IMMUNE RESPONSES TO *BABESIA DIVERGENS* GROWN IN HUMAN AND BOVINE ERYTHROCYTES.

by

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ABSTRACT

"J" and Weybridge strains of *Babesia divergens* isolated from human and bovine infections respectively, were maintained in microaerophilous stationary phase cultures in both human and bovine erythrocytes. Parasites from these were used to study the human immune response to *B. divergens* in immunocompetent individuals.

Antigenic differences were found between merozoites of the same strain of *B. divergens* when raised in different erythrocytes. A surface antigen recognised by a monoclonal antibody raised against merozoites from bovine cultures was found to be absent/altered on merozoites isolated from human erythrocytes.

Merozoites from both human and bovine cultures were used in the following assays to investigate the human immune response to *B. divergens* in unexposed donors. No difference in immunogenicity was found between the two merozoite groups.

Studies of the human T-cell response to *B. divergens* merozoites revealed no mitogenic or antigenic stimulation in immunocompetent donors.

Enzyme-linked immunosorbent assays (ELISAs) and immunoblots carried out using merozoites as antigens showed there was no reactive IgG in normal human sera. However, both immune and non-immune sera inhibited parasite entry into erythrocytes in merozoite neutralisation assays. EBV transformation of B-cell precursors from normal peripheral blood revealed the presence of B-cells capable of secreting IgM reactive against merozoites in some donors.

Assays investigating the normal human blood monocyte response to *B. divergens* showed the efficiency of non-specific immune responses in inhibition of parasite growth. Monocytes and their culture supernatants significantly retarded the growth of *B. divergens* without prior activation. Merozoites were found to induce the release of reactive oxygen intermediates in these adherent cell populations. This response was enhanced following incubation of the merozoites with both normal and immune serum.

Responses of peripheral blood mononuclear cells from species susceptible to *B. divergens* were also investigated. Merozoites isolated from both human and bovine cultures did not induce mitogenic or antigenic responses in non-immune bovine T-cells. Experiments using peripheral blood

unguiculatus) revealed an enhancement of parasite growth following the introduction of adherent cell populations into *B. divergens* cultures. Supernatants from these cells had no effect upon parasite growth, but following activation with lipopolysaccharide both the cells and their supernatants proved inhibitory. Activated gerbil peritoneal macrophages failed to produce tumor necrosis factor in response to *B. divergens* merozoites or to supernatants from infected gerbil erythrocytes.

TABLE OF CONTENTS

Page No:

Page No:

Page No:

Page No:

TABLE LEGENDS

Page No:

FIGURE LEGENDS

Page No:

Page No:

Page No:

GENERAL INTRODUCTION

Since the first report of a case of human babesiosis in Yugoslavia by Skrabalo and Deanovic (1957), there have been a further 18 documented cases in Europe (Brasseur and Gorenflot, 1991). In all but 2 of these, a bovine *Babesia* species has been identified as the aetiological agent. Splenectomy or some form of immunological insufficiency have been common factors linking all the cases, of which 60 % have proved fatal (Gorenflot *et al.*, 1987).

Experiments on chimpanzees by Garnham and Bray (1959) showed the importance of the spleen in the outcome of an infection with *B. divergens*: splenectomy rendered the chimpanzees susceptible to infection whereas intact animals were refractory even to heavy inocula of the parasite. Alteration of innate resistance to many species of *Plasmodium*, resulting from splenectomy has also been well documented. In intact chimpanzees, the human malaria parasites *P. ovale*, *P. vivax* and *P. falciparum* are able to invade the erythrocytes but are rapidly eliminated. Splenectomised chimpanzees, however, are vulnerable to all of these species of *Plasmodium* (Bray, 1957a , b ; Bray, 1958).

Serological surveys have shown an antibody response to *Babesia* in man (Leeflang, 1976; Osorno, *et al.*, 1976; Gorenflot, 1987.) As it seems unlikely that splenectomy would render an individual more susceptible to tick bites, this indicates the opportunistic nature of clinical human babesiosis. There has been an increase in the numbers of reported cases in the past 15 years, probably resulting from improved identification and diagnosis, coupled with a rise in the number of splenectomies and the use of immunosuppressive drugs.

Although splenectomy is known to be a factor in increased susceptibility of man to numerous infections (Munro, 1985), it is not clear exactly how this takes effect. Studies of the human immune system are problematic, not least because of the constant barrage of antigens from the environment and interactions between infecting organisms themselves (Cox, 1987). When human immune responses to acute *B. microti* infection were investigated, it proved difficult to link fluctuations in T cell ratios and percentages with that particular infection (Benach, *et al.*, 1982). This is further complicated in human babesiosis of bovine origin where levels of immunoglobulins may be altered by splenectomy (Chaimoff, *et al.*, 1978)

and indeed by the condition necessitating the splenectomy, such as hairy cell leukaemia (Magee *et al.*, 1985).

In contrast to the increased infectivity and pathogenicity of some species of *Plasmodium* and *Babesia* caused by splenectomy of normally refractory hosts, the absence of a spleen may decrease the virulence of other parasitic protozoa. *P. cynomolgi* has been reported to be less virulent in splenectomised Rhesus monkeys than in intact monkeys (Garnham, 1970). Also, there is a reduction in the severity of *B. bovis* infections in cattle after splenectomy, which is manifested by a decrease in the numbers of animals developing cerebral babesiosis, a fatal complication due to sequestration of infected erythrocytes in brain capillaries (Wright *et al*, 1988). Reduction in virulence of *B. bovis* occurs during rapid passage of infection through splenectomised calves (Callow *et al*, 1979). This has lead to the production of live attenuated vaccines which are now widely used in Australia.

Clearly the effects of splenectomy on the course of babesial infection are dependent upon the host /parasite combination and parasite virulence. In cattle, it is believed that an antigenic shift occurs in the absence of a spleen, with selection for avirulent, immunogenic antigens, or against virulent immunosuppressive ones (Callow *et al*, 1979). In addition, there is evidence that alteration in virulence of *B. bovis*, *Trypanosoma brucei* and *Plasmodium berghei* is correlated with emergence of different antigens (Kahl *et al.*, 1982).

The morphology of *B. divergens* in bovine erythrocytes differs from that seen in abnormal hosts' cells such as those of man, chimpanzees and the Mongolian gerbil *Meriones unguiculatus* (Garnham and Bray, 1959 ; Canning *et al*, 1976). As well as an increase in size of the parasites, perhaps due to the larger erythrocytes of the non-bovine hosts, the present study revealed antigenic differences. A monoclonal antibody, 13.4, which binds to a surface antigen on *B. divergens* merozoites and is associated with erythrocyte invasion (Winger .*et al*, 1987a), has been found to bind only to merozoites from bovine culture and not those grown in human erythrocytes (Flory *et al*, 1990)

In the present study, the "J" strain of *B. divergens*, isolated from a human case of babesiosis in Scotland (Entrican *et al*., 1979) has been grown *in vitro* in both human and bovine erythrocytes. Investigations into the *in vitro* human T-cell, B-cell and monocyte responses to *B. divergens* have

been carried out using cells from unexposed donors, as well as those from one exposed donor. A comparison has been made between the responses to parasites raised in human and bovine cultures in order to ascertain potential differences in immunogenicity. Also, these experiments have been extended to investigate some of the responses in susceptible hosts, the Mongolian gerbil and cattle, in order to assess host-related differences in response.

1. REVIEW OF THE LITERATURE

1.1 BABESIOSIS

This is a tick borne disease, caused by protozoa of the genus *Babesia*. These parasites are capable of infecting a wide range of vertebrate hosts throughout the world, but the greatest economic loss is due to *Babesia* infections in cattle, notably in developing countries. The disease is characterised by pyrexia, haemolytic anaemia, haemoglobulinuria and the presence of parasites in the hosts' erythrocytes. Different *Babesia* species can cause a range of disease manifestations and severity, including the occurrence of a cerebral form which is frequently fatal. This is reflected in the variety of terms used for babesiosis, which include "cattle malaria", "red water fever", "Texas fever", "splenic fever" and "piroplasmosis" among others.

Babes (1888) first identified these parasites while examining cattle with the above symptoms, naming them *Haematococcus bovis*, later to be renamed *Babesia bovis*. Smith and Kilborne (1893) identified the aetiological agent of Texas fever, *B. bigemina*, but more importantly, were the first to establish that a protozoon could be transmitted by an arthropod vector (*Boophilus annulatus*). In 1911, M'Fadyean and Stockman described the occurrence of a piroplasm in the blood of British cattle. This was named *Piroplasma divergens* because the paired parasites had a wide angle of divergence and were often found on the periphery of the erythrocytes.

In general, there are two morphologically distinct forms of *Babesia* species. One is small, rounded or pear shaped, with dividing forms occurring at an obtuse angle. This group includes *B. bovis*, which measures approximately 2.0 x 1.5 µm (Rieck, 1966) and *B. divergens*, measuring 1.5 x 0.4 µm (Joyner *et al.*, 1963). Species in the second group are larger, with the paired forms being at a more acute angle. These include *B. bigemina*, which measures approximately 4.5 x 2.0 µm (Koch, 1966), and another cattle parasite *B. major*.

There are typically at least one large and one small *Babesia* species found in each susceptible vertebrate host. A notable exception to this is man where the bovine species identified have both been small i.e. *B. divergens* and *B. bovis*. Numerous human infections with the rodent parasite *B. microti* have also been recorded. This again is a small species, measuring 1.5-2.0 µm (Mehlhorn and Schein, 1984), but there is evidence that this and the equine species *B. equi* are more closely related to

parasites of the genus *Theileria* on the grounds of their transstadial mechanism of transmission and the pre-erythrocytic development in lymphocytes.

1.1.1 Life cycle and morphology

The geographical distribution of different *Babesia* species must correlate with that of its tick vector. Thus *B. bigemina* and *B. bovis*, which are transmitted by ticks of the genus *Boophilus*, are both found in Central and South America, Southern Europe, Africa, Asia and Australia (Riek, 1966). *B. divergens* is found in Western and Central Europe along with its tick vector *Ixodes ricinus* (Joyner *et al*, 1963), while *B. major* occurs in Western and Southern Europe and in North-West Africa, being transmitted by *Haemaphysalis punctata* (Morzaria *et al*, 1978). *B. microti* is transmitted by *Ixodes* species and is found in Europe and North America (Friedhoff and Smith, 1981).

All true species of *Babesia* are transmitted transovarially from infected female ticks to their progeny. The adult females acquire a primary, alimentary infection through feeding on an infected animal. The majority of parasites die within the gut. Recent work by Mackenstedt, *et al*, (1990) , has shown the existence of gamonts in host erythrocytes and it is thought that these are the stages which continue development in the gut. These differentiate to form "Strahlenkorper" or ray bodies, which are believed to be the gametes (Rudzinska *et al*, 1983; Melhorn and Schein, 1984; Mackenstedt *et al*, 1990). Within 2-4 days after the tick has engorged, these fuse to form a spherical zygote (Freidhoff, 1988) which then undergoes transformation to form a uninucleate motile kinete (7-8 µm). These initiate cycles of multiple fission in the epithelial cells of the gut, releasing sporokinetes which enter the haemolymph where they invade haemocytes, Malpighian tubule cells, muscle fibres, ovarian cells and oocytes and eventually the salivary glands of the larvae. The feeding patterns of the various tick vectors influence at which stage the sporozoites produced in the salivary glands are transmitted to the mammalian host. The various stages of *Boophilus* species feed on only one host and it has been found that *B. bovis* transmission occurs by the larval stage of the next generation but *B. bigemina* is transmitted by both nymphs and adults of the next generation. In *Ixodes ricinus*, which is a three host tick, *B. divergens* sporozoites are transmitted by all the developmental stages (Young and Morzaria, 1986).

With *B. microti* and *B. equi*, transstadial transmission occurs where parasites are ingested by tick larvae or nymphs and then transmitted by the succeeding stage only. As a result, the infection is lost after transmission. Sporozoites of true *Babesia spp.* attach to and penetrate erythrocytes directly following transmission. The intraerythrocytic sporozoite develops into a feeding stage trophozoite, which then undergoes merogony to form two daughter cells called merozoites. Infrequently, more than two merozoites can be produced and "Maltese Cross" tetrads are seen.

Erythrocyte invasion by merozoites has been well studied (Rudzinska *et al*, 1976) and occurs by endocytosis, as in *Plasmodium*. Following attachment via the apical end, the rhoptries discharge their contents and the merozoite becomes interiorized, leaving the surface coat outside the erythrocyte. The parasite then lies in a parasitophorous vacuole. In *Babesia*, unlike *Plasmodium*, the erythrocyte membrane forming this vacuole disintegrates and the parasite then lies in direct contact with the erythrocyte cytoplasm. Development and asexual division occur as for the sporozoite and merozoites will continue invading new erythrocytes until the host dies or eliminates the parasites. Gamonts are produced in some cases which will undergo no further development in the erythrocyte until ingested by a tick thus completing the cycle (Mackenstedt, 1990).

1.1.2. Host specificity

This applies to both the tick vector and the *Babesia* species concerned. Where a one-host vector is reponsible for transmission, such as for *B. bovis* and *B. bigemina*, the vertebrate host specificity for the parasites is equally rigid. *Boophilus* species of ticks very rarely attack man (Osorno, 1976), and although some of the European cases of babesiosis were, at the time, identified as *B. bovis* infections, this now seems unlikely (Brocklesby, 1979).

B. divergens is transmitted by the three-host tick *Ixodes ricinus*. Since all stages are thought to be able to transmit the parasite (Donnelly and Pierce, 1975), the range of potential mammalian hosts is increased. Experiments by Lewis and Young (1980) confirm that *B. divergens* can be transmitted to hosts other than cattle, and it has been found in red deer and reindeer (Adam, *et al*, 1976 ; Nilsson, *et al*, 1965). Infected cattle and ticks have been epidemiologically related to the acquisition of human infections (Skrabalo and Deanovic, 1957 ; Garnham *et al*, 1969; Entrican *et al*, 1979). Also a human serological survey has shown the presence of

antibodies to *B. divergens* in 15 out of 508 volunteers tested from the West of France where bovine *B. divergens* infections are common (Gorenflot, 1987).

With regard to *B. microti*, the geographical distribution of one of the tick vectors has been shown to correspond neatly with the occurrence of human infections (Piesman and Spielman, 1980). In Europe the vectors of *B. microti* are *I. ricinus* and *I. trianguliceps* (Freidhoff, 1988) but these have been found to transmit *B. microti* only to small rodents. In North America the vector is *I. dammini*, the Northern deer tick (Spielman, *et al.*, 1979) This feeds primarily on deer mice and the American field vole as larvae but also on deer, man and domestic animals in the nymphal stages (Piesman and Spielman, 1980). The introduction or increase of deer populations on Nantucket island and other islands off the Massachusetts coast, in Wisconsin and the Long Island complex in New York, has been associated with the increase in human infection in these sites (Hoogstral, 1981). The deer are not infected with the parasite but act as a blood meal for the nymphs and adults. However, the nymphal *I. dammini* readily attach to humans in whom the infection will develop. The subclinical nature of this infection in many healthy individuals has resulted in additional modes of transmission : there have been several cases of transfusion transmitted *B. microti* infection (Jacoby *et al.*, 1980 and Cahill *et al.*, 1981) and one case of transplacental transmission (Esernio-Jensen *et al.*, 1987).

1.2. HUMAN BABESIOSIS

Surveys have demonstrated serological evidence of human exposure to *Babesia* parasites (Leeflang, 1976 ; Osorno *et al.*, 1976 ; Gorenflot, 1987 ; and Popovsky *et al.*, 1988), without any associated illness reported. Thus, with all the documented cases of clinical babesiosis occurring in individuals with predisposing factors such as splenectomy, immunodeficiency or old age, the clinical form of the disease must be viewed as an opportunistic infection. There are two distinct forms of this disease in humans, related to the species involved and its epidemiology. The first human cases were found in Europe and these are associated with babesias of bovine origin. The second type is found in North America, caused by the rodent parasite *B. microti* Also, apart from the geographical differences, there is a marked distinction in the severity and outcome of the diseases in humans.

1.2.1. European babesiosis

These cases have all occurred in splenectomised or immunocompromised individuals and have generally proved fatal. Skrabalo and Deanovic (1957) published the first documented case of human babesiosis. This was in a 33 year-old Yugoslavian, who had been splenectomised 11 years previously and the outcome was fatal. *B. bovis* was thought to be the species infecting cattle in the area and the tick vector was also found to be present.

The second case occurred in Ireland in 1968 in a deep sea fisherman (Fitzpatrick *et al*, 1968 and 1969). The patient had been splenectomised three months previously and again the infection proved fatal. Bovine red water fever was found in the area where he had been on holiday shortly before becoming ill. The infective agent was later identified as *B. divergens*. A further infection was then reported in a Yugoslavian from the same area as the first case (Skrabalo, 1971). Again the patient had been splenectomised and the outcome was fatal. The organism responsible in this case was identified as *B. divergens* and not *B. bovis*. There may have been confusion in the identity of the *Babesia* sp. responsible for the first case possibly because of the unusual morphology of the parasite in human erythrocytes (Brocklesby, 1979).

The first two non fatal infections occurred in France in 1976 (Bazin *et al*, 1976 and Gorenflot *et al*, 1976). Both patients had been splenectomised and were treated with chloroquine and exchange blood transfusion and both eventually recovered. *B. divergens* was believed to be the aetiological agent.

In 1979, Entrican *et al*, described the first human case of babesiosis in Scotland. The patient had been splenectomised and died despite treatment with antimalarial drugs and a blood transfusion. Infected blood from the patient was inoculated intraperitoneally (IP) into rats, guinea pigs, mice, nude mice, hamsters and gerbils but an infection only ensued in the gerbils. The parasite was identified as *B. divergens* by inoculation of the patient's blood into a splenectomised calf and from immunofluorescent antibody titres of the human and bovine sera against other *Babesia* species. This was the first time a bovine *Babesia* species ("J" strain) had been maintained in a laboratory animal and it was found that bovine-derived B. *divergens* strains were also infective to gerbils (Lewis and Williams, 1979). In addition, adult *Ixodes ricinus* were shown to transmit the disease

transovarially, with the resulting larvae introducing infections into gerbils and a splenectomised calf (Lewis and Young, 1980).

Since that time a further fatal case has been described in a splenectomised Russian woman (Rabinovich *et al.*, 1978), five more cases have been recorded in France (Gorenflot *et al.*, 1987) and two in Spain. The first Spanish case was described by Woessner *et al.*, (1986), where a 26 year-old male was found to have an asymptomatic infection with an unidentified *Babesia* species. The man was neither splenctomised nor immunocompromised but had a slight imbalance in his T-cell subpopulations and the outcome was not fatal. The second Spanish case again was not splenectomised but suffered from diabetes type II and died of the infection (Calvo de Mora *et al.*, 1985). The parasites were identified as *B. bovis* from blood smears.

There have been two recent cases of babesiosis in Ireland (Clarke *et al.*, 1989), both from the Galway region, where the infection reported by Fitzpatrick *et al.* (1968) was contracted. Both patients had been splenectomised and one died of the infection. The surviving patient had been splenectomised as part of the treatment for hairy cell leukaemia but was treated with the cattle antibabesial drug Imidocarb and made a complete recovery (Dr. L. Egan, personal communication).

1.2.2. American babesiosis

The first case in North America occurred in a splenectomised man from San Fransisco (Scholtens *et al.*, 1968). This, like many of the European cases, was initially diagnosed as a malarial infection and chloroquine therapy was followed by recovery. The next case occurred in 1969 (Western *et al.*, 1970) but the patient had not been splenectomised and recovered following chloroquine administration. Moreover, the parasite was isolated into hamsters and identified as B. *microti*. Since these reports, nearly 100 cases of human *B. microti* infection have been documented in the United States. The vast majority of patients survived and the few that died had intact spleens.

The prevalence and course of the disease in America is thus quite distinct from that seen in Europe. *B. microti* is of low virulence in its natural rodent hosts and appears to be so in humans also. The absence of a spleen is clearly not necessary for clinical infection to occur and generally the degree of immunocompromise necessary for infection to develop is less with, for example, old age sufficing. There is also a belief that *B. microti*

infection in most human cases is self-limiting (Brockelsby, 1979) and indeed the initial recoveries seen in patients given chloroquine may not have been related to the drug (Miller *et al*, 1978).

1.3. THE SPLEEN

1.3.1. Role of the spleen

The absence of a spleen is clearly an important predisposing factor for clinical *B. divergens* infection in humans. This is not the case for *B. microti* infections where it has been shown that the spleen facilitates recovery from *B. microti* infections in mice but is not essential (Allison *et al*, 1979). A possible reason for this, apart from the lower parasite virulence of *B. microti*, may be linked to the initial schizogony phase which *B. microti* undergoes in T lymphocytes. The spleen is an important first line of defence against blood borne parasites and in this case will be of decreased importance initially as the parasites are removed to local lymph nodes to form schizonts in the lymphocytes (Rehbein *et al.*, 1982).

The relative dependence upon the spleen for immunity varies with the host species and the virulence of the parasite within that host. With bovine babesias of low or moderate pathogenicity such as *B. major* and *B. divergens* respectively, splenectomy can greatly exacerbate the infection, indeed *B. major* infections frequently go unnoticed in intact cattle (Brocklesby and Sellwood 1976). *B. divergens* will not normally produce infections in laboratory rats, mice or hamsters (Canning *et al.*, 1976), but Phillips (1984) has managed to maintain the parasite in splenectomised rats by repeated passage. The occurrence of clinical infection with *B. divergens* in splenectomised humans has already been discussed and it has also been found that goats and deer can be rendered susceptible by splenectomy (Enigk and Freidhoff, 1962).

Infections of cattle with *B. bovis*, however, exhibit a different pattern with regard to splenic dependence. This is a virulent species of *Babesia* in cattle, with cerebral sequestration commonly occuring as well as the development of an Adult Respiratory Distress Syndrome (ARDS) as seen in acute malaria (Wright and Goodger, 1983). These major causes of death in *B. bovis* infections develop less readily in splenectomised animals, even though the parasitaemias are significantly higher (Wright *et al*, 1988). In cerebral malaria adherence to endothelial cells allows sequestration of the parasite, effectively avoiding passage through the spleen. "Knobs" found on erythrocytes harbouring the trophozoites were

thought to be responsible for this cytoadherence and it has been shown for *P. fragile* that, when infected monkeys are given antibodies against these knob antigens, a large release of mature infected cells into the bloodstream occurs. These cells are no longer adherent and, like avirulent strains which induce knobless cells (Langreth and Peterson, 1983), are thus subject to destruction in the spleen. Such knobs, which have been seen on cells harbouring *B. bovis*, are most prevalent with virulent forms (Aikawa *et al*, 1985) and infected erythrocytes have been shown to form attachments with cerebral endothelial cells at the site of these knobs (Aikawa, 1991). Current evidence suggests that for *P. falciparum*, at least *in vitro*, the knob structures themselves are not sufficient or necessary for cytoadherence (Barnwell, 1989) and that this is due to the formation of receptors, possibly mediated by the release of IFN-gamma and TNF (Clark, 1987 and Clark *et al*, 1989). However, in light of the similarities which exist between the manifestations of the two diseases and antigenic cross-reactivities found (James *et al*, 1987), it is likely that a similar mechanism involving some form of membrane alteration exists in both. With the occurrence of cerebral forms of both diseases showing a dependence upon an intact spleen (David *et al*, 1983 and Wright *et al*, 1988) clearly the role of the spleen in the immunology and pathology differs widely from that found in *B. divergens* infections.

Infection of the Mongolian gerbil with *B. divergens* occurs readily in intact animals where it is frequently fatal. Experiments by Lewis and Williams (1979) showed the spleen to be of little importance in controlling *B. divergens* infections in gerbils, with splenectomised animals being no more susceptible to infection. In addition, splenectomy of gerbils recovering or recovered from low grade parasitaemias did not induce further development. This is in direct contrast to the situation occurring with *B. divergens* infections in cattle where a recrudescence of the disease is produced in carriers which are splenectomised. Also, donkeys which are carriers of *B. equi* have been shown to develop fatal infections following splenectomy (Selim *et al*, 1987). Thus, even though splenectomy generally leads to increased parasitaemias (Allison *et al*, 1979), the importance of the spleen in the disease outcome depends upon both host and parasite factors.

