2×10^6 cells in 0.5mls medium were dispensed into each well of a 24 well culture plate (Nunc) containing plastic cover slips to which macrophages adhere. 0.1ml of test dilutions (with medium 199) of hyperimmune serum or control serum (normal serum) were then added to each well. A positive control of fluorescent latex beads (kindly provided by Dr. J. Kusel, Biochemistry Department, Glasgow University) was included. Hyperimmune serum and normal serum were tested at dilutions of 1 in 2, 1 in 10 and 1 in 100. The plate was incubated for two hours at 37°C in a CO_2 incubator, then 5×10^7 PRBC's or free parasites were added into each well. The plate was reincubated in the CO_2 incubator and two cover slips from both hyperimmune sera and normal sera wells at

different dilutions were removed within the next 24 hours at 20 minutes of two hourly intervals. The cover slips were rinsed with saline (Appendix A) then fixed in 100% methanol and stained with Giemsa's stain for microscopical examination. Cover slips in wells containing latex beads were washed with the medium and examined with the fluorescence microscope.

Preparation of peripheral blood mononuclear cells and spleen cells for culture

Intact rats were injected with 2.5×10^9 PRBC. Peripheral blood mononuclear cells and spleen cells were collected as follows.

Peripheral blood mononuclear cells

Both immune and normal rats (controls) were bled aseptically by cardiac puncture. The blood was defibrinated using sterile glass beads and transferred into sterile plastic universals. Peripheral blood mononuclear cells were collected by layering the blood onto Ficoll hypaque (see Appendix F) and centrifugation at 300g for 30 minutes at $4^{o}C$. The layer containing the mononuclear cells was centrifuged at 250g for five minutes and the pellet resuspended in incomplete medium (Appendix B) and washed twice as above and resuspended in complete medium (Appendix B). Peripheral blood mononuclear cells were counted using a haemocytometer after dilution in white cell diluting fluid (Appendix D). The cells were kept on ice until used.

Spleen cells

Spleens were removed aseptically from immune and normal rats into 5ml incomplete medium (Appendix B). The spleens were disrupted by rough chopping with scissors. Spleen pieces were forced through a fine mesh sieve and then through a 21g needle. Cell clumps were allowed to settle out and the remaining cells washed three times in 15 ml incomplete medium by centrifugation (250g for five minutes) and resuspension in complete medium. Spleen cell numbers were counted in a haemocytometer using white cell diluting fluid after lysis of the contaminating erythrocytes with Tris ammonium chloride (Appendix A). Briefly 9ml of Tris ammonium chloride were added to each ml of spleen cell suspension and incubated for 5-7 minutes at room temperature. Spleen suspensions were washed three times with incomplete medium at 250g for five minutes and resuspended. The counts were adjusted after cell viability was determined using the trypan blue exclusion test (see below).

Trypan blue exclusion test

The viability of spleen cells were estimated by diluting the cells 1 : 1 in 0.2% w/v Trypan blue in PBS (pH 7.2) (Appendix A). The cells were observed by phase contrast under oil immersion (x 100 objective x 10 eye piece). Dead cells were unable to exclude the dye and stained blue. The proportion of live to dead cells was expressed as a percentage viability.

CHAPTER THREE

THE COURSE OF INFECTION AND HAEMATOLOGICAL OBSERVATIONS IN SPLENECTOMISED RATS

Chapter Three

The course of infection and haematological observations in splenectomised rats

INTRODUCTION

In 1979, Lewis and Williams reported that the mongolian gerbil Meriones unguiculatus proved to be useful for the isolation and establishment of bovine strains of B. divergens by direct inoculation of blood from splenectomised experimentally infected bovines, and the course of infection in gerbils was well documented (Liddel et al., 1980; Lewis et al., 1980**b**,1981**b**). Recently, Phillips (1984) reported that B. divergens can be adapted to splenectomised rats by passaging infected bovine blood through gerbils then to splenectomised rats, and preliminary observations on the course of primary parasitaemia, morphology, and haematology in splenectomised rats were described.

In this chapter the course of infection in splenectomised rats and a brief description of the morphology of the parasite during the primary infection are described. The haematological changes that accompany the infection, the virulence of the parasite together with a brief description on the use of the babesiacidal drug amicarbalide, and the development of acquired immunity after challenge are also described. The different observations made on the rat model are compared with those described for the mongolian gerbil infected with the same parasite. The results are also compared with those described for B. divergens in cattle.

The course of infection

The course of infection is illustrated by two experiments.

Experiment 3 (i)

Cryopreserved B. divergens rat blood was passaged through two four week old splenectomised rats before it was inoculated into adult splenectomised rats as follows:

Twenty-one 4 1/2 month old female rats were injected with 1.5×10^8 PRBC, i.v., one week after they were splenectomised. The rats were divided into three groups of seven. In one group the course of parasitaemia was followed in blood smears taken daily from the tail vein. The other two groups were used as serum donors as will be described in Chapter Four. The course of infection in the first group is illustrated in Figure 2A. Parasitaemia was patent on day 1 post infection and increased subsequently reaching a peak on day 3 (14-18%). Six out of the seven rats recovered and the parasitaemia became subpatent on day 5. No parasites were detected over 21 days of further observation. The seventh rat in this group had a peak parasitaemia on day 4 (43%) and died on day 5 post infection.

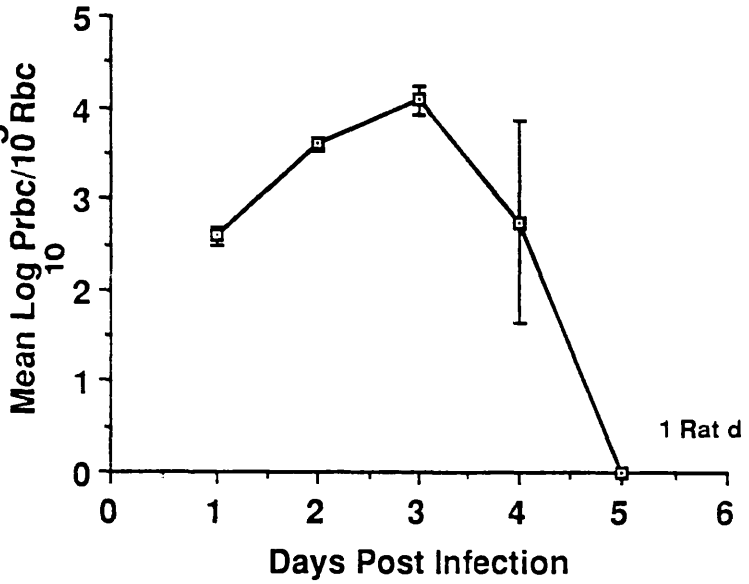
Experiment 3 (ii)

Fresh blood collected from a passage rat was used to inoculate adult splenectomised rats as follows:-

Twenty four four month old female rats were inoculated with the same number of parasites as in experiment 3 (i) (i.e. 1.5×10^8 PRBC) i.v. The rats were divided into one group of ten and two groups of seven and the course of parasitaemia was followed in the first group. The other two groups were used as

Figure 2A:

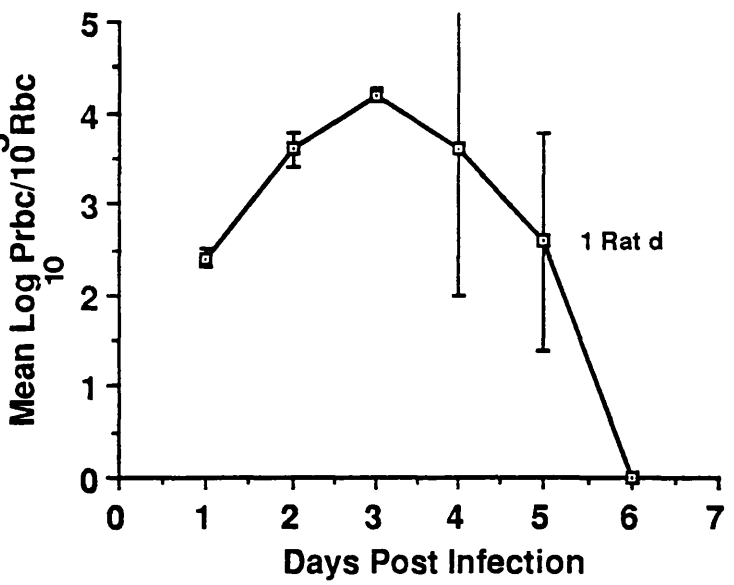
The course of infection in 7-17 week old splenectomised rats injected with $1.5x10^8$ PRBC i.v.



(A)

Figure 2B:

The course of infection in 10-16 week old splenectomised rats injected with $1.5x10^8$ PRBC i.v.



(B)

serum donors as will be described in Chapter Four. The first group developed a patent parasitaemia on day 1 and increased subsequently, reaching a peak on day 3 (16-50%) (Figure 2B). Eight out of ten rats recovered and became **subpatant** by day 6. No parasites were detected in blood smears taken over 21 days of further observation. The other two rats in this group were found dead on day 5 post infection.

Morphology of the parasite:

Experiment 3 (iii)

The appearance of B. divergens in Giemsa's stained rat blood smears was similar to that described by Phillips (1984). Typically divergent forms, rings, oval forms and maltese cross forms were seen (Figure 3). The parasite measured approximately (3.4 um x 0.57 um). At high parasitaemia, multiple parasitisation of the red cells was seen and up to four parasites were noted in one cell. Free parasites were also seen in blood smears with high parasitaemias.

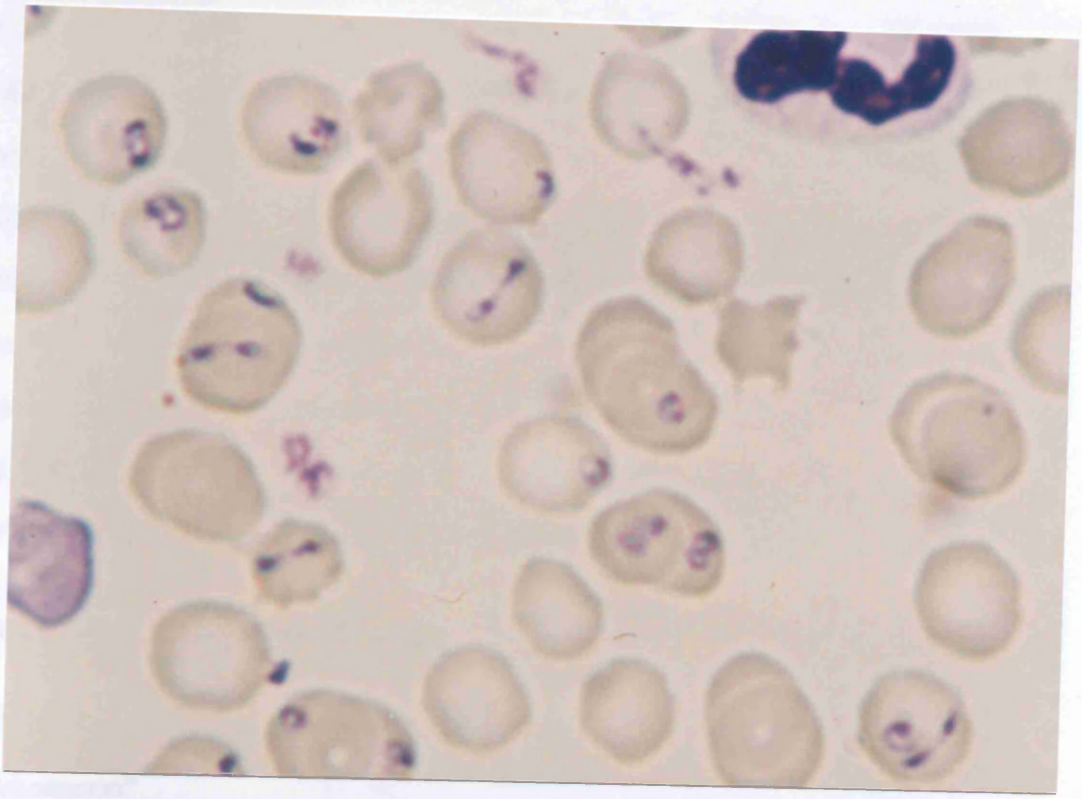
Experiment 3 (iv) and 3 (v)

Haematological observations:

Red blood cell counts and total white blood cell counts were made by collecting blood in heparinised capillary tubes from the tail veins of rats used in experiments 3 (i) and 3 (ii) above. Other experiments not reported here, confirmed these results. Differential white blood cell counts were made by counting 100 leukocytes in blood smears taken 2-6 days preinfection, during

Figure 3:

B. divergens in the blood of infected rats.



the infection and 1-18 days after recovery. The data for differential white blood cell count is given for individual animals or as groups.

The acute parasitaemia was accompanied by a drop in the red blood cell count, between days 2 and 4 (Figure 4A) and days 3-5 (Figure 4B). Red cell counts started rising immediately after recovery from the infection (from day 5 - Figure 4A and day 6 - Figure 4B). The white cell count fell slightly at peak parasitaemia, but rose continuously from day 4 and reached a peak by days 5-6, then fell to preinfection levels by day 22 post infection (Figure 4A and 4B). The pronounced leukocytosis after recovery was predominately a lymphocytosis and the lymphocyte number and percentage correlates with the white cell count described above (Table 1 and Figure 5). Neutrophil percentages rose during peak parasitaemia (day 3) and fell rapidly by day 6, then rose again from day 6. The percentage of monocytes rose slightly by days 4-5, then remained relatively low after recovery (Figure 5).

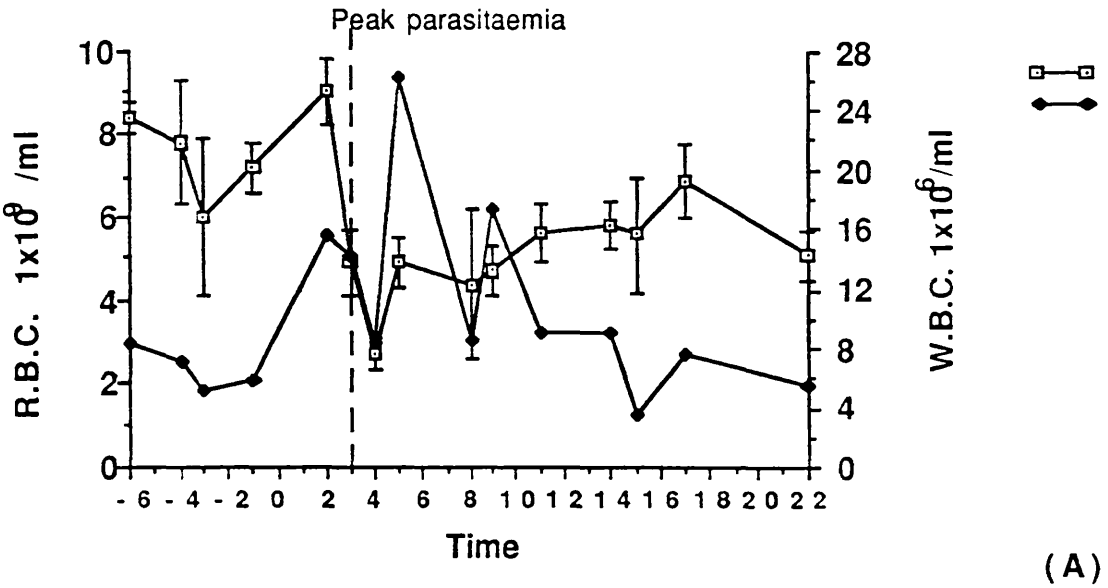
Experiment 3 (vi) and 3 (vii)

Virulence of the parasite and the use of the babesiacidal drug "Diampron"

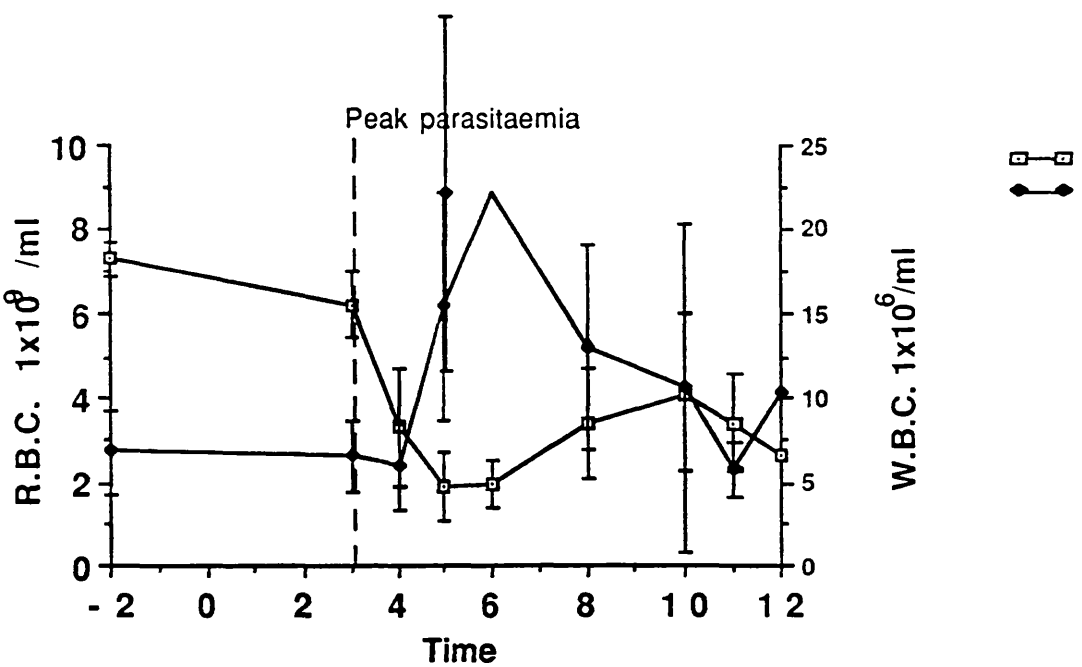
It was shown at the beginning of this chapter that the i.v. injection of 1.5×10^8 PRBC into splenectomised rats resulted in an infection from which most rats recover. The parasite, however, became more virulent with increasing passages and the injection of 1.5×10^8 PRBC resulted in a fatal disease. Although the inoculation of a decreasing number of the virulent parasites

Figure 4A & 4B:

RBC and total WBC counts in rats infected with *B. divergens.*



(A)



(B)

Figure 5:

% Differential Wbc Counts In Individual Rats

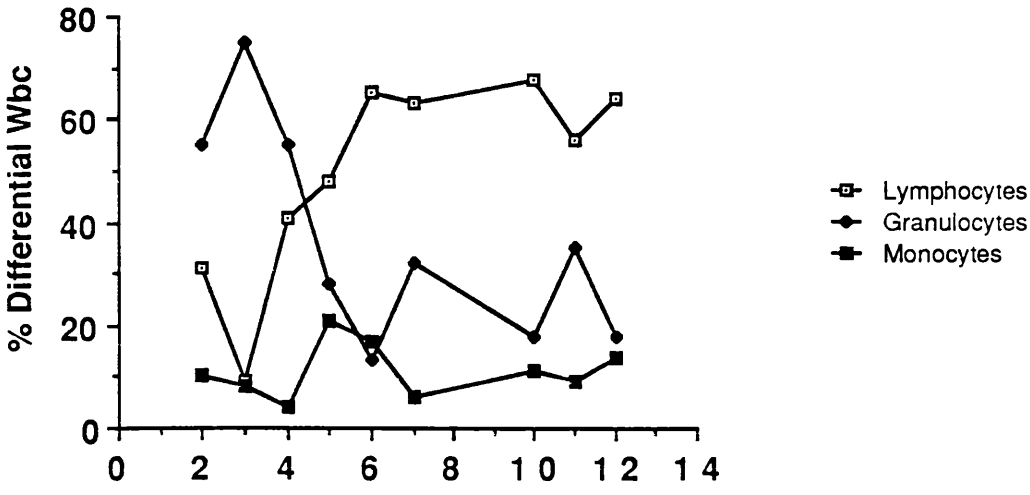


Figure A

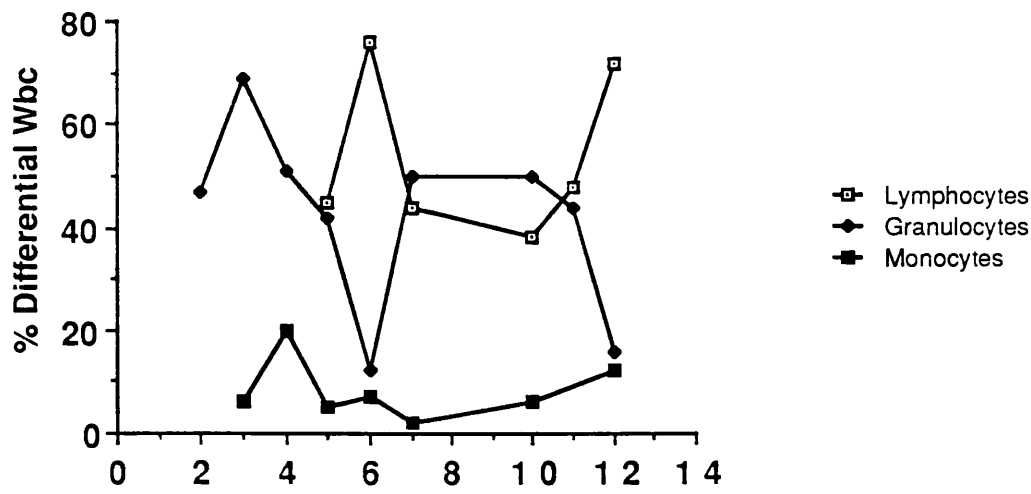


Figure B

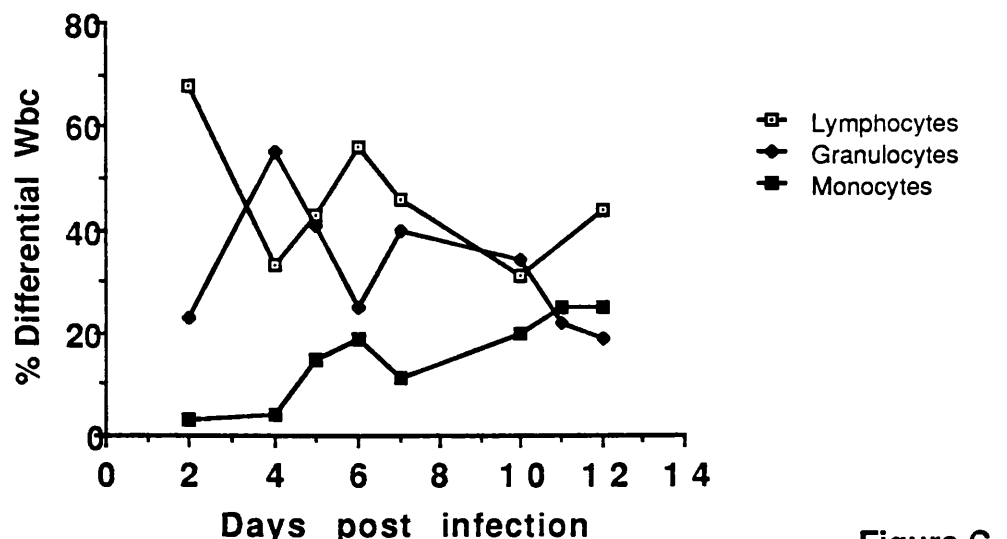


Figure C

TABLE 1

Total W.B.C Counts and absolute lymphocyte count in splenectomised rats infected with *B. divergens.*

Days of infection	White cell counts (x 10^6 /ml)	Lymphocyte counts (x 10^6 /ml)
- 2	6.8 x 10^6	—
3	6.5 x 10^6 (2.1)	2.1 x 10^6
4	5.9 x 10^6 (2.6)	2.4 x 10^6
5	14.8 x 10^6 (6.8)	7.1 x 10^6
6	22.1 x 10^6 (10.5)	14 x 10^6
8	13 x 10^6 (6.1)	—
10	10.6 x 10^6 (9.8)	3.2 x 10^6
11	5.6 x 10^6 (1.6)	1.5 x 10^6
Control	8.2 x 10^6	2.7 x 10^6

resulted in increased prepatent periods, the disease was still severe and most rats died from the infection. It was necessary, therefore, in some experiments to control the parasites with the babesiacidal drug amicarbalide (Diampron). In the following two experiments, a comparison was made between the effect of the injection of 1×10^4 of infected blood derived from an avirulent stabilate and the effect of inoculation of 1×10^4 virulent parasites and the use of the drug amicarbalide for the treatment of some infected rats:-

Experiment 3 (vi):

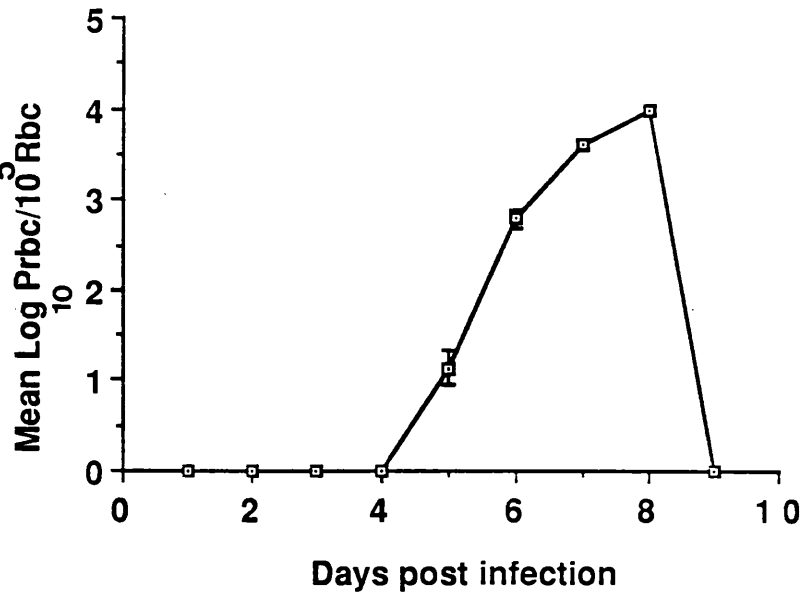
The use of avirulent stabilates:

Eight (four males and four females) five month old splenectomised rats were injected with avirulent parasites as follows:-

An avirulent cryopreserved infected blood (WEP 37) was passaged twice into four week old male splenectomised rats before it was injected into the adult rats. Each rat was injected with 1×10^4 PRBC, (five rats i.v. and three rats i.p.) and the course of infection was followed (Figure 6). No parasites were detected in blood smears taken for four days. Parasitaemia was patent on day 5 and all rats had a mild infection which lasted for four days after which they recovered. No parasites were detected in blood smears taken for 21 days after recovery. This experiment was repeated twice and the same results were obtained.

Figure 6:

The course of infection in 8-5 month old splenectomised rats injected with $1x10^4$ PRBC i.v. (avirulent blood).



Experiment 3 (vii):

The use of virulent infected blood:

The avirulent stabilate used in experiments 3 (i) and 3 (ii) became virulent after approximately 48-50 passages. Fresh infected blood collected from a passaged rat was used to inject adult splenectomised rats as follows:-

Thirteen (six males and seven females) four month old splenectomised rats were injected with 1 x 10^4 PRBC i.v. and the course of parasitaemia was followed (Table 2). In some animals the parasitaemia was subpatent for 8 days although most rats were patent on day 3-4, and five out of 13 rats were treated with 30 mg/kg diampron subcutaneously, some at the onset of haemoglobinuria and some when the parasitaemia reached 18%. Two rats were kept untreated and were used as controls. In the treated rats, recovery was rapid and parasitaemia was subpatent 2-3 days after treatment. The parasitaemia in the untreated rats increased subsequently and they died on day 8 post infection. In the six remaining rats the parasitaemia peaked on day 7 (between 7-21%), but started decreasing on day 8, and they all recovered naturally without treatment. Infected rats were treated on occasions in other experiments as required.

Experiment 3 (viii):

Challenging of normally recovered and drug treated rats:

Rats that recovered naturally from the infection and those treated with diampron, were challenged a month after the primary infection with 1 x 10^8 PRBC i.v. Two control splenectomised rats

Table 2

The injection of virulent blood into splenectomised rats and the use of the drug Diampron.

% Parasitaemia

Drug tested

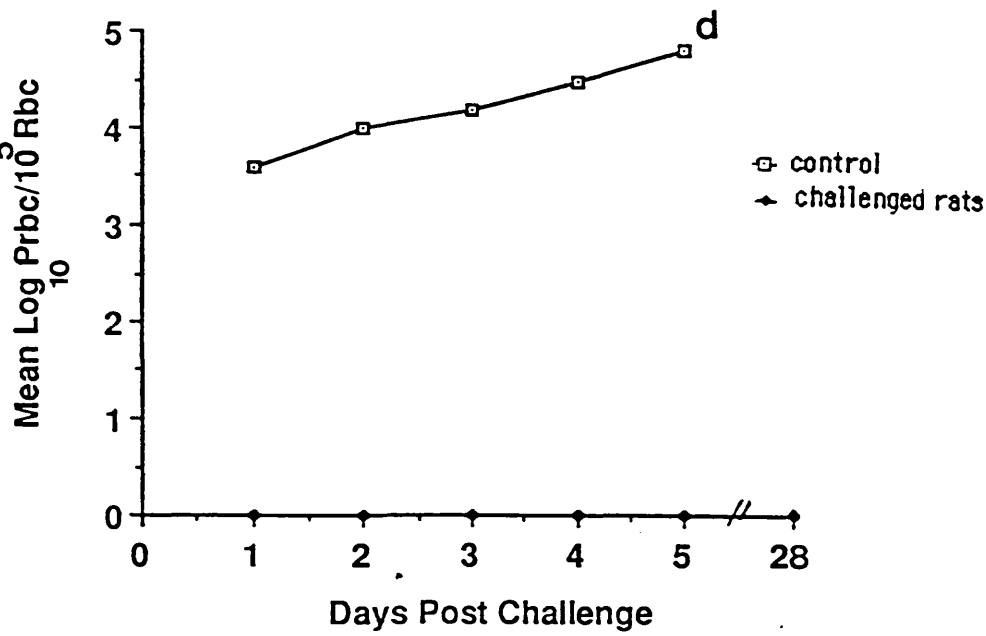
Untreated (control) Rats

Rats naturally recovered

Rat No.→ / DPI ↓	1	2	3	4	5	6	7	8	9	10	11	12	13
1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3	-ve	-ve	-ve	-ve	-ve	-ve	0.04	-ve	-ve	-ve	-ve	-ve	0.03
4	0.04	0.04	0.02	-ve	-ve	0.07	0.09	0.05	0.02	0.05	0.06	0.05	0.09
5	0.2	0.2	0.21	-ve	-ve	0.2	0.5	0.23	0.3	0.3	0.4	0.43	2
6	2.5	3	3	-ve	-ve	4.8	5.9	2.8	3.8	5.7	3.9	5.2	5.2
7	10.8	12.7	7.9	0.05	-ve	21.4	11.2	6.7	11.6	20.9	9.7	19.8	7
8	18.7 +DRUG	24.6 +DRUG	21 +DRUG	0.3	-ve	D	35 D	2.8	0.24	5.2	0.04	1.5	-ve
9	2.2	3.1	4	1.2	0.3	/	/	-ve	-ve	0.2	-ve	0.1	-ve
10	-ve	0.04	0.03	2.8	2.6	/	/	-ve	-ve	-ve	-ve	-ve	-ve
11	-ve	-ve	-ve	11.6 +DRUG	7.8 +DRUG	/	/	-ve	-ve	-ve	-ve	-ve	-ve
12	-ve	-ve	-ve	10.2	0.7	/	/	-ve	-ve	-ve	-ve	-ve	-ve
13	-ve	-ve	-ve	4	0.01	/	/	-ve	-ve	-ve	-ve	-ve	-ve
14	-ve	-ve	-ve	-ve	-ve	/	/	-ve	-ve	-ve	-ve	-ve	-ve

Figure 7:

Challenging of normally recovered and drug treated rats with $1x10^8$ PRBC, 4 weeks after the primary infection.



that were not infected previously were included. All challenged rats (naturally recovered and drug treated) had a detectable parasitaemia five minutes after challenge (parasitaemias not shown on figure). Smears taken five hours after challenge showed that the parasitaemias were declining and no parasites could be detected the day after challenge (Figure 7). Blood smears were taken daily for three weeks afterwards and no parasites were detected. The controls had a patent parasitaemia immediately after they were injected with the parasites. The parasitaemia increased subsequently and peaked on day 7 (50%). Both controls were found dead on day 5 post infection.

Discussion

In this chapter, the course of infection in splenectomised rats, the morphology and the size of the parasite, and the haematological changes that accompany the infection were investigated. The virulence of the parasite, the infection with different doses and some preliminary observations on the effect of using the babesicidal drug "amicarbalide" for the treatment of infected animals, and the development of acquired immunity in animals naturally recovering from the infection and in drug treated animals were investigated. The data showed that the course of infection in splenectomised rats is short lasting (6-8 days) and no recrudesences were observed after the primary patent parasitaemia. The course of infection was similar to that described previously by Phillips (1984) when adapting the parasite to splenectomised rats. Early in the experimental work, the i.v. injection of 1.5×10^8 PRBC into splenectomised rats

produced a patent parasitaemia on day 1 post infection. The parasitaemia rose steadily and peaked between day 3 (16-50%) and the animals either died or recovered from the infection. The pattern of infection in splenectomised rats differed, however, from that described for bovine babesia. Davies and colleagues (1958) described the course of infection in splenectomised calves of what was thought to be B. divergens when studying the British species of babesia. They reported that the inoculation of 1×10^5 PRBC subcutaneously into splenectomised calves resulted in a prepatent period of six days and a patent infection which lasted for 10-12 days. The parasitaemia, however, reappeared in the circulation in some calves after recovery from the primary patent parasitaemia, at an interval of 11-16 days. In this secondary peak of parasitaemia fewer parasites were observed.

Phillips (1984), reported that B. divergens cannot be adapted to intact rats. In the present study, it was observed that the inoculation of B. divergens infected red cells into intact rats resulted in the disappearance of the parasites from the circulation within 7-8 hours (results not shown). Joyner and Davies (1967) working on the same parasite as in the present study but in calves, described the course of infection in splenectomised calves as reported by Davies et al. (1958), but found that the inoculation of B. divergens infected blood into an intact calf resulted in the lack of development of patent parasitaemias. Passage of blood from this animal to other susceptible calves produced, however, an infection six months later and continued to do so for over three years. In the present study, no attempt was made to passage blood from intact

infected rats to other susceptible rats.

B. divergens was also adapted to mongolian gerbils by Lewis and Williams (1979) and the course of infection in the gerbils was described by Liddel et al. (1980) and Lewis et al. (1980b, 1981b). Liddel et al. (1980), found that the i.p. injection of mongolian gerbils with 4.5 x 10^8 PRBC of the human derived "J" strain of B. divergens resulted in a patent parasitaemia two hours post infection, and a fatal infection after continuous passage which resulted in the death of the animals within 48 hours. Lewis et al. (1981b) when titrating the infective dose of B. divergens (derived from gerbil cryopreserved blood) in the mongolian gerbils, found however, that the injection of gerbils with 1 x 10^5 PRBC i.p. resulted in a prepatent period of 1-4 days and an infection which lasted for 6-7 days after which the animals either died or recovered. No recrudesences have been reported for B. divergens in the gerbil. It can be concluded therefore that the pattern of infection depends on the host used, the number of parasites injected, the route of injection and the strain of B. divergens used.

The appearance of B. divergens in blood smears was similar to that described in cattle (Davies et al., 1958) and in gerbils (Liddel et al., 1980). Typically divergent forms, rings, oval forms and maltese cross forms were seen which measured about (3.4 x 0.57um) with occasional pairs located peripherally as in bovine red blood cells. No direct comparison was, however, made between the bovine and the rat propagated B. divergens. Davies et al. (1958) compared the British species of Babesia in cattle

(B. divergens) with the European bovine species (B. bovis) and found that they differ morphologically. The European species differed from the British species in being markedly larger with the absence of divergent forms. On the other hand, Liddel et al. (1980) thought that the gerbil propagated parasites were larger than those found in bovine infected red blood cells and larger non peripheral non divergent forms were observed by these workers in the gerbil propagated parasites.

Several workers have also reported morphological changes in B. divergens in their attempt to adapt the parasite to different laboratory hosts. Canning et al. (1976) reported morphological changes in transient B. divergens parasitaemia in rats and hamsters from typically darkly staining forms to vacuolated forms which were less densely stained. Similar findings have been reported by Adam and Blewett (1974) and Irvin et al. (1978), in their attempts to adapt B. divergens to mice. The morphology of the parasite was also found to differ in different strains of B. divergens. Lewis et al. (1980b) made a morphometric comparison between B. divergens parasites in blood smears infected either with a human or bovine strain and found more amoeboid forms in smears taken from calves infected with a strain of bovine origin. The parasites of human origin were found to be also significantly larger than the bovine originated strains. In the present study, multiple parasitisation of the red cells was very common and up to four parasites were seen in a single red cell. Liddel et al. (1980) reported that multiple parasitisation of red blood cells was a feature of human and gerbil red cells infected with the "J" strain of B. divergens, and up to six parasites in the human

cells and nine or more have been observed by these workers in the gerbil erythrocytes. Such high numbers of parasites in one cell were never noted in the rat erythrocytes. With high parasitaemias a large number of free parasites were always observed. These free parasites were apparently released from the red cells after destroying them, to invade other red blood cells.

The acute parasitaemia was accompanied by severe anaemia evident as a drop in RBC counts. RBC counts started rising again immediately after recovery. It should be noted, that only preliminary observations were made on the anaemia that accompanies the infection, but its cause was not investigated. It is expected, however, that some anaemia must have been caused by the vascular destruction of RBC as the parasites leave the red cells to invade other cells, as was reported for most acute cases of babesiosis (Koch, 1968; Wright, 1973; Callow and Pepper, 1974). Anaemia has been reported during most babesia infections. It was reported for B. canis in the dog (MaeGraith et al., 1957), for B. bovis and B. bigemina in cattle (Wright, 1973), for B. divergens in cattle (Davies et al., 1958; Purnell et al., 1977b), and in the gerbil (Liddel et al., 1980; Lewis et al., 1981b). The cause of this anaemia may be due to a number of mechanisms. It might be due to the intravascular destruction of erythrocytes by escaping babesia as described above, or due to immunological factors. These factors include phagocytosis of both normal and PRBC either in the peripheral blood or in the RES (Hildebrandt, 1981), autohaemagglutinins or opsonins which are present in the serum of babesia infected animals and can react

with infected and even normal erythrocytes and cause anaemia as was reported for B. rodhaini (Shroeder et al., 1966; 1968) and P. berghei (Zuckermann, 1977) infections in the rat. Hussein (1976) using radioactive labelling techniques found that both B. hylomysci infected and uninfected RBC were destroyed in infections in mice and related this to autoimmune reactions.

T-lymphocytes were also reported to cause the activation of macrophages which resulted in erythrophagocytosis in rodent malaria and cause anaemia. Roberts and Weidanz (1978) compared anaemia and splenomegaly in athymic "nude" mice, B-cell deficient mice, and immunologically intact mice, infected with P. berghei. They found enhanced phagocytosis and anaemia in B-cell deficient mice and immunologically intact mice. On the other hand infected athymic "nude" mice had minimal splenomegaly and failed to develop anaemia. They concluded that phagocytosis, anaemia, and splenomegaly are thymus dependent in P. berghei infection and related this to T-lymphocytes which caused activation of macrophages which subsequently resulted in erythrophagocytosis and finally anaemia.

Antibodies to soluble antigens on the surface of infected and normal erythrocytes were also found to be effector mechanisms for the removal of erythrocytes in some Babesia and malaria infections. Sibinovic (1966), Sibinovic et al. (1967; 1969), reported that anaemia in B. canis and B. rodhaini infections was mediated by an antigen (antigen B) which appeared in the serum during acute parasitaemia. They found that this antigen became attached to erythrocytes within a few minutes of intravenous injection, after which most of the erythrocytes to which the

antigen was bound were removed rapidly from the peripheral circulation, by a process which involves binding of antibodies to the antigens and triggering complement or cell dependent effector mechanisms. Similar findings have been reported for some malaria infections (reviewed by Smith et al., 1972) which suggest the protective role of soluble plasma antigens in infections caused by Babesia and Plasmodium species. Finally, immune complexes were found to be important in the development of anaemia in some Babesia and malaria infections. Immune complexes were demonstrated in rats infected with B. rodhaini (Annable and Ward, 1974) in P. falciparum (Wilson et al., 1975) and in P. berghei infections in mice (June et al., 1979). These immune complexes were found bound to normal and infected erythrocytes and caused anaemia.

Recovery from the infection was immediately followed by leukocytosis which was predominately a lymphocytosis. It was not known whether lymphocytosis was due to newly formed lymphocytes entering the blood or due to altered patterns of migration to the different lymphoid organs. The first possibility that lymphocytosis could have been due to the rapid production of new lymphocytes was not investigated but the possibility that lymphocytes might be accumulating in the liver was investigated and will be described and discussed in Chapter Six. Leukocytosis has also been reported for B. canis infections (Sanders, 1937; Siebold and Bailey, 1957; Alperin and Bevins, 1963), B. bovis and B. bigemina infections in cattle (Wright, 1973), and B. divergens in splenectomised calves (Purnell et al., 1977b).