1.3.2. Antigenic variation

An intact host spleen is believed to be involved in variant antigen expression in *P. knowlesi* and *P. falciparum* infections (Howard, 1984). It

was shown by this author that the presence or absence of a spleen influences parasite-induced phenotypic alterations of the infected red cell membrane. These antigens are variant in different isolates and also undergo variation during the course of a natural blood infection with cloned parasites. Furthermore, the spleen has also been shown to be required for alteration of one antigenic phenotype to another in conjunction with the presence of antibody (Barnwell *et al*, 1983). Antibodies against these antigenic variants significantly inhibit the growth and maturation of the late erythrocytic stages *in vitro*, and this inhibition is variant specific. Because this variation occurs as a result of immune pressure, antigenic polymorphism is believed to be an important mechanism for immune evasion (Mendis *et al*, 1991). This is consistent with the poor immunity evoked in humans where a non-sterile, partially protective immunity results after repeated infections. Antigenic variation has been known to occur in *Plasmodium spp.* for some time (Brown, 1976) and is a factor associated with prolonged and chronic infections in malaria. Since a chronic infection normally ensues after the acute phase of a *Babesia* infection, there may be a similar mechanism of immune evasion operating. Phillips (1969 ; 1971) demonstrated the existence of antigenic variation in *B. rodhaini* and later also reported antigenic diversity in *B. divergens* (Phillips *et al*, 1987). These experiments also indicated that the recrudescence of the disease, which occurs following splenectomy of rats with subpatent infections, is made up of a population showing antigenic differences from the infecting population. Therefore the influence of the spleen upon the parasites would appear to include the promotion of changes beneficial to parasite survival in many circumstances as well as those involved in parasite removal.

1.3.3. Splenic anti-microbial activity

One means by which the spleen is thought to act in resolution of parasitaemias is non-specific and has been observed in malaria where it is known as "pitting" (Schnitzer *et al.*, 1972) This is dependent upon the spleen's functional anatomy; on entering the spleen erythrocytes course through the splenic cords into the sinuses, passing through narrow fenestrations. Infected erythrocytes are less deformable than normal and the portion of the cell containing the parasite becomes trapped, then separated, and is ingested by resident splenic macrophages.

There has been much documentation describing the increased risks of infection following splenectomy in humans (Munro, 1985 ; Schwartz *et al.*,

1978 ; Constantopoulos *et al.*, 1973). There are several factors suggested to contribute to this susceptibility apart from the loss of an organ which can directly remove blood-borne organisms by for example pitting. A reduction in serum Immunoglobulin M (IgM) levels has been observed (Chaimoff *et al.*, 1978) and it was suggested that this was due to a reduction in IgM producing cells previously found in the spleen. Also, a defect in C3 activation via the alternative pathway has been found which could produce an impairment of the initial response, before development of specific antibodies enables classical pathway activation (de Ciutis *et al.*, 1978). Tuftsin is a peptide synthesised in the spleen which stimulates polymorphonuclear cells (PMNs) and is thus deficient in splenectomised subjects or those with functional asplenia such as sufferers of sickle cell anaemia (Constantopoulos *et al.*, 1973). In individuals with tuftsin deficiency, increased rates of infection are observed which are more severe and recurrent than in normal subjects.

There is evidence for a return of some splenic functions after splenectomy, seen from a reduction in associated bacterial infections. This is produced by splenosis, where nodules of splenic tissue form on the peritoneum (Pearson *et al.*, 1978). Notably, this has only been found where the splenectomy was for trauma , i.e. where disruption of the splenic capsule occurred prior to removal. The relevance of this with regard to babesial susceptibility is questionable however, as many of the patients had been splenectomised for trauma, one of them 11 years before the infection (Skrabalo and Deanovic, 1957).

1.4 IMMUNOLOGY OF BABESIOSIS

As already discussed, the course of a *Babesia* infection depends partly upon innate characteristics of the host species. In cattle, genetic differences in susceptibility to *B. bovis* have been found (Wright and Goodger, 1988). This is also known to occur in human malaria infections. Those individuals who are heterozygous carriers of the sickle cell allele enjoy an immunity to malaria, as do carriers of the alleles for thalassaemia and glucose-6-phosphate dehydrogenase deficiency (Motulsky, 1960). Certain HLA antigens, common in West Africa, have recently been associated with protection from severe *P. falciparum* malaria. These are a class I antigen and a class II haplotype which are rare in groups other than West Africans and are believed to account for a reduction in the incidence of the disease to the extent of that achieved by the sickle-cell trait (Hill *et*

al, 1991). Age related resistance is another factor known to influence the course of these diseases. Calves have a natural resistance to *B. bovis* infections (Levy *et al*, 1982) and the severity of human infections with *B. microti* is also strongly age related, with severe infections generally being found only in persons over 49 years of age (Ruebush, 1980).

The role played by the immune system in combating an infection will thus vary in different host/parasite combinations. In general however, where clinical infections are non-fatal a common pattern is seen in the course of an infection. The initial acute phase of parasite growth is controlled and eventually degenerating parasites known as "crisis" forms are seen within the circulating erythrocytes. This is a sign of a developing immune response and is followed by a period of low level infection known as premunition where the host is immune to challenge but is still a carrier.

1.4.1 Specific Immunity

Protective immune responses in cattle against *Babesia* involve both cellular and humoral mechanisms (Carson and Phillips, 1981). Passive transfer of immune serum into susceptible animals has been shown to confer partial protection upon challenge (Zwart and Brockelsby, 1979). Also Levy and Ristic (1980) demonstrated inhibition of merozoite invasion into erythrocytes *in vitro* following preincubation of merozoites with immune serum. It is believed that *B. bovis* possesses surface antigens capable of eliciting both thymus-dependent (Td) and thymus-independent (Tind) antibody responses (James, 1984). Tind antigens will induce IgM which will function as an opsonin and agglutinin, whereas Td will promote IgG production which is dependent upon T helper cell involvement. However, exceptions to this are believed to occur. Naturally occurring Ig to bacterial Tind antigens is of the IgG isotype (Schofield, 1991), as is Ig reactive with Tind antigens on the CS protein of *Plasmodium* sporozoites (Schofield and Uadia, 1990). Aside from these differences in IgG production procedures, there is a correlation between a rise in specific IgG and IgM antibabesial antibodies and resolution of the infection and these are believed to act by neutralisation of merozoite invasion and opsonisation and subsequent ingestion by phagocytes.

T-cell dependence in the development of immunity to *B. microti* in mice has been demonstrated by Ruebush and Hanson (1980) and their results suggested that immunological memory was modulated by the T-cell. They also showed that immune serum transferred from recovered mice was

not protective. Therefore for mice, it would appear that recovery from *B. microti* infection is T-cell dependent. This was confirmed by infections in nude mice which persisted for the remainder of their lifespan unless reconstituted with syngeneic thymus cells (Allison *et al*, 1979). Infections of mice with *P. chabaudi* have also revealed a dependence upon the generation of specific CD4+ cells for effective protective immunity (Langhorne, 1988). Moreover, roles have been assigned for the contribution of the two CD4+ subsets T_H1 and T_H2 towards control of the infection. In the first phase of the immune response, where a rapid decrease in parasitaemia occurs, specific T_H1 cells are believed to induce an inflammatory type response, producing gamma-interferon which will activate macrophages and interleukin-2 (IL-2) which acts as a growth factor for T_H2 cells. The frequency of T_H2 cells increases as the infection progresses towards low or subpatent levels of parasite and these cells release IL-4 and IL-5 which cause activation and differentiation of B-cells into antibody producing cells. There is evidence that similar responses operate in humans infected with *P. falciparum* (Perlman *et al*, 1991).

The human immune response to clinical babesiosis has been studied for *B. microti* by Benach *et al*, (1982). They found a supression of non-specific mitogenic responses during acute babesiosis along with polyclonal hypergammaglobulinaemia and decreased numbers of lymphocytes. There was also a significant increase in C1q binding of immune complexes and a depression in C3 and C4 serum levels. In a follow up study of convalescent lymphocytes, the mitogenic response was increased (Rowin *et al*, 1984) although when incubated with acute phase serum the response decreased again, which indicates the presence of soluble inhibitory factors. Asplenic patients were found to have the lowest lymphocyte responses and the highest Ig levels. However, higher parasitaemias are found in asplenic patients and so these features cannot be directly related to the absence of a spleen. There is also evidence for the development of autoimmunity to red blood cells in *B. microti* infection in asplenic patients (Wolf *et al*, 1982). It is suggested that this is related to the observed functional T-cell depression and subsequent inhibition of supressor T-cells.

Recent experiments carried out by Roussilhon *et al*, (1990) have investigated the human T-cell response of donors previously exposed to and cured of acute *B divergens* infection. These individuals (all asplenic) showed specific T-cell proliferative responses to culture exoantigens, even 15 years after exposure in one case. Proliferation was seen at day 7 only in

previously exposed donors, with no response being produced to *B. microti* suggesting that the response was species specific. No mitogenic stimulation was observed in these or in the control donors lymphocytes. Antigen preparations of *P. falciparum* have been found to be mitogenic and to induce an antigenic response in lymphocytes from unexposed donors as well as patients (Troye-Blomberg *et al*, 1983a, 1983b). More recent experiments by Jones *et al*, (1990) have, however, indicated that the proliferation found to *P. falciparum* is of an antigen-specific nature, reflecting *in vivo* priming by a common cross-reactive immunogen. Thus, although the existence of a mitogenic response to *P. falciparum* in unexposed donors is in question, an antigenic response is found, which contrasts with the results found for *B. divergens* by Roussilhon *et al* (1990).

A role has been suggested for natural killer (NK) cells in the cellular immune responses to babesial infection. It was found that differences in susceptibility to *B. microti* infection could be correlated with the relative NK activity of that particular strain of mice (Eugui and Allison, 1980). However, Wood and Clarke (1982) have shown that the increase in NK activity seen in *B. microti* infected mice is not associated with a protective host response. Thus the role played by NK cells is not clear.

1.4.2. Non-specific Immunity

This is the first line of defence and its success in dealing with the parasite is of vital importance to the progression of an infection. This can be seen to act in several different ways.

Calves have an innate resistance to *B. bovis* infections (Levy *et al*, 1982). This involves a red blood cell factor which is related to the presence of foetal haemoglobin, and a serum component which is not believed to be antibody from colostrum and is thought to kill intracellularly.

Non-specific activation of the reticuloendothelial system by live *Mycobacterium bovis* (BCG), has been shown to protect mice against subsequent *B. microti* and *P. vinckei* challenge (Clark *et al*, 1976). This is believed to act by inducing the release of mediators such as interferon (IFN) or activating NK cells. Recent experiments by Langley and Gray (1989) have shown that inoculation of gerbils with BCG protects the animals from subsequent challenge with *B. divergens*, although much higher doses than those used by Clark *et al*, (1976) were required to elicit this protection.

Such non-specific killing was also demonstrated by Cox (1978), who found that prior infection with *B.microti* would protect mice against a challenge with *P.vinckei*. This is thought to act in the same way as BCG and not through any specific immunity developed from cross-reactivity. Attempts to protect cattle against *B. divergens* in this way proved unsuccessful, with intravenous inoculation of BCG failing to protect splenectomised or intact calves against challenge (Brocklesby and Purnell, 1977).

Mononuclear phagocytes from mice have been shown to be active against *Babesia* parasites *in vitro* (Bautista and Krier, 1980). The phagocytes were inhibitory to the growth of *B. microti* which was believed to be mediated by soluble factors, as the monocyte culture supernatant was also inhibitory. Killing was enhanced by the addition of immune serum which was thought to act through prevention of erythrocyte entry and thus increased exposure of the parasite to the phagocytes. It has also been suggested that IgG preparations from patients immune to *P. falciparum* are dependent upon monocytes for their anti-parasite activity (Bouharoun *et al.*, 1990). Although these authors used pooled IgG and much of the activity seen may have been directed against erythrocyte antigens, other authors have associated the presence of immune serum with neutrophil activation (Salmon *et al.*, 1986). *In vitro* experiments using *P. falciparum* -infected erythrocytes have shown that an oxidative burst in human polymorphonuclear cells was produced only in the presence of immune serum (Salmon *et al.*, 1986). However, increased production of superoxide by human monocytes and neutrophils was found to occur when stimulated with *P. falciparum* merozoites or exoantigens, which had been preincubated with immune or normal serum (Kharazmi *et al.*, 1987). These results suggest that responses occurred or were enhanced by both classical and alternative complement pathways, involving opsonisation both by specific IgG and C3b.

The effects of normal bovine blood monocytes upon the growth of *B. bovis in vitro*, has been studied by Montealegre *et al.*, (1985). Soluble factors released from these cells upon stimulation with exoantigens or complexes of these antigens with immune serum, inhibited the growth of *B. bovis* cultures. This also occurred with monocytes treated with immune serum alone but to a lesser extent. The appearance of damaged intraerythrocytic parasites or "crisis forms" in the cultures was most evident when supernatant from exoantigen or immune complex-treated monocytes was added, suggesting the importance of non-antibody factors in this case. Butcher and Clancy (1984) studied the effects of normal human

monocytes upon the *in vitro* growth of *P. falciparum* and found similar results, with the inhibitory factor produced again being effective against intracellular forms.

Interferon-gamma (IFN-gamma), released from activated T-lymphocytes is a factor known to produce monocyte/macrophage activation (Nathan *et al.*, 1983). The presence of this lymphokine has been shown to be necessary for the induction of crisis forms in *P. falciparum* cultures by human monocytes (Ockenhouse *et al.*, 1984) and although other authors have failed to reproduce these results (Butcher, personal communication), the presence of elevated serum levels of IFN-gamma has been associated with moderation of *P. falciparum* malaria.

Antimicrobial activity by monocytes is known to be produced in several ways. One mechanism operates via their secretion of many complement components, notably C2, the concentration of which is most likely to be rate-limiting at an inflammatory site. Another means was demonstrated using serum collected two hours after injection of endotoxin into animals previously given BCG or *Corynebacterium parvum*. This serum was shown to kill several *Plasmodium* species *in vitro* (Taverne *et al.*, 1986) and the component believed to be responsible for parasite death was tumour necrosis factor (TNF), a monokine produced by activated macrophages. Hence the serum was named tumour necrosis serum (TNS). However, this has been disputed because recombinant TNF failed to produce crisis forms in *P. falciparum* cultures (Jensen *et al.*, 1987). Recombinant mouse TNF inhibited *P. yoelii* infection *in vivo* but not *in vitro* (Taverne *et al.*, 1987), and these authors concluded that TNF acts via a secondary mediator, with another molecule in TNS specifically involved in killing the parasite. Administration of recombinant TNF and IFN-gamma was shown by Clark *et al.*, (1987) to inhibit *P. chabaudi* growth *in vivo* and IFN-gamma is known to potentiate the *in vivo* cytotoxicity of TNF (Clark, 1987) thus demonstrating a degree of interdependence between these cytokines in effecting their antimicrobial activity.

Oxygen-dependent mechanisms, measured by the production of superoxide and hydrogen peroxide, are known to be responsible for the killing of ingested protozoa (Nathan, 1983), and polyamine oxidase released from human monocytes has been shown to be toxic for *Plasmodium* species (Egan *et al.*, 1986). However, induction of oxidative radicals in gerbils infected with *B. divergens* by administration of alloxan monohydrate did not alter the course of infection (Langley and Gray, 1989) and this was also

found for murine *B. microti* infections (Millot and Cox, 1985). Thus rodent *Babesias* would appear to be an exception with regard to their susceptibility to these monocyte defences.

Nitric oxide (NO) has recently been identified as another anti-microbial compound, with levels of nitrite being directly correlated with the *in vitro* killing of *Leishmania major* (Green *et al*, 1990 ; Liew *et al*, 1990) and *P. falciparum* (Rockett *et al*, 1991). The generation of such reactive nitrogen intermediates is a process distinct from that of reactive oxygen species (Iyengar *et al*, 1987) and furthermore its release from macrophages appears to be dependent upon prior activation by cytokines (Stuer and Marletta, 1987). It has been shown that IFN-gamma and TNF-alpha-induced killing of intracellular protozoa is mediated by NO (Green *et al*, 1990 ; Liew *et al*,1990). Liew and Cox (1991) have summarised the effector mechanisms for this process ; these involve specific activation of T-cells via an antigen presenting cell followed by their clonal expansion and subsequent secretion of lymphokines such as IFN- gamma. The activation of macrophages produced by T-cell lymphokines then results in the secretion of IL-1, which continues the activation process, and also TNF and NO which have anti-microbial activities.

Specific immune mechanisms thus play an integral part in the activation and killing processes brought about by macrophages in an ongoing immune response. Specific antibodies are involved in macrophage anti-microbial activity in the form of classical pathway activation and antibody dependent cell mediated cytotoxicity (ADCC). This, coupled with the cytokine activation of monocytes, highlights the potential interplay between specific and non-specific immune responses to protozoa and thus their interdependence in the resolution of a clinical infection.

1.5. EVASION OF THE IMMUNE RESPONSE

For obligate intracellular protozoa such as malaria parasites and babesias, avoidance of host defences must be an important feature. The antigenic variation already discussed is one means by which these parasites can evade destruction by the specific immune response and contributes towards the chronic infections produced by these species. Since erythrocytes do not express major histocompatibility (MHC) antigens the parasites are not at risk from direct T-cell recognition once inside the red cell. However, once released, the merozoites are fully exposed to the immune response and, although they are only free in the blood for a very

short time, they would appear to use various mechanisms for protection. With *P. falciparum,* one means employed was thought to rely upon the presence of repeated and cross-reacting surface antigens (Anders, 1986). These epitopes could modify the development of specific antibodies in the following way ; after promoting the clonal expansion of a reacting B-cell many of the resulting B-cells produced through somatic mutation would be maintained due to some cross-reactivity with the stimulating epitope. Because of this, a larger number of B-cells would be maintained instead of being lost from further proliferation. This would have the consequence of reducing the average affinity of the immune response as well as possibly contributing to the hypergammaglobulinaemia commonly seen. However this theory fails to address certain features. These include the reasons for the repetition of antigens, the finding that quite distinct repeat structures occur among allelic variants of the same protein and the occurrence of a bias in the amino acids present in these repeats even though they have been found in a wide range of proteins with a variety of functions (Schofield, 1991). These and other questions would appear to be answered by a recent hypothesis forwarded by Schofield (1991). Although this hypothesis is based on the same premiss as that of Anders ie immune evasion by induction of low grade and non-neutralizing antibodies, the actual mechanism employed to achieve this is quite different. He believes these repeats act as Tind antigens which induce a T-cell independent response. This results in the production of a Tind B-cell response which is thought to lack affinity maturation and T and B-cell memory formation, all features of the immune response found in malaria infections. These antigens are immunodominant and therefore will act to suppress the formation of antibody to important areas on the same protein. This explains why their amino acid sequence can vary considerably in a given protein as all that is in effect required of them is to act as B-cell epitopes and not, it would seem, as sites cross-reactive with the remaining important sections of the protein .

A further means employed by parasites free in the blood is adherence of host serum proteins to "mask" parasite antigens. This has been reported to occur in schistosomes (McLaren and Terry, 1982), *Brugia pahangi* (Premaratne *et al*, 1989) and in malaria and babesia species (Ristic and Kakoma, 1988). These adhering proteins are derived from host red-cells, IgG and albumin respectively. Where IgG has been found on the parasite surface the majority of this appears to be non-specific (Craig, 1988) and so if this

is not an isotype capable of fixing complement it could have a protective role in masking the parasite against specific IgG antibodies.

1.6. IMMUNOPATHOLOGY

While the immune mechanisms discussed above may be specifically induced by the *Babesia* or *Plasmodium* species involved, their effects are often more than just anti-parasite. Reactive oxygen intermediates cause lipid peroxidation of membranes and NO has been shown to affect blood pressure and flow (Vallance *et al*, 1989) as well as inhibiting platelet adhesion and aggregation (Radomski *et al*, 1987). The role of macrophage-derived factors in disease pathology outwith any damage caused directly by the parasite has been noted for some time (Clark, 1978 and Clark *et al*, 1981). The symptoms of illness in acute malaria were seen to be similar to the effects of endotoxin administration and this is now thought to be caused by parasite-derived molecules which resemble endotoxin (Jakobsen *et al*, 1988 ; Playfair *et al*, 1990 ; Riley *et al*, 1991)). Clark (1982) observed the link between levels of parasitaemia reached in malarial and babesial infections and the susceptibility of the host to endotoxin. Humans and cattle have a very low tolerance to endotoxin and become ill at extremely low parasitaemias, whereas mice require much larger doses of endotoxin for macrophage activation to occur and the parasitaemias reached in an infection are consequently much higher. Lizards were found to be refractory to very high doses of endotoxin (Clark, 1982) and correspondingly are found to tolerate densities of malarial parasites as high as 100%. The induction of TNF production by endotoxin is believed to be one of the factors responsible for the pathology seen since it can be prevented by pretreatment with anti-TNF antibodies (Cerami and Beutler, 1988). In contrast, animals, including man, are known to become refractory to TNF on prolonged exposure and this may contribute towards the tolerance seen in children infected with *P. falciparum* to relatively high parasitaemias after repeated infections (Clark, 1987).

Thus, while the anti-parasitic effects of TNF have been widely reported (Taverne *et al*, 1986 ; Clark *et al*, 1987 ; Neifer, 1989 ; Stevenson and Ghadinan, 1989), its production is thought to be associated with many of the clinical manifestations of malarial infections. In human clinical babesia infections, the symptoms reported are consistent with those known to be produced by TNF production. These include pulmonary oedema, severe anaemia over and above that caused by directly by the parasite, diarrhoea

and pyrexia (Entrican *et al*, 1979 ; Rowin *et al*, 1984 ; Calvo de Mora, 1985). This illustrates the similarities seen between clinical infections with *Plasmodium* and *Babesia* and goes some way to explaining the many human babesial infections which were mistakenly diagnosed as malaria. The primary difference between the two diseases in humans is the requirement for some degree of immunocompromise in all *Babesia* infections, particularly those of bovine origin. Studying the response of the various arms of the immune system in normal unexposed donors may produce some insight into the effector mechanisms responsible for this innate immunity.

2. MATERIALS AND METHODS

2.1. CULTURE TECHNIQUES

The "J" strain of *B. divergens*, originally isolated from a human infection (Entrican *et al*, 1979), was kindly donated by Dr. M. Pudney (Wellcome Laboratories, Beckenham) in the form of a frozen stabilate. The Weybridge strain of *B. divergens*, isolated from a bovine infection (Konrad *et al*, 1984 ; Canning and Winger, 1987), was introduced into human erythrocytes by the direct addition of merozoites.

Cultures were maintained using the microaerophilous stationary phase (MASP) technique (Levy and Ristic, 1980 ; Konrad *et al*, 1984 ; Pudney, 1984 ; Canning and Winger, 1987) in human erythrocytes in RPMI 1640 (Imperial Laboratories), supplemented with 4.5g/L glucose, 15mM Hepes, 50 mM glutamine, 50µM Gentamycin (Gibco) and 20% human serum. The two strains were also maintained in bovine erythrocytes under the same conditions as the human cultures but with the substitution of 40% adult bovine serum (Seralab).

2.1.1. Preparation of human blood

AB+ or AB- human erythrocytes were kindly provided by the South London Blood Transfusion Service in Single donor units 4 weeks old. These were washed four times in RPMI 1640 at 800g to remove the anticoagulants and preservatives, citrate phosphate dextrose and saline adenine glucose-mannitol solutions. The packed cell volume (PCV) was estimated by spinning the cells in a capilliary tube in a haematocrit centrifuge (Hawksley and Sons Ltd.) and measuring the packed cell level on a microhaematocrit reader. The washed erythrocytes were stored at 4°C in RPMI 1640 for up to 4 weeks for use in the cultures.