Liddel et al. (1980) reported, however, that leukocytosis in gerbils infected with B. divergens was absent or minimal. The leukocyte picture, during Babesia infections varied from one author to the other, for example Rodriguez and Rivas (1971) described leukopenia with B. bigemina infections and leukocytosis with B. bovis. Wright (1973) on the other hand noticed that leukopenia occurred at the beginning of both B. bovis and B. bigemina infections, and was followed by terminal leukocytosis after the acute haemolytic phase had passed. There were also authors who considered the leukocyte picture during B. bovis and B. bigemina infections is within the normal range (Sergent et al., 1945; Hugoson, 1969).

In the present study, the percentage of absolute numbers of neutrophils rose during peak parasitaemia (day 3-4) and fell rapidly at recovery (day 5-6), and then it was followed by lymphocytosis. The percentage of absolute number of monocytes remained low during the course of the infection and after recovery. The differential count in various Babesia species also varied. Reusse (1954) for example, reported lymphopenia in B. canis infection, while Alperin and Bevins (1963) reported lymphocytosis. In B. bovis and B. bigemina infections, a decline in the percentage of the absolute number of neutrophils followed by lymphocytes was described by Wright (1973). Similar findings have been reported by Purnell et al. (1977b) for B. divergens in splenectomised calves. Lymphocytosis has also been reported for other protozoal infections such as P. yoelii in mice (Playfair and De Sousa, 1982), Trypanosoma vivax in cattle (Esievo and Saror, 1983) and P. chabaudi in mice (Kumararatne et al., 1987).

B. divergens in splenectomised rats become virulent with increasing passages and the i.v. injection of 1.5×10^8 PRBC resulted in a fatal infection and death of the animals, although previously the injection of 1.5×10^8 PRBC of the avirulent parasites resulted in an infection from which most rats recovered. The inoculation of a decreasing number of virulent parasites, resulted in an increased prepatent period but with a fatal infection even when 1×10^4 PRBC were injected. Similar findings have been reported for B. rodhaini in mice (Overdulve and Antonisse, 1970), for B. bovis in calves (Mahoney et al., 1973b) for B. divergens in cattle (Purnell et al., 1977a) and in gerbils (Lewis et al., 1981b). Purnell et al. (1977a) titrated B. divergens in splenectomised calves (10^3 - 10^9 parasites) and found that B. divergens retains its pathogenic identity regardless of the number of parasites inoculated. Lewis et al. (1981b) reported, however, that the same parasite produced a less severe disease when 1×10^4 infected erythrocytes were inoculated. The present data with the virulent B. divergens is therefore in agreement with that described for splenectomised calves (Purnell et al., 1977a) and differs from that described for gerbils (Lewis et al., 1981b). The host parasite relationship apparently differs between different hosts. Splenectomised rats that were infected with 1×10^4 PRBC, but with blood derived from an avirulent cryopreserved stabilate, normally produced an infection with a prepatent period of 3-4 days and a less severe disease which was followed by recovery.

Rats that received 1×10^4 parasitised fresh red blood cells

and were treated with the drug diampron when they showed haemoglobinuria (at parasitaemias of 8-10%) or higher parasitaemias (18-20%), recovered 2-3 days after treatment. Only a single injection at a dose rate of 30 mg/kg of the drug was injected subcutaneously and the data showed that a single injection was enough to cure the animals. Challenging these animals with a large number of parasites (1×10^8) i.v. one month after treatment showed that they were strongly immune to reinfection. The duration of immunity after drug treatment was not, however, investigated. Diampron was also used for the treatment of rats infected with B. rodhaini (Phillips, 1968) and for the treatment of gerbils infected with the same parasite as in the present study (Gray, 1983). Rats treated against B. rodhaini and gerbils treated against B. divergens were also found to be immune to challenge. Rats naturally recovering from a B. divergens infection were strongly resistant to reinfection when challenged with a large number of parasites i.v. one month after recovery. The duration of immunity was not followed but preliminary observations have indicated that recovered rats that were challenged two months after recovery were resistant to reinfection. Phillips (1984) reported that resistance to reinfection in splenectomised rats lasts for at least three months. Previously Davies et al. (1958) and Joyner and Davies (1967) reported that challenging cattle that have naturally recovered from B. divergens infection showed no evidence of reinfection after periods varying from 3-7 years and suggested that sterile immunity may play a part in the resistance of cattle to reinfection. Sterile immunity was also reported for other

Babesia infections after drug treatment. It was reported for B. microti in mice (Cox and Young, 1969) and for B. bigemina in cattle (Callow, 1967; Lohr, 1972).

The persistence and development of acquired immunity was apparently not dependent on the presence of the spleen and this was evident in challenging experiments. The spleen, however, appears to be less important in controlling B. divergens infection in gerbils (Lewis and Williams, 1979). Splenectomised gerbils were no more susceptible to infection. The role of the spleen in acquired immunity will, however, be discussed in a later chapter.

CHAPTER FOUR

THE PROTECTIVE ACTIVITY OF IMMUNE SERA AND THE ANTIBODY LEVELS AS MEASURED BY THE INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

Chapter Four

The protective activity of immune sera and the antibody levels as measured by the indirect fluorescent antibody test (IFAT)

Introduction

Nocard and Motas (1902), were the first to report the passive protective activity of immune serum against babesias. They demonstrated that the serum of a dog which had recovered from B. canis, had a protective activity. Robertson (1906) and Kikuth and Mudrow (1939) however, were unable to confirm this finding. The protective activity of immune sera in some other Babesia species have been reported by several workers: B. rodhaini in rats (Matson, 1964; Phillips, 1969b) and in mice (Abdalla et al., 1978) and B. argentina (= B.bovis) in splenectomised calves (Mahoney et al; 1979). There are, however, no reports of experiments demonstrating the protective role of antibody in cattle infected with B. divergens although the presence of anti B. divergens antibodies in cattle has been demonstrated by several serological tests (Donnelly et al., 1972; Purnell et al., 1976). Antibodies to B. divergens have also been detected in recovered gerbils (Liddel et al., 1982), in recovered rats and in the offspring of recovered mothers (Phillips, 1984), but a definite role for antibody in protection was not demonstrated. In this chapter the role of antibody in protection was investigated in splenectomised rats infected with B. divergens. The antibody levels were measured in sera collected during the course of infection and at different intervals after recovery, by the indirect fluorescence antibody

test (IFAT). The protective activity of the same sera was investigated using passive transfer experiments and compared with the antibody levels as measured by IFAT.

The indirect fluorescence antibody test (IFAT)

Sera for IFAT and passive transfer studies were raised in two experiments as follows:-

Experiment 4 (i):

Twenty one, four and a half month old female splenectomised rats were injected with 1.5×10^8 PRBC i.v. The rats were divided into three groups of seven rats. The course of infection in one group is shown in Figure 2A (see Chapter 3). Rats that survived the infection from the three groups were pooled and divided into groups of three rats. A group was sacrificed on the following days : day 4 (during the infection), day 7 (immediately after recovery), day 10, 15, 22 and 40 post infection and their sera collected. Normal uninfected rats were also bled and their sera collected. These sera were used as controls. The different immune sera were aliquoted and kept at $-20^{\circ}C$, to be used for passive transfer experiments. Small volumes of the sera were also aliquoted in eppendorfs and were used for IFAT. Three to four different sera with a control (normal serum) were tested each time and a 10 fold dilution (from 1/5 to 1/500,000) of immune and normal sera were prepared for the first IFA tests. Two fold dilutions (from 1/5 to 1/10,240) were prepared for the rest of the test sera. All dilutions were made in microtitre plates. A 1/400 dilution of FITC conjugated rabbit antiserum to rat Ig in PBS containing evan's blue was found to be suitable for

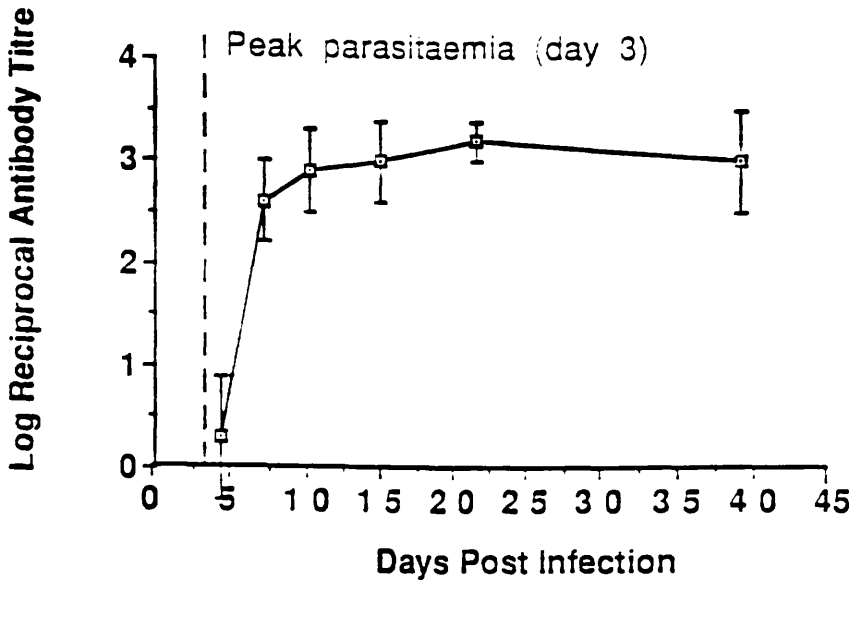
all tests. The mean antibody titre in the sera is shown in Figure 8A. The results showed that no antibodies could be detected at peak parasitaemia (day 4). The antibody titre rose from day 4 and reached a peak on day 22 at which level it remained until day 40. No sera was tested after this time in this experiment.

Experiment 4 (ii)

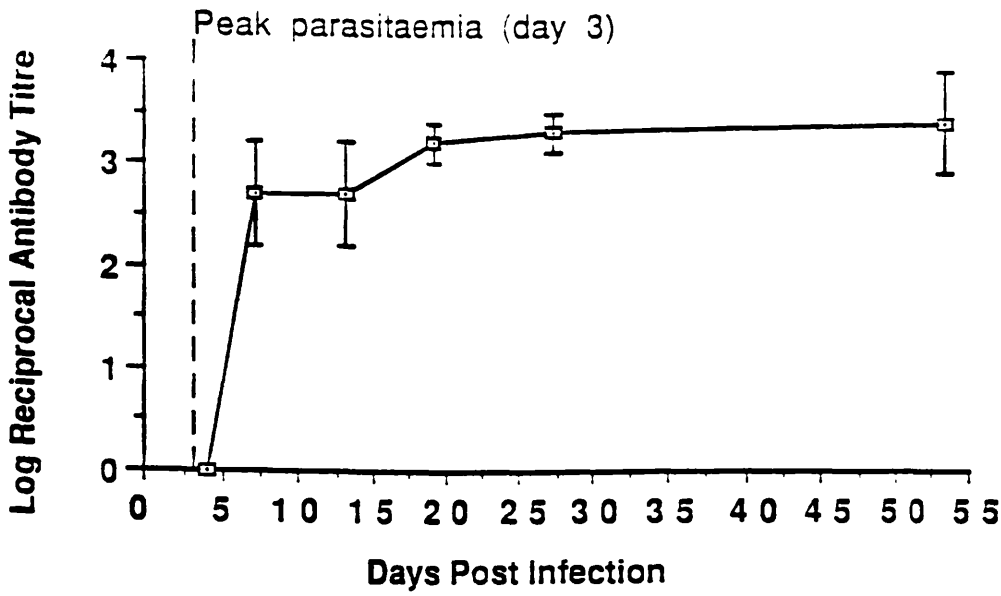
Twenty four, four month old female splenectomised rats were inoculated with 1.5×10^8 PRBC i.v. The rats were divided into one group of ten and two groups of seven rats. The course of parasitaemia was followed in the first group as shown in Figure 2B (Chapter 3). Rats that survived the infection from all groups were pooled and divided into groups of three rats and were sacrificed on the following days: day 4 (during the infection), day 7 (immediately after recovery), day 13, day 19, day 27 and day 53 post infection and their sera collected. The sera were aliquoted and tested for IFAT as in experiment 4 (i) above, except that a two fold dilution (from 1/5 to 1/10,240) was prepared for all the sera tested. The mean antibody titre in the sera is shown in Figure 8B. The results showed that no antibodies were detected at peak parasitaemia (day 4) as was shown in experiment 4 (i) (see above). The antibody titre rose from day 5 and plateaued between day 7 and day 13, then rose again and reached a peak on day 19 at which level it remained for seven weeks (until day 53). No sera were collected or tested after this period.

Figure 8A & B:

The antibody titre as measured by IFAT in splenectomised rats infected with *B. divergens*.



(A)



(B)

Passive transfer experiments

Sera collected in Experiment 4 (i)

Experiment 4 (iii)

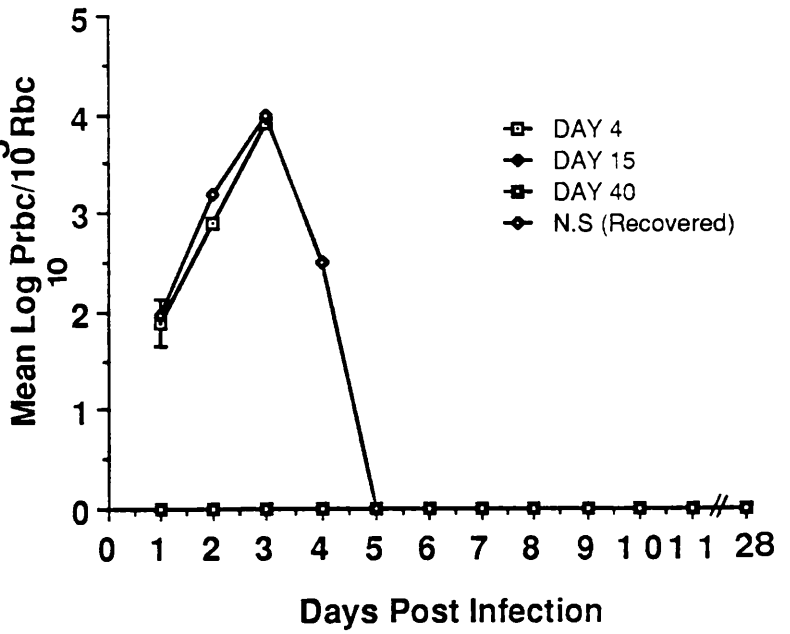
The protective activity of day 4, 15 and 40 sera: Preliminary experiment

In this preliminary experiment, the protective activity of day 4, 15 and 40 sera was investigated. A volume of 2 ml of serum was tested in splenectomised infected rats as follows:-

Twelve (eight females and four males) 7-8 week old splenectomised rats were infected with 1 x 10^7 PRBC i.v. The rats were pooled and then groups of 2-3 rats were given 2 ml of day 4, 15 or day 40 immune sera i.v. Controls receiving normal serum or a highly protective serum (positive control = hyperimmune serum) were also included. The parasitaemia in day 4 serum recipients was patent on day 1 and subsequently increased at the same rate as the normal serum recipients (Figure 9). All day 4 serum and normal serum recipients (except one normal serum recipient) died on day 4 post infection. One normal serum recipient recovered. No parasites were detected in blood smears taken on day 5 post infection. In the day 15, 40 and the positive control serum recipients, no parasites were detected in blood smears taken during 28 days of observation.

Figure 9:

The protective activity of sera collected on days 4, 15 and 40 using 2ml of serum.



Experiment 4 (iv)

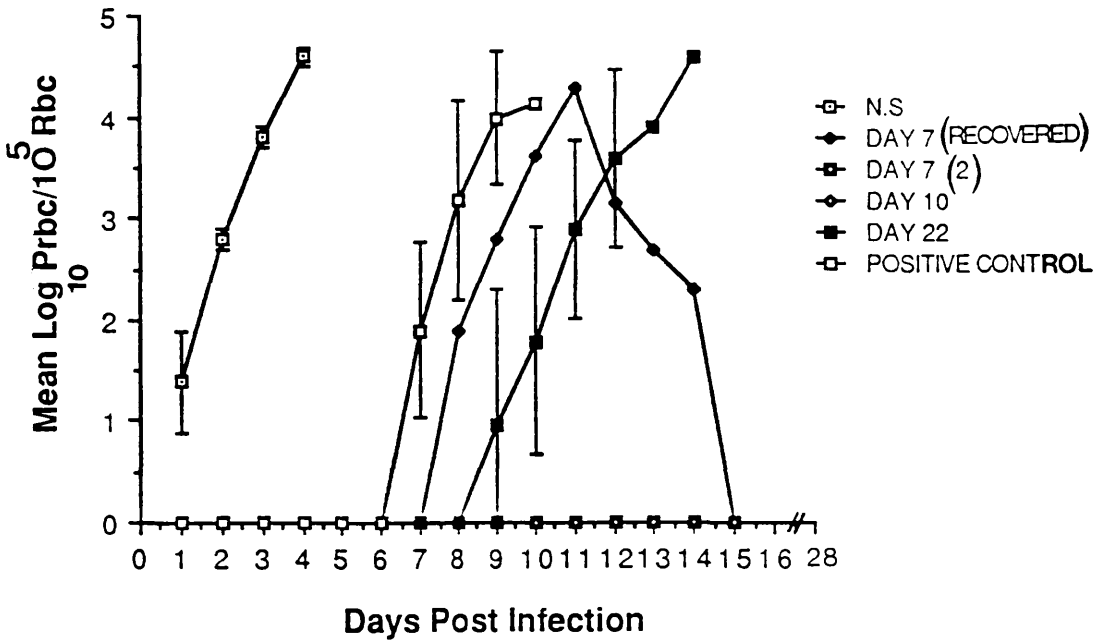
The protective activity of day 7, 10 and 22 sera

In the above experiment, it was shown that 2 ml of serum could completely protect the rats and no difference could be made between the protective activity of immune sera (i.e. day 15 and 40). In this experiment, it was decided to reduce the volume of the serum injected from 2 ml to 0.5 ml to test the protective activity of day 7, 10 and 22 sera.

Ten male 8-9 week old splenectomised rats were injected with 1×10^7 PRBC i.v. The rats were pooled, then groups of two rats were given 0.5 ml of day 7, 10 or day 22 sera i.v. Controls receiving normal serum and a highly protective serum (hyperimmune serum) were also included. In one day 7 recipient and both day 10 serum recipients, no parasites were detected in blood smears taken during 28 days of observation (Figure 10). The other day 7 serum recipient had a subpatent parasitaemia for seven days which became patent on day 8 and peaked on day 11 (21%) after which it decreased. No parasites were detected in a blood smear taken on day 15 post infection. In day 22 serum recipients no parasites were detected in blood smears taken for 8-9 days. Parasitaemia was patent on days 9-10 and subsequently increased, peaking on day 12 in one rat (17%), which died on day 13 post infection, and on day 14 in the other rat (38%), which died on day 15 post infection. In rats which received a highly protective serum, no parasites were detected in blood smears taken for six days. The parasitaemia was patent on day 7 and peaked on day 9 in one rat (16%), which died on day 10 post

Figure 10:

The protective activity of sera collected on days 7, 10 and 22 using 0.5ml of serum.



infection, and on day 10 in the other rat (14%) which died on day 11 post infection. Normal serum recipients had a patent parasitaemia on day 1 which increased subsequently and peaked on day 4 (31-41%). The rats died on day 5 post infection.

Sera collected in Experiment 4 (ii)

Experiment 4 (v)

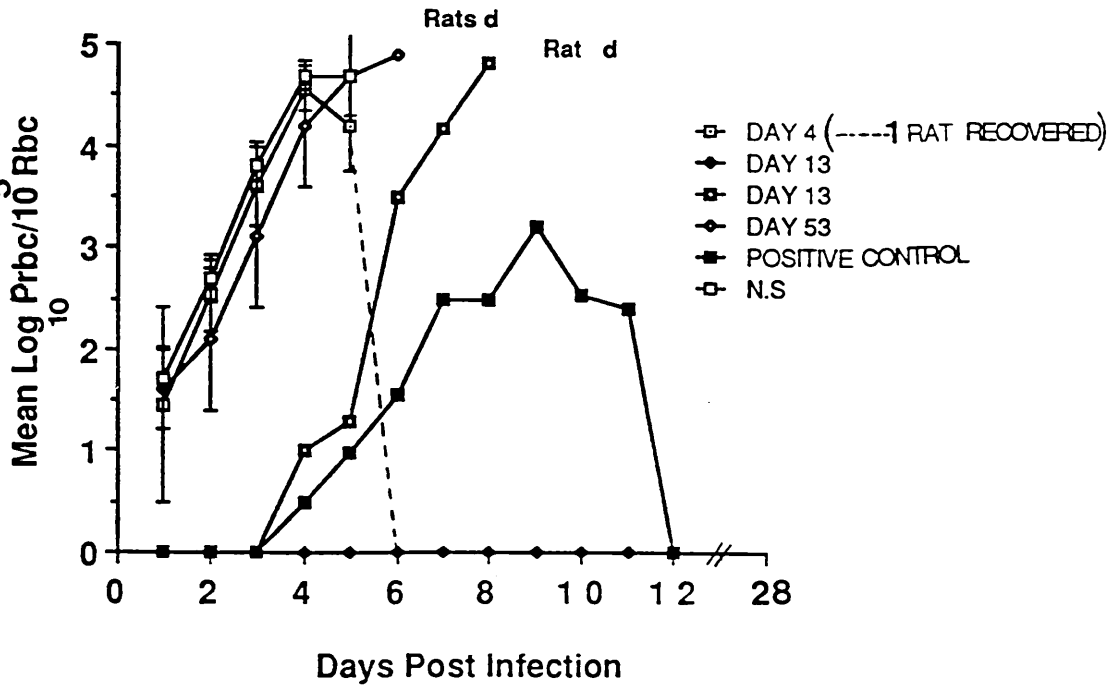
The protective activity of day 4, 13 and 53 sera

In the above experiment, it was shown that 0.5ml of serum was completely protective in most rats. In this experiment, it was decided to reduce the volume of serum injected from 0.5 ml to 0.4 ml to test the protective activity of day 4, 13 and 53 sera. It should be noted that both parasites and serum were injected i.p. because i.v. injections were not possible at that time. The protective activity of day 4, 13 and 53 sera using 0.4 ml of sera was tested in infected splenectomised rats as follows:

Twenty female 7-9 week old splenectomised rats were injected with 1×10^7 PRBC i.p. The rats were pooled and then groups of four rats were given 0.4 ml of day 4, 13 or 53 sera i.p. Controls receiving normal serum and a highly protective serum (hyperimmune serum) were included. Three of the four day 4 serum recipients and three of the four day 53 serum recipients had a patent parasitaemia on day 1 (Figure 11). In one day 53 serum recipient no parasites were detected on day 1. Parasitaemia was patent on day 2. Parasitaemia in the three day 4 serum recipients and the four day 53 serum recipients increased subsequently at the same rate as the normal serum recipients and

Figure 11:

The protective activity of sera collected on days 4, 13, and 53 using 0.4 ml of serum.



peaked between days 4-6 to reach 33-65%. Both day 4 and day 53 serum recipients died between days 5-7 post infection as did the normal serum recipients. In one day 4 serum recipient no parasites were detected in blood smears taken on day 1. The parasitaemia was patent on day 2 and the rat had a mild infection which lasted for four days after which it recovered. In three of the four day 13 serum recipients, no parasites were detected in blood smears taken over 28 days of observation. In the remaining day 13 serum recipient, no parasites were detected in blood smears taken for three days. The parasitaemia was patent on day 4 post infection and subsequently increased peaking on day 8 at 65%, and the rat died. No parasites were detected for 3-6 days in three of the four rats which received a highly protective serum (hyperimmune serum). The parasitaemia was patent on days 4-7 and subsequently increased. Two of the three rats died on days 8 and 12 respectively, and one rat recovered. No parasites were detected in blood smears taken from this rat on day 12 post infection. The fourth rat in this group had a patent parasitaemia on day 6 which increased subsequently and reached a peak on day 9 post infection, and the rat died on day 10 post infection.

Experiment 4 (vi)

The protective activity of day 7, 13, 19 and 27 sera

In this experiment, the protective activity of day 7, 19 and 27 sera was tested using the same volume of sera as in the above experiment (i.e. 0.4 ml). It was shown in the previous experiment that day 13 serum was highly protective. Three of the

four day 13 serum recipients did not show any parasites in blood smears taken for 28 days. One day 13 serum recipient had, however, a patent parasitaemia on day 3 and died on day 8 post infection. In this experiment it was decided to test the protective activity of day 13 serum again using the same volume as above to confirm the protective activity of this serum. Both parasites and serum were again injected i.p. since i.v. injections were not possible at that time:

Twenty female 4-6 week old splenectomised rats were injected with 1×10^7 PRBC i.p. The rats were pooled, then groups of four rats were given 0.4 ml of day 7, 13, 19 or 27 sera i.p. A group of rats receiving normal serum was included as a control. Two of the day 7 serum recipients had a patent parasitaemia on day 1 and 3 respectively (Table 3). One of the recipients had a mild infection which lasted for four days and then it recovered. In the other recipient, the parasitaemia increased subsequently and peaked on day 4 at 50% and the rat died on day 5 post infection. The other two day 7 serum recipients had no detectable parasitaemia in blood smears taken for 28 days. One of the day 13 serum recipients had a patent parasitaemia on day 1 which increased subsequently and peaked on day 4 at 58% and the rat died on day 5 post infection. In the other three day 13 serum recipients, no parasites were detected in blood smears taken for 28 days. In two of the day 19 serum recipients, no parasites were detected in blood smears taken for three days in one recipient and for nine days in the other recipient. Parasitaemia was patent on day 4 in one recipient and on day 10 in the other

Table 3

The protective activity of day 7, 13, 19 and 27 after I.P. injection of both parasites and serum.

% Parasitaemia	Day 7				Day 13				Day 19				Day 27				Control (NS)			
Rat No.→ / DPI ↓	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	-ve	-ve	0.04	-ve	-ve	-ve	-ve	0.22	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	0.05	0.12	-ve	0.12
2	-ve	-ve	0.73	-ve	-ve	-ve	-ve	0.52	-ve	-ve	-ve	-ve	-ve	-ve	0.72	-ve	0.24	0.72	0.6	3
3	-ve	0.2	8.7	-ve	-ve	-ve	-ve	11.9	-ve	-ve	-ve	-ve	-ve	-ve	12.7	-ve	4.6	9.1	8.4	15.5
4	-ve	0.7	50.4	-ve	-ve	-ve	-ve	57.5	-ve	0.3	-ve	-ve	-ve	-ve	65.6	-ve	26.2	38.8	75.7	60.2
5	-ve	11.1	D	-ve	-ve	-ve	-ve	D	-ve	1.1	-ve	-ve	-ve	-ve	D	-ve	50.2	61.2	D	D
6	-ve	10.1	/	-ve	-ve	-ve	-ve	/	-ve	14.7	-ve	-ve	-ve	-ve	/	-ve	D	D	/	/
7	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	61.5	-ve	-ve	-ve	-ve	/	-ve	/	/	/	/
8	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	D	-ve	-ve	-ve	-ve	/	-ve	/	/	/	/
9	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	/	/	/
10	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	0.04	-ve	-ve	-ve	/	-ve	/	/	/	/
11	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	0.7	-ve	-ve	-ve	/	-ve	/	/	/	/
12	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	8.6	-ve	0.01	-ve	/	-ve	/	/	/	/
13	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	41.7	-ve	0.1	-ve	/	-ve	/	/	/	/
14	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	D	-ve	2.3	-ve	/	-ve	/	/	/	/
15	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	/	-ve	8.6	-ve	/	-ve	/	/	/	/
16	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	/	-ve	61.7	-ve	/	-ve	/	/	/	/
17	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	/	-ve	D	-ve	/	-ve	/	/	/	/
18-28	-ve	-ve	/	-ve	-ve	-ve	-ve	/	-ve	/	/	-ve	/	-ve	/	-ve	/	/	/	/

recipient which increased subsequently to reach 62% and 42% on days 7 and 13 respectively. These both died, on days 8 and 14 respectively. In the other two day 19 serum recipients, no parasites were detected in blood smears taken over 28 days of observation. Three of the day 27 serum recipients had a patent parasitaemia on days 2, 11 and 12 respectively which increased subsequently and peaked at 66, 36 and 62% on days 4, 13 and 16. All three of these recipients died, on days 5, 14 and 17 respectively. In the fourth recipient in this group, no parasites were detected in blood smears taken over 28 days of observation. All normal serum recipients (controls) had a patent parasitaemia on day 1 which increased subsequently and they all died between days 5 and 6 post infection.

Experiment 4 (vi)

The protective activity of day 7, 13, 19 and 27 sera

In experiments (v) and (vi), it was shown that there was variation in the results and the protective activity of some sera (for example day 7 and day 13) was not confirmed. This variation was due to the fact that both parasites and serum were injected i.p. The disadvantage of i.p. injections is that it is possible that not all the parasites or the serum injected enter the blood cirulation. Some might for example enter the gut. It was decided, therefore, to repeat the experiment. In this experiment, both parasites and serum were injected i.v. and the protective activity of day 7, 13, 19 and 27 serum was tested in splenectomised rats as follows:

Twenty male five week old splenectomised rats were injected

with 1 x 10^7 PRBC i.v. Of these three groups of four rats were given 0.4ml of day 7, 13, 19 or 27 i.v. A group of rats which received normal serum was included as a control. In all day 7 and day 13 serum recipients, no parasites were detected in blood smears taken during 28 days of observation (Figure 12). In all day 19 and day 27 serum recipients no parasites were detected in blood smears taken for 6 days and 7 days respectively. The parasitaemia in groups was patent on days 7 and 8 respectively, which increased subsequently and peaked between days 10 and 13 post infection (26-86%). All rats in both groups died between days 11 and 13 post infection. All normal serum recipients had a patent parasitaemia on day 1 which increased subsequently and peaked on day 3 (19-40%). All rats in this group died on day 4 post infection.

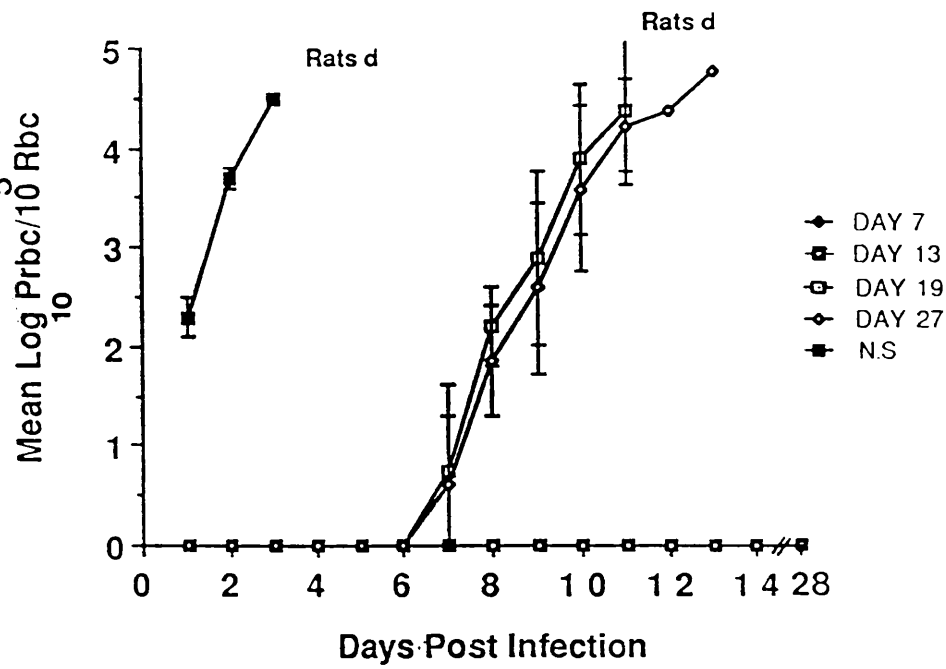
Experiment 4 (vii)

The protective activity of day 7, 13, 27 and 53 sera

In the previous experiment, it was shown that both day 7 and day 13 sera were strongly protective when 0.4 ml of either sera were used, and no difference could be found between the protective activity of the two sera. It was decided, therefore, to reduce the volume of serum injected from 0.4 ml to 0.2 ml, to test which of the sera (day 7 or day 13) is more protective, and to compare the protective activity of these sera with the protective activity of day 27 (which was partially protective when 0.4 ml of serum was used) and day 53 serum (which was not protective when 0.4 ml of serum was used) using 0.2 ml of serum.

Figure 12:

The protective activity of sera collected on days 7, 13, 19 and 27 using 0.4ml of serum.

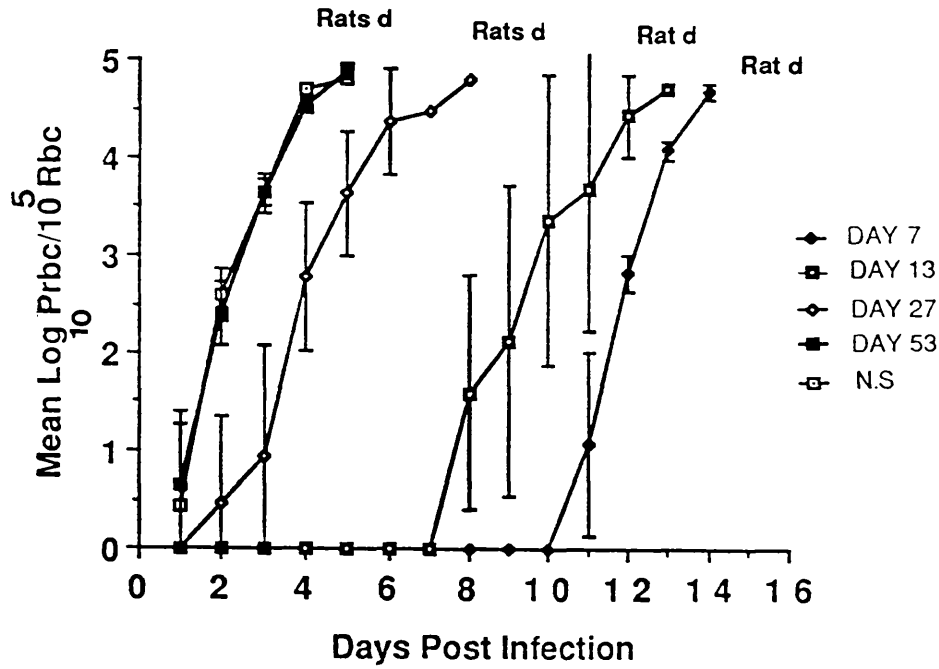


The protective activity of day 7, 13, 27 and 53 sera was tested in splenectomised infected rats as follows:

Twenty female, 5-6 week old splenectomised rats were injected with 1 x 10^7 PRBC i.v. The rats were pooled and then groups of four rats were given 0.2 ml of day 7, 13, 27 or 53 sera i.v. A group of rats which received the same volume of normal serum was included as a control. Three of the day 7 serum recipients had a prepatent period of 10-11 days. Parasitaemia was patent on days 11-12 which increased subsequently and peaked on day 14 (39-56%) (Figure 13). All three recipients died on day 15 post infection. The fourth recipient in this group had no detectable parasites in blood smears taken for 28 days. Day 13 serum recipients had a prepatent period of 7-9 days. Parasitaemia was patent on days 8-10 which increased subsequently and peaked between days 10-13 (41-65%). All recipients in this group died between day 11 and 14 post infection. Day 27 serum recipients had a prepatent parasitaemia of 1-3 days. Parasitaemia was patent on days 2-3 which increased subsequently and peaked on days 6-8 and the rats died between days 7 and 8 post infection. Two of the day 53 serum recipients and three of the normal serum recipients had a prepatent period for one day. The other two day 53 serum recipients and one normal serum recipient had a patent parasitaemia on day 1 which increased subsequently. All rats in both groups died on days 5 and 6 post infection.

Figure 13:

The protective activity of sera collected on days 7, 13, 27 and 53 using 0.2 ml of serum.



Experiment 4 (viii)

Challenging of serum recipients:

In order to test whether immune serum was still protective four weeks after serum administration, serum recipients were challenged on day 28 with 1 x 10^8 PRBC i.v. A splenectomised rat that received no serum at all was included as a control. Figure 14 shows the results of one challenge experiment (Experiment 4 (iii) - day 15 and 40 serum recipients). Day 15 and 40 serum recipients had a patent parasitaemia on day 1 which increased subsequently at the same rate as the control rat and reached a peak on day 3 (44-56%). All challenged serum recipients died on day 4 post challenge as did the control rat.

A comparison between protection in passive transfer experiments and the antibody titre as measured by IFAT

Passive transfer experiments showed that the levels of serum protection rose rapidly from day 6 to reach a peak between day 7 and 13 and thereafter the levels declined rapidly. On the other hand the antibody titre as measured by IFAT (see experiments 4 (i) and 4 (ii)) rose from day 5 and reached a peak on day 19 at which level it remained for at least seven weeks. It was not possible therefore to correlate protection with antibody levels as measured by IFAT. Figure 15 shows a comparison between serum protection in passive transfer and the antibody titre as measured by IFAT.

Figure 14:

Challenging of serum recipients with $1x10^8$ PRBC, 4 weeks after serum administration.

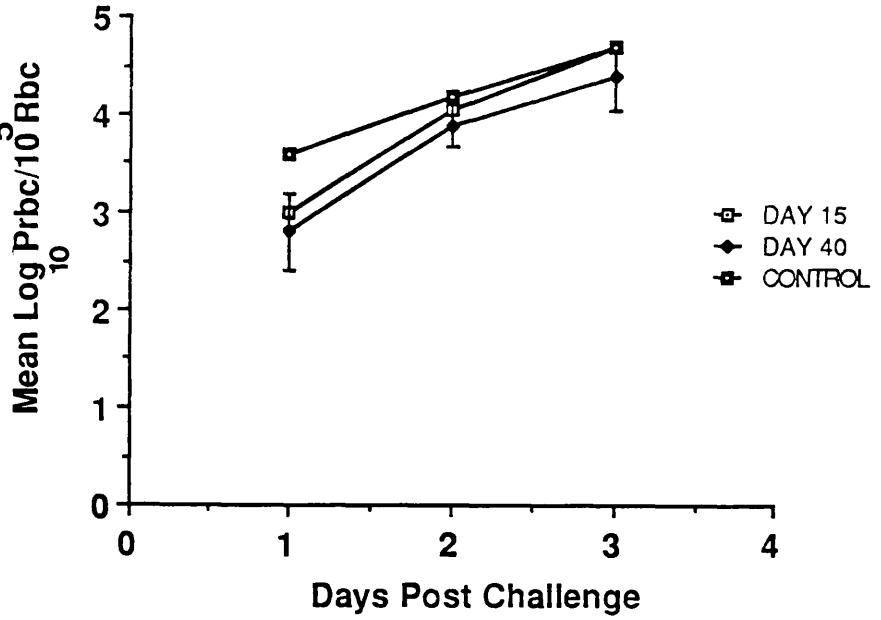
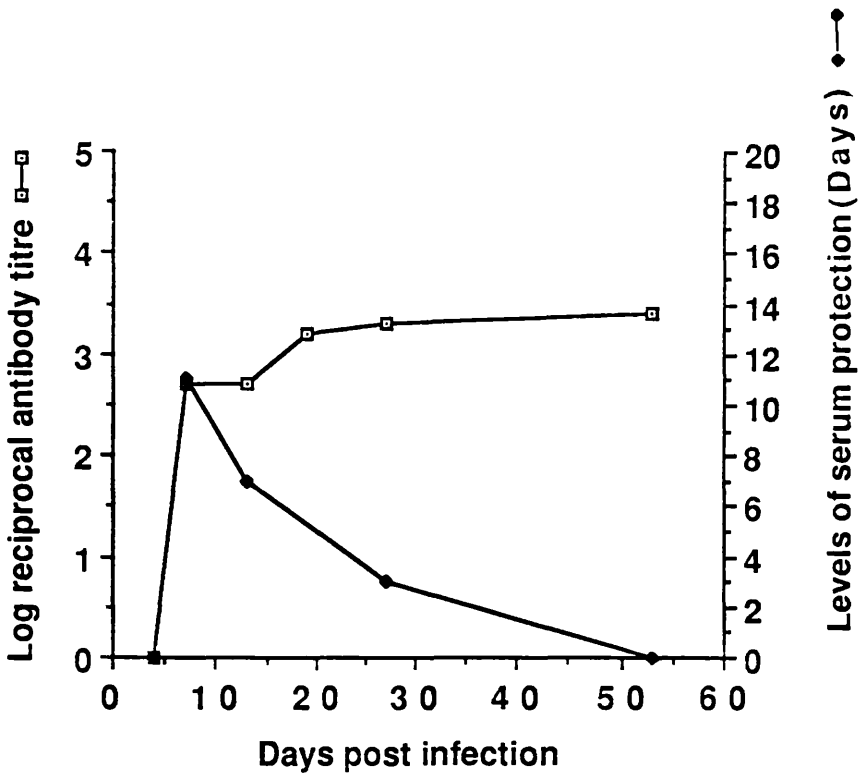


Figure 15:

A comparison of protective in passive transfer experiments and the antibody titre as measured by IFAT.



Discussion

In this chapter the possible participation of humoral factors in acquired immunity to B. divergens was investigated in splenectomised rats, using passive transfer experiments. Antibodies to B. divergens were first detected by the indirect fluorescence antibody test (IFAT). This test has also been used for the detection of antibodies to B. divergens in rhesus monkeys (Garnham and Voller, 1965), cattle (Donnelly et al., 1972; Blewett and Adam, 1978), deer (Adam et al., 1976), gerbils (Liddel et al., 1982) and in rats (Phillips, 1984). The antibody titre as measured by this test rose rapidly after peak parasitaemia (day 4), and then continued to rise while parasitaemia fell from day 5. This rise continued until the parasites had disappeared from the blood, and reached a peak between days 19 and 22 at which level it remained for seven weeks.

The protection in splenectomised rats with immune serum in passive transfer experiments has confirmed the participation of humoral factors in acquired immunity. In general the protective activity of the serum rose from days 5-6 (at recovery) and reached a peak between day 7 and day 13 after which it declined rapidly. It can be concluded, therefore, that it is not possible to correlate protection with antibody levels as measured by IFAT, indicating that some antibodies to B. divergens were not protective. Similar findings have also been reported for B. microti in mice (Cox and Turner, 1970a) and previously for some murine malaria such as P. vinckei and P. chabaudi in mice (Cox et al., 1969). Depending on the volume of the serum

injected, immune serum was either responsible for the death of all parasites which was reflected in the lack of patent parasitaemia, the temporary inhibition of the parasite, or the death of some parasites which was reflected in an increased prepatent period. Another possibility is that the antiserum might have had a cytostatic action on the parasite so that their multiplication rate is reduced to a very low level that could not be detected in blood smears. In a preliminary experiment, a volume of 2 ml of immune serum was used to compare the protective activity of day 4, 15 and 40 sera. Day 4 serum was not protective. The results were expected since the IFA test using this serum did not show any detectable antibody titres, although the antibody titres as measured by IFAT cannot be correlated with protection as was mentioned before. All day 4 serum recipients had a patent parasitaemia on day 1 and a severe infection which ended with their death at the same time as the controls. This serum was also non-protective when the volume of the serum was reduced as will be mentioned later in the text. On the other hand, day 15 and day 40 sera were strongly protective. The data have shown that this volume (i.e. 2 ml) was apparently responsible for the rapid elimination of all parasites in day 15 and day 40 serum recipients.

Since a volume of 2 ml of serum completely protected the recipients, and no difference could be found between the protective activity of day 5 and day 40 serum, it was decided therefore, to reduce the volume of serum injected from 2 ml to 0.5 ml, to compare the protective activity of day 7, 10 and 22

sera. Day 7 and day 10 sera were apparently more protective than day 22 serum using this volume. In one day 7 serum recipient and both day 10 serum recipients, no parasites were detected in blood smears taken for 28 days. In the second day 7 serum recipient, no parasites were detected in blood smears taken for seven days; the parasitaemia was however, patent on day 8 and the rat had a mild infection which lasted for seven days after which it recovered. It is possible that the variation in the results in the two day 7 serum recipients might have been due to a fault in the injection of either the parasite or the serum in the second day 7 recipient, so that a smaller number of the parasites or the serum than the original inoculum were actually injected, since the same serum proved to be strongly protective when a reduced volume of serum was injected as will be mentioned later. Day 22 serum was partially protective. There was a delay in the onset of parasitaemia for 8-9 days. Parasitaemia was patent on days 10-11 which was followed by a severe infection and finally the death of all the rats in this group.

Since 0.5 ml of serum completely protected most day 7 and day 10 recipients, it was decided in the following two passive transfer tests to reduce the volume of serum injected from 0.5 ml to 0.4 ml. In both passive transfer tests, both the parasite and the serum were injected i.p. because i.v. injections were not possible at that time. In these two experiments there was the possibility that not all the parasites injected (i.e. 1×10^7) had entered the blood circulation. Some may, for example, have entered the gut for example as a result of an i.p. injection, and therefore a smaller number of the parasites or volume of serum

than the original inoculum (1 x 10^7 PRBC or 0.4 ml of serum) had actually entered the circulation. This was reflected in the variation in the results between the protective activity of the different sera. In one of these tests, the protective activity of day 4, 13 and 53 sera was compared after the i.p. injection of both parasites and serum. Although there was some variation in the results, a comparison between the protective activity of these sera was possible. Day 4 and day 53 sera were apparently not protective. Most recipients in these two groups had a patent parasitaemia on day 1-2 and a severe infection, and they died at the same time as the controls. Day 13 serum was strongly protective. In most recipients no parasites were detected in blood smears taken for 28 days.

In the second passive transfer test where both the parasite and the serum were injected also i.p., the protective activity of day 7, 13, 19 and 27 serum was compared using 0.4 ml of serum. In this test, there was considerable variation between the results. It was decided, therefore, to repeat the test using the same sera, but this time both parasite and serum were injected i.v. The results have shown that day 7 and day 13 sera were strongly protective using this volume of serum (0.4 ml). No parasites were detected in blood smears taken from both serum recipients for 28 days. Day 19 and day 27 sera were partially protective. In all recipients of both groups, there was a delay in the onset of parasitaemia for 6-7 days. Parasitaemia was patent on day 7-8 followed by a severe infection. All recipients in both groups died between days 11-13 post infection.

Since no difference could be made between the protective activity of day 7 and day 13 sera when 0.4 ml of serum was injected into recipients, and they both appeared strongly protective, it was decided to reduce the volume of serum injected from 0.4 ml to 0.2 ml, to test which of the sera is more protective. In the final passive transfer test, the protective activity of day 7, 13, 27 and 53 was compared using 0.2 ml of serum. The results have indicated that day 7 serum was more protective than day 13 serum. There was a delay in the onset of parasitaemia for 11-12 days in day 7 serum recipients, and a delay of 7-9 days in the onset of parasitaemia in day 13 serum recipients. Most rats in both groups had a severe infection and died on day 15 (day 7 recipients) and between days 11-14 (day 13 serum recipients). Day 27 serum appeared to be less protective than day 7 and 13 sera, and more protective than day 53 serum. There was a delay in the onset of parasitaemia for 2-3 days in day 27 serum recipients, and a severe infection followed by their death on days 7 and 8 post infection. The results for day 53 serum were expected. This serum was apparently less protective even when a larger volume of serum was used (2 mls). All serum recipients in this group had a patent parasitaemia between days 1 and 2 as did the controls, and a severe infection, resulting in death on day 5-6 at the same time as the controls.

It can be concluded, therefore, that maximum protective activity was obtained between the serum collected immediately after recovery (day 7), and day 13 serum, and the protective activity of the serum apparently wanes by day 53. In the passive transfer experiments, the administration of a single dose of

immune serum after the injection of the parasite was effective and it either killed all the parasites or delayed their appearance depending on the serum and the volume used. In B. bovis in cattle (Mahoney, 1967a) and B. rodhaini in rats (Phillips, 1969b), it was found that the daily injection of immune serum was more effective than the administration of a single large dose of serum. In the present study, the effect of the daily injection of immune serum was not tested. Mahoney (1967a) suggested that the timing of injection of serum could be important in conferring a high degree of protection as the parasites may be vulnerable to antibody attack when passing from one cell to the other at the multiplication phase. Passive transfer experiments have shown that the injection of immune serum reduced only the infective dose, but the subsequent parasitaemia increased at the same rate as the controls receiving normal serum. Similar findings were also observed with rat and mouse serum against B. rodhaini (Phillips, 1969b; Abdalla et al., 1978). Previously, Roberts and Tracey Patte, 1975b, reported however, that parasites surviving the lethal effect of immune serum in B. rodhaini infected mice, were found to have a reduced multiplication rate.

The data have shown that the transferred serum had no lasting protective activity. This was apparent when the serum recipients were challenged with a large number of parasites four weeks after serum administration. Rats which showed no patent parasitaemia after serum administration, had a patent parasitaemia on day 1 when challenged, and a severe infection

developed. It appeared that no immunity had been acquired from the antigenic stimulus of the primary parasitaemia and confirmed that all parasites were killed after serum administration. In addition, the transferred serum had no lasting protective activity. It is also possible that while the administration of immune serum mediated parasite destruction it might have inhibited the active development of the host's immune response. It has been shown by some workers (Uhr and Baumann, 1961; Neiders et al., 1962), that it is possible to depress the animal's humoral antibody by passively immunising it with homologous antisera to an antigen such as sheep red blood cells, before or after antigen administration. Phillips (1969b), working on B. rodhaini in rats, reported that the secondary parasitaemia in rats treated with immune serum was severe and it appeared that no immunity had been acquired from the antigenic stimulus of the primary parasitaemia. Phillips (1969b) compared this secondary parasitaemia with the secondary parasitaemia in young rats which were treated with the babesiacidal drug babesan and found that drug treated rats rarely suffered a secondary parasitaemia and if they do, it remained at a very low level, although babesan has no long term activity at the dose level this author used. Phillips (1969b) suggested that either the injection of serum after the injection of the parasite in passive transfer experiments might have inhibited protective antibody production, or that antigenic variation might have played a part in protection, so that a population of parasites can be vulnerable to the action of antiserum, and another population can be resistant. Antigenic variation in B. rodhaini was also reported by Thoongsuwan and Cox

(1973) and Roberts and Tracey Patte (1975a). It was also reported for B. bovis in cattle (Curnow, 1973a) although Mahoney et al.,1979 reported that variation of protective antigens in cattle infected with B. bovis had little effect on the host's immune response. Recently, antigenic diversity was reported for B. divergens in rats (Phillips et al., 1987) which suggests that it may play a role in the host's immune response against the infection.

The mechanisms of action of immune serum are not known. The protective activity of the immune serum might have been due to its direct action on the parasite or the initiation of some responses in the host against the parasite, such as enhanced phagocytosis of PRBC's (i.e. opsonic antibody was involved). Shroeder et al. (1966) reported that the protective activity of immune serum in B. rodhaini infection in rats may be due to autoimmune antibodies that opsonise the infected erythrocytes and cause their phagocytosis by macrophages. Rogers (1974) also correlated opsonic activity of antiserum to B. rodhaini with its ability to protect rats in passive transfer tests. Abdalla and colleagues (1978), working on the same parasite in mice (i.e. B. rodhaini), injected mice with parasites mixed with serum and found that although antibody may kill some parasites, it does not eliminate them. They suggested that free parasites represent direct targets for the protective antibodies which prevent their penetration into red cells and cause their phagocytosis. Mahoney et al.,1979 reported that when cattle are passively protected with hyperimmune serum, free parasites as well as infected

erythrocytes were targets for antibody attack. Bautista and Kreier (1979), working on B. microti in hamsters in vitro also suggested that immune serum inhibits parasite multiplication by preventing the invasion of red cells by the parasite. In the present study, it is not known at this stage whether opsonic antibody has a role in protection against B. divergens in rats. The possible role of opsonic antibody in protection will be described and discussed in chapter seven.

Complement fixing antibody may also play a role in protection, but this has not been investigated. Mahoney (1964), however, reported that the level of complement fixing antibody in the serum of cattle recovered from B. bovis infection fell to a low level within a few weeks of recovery. Similar findings have been reported for B. rodhaini in rats (Phillips, 1968). In a later work, Mahoney (1967a) found that protection was obtained only with serum with a low level of complement fixing antibody collected from cattle that have been superinfected with B. bovis. It was concluded therefore that complement fixing antibodies are unlikely to play a part in protective immunity against B. bovis and B. rodhaini infections (Mahoney, 1967a; Phillips, 1968; 1969b).

Passive transfer experiments have shown that splenectomy did not effect the action of passively administered serum. Similar findings have been reported for B. bovis in cattle (Mahoney, 1967a) and in B. rodhaini in mice (Abdalla et al., 1978). The role of the spleen, will be, however, discussed in detail later.

The protection of splenectomised rats with immune serum has demonstrated the importance of humoral factors in acquired

immunity and has suggested that the mechanisms of immunity in B. divergens in rats is dependent at least on the presence of protective antibodies. They are thus in agreement with conclusions made on other Babesia (Mahoney, 1967a; 1968; Phillips, 1969b; Abdalla et al., 1978). It remains now to confirm that antibody does play a role in protection. In the following chapter, immune and hyperimmune serum were fractionated, to demonstrate that protection was associated with serum gamma-globulins and to determine the class or classes of antibody responsible for protection.

Summary Table of Experiments

CHAPTER FIVE

FRACTIONATION OF IMMUNE AND HYPERIMMUNE SERA AND THE PROTECTIVE ACTIVITY OF IgG AND IgM ANTIBODIES

Chapter Five

Fractionation of immune and hyperimmune sera and the protective activity of IgG and IgM antibodies

INTRODUCTION

In Chapter Four, passive transfer experiments showed that humoral factors play an important role in acquired immunity to B. divergens in splenectomised rats and indicated that antibody might be involved in protection. Similar findings have been reported for some other Babesia species (reviewed by Callow and Dalgliesh, 1982). Mahoney et al;1979, working on B. bovis in cattle reported that serum collected after 2-3 infections (hyperimmune serum) was strongly protective when injected into splenectomised infected calves, and that protection was contained in the IgG fraction of the serum. There are, however, no other reports specifically demonstrating the class or classes of immunoglobulins involved in protection in the different Babesia species. In order to confirm that antibody plays a role in acquired immunity to B. divergens in splenectomised rats, it was necessary, therefore, to fractionate the immune sera to demonstrate that protection was associated with serum gamma globulins and to determine the class or classes of antibodies responsible for protection. In this chapter, the protective activity of IgG and IgM fractions of hyperimmune serum and of day 7-8 sera (sera collected immediately after recovery), and the IgG fraction of day 10 sera (sera collected 3-4 days after recovery) was investigated.

Experiment 5 (i)

The protective activity of hyperimmune serum (preliminary experiment)

In this experiment, the protective activity of hyperimmune serum prior to fractionation was investigated. A volume of 0.35 ml of serum was tested in splenectomised infected rats as follows:

Four (two males and two females) five month old splenectomised rats were injected with 1 x 10^7 PRBC i.v. The rats were pooled and then a group of two rats was given 0.35 ml of hyperimmune serum i.v. The other group received the same volume of normal serum i.v. Hyperimmune serum recipients had a patent parasitaemia on day 1 which increased subsequently at the same rate as the normal serum recipients and both rats in this group died on day 6 post infection as did the controls (Figure 16).

Experiment 5 (ii)

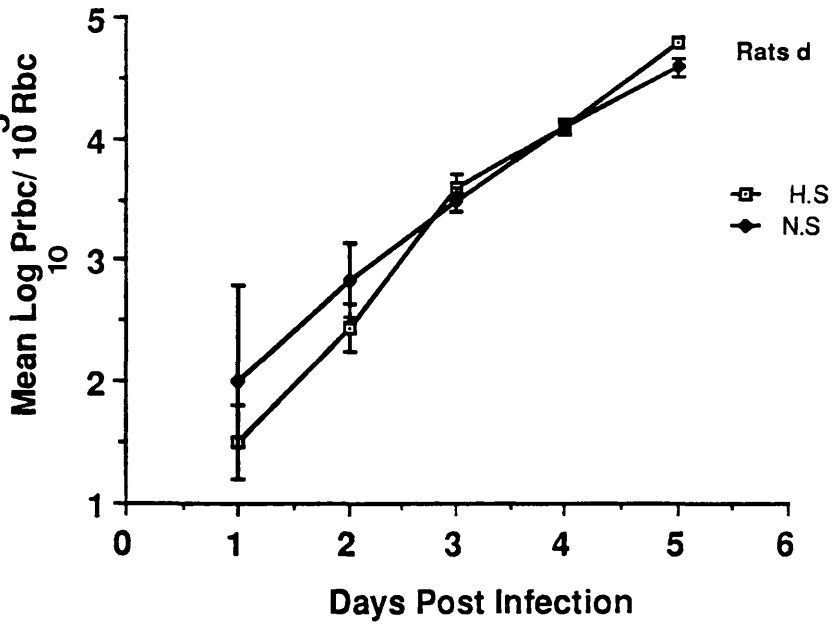
The protective activity of hyperimmune serum using 2 ml of serum

In the above experiment it was shown that using 0.35 ml of hyperimmune serum was not protective. It was decided, therefore, to increase the volume injected from 0.35 ml to 2 ml. A volume of 2 ml of serum was tested in splenectomised infected rats as follows:

Four female 6-7 weeks old splenectomised rats were injected with 1 x 10^7 PRBC i.v. The rats were pooled and a group of two rats were given 2 ml of hyperimmune serum i.v. The other group

Figure 16:

The protective activity of whole hyperimmune serum using 0.35ml of serum.



received the same volume of normal sera (one i.v. and the other i.p.). Hyperimmune serum was strongly protective. No parasites were detected in blood smears taken from hyperimmune recipients during 28 days of observation (Figure 17). On the other hand, normal serum recipients had a patent parasitaemia on days 1-2 and a severe infection. Both rats in this group died on days 5-6 post infection.

Experiments 5 (iii)

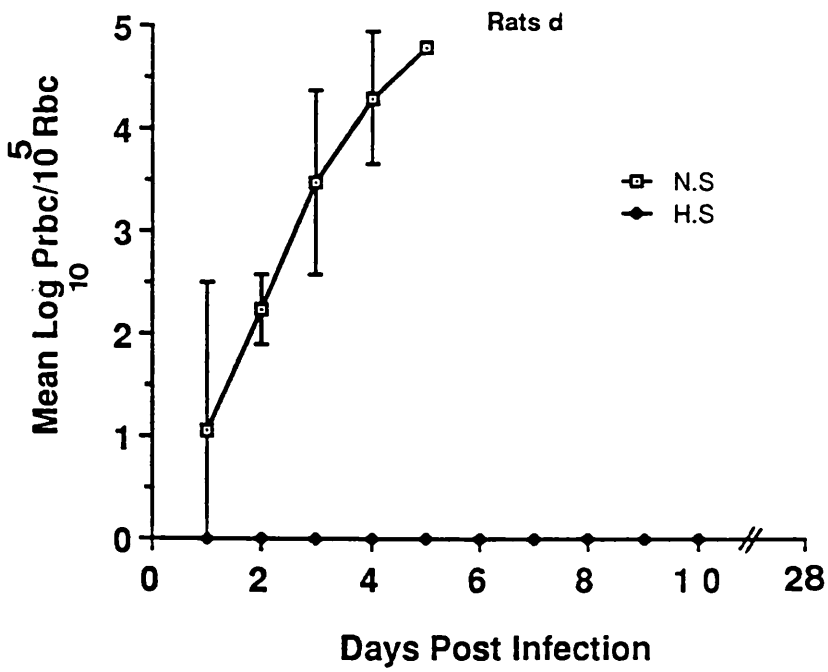
The protective activity of day 4, 5, 6 and 8 sera

It was shown in Chapter Four that sera collected during the infection (day 4-5) were not protective in passive transfer tests. Sera collected after recovery from the infection (day 7-13) were, however, strongly protective. In this experiment, it was decided to test again the protective activity of sera collected during the infection (day 4 and 5), at recovery with a very low parasitaemia (day 6) and 1 day after recovery (day 7 or 8 depending on when the rats recover from the infection), prior to fractionation of day 7 or 8 sera and day 10 sera (3-4 days after recovery). It should be noted that this experiment was done at a time when the parasite was avirulent (or had just started to become virulent) and rats normally recover from the infection when receiving 1 x 10^5 PRBC. Day 4, 5, 6 and 8 sera was raised in splenectomised infected rats as follows:

Eight (five males and three females), 6 1/2 month old splenectomised rats were infected with 1 x 10^5 PRBC i.v. and the course of infection was followed. There was a delay in the onset of parasitaemia of two days in all rats. The parasitaemia was

Figure 17:

The protective activity of whole hyperimmune serum using 2ml of serum.

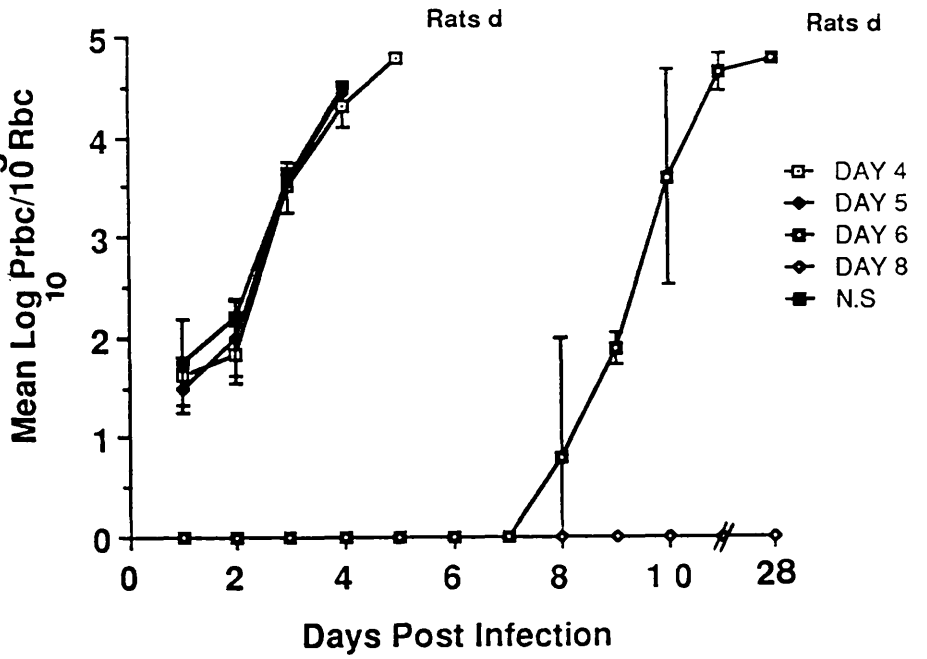


patent on day 3 post infection which increased subsequently. The rats were divided into groups of two rats and a group was sacrificed on day 4 (parasitaemia = 17-19%), day 5 (parasitaemia 38-39%), day 6 (at recovery) (parasitaemia 0.2-0.5%) and day 8 (one day after recovery), and their sera collected. The protective activity of day 4, 5, 6 and 8 sera was tested as follows:

Fifteen (eight female and seven male), 4-5 week old splenectomised rats were injected with 1×10^6 PRBC i.v. It was shown in Chapter Four when testing the protective activity of day 4 and day 7 sera, that 0.4 ml of serum was not protective with day 4 serum and was protective with day 7 serum. It was decided in this experiment to inject 1 ml of serum into each recipient. Groups of 2-3 rats were injected with 1 ml of day 4-5, 6 or 8 sera immediately after the injection of the parasites. A control group which received the same volume of normal serum was included. Day 4 and 5 sera were not protective (Figure 18). Day 4 and 5 sera recipients had a patent parasitaemia on day 1 which increased subsequently at the same rate as the normal serum recipients and they died on day 5 post infection as did the controls. Day 6 serum was partially protective. There was a delay in the onset of parasitaemia for 7-8 days in day 6 serum recipients. The parasitaemia was patent on day 8-9 which increased subsequently. All rats in this group died on days 12-13 post infection. Day 8 serum was strongly protective. No parasites were detected in blood smears taken over 28 days of observation, thus confirming that sera collected immediately after recovery were strongly protective.

Figure 18:

The protective activity of sera collected on days 4, 5, 6 and 8 using 1ml of serum.



Experiment 5 (iv)

Isolation of IgG from hyperimmune serum, day 7, day 10 and normal serum (N.S.)

The method for isolation of IgG from the different sera was described in the materials and methods chapter (see Chapter Two). Twenty ml of serum were used for each fractionation (except day 7 serum where 15 ml of serum were used). The Ig fraction of the sera was first precipitated by sodium sulphate. The precipitate was resuspended in 10 ml of PBS (pH 7.2) (Appendix A), dialysed against PBS overnight and poured into a column of DEAE Cellulose to isolate the Ig fraction of each serum. IgG was eluted by the starting buffer (i.e. phosphate buffer pH = 7) in a single asymmetric peak (Figure 19 fraction B). The relative concentration of IgG was estimated using a spectrophotometer at an absorbence of 280 nm.

Experiment 5 (v)

Agar gel electrophoresis

Rat IgG in fraction B (Figure 19) was demonstrated by agar gel electrophoresis as described in the materials and methods chapter against neat anti-rat whole serum (Sigma), sheep anti-rat IgGAM and L chains and rat IgG F_c. Figure 20 shows IgG precipitation arcs for hyperimmune serum, compared to whole normal serum.

Figure 19:

Isolation of IgG from rat serum on DEAE cellulose in 0.07M sodium phosphate pH7 following sodium sulphate precipitation.

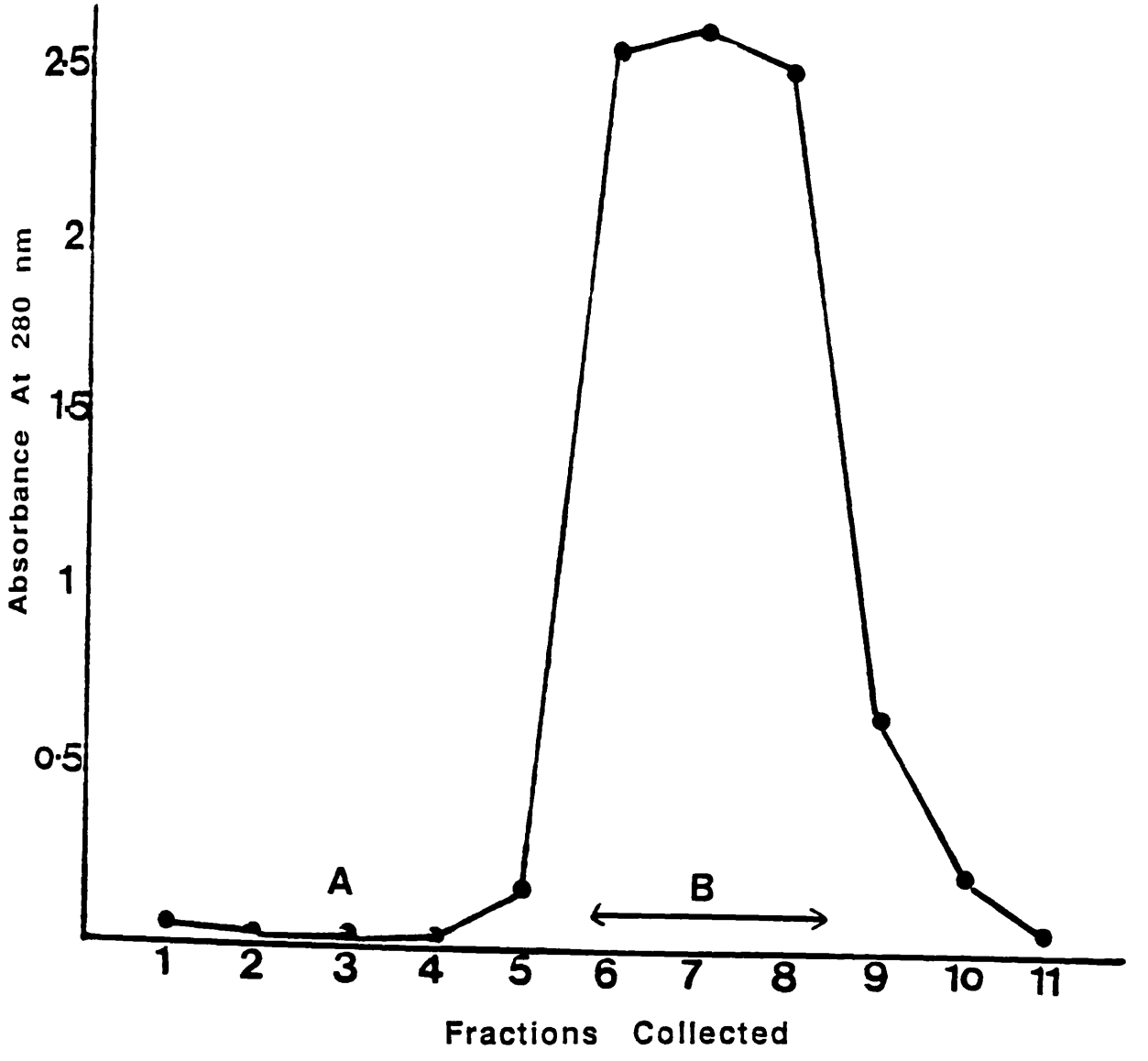
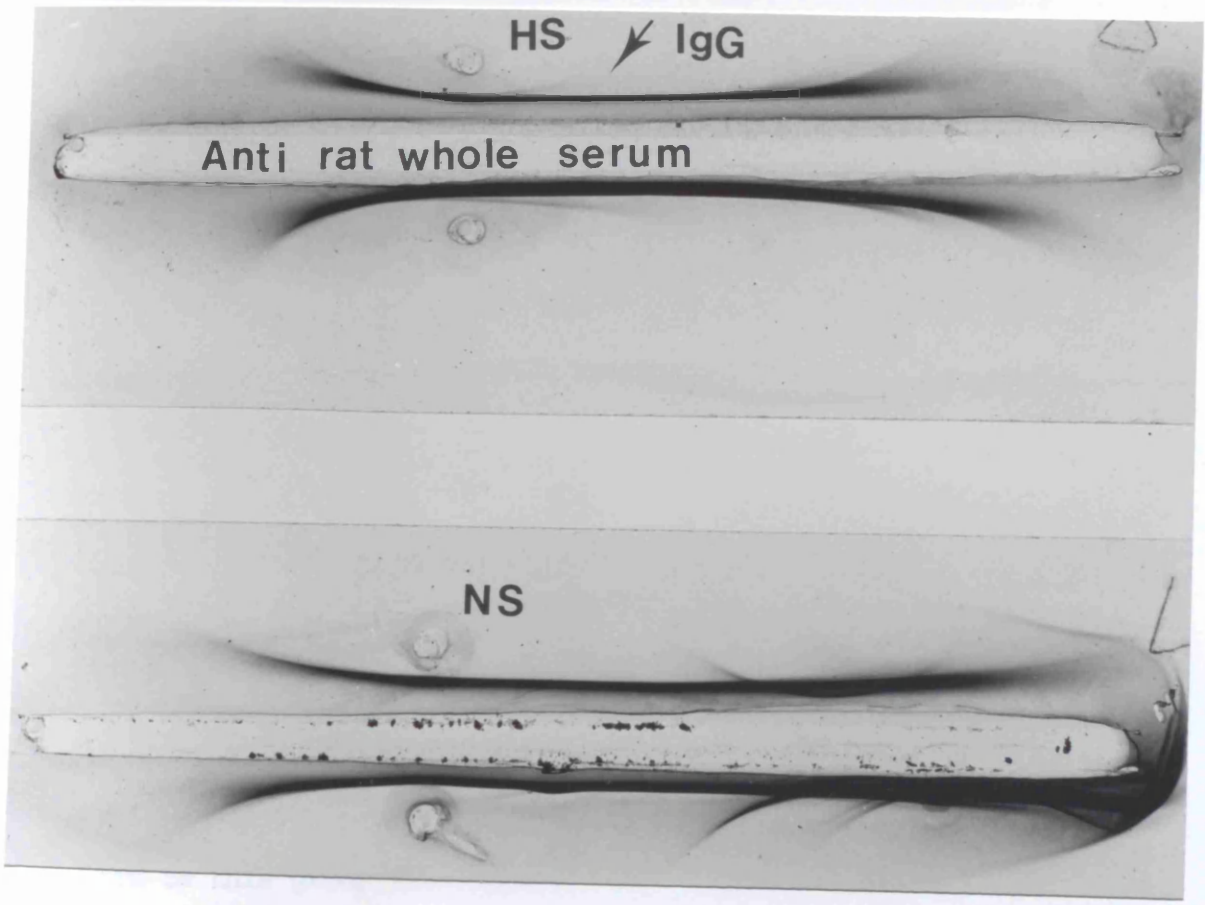


Figure 20:

Immunoelectrophoretic analysis of hyperimmune serum IgG eluted on DEAE cellulose compared to whole, normal serum.



Experiment 5 (vi)

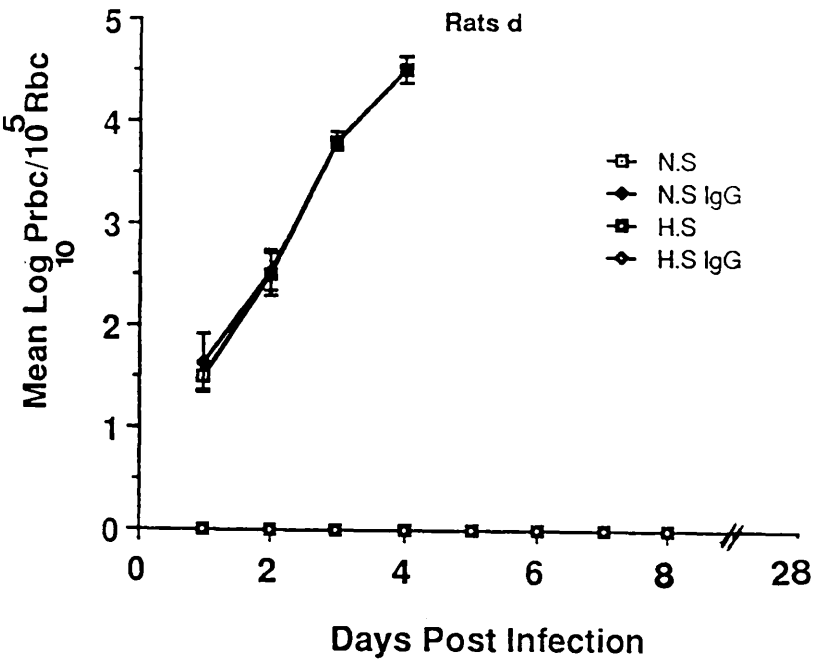
The protective activity of hyperimmune serum IgG compared to hyperimmune serum (whole) and normal serum IgG

It was shown in a previous experiment that 2 ml of hyperimmune serum was strongly protective (see experiment 5 (ii)). In this experiment, the volume of hyperimmune serum injected was reduced from 2 ml to 1.5 ml and the protective activity of hyperimmune serum IgG, hyperimmune serum (whole) and normal serum IgG was compared as follows:

Sixteen (11 females and 5 males) five week old splenectomised rats were injected with 1×10^6 i.v. The rats were pooled and then groups of four rats were given 1.5 ml of hyperimmune serum IgG, hyperimmune serum (whole) or normal serum IgG. A control group receiving the same volume of normal serum was also included. Hyperimmune serum IgG and hyperimmune serum (whole) were strongly protective (Figure 21). No parasites were detected in blood smears taken from both sera recipients during 28 days of observation, thus confirming the protective activity of hyperimmune serum IgG and hyperimmune serum (whole) in a previous experiment (results not shown), where 2 ml of either serum was injected into recipients and proved to be highly protective. In contrast, normal serum IgG was not protective. All rats in this group had a patent parasitaemia on day 1 which increased subsequently at the same rate as the normal serum (whole) recipients, and died on day 5 post infection as did the whole normal serum recipients.

Figure 21:

The protective activity of hyperimmune serum compared to whole hyperimmune serum and normal serum IgG.



Experiment 5 (vi)

The protective activity of day 10 IgG compared to day 10 (whole serum)

In order to check whether IgG was responsible for protection in sera collected 3-4 days after recovery (day 10), the protective activity of day 10 IgG was investigated and compared with the protective activity of day 10 (whole serum). A volume of 1.5 ml of either day 10 IgG or day 10 (whole serum) was injected into splenectomised infected rats as follows:

Twelve (ten females and two males) 5-6 week old splenectomised rats were injected with 1 x 10^6 PRBC i.v. The rats were pooled and then groups of 3-4 rats were injected with 1.5 ml of day 10 IgG or day 10 (whole serum) i.v. A control group which received the same volume of normal serum was included. Day 10 IgG and day 10 whole serum were strongly protective. No parasites were detected in blood smears taken during 28 days of observation (Figure 22). Normal serum recipients had a patent parasitaemia on day 1 which increased subsequently and they all died on day 5 post infection.

Experiment 5 (vii)

Challenging of serum recipients

To test whether whole hyperimmune serum, hyperimmune serum IgG, whole and day 10 serum and day 10 IgG had any residual protective activity four weeks after administration, serum or IgG recipients were challenged on day 28 with 1 x 10^8 PRBC i.v. An eight week old splenectomised rat that had received no serum at

Figure 22:

The protective activity of day 10 IgG compared to whole day 10 serum.

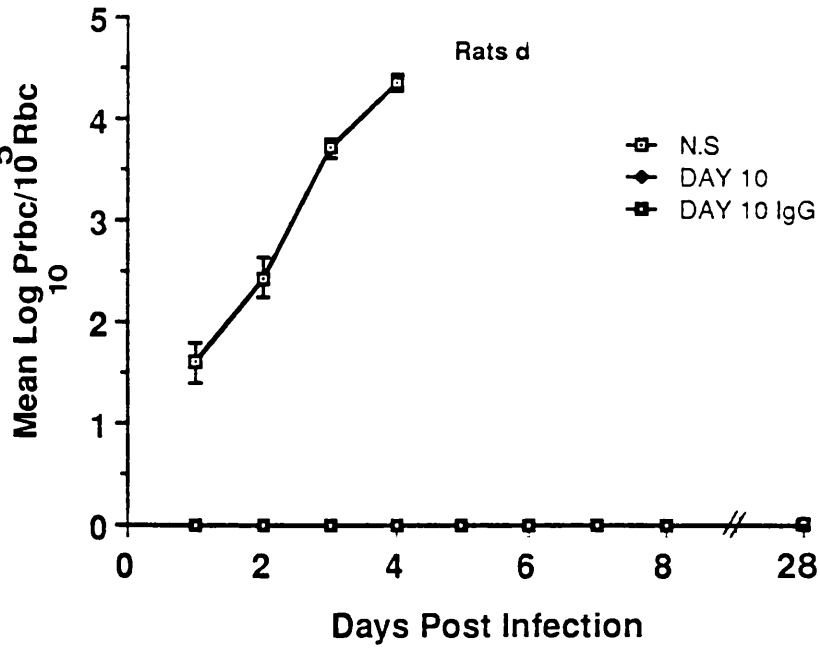
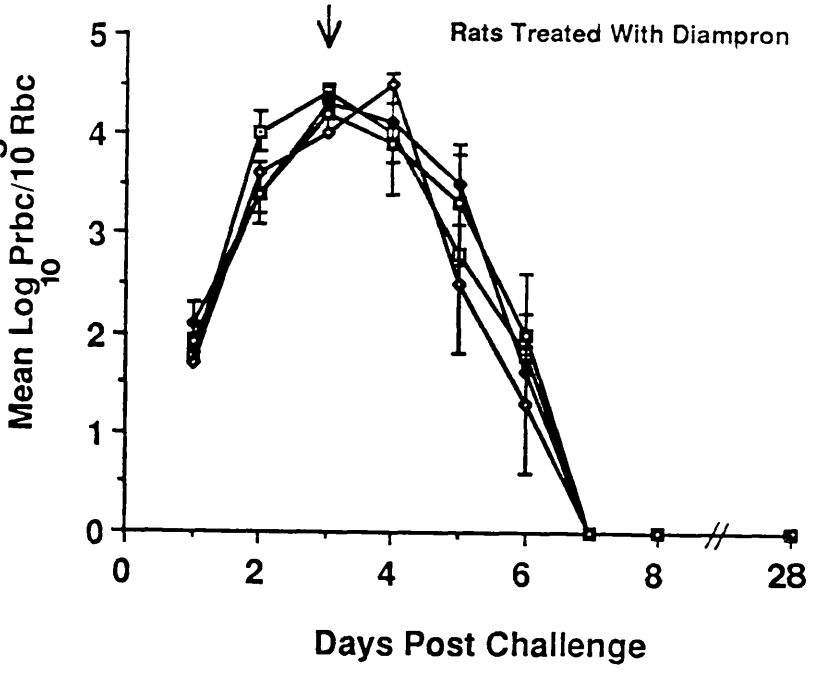


Figure 23:

Challenging of hyperimmune serum IgG, whole hyperimmune serum, day 10 IgG and whole day 10 recipients with $1x10^8$ PRBC 4 weeks after serum administration.



all was included as a control. All challenged recipients had a patent parasitaemia on day 1 which increased subsequently at the same rate as the control rat (Figure 23). Rats in all groups including the control rat were treated on days 2 or 3 (parasitaemia between 9% and 33%) post challenge with 30 mg/kg Diampron subcutaneously. The parasitaemia in the treated rats started decreasing 1-2 days after treatment, and all rats recovered subsequently. No parasites were detected in blood smears taken 3-4 days after treatment.