2.1.2. Preparation of bovine blood

Bovine erythrocytes were obtained every 2 weeks from the blood of an adult cow held at the Central Veterinary Laboratory, Weybridge. The bovine blood, previously tested *in vitro* for its ability to support *B. divergens* parasites in culture, was taken by jugular venipuncture and defibrinated by shaking with glass beads. The serum was removed after centrifugation of the blood at 800g for 10 min. The erythrocytes were then washed and the PCV measured in the same way as for the human blood.

2.1.3. Human serum

Single donor units of this were kindly provided by the south London Blood Transfusion Service. AB+ or AB- serum was used and most proved suitable for culture maintenance. This was aliquoted and stored at -20°C, being used for up to two weeks once thawed. Although reasonable parasite growth occured using 10% serum serum in the cultures, 15-20% was used as growth was improved and a good supply of serum was available. Single donor units of plasma were also used in culture maintenance following removal of the clotting components. This was carried out according to Butcher (1985), which involved a 4-5 hour incubation of the plasma at 37°C with 1M $CaCl_2$ (0.5ml per 50 ml plasma) and bovine thrombin (Sigma) used at 20 units per 50ml plasma. The clot was then removed and the serum stored at 20°C until required. Growth was comparable to that achieved with the serum units above.

2.1.4. Culture conditions

The PCV of the blood was adjusted to 9% by the addition of RPMI 1640 and serum at ratios calculated to produce a final serum level of 20% for human cultures, or 40% for bovine cultures. The cultures were initiated and maintained in 24 well Costar tissue culture plates (Nunc, Gibco) and, once established, were scaled up to $25cm^2$ (50ml) tissue culture flasks (Gibco). From the results obtained by Levy and Ristic (1980), volumes of 1.25 ml and 15ml respectively were used to provide the correct depth of culture to ensure the oxygen tension remained low.

Whilst being established, the cultures were grown in a humidified desiccator which had been gassed with a mixture of 4% CO_2, 3% O_2 and 93% N_2 and placed at 38°C. Once in tissue culture flasks, the cultures were maintained in a mixture of 5% CO_2 in air in a humidified incubator at 38°C.

2.1.5. Subculture

The culture medium was changed daily by removal and replacement of 6ml of supernatant from the settled erythrocytes in a $25cm^2$ flask using a syringe. Parasitaemias were estimated by making blood smears , staining with 20% Giemsa (BDH Chemicals) for 15 min, and counting the number of parasitised erythrocytes per 1000 cells. The cultures were subcultured when the parasitaemias reached 10-20%. This was carried out by dilution of the infected culture to around 1-4% with the appropriate mixture of blood,

serum and medium. These were then left undisturbed in 5% CO_2 in air in a humidified incubator at 38°C for 48 hours.

When a continuous supply of parasites was not required, cultures were maintained by sealing the flasks and placing them at 4°C where they remained viable for up to 2 weeks.

2.1.6. Cryopreservation of infected blood

Cultures were used for cryopreservation at a parasitaemia of at least 10%. The culture was centrifuged at 400g for 10 min at 4°C, the supernatant removed and replaced with serum at a ratio of 1:1 with the pellet. Dimethylsulphoxide (DMSO) (Sigma) diluted to 30% in RPMI 1640 was added to give a final concentration of 15% and to give ratios of 1:1:2 (pellet : serum : DMSO). Aliquots of 1ml were immediately placed in sterile cryotubes (Nunc, Gibco) and snap frozen by plunging into liquid nitrogen.

2.1.7. Initiation of cultures from cryopreserved infected blood

Two methods have been tried for this and both proved successful. The first method is the simpler but requires that the initiation takes place in the morning so the medium can be changed in the afternoon once the erythrocytes have settled.

(i) A vial of frozen culture was thawed in a water bath at 38°C and 0.6ml added to each of two wells of uninfected culture in a 24 well costar plate prewarmed to 38°C. The erythrocytes in the infected culture lyse on thawing, so to ensure a final PCV of 9%, 0.6 ml of infected culture was added to 0.6 ml of uninfected culture with a PCV of 18. 0.8ml of medium was removed once the erythrocytes had settled and this was replaced with 20% serum in RPMI 1640. This was repeated twice daily over the following 2 days to remove any remaining DMSO.

(ii) The thawed culture was centrifuged at 1000g for 10 min at 20°C and made up to 1.2ml with RPMI 1640. This was added to the uninfected culture mixture in the same way as the first method but the medium was not exchanged until the following day.

2.1.8. Initiation of Weybridge strain in human blood

Merozoites isolated from bovine MASP cultures deprived of CO_2 for 6 hours (section 2.2.1) were made up to 0.5ml with medium and added to 0.75 ml of uninfected human cultured erythrocytes as in 2.1.7. Attempts to

introduce *B. bovis* into human erythrocytes were carried out in this way without success.

2.1.9. Initiation of "J" strain in bovine and murine blood

Merozoites isolated from human MASP cultures (section 2.2.1.) were added to bovine culture mixture in the same way as described in 2.1.8.

Attempts were also made to introduce the "J" strain of *B. divergens* into mouse erythrocytes *in vitro*. Two Balb/c mice (Olac Ltd.) were anaesthetised with halothane (May and Baker), blood was removed by cardiac puncture and the mice then sacrificed by cervical dislocation. The blood was defibrinated by means of glass beads in a sterile glass bijou bottle, centrifuged at 800g to collect the serum and then washed twice in RPMI 1640. The blood was resuspended to its original volume with medium and the PCV determined as for human and bovine blood. "J" strain merozoites collected from human erythrocyte cultures (section 2.2.1.) were added directly to a culture mixture of mouse erythrocytes in two wells of a 96-well tissue culture plate (Nunc, Gibco). Both wells contained blood, serum and medium in the same proportions as the human cultures, but one well contained autologous mouse serum and the other human serum which had been heat-inactivated (HI) by incubation at 56°C for 30 min. The volumes for cultures in 96-well plates were maintained at 130µl, with 80µl of supernatant exchanged at 24 hour intervals. Human cultures were set up in parallel as a control. All cultures were placed in a humidified atmosphere of 4% CO_2 , 3% O_2 and 93% N_2 at 38°C.

2.2. CULTURE DERIVED ANTIGENS

2.2.1. Merozoites

Cultures in 25cm^2 flasks with parasitaemias of 10 % or more were placed in a desiccator containing soda lime (BDH Chemicals) for 6-18 hours at 38°C. The cultures were pooled into 50ml Falcon tubes (Phillip Harris) and the merozoites isolated using a modification of the method described by Levy and Ristic (1980). These were centrifuged at 200g for 10 min at 4°C, the supernatant transferred to another 50ml tube on ice and centrifuged at 400g for 10 min at 4°C. The resulting supernatant was then spun at 1000g for 20 min at 4°C, the pellet resuspended in 15ml of cold RPMI 1640 and spun again at 1000g. The pellet was then resuspended in 2 volumes of medium. There are roughly 12 x 10^7 merozoites in 200µl of this final

product as estimated by Coulter Counter (C. Winger, personal communication).

2.2.2. Parasite enriched erythrocyte lysis preparation (PELP)

This was used to obtain free parasites of all different developmental stages, plus membranes from infected erythrocytes (Winger *et al*, 1987b). Cultures at 10% parasitaemia or more were utilised. These were centrifuged at 750g for 5 min, the supernatant removed and the cells resuspended to 5ml in phosphate-buffered saline (PBS) (Appendix 1), diluted 1:1 with deionised distilled water. This was incubated at 37°C for 5 min to allow hypotonic lysis of the more fragile infected cells and the volume made up to 15ml with PBS. It was then centrifuged at 200g for 10 min at 4°C, the supernatant removed down to the last 2ml and then spun at 300g for 10 min at 4°C. The resultant supernatant was centrifuged twice at 1000g for 20 min at 4°C and the pellet treated as for the merozoite preparation.

2.2.3. Preparation of culture supernatants

These were prepared from both infected gerbil and human blood in order to compare parasites from susceptible and non-susceptible hosts respectively. To obtain infected gerbil blood, 0.5 ml of human "J" strain culture at 10-15% parasitaemia was injected intraperitoneally (IP) into a Mongolian gerbil (*Meriones unguiculatus*) and 96 h later parasitaemias of 45-50% were reached. The infected gerbil was anaesthetised with 0.1ml Rompun (Beyer) and 0.05ml Vetalar (Parke-Davis) injected intramuscularly into the flank. Blood was removed by cardiac puncture and agitated in a sterile glass bijou containing glass beads to promote defibrination.

The infected gerbil blood and human blood from cultures with high parasitaemias (15-20%) were then washed twice in medium by centrifugation at 800g and resuspended to 10^8 infected cells/ml in sterile PBS. This was incubated for 24 hours at 38°C in a gas mixture of 3% O_2 , 4% CO_2 and 93% N_2 in a shaking incubator (Gallenkamp). These cultures were centrifuged at 800g for 10 min and the supernatants removed and then boiled for 5 min in order to precipitate the serum proteins present from the culture medium. The supernatants were centrifuged as before and then filtered through a 0.2µm Millipore filter (Flow). These were stored at 4°C until use.

Supernatants direct from the cultures were also used. These were boiled for 5 min and centrifuged as above. In addition, since the number of infected cells/ml in normal culture conditions are less than those used to prepare the above supernatants, those from normal cultures were concentrated 10 times by dialysis against polyethylene glycol (PEG) (mol. wt. 20,000) (BDH Chemicals), followed by dialysis against PBS overnight with one change of medium. They were then filtered and stored as above.

2.2.3.1. Papain treatment of supernatants

This was carried out when supernatants failed to elicit a response in the macrophage assays. The supernatant was incubated for 1 h at 37°C with 10µg/ml papain (Sigma), then boiled for 5 min, centrifuged at 800g for 10 min, dialysed against PBS with one medium change and filtered as above (J. Taverne, personal communication).

2.2.4. Controls

These were carried out for each of the above procedures using uninfected blood cultures and their supernatants treated in an identical fashion.

2.2.5. Preparation of *B. divergens* culture for transmission electron microscopy (TEM)

Human "J" strain culture at 15% parasitaemia was used, following a method based on that of Aikawa *et al* (1985). A 10µl pellet of culture in an eppendorf tube (BDH) was suspended in a primary fixative consisting of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer with 4% sucrose, at pH7.4. This was placed on ice for 1 h. The samples were washed three times for 10 min each in wash buffer (0.1M sodium cacodylate (Agar Aids) with 4% sucrose, pH7.4). The pellet was resuspended in secondary fixative (1% osmium tetroxide in 0.1M sodium cacodylate, pH7.4) and left for 1 h on ice. Washing was carried out as before and the pellet dehydrated through an acetone gradient of 20%, 35%, 50%, and 70% for 10 min each on ice, with final exposure to 90% and 100% acetone being carried out at RT.

Araldite (CY212) (Agar Aids) 1:1 with acetone was added to the pellet and left overnight on a rotator at RT followed by a further 24 h with the caps off the eppendorf tubes. The pellets were then embedded in fresh Araldite and this was polymerised at 60°C for 24 h.

Thin sections (90-120nm) were prepared and were stained using uranyl acetate and Reynolds lead citrate (Appendices 2 and 3). The sections were placed in the uranyl acetate solution for 30 min, shaded from direct sunlight and then washed in warm distilled water. The grids were allowed to dry and were then placed in the lead citrate solution for 15 min. Excess stain was removed by first washing in 0.02M sodium hydroxide (BDH Chemicals) and then washing thoroughly in distilled water.

2.3. CHARACTERISATION OF *B. divergens* IN HUMAN AND BOVINE CULTURES

These assays were carried out to ascertain the extent of antigenic differences between merozoites raised in human and bovine erythrocytes.

2.3.1. Enzyme-linked immunosorbent assays (ELISA)

Because the alkaline phosphatase (AP) conjugated goat anti-human IgG second antibody (G∝HIgG) was found to bind directly to merozoites raised in standard human cultures, ELISAs were initially carried out with and without a first antibody to determine the extent of this binding. Where this first layer was included, normal human serum was used from a starting dilution of 1 in 50. A goat anti-human IgM (G∝HIgM) second antibody (Sigma) was also tested for anti-merozoite activity in this way.

96-well rigid polystyrene ELISA plates (Flow) were coated with 100µl/well of merozoites isolated from human cultures (100µl of the standard merozoite preparation made up to 10ml with PBS). The plates were stored at 4°C overnight and then placed at 37°C for 1 h. The assay was performed as described by Voller *et al.* (1976), using AP-conjugated second antibodies and nitrophenyl phosphate (NPP) (Sigma) as the substrate.

After coating with antigen, the plates were washed 3 times with PBS and blocked for 1 h at 37°C with 200µl/well PBS containing 1% bovine serum albumin (BSA) (Janssen). The plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween) (BDH Chemicals) and 100µl/well of human sera added, where appropriate, in doubling dilutions from 1 in 50. These were incubated for 1 h at 37°C and then washed as before in PBS-Tween. 100µl AP conjugated G∝HIgG or G∝HIgM at dilutions of 1in 1000 in PBS-Tween were added to each well. These were incubated at 37°C for a further hour, washed 3 times in PBS-Tween followed by a further wash in bicarbonate buffer (Appendix 4). 100µl NPP diluted to 1mg/ml in bicarbonate buffer was added to each well and the plates incubated at 37°C for 30 min. The optical densities were read at 405nm on an ELISA reader (Biorad).

Where mouse ascites were to be tested, the merozoites used had been isolated from human and bovine cultures, both supplemented with HI bovine serum. The standard procedure was followed with the exception that the AP-conjugated second antibody used was sheep anti-mouse IgG (S∝MIgG). The ascitic fluid used contained an IgG1 monoclonal antibody (Mab) 13.4 which reacts with surface coat proteins of *B. divergens* merozoites. This was used in doubling dilutions from 1 in 100.

2.3.2. Indirect immunofluorescence antibody test (IFAT)

A live assay was used for this, where merozoites were suspended in the antibodies to be tested. 10µl pelleted merozoites (section 2.2.1) were resuspended in 100µl of a 1 in 100 dilution of the same ascites used in the ELISA. MOPC 21 (mouse IgG1) (Sigma) was used as a control and these were both left for 30 min on ice. The merozoites were washed 3 times by alternate spinning in a microcentrifuge and resuspension in PBS at 4°C and were finally resuspended in 50µl biotinylated horse anti-mouse IgG (H ∝ MIgG) at 1 in 200 (Vector Laboratories). This was left for 30 min on ice followed by three washes in PBS as before. The merozoites were then resuspended in 50µl of an avidin-fluorescein isothiocyanate (FITC) conjugate at 25mg/ml and left on ice for a further 30 min. This was followed by a further 4 washes in PBS before the pellet was resuspended in 20µl of mountant medium (Citifluor). Drops of this were placed on slides and the fluorescence viewed under coverslips with a Zeiss microsope using dark background UV illumination at 330-500nm.

2.3.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix 5)

In order to assess strain related antigenic differences, SDS-PAGE was carried out on "J" strain and Weybridge strain merozoites isolated from human and bovine cultures and on their corresponding blood and serum controls. A modification of the Laemmli system was used (Laemmli, 1970) where samples were resuspended 1:5 in modified Laemmli sample buffer with 5% 2-mercaptoethanol (Sigma). A few grains of bromophenol blue (Edward Gurr Ltd.) and sucrose were added to each sample which was then boiled for five min. To remove any particulate matter, the samples were spun in a microcentrifuge at 14,000g for 5 min. The samples were loaded onto the gel using a Hamilton syringe, along with a set of low molecular weight protein standards (Sigma). Pre-stained molecular weight markers

(Biorad) were used where the proteins were to be transferred onto nitrocellulose for immunoblotting.

Two gel systems were used : a water-cooled Biorad protein dual electrophoresis cell and a fan-cooled mini-gel apparatus (Gallenkamp). With the former system, 10µl of each sample was loaded and these were run into the 5% stacking gel at 25mA. The main 15% gel was electrophoresed overnight at 8mA with a maximum voltage of 15mA. When using the mini gel system, 5µl of each sample was loaded onto a 10% gel and electrophoresis carried out for about 50 minutes at 20-25mA, 250-300 volts.

Following electrophoresis, the gel was removed and stained with Coomassie brilliant blue (Sigma) (Appendix 6). It was then destained in 10% acetic acid containing 40% methanol and stored in 10 % acetic acid containing 10% methanol. For long-term storage the gel was placed in 7% acetic acid.

2.3.4. Western transfer and immunoblotting (Appendix 7)

This was carried out immediately after running the gel. The proteins in the gel were transferred to nitrocellulose paper (Biorad) in a water-cooled Trans-blot cell (Biorad) for 3-4 hours at 20mA, 70 volts.

The molecular weight standards were then set aside and the sample tracks blocked using a solution of 3% gelatin (Biorad) in tris-buffered saline (TBS) at RT on a rocker for 1 h or overnight. The tracks were washed 3 times for 5 min each in TBS containing 0.05% Tween (TBS-T). The nitrocellulose strips were incubated with immune human sera or Mab 13.4 ascites diluted to 1 in 200 in TBS-T with 1% gelatin for 1 to 2 h on a rocker at RT. After washing as before, the corresponding horseradish peroxidase-conjugated anti-human or anti-mouse IgG (Biorad) was added, diluted 1 in 3000 and the tracks left on a rocker at RT for 1 to 2 hours. This was folowed by 3 washes in TBS-T followed by an additional 5 min wash in TBS alone. The strips were then placed in 50ml of the substrate 3,3-diaminobenzidine (DAB) (Sigma) at 1mg/ml in 0.1M tris Hcl, pH 8, containing 12µl hydrogen peroxide (Sigma) and 20mg cobolt chloride. Following colour development, the strips were washed for 30 min in double distilled water and air dried.

MOPC 21 and normal human serum were used as controls for the mouse ascites and human serum.

This assay was also carried out in order to confirm the results from the ELISAs which suggested that there was binding of the anti-human IgG second antibody directly to merozoites grown in human blood and serum. Merozoites were prepared as usual and then washed a further 1, 2 or 3 times to remove any IgG present in the medium. These merozoite groups were then treated as above but with the omission of the first antibody layer. An additional experiment was carried out in this way, using merozoites isolated from standard human cultures, human cultures supplemented with HI bovine serum and standard bovine cultures in order to assess the involvement of human IgG present in the culture medium in this binding.

2.3.5. Merozoite neutralisation assays

The monoclonal antibodies 13.4 and 13.5, raised against *B. divergens* merozoites from bovine cultures by C. Winger were tested for their ability to inhibit merozoite invasion of human and bovine erythrocytes. The method followed was that of Winger *et al.* (1987a). Briefly, merozoites isolated from human and bovine cultures under sterile conditions were resuspended in 1ml of either

(i) 13.4
(ii) 13.5
(iii) RPMI 1640.

Before use, ascites containing the Mabs was heat-inactivated by incubation at 56°C for 30 min and then filtered through a 0.2µm millipore filter. The ascites was diluted to 1 : 100 in RPMI 1640 at 4°C. These were kept on ice for 30 min then washed once in 10 ml cold RPMI 1640 by centrifugation at 1000g for 20 min. The merozoites were resuspended in an appropriate volume of RPMI 1640 containing the supplements described in section 2.1 and plated out in triplicate into a 96-well plate, containing the correct proportions of blood and serum to give a final PCV of 9% and 20% serum.

The cultures were incubated in a humidified atmosphere of 3%O_2 , 4% CO_2 and 93% N_2 at 38°C and smears made at 16 and 40 h. Parasitaemias were estimated in a single blind trial by counting the number of parasites in 10,000 erythrocytes in Giemsa-stained smears of the cultures.

2.3.6. Production and assay of mouse anti-sera to human and bovine isolated merozoites.

2.3.6.1. Immunisation of mice

6-8 week old CBA mice (Olac Ltd.) received IP injections of 0.5ml of an oil in water emulsion of $6x10^7$ merozoites in complete Freunds adjuvant (CFA) (Sigma). Both "J" and Weybridge strain merozoites each raised in human and bovine erythrocytes were used, giving 4 groups in all with 2 mice in each group. The mice were immunised again 2 weeks later using incomplete Freunds adjuvant and 1 week later the mice were bled by cardiac puncture under terminal halothane anaesthesia and the serum collected.

2.3.6.2. ELISA

The procedure in 2.3.1 was followed with the exception that the AP-conjugated second antibody used was sheep anti-mouse IgG. The mouse sera were tested on all 4 groups of merozoites in doubling dilutions from 1 : 50 to 1 : 3,200. Reactivity of the sera to human and bovine blood and serum controls were also tested to determine background binding. Normal mouse serum was included on all plates as a control.

2.3.6.3. IFAT

Immunofluorescence was carried out using live merozoites and fixed blood smears from infected bovine and human cultures with parasitaemias greater than 10%. The live assay was carried out as in 2.3.2. with the exception that the merozoites were resuspended in immune or normal mouse sera at 1 : 100 dilution instead of the ascites.

For the fixed assay, the smears were fixed in acetone (BDH Chemicals) for 3 min and air-dried. 100µl of serum raised in mice against both human and bovine merozoites was placed on the slides at 1 : 100 dilution and these were left at room temperature (RT) in a humidified sandwich box for 1 h. The slides were then washed 3 times by dipping into coplin jars of PBS. 50µl of biotinylated HκMIgG was added at a dilution of 1 : 200. This was incubated as previously for 30 min followed by 3 washes in PBS. 50µl of avidin-FITC conjugate at 25mg/ml was placed on the slides and left for a further 30 min in a humidified sandwich box, before finally washing 4 times in PBS. 10µl citifluor was added to each smear with a coverslip to prevent drying and the fluorescence viewed as for the live assay.

2.3.6.4 Western transfer and immunoblotting

This was carried out as in 2.3.4., using mouse sera pooled from each group on blots of all 4 merozoite preparations. Normal mouse serum was included as a control and all the sera were used at 1 : 200.

2.3.6.5 Merozoite neutralisation assay

Mouse anti-sera raised against "J" strain merozoites from human and bovine blood cultures were tested for their ability to inhibit invasion in both human and bovine cultures. The method in 2.3.5. was followed, with normal mouse serum used as a control and sera being tested at 1 : 10 and 1 : 100 dilutions

2.4. T-CELL ASSAYS

2.4.1 Separation of human peripheral blood mononuclear cells (PBM)

Venous blood from healthy volunteers was drawn into a 50 ml syringe containing 0.5ml sterile heparin (Sigma) at 500 units/ml. The blood was centrifuged at 800g for 10 min at 4°C to collect the serum. The remainder was then resuspended to twice the original volume with RPMI1640 containing 5% autologous serum or 5% foetal calf serum (FCS) (Imperial Laboratories) and heparin at 10 units/ml. Single donor units of human buffy coats were also used (South London Blood Transfusion Service and North London Blood Transfusion Service) and these were treated in the same way except for the omission of the step for serum removal.

The resuspended cells were layered gently over Lymphoprep (Nycomed) at a ratio of 1:1 and then centrifuged at 800g for 15 min at 20°C. The layer of mononuclear cells produced at the interface was removed and washed twice with RPMI 1640 by centrifugation at 150g for 10 min and viable cell numbers were counted using trypan blue dye exclusion (Appendix 8)

2.4.2. Separation of bovine PBM

This was carried out as for the human cells using bovine blood which had been defibrinated by shaking with glass beads.

2.4.3. T-cell proliferation assays

PBM separated as above, were resuspended to 2 x 10^6 cells/ml in RPMI 1640 supplemented with 15mM Hepes, 50mM glutamine and 20% FCS and 100µl/well added to 96-well tissue culture plates. 100µl of the

antigen or mitogen was added to triplicate wells at the following concentrations;

(i) Merozoites - $2x10^5$, $2x10^3$ or $2x10^1$ freshly harvested merozoites per well.

(ii) Blood control - lysed uninfected culture prepared and diluted as the merozoite preparation to represent the equivalent of the above numbers.

(iii) Mitogen - Phytohaemagglutinin (PHA) (Sigma) was used at 50µg/ml.

Control wells received 100µl of medium alone. The cells were placed at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.4.3.1. Culture Media

Once an exposed donor became available, 10% autologous serum, and 10% FCS were assayed for effects on proliferation in the human T-cell assays. However, in the absence of any autologous serum with the buffy coats, 10% FCS was used in all other assays and also in the bovine proliferation assay.