Experiment 5 (viii)

The protective activity of day 7 IgG compared to whole day 7 serum

In this experiment, the protective activity of the IgG fraction of day 7 serum was tested. In this test, rats were injected with the corrected volume of the fractionated serum to allow for dilution during fractionation as shown in Table 4. The protective activity of day 7 IgG and whole day 7 serum using volumes of either IgG fraction or whole serum described in Table 4, was compared in splenectomised infected rats as follows:

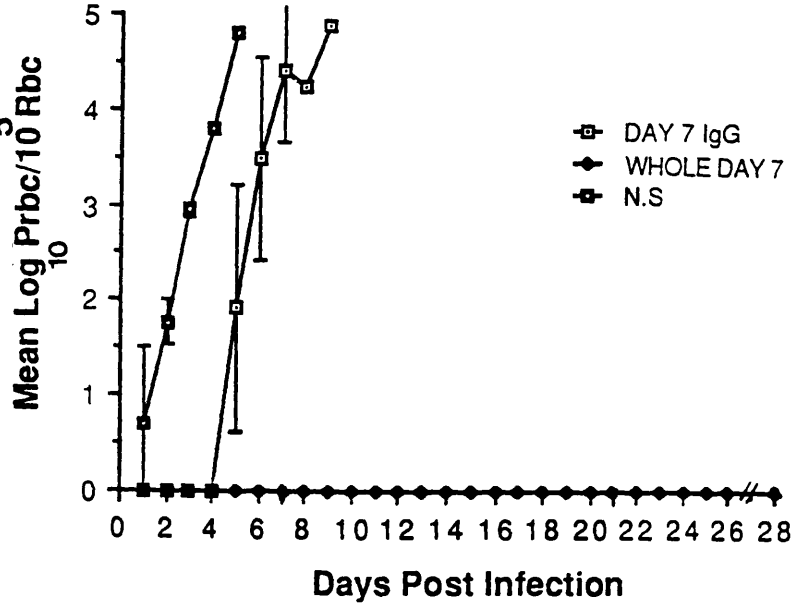
Twelve (six males and six females) 4-5 week old splenectomised rats were injected with 1 x 10^6 PRBC i.v. The rats were pooled and groups of four rats were injected with 1.5 ml of day 7 whole serum or 0.65 ml of day 7 IgG. A control group which received 1.5 ml of normal serum was included. Day 7 IgG was partially protective (Figure 24). There was a delay in the onset of parasitaemia for 4-5 days. Parasitaemia was patent on day 5 and day 6 which subsequently increased and all day 7 IgG

Table 4:

Volume of day 7 serum originally fractionated	15ml
Volume of day 7 IgG fraction obtained after concentration of pooled IgG fractions	6.5ml
Volume of day 7(whole serum) injected into each recipient1.5	1.5ml
Volume of day 7 IgG injected into each recipient	0.65ml

Figure 24:

The protective activity of day 7 IgG compared to whole day 7serum.



recipients died on days 8-9 post infection. Day 7 (whole serum) was strongly protective. No parasites were detected in blood smears taken during 28 days of observation. Normal serum recipients had a patent parasitaemia on day 1 which increased subsequently and they all died on day 6 post infection.

Experiment 5 (ix)

Isolation of IgM from hyperimmune serum, normal serum and sera collected on day 7 (immediately after recovery) by gel filtration

The method for the isolation of IgM from hyperimmune serum, normal serum and day 7 serum by sephadex G-200 and sephacryl S-300 was described in the materials and methods chapter. Two x 20ml of pooled hyperimmune serum were used for two fractionations. Fourteen ml of pooled normal serum and 6 ml of pooled day 7 serum were used, for one fractionation each. In each fractionation, hyperimmune serum, normal serum and day 7 serum were eluted from a column of sephadex G-200 with borate buffer (pH 8). Three main peaks were obtained (Figure 25). The relative concentration of fractions in each peak was estimated using a spectrophotometer at an absorbence of 280 nm. Rat immunoglobulins of pooled fractions of each peak obtained were identified by double diffusion. Day 7 IgM was isolated using sephacryl S-300. Six ml of pooled day 7 serum were eluted from a column of sephacryl S-300 with borate buffer pH 8 and 3 ml of the same pooled serum were eluted using the same column but with tris Nacl (pH 8). Four main peaks were obtained from each fractionation (Figure 26). Rat immunoglobulins of pooled fractions of each peak obtained were identified by double

Figure 25:

Elution of IgG and IgM from a column of sephadex G-200.

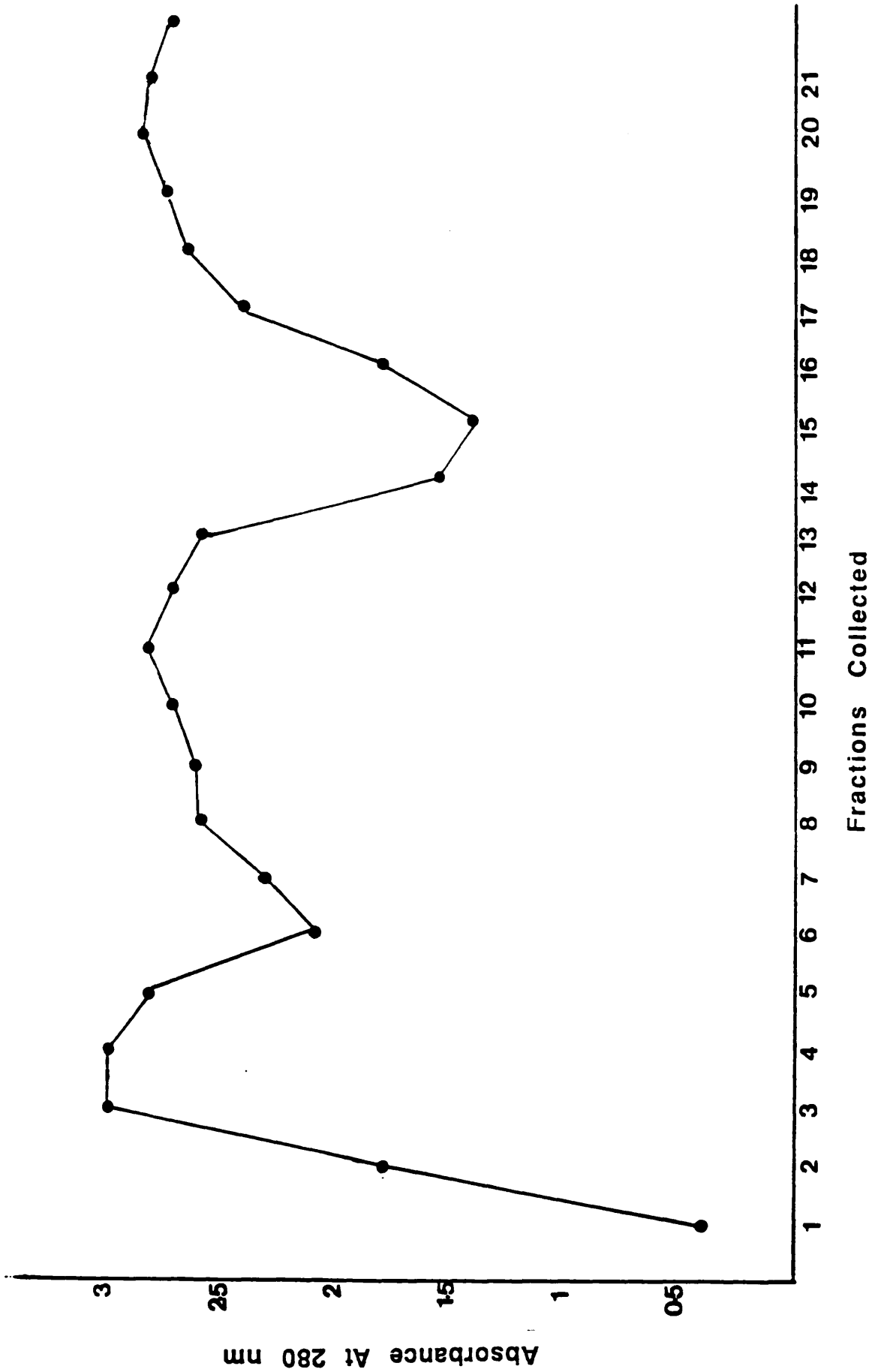
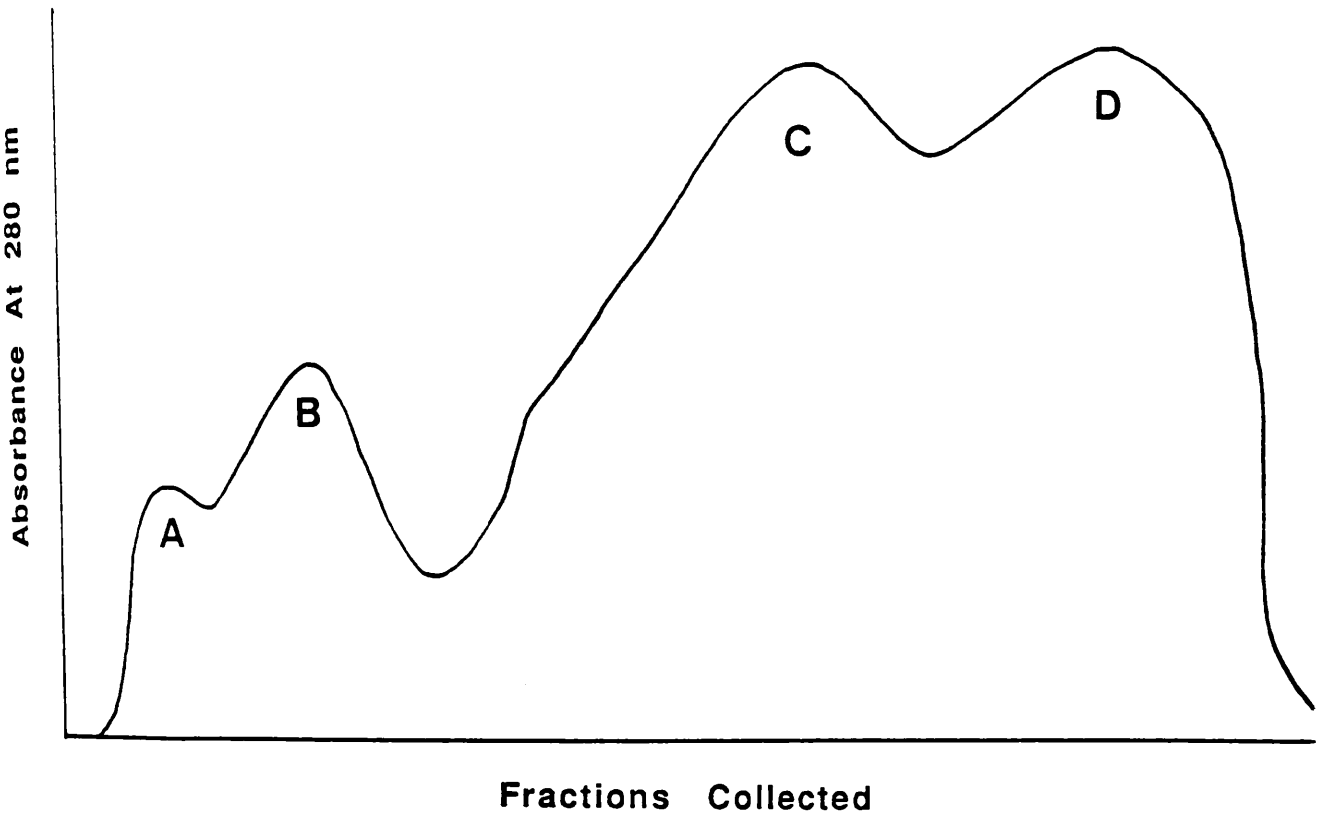


Figure 26:

Elution of serum proteins from a column of sephacryl S-300.



diffusion.

Experiment 5 (x)

Ouchterlony (Double diffusion) test on IgG and IgM fractions

Rat immunoglobulins of pooled fractions of each peak obtained after fractionation of hyperimmune serum, day 7 serum or normal serum identified on sephadex G-200 or after fractionation of day 7 serum with sephacryl S-300 were identified by double diffusion test, against IgG, IgM and IgA. The fractions were tested either neat or diluted 1 in 10 or 1 in 100 in PBS. It was found that the first peak obtained in the fractionation of hyperimmune serum, day 7 and normal serum on sephadex G-200 was IgM (Fraction A), the second peak was IgG (Fraction B) and the third peak was albumin (Figure 26). The first and second peak obtained after fractionation of day 7 sera on sephacryl S-300 was IgM (Fractions A and B) and the third and fourth (Fractions C and D) was IgG (Figure 26). Some of the immunoglobulins could not be demonstrated when neat undiluted fractions were tested but could be when the fractions were diluted 1 in 10 (see Figure 27). The reason for this will be explained later in the discussion.

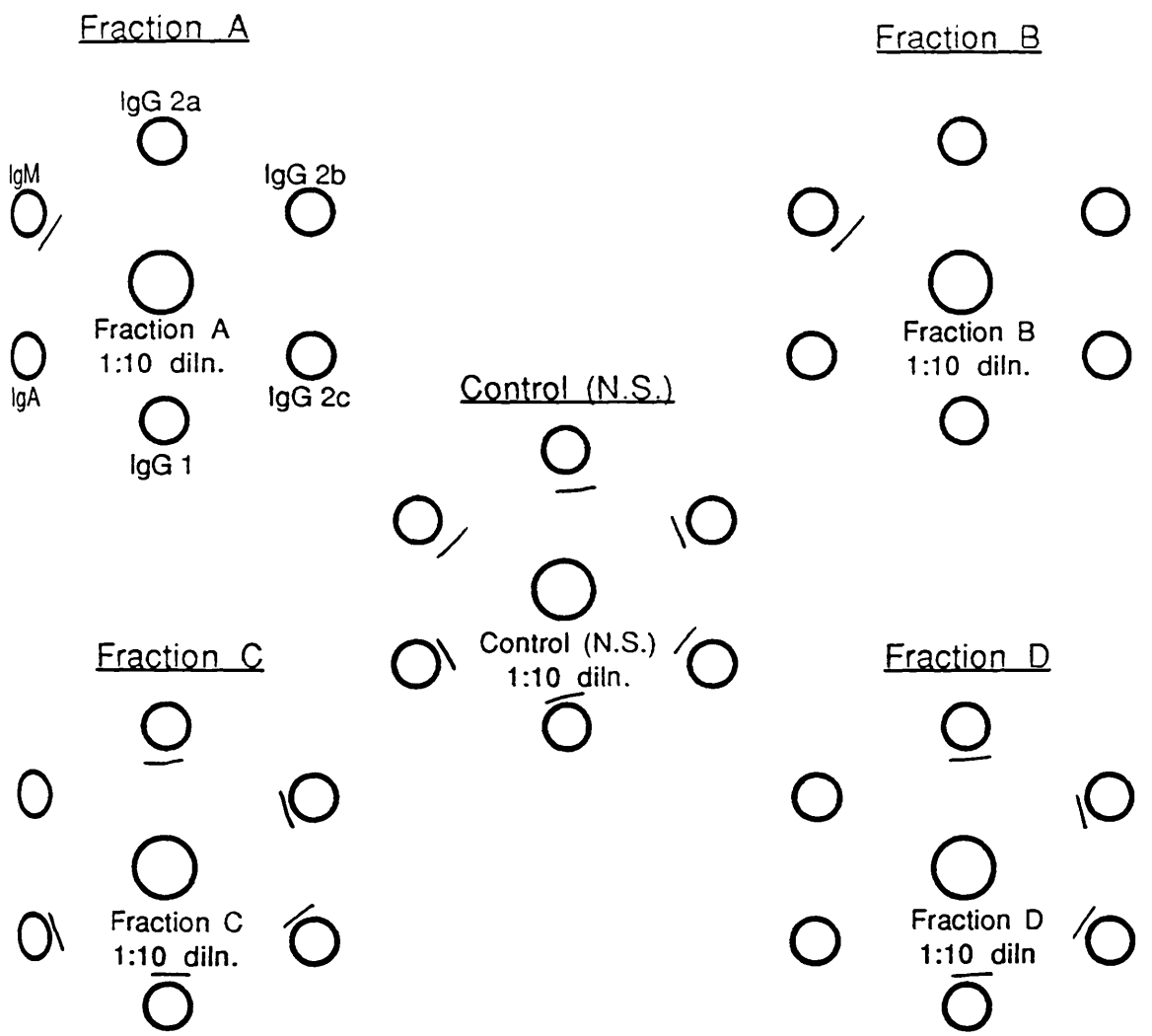
Experiment 5 (xi)

The protective activity of hyperimmune serum IgM compared to hyperimmune serum IgG and hyperimmune serum whole

The protective activity of the IgM fraction (Fraction A) and the IgG fraction of hyperimmune serum which was obtained in the same fractionation with sephadex G-200 (Fraction B) was compared in two experiments as follows:

Figure 27:

Ouchterlony (double diffusion) test on day 7 IgG and IgM eluted from a column of sephacryl S-300 compared to whole, normal serum.



Outer wells = Antisera
Central hole = Fraction tested

Experiment A

It was shown in a previous experiment that 1.5 ml of hyperimmune sera IgG and 1.5 ml of whole hyperimmune sera were protective. In this preliminary experiment, the same volume was used to compare the protective activity of hyperimmune sera IgM, hyperimmune sera IgG and whole hyperimmune sera as follows:

Twelve (nine female and three male) 4-5 week old splenectomised rats were injected with 1 x 10^6 PRBC i.v. The rats were pooled and groups of three rats were injected with 1.5 ml of hyperimmune sera IgM, hyperimmune sera IgG or whole hyperimmune sera. A control group which received the same volume of normal sera was included. Hyperimmune sera IgG and whole hyperimmune sera were completely protective as described before. Hyperimmune sera IgM was partially protective. There was a delay in the onset of parasitaemia for 3-4 days (Figure 28A). The parasitaemia was patent on days 5-6 which increased subsequently. All hyperimmune sera IgM recipients had a severe infection and died on days 8-9 post infection. Normal serum recipients had a patent parasitaemia on day 1 and a severe infection and they all died on days 4-5 post infection.

Experiment B

In the above experiment, it was shown that 1.5 mls of hyperimmune sera IgM was partially protective. In this experiment hyperimmune sera IgM recipients were injected with the corrected volume of another batch of fractionated hyperimmune serum (fractionated with sephadex G-200) to allow for dilution during fractionation as shown in Table 5. The protective

activity of hyperimmune sera IgM, IgG and whole hyperimmune sera was compared using volumes of IgG or IgM fractions of whole serum as mentioned in Table 5 as follows:

Eighteen (ten female and eight male) 5-6 weeks old splenectomised rats were injected with 1 x 10^6 PRBC i.v. The rats were pooled, then groups of 4-5 rats were injected with 0.9 ml of hyperimmune sera IgM, 1.4 ml of hyperimmune sera IgG or 2 ml of whole hyperimmune sera. A control group which received 2 ml of normal sera was included. Hyperimmune sera IgG and whole hyperimmune sera were strongly protective. No parasites were detected in blood smears taken over 28 days of observations. Hyperimmune sera IgM was partially protective (Figure 28B). There was a delay in the onset of parasitaemia for 4-6 days. The parasitaemia was patent between days 5-7 which increased subsequently and most hyperimmune sera IgM recipients died on days 10 and 11 respectively. Normal sera recipients had a patent parasitaemia on day 1 and a severe infection and they all died on days 5 and 6 post infection.

The protective activity of day 7 IgM compared to day 7 IgG and day 7 whole serum

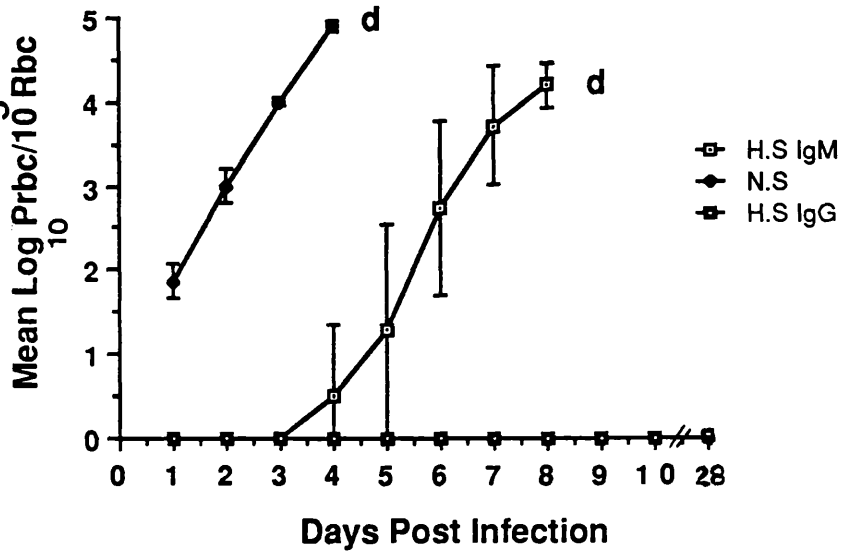
The protective activity of the IgM fraction of day 7 serum was compared in three experiments as follows:

Experiment 1

In this experiment, the protective activity of the IgM fraction of day 7 serum and of the IgG fraction of the same serum which was obtained in the same fractionation with sephadex G-200

Figure 28A:

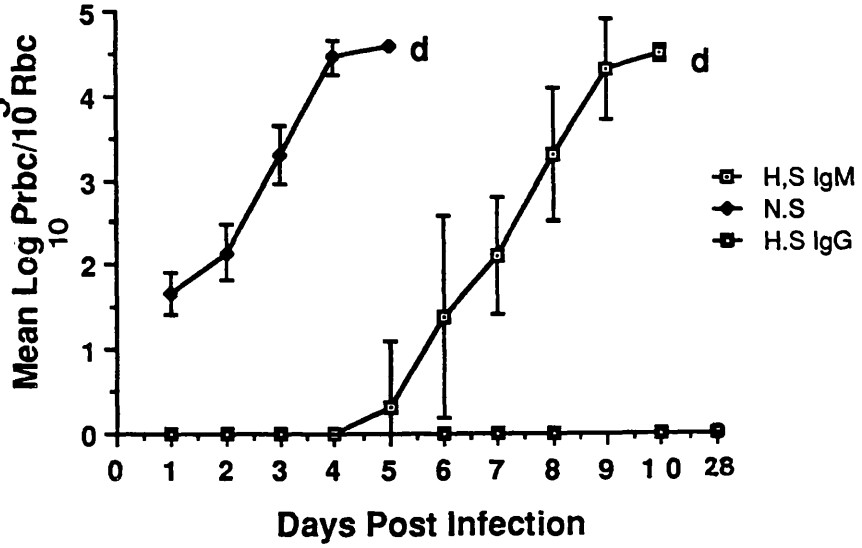
The protective activity of hyperimmune serum IgM compared to hyperimmune serum IgG and whole hyperimmune serum using 1.5ml of serum.



(A)

Figure 28B:

The protective activity of hyperimmune serum IgM compared to hyperimmune serum IgG and whole hyperimmune serum.



(B)

was compared. The protective activity of day 7 IgM and IgG was also compared with the protective activity of normal serum IgM which was also fractionated with sephadex G-200. The corrected volume of the fractionated sera was used as shown in Table 6.

The protective activity of day 7 IgM, IgG, whole serum and normal serum IgM was compared using the volumes of whole serum or IgG or IgM fractions of day 7 or normal serum mentioned in Table 6 as follows:

Twenty, five week old male splenectomised rats were injected with 1×10^7 PRBC i.v. The rats were pooled and groups of four rats were injected with 0.9 ml of day 7 IgM fraction, 1 ml of day 7 IgG fraction, 1 ml of day 7 whole serum, 0.55 ml of normal serum IgM fraction or 1 ml of whole normal serum. Day 7 IgM was strongly protective. Two of the four day 7 IgM recipients did not show any parasites in blood smears taken over 28 days of observation (Figure 29A). There was a delay in the onset of parasitaemia for 15 days in one day 7 IgM recipient. The parasitaemia was patent on day 16, subsequently increased, and the rat died on day 19 post infection. There was a delay in the onset of parasitaemia of 21 days in the other day 7 IgM recipient. The parasitaemia was patent on day 22 which was followed by a severe infection and the rat died on day 26 post infection. Day 7 IgG was partially protective. There was a delay in the onset of parasitaemia for five days (there was a delay in the onset of parasitaemia for four days when 0.65 ml of day 7 IgG was used in a previous experiment). Parasitaemia in all day 7 IgG recipients was patent on day 6. This subsequently increased and all recipients died between days 9 and 11 post

infection. Day 7 (whole serum) was completely protective as was shown previously. Normal serum IgM recipients had a patent parasitaemia on day 1. This increased subsequently at the same rate as the normal serum recipients, and all rats in both groups died on day 4 post infection.

Experiment 2

In this experiment, the protective activity of the IgM fraction of day 7 sera and of the IgG fractions of the same sera which were obtained from the same fractionation with sephacryl S-300 was compared. It was shown in Chapter Three that 0.2 ml of whole day 7 serum was partially protective (there was a delay in the onset of parasitaemia for 11 days). In this experiment it was decided to use the same volume of day 7 whole serum (i.e. 0.2 ml) and the corrected volumes of the fractions were worked out from this as shown in Table 7. The protective activity of day 7 IgM, day 7 IgG (fraction C), day 7 IgG (fraction D) and day 7 whole serum, was compared using the volumes of IgM or IgG fractions or whole serum recorded in Table 7 as follows:

Twenty (14 female and 6 male) 11 week old splenectomised rats were injected with 1×10^7 PRBC i.v. The rats were pooled, and groups of four rats were injected with 0.35 ml of day 7 IgM (Fractions A & B), 0.18 ml of fraction C, 0.2 ml of fraction D or 0.2 ml of whole day 7 serum. A control group which received 0.2 ml of normal serum was included. Day 7 IgM was partially protective using this volume (0.35 ml) (Figure 29B). There was a delay in the onset of parasitaemia of 6-8 days (day 7 IgM was strongly protective when 0.9 ml were used in a previous

experiment). Parasitaemia in all recipients was patent on days 7-9 and two of the day 7 IgM recipients had a severe infection and died on days 12 and 13 post infection. The other two day 7 IgM recipients had a mild infection which lasted for five days and they subsequently recovered. No parasites were detected in blood smears taken from these two recipients on days 13 and 14 post infection. Day 7 IgG (Fraction C and Fraction D) were both not protective. Recipients in both groups had a patent parasitaemia on day 1 which increased subsequently at the same rate as the normal serum recipients and rats in both groups died on day 5 post infection as did the normal serum recipients. Whole day 7 serum was strongly protective. Three of the four recipients did not show any parasites during 28 days of observation. One recipient however, had a patent parasitaemia on day 15 and a mild infection which lasted for four days, and it subsequently recovered. No parasites were detected in blood smears taken on day 18 post infection.

Experiment 3

In order to confirm that day 7 IgM was protective, the above experiment was repeated using a different batch of pooled whole day 7 serum which was also fractionated with sephacryl S-300. In this experiment, a group of recipients which received pooled fractions of day 7 (fraction A, B, C and D) was included. This was to test whether the pooled fractions would give the same protection as the IgM fraction, since it was shown in the previous experiment that day 7 IgG (fractions C and D) was not protective, and to confirm therefore that protection in the

pooled fractions was contained in the IgM. The same volume of whole day 7 serum (i.e. 0.2 ml) was injected into recipients as above and the corrected volume of the fractions injected was worked out from this as shown in Table 8.

The protective activity of day 7 IgM (fraction A and B), day 7 IgG (fraction C and fraction D), day 7 (fractions A, B, C and D pooled) and day 7 whole serum was compared as follows:

Twenty two (13 female and nine male), 5-6 week old splenectomised rats were injected with 1 x 10^7 PRBC i.v. The rats were pooled and groups of 2-4 rats were injected with 0.2 ml of day 7 IgM, 0.4 ml day 7 IgG (fraction C), 0.27 ml of day 7 IgG (fraction D), 0.87 ml of day 7 pooled fractions or 0.2 ml of day 7 whole serum. A control group which received 0.2 ml of normal serum was included. Day 7 IgM was partially protective using this volume (i.e. 0.2 ml). There was a delay in the onset of parasitaemia of 4-8 days although there was a delay in the onset of parasitaemia for four days in most recipients (Figure 29C). Parasitaemia was patent on day 5-9 (day 5 in most recipients), and subsequently increased. Most rats died on day 9 post infection, one rat died on day 12 post infection. Day 7 pooled fractions was also partially protective. There was a delay in the onset of parasitaemia for 5-7 days. Parasitaemia was patent on days 4-9 which subsequently increased and both rats in this group died on days 10 and 12 post infection. Day 7 IgG (fraction C and fraction D) were both not protective. Recipients in both groups had a patent parasitaemia on day 1 which increased subsequently at the same rate as the normal serum recipients and

Table 5

Volume of H.S. originally fractionated	20ml
Volume of H.S. IgM (frac. 1) obtained after concentration of pooled peak	9ml
Volume of H.S. IgG (frac. 2) obtained after concentration of pooled peak	14ml
Volume of H.S. (whole) injected into each recipient	2ml
Volume of H.S. IgM injected into each recipient	0.9ml
Volume of H.S. IgG (frac. 1) injected into each recipient.	1.4ml

Table 6

	Day 7		N.S.
Volume of serum fractionated	6ml		14ml
Volume of fraction obtained after concentration of peaks	FRA 1 IgM ↓ 5.5ml	FRA 2 IgG ↓ 6ml	FRA 1 IgM ↓ 7.5ml
Volume of whole serum injected into each recipient	1ml		1ml
Volume of fraction injected into each recipient	IgM 0.9ml	IgG 1ml	IgM 0.55ml

Table 7:

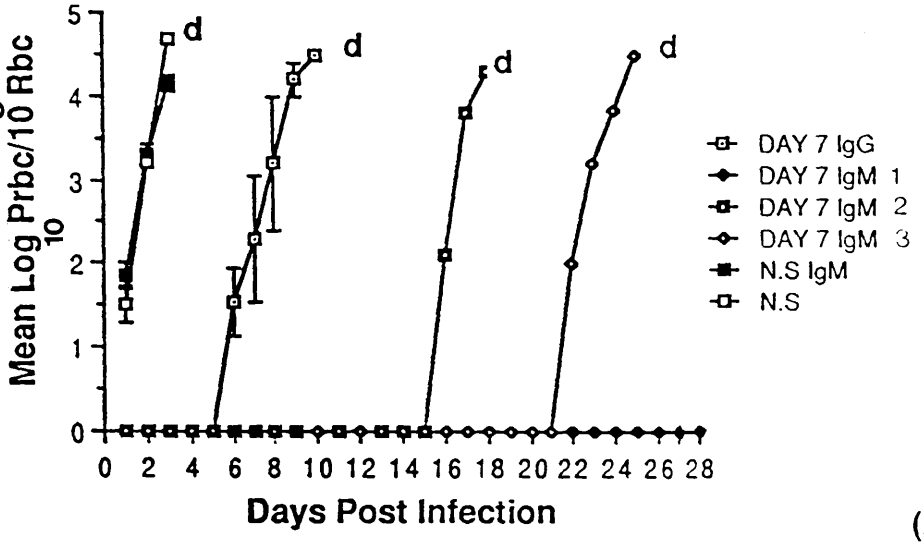
Volume of serum fractionated	6ml			
Volume of fraction obtained after concentration of each peak	Fractions obtained with sepha ? S-300			
	FRA 1 IgM	FRA 2 IgM	FRA 3 IgG	FRA 4 IgG
	5.2ml	5.2ml	5.4ml	6ml
Volume of fraction injected into each recipient	Pooled ↓ 0.35ml		↓ 0.18ml	↓ 0.2ml
Volume of whole day 7 serum injected into each recipient	0.2ml			

Table 8:

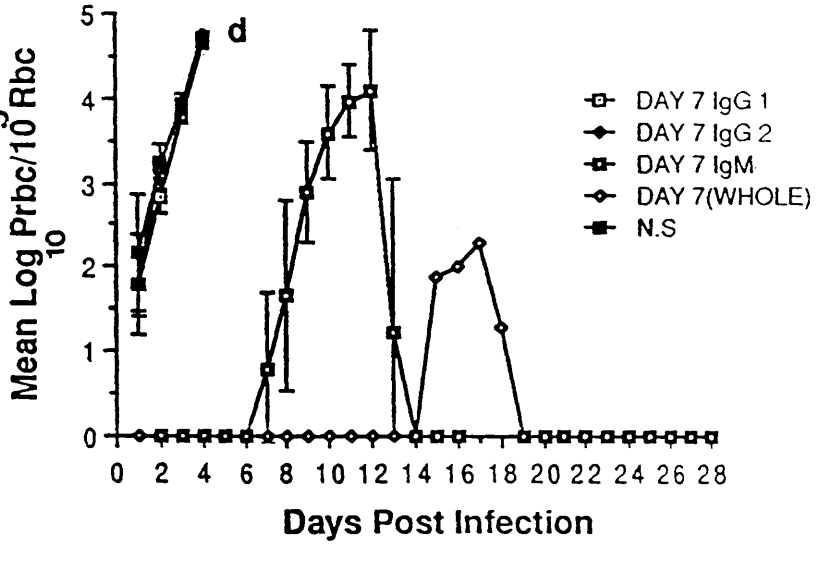
Volume of serum fractionated	3ml		
Volume of fractions obtained after concentration of pooled peaks	FRA 1&2 IgM 3ml	FRA 3 IgG 6ml	FRA 4 IgG 4ml
Volume of whole day 7 serum injected into each recipient	0.2ml		
Volume of each fraction injected into each recipient	0.2ml	0.4ml	0.27ml
Volume of pooled fractions injected into each recipient	0.87ml		

Figure 29:

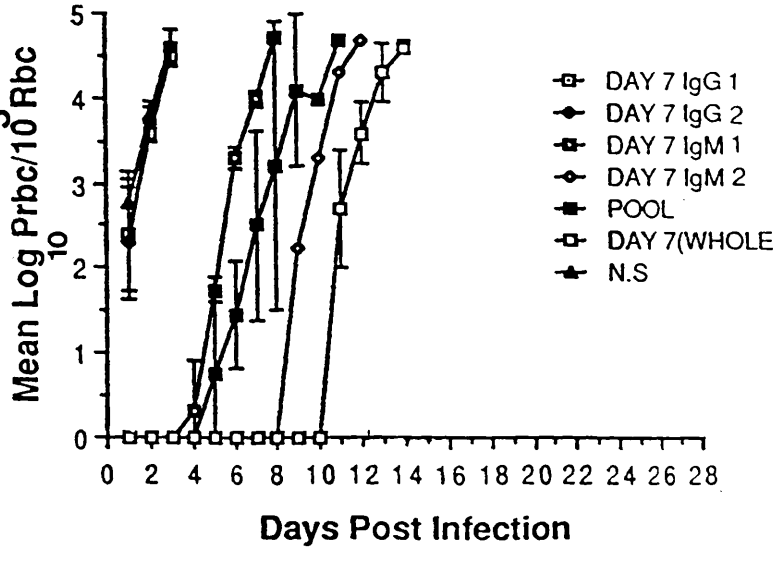
The protective activity of day 7 IgM compared to day 7 IgG and whole day 7 serum using different volumes of sera.



(A)



(B)



(C)

all rats in both groups died on day 4 post infection as did the controls. There was a delay in the onset of parasitaemia of ten days in the whole day 7 serum recipients. Parasitaemia was patent on day 11 and the rats suffered a severe infection. All rats in this group died days 14 and 15 post infection.

Protein assay

The concentration of IgG and IgM in day 7 serum was preliminary determined with fractions collected after fractionation of day 7 serum on sephacryl S-300. It was found that there is approximately 1.5 mg/ml of IgM in fractions A and B 6 mg/ml of IgG in fraction C and 13 mg/ml in fraction D.

Discussion

In this chapter, the role of antibody in acquired immunity to B. divergens was investigated in splenectomised rats. The protective activity of IgM and IgG fractions of hyperimmune sera and of sera collected immediately after recovery from a primary infection (day 7-8), and the IgG fraction of sera collected 3-4 days after recovery from a primary infection (day 10) was compared. The protective activity of the IgG and IgM fractions of hyperimmune sera was first investigated. The data showed that the protective activity of hyperimmune serum was mainly contained in the IgG fraction of the serum. Similar findings have been reported for B. bovis in cattle (Mahoney, 1967a; 1979a). Mahoney (1979a) reported that there is a direct relationship between the protective activity of the antibody and its concentration in the serum. Although the concentration of antibody in whole hyperimmune serum was not determined, it was found however that a

volume of 0.35 ml of hyperimmune serum was not protective. A volume of 1.5 ml and of 1.4 ml of both hyperimmune serum and hyperimmune serum IgG were completely protective. No parasites were detected in blood smears taken from both hyperimmune serum and hyperimmune serum IgG recipients during 28 days of observation.

The protective activity of hyperimmune serum by IgG antibody has also been reported for some malaria infections such as P. falciparum and P. malariae in humans (Cohen et al., 1961), P. knowlesi in rhesus monkeys (Cohen and Butcher, 1970) and in rodent malaria in P. berghei infection (Diggs and Osler, 1969; Phillips and Jones, 1972; Green and Kreier, 1978).

The protective activity of the IgM fraction of hyperimmune serum was also investigated. The results have shown that hyperimmune serum IgM was partially protective compared to hyperimmune serum IgG. The degree of protectivity of hyperimmune serum IgM was, however, dependent on the volume administered. For example, a volume of 1.5 ml delayed the onset of parasitaemia for 4-6 days, while a volume of 0.9 ml delayed the onset of parasitaemia for 3-4 days. Hyperimmune serum IgM recipients, which received either 1.5 ml or 0.9 ml, suffered, however, a severe infection and they all after died 4-5 days of patency. The concentration of IgG or IgM in the fractions injected was related to their physiological concentration in the whole unfractionated serum.

To test whether IgG in immune sera rather than hyperimmune serum was also protective the protective activity of the IgG

fractions of immune sera collected on day 7 (immediately after recovery) and day 10 (3-4 days after recovery) was compared. The results have shown that day 10 IgG was strongly protective.

In order to check whether day 10 IgG or hyperimmune serum IgG retained any protective activity in the recipients, day 10 IgG, hyperimmune serum IgG and whole hyperimmune serum recipients were challenged with a large number of parasites (1×10^8) 4-5 weeks after IgG or whole serum administration. All challenged recipients had a patent parasitaemia on day 1 which subsequently increased at the same rate as the control rat that had no serum at all, and all rats had to be treated on day 3 post challenge with the drug Diampron. Two conclusions were drawn from the challenge experiment. Firstly, the results have indicated that IgG in either day 10 or hyperimmune serum had no residual activity four weeks after administration. Campbell et al. (1956) reported that the half life of passively transferred rat gamma globulin is short lasting (6-6.5 days). Secondly, the results have also shown that volumes of 1.5 and 1.4 ml of hyperimmune serum IgG and a volume of 1.5 ml of day 10 IgG were responsible for the death of all parasites and it was apparent that no immunity had been acquired from the antigenic stimulus of the primary parasitaemia, as all challenged recipients suffered as severe an infection as the control rat.

The IgG fraction of sera collected immediately after recovery (day 7) was partially or not protective depending on the volume injected. For example, there was a delay in the onset of parasitaemia of five days when 1 ml was administered and a delay of four days when 0.65 ml were administered. Day 7 IgG

recipients which received either 1 ml or 0.65 ml of IgG suffered, however, a severe infection and they all died 4-5 days after patency. Volumes of 0.4 ml and 0.18 ml were not protective, all recipients having a patent parasitaemia on day 1 and a severe infection which ended fatally. Day 7 whole serum was, however, more protective than day 7 IgG; a volume of 1 ml serum was strongly protective. A volume of 0.2 ml delayed the onset of parasitaemia for ten days. It should be noted that factors including the number of parasites in the inoculum at the time of administration of immune serum and the concentration of antibody in the serum might directly relate to the protective activity of antibody as was reported for B. bovis in cattle (Mahoney, 1979a).

The above results have shown that day 7 IgG was partially protective compared to whole day 7 serum. It is expected that during fractionation of the serum some of the immunoglobulins might be lost and this might affect some of the results, or there might be other factors in the whole serum which are responsible for protection other than IgG. The possibility that the IgM fraction of day 7 might be protective was investigated. The results indicated that the protective activity of sera collected immediately after recovery (day 7) was mediated by IgM antibody. No reports on Babesia or malaria infections have specifically demonstrated the role of this class of immunoglobulins in protection.

The degree of protection of IgM was dependent on the volume injected. The concentration of day 7 IgM fraction was determined and was found to be 1.5 mg/ml. The concentration of IgM in day 7 serum was not, however, compared with its concentration in normal

serum. Strong protective activity was observed when 0.9 ml of day 7 IgM was injected. The IgM fraction must have been responsible for the death of all or most parasites. The few parasites that remained in the circulation were not, however, affected by the protective effect of IgM antibody and multiplied normally, and led to the death of some hosts. This would explain the death of two hosts in this experiment on days 19 and 26. This is a common feature of immune sera as explained in the previous chapter and was also reported for other Babesia and malaria infections (Callow and Dalgliesh, 1982). The protective activity of day 7 IgM was confirmed when pooled fractions of day 7 serum which included IgM and IgG immunoglobulins were injected into recipients. The pooled fractions gave approximately the same protection as the IgM fraction (there was a delay in the onset of parasitaemia for 5-7 days) and confirmed that protection was mediated by IgM since it was shown that day 7 IgG was not protective.

Immunoelectrophoresis and double diffusion tests have illustrated the presence of IgG and IgM antibodies after fractionation of day 7 serum. In some double diffusion tests, the presence of IgG or IgM could not be demonstrated when neat IgG or IgM fraction was used. This might be due to the presence of the highest level of antibody which is known as a prozone. IgG and IgM were demonstrated in this test when the IgG and the IgM fractions tested were diluted 1 in 10.

Passive transfer tests with the fractionated sera has, therefore, confirmed the role of serum gamma globulins in acquired immunity to B. divergens in splenectomised rats. The

protective activity of sera collected immediately after recovery (day 7) was mediated by IgM antibodies. The protective activity of sera collected 3-4 days after recovery (day 10) and of hyperimmune serum was mediated mainly by IgG antibody. IgM antibody in hyperimmune serum was, however, partially protection. Liddel et al. (1982), working on B. divergens "J" strain in gerbils reported a minor role for antibody in protection. They measured the antibody response in infected gerbils using IFAT and ELISA techniques and found that gerbils which received 10^6-10^9 PRBC died before an antibody response could be mounted. Animals which received less than 10^6 PRBC suffered prolonged infections which resulted in either death with little or no antibody formation, or death with significant levels of specific antibody, or recovery with no circulating antibody detectable. They suggested that these animals were able to eliminate infected cells before parasites were released and suggested the role of cell mediated immunity in eliminating infected erythrocytes. They also demonstrated that antibody was protective only when gerbils were immunised with inert Babesia antigens which were provided from cryostabilates, either prior to or concurrently with infection with intact viable parasites. Previously other workers also suggested a minor role for antibody in protection to some Babesia infections such as B. rodhaini (Roberts, 1968; Mitchell et al, 1978 and B. microti (Allison et al., 1978) infections and a role of T cells has been suggested for protective immunity in these infections.