2.4.3.2 Determination of T-cell proliferation

This was assessed using incorporation of ^{3}H-thymidine (Amersham). Proliferation was initially tested daily from 2 to 7 days in culture by the addition of 1µCi of ^{3}H-thymidine to each well 18 hours before harvesting onto glass fibre filter paper (Whatman) in an automated cell harvester (Dynatech). Filters were then placed in 4ml scintillation fluid (PCS) (Amersham) and assayed for uptake by ß-emission.

2.5. HUMAN B-CELL ASSAYS

2.5.1. Serum antibody responses

The presence of antibodies in normal human sera which may bind to *Babesia* antigens was assessed by ELISA and immunoblotting. Functional inhibition of parasite growth by these sera was tested by carrying out using merozoite neutralisation assays. Serum from the Irish patient was used in all these assays as a positive control.

2.5.1.1 ELISA

These were carried out as described in 2.3.1. using merozoites raised in human red cell cultures supplemented with HI bovine serum. Serum from 17 unexposed donors was compared with that from the Irish patient.

2.5.1.2 Immunoblotting

This was carried out as described in 2.3.4.

2.5.1.3 Merozoite neutralisation assays

These were carried out as described in section 2.3.5. Sera were heat inactivated before use, filtered through 0.2µm Millipore filters and used at a dilution of 1 : 50.

2.5.2. B-cell transformations

To establish whether normal human donors have B-cell precursors capable of producing antibodies which recognise merozoite antigens, B-cells from peripheral blood were transformed into continuously growing lymphoblastoid cell lines by exposure to Epstein-Barr virus (EBV).

2.5.2.1 Isolation of B-cells

PBM were isolated from whole blood in the way described in section 2.4.1. and resuspended to 10^7/ml in RPMI 1640 supplemented with 5% FCS. The T-cells were then removed by rosetting with sheep erythrocytes. (Pellegrino *et al*, 1975)

2.5.2.2 Erythrocyte (E) rosette formation and T-cell separation

One volume of cells was added to one volume of 4% 2-aminoethylisothiouronium bromide hydrobromide (AET) treated sheep red blood cells (AET sRBCs) (Appendix 9) in round-bottomed universals (Nunc, Gibco). This was centrifuged at 250g for 5 min and left on ice for 1 h or 4°C overnight. The supernatant was then removed to give a workable meniscus and the rosettes resuspended by gentle rotation (the percentage of rosetting cells was determined at this stage by viewing a drop of the cell suspension under a haemocytometer).

Erythrocyte positive (E+) and erythrocyte negative (E-) lymphocytes were separated by underlayering with Percoll (Density 1.080g/ml, Appendix 10) (Sigma) at a ratio of 1 Percoll : 2 rosetted mixture followed by centrifugation at 1500g for 20 min at 20°C. E- cells were harvested from

the interface using a pasteur pipette, resuspended in RPMI 1640 containing 5% FCS and centrifuged at 250g for 10 minutes to remove the Percoll. They were resuspended and viable cell numbers assessed by trypan blue dye exclusion.

2.5.2.3 Removal of sheep red blood cells (sRBCs)

If large numbers of free sRBCs remained in the E- pellet, these were lysed by the addition of Gey's solution (Appendix 11) for 3 min, followed by a further wash.

2.5.2.4 Panning for adherent B-cells

This was carried out in order to enrich the B-cell population to be transformed, with the B-cells posessing receptors which recognise merozoite antigens. (Winger *et al*, 1983)

2.5.2.4.1 Coating petri-dishes with merozoites

The petri-dish (Sterilin) was pretreated with 2ml of poly-L-lysine (Sigma) at 100µg/ml. This was removed after 5 min and the dish rinsed twice with sterile double distilled water. 400µl of freshly harvested merozoites from human cultures were added to the petri-dish and made up to 10 ml in RPMI 1640 at 4°C. These were placed at 4°C overnight or for 2-3 days to allow settling and adherence. The medium was then aspirated and the dishes washed vigorously 3 times with medium at 4°C. Dishes were blocked with 10ml of 10% FCS in RPMI 1640 at 4°C for 1 h and the blocking solution aspirated before use.

2.5.2.4.2 Panning procedure

10^7 E- cells in RPMI 1640 supplemented with 5% FCS at 4°C were added to the prepared petri-dish and placed at 4°C for 1 h. Non-adherent cells were then aspirated, 5ml of 5% FCS at 4°C was added and this was swirled around and the medium aspirated again. 5ml of medium was then added as before and pipetted up and down vigorously to remove attached cells. this was carried out 3 times, saving the aspirated cell suspension each time. These adherent cells were then centrifuged at 250g for 10 min, resuspended in 0.5ml medium and counted.

2.5.2.5. B-cell scattergun experiments

To determine the effects of panning in the enrichment of the B-cells which recognised merozoite antigens, a population of E- cells were transformed with EBV without previously being panned.

2.5.2.6. EBV transformation

The adherent populations of enriched E- cells and 10^7 un-enriched E- cells were spun at 250g for 5 min and each was resuspended in 1ml of EBV supernatant from the marmoset cell line B95-8 (Miller and Lipman, 1973) (kindly donated by Dr. D. Crawford). These were incubated for 3-5 h or overnight. The cells were then centrifuged for 5 min at 250g and resuspended in RPMI 1640 containing 20% FCS.

2.5.2.7. Preparation of feeder layers

These were made with all remaining E- cells, not necessarily autologous. The cells were seeded in 96-well tissue culture plates at 2 x 10^5 /well in 0.1 ml of RPMI 1640 containing 10% FCS and received 4500 Rads on ice from a cobalt 60 source before addition of 0.1ml of the transformed cells /well.

2.5.2.8. Culture procedure

For the scattergun experiments, the transformed cells were seeded at 10^5/well, 10^4/well and 10^3/well. The panned cells were seeded at $3x10^3$/well, 10^3/well, 10^2/well and 10^1/well. The cultures were then left undisturbed for 8-9 days at 37°C in a humidified atmosphere of 5% CO_2 in air. 100µl medium was then exchanged for fresh RPMI 1640 supplemented with 20% FCS and this was repeated weekly until the clones were large enough to test for antibody production (around 4 weeks after transformation).

2.5.2.9. ELISA to test for antibodies from EBV transformed B-cells

Merozoites were isolated from human erythrocyte cultures supplemented with 40% heat-inactivated (HI) adult bovine serum instead of 20% human serum to ensure the absence of human immunoglobulins in the merozoite preparation.

The assay was performed as described in 2.3.1. by adding 50µl of the undiluted B-cell supernatants to each well. The second antibody used was alkaline phosphatase-conjugated goat anti-human Ig A,G and M (G∝HIgAGM) (Sigma) at a dilution of 1 : 350. This was used in the first screening of the supernatants to assess the number of positive wells and thereafter positive wells were tested using G∝HIgM and G∝HIgG (Sigma), both used at 1 : 1000, to ensure that clones producing IgG were not missed.

To test the specificity of positive wells, the supernatants were also assessed for binding to HI bovine serum (100µl of a 0.1% solution was used to coat each well) and also to lysed human blood (this was lysed by the addition of a 1:2 solution of PBS:distilled water and treated in an identical fashion to the merozoite preparation). RPMI 1640 containing 10% FCS was used as a control for the supernatants and, once available, immune human serum was used as a positive control in doubling dilutions from 1 in 100.

2.5.2.10. Culture maintenance

Wells containing clones which gave a positive result in the ELISAs were divided into wells without feeders to prevent overcrowding. At the same time a portion of these cells was collected and frozen in liquid nitrogen in 1ml aliquots of RPMI 1640 containing 10% FCS and 10% DMSO. Cells from wells containing positive clones were frozen in this way 2 or 3 times. The clones were scaled up into 24-well Costar plates when sufficiently expanded, to provide the larger volumes of supernatant required for specificity testing by ELISA.

2.5.2.11. Merozoite neutralisation assay

This was carried out as described in section 2.3.5. The merozoites were resuspended in 0.5ml of the supernatant to be tested which was used undiluted.

2.6. MONOCYTE ASSAYS

2.6.1. Human monocyte assays

Following Percoll separation, the E- cells were washed once and resuspended to 2-4 x 10^6/ml. 15ml of this cell suspension was added to a gelatin coated tissue culture flask (25 cm^2) (Flow).

2.6.1.1. Isolation of monocytes using gelatin adherence

This was carried out as described by Hassan *et al* (1986). A 2% gelatin solution (100 bloom, Sigma) was made up in double distilled water and autoclaved at 110°C for 40 min. This was brought to room temperature and 10ml added to a 25cm^2 tissue culture flask. The flasks were incubated at 37°C in a dry incubator for 2 h, the gelatin removed by suction and the flasks stored at 37°C in a dry incubator for 48 h before use. These flasks could be used for up to 2 weeks and were washed twice with RPMI 1640 before use.

The mononuclear cell suspension was incubated in the gelatin coated flasks at 37°C for 2 h in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were then removed by suction and the flasks washed gently 3 times with RPMI 1640 prewarmed to 37°C. In order to detach the adherent cells, the medium was replaced with 15ml of a 1:1 mixture of PBS containing 3.3mg/ml disodium ethyleneaminetetraacetic acid (EDTA) (BDH Chemicals) and RPMI 1640 supplemented with 20% FCS, prewarmed to 37°C. This was incubated at 37°C for 10-15 min and the detached cells were then collected and centrifuged at 300g for 10 min. The cells were resuspended in RPMI 1640 containing 20% FCS and counted.

2.6.1.2. Assay of *in vitro* killing of *B. divergens* by peripheral blood mononuclear cells (PBM)

This was based on the method used by Butcher and Clancy (1984) for assaying non-specific immunity to *P. falciparum*. 96-well tissue culture plates were used with each well receiving 50µl of human erythrocytes at $2x10^8$/ml and 1% parasitaemia, and 40% HI human AB serum. Three groups of PBM in RPMI 1640 were added to these wells in 50µl quantities ;

(i) Whole population of PBM

(ii) PBM which did not adhere to gelatin coated flasks

(iii) Adherent PBM.

Ratios of 10:1, 5:1, 1:1 and 0.1:1 PBM to parasites were used at the initiation of each culture. These were set up in triplicate and control wells without PBM received 50µl medium.

The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 3 days. The medium in each well was renewed daily and on the third day Giemsa stained smears of each well were used to assess parasite growth from counts of 2000 erythrocytes.

2.6.1.3. Production and assay of supernatants from activated monocytes

In order to determine whether killing by monocytes occurred directly or by the release of soluble mediators, supernatants from lipopolysaccharide (LPS) treated and control monocytes were collected and added to cultures of *B. divergens*. Monocytes were seeded at $2x10^5$/well in 96-well tissue culture plates in RPMI 1640 containing 10% HI human serum. 10µg/ml LPS (phenol extract of *Escherichia coli* 055:B5) (Sigma) was added to half the wells and the plates placed in a humidified atmosphere of 5% CO_2 in air for 24 h. The supernatants were removed, dialysed against RPMI

1640 and passed through a 2µm Millipore filter (Flow). These were either used immediately or stored at -80°C until required.

The effect of the supernatants upon parasite growth was tested by their addition to cultures in place of medium. The supernatants were added at 100%, 50% and 10%, with control wells receiving normal culture medium. The cultures were left undisturbed at 38°C for 48 h in a humidified atmosphere of 5% CO_2 in air, after which Giemsa stained smears were used to assess parasitaemias.

2.6.1.4.Assay of killing by activated monocytes

This was carried out using those cells treated with LPS in the previous section and their untreated controls. Once the supernatants had been removed, the medium was replaced with erythrocytes at 1% parasitaemia in normal culture medium containing 20% HI human serum to give a final ratio of 1:1 monocyte to parasite. Control wells were set up without monocytes and the plates were left undisturbed for 48 h in a humidified atmosphere of 5% CO_2 in air. Parasitaemias were assessed as above.

2.6.1.5.Assay for release of superoxide anion from human monocytes.

This was based on the method of Rook *et al* (1985) which uses reduction of nitroblue tetrazolium (NBT) as a marker for superoxide release. Peripheral blood monocytes were resuspended to 5 x 10^5/ml in RPMI 1640, containing 20% FCS and 0.2 ml/well placed in a flat-bottomed 96-well tissue culture plate. These were placed in a humidified atmosphere of 5% CO_2 in air at 37°C for 24 h. The cells were then washed twice with RPMI 1640 prewarmed to 37°C and the medium replaced with 0.1ml prewarmed RPMI 1640 and 0.1ml NBT (Sigma) at 2mg/ml in RPMI 1640 with either phorbol myristate acetate (PMA) (Sigma) at 20µg/ml as a positive control or *B. divergens* merozoites at roughly 10^5 /well. Control wells contained monocytes in medium and NBT only. The plate was incubated for 30 min at 37°C, the medium removed from all the wells and the cells were fixed with 70% methanol followed by 100% methanol.

The black formazan deposit which occurred in reactive monocytes could be viewed under an inverted microscope and the percentage of cells producing this in the PMA positive control was used as a means of assessing the purity of the preparation with regard to contaminating lymphocytes.

The formazan deposits in reacting wells were then solubilised in order to compare the amounts present in each well by optical density readings. Each well was washed thoroughly with methanol and air dried. 120µl of 2M KOH was added to each well and then 140µl of DMSO. These were mixed together with a Gilson pipette and the optical density (OD) read at 630nm on an Elisa reader (Dynatech). A well containing KOH and DMSO but no cells was used to blank the machine.

In order to relate absorbance at 630nm to nanomoles of NBT reduced, a calibration of the reduction was carried out by dissolving a known quantity of NBT in 2M KOH and adding DMSO in the same way as above to various dilutions of this solution.

2.6.1.6.Opsonisation of merozoites

This was carried out using normal human serum and serum from a patient recovered from an infection with *B. divergens* and was based on the method of Kharazmi *et al* (1987). Freshly isolated merozoites were incubated for 30 min at 4°C with a 30% solution of the appropriate serum. These were then centrifuged at 1000g for 20 min at 4°C and the merozoites added to the monocyte preparations as described above. For serum controls without merozoites, 30% serum solutions were added to monocyte wells in an identical fashion to the serum-treated merozoite preparations.

2.6.2. Gerbil monocyte asays

In vitro cytotoxicity assays were carried out using blood obtained by cardiac puncture from terminally anaesthetised gerbils, as described in 2.2.3. Ten gerbils of ages ranging from 8 weeks to 2 years were used. The blood was taken up into syringes containing sufficient sodium heparin to give a final concentration of 5 units/ml and was then pooled.

The gerbil PBM were separated from the whole blood as described for human PBM (section 2.4.1.) and gelatin adherence used to isolate the monocytes. All the above human monocyte assays, except the NBT reduction, were carried out in parallel with a gerbil assay using the same technique but with the exception that 50µg/ml LPS was used to activate the gerbil monocytes as opposed to 10µg/ml in the human assay.

2.6.3. Assay for TNF release

2.6.3.1. Preparation of human monocyte cultures

Monocytes isolated as in 2.5.1 were dispensed into flat bottomed 96-well microtitre plates at $5x10^5$ cells/well in 0.1ml RPMI 1640. 100µl of medium containing 200 units/ml of recombinant human gamma-IFN (kindly donated by Dr. J. Taverne) and indomethacin at 2 µg/ml (Sigma) were added to each well and the cells incubated for 3 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was then replaced by 0.2ml volumes of RPMI 1640 containing 5µg/ml polymyxin B (Sigma) and *B. divergens* merozoites at levels roughly equivalent to 10 merozoites / monocyte, 1 merozoite / monocyte and 0.1 merozoite / monocyte. Uninfected lysed blood controls were also included in equivalent amounts. Medium only controls with and without polymixin B and LPS at 1µg/ml, 0.1µg/ml, 0.01µg/ml and 0.001µg/ml were also assayed.

2.6.3.2. Preparation of gerbil monocytes cultures

Gerbils of 1-2 years of age were used . The monocytes were activated by intraperitoneal (IP) injection of 3mls of 4% thioglycollate (Difco) in sterile PBS 3 days prior to collection. The gerbils were then sacrificed by halothane inhalation and the peritoneal cavities flushed with 15ml PBS containing 1 unit/ml sodium heparin and 5µg/ml polymyxin B. The pooled peritoneal washes were centrifuged at 300g at 4°C for 5 min and the cells counted. The cells were resuspended to 10^7/ml in RPMI 1640 with polymyxin B and plated out in 0.1ml volumes into a 96-well microtitre plate. These were incubated for 3 h at 37°C in a humidified atmosphere of 5% CO_2 in air and then 0.1ml/well medium containing 2µg/ml indomethacin added and incubated for a further 30 min.

Non-adherent cells were washed away using RPMI 1640 free of polymyxin and the medium replaced with 0.2 volumes of the stimulants to be tested as in the human assay. Culture supernatants were tested using dilutions from 1:5 to 1:80.

The cells were incubated overnight as previously described and the supernatants removed for testing the following morning.

2.6.3.2.Assay for TNF

The supernatants were collected from the monocytes the following morning and taken for testing. This was carried out at the Middlesex Hospital by Dr. J. Taverne and involved the use of a cytotoxicity assay for

both human and gerbil supernatants (Bate *et al*, 1989) and also an ELISA assay for re-testing human supernatants (Moreno *et al*, 1989).

3. RESULTS

3.1. MASP CULTURES

3.1.1. Serum requirements

The serum used in the human "J" strain cultures of *B. divergens* was taken from single donor units which had been obtained frozen. These were thawed, aliquoted and refrozen and could be used for up to 6 months. Only one batch of serum failed to support growth, but this was not because of the presence of anti-babesia antibodies, as determined by ELISA.

To determine the effects of serum concentrations upon growth of the human "J" strain cultures, wells containing from 5 - 80% human serum in two-fold increases were monitored for 5 days and the parasitaemias assessed throughout (Appendix table 1). After an initial delay, the presence of 80% serum was not prohibitive, at least in the short term, and adequate growth was achieved using as little as 5% serum. The serum levels in the human cultures are therefore less rigid than those found to be required in the bovine cultures of *B. divergens* (C. Winger, personal communication), where a reduction in the level of serum used from 40% to a minimum of 25% was achieved only by 5% reductions over a period of several weeks.

3.1.2. Initiation of *Babesia* species in different host erythrocytes

Weybridge strain merozoites, isolated from long term culture in bovine erythrocytes, were used to infect human erythrocytes. These were maintained in continuous culture for 12 months and exhibited growth rates similar to those seen for the "J" strain. "J" strain merozoites raised in human erythrocytes were used to initiate bovine cultures in the same way.

Three attempts were made to initiate an infection in human erythrocytes using *B. bovis* merozoites. Although a few dividing forms were seen, indicating that some parasites were capable of invasion, there was no further progression. The gradual addition of human erythrocytes and HI human serum to a bovine culture of *B. bovis* also failed to establish a human infection, with the parasitaemias dropping to zero as the proportion of bovine erythrocytes decreased.

An attempt to infect mouse erythrocytes with the "J" strain of *B. divergens* was made by the direct addition of merozoites to mouse erythrocytes *in vitro*. When mouse serum was used in the culture no invasions were noted but where HI human serum was used, some dividing

forms and trophozoites were seen within the erythrocytes. However, the parasites did not replicate any further.

Attenuation of *B. divergens* produced by long-term culture has been reported for bovine cultures of the Weybridge strain (Winger *et al*, 1989). To determine whether the "J" strain raised in human erythrocytes was still infective for gerbils and also its relative virulence, a gerbil was injected with 4×10^7 infected human erythrocytes, which had been maintained through 150 passages. This produced a parasitaemia of 43% on the fourth day and the animal died on the fifth day, criteria which are in accordance with the virulent form of the bovine Weybridge cultures. Thus long-term culture in human erythrocytes has no attenuating effects and the parasite has no difficulty in adapting from human to gerbil erythrocytes in sufficient time to overcome the host immune system

3.1.3. Culture conditions

The depth of culture has been reported to be important for parasite growth with regard to bovine cultures of *B. divergens* because of its relationship with the oxygen concentration at the level of the settled erythrocytes (Konrad *et al*,1984). Less rigid conditions were found to suffice in human cultures of *B. divergens* with parasitaemias not being affected by reductions in depth up to 30% even without an accompanying decrease in the atmospheric oxygen tension.

Parasitaemias regularly reached 20 - 30% in human MASP cultures, again a contrast to the bovine cultures, where levels rarely exceed 10%.

3.2. CULTURE DERIVED MEROZOITES

Figure 1. shows a Giemsa stained preparation of "J" strain merozoites isolated from human MASP cultures, which had been deprived of CO_2 for 5 hours. The background shows the presence of erythrocyte stroma and membrane fragments in the merozoite preparation but no intact erythrocytes.

3.2.1. Identification of Ig in merozoite preparations

ELISAs, carried out using merozoites raised in human cultures as the antigen, produced very high backgrounds when testing human sera for reactivity. This was found to be due to the direct binding of the alkaline phosphatase (AP) conjugated goat anti-human Ig to the merozoite preparation. Figure 2. illustrates this binding and shows the target antigen

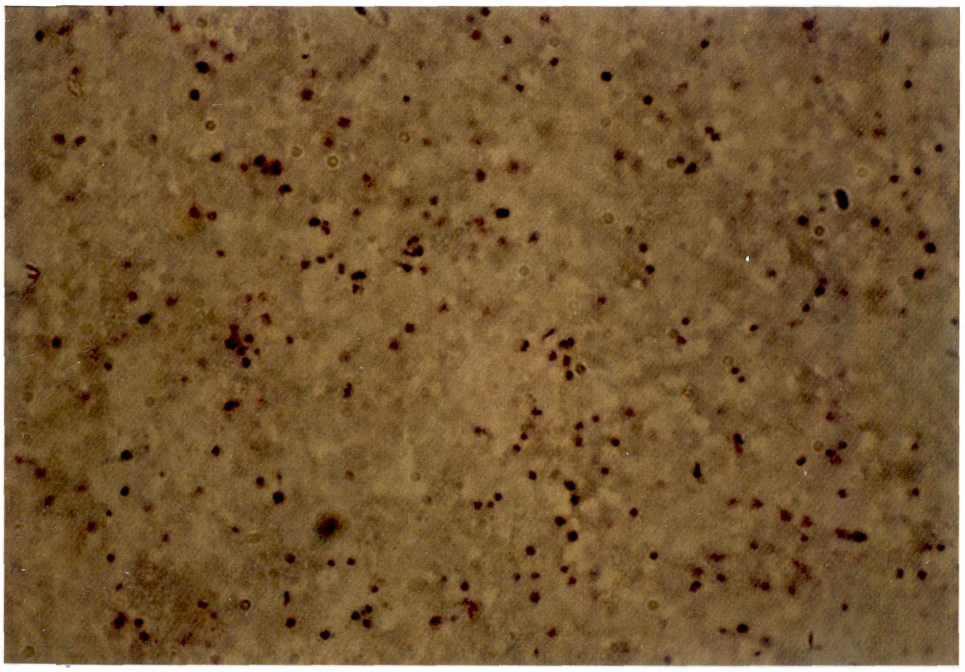

Figure 1 "J" strain merozoites isolated from human MASP culture by CO_2 deprivation. Giemsa stain. Magnification x 4,800

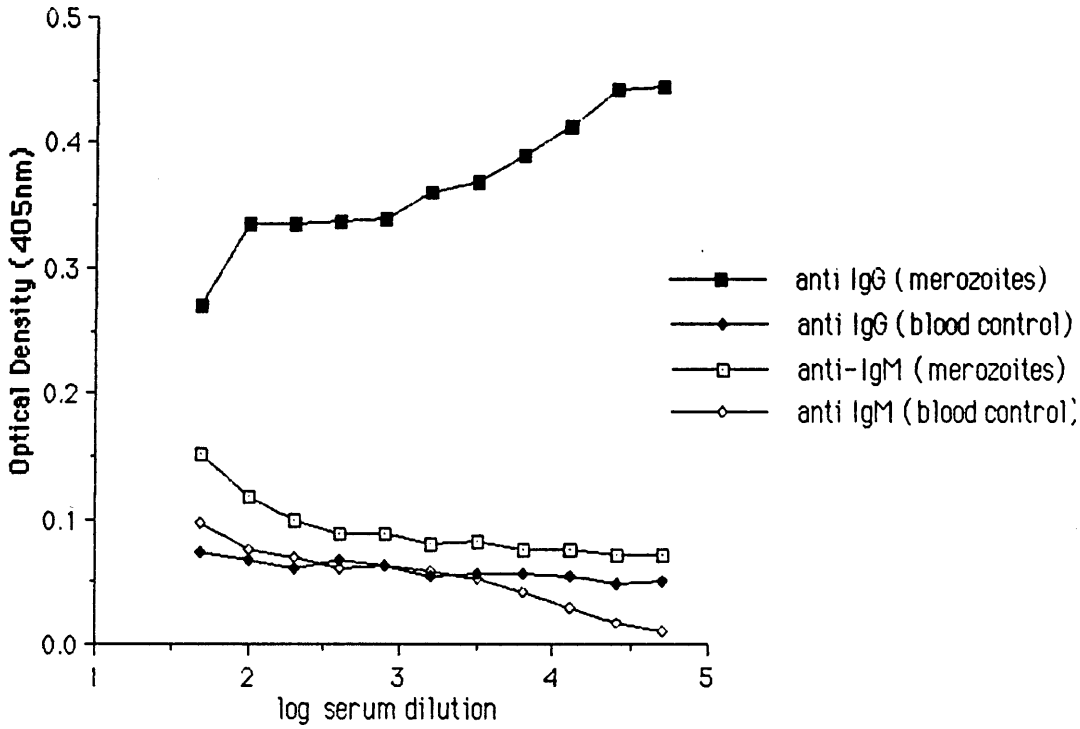

Figure 2. ELISA demonstrating binding of second antibody to merozoites

In control wells where the second AP-conjugated antibody was added without any serum the following readings were obtained : for anti-IgG alone OD^{405} = 0.446 ± 0.029 and for anti-IgM alone OD^{405} = 0.070 ± 0.003.

to be IgG and not IgM, since decreasing dilutions of the normal human serum used as the first antibody did not decrease the corresponding binding of the second antibody, but rather allowed a slight increase, suggesting the serum is actually inhibiting its binding to some extent. There was no reactivity with merozoites in ELISAs carried out using merozoites isolated from human cultures which were supplemented with HI bovine serum instead of human serum.

To determine whether the IgG is bound to the merozoites or is merely present in the merozoite preparation, SDS PAGE and immunoblotting after Western transfer were carried out using merozoites raised in human erythrocytes. These were prepared as usual and then washed a further 1, 2, or 3 times. Immunoblotting using HRP conjugated anti-human IgG shows the binding to be strong even after extensive washing. The absence of any gamma light chains in the blood control suggests that this binding is not to erythrocyte fragments present in the merozoite preparation which indicates that the IgG in the merozoite preparation is bound directly to the merozoites (Figure 3).

In order to ascertain if this phenomenon occurs during the prolonged exposure of extracellular merozoites to the human serum as a result of CO_2 deprivation, or if it is found under normal culture conditions, further SDS-PAGE, Western transfer and immunoblots were carried out. Here the merozoites used were prepared both from cultures deprived of CO_2 for 5 hours and directly from normal cultures. Figure 4. illustrates the similarities betwen these two groups of merozoites with regard to IgG presence and implies that such binding exists normally in the cultures, although the possibility that binding occurs during the actual merozoite separation procedure cannot be discounted.

3.2.2. Antigenic profiles of "J" and Weybridge merozoites

Since the "J" strain of *B. divergens* was originally isolated from a human infection and the Weybridge strain from a bovine infection, merozoites from human and bovine cultures of each were run on reducing 15% SDS-PAGE to compare antigens. Figure 5. shows a Coomassie Blue-stained gel which clearly illustrates the similarity between the two strains when in human or bovine culture.

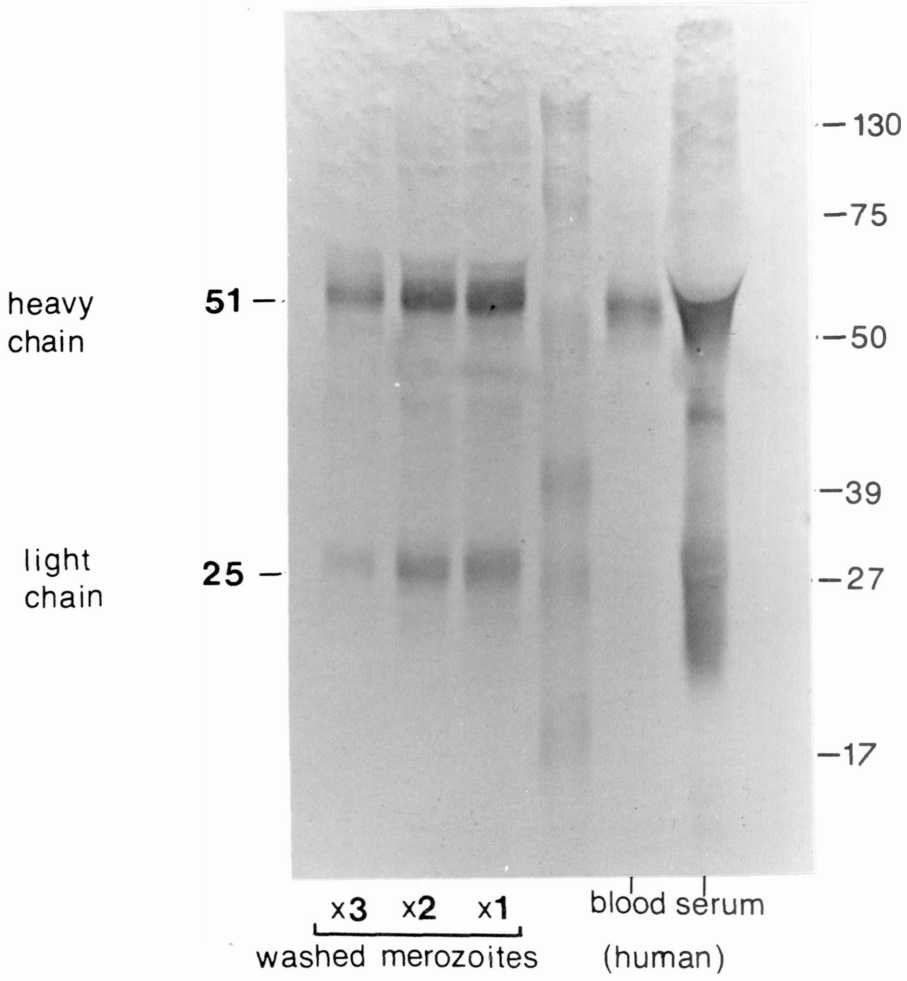

Figure 3 Immunoblot of 15% reducing SDS-PAGE protein profiles of "J" strain merozoites raised in human erythrocytes demonstrating binding of anti-IgG antibody following washing.

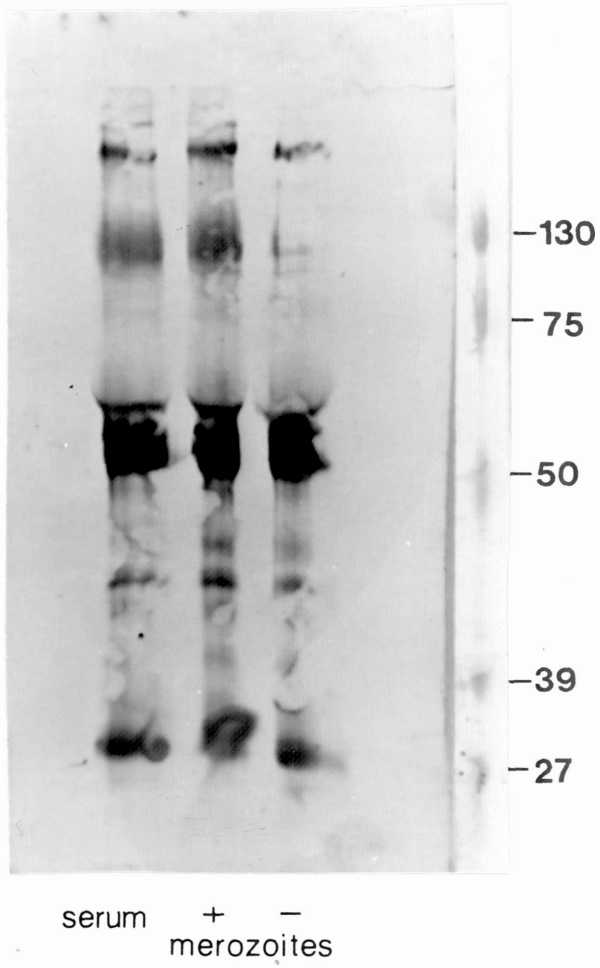

Figure 4 Immunoblot of 10% reducing SDS-PAGE protein profiles of "J" strain merozoites raised in human erythrocytes demonstrating binding of anti-IgG antibody to merozoites isolated with (+) and without (-) CO_2 deprivation.

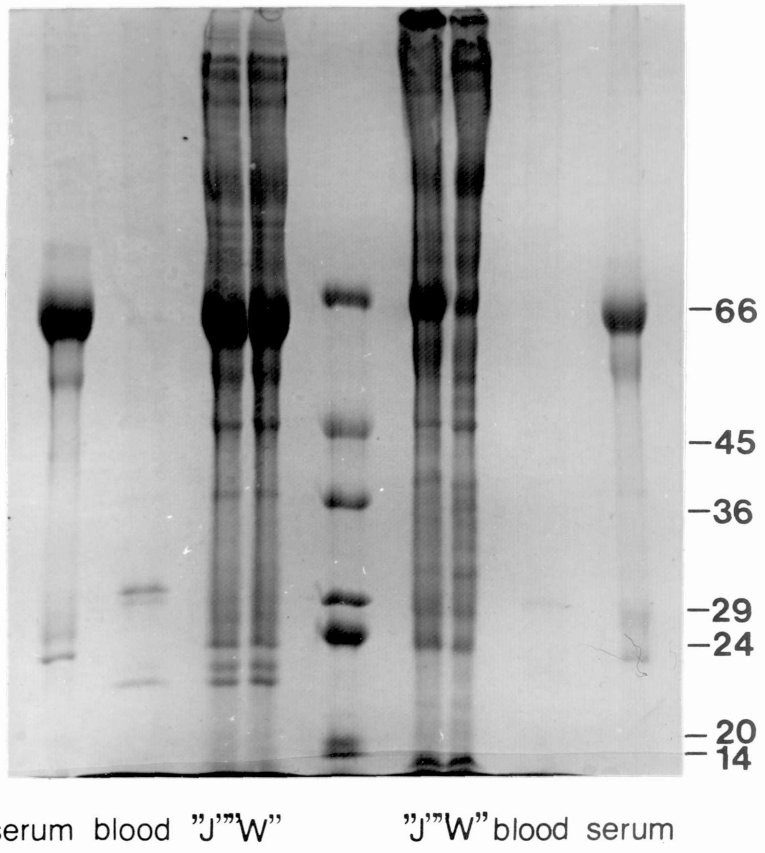

Figure 5 10% reducing SDS-PAGE on "J" and Weybridge ("W") strain merozoites isolated from human and bovine cultures.

3.3. CHARACTERISATION OF *B. divergens* IN DIFFERENT HOST ERYTHROCYTES

3.3.1. Morphology of the parasites

The size and morphology of *B. divergens* in human, bovine and gerbil blood are quite distinct (Figures 6 - 10). In the human and gerbil blood, the parasite is much larger, in accordance with the larger erythrocyte diameter, measuring in the order of 1.5 - 2.9 µm as compared to the 0.8 - 2.3 µm seen in bovine cultures. In addition, maltese-cross tetrads, even division into 8 and multiple invasions were commonly seen, with as many as 12 parasites being recorded in one human erythrocyte.

Transmission electron micrographs of the "J" strain in human blood show the basic parasite morphology to be the same as that recorded for parasites raised in bovine erythrocytes (Winger *et al*, 1990). Figures 11, 12 and 13 show sections through a dividing form.

3.3.2. Antigenic differences

A monoclonal antibody (Mab) 13.4, raised against Weybridge merozoites from bovine blood (Winger *et al*, 1987a), was tested for its ability to bind to merozoites isolated from human and bovine blood cultures using several different identification procedures. All the merozoites used in these procedures were raised in cultures supplemented with HI bovine serum to remove any differences occurring as a result of background binding to serum components.

3.3.2.1. ELISA

Mab 13.4 was tested against merozoites raised in human and bovine cultures using AP-conjugated sheep anti-mouse IgG. Both "J" and Weybridge strain merozoites were used to determine whether there were strain differences. Mab 13.4 was found to bind only to those merozoites raised in bovine erythrocytes and not those raised in human erythrocytes, regardless of the strain used (Figure 14.).

3.3.2.2. IFAT

This was carried out with live "J" strain merozoites isolated from human and bovine cultures using Mab 13.4 and biotinylated horse anti-mouse IgG and FITC-avidin. Figures 15 - 18 show clearly the actual differences in binding to the merozoites suggested from the ELISA results.

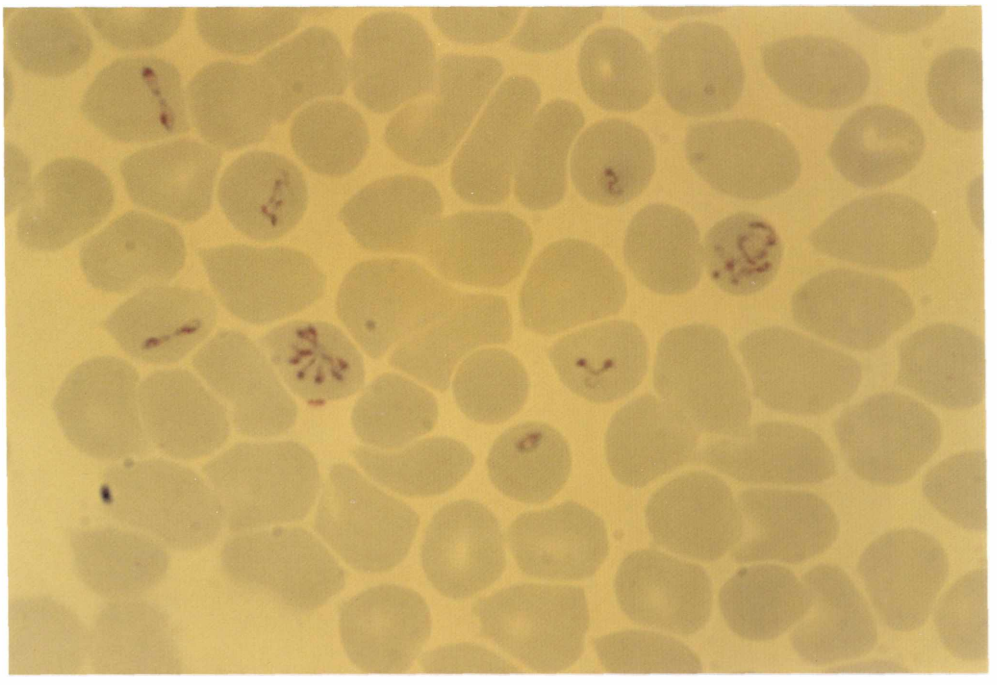

Figure 6 MASP culture of the "J" strain of *B. divergens* in human erythrocytes. Giemsa stain. Magnification x 3,400

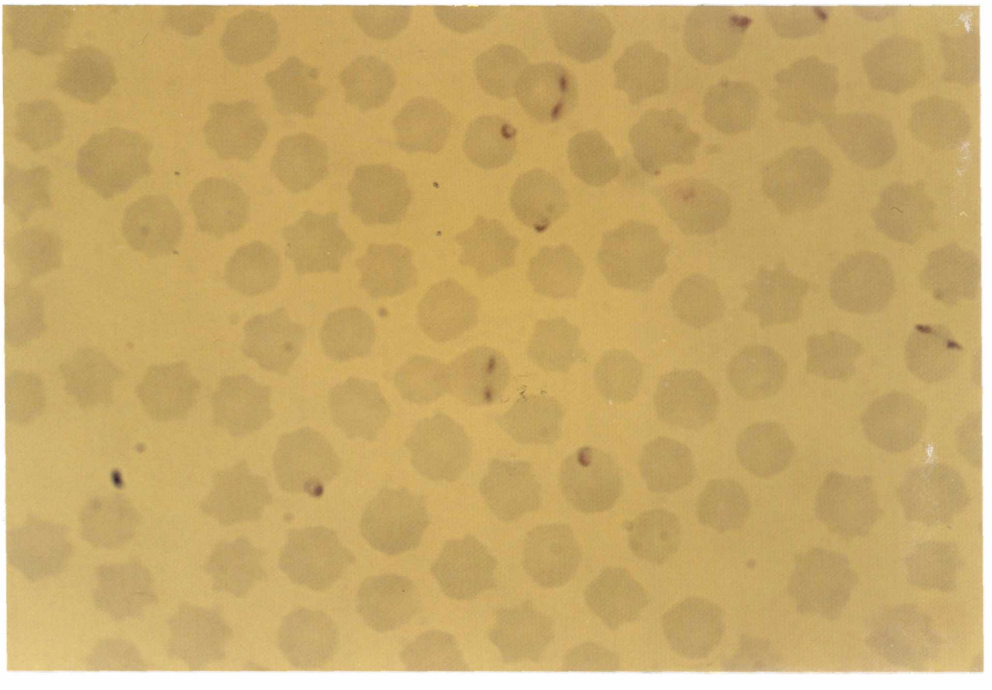

Figure 7 MASP culture of the "J" strain of *B. divergens* in bovine erythrocytes. Giemsa stain. Magnification x 3,400

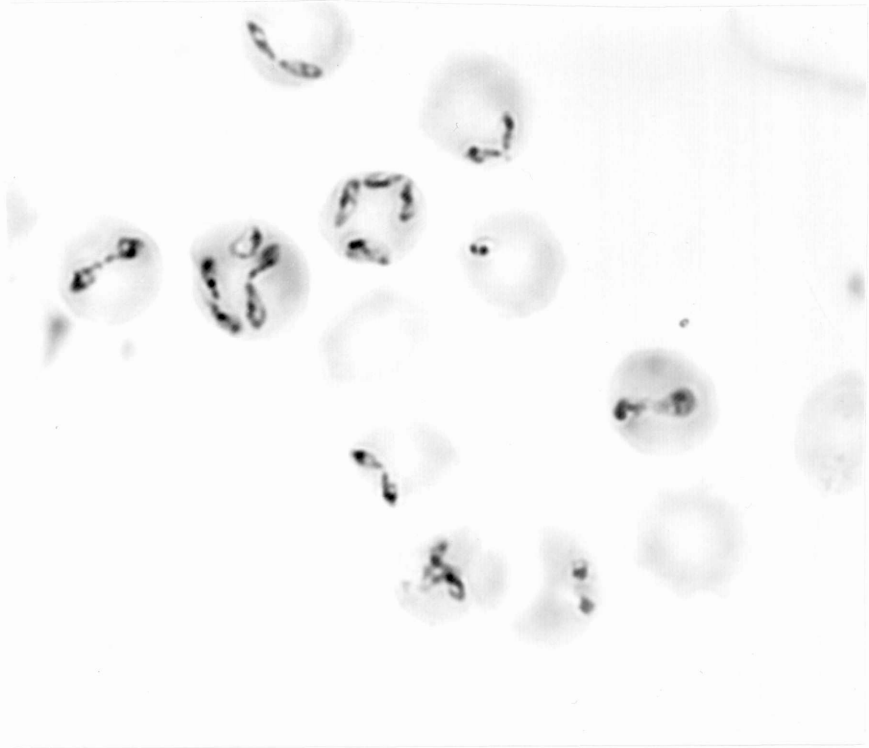

Figure 8 MASP culture of the "J" strain of *B. divergens* in human erythrocytes at 50% parasitaemia. Giemsa stain. Magnification x 4,800

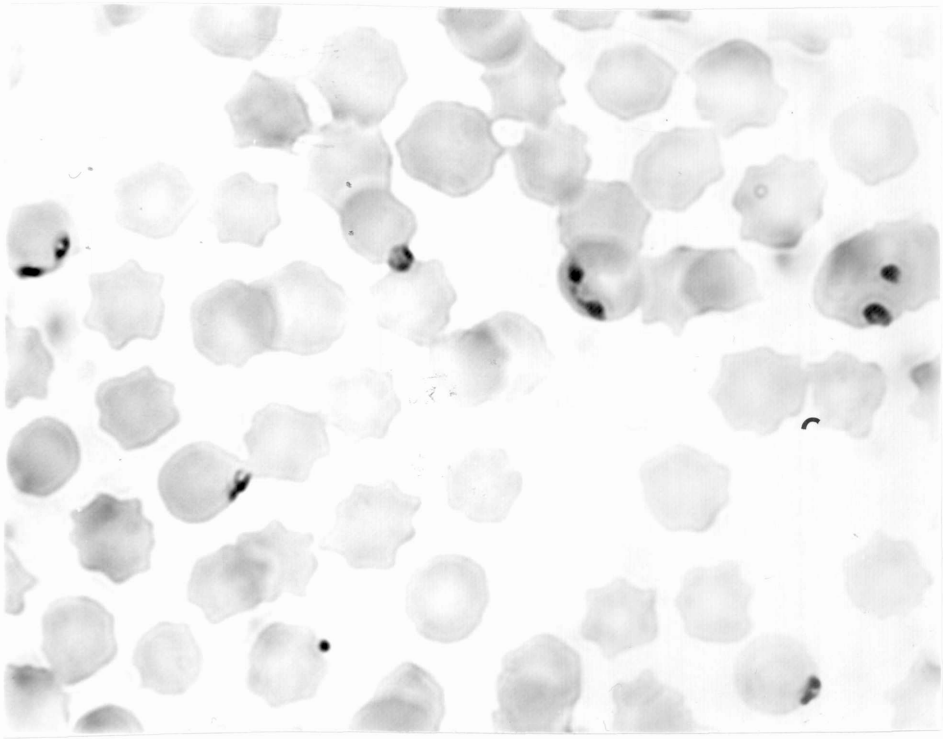

Figure 9 MASP culture of the "J" strain of *B. divergens* in bovine erythrocytes. Giemsa stain. Magnification x 4,800

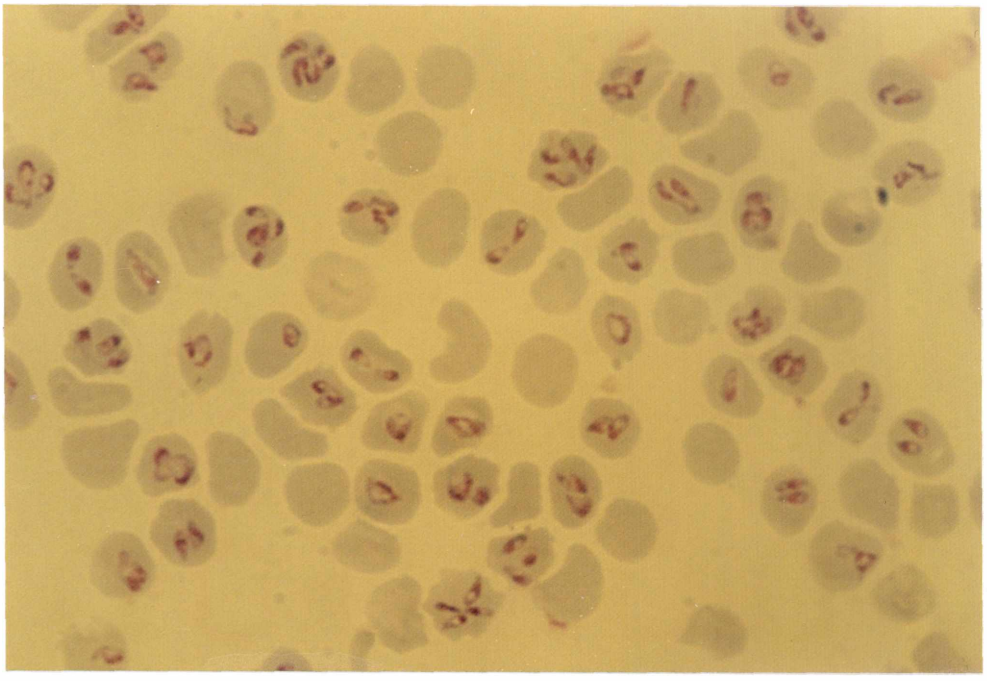

Figure 10 Blood smear from a gerbil infected with the "J" strain of *B. divergens* at 60% parasitaemia. Giemsa stain. Magnification x 2,800