It can be concluded that antibody plays an important role in

protective immunity against B divergens in vivo. Its mode of action is, however, unknown. Some aspects of the mechanism of action of antibody will be described in Chapter Seven.

In the next Chapter, the possibility that non splenic sources might take over antibody production and that other parts of the RES would have a major role in phagocytosis in splenectomised rats will be investigated.

Summary Table of Experiments

CHAPTER SIX

LIVER HISTOLOGY AND PRELIMINARY INVESTIGATIONS ON THE ROLE OF THE LIVER IN REMOVING PRBC'S

Chapter Six

Liver histology and preliminary investigations on the role of the liver in removing PRBC's

INTRODUCTION

It was shown in Chapter Three that the course of a B. divergens infection in splenectomised rats was short-lived, and that the animals either died from the infection or recovered and were immune to challenge. The acquisition of acquired immunity was therefore not dependent on the presence of the spleen. The mechanisms by which the parasites or PRBC were removed from the circulation of immune splenectomised rats were, however, not known. It was also shown in a previous chapter, that antibody plays an important role in protection against the infection in splenectomised rats. In this chapter, the possibility that the liver is the major site of antibody production and that other parts of the RES would have an important role in phagocytosis were investigated in splenectomised rats that had recovered from the infection.

Since the liver is considered to be the second most important organ after the spleen where PRBC might be killed and/or phagocytosed (Jandl et al., 1965; Weiss, 1985), the possible removal of B. divergens infected cells or free parasites by the liver was investigated. This was by histological examination and by following the clearance of Cr^{51} labelled B. divergens infected cells from the blood and their possible uptake by the liver in immune splenectomised rats.

In this chapter, the effect of injecting irradiated and non-

irradiated parasites into immune splenectomised rats was also compared, in order to determine whether it is the intracellular parasite or the free merozoite which is cleared by the immune animals. It is thought that irradiation of babesia parasites would inhibit parasite division, and therefore reduce the number of merozoites leaving the erythrocytes. That is, the injection of irradiated parasites into immune splenectomised rats should provide some evidence on whether infected cells and/or merozoites are phagocytosed.

It was shown in a previous chapter, that recovery from B. divergens infection in splenectomised rats was immediately followed by a pronounced leukocytosis which was predominately a lymphocytosis. It was not known whether lymphocytosis was due to newly formed lymphocytes entering the blood or due to altered patterns of migration to the different lymphoid organs. The possibility that lymphocytosis was due to newly formed lymphocytes was not investigated, but the possibility that lymphocytes might be accumulating in livers of recovered immune splenectomised rats was investigated through histological studies. Some preliminary immunohistological studies were also carried out on the livers of recovered splenectomised rats and will be briefly described in this chapter.

Experiment 6 (i):

Clearance and organ uptake of Cr^{51} labelled erythrocytes:

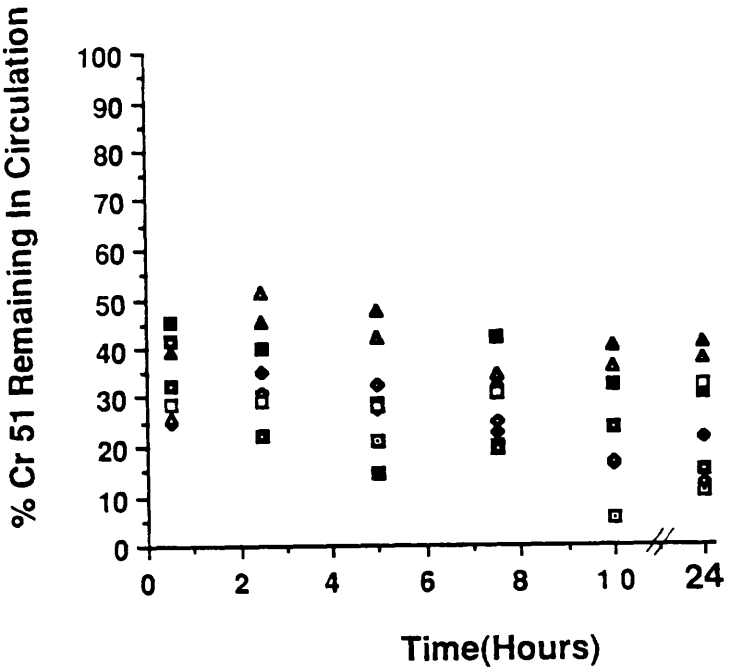
The aim of this experiment was to determine where PRBC were removed in splenectomised rats which survived the primary parasitaemia. Infected erythrocytes were labelled with Cr^{51} and

their clearance from the circulation of immune splenectomised rats and possible uptake by the liver and lung was investigated as follows:

Twenty four (12 males and 12 females) 4-5 month old splenectomised rats were injected with 1 x 10^5 PRBC i.v. Blood smears were taken daily and the parasitaemia was followed. All rats had a patent parasitaemia on day 4 and a mild infection where parasitaemia did not exceed 8-10%, after which they recovered between days 8-9 post infection. Two weeks after recovery, the rats were divided into two groups and injected intravenously with 5 x 10^8 Cr^{51} labelled PRBC, or NRBC equivalent to the total number of erythrocytes in the infected RBC inoculum (see materials and methods for labelling and handling the RBC). A group of 24 (12 males and 12 females) naive splenectomised rats were included as controls. These control rats were also divided into two groups and injected i.v. with 5 x 10^8 labelled PRBC or labelled normal erythrocytes equivalent to the total number of erythrocytes in the infected RBC inoculum. Two rats from each group were sacrificed at 30 minutes, 2.5, 5, 7.5, 10 and 24 hours post infection, and blood, livers and lungs were collected. Blood smears were also taken from the rats every 15-30 minutes. The clearance of Cr^{51} from the circulation was calculated. The liver and lung uptake of labelled erythrocytes was also calculated as described in the materials and methods chapter. The results showed that there was no significant difference in the clearance of Cr^{51} labelled PRBC or NRBC from the circulation of both immune and non immune rats (Figure 30). The clearance of

Figure 30:

Clearance of Cr^{51} labelled PRBC after i.v. injection in immune ▫ & ◆ and non immune ■ & ◆ splenectomised rats. Clearance of Cr^{51} labelled NRBC is represented by ■ & □ in immune rats and by ▲ & ▲ in non immune rats.



Cr^{51} was gradual in both groups of rats that received either Cr^{51} labelled PRBC or NRBC. The results shown in the figure indicated, however, that at 24 hours the percentage of Cr^{51} in the circulation of immune or control rats that received labelled PRBC was less than (11-22%) the percentage of Cr^{51} that remained in the circulation of immune or control rats that received labelled NRBC (31-38%). Parasitaemias, as determined from blood smears, rose in non immune splenectomised rats, but decreased in immune rats and no parasites were detected in blood smears taken at 24 hours (Figure 31). There was also no difference in the uptake of Cr^{51} by the livers of immune and non immune rats that received Cr^{51} labelled PRBC (Figure 32). There was, however, a higher uptake of Cr^{51} by the livers of immune and non immune rats that received Cr^{51} labelled infected cells compared to the uptake of Cr^{51} by the livers of both immune and non immune rats that received Cr^{51} labelled NRBC (Figure 32). No noticeable uptake of Cr^{51} was observed in the lungs of immune and non immune rats (Figure 33). This experiment was repeated twice and the same results were obtained.

Experiment 6 (ii):

The effect of injection of irradiated parasitised red cells into immune and non immune splenectomised rats

In the previous experiment, it was shown that it was not possible to determine whether the infected erythrocytes were removed by the livers of immune rats. The aim of the following experiment was to examine whether it was intact PRBC or free merozoites which are cleared by the immune rats. Irradiated and

Figure 31:

Percentage parasitaemia resulting from the inoculation of Cr^{51} labelled PRBC in immune and non immune splenectomised rats.

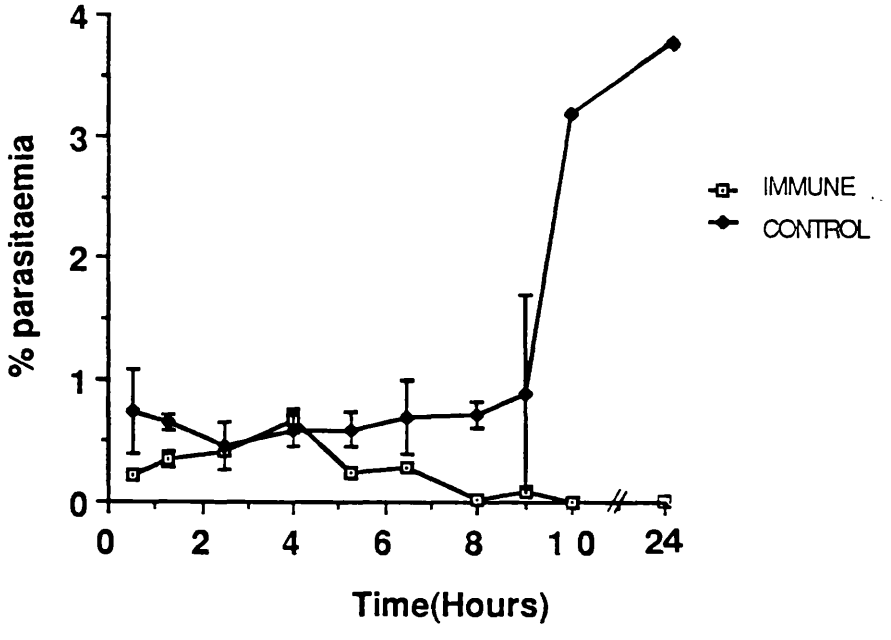


Figure 32:

Liver uptake of Cr^{51} labelled PRBC after i.v. injection in immune ▫ & ◆ and non immune ▫ & ◆ splenectomised rats. Uptake of Cr^{51}1 labelled NRBC is represented by ■ & ▫ in immune rats and by ▲ & △ in non immune rats.

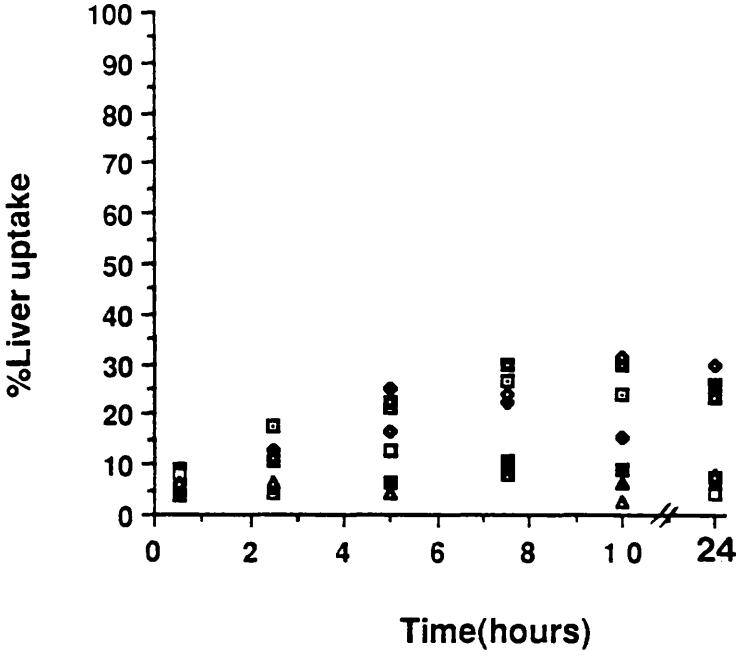
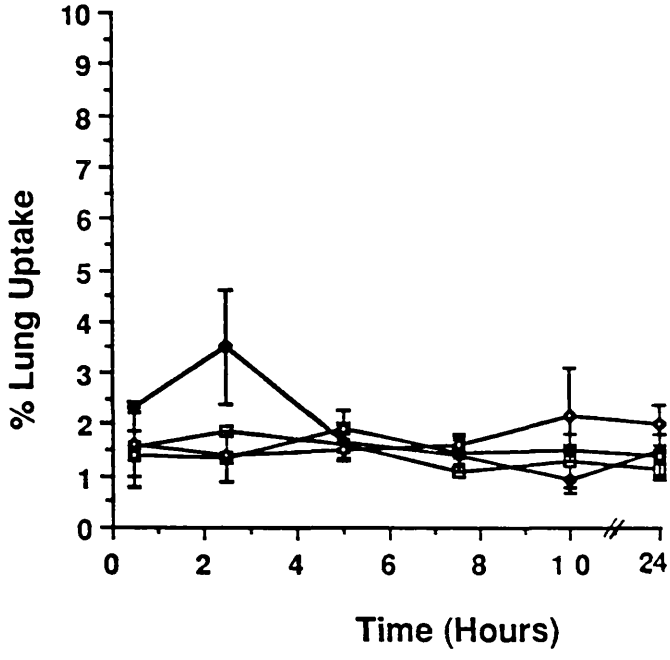


Figure 33:

Lung uptake of Cr^{51} labelled PRBC after i.v. injection in immune ⊡—⊡ and non immune ◆——◆ splenectomised rats. Uptake of Cr^{51}1 labelled NRBC is represented by ▫——▫ in immune rats and by ◇——◇ in non immune rats.



non-irradiated parasites were injected as follows:

1. Preparation of immune rats

Four (three male, one female) 4 month old splenectomised rats were injected with 1 x 10^4 PRBC i.v. Blood smears were taken daily and the parasitaemia was followed. All rats had a patent parasitaemia on day 4 and a mild infection where parasitaemia did not exceed 10% after which they recovered on days 10-11 post infection.

2. Irradiation of parasites

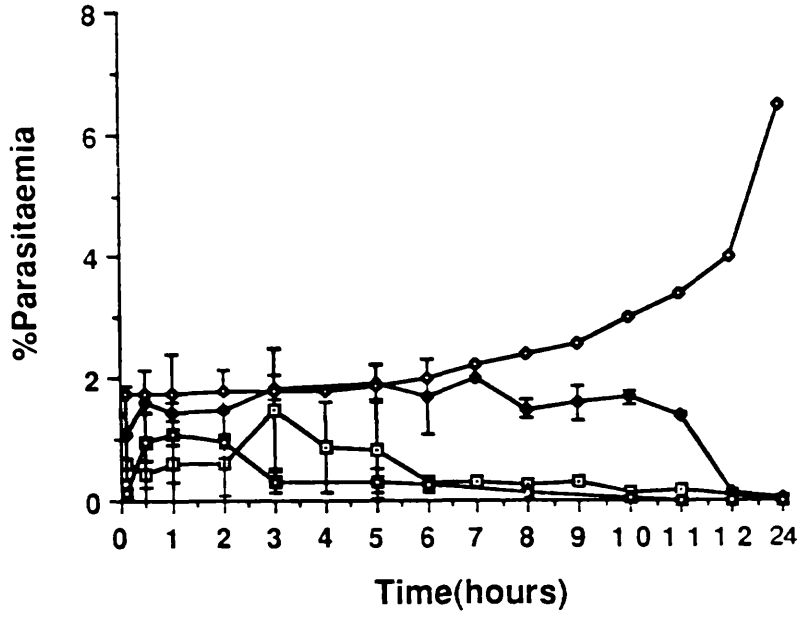
Infected blood was irradiated as described in the materials and methods chapter. Some infected blood was not irradiated and received the same handling as the irradiated blood before it was injected into the rats.

3. Injection of parasites and collection of data

The immune rats were divided into groups of two. Four (two male and two female) splenectomised naive rats were included as controls and were also divided into groups of two. One group of immune and one group of control rats were injected with 1.2 x 10^9 irradiated PRBC i.v. The other group of immune and control rats received the same number of non irradiated PRBC i.v. A blood smear was taken immediately from each rat and then every 15-30 minutes for 12 hours and at 24 hours after the start of the experiment. The results shown in Figure 34 indicate that immune rats cleared irradiated parasites more rapidly than did normal rats. There was no obvious difference between the clearance of non irradiated and irradiated parasites in immune rats. Non

Figure: 34

The effect of injecting Co^{60} irradiated parasites into immune ⊡—⊡ and non immune ◆—◆ splenectomised rats. Normal(Non irradiated)parasites are represented by ■—■ in immune rats and by ◇—◇ in non immune rats.



irradiated parasites multiplied normally in non immune rats. This experiment was repeated twice and the same results were obtained.

Experiment 6 (iii):

Histological and histochemical studies

It has been reported that the spleen is an important organ in the initiation of primary antibody response and it is the main site of phagocytosis of infected and uninfected erythrocytes (Phillips, 1969c). It has also been reported that splenectomy reduces the level of antibodies against various antigens present in the blood circulation (Winebright and Fitch, 1962; De Carvalho et al., 1967). Since the liver was reported to be the second most important organ in the RES where PRBC sequester in rats (Ultmann and Gordon, 1965), the possibility that the liver would take over the function of the spleen in antibody production and phagocytosis of infected erythrocytes and/or free parasites was examined in splenectomised rats. It was shown previously that recovery from the infection in splenectomised rats was followed by lymphocytosis. The possibility that lymphocytes might be accumulating in the liver of immune rats was also investigated. Histological studies were carried out on livers of splenectomised rats that recovered from the primary infection and on livers of immune reinfected rats as follows:

Experiment 6 (iii) A:

Liver histology in immune rats

A group of 10-12 male and female 4-5 month old splenectomised rats were injected with 1 x 10^5 PRBC i.v. The rats had a patent parasitaemia on day 4 and a mild infection with a parasitaemia not exceeding 7-9% after which they recovered on day 10-11. The rats were reinfected one day after recovery with 1 x 10^9 PRBC i.v. Blood smears were taken from the tail vein of the rats at 30 minute intervals. The course of parasitaemia is shown in Figure 35. Two rats were sacrificed at two hour intervals for 12 hours and their livers collected (see Figure 35). Impression smears were made from the livers of challenged rats and the rest of the tissue was cut into small pieces and processed for histology as described in the materials and methods chapter. Parasitaemia was 3.3 - 3.5% at 30 minutes (see Figure 35) and then started decreasing subsequently until no parasites were detected in blood smears taken 10 hours post challenge. In the liver sections of these rats an accumulation of cells was observed around the central veins (Figure 36A & B). All sections collected between 2 and 12 hours contained these accumulations, but they appeared large at seven hours. No parasitised cells or parasites were detected in impression smears made from the livers at the same intervals. Livers collected from naive splenectomised rats did not show any accumulation of cells (Figure 36D).

Figure 35:

The course of parasitaemia in splenectomised rats challenged with $1x10^9$ PRBC one day after recovery from the primary infection.

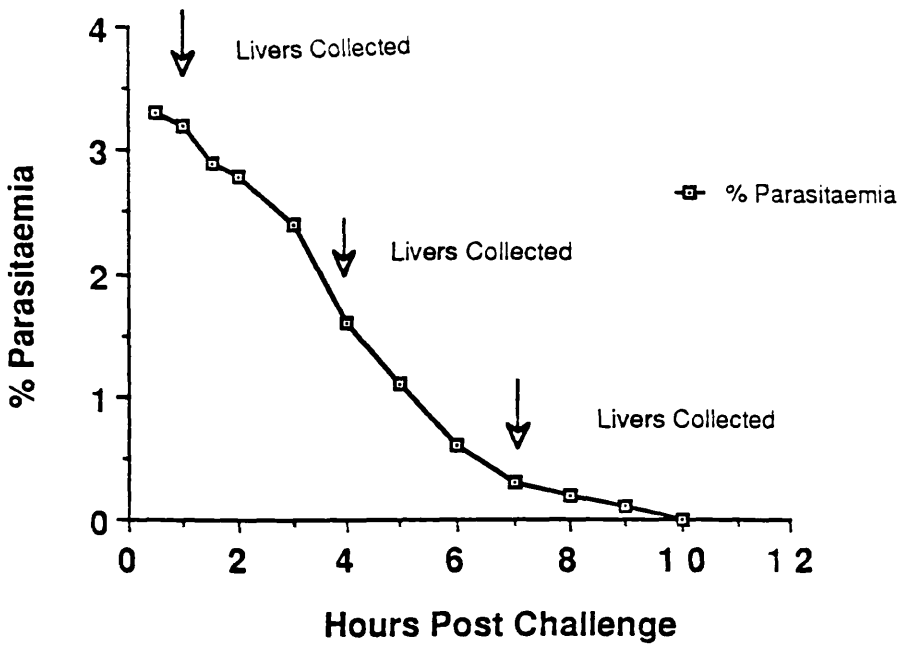
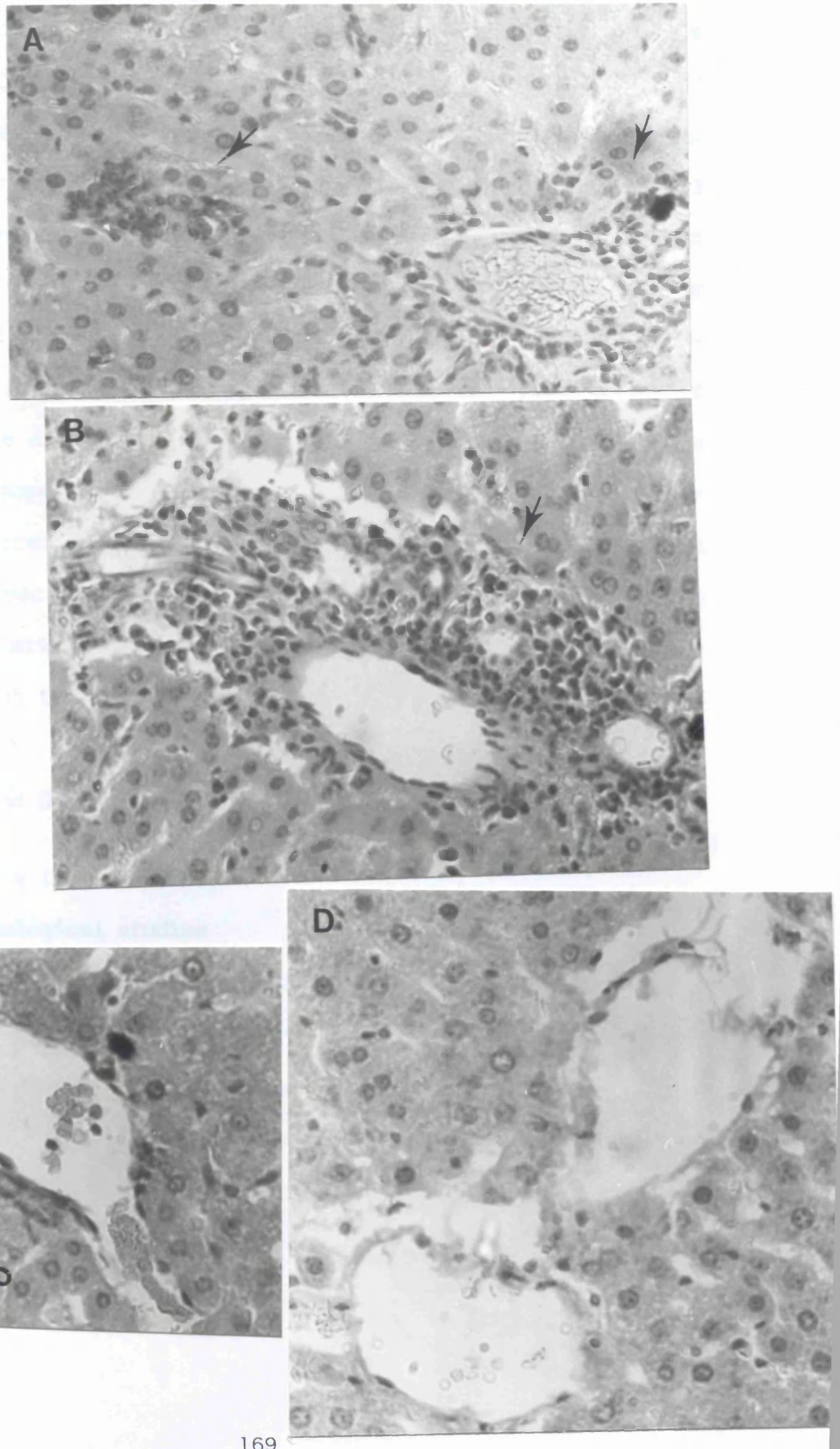


Figure 36:

Liver histology:
Accumulation of cells in livers of rats which recovered from the primary infection and in immune rats compared to naive rats.



Experiment 6 (iii) B:

Liver histology in primary infection

In a series of experiments, groups of 4-5 month old splenectomised rats were injected with 1 x 10^4 PRBC (avirulent blood). The parasitaemia in the rats was followed in blood smears taken daily. The rats normally had a mild infection which lasted for 4-5 days after which they recovered. Groups of 2-3 rats were sacrificed during the infection (day 3) and at different times after recovery from day 6 to day 368 and their livers collected. The livers were processed for histology as described in the materials and methods chapter. Some pieces of livers were also snap frozen in liquid nitrogen after they were fixed in isopentane and then stored at -70°C. The liver sections showed accumulations of cells around the central veins as those described for immune rat (Figure 36A & B). All livers collected between day 3 and day 330 contained these accumulation of cells but they appeared large and most frequent between day 10 and day 22. By day 368, these accumulations had largely disappeared (Figure 36C).

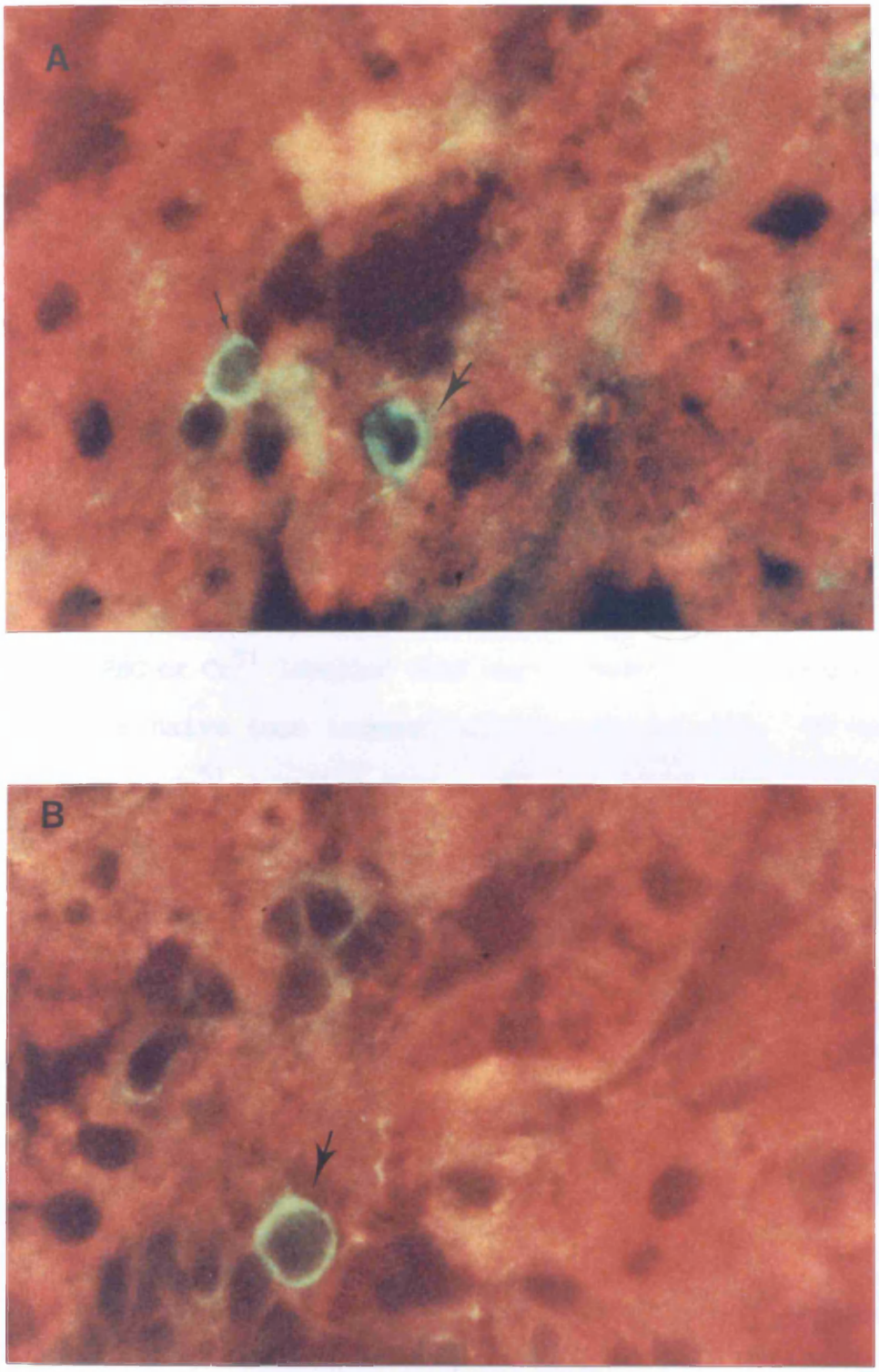
Experiment 6 (iv):

Immunohistological studies

Preliminary immunohistological studies were carried out on livers collected on day 10 and 13 after the primary infection. The method followed is described in chapter three. The results showed that in livers collected on both days (i.e. day 10 and day 13) the cell accumulations consisted of B and T cells (Figure 37A & B).

Figure 37:

Immunofluorescence for analysing B and T lymphocytes in livers of immune rats.



Discussion

It has been shown in a previous chapter that splenectomised rats either died from the infection or recovered and were immune to challenge and it was concluded that the development and persistence of acquired immunity was not dependent on the presence of the spleen. In this chapter, the possible mechanisms by which splenectomised rats survived the primary parasitaemia were investigated. It has been reported that the spleen and the liver are the major site of sequestration of infected red cells in rats (Ultmann and Gordon, 1965). The possible removal of B. divergens PRBC by the livers of immune splenectomised rats was, therefore, investigated. To study this possibility, the clearance of Cr^{51} labelled B. divergens infected erythrocytes was followed in splenectomised rats. The aim of this was to test whether Cr^{51} labelled PRBC are cleared from the circulation of immune rats, through uptake and phagocytosis by the liver. Cr^{51} labelled PRBC or Cr^{51} labelled NRBC were transferred to recovered (immune) or naive (non immune) splenectomised rats. It was expected that Cr^{51} labelled PRBC would be removed more rapidly from the circulation in immune rats than in non immune rats, and they would be taken up by the livers of immune rats. The results showed that there was no significant difference in the clearance of Cr^{51} from the circulation of immune and non immune rats which received either Cr^{51} PRBC or NRBC, although the parasitaemia in immune rats decreased subsequently until no parasites were detected at 24 hours. In contrast parasitaemia in the non immune rats continued to rise. The results, however, indicated that at 24 hours, the percentage of Cr^{51} in the circulation of immune or

control rats that received labelled PRBC was less than (11-22%) the percentage of Cr^{51} that remained in the circulation of the immune or control rats that received labelled NRBC (31-38%).

There was also no difference in the uptake of Cr^{51} by the livers of immune and non immune rats that received Cr^{51} labelled PRBC. There was, however, a higher uptake of Cr^{51} by the livers of immune and non immune rats that received Cr^{51} labelled PRBC compared to the uptake of Cr^{51} by the livers of both immune and non immune rats that received Cr^{51} labelled NRBC. This would therefore explain why the percentage of Cr^{51} that remained in the circulation of the immune or the control rats that received Cr^{51} labelled PRBC was less than the percentage of Cr^{51} that remained in the circulation of the immune or the control rats that received Cr^{51} labelled NRBC. It is known from other studies (Ebaugh et al., 1953) that Cr^{51} labels the erythocyte haemoglobin. It is also known that the erythrocyte haemoglobin is removed in the liver after the disintegration of the red cells where it is excreted as one of the bile pigments in the faeces. It is possible, therefore, that the small uptake of Cr^{51} observed in the livers of immune rats that received Cr^{51} labelled NRBC was because of the rapid excretion of the released Cr^{51} after it was removed from the liver. On the other hand, the higher uptake of Cr^{51} in immune rats which received Cr^{51} labelled PRBC may have been either due to the removal of PRBC by the liver where they were phagocytosed, or that the free merozoites were destroyed by antibody as they emerged from the erythrocytes and the damaged red cells containing the chromium were removed by the liver. The

higher uptake of Cr^{51} by the livers of immune rats which received labelled PRBC might have resulted from the uptake into their livers of ruptured erythrocytes. The released merozoites must have invaded unlabelled erythrocytes and this was reflected in the rising parasitaemia in these rats. In immune rats, it is possible that the process of removal of infected erythrocytes or free merozoites happens very quickly. This may occur in the peripheral blood, as it was reported for P. falciparum (Trubowitz and Masek, 1968) in which the ingestion of merozoites by polymorphonuclear leukocytes was observed in blood smears taken from infected patients, or, by phagocytosis of parasitised erythrocytes by peripheral monocytes as was reported by Vernes (1980) in blood smears taken from children infected with P. falciparum in Gabon. Alternatively the PRBC could have been damaged through an ADCC through monocytes. An indirect evidence of ADCC has been reported for P. chabaudi in mice (McDonald and Phillips,1978). Blood smears taken from rats that recovered from a B. divergens infection, did not, however, show any phagocytosed parasites or parasitised erythrocytes and impression smears taken from livers of immune rats did not show any parasites or parasitised cells phagocytosed by macrophages.

Since the Cr^{51} experiment did not show evidence that PRBC were removed by phagocytosis in the liver, irradiated parasites were injected into immune rats in another experiment to determine the stage of the parasite which is cleared by the immune rats. It is thought that irradiation of babesia parasites would inhibit parasite division and therefore reduce the number of merozoites leaving the erythrocytes. The results showed that immune rats

cleared parasites slightly more rapidly than did normal rats. The similar rates of elimination of normal and irradiated parasites by immune rats might indicate that although the parasite is prevented from leaving the cell, it is still cleared from the circulation at the same rate as in the rats that were injected with normal non irradiated parasites. It is possible therefore that protective antibodies reacted with infected cells. The red blood cells and the parasites were then removed perhaps by lysis or phagocytosis and it was not necessary for the parasites to be extracellular to be destroyed. That is, immune splenectomised rats were therefore able to clear PRBC from the blood stream and that immunity was not specifically directed at merozoites. This would explain the higher uptake of Cr^{51} by the livers of immune rats and indicate that infected red cells might have been phagocytosed in their livers. Roberts and Tracey Patte (1974) working on B. rodhaini in mice did similar work to that described above with irradiated parasites, and although they were uncertain about the method by which the parasites were killed in the infected mice, they speculated that parasites inside red cells were removed by lysis or phagocytosis.

The non specific RES system clearance has also been suggested in malaria, by examining the intravascular clearance of inert particles such as colloidal carbon, I^{125} microagregated albumin, and ^{99m}Tc sulphur colloid (Lucia and Nussenzweig, 1969; Sheagren et al., 1970; Kitchen and Diluzio, 1971; Loose and Diluzio, 1976), but, in Babesia, there are no reports of intravascular clearance of parasitised erythrocytes by the RES.

Quinn and Wyler (1979) working on P. berghei in rats did similar work as described at the beginning of the discussion, where they examined the clearance of Cr^{51} labelled P. berghei infected erythrocytes in intact rats. They found that P. berghei infected erythrocytes were removed more rapidly from the circulation than uninfected erythrocytes. The accelerated clearance was more rapid in rats immune to P. berghei than in non immune infected rats. They observed that this accelerated clearance was dependent upon splenic uptake, but all differences in clearance rates were, however, abolished by splenectomy, since they found that the clearance rates were similar in both immune and non immune splenectomised rats. They also observed that the parasitaemia in splenectomised immune rats did not rise after challenge, while there was a continuous rise of parasitaemia in non immune splenectomised rats as was observed in the present study. These authors concluded that the ability of immune rats to resist challenge with P. berghei might depend upon mechanisms which are largely spleen independent. They are therefore in agreement with observations made in the present study, although no clearance of Cr^{51} was observed both in rats immune to B. divergens and in non immune rats. Cr^{51} appeared to be accumulating in the circulation and the reason for this is not known.

In the present study, the reason why there was no significant difference between the clearance of parasites by immune rats and non immune rats when irradiated parasites were injected into both groups of rats is not known. Studies on B. rodhaini (Phillips, 1970a;1971), B. bovis (Mahoney et al.,

1973a), B. bigemina (Bishop and Adams, 1974), B. major (Purnell et al., 1978; 1979) and B. divergens (Lewis et al., 1979a), have demonstrated that when these Babesias were irradiated with a dose of ionising radiation sufficient to stop them reproducing, they still retained their capacity to induce a protective immune response in susceptible hosts. Wright et al. (1980), working on B. bovis in splenectomised calves concluded that the cause of mild infections in these animals was due to the selection by irradiation of an avirulent parasite population, by killing the more virulent individuals. Similarly, Waki and colleagues (1982), working on P. berghei in mice reported that a strain of P. berghei consisted of heterologous populations different in virulence and susceptibility to irradiation and concluded that parasites attenuated by irradiation lack a certain metabolic function. The same can be applied to the irradiated B. divergens and it could be that B. divergens parasites attenuated by irradiation differ in their metabolic activity and this perhaps would explain the lack of remarkable difference between the clearance of irradiated parasites by both immune and non immune rats.

It has been shown in Chapter Four that antibody plays an important role in protection. It is well known that the spleen plays an important role in antibody production and it is the major site of phagocytosis of infected and non infected cells as was reported for B. rodhaini in rats (Phillips, 1969c). Phillips (1969c) suggested that in splenectomised rats, extra splenic sites in the rat can function in synthesis of antibabesial

antibody. In the present study, in the absence of the spleen, non splenic sources must have taken over antibody production and other parts of the RES must have an important function in phagocytosis, but as was mentioned in the beginning of the discussion impression smears taken from livers of immune rats did not show any parasites or PRBC phagocytosed by macrophages. It was shown, however, in Chapter Three, that recovery from the infection was immediately followed by leukocytosis which was predominately a lymphocytosis. Histological studies on livers collected from immune rats gave evidence that lymphocytes are accumulating in this organ. Accumulation of cells at the central veins that had the appearance of "pseudofollicles" as described by Weiss (1985) in splenectomised gerbils infected with P. berghei, were observed in livers collected from immune rats, recovering from a primary parasitaemia and in immune challenged rats. In livers collected during or after recovery from the primary parasitaemia the accumulation of cells appeared in all livers collected between day 3 and day 330, but they appeared large between day 10 and day 22. By day 368, areas devoid of cells were observed but few cells could still be seen. In immune challenged rats, the accumulation of cells was seen in livers collected between two hours and twelve hours post challenge but they appeared large at seven hours.

Weiss (1985) reported that in immune splenectomised gerbils, the secondary splenic germinal centre function appeared to be taken over by these pseudofollicles. She reported that these pseudofollicles consisted of B cells, T cells and macrophages containing malarial pigments that were similar to pigment

containing macrophages appearing in splenic germinal centres and concluded that the germinal centres and the hepatic follicles have a similar function i.e. secondary antibody response. In the present study preliminary histological studies on livers collected from immune rats have indicated that these accumulations of cells also consisted of B and T cells. The macrophage activity in these accumulations was not, however, tested. It was concluded therefore, that the leukocytes accumulating in this organ (i.e. the liver) might be very important in the development of acquired immunity to B. divergens in splenectomised rats. Similar observations have been reported for P. yoelii (Playfair and DeSousa, 1982) and P. chabaudi infections (Kumararatne et al., 1987).

It has been reported for malaria infections that toxic oxygen metabolites produced by activated macrophages play an important role in the in vivo destruction of malaria parasites (Allison and Eugui, 1983; Dockrell and Playfair, 1984; Brinkman et al., 1985). Brinkman et al. (1985) reported that the production of these toxic oxygen metabolites by macrophages is enhanced by lymphokines secreted by antigen specific T lymphocytes (i.e. through a delayed type of hypersensitivity). Kumararatne et al. (1987) speculated that the accumulation of cells observed in mice controlling a P. chabaudi infection might be the major site for parasite destruction via a delayed type of hypersensitivity response. Similar mechanisms might therefore be important for the destruction of parasites in splenectomised rats controlling a B. divergens infection.

CHAPTER SEVEN

THE ADAPTATION OF THE RAT ADAPTED STRAIN OF *Babesia divergens* TO CONTINUOUS CULTURE IN RAT ERYTHROCYTES AND SOME ASPECTS ON THE MECHANISMS OF ACTION OF ANTIBODY *in vitro*

Chapter Seven

The adaptation of the rat adapted strain of *Babesia divergens* to continuous culture in rat erythrocytes and some aspects on the mechanisms of action of antibody *in vitro*

INTRODUCTION

It was shown in Chapters Four and Five that immunity can be transferred with serum and that antibody plays an important role in protection in splenectomised rats. In recent years, methods for *in vitro* cultivation of malaria and *Babesia* have been developed and proved to be a laboratory tool for investigation of various aspects of human malaria and bovine babesiosis that were not possible before (Trager and Jensen, 1976; Levy and Ristic, 1980).

In order to compare the protective activity of immune serum *in vivo* with its effect on parasite growth *in vitro*, a method for cultivation of *B. divergens* in rat erythrocytes was established. In this chapter, the adaptation of the rat adapted strain of *B. divergens* to continuous culture in rat erythrocytes is described. Some aspects on the mechanisms of action of antibody and on cellular immunity were also investigated *in vitro*, and the results will be described and compared with those reported for other *Babesia* and malaria species.

Experiment 7 (i)

Cultivation procedure

The rat adapted strain of *B. divergens* was obtained from a stabilate (WEP 37) which had been passaged several times in

splenectomised rats before it was put into culture. The method for cultivation of B. divergens, using the Candle Jar technique (Trager and Jensen, 1976) was described in the materials and methods chapter. B. divergens was cultured continuously for six months in rat erythrocytes using this technique (Figure 38). The cultures were initiated with defibrinated blood from a splenectomised infected rat. The use of standard anticoagulants in blood collection, such as heparin, have not been tested, but it was found that cultures where blood was collected in heparin grew as well as those initiated with defibrinated blood (Phillips, personal communication). Parasitaemias exceeding 35% infected erythrocytes have been observed in cultures which were initiated with 2% infected red cells (Figure 39). At high parasitaemia the cultures became dark. Subcultures were made every 48-72 hours by diluting cultures which had reached a 10-12% parasitaemia, by 1 in 5 or 1 in 6 with normal rat erythrocytes. After eight weeks in culture (approximately 24-26 subcultures) the parasites still caused a severe disease in a splenectomised rat (Figure 40). The morphology of the parasite in Giemsa's stained blood smears was similar to that described in rat blood smears. Typically divergent forms, oval, rings and maltese cross forms were seen (Figure 39). At high parasitaemias, a large number of extracellular merozoites were noticed in blood smears taken from these cultures (Figure 39). These free merozoites were collected then frozen or used for other experiments as will be described later in the text.

Figure 38:

Growth of *B divergens* (rat adapted strain) in continuous culture. Arrows indicate subcultures (cultures diluted to 2%)

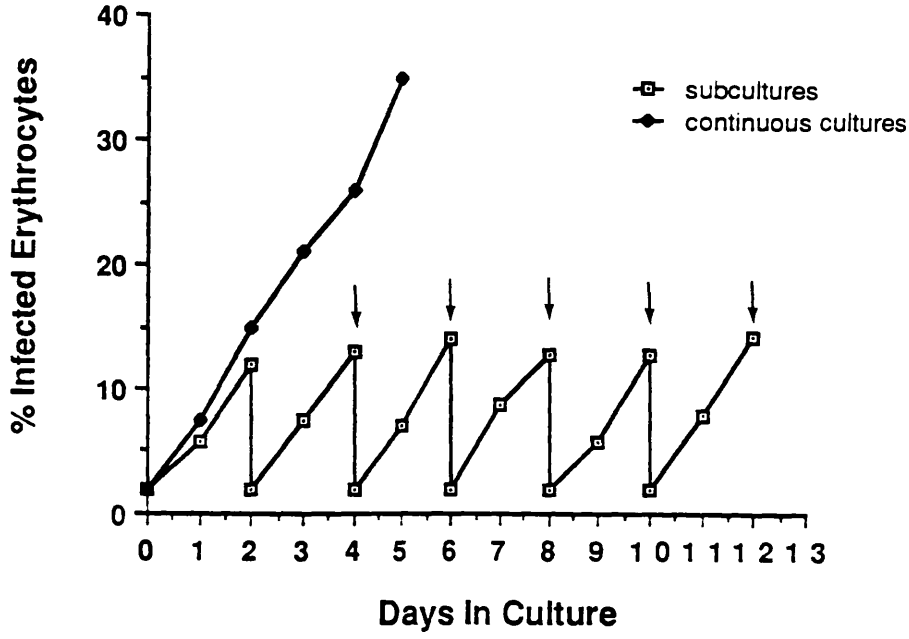


Figure 39:

The morphology of *B divergens* in cultures with a parasitaemia of 35%.

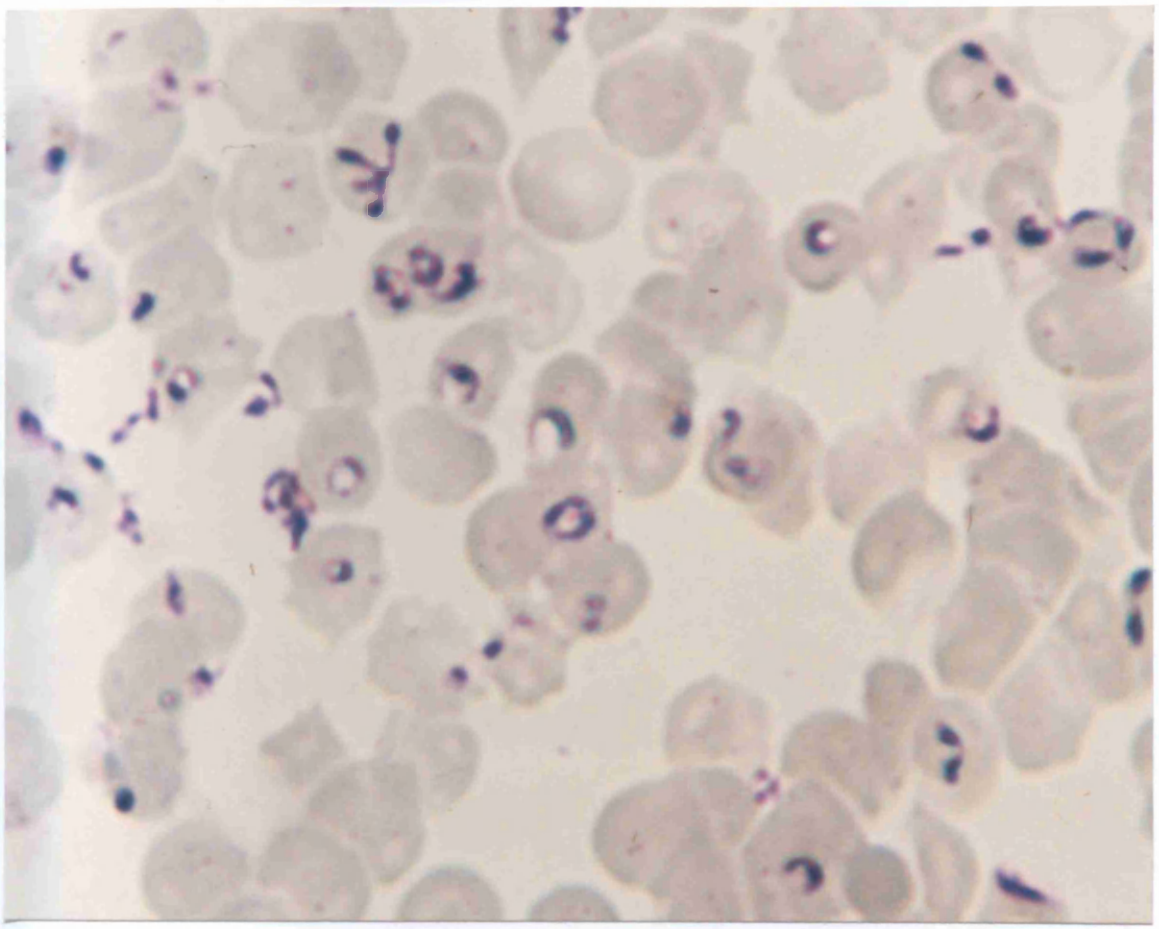
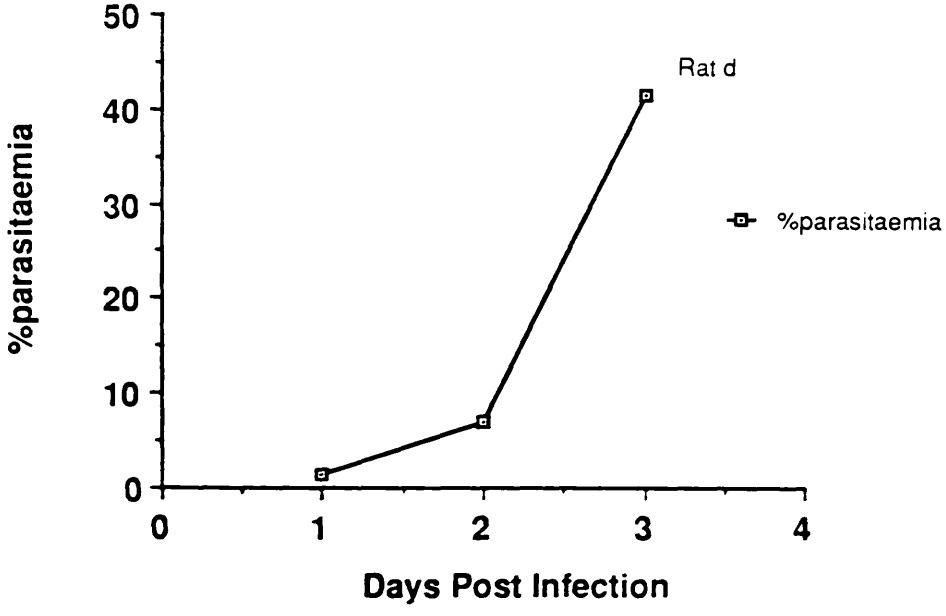


Figure 40:

The effect of injecting 8 week *B. divergens* cultures into a splenectomised rat.



Experiment 7 (ii)

Modification of foetal calf serum (FCS)

Only two batches of FCS were tested in culture. Complete RPMI medium containing 20% of either FCS batch no.10F154SA for general use or heat inactivated FCS were tested. Both sera appeared to support parasite growth in culture and no difference in parasite growth was observed in either cultures (Figure 41). It was decided therefore to use one batch of FCS (10F154SA) for preparing complete media for feeding the cultures.

Experiment 7 (iii)

Length of storage of normal rat erythrocytes

The normal rat erythrocytes used for diluting the cultures were used either as fresh i.e. collected from a normal rat on the same day, or they were kept at 4°C for 1-3 weeks before they were used for diluting the cultures. Normal erythrocytes were washed three times in incomplete medium (Appendix B) at a speed of 300 g for five minutes. It was found that cultures diluted either with fresh rat erythrocytes or erythrocytes stored for 1-3 weeks both grew very well and no difference was observed in parasite growth in both cultures (Figure 42).

Experiment 7 (iv)

Recovery of cryopreserved cultures

The method for cryopreservation and recovery of Babesia cultures was described in the materials and methods chapter. Attempts to recover the cultures using the described method were

Figure 41:

A comparison of the growth of *B. divergens* in cultures containing FCS or heat inactivated FCS.

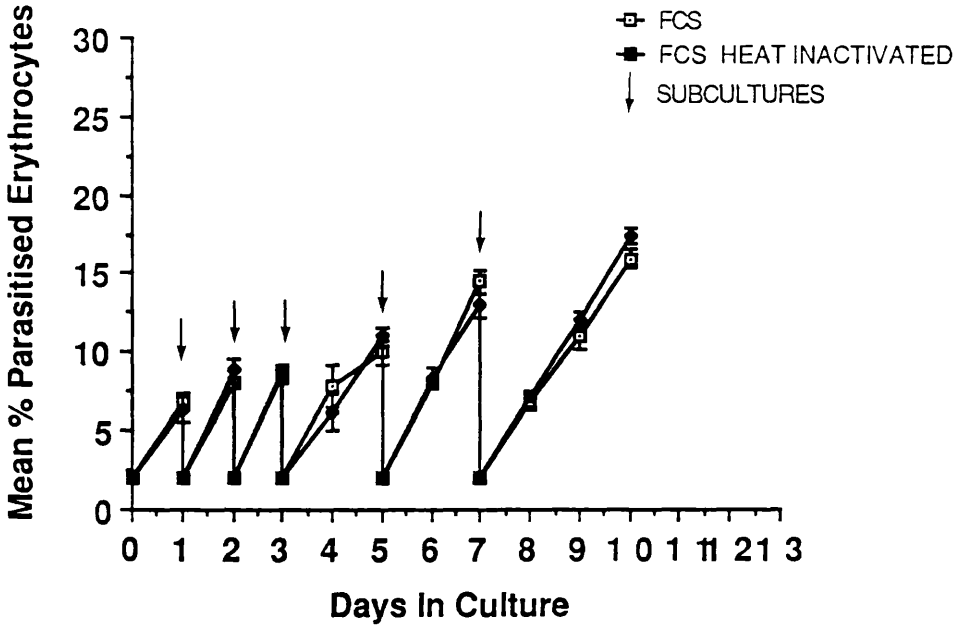
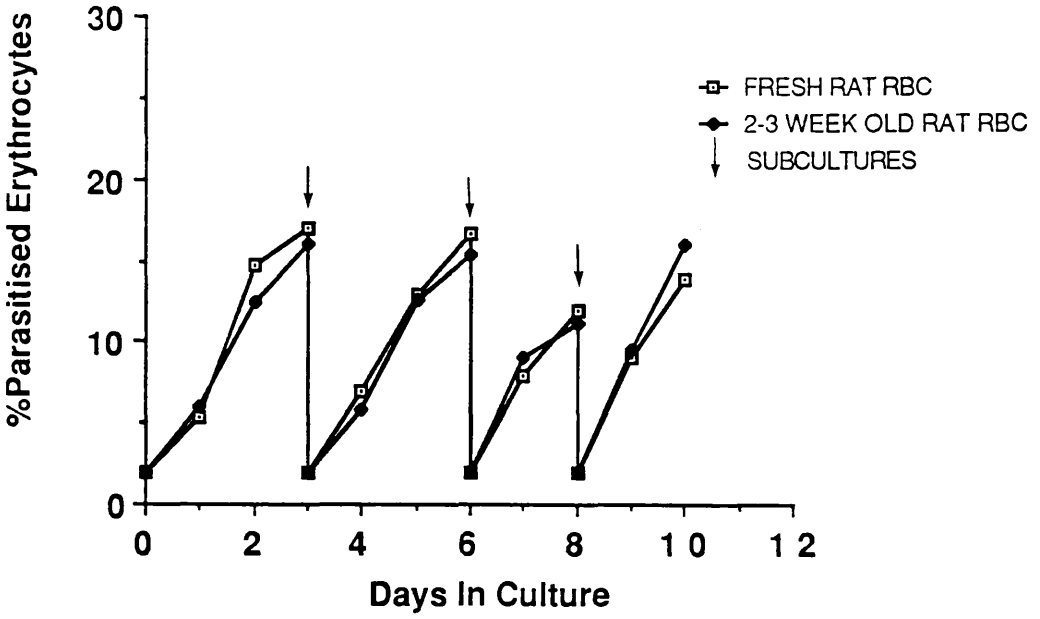


Figure 42:

A comparison of the growth of of *B. divergens* in cultures containing fresh, normal rat erythrocytes or 2-3 week old rat erythrocytes.



unsuccessful. After the addition of 0.5 mls of 4.5% saline and 4.5 mls of 3.5% saline then centrifugation, a lot of haemolysis was observed. There was difficulty in resuspending the pellets of erythrocytes in incomplete medium since they tend to clump and even when they were resuspended in the medium, they all lysed after centrifugation (N.B.: parasites cryopreserved by this method were viable in vivo).

Experiment 7 (v)

Recovery of cultures that were kept at low temperature ($4^{o}C$)

It was shown above that it was not possible to recover cryopreserved cultures. It was decided therefore to test the effect of low temperature ($4^{o}C$) on the growth of cultures as follows:

Cultures with 15-20% infected erythrocytes were emptied into sterile tubes, spun at 300 g for ten minutes and the supernatant removed. The cultures were resuspended with fresh complete medium and kept at $4^{o}C$. They were then recovered after 4, 5, 8 or 11 days. The cultures were spun and the supernatant was removed. A blood smear was taken from each culture where a large number of degenerated parasites could be seen. 50-100 ul of normal rat erythrocytes were added and a 10% suspension was made with complete medium. The cultures were placed in 35 mm petri dishes with 1.5 ml in each, and put in a candle jar and in a $37^{o}C$ incubator. Blood smears were taken daily and the growth of the parasite was followed. The parasites started appearing on days 3-5 in cultures that were kept for 4-5 days at $4^{o}C$, and on days 6-8 in cultures which were kept for 8-11 days at $4^{o}C$

(Figure 43). The parasite started multiplying normally and the cultures subsequently grew normally. A percentage of 20-25 or more of infected erythrocytes was eventually observed.

Experiment 7 (vi)

Collection of free merozoites

Attempts to collect free merozoites from cultures in a CO_2 deprived atmosphere (Levy and Ristic, 1980; Winger et al., 1987a, b) were unsuccessful. Cultures with 8-15% infected erythrocytes were deprived of CO_2 by placing them on soda lime for 6-8 hours. No major decrease in the percentage of infected erythrocytes was observed, and few extracellular parasites were seen in blood smears taken from these cultures. Free merozoites were collected from cultures with a high percentage of infected erythrocytes (25-30%) without depriving them from CO_2 as described in the materials and methods chapter. A large number of merozoites can be obtained in these cultures and they can be easily seen moving when a drop of the resuspended pellet is placed on a haemocytometer and a x 100 objective microscrope lens is used.

Experiment 7 (vii)

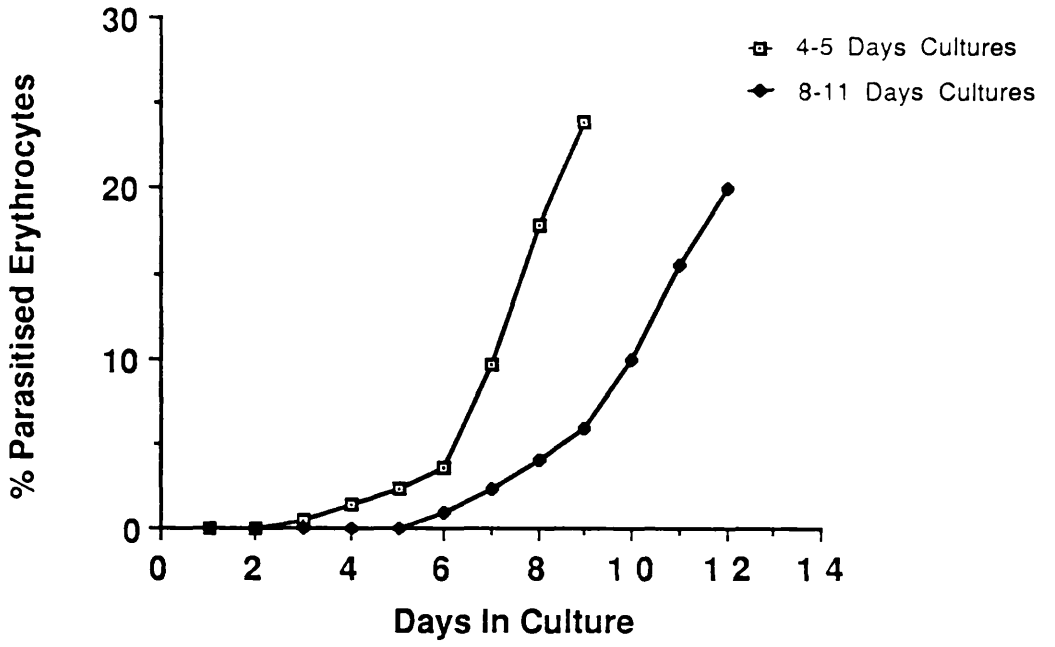
Cryopreservation and recovery of free merozoites

The method for cryopreservation and recovering of free merozoites is as described in the materials and methods chapter. Some of the cryopreserved merozoites were recovered and tested in culture as follows:

A cryopreserved merozoite ampoule was thawed, and the

Figure 43:

The effect of recovering cultures kept at 4°C for 4, 7 and 11 days.



contents emptied into a sterile tube. This was spun at 1000 G for ten minutes, the pellet was resuspended in a small volume of complete medium and then added to a 10% suspension of normal rat eryhrocytes in complete medium. Blood smears were taken daily, and the medium changed daily. One or two free merozoites were seen in blood smears taken on day 3. On day 4, 1-2 infected erythrocytes were seen. The number of infected erythrocytes started increasing (Figure 44), indicating that the parasite was multiplying and the cultures subsequently grew normally.

Experiment 7 (viii)

The effect of addition of immune or normal serum into culture

The protective activity of immune serum in vivo was compared with its effect on parasite growth in vitro. In this preliminary experiment, the effect of adding immune or normal serum into culture was investigated. The cultures were initiated with a 2% infected erythrocytes as described before, and 1.5 ml of cultures were dispersed in 35 mm petri dishes. 0.1 ml of either immune or normal rat serum were added to the cultures at 0, 24 and 48 hours to obtain an approximate concentration of 6-7% of immune or normal serum in culture. Duplicate cultures for testing the effect of immune or normal serum were made. Cultures which contained no serum were included as a control. Smears from the cultures were taken at 24, 48 and 72 hours and the percentage of infected red cells was determined. The results have shown that both immune and normal rat sera inhibited parasite growth (Figure 45). There was a slight increase in the percentage of infected erythrocytes at 24 hours in both immune and normal serum

Figure 44:

The growth of *B. divergens* free merozoites recovered from liquid nitrogen in cultures containing normal rat erythrocytes.

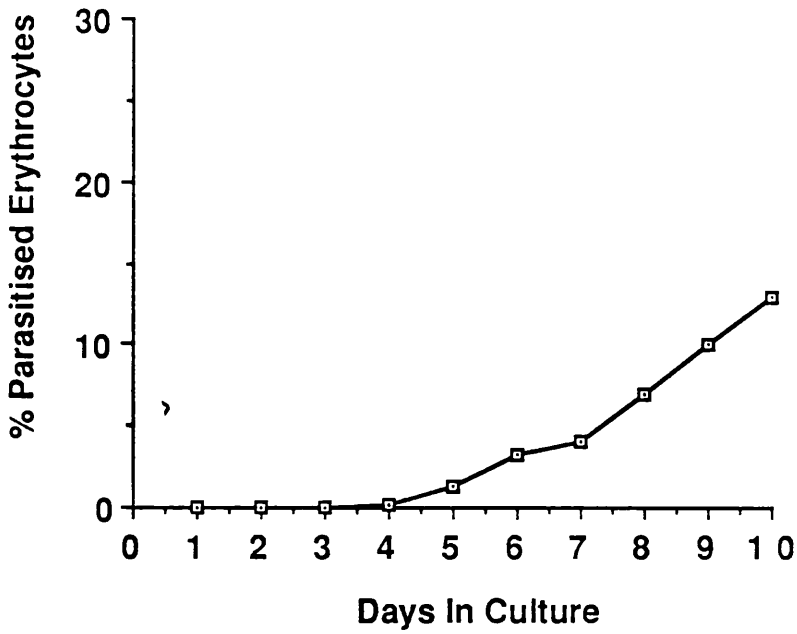
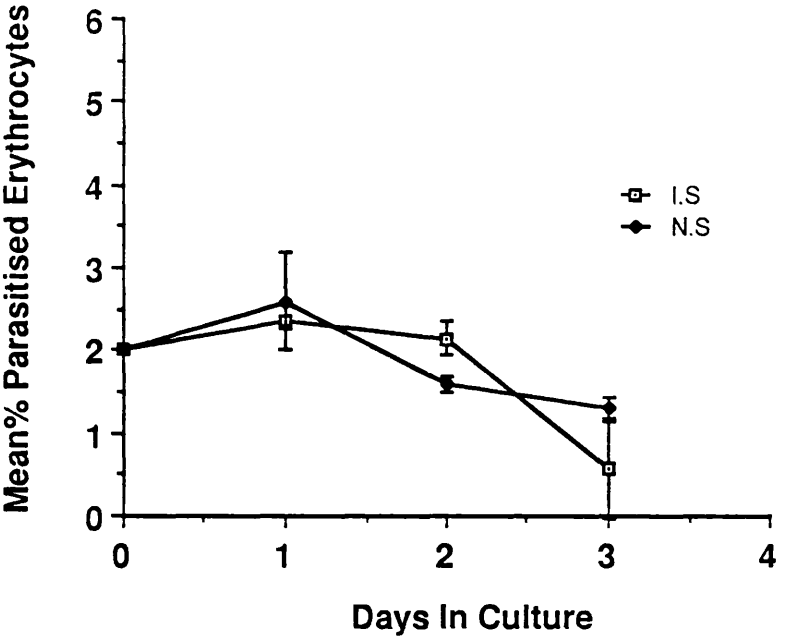


Figure 45:

The effect of adding whole immune serum or normal serum into *B. divergens* culture.



cultures. By day 3 most of the parasites in both cultures looked degenerated and were dying. Cultures that had no serum at all grew normally. This experiment was repeated and the same results were obtained.

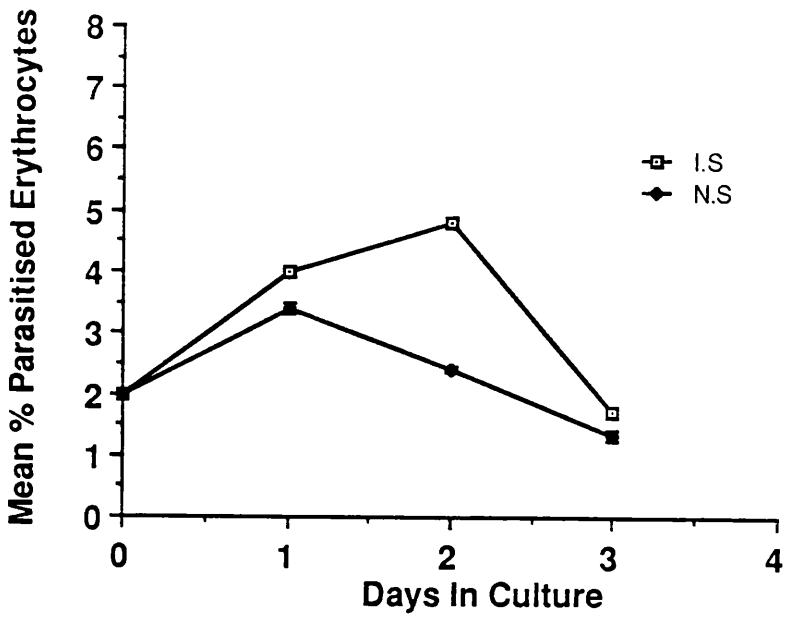
Experiment 7 (ix)

The effect of addition of heat inactivated immune and normal sera into cultures

It was shown in the above experiment that both immune and normal sera had an inhibitory effect on parasite growth in culture. The experiment was repeated, but this time immune and normal sera were heat inactivated at 56°C for half an hour before they were added to culture. This was to test whether destroying the complement activity in the sera has any effect on the inhibitory activity of normal serum in the cultures. 0.15 ml of either heat inactivated immune or normal sera were added into cultures (i.e. the concentration of serum in culture is 10%) at 0, 24 and 48 hours, and the experiment was followed as above. There was an increase in the percentage of infected erythrocytes in cultures containing heat inactivated immune serum from 2% to 4.8% on day 2 (Figure 46). The percentage of infected erythrocytes decreased, however, to 1.7% on day 3. A slight increase in the percentage of infected erythrocytes was noticed in cultures containing normal serum, but it decreased to 1.5% by day 3. It was concluded from the results that both heat inactivated immune and normal sera had an inhibitory effect on parasite growth in culture.

Figure 46:

The effect of adding heat inactivated immune or normal serum into *B. divergens* cultures.



Experiment 7 (x)

The effect of addition of the Ig fraction of immune or normal sera into culture

It was shown in the above experiments, that no difference could be detected between the cultures containing whole immune or normal sera or heat inactivated immune or normal sera. Both sera appeared to have an inhibitory effect on parasite growth, when a concentration of 6-7% or 10% of immune or normal serum was tested in culture although normal serum had no effect on parasite growth in vivo and was completely unprotective in splenectomised infected rats. In a series of experiments, the Ig fraction of immune and normal sera was isolated and its effect on parasite growth was compared in cultures. A brief explanation will be given on the Ig fractions isolated before describing the experiments. The Ig fraction of either immune or normal serum was obtained after sodium sulphate precipitation as was described in the materials and methods chapter. An 18% w/v sodium sulphate in sera gave a precipitate which is thought to contain most of the globulins. When this globulin is redissolved in PBS or RPMI, centrifuged and again precipitated with sodium sulphate to give a 14% w/v solution the precipitate formed is thought to contain pure immunoglobulins. After the precipitation of the 18% and the 14% Ig's, the two supernatants left were not discarded but they were also tested in culture. In addition, whole immune and normal sera and their Ig fractions were dialysed against PBS to test whether the inhibitory activity in the sera or Ig fractions can be lost after dialysis. In a series of

experiments, the effect of adding dialysed and non-dialysed whole immune and normal sera, dialysed Ig fractions of both sera (18% and 14%), and the supernatants obtained after each precipitation in cultures was compared as follows:

Experiment 1

In the following experiment, the effect of addition of normal serum Ig's (18% and 14%), whole normal serum dialysed (WSD), whole normal serum not dialysed (WSND) and the supernatant obtained after precipitating the 18% Ig's was tested in culture as follows:

Cultures were set up as described before (see experiment 7 (i)) with a starting parasitaemia of 2% and 0.1 ml (i.e. concentration of serum in culture is 6-7%) of either whole normal serum, the Ig fractions, or the supernatant obtained after precipitating the 18% Ig's were added to culture at 0, 24 and 48 hours, and their effect on parasite growth was followed. Duplicates were made for each culture. The percentage of infected erythrocytes in cultures containing (18%) Igs increased slightly, but did not exceed 4% on day 3 (Figure 47A). WSD and WSND and the supernatant obtained after the precipitation of the 18% Ig's were inhibitory to parasite growth. Cultures containing the 14% Ig's of normal serum grew normally and the rate of multiplication of the parasite was approximately the same as in the control cultures that had no serum at all.

Experiment 2

In this experiment immune serum Ig's (18%and14%), immune serum WSD and WSND and the supernatant obtained after the

precipitation of the 18% Ig's were tested in culture as above. 0.1 ml (6-7% serum in culture) of the immune serum or immune serum fractions were added to cultures and their effect on parasite growth was followed. The 18% Ig's, immune WSD and WSDN and the supernatant obtained after the 18% precipitation of Ig's were all inhibitory to parasite growth. In cultures containing the 14% Ig's of the serum, there was an increase in the percentage of infected erythrocytes which reached 10% on day 3, but the rate of multiplication was slower than cultures containing no serum at all (Figure 47B).

Experiment 3

In this experiment, the effect of addition of immune serum Ig's (18% or 14%) and immune WSND into cultures was compared with the effect of addition of normal serum Ig's (18% or 14%), and normal WSND. The volume of immune or normal serum Ig's (18% or 14%) or immune or normal WSND added was, however, increased from 0.1 ml to 0.15 mls (i.e. concentration of serum in culture increased from 67% to 10%). Cultures were set up as described in experiment 47A). Cultures containing no serum were included as a control. The results have shown that both immune serum and normal serum Ig's (18%) and immune and normal WSND were inhibitory to cultures (Figure 47C). In cultures containing immune or normal serum Ig's (14%), there was an increase in the percentage of infected erythrocytes which reached 12% on day 3 in cultures containing 14% Ig's of immune serum and 9% in cultures containing 14% Ig's of normal serum. Cultures containing no serum had a parasitaemia of 18% on day 3.

Experiment 4

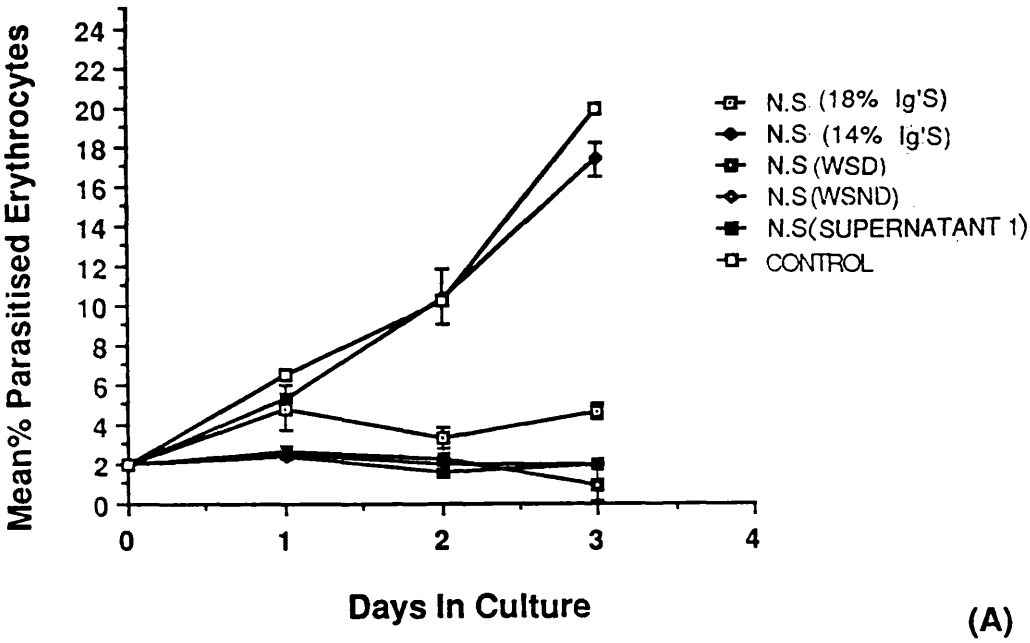
In this experiment, the effect of addition of immune serum (day 13) Ig's (18% and 14%) and immune WSD or WSND into culture was tested as above, but the volume of the cultures was reduced and the volume of immune serum or fractions of immune serum was increased. This is to test whether a difference can be found between cultures containing the 14% Ig's of immune serum and the 14% Ig's of normal serum since both immune serum Ig's and normal serum Ig's were found to support the growth of the parasite in culture (see experiments 1, 2 and 3). Cultures were set up as follows: 24 sterile well plates were used for preparing the cultures instead of the 35 mm petri dishes. A total volume of 500 ul of culture was added to each well which contained 50% (50 : 50 culture : serum) of whole serum or Ig fractions of immune serum. The effect of adding immune serum Ig's (18% or 14%), immune WSD and WSND and the supernatants obtained from the first and second Ig precipitations (18% and 14%) was compared in culture. The results have shown that all Ig's (including 14%), immune WSD and WSND and the two supernatants obtained after precipitating the 18% and the 14% Ig's were inhibitory to parasite growth (Figure 47D). On day 3 a small number of degenerated parasites were seen in blood smears taken from all cultures. The control cultures that had no serum grew normally. A parasitaemia of 21% was obtained on day 3.

Figure 47A:

A comparison of adding 0.1ml of whole normal serum (WD or WND) immune serum 18% Ig's, normal serum 14% Ig's or normal serum (supernatant 1) to *B. divergens* cultures.

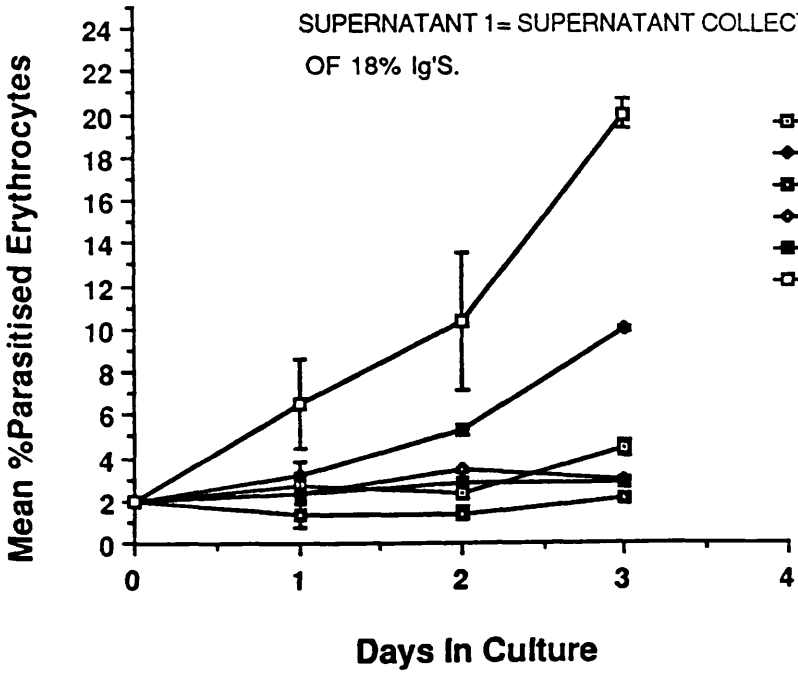
Figure 47B:

A comparison of adding 0.1ml whole immune serum (WN or WND) immune serum 18% Ig's, immune serum 14% Ig's or immune serum (supernatant 1) into *B. divergens* cultures.



(A)

SUPERNATANT 1= SUPERNATANT COLLECTED AFTER THE PRECIPITATION OF 18% Ig'S.



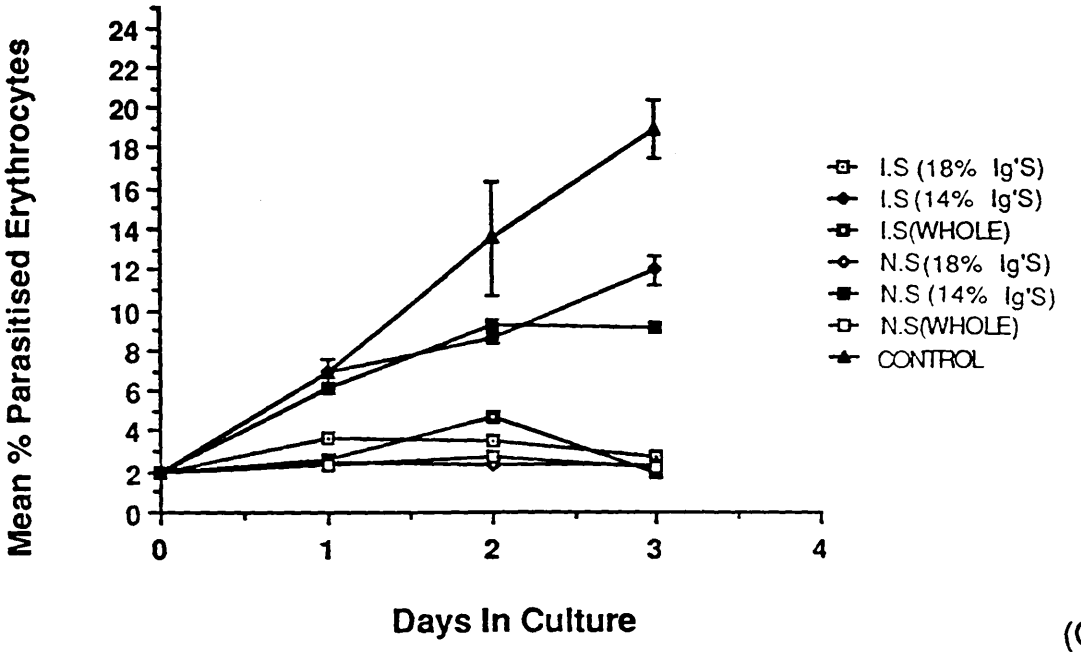
(B)

Figure 47C:

A comparison of adding 0.15ml immune or normal serum (WSND), immune or normal serum 18% or 14% Ig's into *B. divergens* cultures.

Figure 47D:

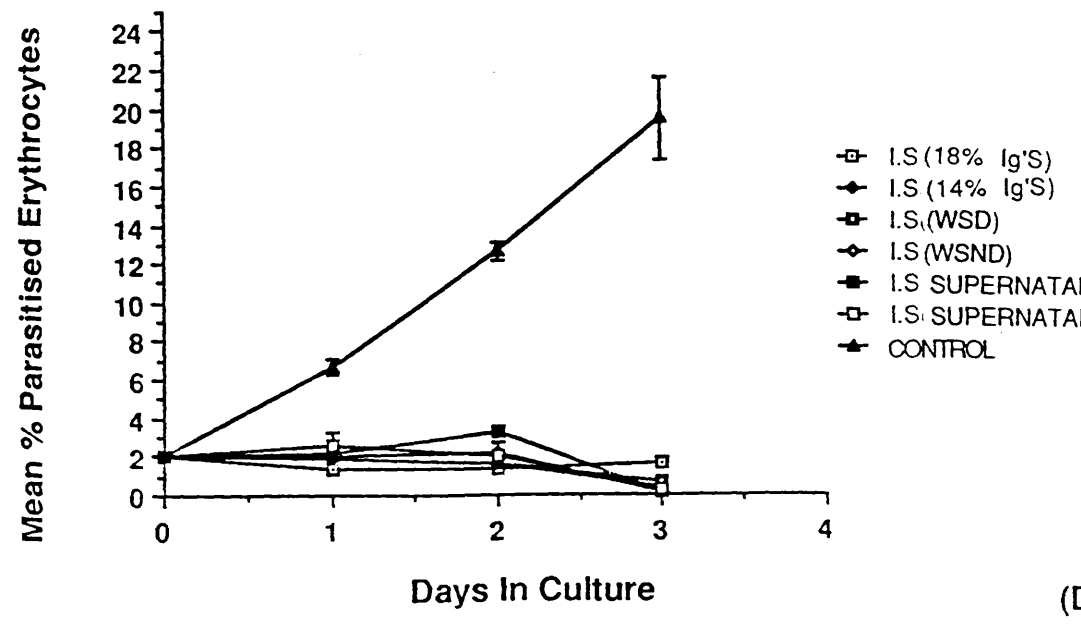
A comparison of adding 50:50 (serum to culture) immune serum 18% or 14% Ig's, immune serum supernatant 1 or immune serum supernatant 2. into *B. divergens* cultures.



(C)

SUPERNATANT 1= SUPERNATANT COLLECTED AFTER THE PRECIPITATION OF 18% Ig'S.

SUPERNATANT 2 = SUPERNATANT COLLECTED AFTER THE PRECIPITATION OF 14% Ig'S.