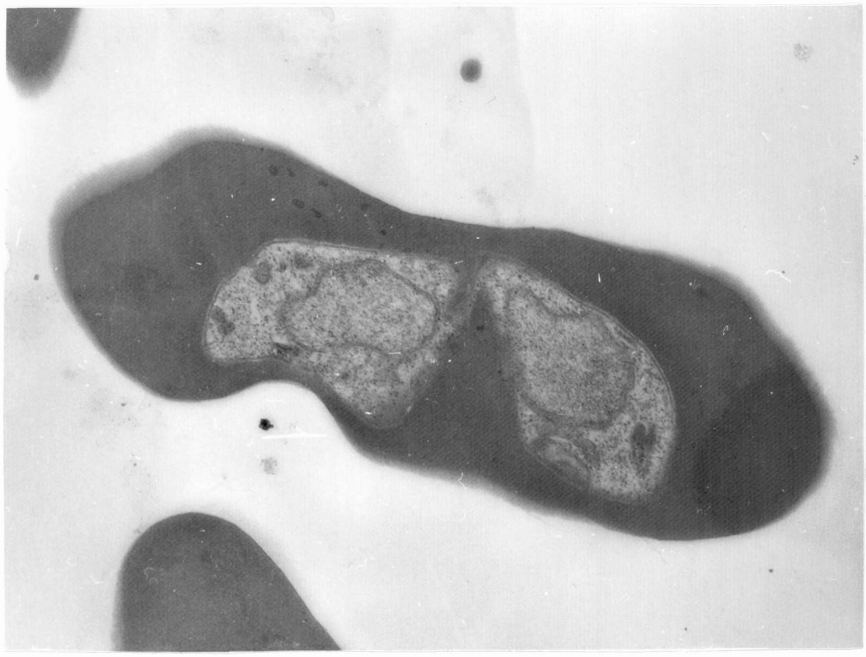

Figure 11 T.E.M. of the "J" strain of *B. divergens* in a human erythrocyte. Magnification X 9,100

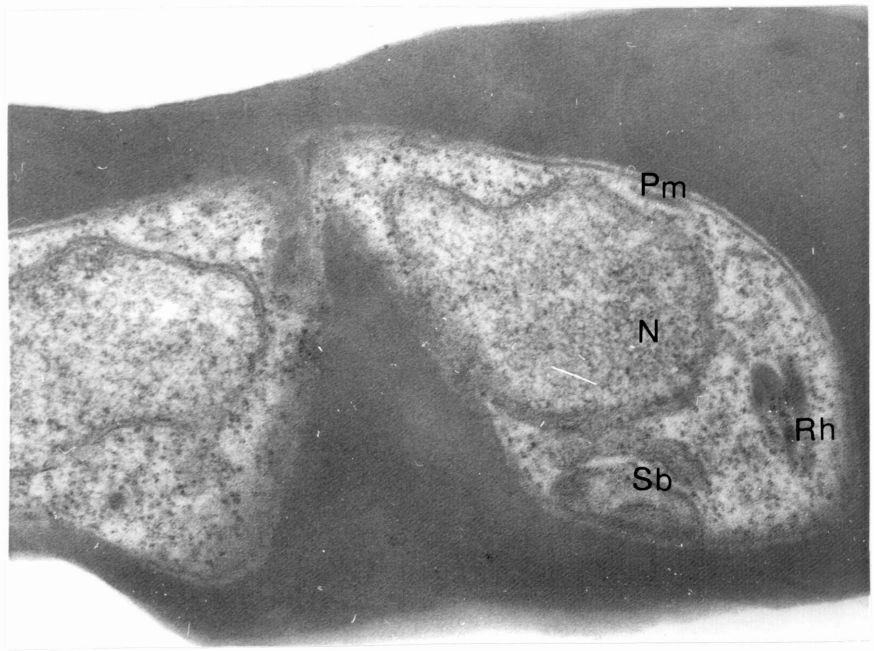

Figure 12 x 25,000 magnification of Fig. 11.
Pm = plasma membrane ; N = nucleus ; Rh = rhoptries ;
Sb = spherical body

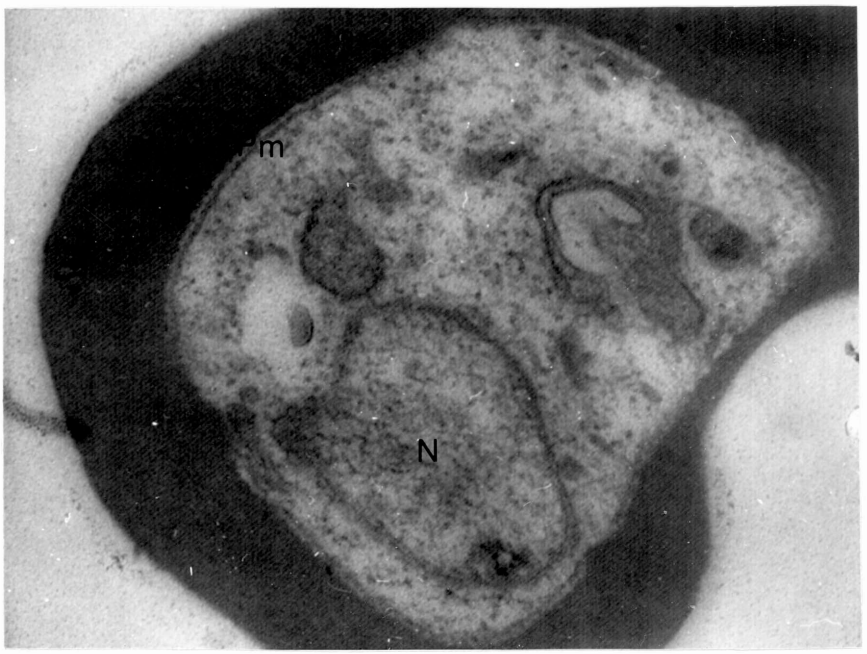

Figure 13 T.E.M. of the "J" strain of *B. divergens* in a human erythrocyte. Magnification x 25,000
N = nucleus ; Pm = plasma membrane

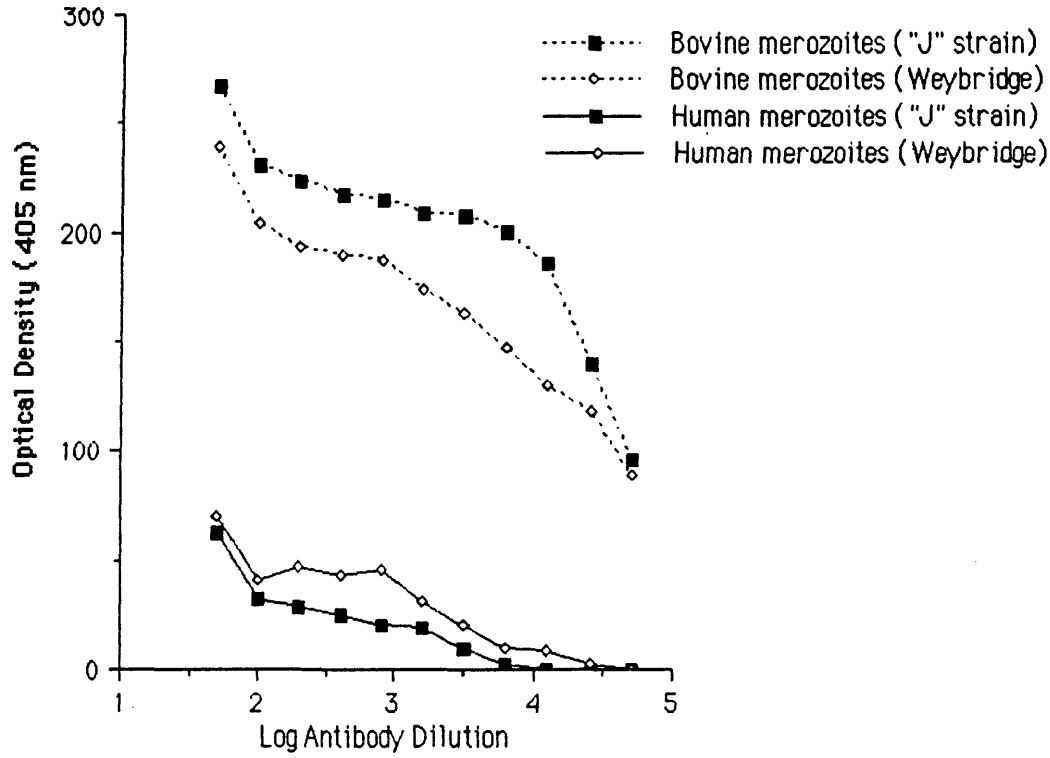

Figure 14. ELISA demonstrating binding of Mab13.4 to merozoites raised in human and bovine cultures.

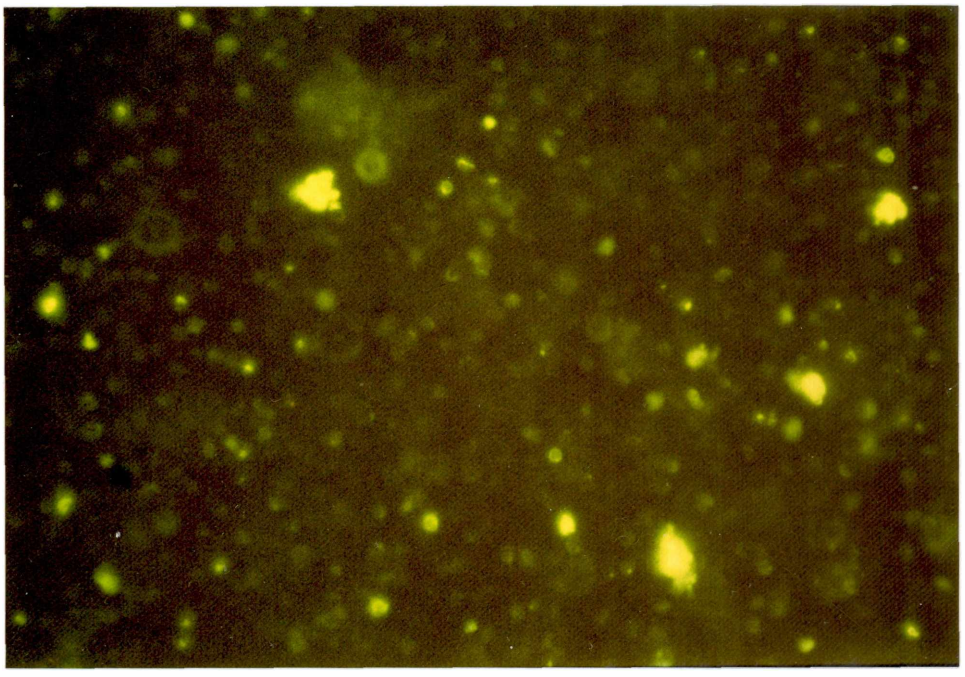

Figure 15 IFAT : binding of Mab 13.4 to live merozoites raised in bovine erythrocytes. Magnification x 2,000

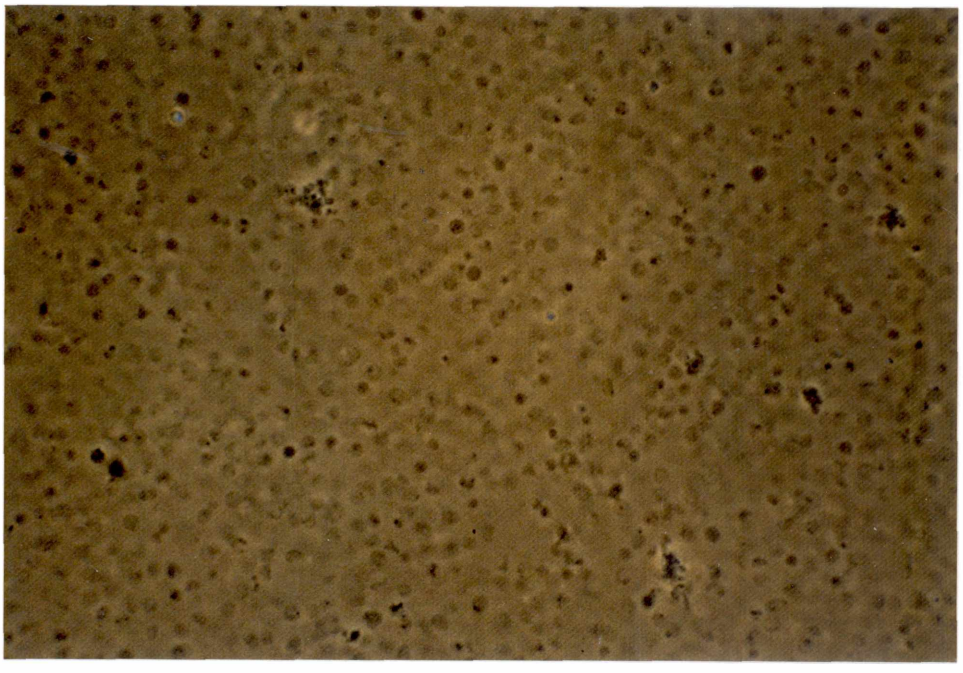

Figure 16 Phase contrast view of Figure 15.

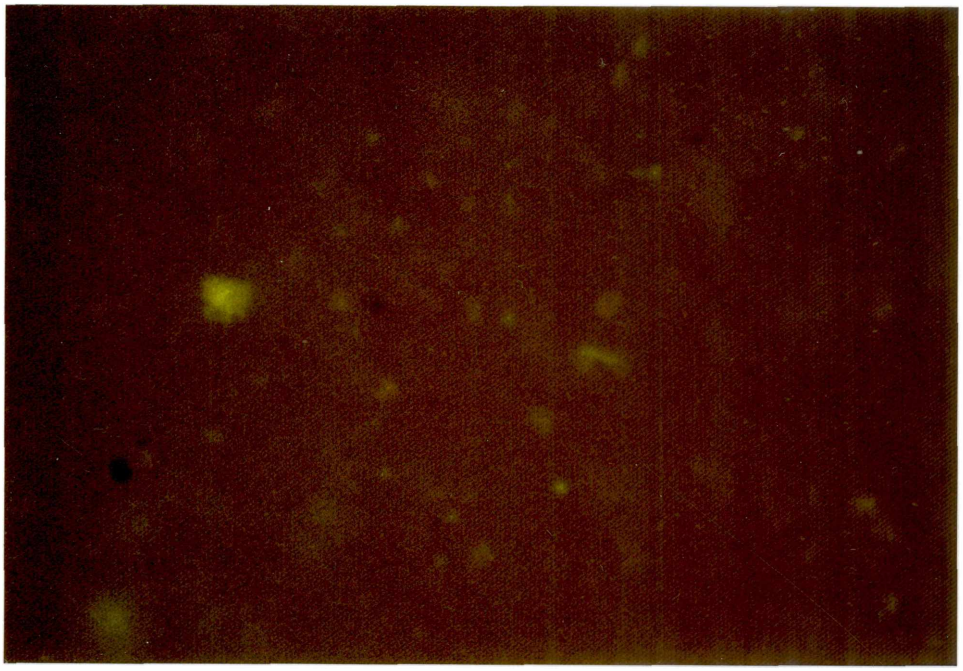

Figure 17 IFAT : binding of Mab 13.4 to live merozoites raised in human erythrocytes. Magnification x 2000

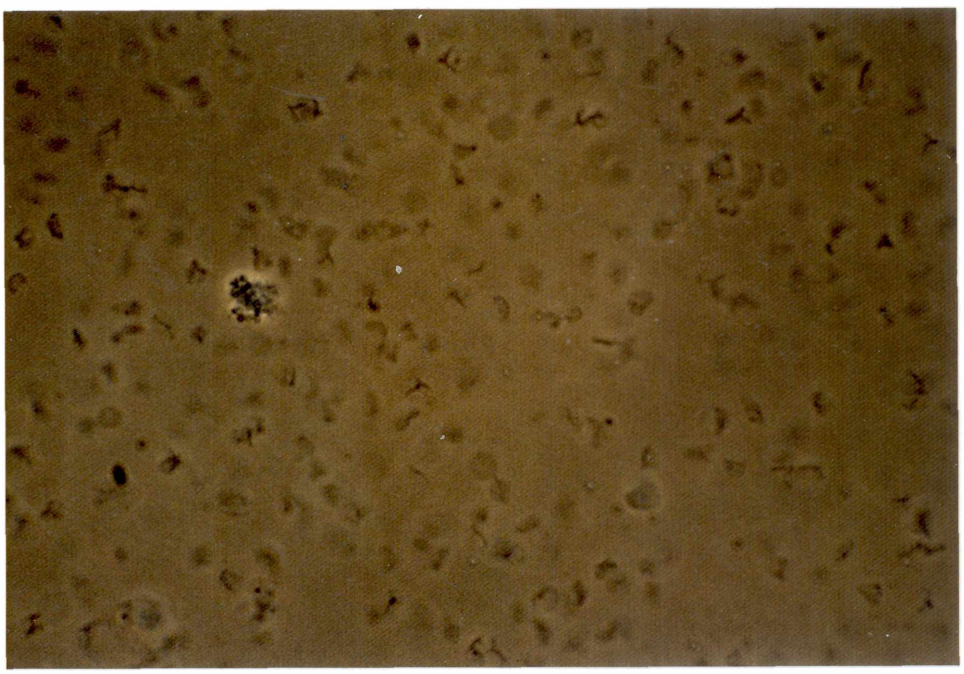

Figure18 Phase contrast view of Figure 17.

3.3.2.3. Immunoblotting

Both "J" and Weybridge strain merozoites were immunoblotted with Mab 13.4 The results confirm that differences in binding of Mab13.4 did not result from antigenic differences in the strain used to immunise for the Mab production. SDS-PAGE protein profiles of merozoites of each strain, raised in both human and bovine erythrocytes, were immunoblotted after Western transfer using Mab13.4 and HRP-conjugated anti-mouse IgG second antibody (Fig. 19.). The results show that Mab 13.4 recognises antigens in both strains of *B. divergens* but only when raised in bovine erythrocytes. There is a small degree of binding above control levels to antigens from both Weybrige and "J" strain human preparations However, this is no more distinct than the reactivity found to occur in the bovine preparations with the control antibody and could therefore be attributable to non-specific binding. These results show that the differences in binding are host-cell rather than strain related.

3.3.2.4. Merozoite neutralisation assay

Mab 13.4 has been shown to inhibit invasion of Weybrige strain into bovine erythrocytes (Winger *et al*, 1987a) and its recognition of surface antigens has been shown by immunogold electron microscopy (Winger *et al*, 1990). To confirm the previous results in a functional assay, inhibition of merozoite entry into human and bovine erythrocytes by Mab13.4 was tested using human and bovine raised merozoites respectively. Again both "J" and Weybridge strain merozoites were used and the results are shown on Table 1. The inhibition produced by Mab 13.4 is expressed as a percentage of the values produced after incubation with Mab 13.5 which recognises an internal antigen (Winger *et al*, 1990) and was used as a control. There was no significant difference found between Weybrige and "J" strain merozoites with 76% inhibition of invasion of bovine erythrocytes occurring for both strains following incubation with Mab 13.4. In contrast, Mab 13.4 produced only 19.5% and 24.2% inhibition of entry into human erythrocytes for "J" and Weybrige strains respectively.

3.4. REACTIVITY OF MOUSE SERA RAISED AGAINST HUMAN AND BOVINE MEROZOITES

These antisera were raised to investigate whether antibodies against merozoites isolated from human blood would show any selectivity in binding to human as opposed to bovine raised parasites. The tests were

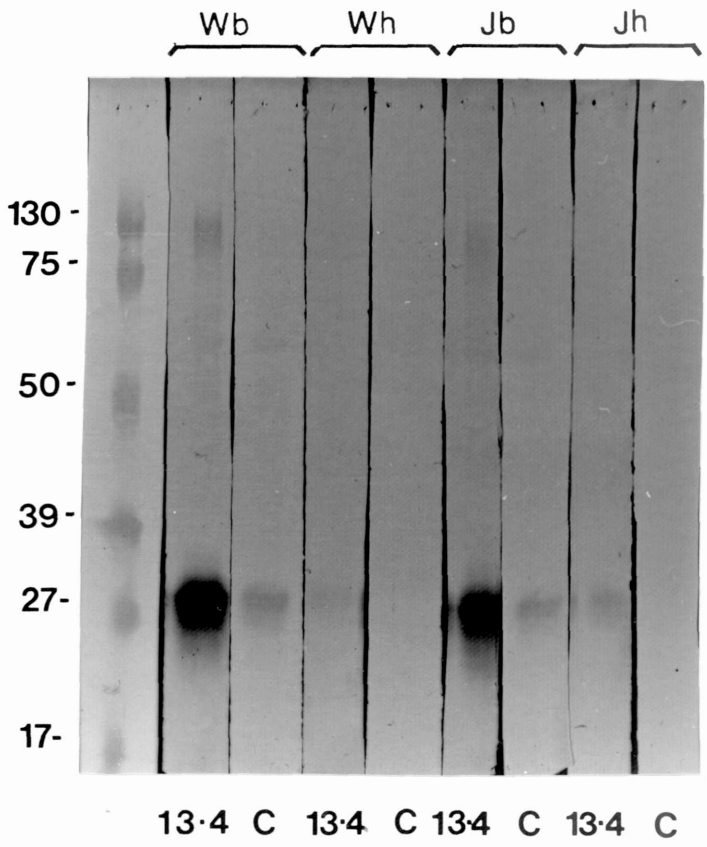

Figure 19 Immunoblot of 15% reducing SDS-PAGE protein profile of "J" strain merozoites demonstrating binding of Mab 13.4 to merozoites raised in human and bovine erythrocytes.

Wb = Weybridge strain merozoites raised in bovine blood.
Jb = "J" strain merozoites raised in bovine blood.
Wh = Weybridge strain merozoites raised in human blood.
Jh = "J" strain merozoites raised in human blood.

"C" is a mouse IgG1 ascites used as a control.

TABLE 1.

MEROZOITE NEUTRALISATION ASSAY USING MAB13.4

"J" and Weybridge strain merozoites raised in both human and bovine cultures were incubated for 30 min with Mab 13.4 or with Mab 13.5, which recognises an internal antigen of *B. divergens*; then added back to the erythrocyte type from which they had originally been isolated.

MEROZOITE TYPE	MAB	PARASITAEMIA (%) after 40 hours x ± SE (n=3)	INHIBITION (%)
Raised in human red cells	medium control	**0.100 ± 0.028**	-
		0.173 ± 0.009	-
	13.5	**0.103 ± 0.033**	**0**
		0.153 ± 0.022	0
	13.4	**0.083 ± 0.012**	**19.5**
		0.116 ± 0.023	24.2
Raised in bovine red cells	medium control	**0.053 ± 0.009**	-
		0.093 ± 0.02	-
	13.5	**0.083 ± 0.016**	**0**
		0.076 ±0.004	0
	13.4	**0.020 ± 0.008**	**76.0**
		0.033 ± 0.018	76.6

* Parasitaemias for "J" strain merozoites are shown in bold type and those for the Weybridge strain are shown in normal type.

carried out using both "J" and Weybridge strain merozoites raised in human (Jh, Wh) and bovine (Jb, Wb) cultures, however, in most cases only the results for the "J" strain are shown as the results produced for each were the same.

3.4.1. ELISAs using mouse anti-sera against human and bovine raised merozoites.

The degree of binding of the anti-sera to the two groups of merozoites was found to correspond to the immunising fraction used (Figs 20 and 21). There was strong binding of mouse antisera raised against "J" strain merozoites isolated from bovine culture (Jb antisera) with Jb merozoites and the uninfected bovine erythrocyte control (Fig 20). The binding was weaker with Jh antisera on Jb merozoites and bovine blood. The reactivity of Jb and Jh antisera on merozoites raised in human culture and on the human red blood cell control followed the same pattern, with strong binding to these antigens occurring with Jh antisera and weaker binding occurring with Jh antisera (Fig 21). It is evident therefore that much of this binding can be attributed to selective reactivity against the different blood antigens also present in the merozoite preparations. Because of the polyclonal nature of the antisera, no clear answer could be elucidated from the ELISA results.