(D)

Experiment 7 (x)

Opsonisation test

The possible role of opsonising antibody in protection was investigated using hyperimmune serum and B. divergens infected cells or free parasites in the presence of peritoneal macrophages from normal rats. The results have shown that peritoneal macrophages did not phagocytose parasitised erthrocytes or free parasites in the presence of hyperimmune serum. No erythrophagocytosis of Babesia infected erythrocytes was detected in any of the serum dilutions used during 24 hours of observation. This experiment was repeated twice and the same results were obtained.

Experiment 7 (xi)

Merozoite neutralisation test

In the merozoite neutralisation test, the free merozoites were incubated with either immune or normal serum in a CO_2 incubator at $37^{o}C$ for one hour before they were added into cultures. This is to test whether antibody in immune serum will neutralise the merozoites and to compare this with normal serum. Following the period of incubation, 20 ul of merozoites (2×10^6 merozoites) in serum suspension, were added to 200 ul of cultures containing normal erythrocytes (the number of erythrocytes = 1.9×10^8/well) using a 96 sterile well plate. The plate was incubated for 17 hours after which a blood smear was taken from each well. Blood smears were taken daily afterwards for 8-9 days and the medium in each well was also

changed daily and replaced by fresh complete medium (Appendix B). The percentage of infected erythrocytes was compared with that of the control cultures containing no immune or normal serum. Infected erythrocytes were detected on day 3 in blood smears taken from cultures with immune serum. On the other hand, infected erythrocytes were detected on day 1 in blood smears taken from cultures with normal serum (Table 9). This experiment was repeated and the same results were obtained.

Experiment 7 (xii)

The effect of addition of immune spleen cells and peripheral blood mononuclear cells (PBMC)in the presence of immune serum in culture (The ADCC assay)

It was shown in previous experiments that no difference could be observed between the effect of addition of immune or normal serum on parasite growth in culture. With immune serum there was no evidence that antibody agglutinates merozoites and prevents their invasion in vitro. Evidence that antibody neutralises merozoites and prevents their invasion in vitro was demonstrated only by preincubating free merozoites with immune serum before adding them to cultures containing normal rat erythrocytes (see experiment 7(xi)). In the following experiment, the possibility that antibodies might only be protective in the presence of cellular elements was investigated. An antibody dependent cellular cytotoxicity assay (ADCC) using immune spleen cells or PBMC was set up in culture as follows:

Two five month old male intact (non splenectomised) rats were injected with 2.5×10^9 PRBC i.v. The parasitaemia in the

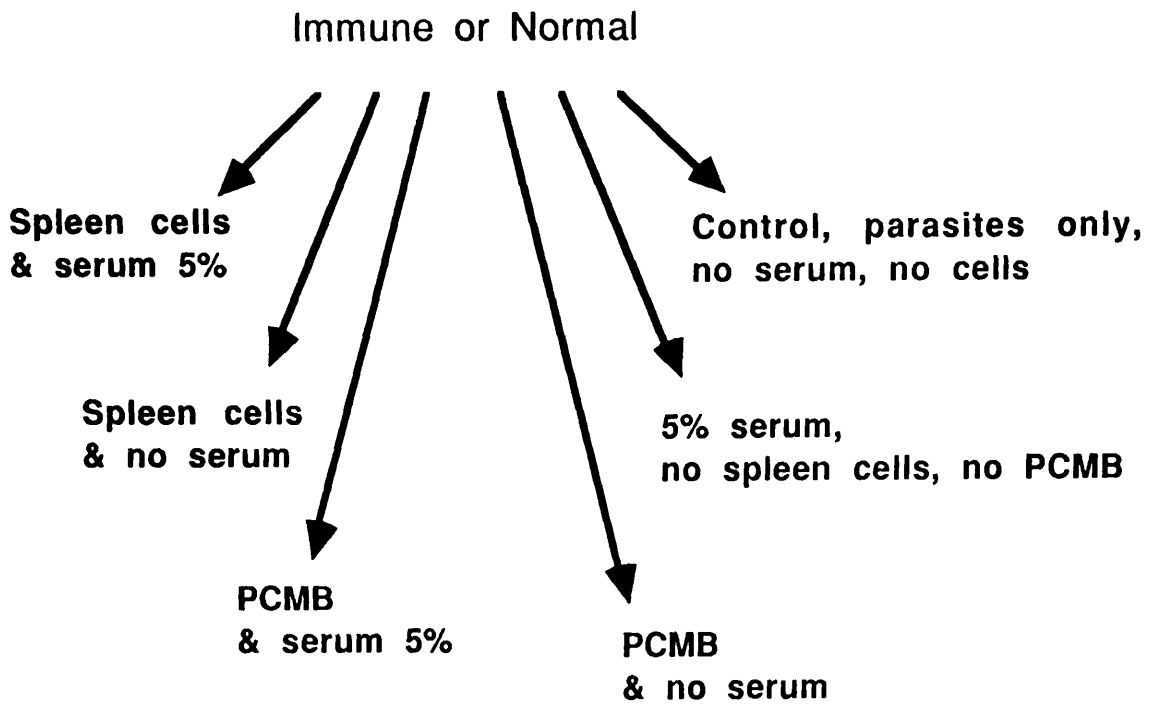
Table 9:
The Merozoite Neutralisation Test

% Parasitised erythrocytes

Culture No. → / Days in culture ↓	I.S.			N.S.			Control		
	1	2	3	1	2	3	1	2	3
1	0	0	0	0.01	0.023	0.04	0.09	0.06	0.06
2	0	0	0	0.02	0.03	0.045	0.1	0.1	0.2
3	0.01	0.02	0.01	0.02	0.03	0.05	0.22	0.32	0.3
4	0.01	0.03	0.02	0.024	0.06	0.07	0.4	0.4	0.5
5	0.5	0.6	0.4	0.7	0.8	1	2.5	2	4.3
6	1.8	1.3	1.2	1.7	1.5	1.4	8.6	8	11.8
7	3.2	2.9	4.3	3	4	3	20.7	17.5	20.8
8	10	6.5	5.5	6.7	6.5	9.2	23	18.6	22
9	16.8	12	10	14.5	13.2	14.4	25	24	25

rats was followed in blood smears taken daily. The highest parasitaemia was observed on day 2 post infection (3-4%) and by day 3 the rats had recovered. On day 7 post infection both rats showed lymphocytosis. They were bled and their spleens removed aseptically. The spleens were considerably enlarged. Two normal uninfected rats were also bled and their spleens removed aseptically. Spleen cells and PMNC were prepared as described in the materials and methods chapter. The plasma of both immune and normal blood was also kept after the separation of the PMNC. The trypan blue exclusion test has shown that 90% of either immune or normal spleen cells were viable. The parasites in the culture were adjusted to a starting parasitaemia of 1% and a 10% suspension was made with complete medium as described before. The parasite suspension was then dispensed in a 96 well sterile microtitre plate, and the appropriate number of spleen cells or PBMC (see Table 10) were added to make a total culture of 200 ul in each well. The plate was then incubated at 37^{o}C in a candle jar. The medium was changed daily and an appropriate number of spleen cells or PBMC were added to each well at 24, 48 and 72 hours. Blood smears were taken daily. A ratio of 5 : 1, 2 : 1 and 1 : 1 of immune or normal spleen cells or PMBC's to parasites were examined. Triplicate cultures were made for each ratio. The degree of the ADCC was determined by assessment of the rate of parasite multiplication in the presence of immune spleen cells or immune PMNC and serum and immune spleen cells or PBMC alone (i.e. cellular cytoxicity assay - CMC), compared to parasite growth in serum alone. It should be noted that in a previous experiment (results not shown), it was shown that 5% ratio of

Table 10: The ADCC assay: cultures containing 5:1, 2:1, 1:1 spleen cells or PMNC to parasites. Triplicate cultures were made for each



immune serum culture proved to be not inhibitory to parasite growth, and this was the concentration of immune or normal serum used in this experiment. The results showed that there was a delay in the appearance of infected erythrocytes for 5-6 days in cultures containing immune or normal serum and immune or normal spleen cells at a ratio of 5 : 1 with parasites. The percentage of infected erythrocytes increased subsequently every day in wells containing immune or normal serum and immune or normal spleen cells (Table 11). Infected erythrocytes were detected in cultures containing immune or normal serum and immune or normal spleen cells at a ratio of 2 : 1 and 1 : 1 with parasites on day 1 but the rate of multiplication in wells containing immune or normal serum and immune or normal serum at a ratio of 2 : 1 with parasites was relatively slow compared to those containing immune or normal serum and immune or normal spleen cells at a ratio of 1 : 1 with parasites. Similar results were obtained in wells containing 5 : 1, 2 : 1 and 1 : 1 immune or normal spleen cells : parasites without the presence of immune or normal serum (CMC assay). No difference could therefore be found between the effect of addition of immune or normal spleen cells into culture. Parasites in wells containing immune or normal serum without cells and wells containing immune or normal PBMC with or without sera grew normally at a similar rate as the control cultures that contained no serum and no cells. A 26-30% infection of erythrocytes was obtained on days 8-9.

Table 11:
The ADCC & CMC Assays Using Immune Spleen Cells and PBMC

	Immune spleen cells and serum						Immune spleen cells and no serum						Normal spleen cells and normal serum						Normal spleen cells and no serum						Immune PBMC & serum		Normal PBMC & serum		Immune PBMC no serum		Normal PBMC no serum		Control		Immune serum no cells		Normal serum no cells	
	5:1		2:1		1:1		5:1		2:1		1:1		5:1		2:1		1:1		5:1		2:1		1:1															
Days in culture ↓	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	-ve	-ve	1.2	1.3	2	2	-ve	-ve	0.09	0.3	2.1	2.3	-ve	-ve	1.2	1.5	1.5	1.6	0.14	0.1	2	1.5	1.3	1.2	2.5	2.2	2.6	2.4	3	3.1	2.1	2.5	3.5	3	2.5	2.6	2.2	2.4
2	-ve	-ve	1.4	1.6	3.8	3	-ve	-ve	0.17	0.13	3.3	4.1	-ve	-ve	1.7	2	2.3	2.7	0.06	0.01	1	0.25	2.6	2.5	3.3	2.8	3	3.2	5.6	4.2	3	3.4	4.2	4.5	3.1	3.2	4	3.4
3	-ve	-ve	1.7	1.7	4.8	6.4	-ve	-ve	1.5	1.4	5.4	6	-ve	-ve	1.7	2.1	2.3	2.8	0.1	0.03	0.5	0.2	2	2.1	4.9	4.1	4.5	4	6.1	5.3	5.2	4	6	6.3	5	6.2	5.2	4.5
4	-ve	-ve	3.1	2.5	10	12	-ve	-ve	2.1	3.2	9	10.5	-ve	-ve	4	3.5	4.8	5	0.2	0.08	5.6	3.3	6	7.4	6.2	5.3	4.8	4.5	7	6.2	6.7	5.9	8.5	8.2	7.6	8.1	7.8	5.9
5	-ve	0.1	12.5	8	12	14	0.15	0.2	6.4	8.6	11	13.3	-ve	-ve	6.2	7	10	12	0.5	0.3	15	8.5	12	11.6	9.3	9.8	8	8.7	9.2	9.8	8.6	8.2	9	9.6	14.6	10.7	9.5	8.1
6	0.05	0.04	14	10	18	20	0.07	0.26	10.5	12	17.5	18.3	0.01	0.02	10	12	12	14	0.52	0.4	16.3	14.4	15.6	14.3	12	11.6	11.5	12.1	11	11.5	11.9	10	12.3	13.2	16	12.4	12.5	12
7	0.07	0.06	16.2	13	-	-	0.2	0.33	13	14.5	-	-	0.02	0.02	15.3	14	-	-	0.6	0.45	18.5	16	-	-	14.6	13	13.1	14	11.6	11.8	12.2	12.6	17.5	15	18	14	16	15.3
8	0.2	0.2	20	17	-	-	0.3	0.4	22	18	-	-	0.3	0.15	-	-	-	-	0.6	0.5	-	-	-	-	-	-	-	-	12	15.7	-	-	23	21	-	-	-	-
9	0.24	0.3	-	-	-	-	0.3	0.5	-	-	-	-	0.4	0.3	-	-	-	-	0.4	0.43	-	-	-	-	-	-	-	-	17.5	26	-	-	26	24	-	-	-	-
10	0.5	0.66	-	-	-	-	0.4	0.6	-	-	-	-	0.4	0.33	-	-	-	-	0.5	0.45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Discussion

The rat adapted strain of B. divergens was cultured continuously for six months in rat erythrocytes using the candle jar technique (Trager and Jensen, 1976) and provided an opportunity to study some aspects of the parasite which were difficult to examine in the animal host. The candle jar technique has previously been used for the short term cultivation of B. microti (Bautista and Kreier, 1979; 1980), and B. bovis, B. bigemina and B. rodhaini (Timms, 1980). Attempts by Erp and colleagues (1978) to use this technique for the cultivation of B. bovis were, however, unsuccessful. Short term in vitro cultivation of B. bovis (Erp et al., 1978) was achieved in suspension cultures (spinner flasks). Later, continuous growth of B. bovis in suspension cultures was achieved (Erp et al., 1980) but this method was limited as the growth of the parasite was relatively slow and large volumes of culture medium were required. This problem was overcome by the development of the microaerophilous stationary phase (MASP) culture for the continuous cultivation of B. bovis (Levy and Ristic, 1980). This method was used for the cultivation of B. divergens in calf erythrocytes (Vayrynen and Tuomi, 1982) and in bovine or gerbil erythrocytes (Konrad et al., 1984), B. major (Donnelly et al., 1984), B. bigemina (Vega et al., 1985) and B. odocoilei (Holman et al., 1988).

The cultures of B. divergens described in this chapter were initiated with fresh infected rat blood. A stabilate of infected rat blood was subpassaged into a splenectomised rat before introduction into culture. Attempts to recover stabilates of

culture parasites were unsuccessful. Konrad et al. (1984) also used fresh infected bovine or gerbil blood to initiate B. divergens cultures since they were unsuccessful in recovering stabilates of culture parasites. In contrast, cultures of B. bovis (Palmer et al., 1982), B. bigemina (Vega et al., 1985) and B. odocoilei (Holman et al., 1988) have been initiated using cryopreserved cultures.

In the experiments described in this chapter, the use of standard anticoagulants such as heparin in the collection of blood for culture has not been tested and defibrination with glass beads was the only method used for blood collection. It was found in our laboratory, however, that cultures where blood was collected in heparin grow as well as those initiated with defibrinated blood (Phillips personal communication). Levy and Ristic (1980) also used blood defibrinated with glass beads for B. bovis cultures, since they found that collecting infected red blood cells into heparin inhibits parasite growth. Vega et al. (1985) however, used heparin for collection of blood for B. bigemina cultures, since they found that blood collected by defibrination with glass beads haemolysed rapidly and contained fewer infected red blood cells.

B. divergens was cultured in RPMI 1640 medium containing 20% FCS. The cultures were incubated at 38°C in a candle jar under similar conditions described for the cultivation of P. falciparum (Trager and Jensen, 1976) i.e. in reduced oxygen and 5% CO_2 atmosphere. This atmosphere supported the growth of the parasite. Only RPMI 1640 medium was used in the cultures; other

media were not tested. It has been reported, however, that medium 199 also supports the growth of B. divergens in calf erythrocytes (Vayrynen and Tuomi, 1982) and in bovine or gerbil erythrocytes (Konrad et al., 1984) and also for the cultures of B. bovis (Erp et al., 1978; 1980; Levy and Ristic, 1980) and B. odocoilei (Holman et al., 1988).

Both FCS and heat inactivated FCS supported good growth. Konrad et al. (1984) reported that FCS does not support the growth of B. divergens in bovine or gerbil erythrocytes in culture. The same percentage (20%) of deer serum was used for the cultivation of Babesia of the white tail deer (B. odocoilei) (Holman et al., 1988). Levy and Ristic (1980), however, reported that relatively high concentrations (40%) of normal bovine serum were required to support good growth of B. bovis in cultures and found that using a 20% concentration of normal bovine serum inhibited parasite growth. Konrad et al. (1984) also used 40% of bovine serum for B. divergens cultures. Vega et al. (1985) reported that concentrations of fresh normal bovine serum of 20 to 50% were required to sustain growth of B. bigemina.

FCS was normally stored aliquoted at -20°C or kept at 4°C and used within a week. Trager and Jensen (1976) reported that storage of freshly collected human sera for more than 2-3 weeks at 4°C was deleterious to P. falciparum cultures. For B. bovis a comparison was made between cultures containing freshly collected bovine serum and serum stored at 4°C for 2-3 weeks. It was concluded that the ability of serum to support babesial growth decreased with time (Levy and Ristic, 1981).

The use of fresh normal rat cells or cells stored at 4°C for 2-3 weeks for diluting the cultures both supported growth. Trager and Jensen (1976) reported that human cells stored for a long period at 4°C (outdated cells) supported better growth in P. falciparum cultures. Levy and Ristic (1981) found, however, that storage of bovine blood for six days at 4°C resulted in half the babesial growth as compared to freshly collected red cells. Konrad et al. (1984) also reported that fresh bovine red cells were better for the growth of the B. divergens in culture than stored cells. In the present study, B. divergens does not appear to be affected by white blood cells are in culture, and their separation from infected blood was not carried out. Timms (1980) reported, however, that B. bovis in culture was inhibited by white blood cells.

A 10% suspension of infected rat erythrocytes in complete medium supported good growth of B. divergens in culture. This haematocrit was used for the cultivation of P. falciparum (Trager and Jensen, 1976) and for most Babesia species (Levy and Ristic, 1981, Vayrynen and Tuomi, 1982; Konrad et al., 1984, Holman et al., 1988). Timms (1980), however, reported that a 40% suspension of red blood cells gave better results for B. bovis cultures than a 10-12% suspension.

Parasitaemias exceeding 35% have been observed in cultures which were inhibited with 2% B. divergens infected cells. The highest parasitaemia observed with cultures of the same parasite but in calf erythrocytes was 5-10% (Vayrynen and Tuomi, 1982), 10-15% in bovine or gerbil erythrocytes (Konrad et al., 1984) and 5-14% where cultures were initiated and progressively diluted

with gerbil erythrocytes in human or bovine erythrocytes (Pudney, 1984). For other Babesia species, the highest parasitaemia observed was 38% for B. bovis (Levy and Ristic, 1980), 1.9% for B. bigemina (Vega et al., 1985) and 30% in B. odocoilei (Holman et al., 1988).

At high parasitaemia, the cultures became dark red or black. The addition of fresh cells and fresh medium resulted in the reappearance of the bright red cells. Similar observations have been reported for B. bovis (Levy and Ristic, 1980) and B. divergens (Vayrynen and Tuomi, 1982) cultures. Levy and Ristic (1980) suggested that the darkening of erythrocytes in B. bovis cultures indicated a condition of low oxygen tension (deoxyhaemoglobin), and that the depletion of oxygen from the settled cell layer results from the parasite's metabolism since they noticed that cultures with less than 1% infected red cell failed to darken. They suggested that the medium above the cells acts as a barrier to oxygen exchange and its depth is crucial to successful cultivation. They concluded that the culture conditions allowed the parasite to become established as a result of their metabolic activity which reduces the oxygen in the environment. In the present study, cultures with a low percentage of infected erythrocytes (1-5%) also failed to darken, which indicates that the conclusions suggested by Levy and Ristic (1980) for B. bovis cultures might also be applicable to B. divergens cultures in rat erythrocytes.

The morphology of Babesia in culture was identical with that of parasites from the host. Multiparasitisation of red blood

cells was common and four parasites in each erythrocyte were occasionally seen in blood smears taken from cultures with a high parasitaemia. Similar observations have been reported for B. bovis (Erp et al., 1980; Levy and Ristic, 1980) and B. odocoilei (Holman et al., 1988). Pudney (1984) noted, however, that B. divergens cultured parasites were atypical and differed from those seen in the infected gerbil.

B. divergens cultured for eight weeks in rat erythrocytes showed no loss in infectivity and virulence when injected into a splenectomised rat. The rat suffered a severe infection and died. Similar results were obtained after eight weeks in culture for B. bovis, B. divergens and B. bigemina in cultures (Erp et al., 1980; Vayrynen and Tuomi, 1982; Vega et al., 1985). In the present study no cultures after eight weeks (56 days) were specifically tested for their infectivity and virulence. Injection of babesia cultures into a splenectomised rat was, however, occasionally performed after eight weeks in culture and this was for clearing contaminated cultures. The contaminated cultures were inoculated into a splenectomised rat, the parasite multiplied and the contamination cleared. The parasite was then re-isolated in new cultures.

Babesia cultures stored in medium at 4°C for up to 11 days could be used to initiate new cultures. No cultures were kept at 4°C for longer than this period. Erp et al. (1980) reported that B. bovis cultures can be stored at 4°C for up to 30 days. Konrad et al. (1985), stored cultures of erythrocytes parasitised by B. divergens or B. major in medium cooled at 4°C for up to eight weeks. The ability to store Babesia parasites at 4°C provides a

convenient system and eliminates the need for continuous culture to maintain the parasite.

Smears taken from cultures with a high percentage of infected erythrocytes contained a large number of extracellular merozoites. Extracellular merozoites were collected from the cultures without depriving the cultures of CO_2. Attempts to induce release of merozoites by depriving cultures of CO_2 were unsuccessful. Levy and Ristic (1980) reported that extracellular merozoites were rarely observed in cultures during the growth phase and the only way to collect free merozoites was to deprive them of CO_2. Winger et al. (1987a) also collected B. divergens free merozoites by depriving cultures of CO_2 by the method described by Levy and Ristic (1980). In the present study, extracellular merozoites were collected from pooled cultures with a high percentage of infected erythrocytes by differential centrifugation. The final pellet contained a large number of merozoites. The merozoites were either used to initiate new cultures or were frozen in liquid nitrogen. Frozen merozoites remained infectious and would infect rat red blood cells in culture. The isolation of a large number of merozoites from cultures permitted the study of some aspects of the mechanisms of action of antibody as will be described later in the text.

Passive transfer experiments have demonstrated the participation of humoral factors in acquired immunity to B. divergens and confirmed the role of antibody in protection. The mechanisms by which antibody protects infected splenectomised rats are, however, unknown. Antibodies may inhibit the invasion

of erythrocytes by merozoites or function as agglutinins or opsonins against infected erythrocytes or merozoites. Antibodies can also be protective in the presence of cellular elements i.e. through an ADCC mechanism. The possible role of opsonising antibody in protection against B. divergens was first investigated in vitro using hyperimmune serum and B. divergens PRBC or free parasites in the presence of normal peritoneal macrophages. No evidence was found for the role of opsonising antibody in protection. Opsonising antibody has been reported, however, to be protective in vitro in B. rodhaini (Rogers, 1974) and B. canis (Ishimine et al., 1979) infections, and was speculated to be involved in vivo in B. bovis infections (Mahoney, 1979).

The development of a method for the continuous cultivation of B. divergens, has provided an opportunity to study further some aspects of the mechanisms of action of antibody and to compare its protective activity in vivo with its effect on parasite growth in vitro. The effect of addition of immune or normal serum on their Ig fractions into B. divergens cultures on parasite growth was studied. The Ig fractions of either immune or normal serum were precipitated with sodium sulphate to 18% w/v, and then further precipitated with sodium sulphate to 14% w/v. The 18% and the 14% Ig suspensions were not tested for the presence of immunoglobulins. It was expected, however, that the 18% suspension would contain most of the Ig's and the 14% suspension would contain pure Ig's. Whole rat immune and normal serum inhibited parasite growth to the same extent as the 18% immunoglobulin fraction of both sera even with a concentration of

6-7% of Ig fraction in culture. In passive transfer tests *in vivo*, protective activity was observed only in the unfractionated and the immunoglobulin fraction of immune serum. The portion of the serum that remained after the Ig fraction was precipitated with sodium sulphate (18% and 14%), retained inhibitory activity. In contrast, the 14% suspension was partially inhibitory to parasite growth. Cultures containing either 14% Ig suspension of either immune or normal serum grew although cultures containing 14% of immune serum did not grow at the same rate as the control cultures which contained no serum. A concentration of 50% of the 14% Ig's of both immune and normal serum were, however, inhibitory to cultures. The cause of the inhibitory effect of normal serum is not known, and even dialysed normal serum was toxic to the parasite indicating that the inhibitory activity was not lost during dialysis. Heat inactivating the serum did not reduce its non specific inhibitory effect. It was concluded, therefore, that normal serum is toxic to cultures. This was also observed in *P. chabaudi* cultures (Harvey and Phillips personal communication).

Evidence that immune serum (antibody) neutralises merozoites and prevents their invasion *in vitro* was demonstrated only by pre-incubating free merozoites with immune serum before adding them to cultures containing normal rat erythrocytes. The results showed that at least some of the merozoites were neutralised by antibody. This was evident by the fact that no infected erythrocytes were seen in blood smears taken from cultures for 2-3 days, whereas after incubation with normal serum, infected

erythrocytes were detected after 24 hours of culture. Neutralisation of merozoites by antibody was also reported for B. bovis (Ristic and Levy, 1981) and for B. divergens (Winger et al., 1987a) in merozoite neutralisation assays after collection of cell free merozoites from CO_2 deprived MASP cultures.

An important antigenic determinant associated with protection appears to be localised on the surface coat of the merozoites (Miller et al., 1975; Montenegro-James et al., 1983). Miller et al. (1975) reported that merozoite agglutination in P. knowlesi infection was caused by the binding of surface coats of adjacent parasites. This coat which consisted of proteins or glycoproteins appeared in the plasma membrane of merozoites after it was exposed to culture medium both with or without serum. They concluded that antibodies directed against this surface coat are crucial for the reduced invasion of erythrocytes. Ristic and Levy (1981) confirmed the specificity of the merozoite neutralisation assay in B. bovis by pre-incubating immune sera with soluble antigens derived from B. bovis cultures. They observed that there was a loss in the ability of the antibody to prevent in vitro infection of erythrocytes by B. bovis merozoites. Montenegro-James et al. (1983), working on the same parasite also reported that merozoites deprived of their surface coat were incapable of invading erythrocytes.

The addition of immune or normal serum or the Ig fractions into B. divergens cultures, has indicated that it is difficult to correlate the protective activity of immune serum and its Ig fraction in vivo with its action on parasite growth in vitro. In some malaria and babesia infections, attempts have also been made

to correlate functional immunity *in vivo* with the inhibitory activity of immune serum *in vitro*. Cohen *et al.* (1969), studied the effect of immune sera on the erythrocytic cycle of *P. knowlesi in vitro* and found that immune sera had no inhibitory effect of the growth of intraerythrocytic parasites but inhibited growth at the time of merozoite release from infected erythrocytes and reinfection of normal erythrocytes. Similar observations have been reported by Brown *et al.* (1970) and Miller *et al.* (1975). In a later report, Cohen and Butcher (1970) reported that the *in vitro* inhibitory activity of immune sera in *P. knowlesi* infection was concentrated in the IgG and IgM fractions, and that the $F(ab^2)$ fragment of IgG was responsible for this inhibition and not the Fab or the Fc fragments. Butcher and Cohen (1972) reported that immune serum neutralised the merozoites, and that this mechanism may function *in vivo* to eliminate malaria parasites. Bautista and Kreier (1979) working with *B. microti* in the hamster found that immune hamster serum alone inhibited parasite multiplications by preventing the invasion of red cells by the parasite in short term cultures. In further experiments Bautista and Kreier (1980), cultured *B. microti* over peritoneal macrophage monolayers with immune or normal serum. They concluded that opsonisation and phagocytosis is not the major cause of parasite death in the culture and suggested that immune macrophages produce a soluble factor which inhibits growth of *B. microti* in the presence of immune serum. Bautista and Kreier (1979; 1980) did not, however, correlate the inhibitory activity of immune serum with any functional immunity

in vivo.

The importance of antimerozoite antibody in protection in Babesia and malaria infections was confirmed by the development of monoclonal antibodies against merozoites of these two parasites. Monoclonal antibodies were found to be protective in P. yoelii infection when passively transferred to appropriate animal hosts (Freeman et al., 1980), and were inhibitory to merozoite invasion in P. knowlesi (Deans et al., 1982) and B. divergens infections in vitro (Winger et al., 1987a or b).

Although a relationship between functional immunity in vivo and serum inhibitory activity in vitro was reported by Cohen and Butcher (1979) as described above, this correlation had not been demonstrated previously by other workers using the same parasite (i.e. P. knowlesi) (Miller et al., 1977; Butcher et al., 1978). Similarly, immune sera or purified IgG from P. falciparum infected Aotus monkeys (Campell et al., 1979; Chulay et al., 1981) and from chronically infected humans (Wilson and Phillips, 1976; Brown et al., 1982) have been shown to inhibit parasite growth in culture. This inhibition was, however, always incomplete and obtained only with high concentrations of Ig. Fandeur et al. (1984) found that in Saimiri monkeys infected with P. falciparum, some Ig preparations showing high protective activity in vivo provided little or no inhibition of the parasite in vitro. In contrast, inhibitory activity in vitro was present in Ig preparations unable to be protective in vivo. Furthermore, they reported that protective antibodies are not active through a simple neutralizing mechanism, and they confirmed this when they found that the $F(ab)^2$ fragments derived from protective Ig

preparations were not protective, and they suggested the participation of cellular immune mechanisms for the control of the infection.

In the present study the lack of evidence that *in vitro* antibodies agglutinate merozoites and prevent them invading erythrocytes has led to the possibility that antibodies might only be protective in the presence of cellular elements. An ADCC assay using immune spleen cells or immune PMBC's in the presence of immune serum was set up in culture. A cell mediated cytotoxicity assay (CMC) (i.e. in the absence of antibody) using immune spleen cells or immune PMBC was also set up in culture and the results compared with the ADCC assay. In the ADCC assay, it was expected that immune spleen cells or PBMCs would damage target cells (i.e. infected erythrocytes or parasites) in the presence of immune serum. No parasites were detected in blood smears taken for 5-6 days in cultures containing immune serum and immune spleen cells and parasites at a ratio of 5 : 1. Parasites were detected in blood smears taken on days 6-7 which multiplied normally. This indicated that multiplication of *B. divergens in vitro* was reduced by the presence of specific antibody and immune spleen cells. These results, however, were found not to be specific for the immune spleen cells or immune serum, and similar results were obtained with the control cultures i.e. those containing normal serum and normal spleen cells and parasites at a ratio of 5 : 1. The results also showed no evidence of specific ADCC mediated by immune PBMCs. The parasites in these cultures multiplied normally as in the control

cultures which contained no cells and no serum.

There was also no evidence of specific cell mediated cytotoxicity by immune spleen cells or immune PBMCs in the absence of specific antibody as similar results as those described above for cultures containing immune serum and immune spleen cells and parasites at a ratio of 5 : 1 were also obtained with cultures containing only immune spleen cells and parasites at ratio a 5 : 1. Since in the ADCC assay and the CMC assay, the cultures were followed for ten days, it could be expected that the similarity in the results obtained in the two assays might have resulted from the production of specific antibodies by plasma cells which constitute a population of spleen cells in cultures containing only immune spleen cells without immune serum.

The participation of cellular immunity associated with humoral factors in protective immunity has been suggested for a number of plasmodial species but there are no reports of the participation of ADCC in babesial immunity. Indirect evidence of an increased ADCC activity in the spleen of mice during the second week of P. chabaudi infection has been reported (McDonald and Phillips, 1978), and in human peripheral blood mononuclear cells during P. falciparum infection (Greenwood, 1977). Direct evidence of ADCC by PBMCs have been reported for P. falciparum infection in humans (Brown and Smalley, 1980) and by spleen cells in P. berghei infection in rats (Orago and Solomon, 1986).

In the present study, it was observed that the spleens of immune rats from which spleen cells were prepared for the ADCC

assay or the CMC assay were considerably larger than those of normal rats (controls). The cause of splenomegaly was not investigated, and although the studies in the present project were carried out on splenectomised rats, it is expected that in intact rats recovering from a B. divergens infection, splenomegaly might have resulted from the accumulation of cells which would probably include lymphocytes and macrophages in the spleen as a result of immune response to parasite antigens. Splenomegaly has been reported in mice recovering from a B. microti infection (Allison et al., 1978). These authors reported that T lymphocytes were required for recovery. They found that splenomegaly following infection of nude mice is less than in intact animals and suggested that a thymus-dependent immune response to parasite antigens is important in splenomegaly. They confirmed this when they placed parasitised erythrocytes and immune spleen cells in diffusion chambers in the peritoneal cavity and found that the diffusion chambers were surrounded by a dense mass of cell infiltrates indicating the recruitment of these cells from the circulation. Previously Wyler and Gallen (1977) described the presence of a mononuclear chemotactic factor and a macrophage migration inhibition factor in the spleens of mice recovering from malaria infections and confirmed the accumulation of mononuclear phagocytes in the spleens of infected animals. Allison et al. (1978) suggested the possible formation of macrophages from precursors in the spleen, which is a haemopoietic organ in mice, contributes to the splenomegaly in parasitic infections.

It was shown in the present study that antibody plays an important role in acquired immunity to B. divergens in vi (see Chapters Four and Five). It was also shown in chapter six that the livers of immune splenectomised rats showed the appearance of accumulation of cells around the central veins and preliminary histological studies showed that these accumulations of cells consisted of T and B lymphocytes. The lack of evidence that antibody agglutinates merozoites and prevents their invasion in vitro, the failure to demonstrate the role of opsonizing antibody and phagocytosis, CMC and ADCC by immune spleen cells or PBMCs suggests that recovery from B. divergens infection in splenectomised rats is possibly mediated by T lymphocytes. The role of T lymphocytes in cell mediated immunity has been reported for a number of Babesia and malaria infections. T cells may act as helper cells in protective antibody production (Brown, 1971), be cytotoxic to parasites (Coleman et al., 1975) or perhaps be necessary for the release of non antibody factors which have an antiparasitic activity (Clark et al., 1976). Evidence for cell mediated immune mechanisms in Babesia also comes from the results of delayed skin tests (Banarjee et al., 1977), macrophage inhibition tests (Perez et al., 1977) and experiments involving natural killer cells (Eugui and Allison, 1980). In the present study, it is possible therefore that certain populations of T cells (T helper cells) acted as helper cells for the production of protective antibody, or they were responsible for the release of non antibody factors (lymphokines) which can activate macrophages. Non antibody factors released by T lymphocytes (lymphokines) and macrophages (cytokines) can inhibit the

development of parasites in the erythrocytes. Similar suggestions have been reported for B. microti in mice (Allison et al., 1978) and in hamsters (Bautista and Kreier, 1980). Allison et al. (1978) suggested that opsonisation of parasitised erythrocytes for phagocytosis can only be a contributory factor in immunity to B. microti in mice. They also failed to demonstrate lysis of parasites or infected erythrocytes by immune T lymphocytes or by an ADCC assay and suggested the release of non antibody factors by T lymphocytes which would have an antiparasitic activity. Bautista and Kreier (1980) suggested that opsonisation and phagocytosis was not the major cause of parasitic death in B. microti cultures. They suggested that immune macrophages produce a soluble factor which inhibit growth of B. microti and this is facilitated by the presence of immune serum.

Evidence of non antibody mediated immunity to Babesia and plasmodium species came from observations reported by Clark et al. (1977a; 1977b), Herod et al. (1978), Wood and Clark (1984) and Montenegro-James et al. (1985). These authors suggested that a non specific type of immunity mediated by soluble factors secreted by activated mononuclear phagocytes may be important in Babesia and human malaria. Clark et al. (1977a), proposed a hypothesis where they suggested that mononuclear phagocytes become activated by parasite derived antigens in the course of the infection and are stimulated to release products toxic to the parasite. Recently, Montenegro-James et al. (1985), using a microaerophilous stationary phase culture (MASP) for B. bovis

concluded that soluble products secreted by bovine blood mononuclear phagocytes activated by soluble antigens or immune complexes inhibit the *in vitro* growth of *B. bovis*, and suggested that a possible mechanism may involve alteration of parasite metabolism by the restriction of passage of nutrients into the host erythrocytes. These observations may therefore be applicable to *B. divergens*, and future investigations on the activity of these soluble non antibody factors will clarify their role in protective immunity.

CHAPTER EIGHT

THE ADAPTATION OF TWO BOVINE ISOLATES OF *B.divergens* TO CONTINUOUS CULTURE IN RAT ERYTHROCYTES AND THE GROWTH OF *B.divergens* IN NON RAT ERYTHROCYTES IN CULTURE

Chapter Eight

The adaptation of two bovine isolates of B.divergens to continuous culture in rat erythrocytes and the growth of B.divergens in non rat erythrocytes in culture

INTRODUCTION

The development of a method for the long term cultivation of the rat adapted strain of B. divergens in rat erythrocytes (see Chapter Seven) has provided an opportunity to adapt new babesia isolates for long term cultivation. In this chapter the adaptation of two bovine isolates of B. divergens to continuous culture in rat erythrocytes, and the suitability of erythrocytes from non rat species (gerbil and human erythrocytes) to support growth of B. divergens in culture are described.

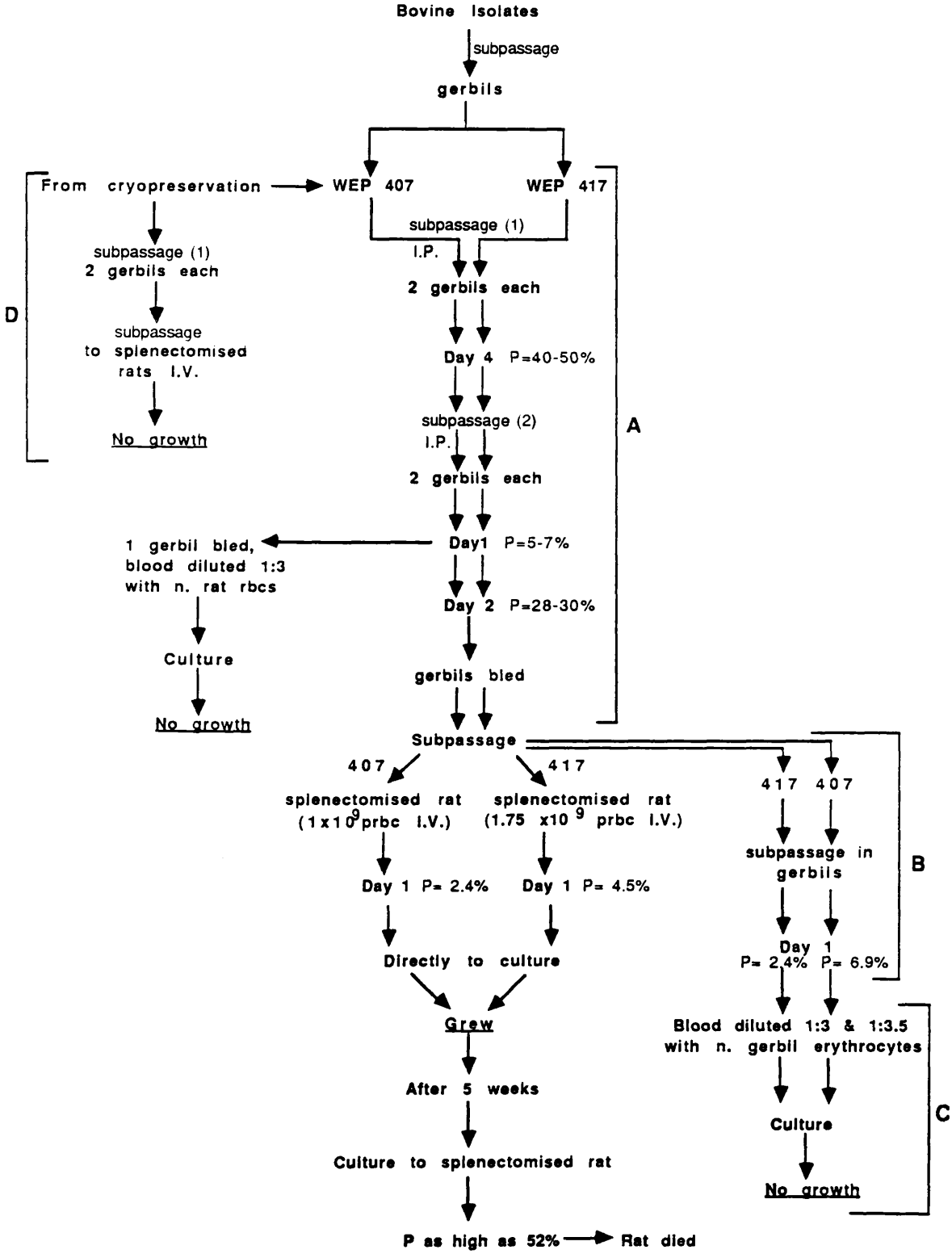
Experiment 8 (i)

The adaptation of bovine isolates to culture

Two isolates of B. divergens have been adapted to continuous culture as follows: Two bovine field isolates which had been passaged into gerbils and then cryopreserved as stabilates designated as WEP 407 and WEP 417 (see Phillips et al., 1987) were used. On recovery from cryopreservation the isolates were subpassaged through two gerbils each (Figure 48), and then either put directly into culture with gerbil erythrocytes (after one more subpassage in a gerbil for each isolate) or rat erythrocytes, or subpassaged through a splenectomised rat for one day before going into culture (Figure 48 Part B) without any additional red cells. The cultures were prepared as described

Figure 48:

The adaptation of two bovine isolates of *B. divergens* to continuous culture.



for the rat adapted strain and triplicate cultures were made for each. Cultures initiated with parasites in rat erythrocytes grew (Figure 48 Part C) and subcultures were made from these every 48-72 hours by diluting them with normal rat erythrocytes (Figure 49). These cultures grew as well as the rat adapted ones, and the parasite was maintained continuously over a period of 75 days through 32 subcultures. The highest percentage of infected erythrocytes obtained was between 26-30% (Figure 50). After five weeks of culture, some cultures were used to infect splenectomised rats successfully (Figure 51), while the same isolates that were injected directly from gerbils to splenectomised rats did not grow (Figure 51) (Figure 48 Part D). No growth was observed in cultures initiated with parasitised gerbil blood in normal rat erythrocytes (Figure 52) or cultures initiated with parasitised gerbil erythrocytes in normal gerbil erythrocytes (Figure 52) (Figure 48 Part C). In blood smears taken from both cultures, the parasites appeared dead and degenerated and the cultures were discontinued from day 6.

The adaptation of the two bovine isolates to continuous culture was repeated and the same results were obtained.

Experiment 8 (ii)

In vitro growth of rat infected erythrocytes in human red blood cells

Some of the rat adapted cultures were initiated with human erythrocytes, and the effect of diluting infected rat erthrocytes with normal human erythrocytes was followed. Rat adapted cultures with a 10-12% infected erythrocytes were diluted 1 in 5

Figure 49:

The growth of 2 bovine isolates of *B. divergens* (WEP 407 and 417) in continuous culture in rat erythrocytes.

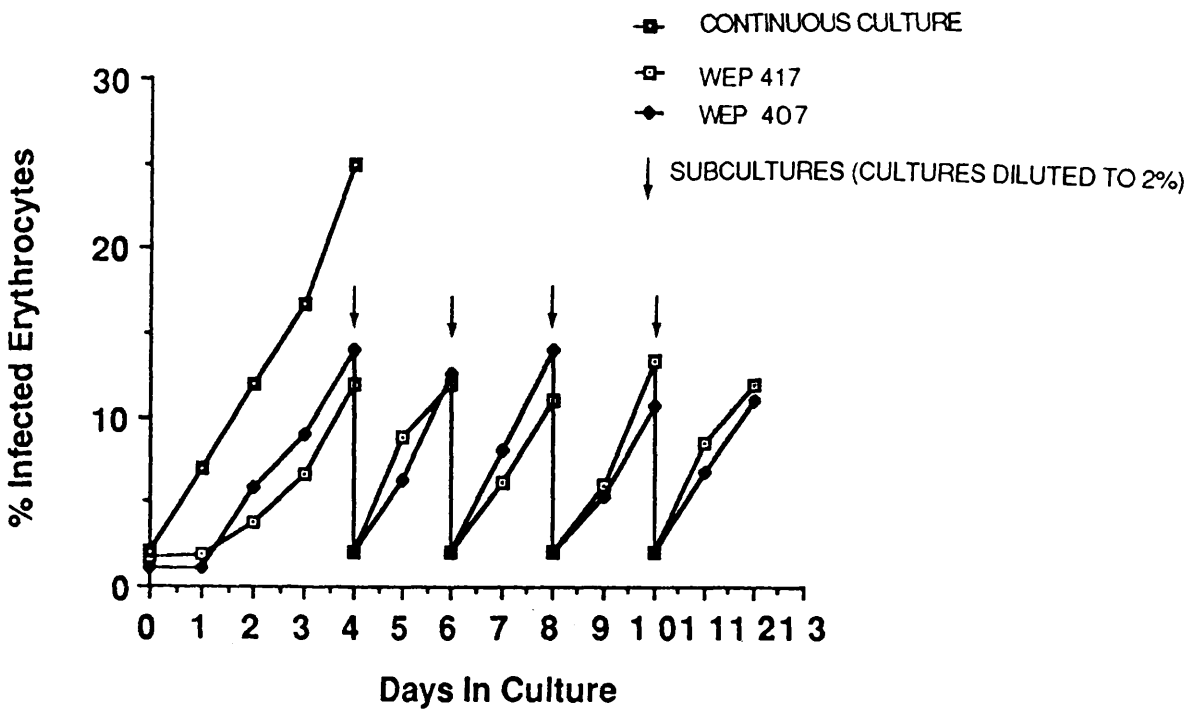


Figure 50:

Morphology of *B. divergens* in cultures of WEP 407 and 417.

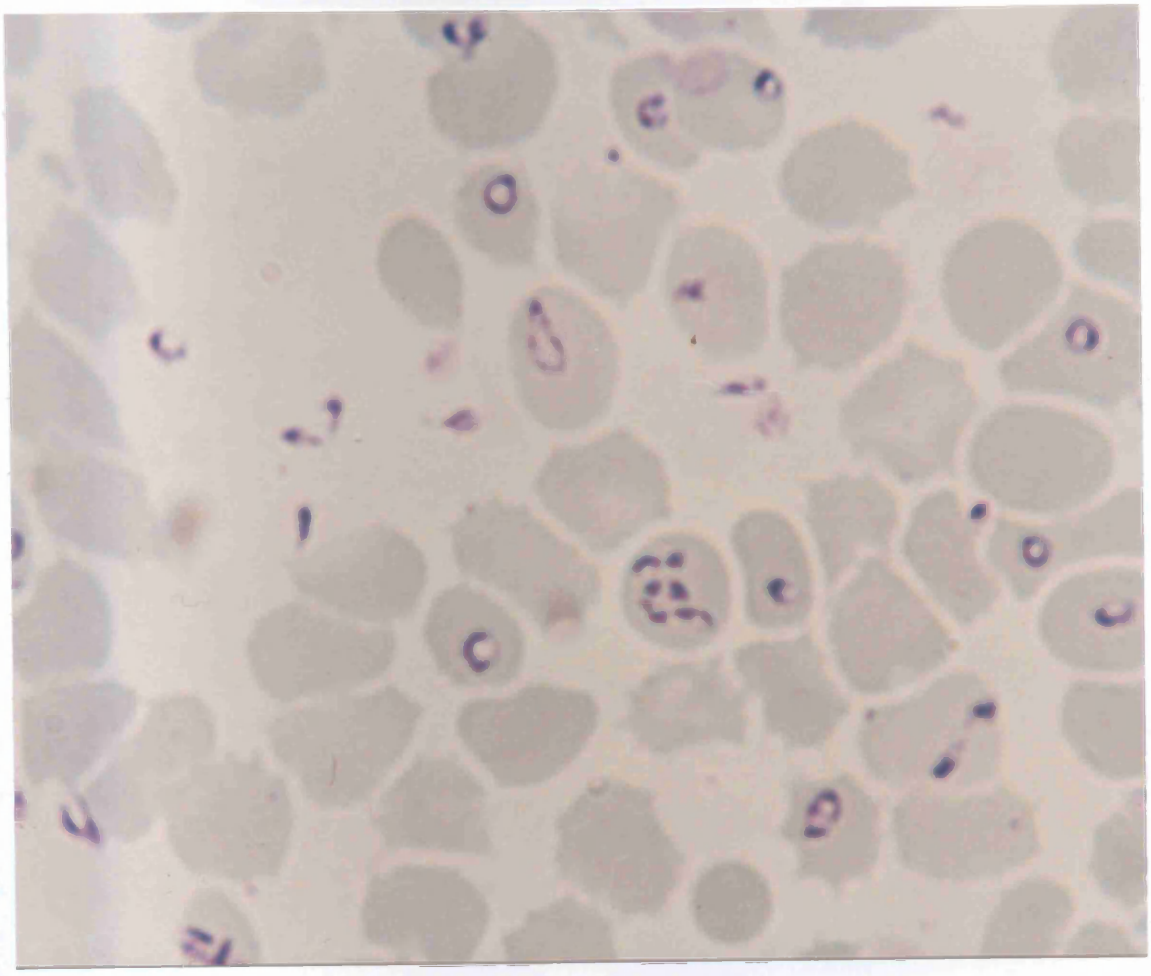


Figure 51:

The effect of passaging WEP 417 isolate and WEP 417 culture (5 weeks) into a splenectomised rat.

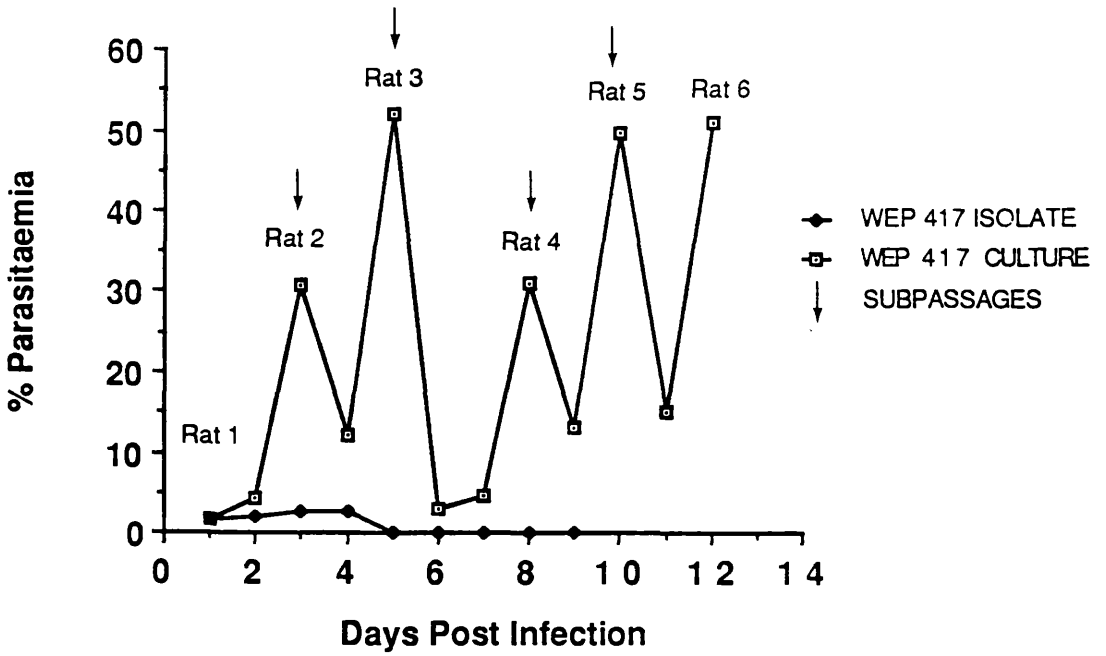
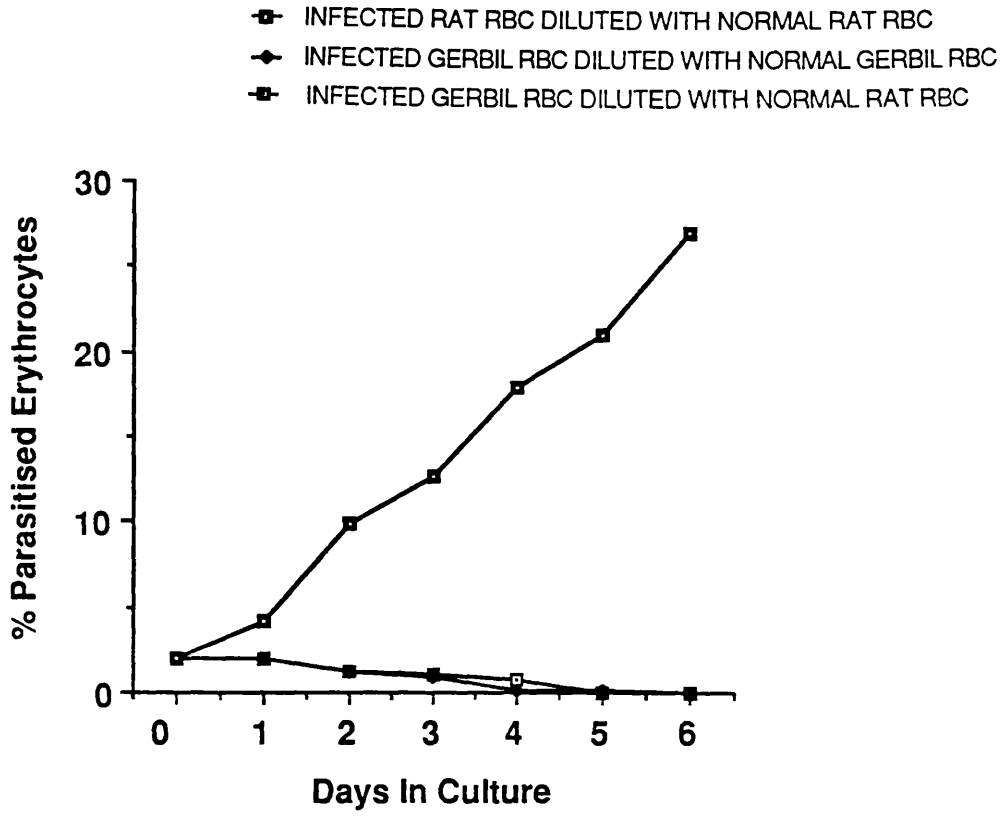


Figure 52:

Dilution of *B. divergens* cultures of infected rat or gerbil erythrocytes with homologous or heterologous cells.



or 1 in 6 with normal O Rh^+ erythrocytes to make a starting parasitaemia of 2%. A 10% suspension was made with complete medium containing 20% FCS, and triplicate cultures were prepared. The percentage of infected erythrocytes increased subsequently from 2% to 11-12% at 48 hours, and subcultures were prepared by diluting these cultures with normal human erythrocytes as described above. Duplicate cultures were prepared. The highest percentage of infected erythrocytes obtained was 3.7% in one culture (five days after subculture) and 11% in the other culture (seven days after subculture) (Figures 53 and 54). Subcultures were made from the culture with 11% infected erythrocytes. The highest percentage of infected erythrocytes in these subcultures was 6% at 48 hours after which the percentage of infected erythrocytes started decreasing, and the parasites looked dead and degenerated. The cultures were discontinued five days after the last subculture. The increase of the number of human erythrocytes in each subculture did not apparently support their growth. This experiment was repeated and the same results were obtained. The modification of the serum (i.e. the use of human serum instead of FCS) in the cultures was not tested.

Discussion

Two bovine field isolates of B. divergens which had been passaged into gerbils and then cryopreserved as stabilates have been adapted to continuous culture in rat erythrocytes. Stabilates that were subpassaged into gerbils and then into splenectomised rats before going into culture grew continuously as well as the rat adapted cultures for 75 days. Some of these

Figure 53:

In vitro growth of *B. divergens* (rat adapted) in human RBC.

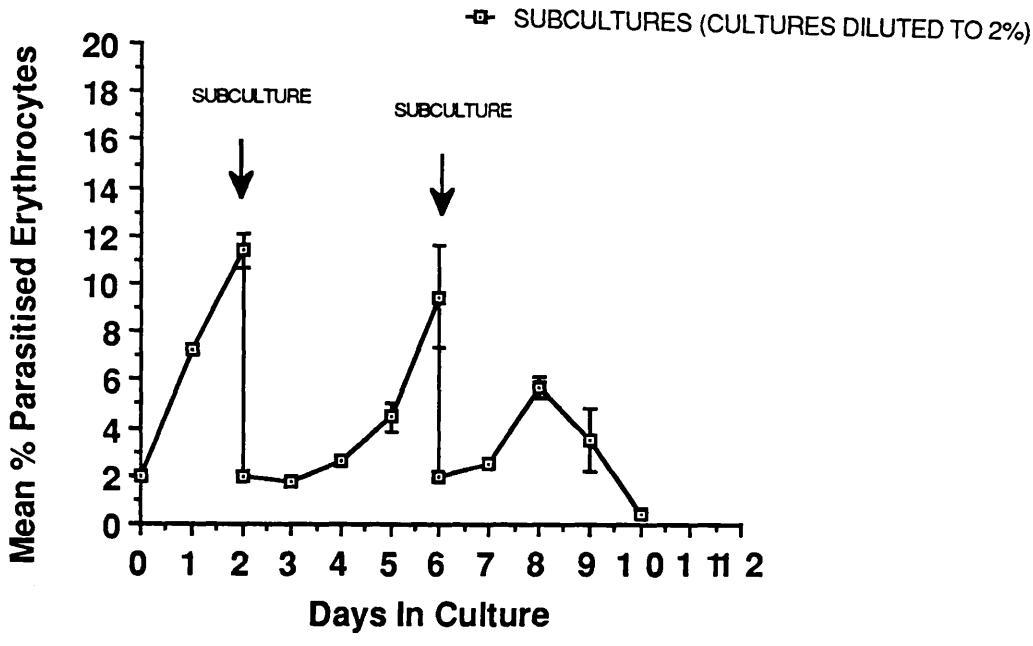
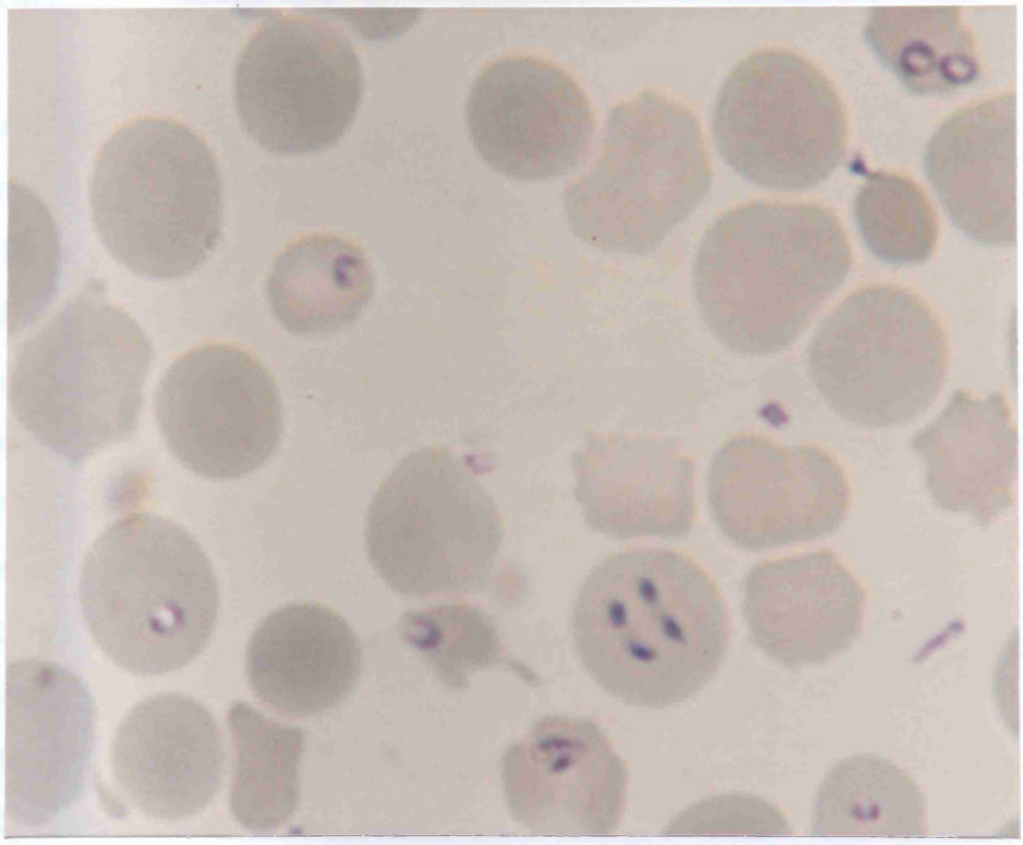


Figure 54:

The morphology of *B. divergens* in cultures containing human erythrocytes.



cultures were used after five weeks to infect splenectomised rats and parasitaemias as high as 52% were obtained. In contrast, parasites of the same isolates which had not been cultured but were injected directly from gerbils to splenectomised rats did not survive long. In addition, cultures that were initiated with parasitised gerbil erythrocytes in normal rat erythrocytes or cultures initiated with parasitised gerbil erythrocytes in normal gerbil erythrocytes did not support the growth of the parasites. It can be concluded therefore that the two bovine isolates that were cryopreserved in gerbil erythrocytes can be maintained continuously in culture only if subpassaged through gerbils into splenectomised rats and then cultured in rat erythrocytes. Cultures initiated with parasitised gerbil blood in rat erythrocytes did not, however, support the growth of the parasite. It appears therefore that gerbil erythrocytes are not suitable for maintaining the parasite in culture. These results are not in agreement with those reported by Konrad et al. (1984) and Pudney (1984) using the same parasite as the present study. Konrad et al. (1984) grew B. divergens in gerbil erythrocytes with bovine serum at a concentration of 40% in the medium. The suitability of human erythrocytes to support B. divergens growth in culture was also investigated. Some of the rat adapted cultures were initiated with human erythrocytes using RPMI 1640 and FCS. Although the parasite was maintained for two weeks in culture, the cultures had to be discontinued after this period as the growth rate of the parasite started decreasing in each subculture, as the number of human erythrocytes used for diluting the cultures increased. It was concluded that human erythrocytes

are unsuitable for the growth of B. divergens in culture using RPMI 1640 and FCS. Pudney (1984) reported that B. divergens can be cultured in human erythrocytes using medium 199 and bovine serum. In the present study, no attempts were made to test the growth of B. divergens in human erythrocytes using other media or sera. It can be concluded therefore that the growth of B. divergens in non rat erythrocytes (gerbil or human erythrocytes) depends on factors which might include the medium or serum used in the cultures, or it might depend on other unknown factors.

CHAPTER NINE

GENERAL DISCUSSION

Chapter Nine

General Discussion

Little is known about the mechanisms of immunity to B. divergens in its normal host, the bovine. The adaptation of this parasite to splenectomised rats (Phillips, 1984), has provided an opportunity to investigate the mechanisms of immunity to it in the rat model. There were two principle aims to this project : firstly, to investigate the mechanisms of immunity to the parasite in vivo, and secondly to develop a method for the in vitro cultivation of B. divergens which would subsequently be used for investigating immune mechanisms in vitro.

B. divergens could not be adapted to intact rats (Phillips, 1984). The course of infection in intact rats lasts only for 7-8 hours after which the parasites disappear from the circulation indicating the importance of the spleen in natural resistance to the parasite. The spleen is a major site for phagocytosis of parasites and PRBC and for the initiation of the primary antibody response (Phillips, 1969c).

The course of infection in splenectomised rats was short lasting (5-6 days) and no recrudescences were observed after the primary patent parasitaemia. The rats either died from the infection which indicates that there was a total or partial failure in the initiation of an effective acquired immunity or they recovered, indicating that antibody production and/or phagocytosis in these animals was taken over by other parts of the RES. The recovered rats were immune to challenge.

B. divergens in splenectomised rats became virulent with

increasing passages and the i.v. injection of 1.5 x 10^8 PRBC resulted in a fatal infection, although previously the injection of 1.5 x 10^8 PRBC of the avirulent parasites resulted in an infection from which most rats recovered. The inoculation of a decreasing number of virulent parasites resulted in an increased prepatent period, but the infection was fatal even when 1 x 10^4 PRBC were injected. 1 x 10^4 PRBC was the lowest dose of parasites tested. It would be preferable, therefore, to titrate B. divergens in splenectomised rats further in order to examine whether the pathogenic effect of the disease would change.

The drug diampron was used to treat splenectomised infected rats after the parasite became virulent. Only a single injection at a dose of 30 mg/kg was enough to cure the animals. Challenging the treated animals with a large number of parasites (1 x 10^8 PRBC) i.v. one month after treatment showed that they were strongly immune. Babesia infections are typically of long duration (Carson and Phillips, 1981). It has been reported that sterile immunity plays an important part in the resistance of cattle to reinfection with B. divergens (Joyner and Davies, 1967), and with B. bigemina (Callow, 1967; Lohr, 1972). Sterile immunity has also been reported for B. microti in mice (Cox and Young, 1969). Joyner and Davies (1967) reported that splenectomised calves were resistant to reinfection for as long as seven years. In the present study, the duration of immunity after drug treatment or in naturally recovered animals was not investigated. It would be worthwhile to examine the duration of immunity in naturally recovered and drug treated rats, in order to test whether sterile immunity plays a role in resistance to

reinfection.

Immunity to Babesia is probably mediated by more than one mechanism (Allison and Clark, 1977). The mechanisms that eliminate babesia from the blood might include humoral factors and/or cell mediated factors. In the present study, passive transfer experiments with immune and hyperimmune sera have demonstrated the participation of humoral factors in acquired immunity to B. divergens in the rat. Passive transfer tests with the fractionated sera have confirmed the role of antibody in protection. Immediately after recovery from the primary infection protection was mainly mediated by IgM antibodies, and 3-4 days after recovery it was mainly mediated by IgG antibodies. The protective activity of hyperimmune serum (2-3 infections) was mainly due to IgG. IgM antibody in hyperimmune serum was, however, partially protective. The concentration of IgG and IgM in hyperimmune and in immune sera was not determined. It is therefore necessary to determine the concentration of these immunoglobulins in immune and hyperimmune sera and to compare it with the concentration of immunoglobulins in normal serum in order to correlate between protection and the concentration of immunoglobulins in hyperimmune or immune sera.

In passive transfer tests with immune sera, the level of serum protection reached a maximum between day 7 (immediately after recovery) and day 13 after which it declined rapidly and no protection was observed on day 53 post infection. It would be of interest to examine the levels of protective antibody after reinfection to check whether these levels change, although it is

expected that the levels of protective antibody would be the same as those detected after the primary infection.

Antibodies to B. divergens were also detected by the IFA test. It was not possible to correlate protection with antibody levels measured by IFAT which demonstrated that part of the anti B divergens antibody repertoire is not protective. Development of a vaccine against B. divergens would require information on those particular antigens which induce the production of protective antibodies. Immunoprecipitation of soluble antigens of B. divergens with immune rat sera, sera with and without significant levels of protective antibody, followed by SDS electrophoresis, might indicate those antigens which specifically react with protective antibodies.

The monoclonal antibody technology invented by Kohler and Milstein (1975) has permitted the identification of molecules relevant to protective immunity. Monoclonal antibodies have been raised against B. bovis (Wright et al., 1983) and B. divergens (Phillips et al., 1987; Winger et al., 1987a). Two of the monoclonal antibodies raised against the rat adapted strain of B. divergens (Phillips et al., 1987) proved to be protective in splenectomised rats. This might provide a means of identifying the protective protein or glycoprotein molecules from B. divergens using affinity chromatography techniques.

Splenectomised rats which recovered from a primary B. divergens infection were immune to challenge. The mechanisms by which parasites or PRBC were removed from the circulation of immune rats were not, however, elucidated. It has been reported that in the rat, the liver is the second most important organ

after the spleen where parasites or PRBC sequester (Ultman and Gordon, 1965). The possible removal of PRBC by the livers of immune rats was investigated using Cr^{51} labelled PRBC. It was concluded from this experiment that either the parasites within the red cells were opsonised and then removed by the liver where they were phagocytosed, or the merozoites may have been destroyed by antibody as they emerged from the erythrocytes and the damaged RBC were removed by the liver. Impression smears taken from immune livers gave no evidence that parasites or PRBC were phagocytosed by macrophages in that organ. In another experiment irradiated parasites were injected into immune rats to determine whether it was intact PRBC or free merozoites that are cleared by the immune rats. It was concluded from this experiment that immune splenectomised rats were able to clear PRBC from the circulation and that immunity was not obviously directed to merozoites. The effector mechanisms which were responsible for the destruction or removal of PRBC are unknown. It is possible that effector cells can recognise PRBC in the circulation, as has been suggested for murine malaria (Allison and Eugui, 1983), through F_cIgG receptors which can bind to PRBC in the absence of antibody, and such binding triggers O_2 production. This would in turn lead to the destruction of PRBC. It is also possible that the liver might be important for the destruction of PRBC and for the development of acquired immunity, since in the present study, histological examinations showed that leukocytes accumulate in this organ. These accumulated cells had the appearance of "pseudofollicles" as described by Weiss (1985) in splenectomised

gerbils infected with P. berghei. Preliminary studies have shown that some of these cells consisted of B and T lymphocytes which indicate that they could be a source of antibabesial antibody. The presence of T lymphocytes indicate that they may act as helper cells in protective antibody production, be cytotoxic to parasites or perhaps be necessary for the release of non antibody factors (lymphokines) which were reported to have an antiparasitic activity (Clark et al., 1976). It is necessary, therefore, to identify the different subpopulations of cells in the liver which might be important for the development of protective immunity to B. divergens and to test the macrophage activity in this organ. The possible participation of T lymphocytes in protection was investigated in vitro as will be mentioned later in the discussion when describing B. divergens cultures.

The acute parasitaemia was accompanied by severe anaemia evident as a drop in RBC counts. The cause of this anaemia was not investigated. It is expected, however, that some anaemia must have been caused by the vascular destruction of the RBC as the parasites leave the cells to invade other cells, or it might be due to immunological factors.

Recovery from the infection was immediately followed by a pronounced leukocytosis which was predominately a blood lymphocytosis. This could be the result of a rapid production of new lymphocytes or indicates that there was a redistribution of the lymphocyte pool as they move from the blood circulation to the lymphoid organs and return back to the blood stream. Similar blood lymphocytosis was described in splenectomised animals (Ford

and Smith, 1979). Histological studies gave evidence that leukocytes are accumulating in the livers of immune rats which indicate that lymphocytosis was due to altered patterns of migration of lymphocytes to the lymphoid organs.

The development of a method for the in vitro cultivation of B. divergens gave the opportunity to investigate some of the immune mechanisms in vitro. B. divergens was cultured continuously for six months in rat erythrocytes using the candle jar technique. Some aspects of the mechanisms of action of antibody were investigated in vitro. The role of opsonising antibody was tested using hyperimmune serum and B. divergens PRBC or free parasites in the presence of peritoneal macrophages. No evidence was found in vitro for a role in protection. Investigations on the role of opsonising antibody can be repeated by altering the culture conditions i.e. using different medium and serum, by using other populations of macrophages such as the kupffer cells in the liver and by testing immune sera collected at different times after recovery.

The protective activity of immune serum in vivo was then compared with its effect on parasite growth in vitro. There was no evidence that antibody agglutinates merozoites and prevent them invading erythrocytes in vitro. Evidence that antibody neutralises merozoites was, however, demonstrated only by preincubating free merozoites with immune serum before adding them to cultures containing normal rat erythrocytes.

Fresh normal rat serum was toxic to the parasite in culture and it was difficult to differentiate between cultures containing

immune or normal serum. Attempts to reduce the toxic effect of normal serum either by heat inactivation or by dialysing it against PBS before it was added into culture were unsuccessful.

The Ig fraction of normal and immune sera was precipitated with sodium sulphate. The Ig fraction was first precipitated with sodium sulphate to give an 18% w/v Ig's and then further precipitated with sodium sulphate to give a 14% w/v Ig's. The 18% of both normal and immune serum were inhibitory to parasite growth in culture. The 14% Ig's of normal or immune serum did not, however, have an inhibitory effect on parasite growth in culture, indicating that the toxic effect of normal serum was lost in the 14% Ig's fraction. Investigations on the toxic effect of normal serum in culture are therefore necessary so that it would be possible to use it as a control in culture. The lack of evidence that antibody can opsonise parasites or PRBC and promote their phagocytosis by peritoneal macrophages, and the failure to demonstrate that antibody agglutinates merozoites and prevents their invasion *in vitro* has led to the possibility that antibody might only be protective in the presence of cellular elements. An ADCC assay using immune spleen cells or PMBC's gave no evidence of specific ADCC damage of parasites *in vitro*. A CMC assay in the absence of antibody also gave no evidence of specific killing of parasites *in vitro*. It is suggested therefore that a non specific type of immunity mediated by soluble factors secreted by activated macrophages might be important in protective immunity to Babesia in splenectomised rats. It has been reported for *B. bovis* that soluble products secreted by mononuclear phagocytes, activated by soluble antigens

or immune complexes inhibit the *in vitro* growth of *B. bovis* (Montealegre *et al.*, 1985). The main advantage of *B. divergens* cultures is the ability to collect a large number of free merozoites in the culture supernatant. This would provide an opportunity to collect a large quantity of soluble antigens. The presence of soluble factors secreted by mononuclear phagocytes could therefore be investigated and their effect on parasite growth could be subsequently tested in the presence of soluble parasite antigens and immune complexes. In addition, the ADCC assay can be repeated, by testing immune sera collected at different times after recovery or hyperimmune serum and by testing different ratios of spleen cells or PMBC's in culture.

It has been suggested that the appearance of intracellular damaged "crisis" parasites in humans infected with *P. falciparum* is due to crisis form factors (Jensen *et al.*, 1982; 1983; 1984; Butcher *et al.*, 1987) and tumor necrosis factor (Carlin *et al.*, 1985) which are present in sera of humans immune to *P. falciparum*. These non antibody factors act as toxins leading to the death of parasites within host erythrocytes. It has been suggested that this factor may be a product of T cell activated macrophages (Jensen *et al.*, 1984). It is possible therefore to investigate the role of these non specific factors (crisis form factor and tumor necrosis factor) using *B. divergens* cultures in the presence of immune serum.

It has been reported that immunogens consisting of soluble exoantigens collected from culture supernatants are prime candidates for babesiosis vaccines, because they are safe,

effective and inexpensive (Levy and Ristic, 1980; Montenegro-James et al., 1987). Vaccination with soluble babesia immunogens has been shown to induce solid clinical protection against homologous (Smith et al., 1979; 1981; Kuttler et al., 1982; 1983) and heterologous (Timms et al., 1983; Montenegro James et al., 1985) parasite antigens. A similar approach could therefore be applied for B. divergens using soluble antigens collected from culture supernatants. The percentage of infected erythrocytes obtained by B. divergens cultures was relatively high (35%). This would provide a high concentration of antigens in the supernatant which could subsequently be used for immunisation trials in splenectomised rats similar to those described for B. divergens, in the mongolian gerbil Meriones unguiculatus (Winger et al., 1987b). B. divergens cultures can also be used to develop drug sensitivity assays similar to those developed for B. bovis cultures (Levy and Ristic, 1981).

The development of a method for the long term cultivation of B. divergens in rat erythrocytes has provided an opportunity to adapt new babesia isolates for long term cultivation. Two bovine isolates of B. divergens were adapted for continuous cultivation in rat erythrocytes. These cultures grew as well as the rat adapted cultures. The adaptation of new isolates of B. divergens to continuous culture would provide a good system for investigation different aspects on babesiosis without the need for the animal host.

The suitability of erythrocytes from non rat species (gerbil and human erythrocytes) to support growth of B. divergens was also investigated. Cultures initiated with gerbil erythrocytes

did not support the growth of the parasite. Cultures initiated with human erythrocytes were maintained for two weeks and had to be discontinued after this period as the growth of the parasite was decreasing. It was concluded that gerbil and human erythrocytes are unsuitable for the growth of B. divergens in culture using RPMI and FCS. The suitability of non rat erythrocytes to support growth of the parasite in culture can be tested again using different medium and serum.

Advances in recombinant DNA technology offer another approach to the production of suitable vaccines. Immunity against the malaria parasite P. yoelii yoelii has been demonstrated using single peptides (Holder and Freeman, 1981). Wright et al. (1983) speculated that the antigen they described for B. bovis, which is both small (44×10^3 daltons) and a single polypeptide chain can be ideal candidate for vaccine production using a recombinant DNA technology. Recombinant DNA technology might therefore provide an opportunity to search for a vaccine which will protect against the pathogenic stage of the infection.

In conclusion, antibody plays an important role in protection in vivo. Its mode of action is, however, unknown. Antibody dependent cellular mediated factors may be involved. Non antibody mediated factors might also play an important role in protective immunity to Babesia. This might be mediated through T cell activated macrophages which would lead to the death of the parasite within the erythrocyte. The liver might play an important role in protective immunity against B. divergens in splenectomised rats. The adaptation of three

isolates of *B. divergens* for long term cultivation would facilitate the study of different aspects of babesiosis, as described in the text.

APPENDIX A

Phosphate buffers (PBS)

1. Giemsa's buffer

   3 g Na_2HPO_4

   0.6 g KH_2PO_4

   Made up to 1 litre with distilled H_2O

   pH adjusted to 7.2 with 1N NaOH

2. Isotonic PBS

   Stock solution

   60 g $Na_2HPO_4.12H_2O$

   13.6 g $NaH_2PO_4.2H_2O$

   8.5 g Nacl

   Made up to 1 litre with distilled water

   0.9 saline

   9 g Nacl

   Made up to 1 litre with distilled H_2O

   Buffer

   40 ml stock

   Made up to 1 litre with 0.9% saline and adjusted to pH 7.2

0.07M Phosphate buffer for isolation of IgG from serum on DEAE cellulose

Stock solution (0.5M)

(a) 78 g $NaH_2PO_42H_2O$ ------> Each made up to 1 litre

(b) 71 g Na_2HPO_4 with distilled H_2O

Solutions (a) and (b) mixed to obtain pH 7

140 ml of mixed stock made up to 1 litre with distilled water to obtain 0.07M solution

Borate buffer pH 8

6.185 g Boric acid (BDH)

9.536 g Borax (Sodium tetraborate) $Na_2B_4O_7.10H_2O$ (BDH)

4.384 g Nacl (Formachem)

Made up to 1 litre with distilled H_2O

pH adjusted to 8 with 1N Hcl

0.1 M Tris Nacl pH 8

12.11 g Tris (hydroxymethyl) amino methane $C_4H_{11}NO_3$

Made up to 1 litre with distilled H_2O

pH adjusted to 8 with 0.5 M Nacl (29.22 g)

Formal saline

8.5 g Nacl (Formachem)

100 ml Formalin (BDH)

900 ml Distilled H_2O

3.5% Saline

3.5 g Nacl (Formachem)

Made up to 100 ml in distilled H_2O and filter sterilised

4.5% Saline

4.5 g Nacl (Formachem)

Made up to 100 ml in distilled H_2O and filter sterilised

0.2% Saline (for lysing PRBC)

2 g Nacl (Formachem)

Made up to 1 litre with distilled H_2O

Tris ammonium chloride

0.83 g NH_4cl (BDH)

Made up to 100 ml with distilled H_2O

Tris (Boehringer Mannheim)

Made up to 100 ml

pH adjusted to 7.6 with

1 part tris and mixed with 9 parts 0.83% NH_4cl

Barbitone buffer (for immunoelectrophoresis) pH 8.6

1. **Tank buffer**

1.38 g Biethylbarbituric acid (BDH)

8.76 g Sodium barbitone (BDH)

0.83 g Calcium lactate (BDH)

Made up to 1 litre with distilled water

2. **Buffer for preparation of gel**

0.55 g diethylbarbituric acid (BDH)

3.5 g sodium barbitone (BDH)

0.51 g calcium lactate (BDH)

Made up to 1 litre with distilled water

Add 10 g agarose 15 (Electron - BDH) to make a 1% (w/v) agarose in barbitone buffer (in a water bath 70-90^{o}C)

APPENDIX B

Media

1. RPMI

Stock

10.4 g RPMI 1640 powdered medium (with L-glutamine) (Gibco)

5.94 g N_2-hydroxyethyl piperazine-N'-2 ethane sulphonic acid (Hepes) (Sigma)

Made up to 960 ml with distilled H_2O and filter sterilised (Millipore filter size 0.22 Um) and aliquoted into 100 ml bottles

RPMI for <u>in vivo</u> use

100 ml RPMI (stock)

4.2 ml 5% w/v $NaHCO_3$ (filter sterilised)

0.25 ml gentomicin sulphate (Sigma)

5 ml foetal calf serum (Gibco)

RPMI for culture

Incomplete RPMI

100 ml RPMI (stock)

4.2 ml 5% (w/v) $NaHCO_3$ (filter sterilised)

0.25 ml gentomycin sulphate (Sigma)

Complete RPMI

80 ml incomplete RPMI

20 ml foetal calf serum (Gibco)

2. Medium 199

9.6 g Medium 199 with Earle's salts and glutamine (Flow)

2 g glucose (BDH)

200,000 units benzil pencillin

100,000 units streptomycin

30 ml 6.6% (w/v) $NaHCO_3$ in distilled H_2O

Made up to 1 litre with distilled H_2O and filter sterilised

APPENDIX C

17.5% Sorbitol for recovering of cryopreserved PRBC

17.5 g Sorbitol (BDH)

Made up to 100 ml with distilled H_2O and filter sterilised

Sorbitol glycerol for cryopreservation of PRBC

380 g glycerol (BDH)

29 g sorbitol (BDH)

6.3 g Nacl (Formachem)

Made up to 1 litre with distilled H_2O and filter sterilised

10% DMSO in puck's saline (for cryopreservation of merozoites)

1 g DMSO (Sigma)

0.2 g glucose (BDH)

Made up to 10 ml with puck's saline (Gibco)

APPENDIX D

White cell diluting fluid

490 ml distilled H_2O

10 ml glacial acetic acid (Analar - BDH)

Few drops crystal violet stain (Edward Gurr Ltd.)

APPENDIX E

Coomasie blue stains and destains (for immunoelectrophoresis)

10 ml glacial acetic acid (BDH)

45 ml Methanol (BDH)

45 ml distilled water

0.5 g Coomassie blue (Sigma)

Destain

10 ml glacial acetic acid (BDH)

45 ml methanol (BDH)

45 ml distilled water

Blue dextran 2000

0.01 g blue dextran 2000 (Sigma)

0.5 g sucrose (BDH)

Add 10 ml distilled water

1% Bromphenol blue

0.1 g bromophenol blue (BDH)

Made up to 10 ml with distilled water

APPENDIX F

Ficoll hypaque (for separation of PBMC)

34% Isopaque

20 ml Isopaque (NyComed UK Ltd.)

24 ml Sterile distilled water

9% Ficoll

9 g Ficoll 400 (Pharmacia)

Made up to 100 ml distilled water

10 ml 34% isopaque are mixed with 21 ml 9% Ficoll and filter sterilised.

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