3.4.2. IFAT using mouse anti-sera.

This was carried out using live merozoites isolated from human and bovine cultures and also with fixed smears from both types of cultures.

3.4.2.1. Live merozoites

Sera raised against human (Jh, Wh) and bovine (Jb, Wb) merozoites were found to exhibit comparable fluorescence against both groups of merozoites irrespective of the merozoite strain used. Again due to the polyclonal nature of the anti-sera any subtle differences in recognition of antigens would have been masked.

3.4.2.2. Fixed culture smears

Figures 22 and 23 show the binding of sera raised against bovine and human "J" strain merozoites respectively to smears of a bovine culture. The background binding to uninfected erythrocytes can be clearly seen when bovine merozoites were used as the immunogen. In contrast there was little

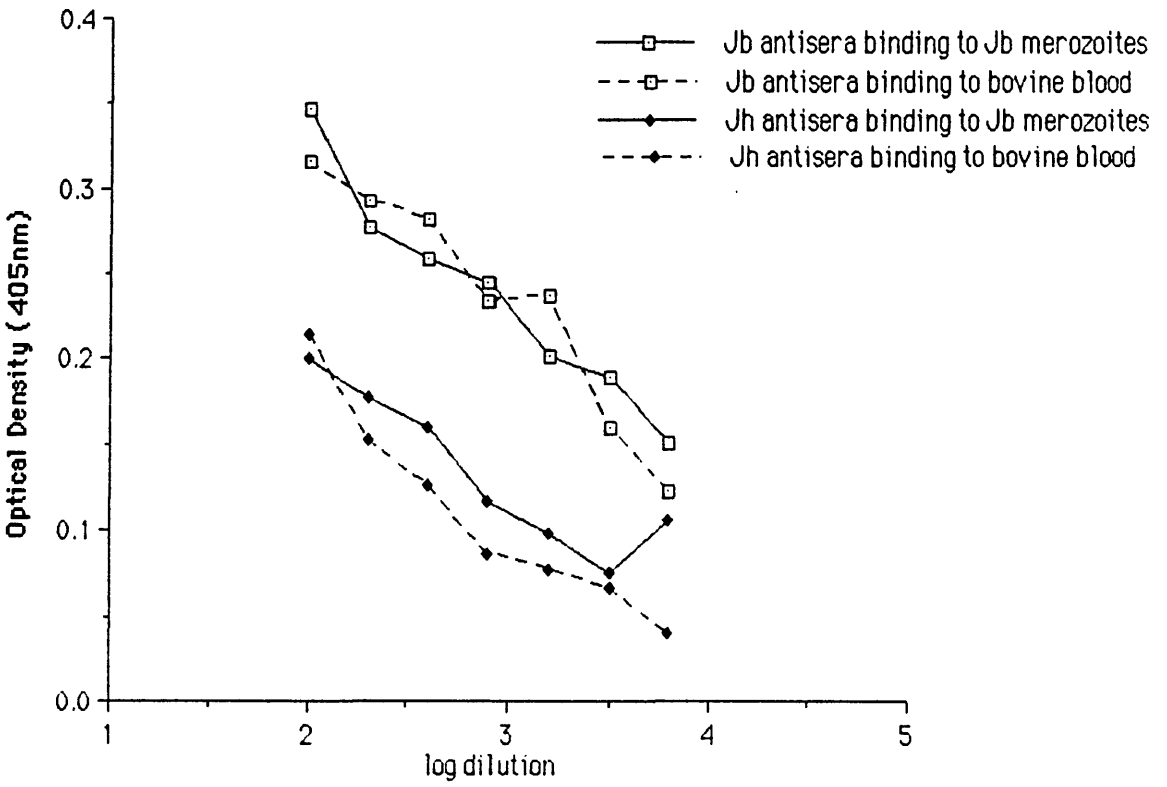

Figure 20. ELISA demonstrating binding of mouse anti-sera to bovine erythrocytes and merozoites raised in bovine erythrocytes.

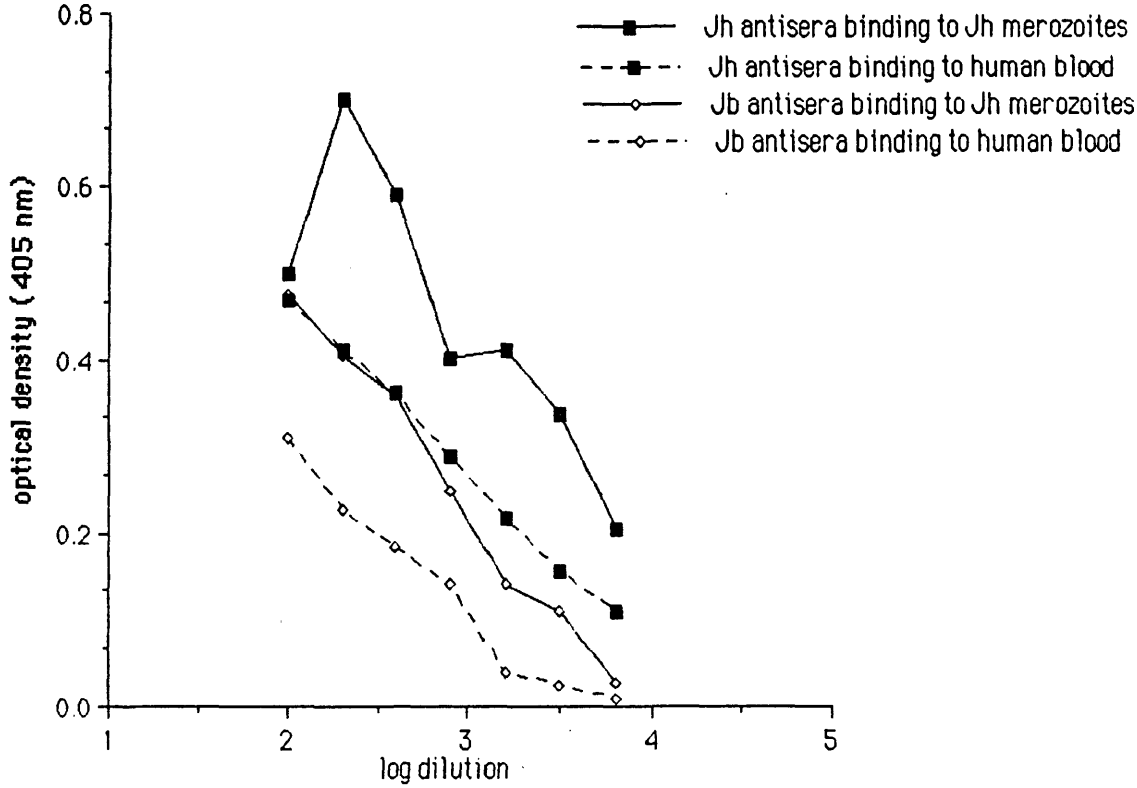

Figure 21. ELISA demonstrating binding of mouse anti-sera to human erythrocytes and merozoites raised in human erythrocytes.

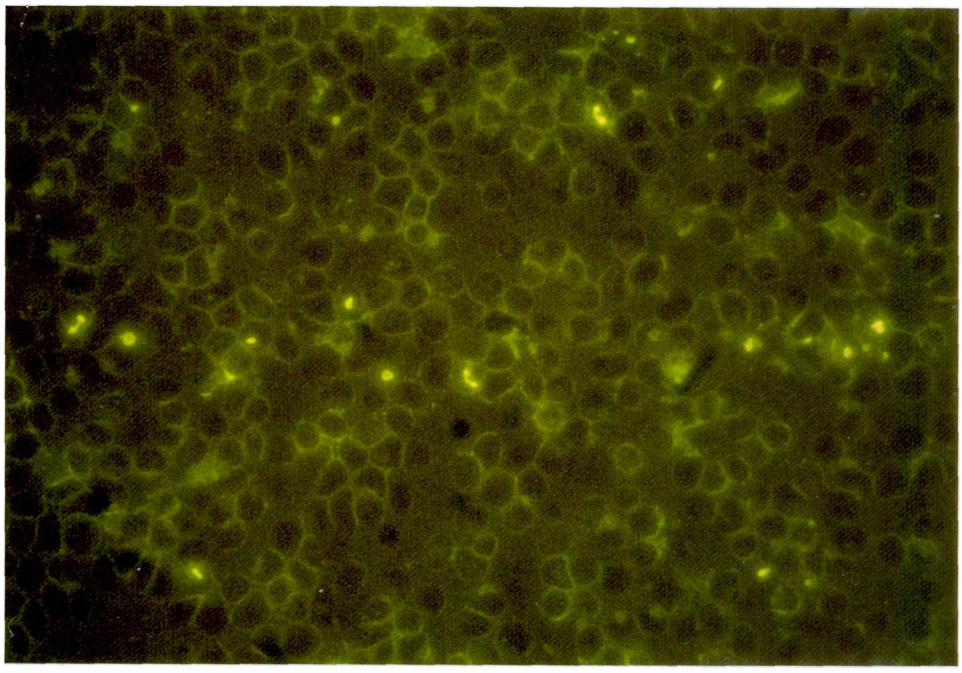

Figure 22 IFAT: Binding of mouse sera raised against bovine merozoites (Jb) to a fixed blood smear from a bovine MASP culture. Magnification x 2,000.

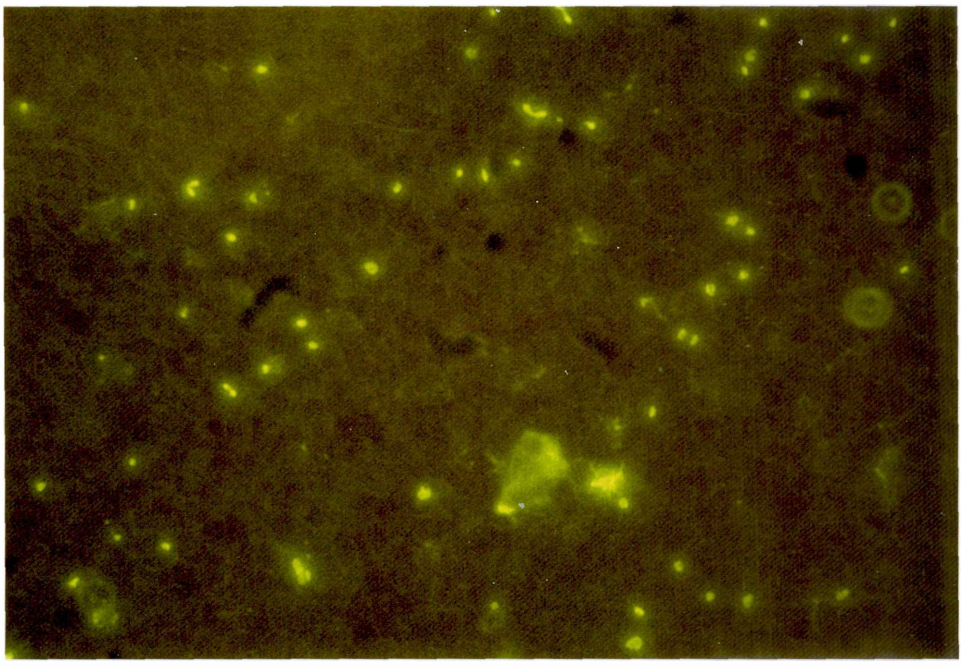

Figure 23 IFAT: Binding of mouse sera raised against human merozoites (Jh) to a fixed blood smear from a bovine MASP culture. Magnification x 2,000.

background binding when human merozoites were used as the immunogen, confirming the ELISA results. Whatever the immunogen, the antisera bound with comparable fluorescence to the intracellular forms in bovine red cells. Thus antigens present in merozoites used to raise the antisera are present also in the intracellular developmental stages.

3.4.3. Immunoblotting

Fig. 24 compares the binding of mouse sera raised against merozoites isolated from human cultures (Jh) to preparations of merozoites of "J" and Weybridge strains from bovine and human cultures. While much background anti-erythrocyte activity can be seen, there also appear to be differences in binding to merozoite antigens. Notably using profiles of "J" and Weybridge merozoites from bovine blood there was no binding of the antiserum to a 26kD antigen which is recognised by Mab 13.4. This confirms its absence or alteration when the strains are grown in human red cells, then used to immunise the mice. There does, however, appear to be binding to an antigen in the profile of both strains grown in human erythrocytes at around 26kD, which suggests that the antigen is altered rather than absent. Also, a slight difference in binding to the bovine and human merozoites is seen at between 50 - 60 kD. A protein of the same molecular weight is recognised by Mab 13.4 and is believed to represent part of a merozoite surface antigen involved in entry into erythrocytes. A control carried out to assess any reactivity in normal mouse serum against these antigens exhibited no binding.

3.4.4. Merozoite neutralisation assay

To determine whether there is any functional relevance to the different antigens expressed by the strains raised in the bovine and human erythrocytes, the ability of mouse anti-sera raised against these two merozoite groups to inhibit invasion of erythrocytes was investigated. The anti-sera used were those raised against "J" strain merozoites grown in both human and bovine blood. Their effects upon merozoite invasion into human and bovine cultures are shown in table 2. There is clearly a host-related difference with regard to the merozoite group used to immunise the mice, with significant inhibition being produced only when merozoites from the corresponding host erythrocyte group were being used to test entry into erythrocytes. Normal mouse serum (NMS) can be seen to have some inhibitory effects upon the merozoites when compared to the medium

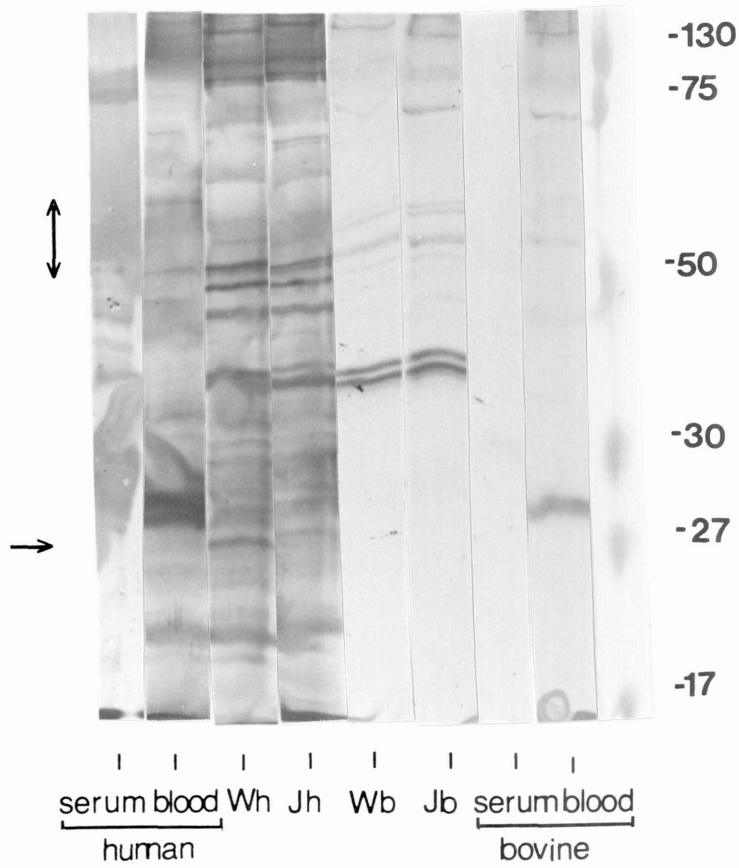

Figure 24 Immunoblot of 10% SDS-PAGE protein profiles of *B. divergens* merozoites demonstrating binding of mouse sera raised against "J" strain merozoites grown in human erythrocytes.

Antigens : Wb = Weybridge strain merozoites raised in bovine blood.
Jb = "J" strain merozoites raised in bovine blood.
Wh = Weybridge strain merozoites raised in human blood.
Jh = "J" strain merozoites raised in human blood.

TABLE 2

MEROZOITE NEUTRALISATION ASSAY USING MOUSE ANTISERA RAISED AGAINST THE "J" STRAIN OF *B. DIVERGENS* GROWN IN HUMAN OR BOVINE ERYTHROCYTES.

MEROZOITE TYPE	MOUSE IMMUNOGEN	PARASITAEMIA (%) after 40 hours x ± SE (n=3)	INHIBITION (%)*
"J" STRAIN FROM HUMAN CELLS	Merozoites from human cells	0.46 ± 0.04	58.3
	Merozoites from bovine cells	0.96 ± 0.09	0
	None (NMS control)	0.96 ± 0.04	0
	None (medium control)	1.19 ± 0.08	-
"J" STRAIN FROM BOVINE CELLS	Merozoites from human cells	0.70 ± 0.10	12.5
	Merozoites from bovine cells	0.30 ± 0.13	62.5
	None (NMS control)	0.80 ± 0.08	0
	None (medium control)	1.10 ± 0.09	-

* Inhibition is expressed as a percentage of the normal mouse serum (NMS) control

control and so the degree of inhibition produced has been expressed as a percentage of the NMS value.

3.5. T-CELL PROLIFERATION ASSAYS

3.5.1. Human T-cell proliferation assays

3.5.1.1. Unexposed donors

These were carried out using PBM from normal volunteers to determine if T-cell recognition of merozoite antigens occurs in unexposed donors. In the absence of an exposed donor, the effects of cell numbers upon proliferation to PHA were tested and as a result 2 x 10^5 cells/well were used in all subsequent assays (Appendix figure 1). Since no differences were found between the "J" and Weybridge strains in any of the previous experiments the "J" strain only was used in all the following white cell assays. For all donors, merozoites isolated from both human and bovine cultures were tested for induction of proliferation to determine whether there is a functional significance to the antigenic differences. None were found and all results shown are for "J" strain merozoites grown in human red cells. Of the 17 normal donors tested no T-cells showed any proliferation to either set of merozoites. Thus no mitogenic stimulation occurs and there is no innate response to *B. divergens* merozoite antigens in unexposed donors. Lymphocyte proliferation to merozoites at day 7 was found in one worker from this laboratory when 2 x 10^5 merozoites/well were present (Fig.25). However, this donor possibly had been exposed to the parasite in the laboratory and, as this proliferation was produced in only 3 out of 5 assays, no firm conclusions could be drawn from the result.

3.5.1.2. Exposed donor

A T-cell proliferation assay was carried out using blood from an Irish patient with hairy cell leukaemia, who had suffered from a *B. divergens* infection three months previously. Figs. 26 and 27 show the results obtained using 10% FCS and 10% autologous serum respectively as medium supplements. However, the presence of leukaemic B-cell clones in many wells on day 7, interfered with triplicate counts. Thus the antigenic stimulation which may seem to have occurred cannot be regarded as significant. Additionally, it became evident following B-cell separation that only 63% of the patient's PBM were T-cells, a value 10 - 15% less than normal, which may also have some bearing on the results. No mitogenic response seen to merozoites was observed, either with FCS or autologous

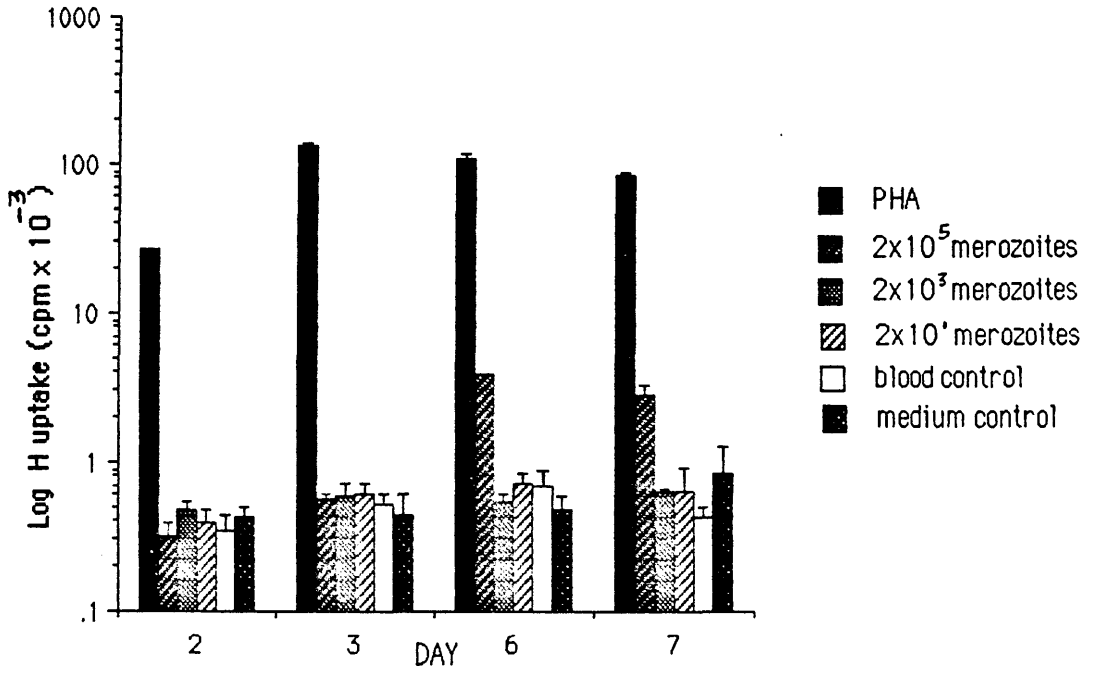

Figure 25. T-cell proliferation assay using PBM from a laboratory volunteer.

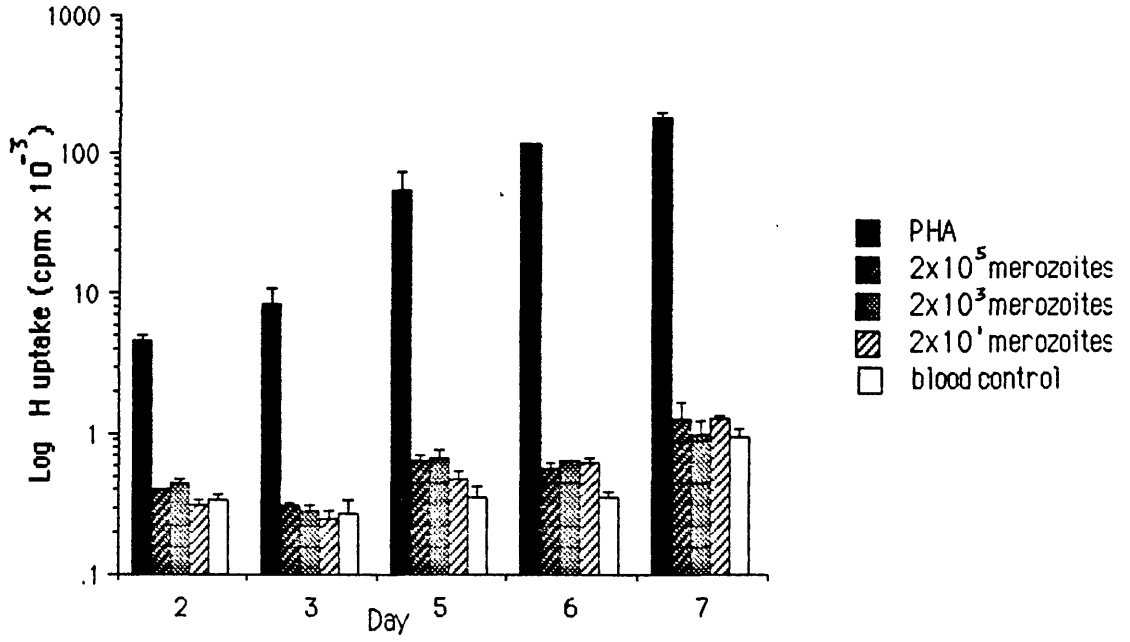

Figure 26. T-cell proliferation assay using PBM from an exposed donor and FCS as supplement.

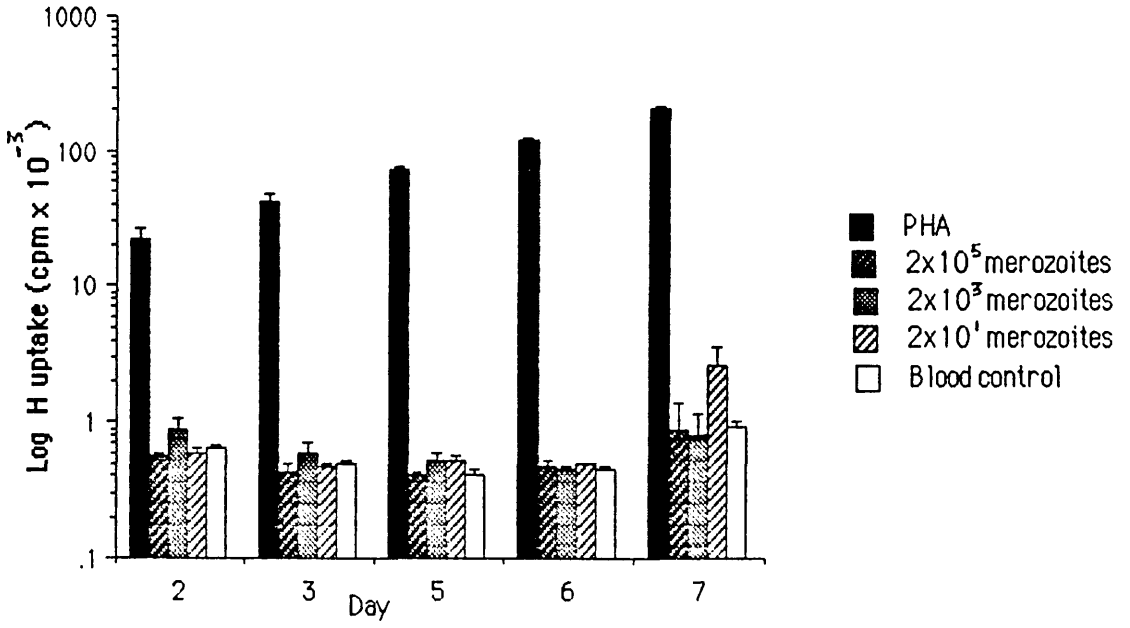

Figure 27. T-cell proliferation assay using PBM from an exposed donor and autologous serum as supplement.

serum, although again the reason for this might be linked to the patients condition and ongoing chemotherapy. The assay could not be repeated with the B-cells removed as no further blood was available.

3.5.1.3.Donor exposed to *P. falciparum*

T-cell proliferation to merozoites was also carried out using PBM from a donor who had suffered from *P. falciparum* malaria 18 months previously (Fig. 28). No proliferation to merozoite antigens was found.

3.5.2. Bovine proliferation assay

Bovine PBM from an unexposed donor cow were assayed for proliferation in response to human and bovine merozoites (Figs. 29 and 30). No mitogenic or antigenic responses were produced and again no difference between responses to human and bovine merozoites was found.

3.6. B-CELL ASSAYS

3.6.1. Antibody responses

These were investigated using serum from 17 unexposed donors and compared with the response found with the serum from the Irish patient.

3.6.1.1.ELISA results

HI bovine serum was used in all these assays to remove the background binding discussed previously (section 3.2.1.). Figure 31 shows the response produced with the serum from the Irish patient who had been infected with *B. divergens*. Anti-IgG and anti-IgM second antibodies were used to detect Ig binding to merozoites raised in human blood and to a human red blood cell control. None of the sera from unexposed donors showed significant binding. A notable difference between exposed and unexposed donor sera was the presence of IgG reactive with merozoite antigens in the exposed donor serum. With normal serum (Figure 32) no reactivity was found above that of the blood control. Additionally, the reactivity of IgG with blood antigens is slightly raised in the Irish serum in comparison with IgG in the unexposed serum.

3.6.1.2.Immunoblotting

Fig. 33 demonstrates the strong binding of IgG in the serum from the Irish donor to merozoite and blood antigens. Merozoite antigens of around 26kD, 30kD, 34kD, 36kD and 55kD are recognised. This is in contrast

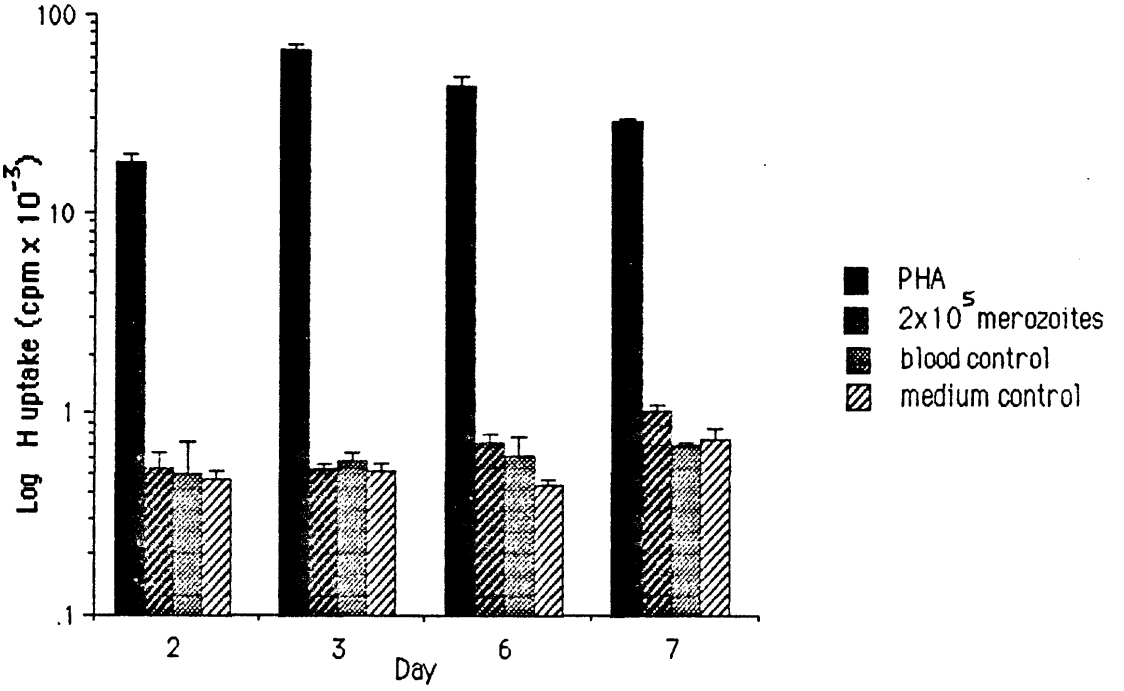

Figure 28. T-cell proliferation assay using PBM from a donor previously exposed to *P. falciparum*.

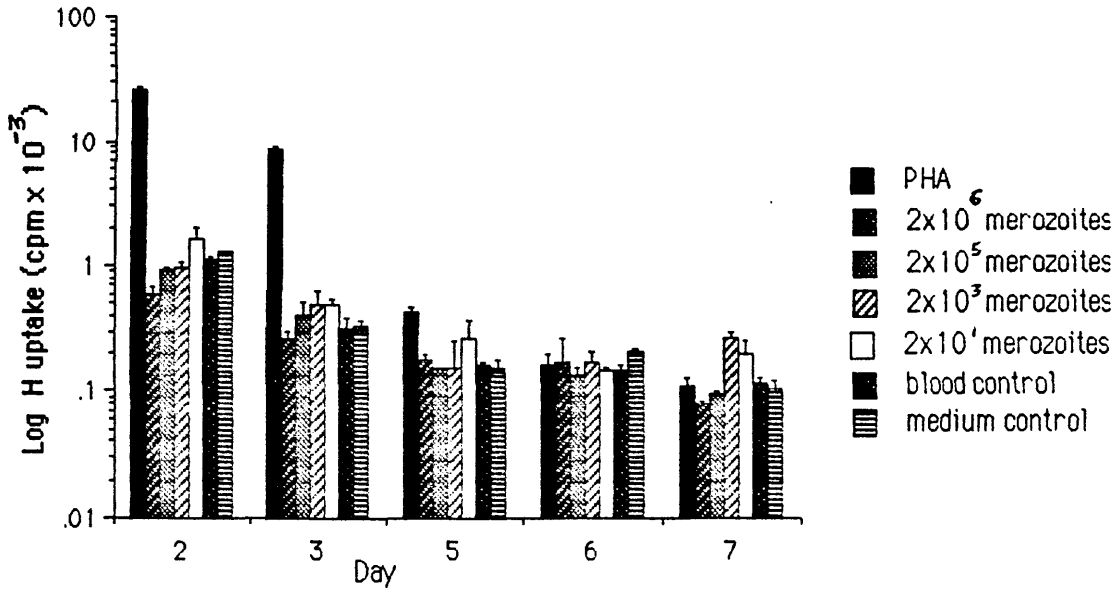

Figure 29. Bovine T-cell assay using merozoites from bovine cultures.

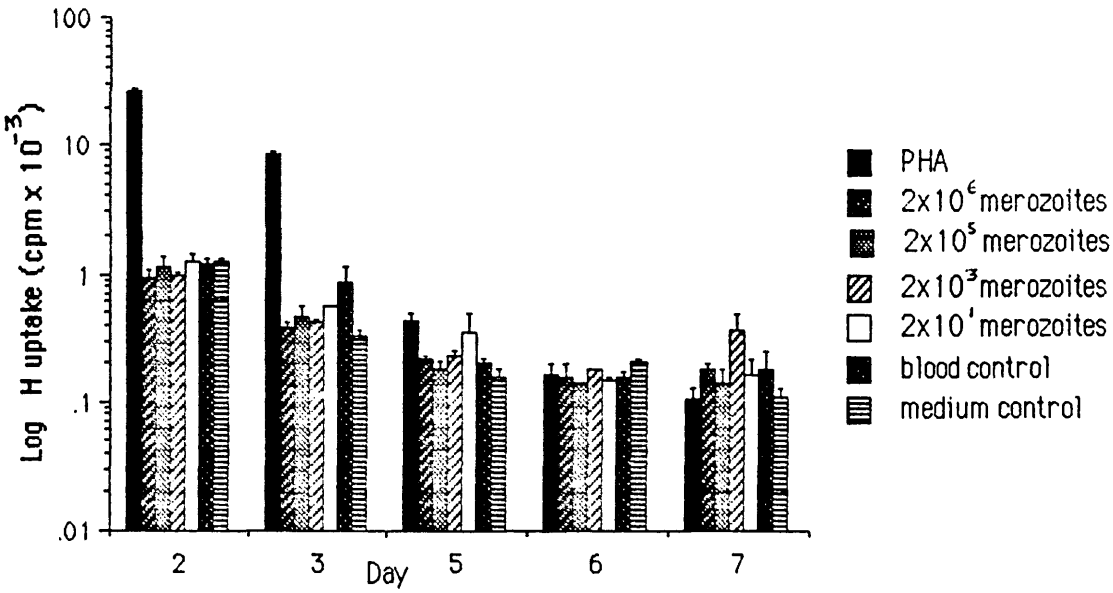

Figure 30. bovine T-cell assay using merozoites from human cultures.

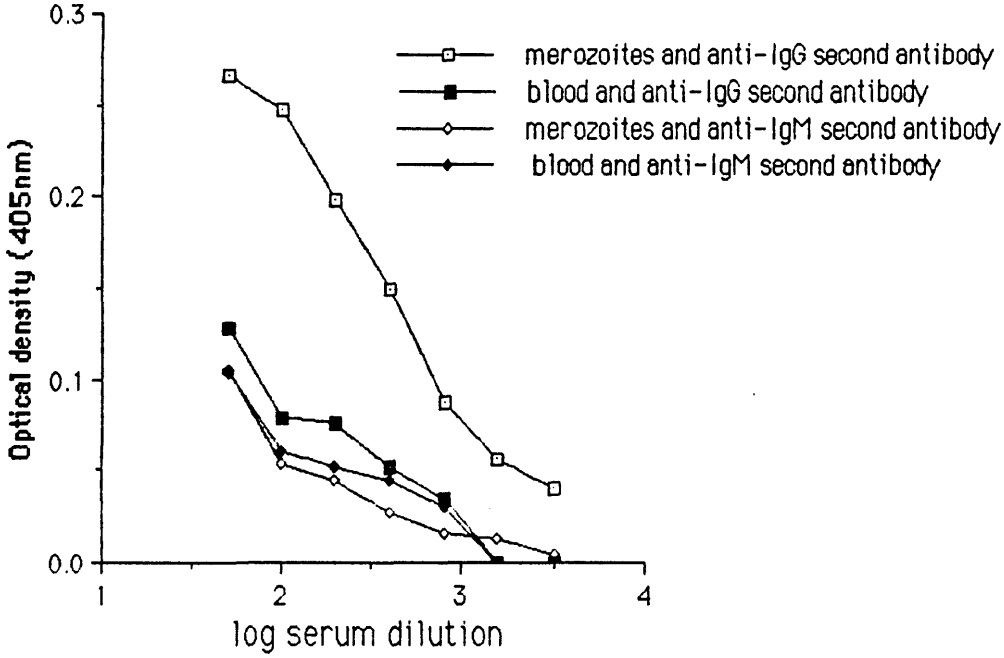

Figure 31. ELISA demonstrating binding of Irish serum to merozoites raised in human MASP cultures and to human erythrocyte controls

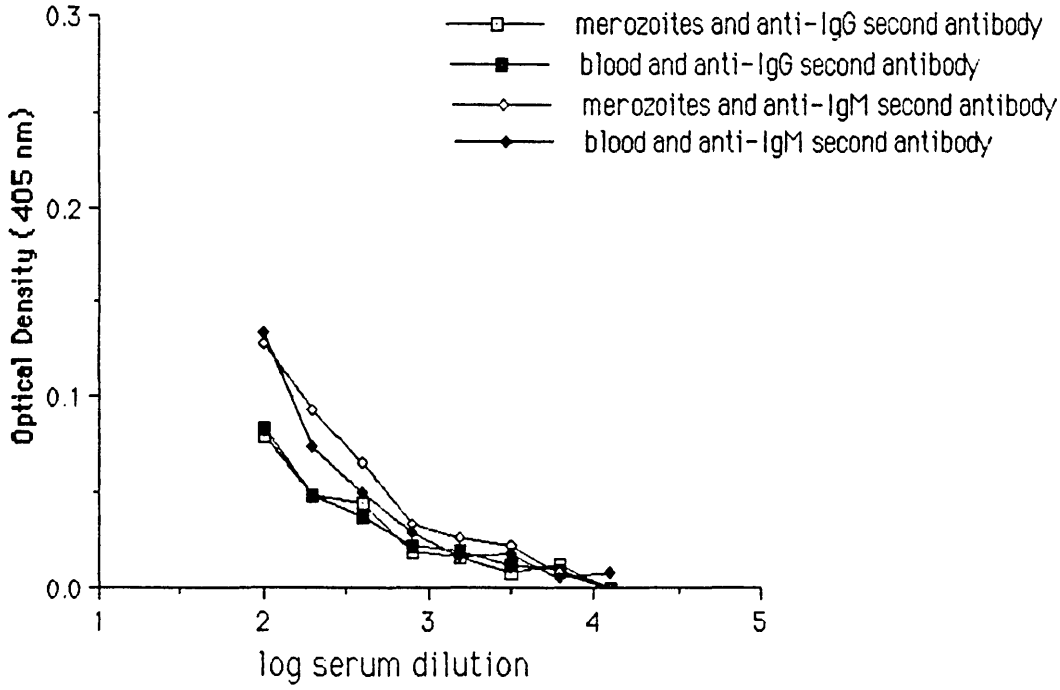

Figure 32. ELISA demonstrating binding of normal serum to merozoites raised in human MASP cultures and to human erythrocyte controls.

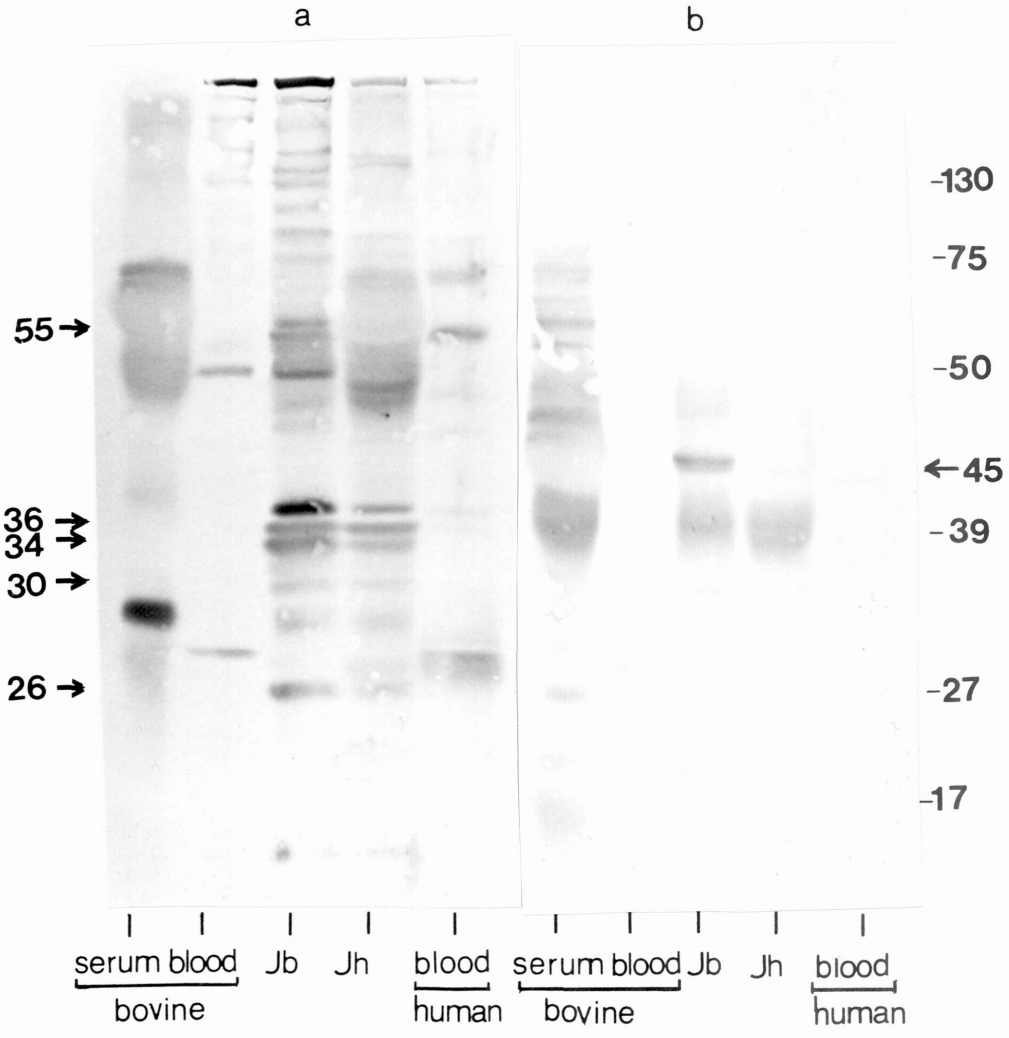

Figure 33 Immunoblot demonstrating binding of IgG from serum of an Irish donor (a) and normal serum (b) to "J" strain merozoites raised in human and bovine erythrocytes. Controls are bovine serum and blood and human blood.

Jb = "J" strain merozoites raised in bovine blood
Jh = "J" strain merozoites raised in human blood

Both groups of merozoites were raised in cultures supplemented with heat inactivated bovine serum.

to the IgG present in normal human serum which binds only to blood and serum antigens present in the merozoite preparations with one exception ; binding of IgG in the unexposed serum to a band at about 45kD in the Jb merozoite track. This is not seen in the track containing Jh merozoites nor in any track using the serum from the Irish patient. Additionally, differences can be seen between the binding of the serum from the Irish patient to tracks of merozoites raised in human blood (Jh) and merozoites raised in bovine blood (Jb). The serum bound to a 55kD protein in Jb but not Jh and there was stronger binding to the Jb merozoite track at 26kD.

3.6.1.3.Merozoite neutralisation assay

The ability of components in normal serum to inhibit "J" strain merozoite invasion in both human and bovine cultures was compared with that of the serum from the Irish patient. Table 3 shows the parasitaemias reached following incubation with the human sera. Although the parasitaemias were all rather low, the Irish and normal sera produced significant neutralisation in both the human and bovine cultures when compared to the medium controls ($p=0.05$, Mann-Whitney Test). However, the difference between the normal and Irish sera was not significant. ELISAs and immunoblots have shown that the Irish serum contains IgG reactive against intact merozoites and that reactive antibodies are absent from normal serum. This suggests that the inhibition seen in the merozoite neutralisation assays is brought about by non-specific factors. There was no significant difference found between the inhibition of human and bovine cultures

3.6.2. B-cell transformations

These were carried out to determine the presence of B-cell precursors in a population of PBM and their potential to recognise and mount an antibody response to merozoites *in vitro*

3.6.2.1.Precursor frequencies

These were estimated from the percentage of the E- cells which bound to the merozoites during the panning procedure. Since this was carried out at 4°C, binding by monocytes present in the preparation should have been minimal. E- cells were found to comprise 22.5 ± 3.2 % of the total PBM population. 10^7 E- cells from each of 14 normal donors were panned and an average of $5.1 \pm 2.8 \times 10^5$ cells adhered, giving an average value of 0.05% for the proportion of adherent cells which will bind to merozoite antigens. However, this can not be taken as an absolute percentage of B-cells present

TABLE 3

MEROZOITE NEUTRALISATION ASSAY USING IRISH AND NORMAL SERA

SERUM	* PARASITAEMIAS HUMAN (%)	INHIBITION (%)	PARASITAEMIAS BOVINE (%)	INHIBITION (%)
IRISH	<0.01, <0.01, 0.01	86.3	0.01, 0.01, 0.02	82.9
NORMAL	0.01, 0.02, 0.02	77.2	0.02, 0.03, 0.03	66.0
MEDIUM CONTROL	0.06, 0.08, 0.08	-	0.07, 0.08, 0.08	-

*Parasitaemias were all counted after 40 hours

in normal peripheral blood potentially reactive against *B. divergens* merozoites because of the red cell antigens present in the merozoite preparation.

3.6.2.2.Frequency of transformation

Frequencies of E- cells from 8 donors which underwent transformation following EBV exposure are shown in Table 4. The susceptibility of different donors B-cells to transformation can be seen to vary substantially. However, in all cases, those E- cells which were panned prior to EBV exposure are clearly enriched with regard to the numbers of B-cells present which then go on to form clones.

3.6.2.3.Antibody production by transformed B-cells

Supernatants from all the clones produced were tested by ELISA for production of IgM or IgG reactive against merozoites, human blood and HI bovine serum. No clones were found to be producing IgG to any of these antigens, suggesting that no previous exposure of the donors to *B. divergens* had occurred. The B-cells from the Irish patient did not produce any antibodies which reacted with the antigens tested. However, hairy-cell clones were present in many wells after only a few days, offering little chance for any newly transformed cells to survive.

Table 5 shows the results from ELISAs for detecting IgM carried out on supernatants from wells containing clones. The proportion of supernatants containing antibodies (IgM) which reacted against all antigens tested are shown and also the number containing IgM which bound to merozoites only. Using these results along with the numbers of cells per well and the total numbers of wells present for each dilution, the number of clones per 10^6 cells producing antibodies reactive with merozoites has been calculated. The B-cells are all from normal donors except number 7 who had recovered from an infection with *P. falciparum* 18 months previously. The results differ from person to person with some producing no specific antibodies at all while other sera reacted sufficiently to suggest that B-cells capable of producing antibodies against merozoites were present in numbers ranging from 11-250 per 10^6 E- cells. This gives a percentage value of between 0.0011-0.025, although this cannot be taken as an absolute figure as the cells were not a true representation of the peripheral blood B-cell population, due to the panning procedure.

TABLE 4

B-CELL TRANSFORMATION FREQUENCIES

	NO. OF CELLS EXPOSED TO EBV	FREQUENCY OF TRANSFORMATION FOR 8 DONORS (%)							
		1	2	3	4	5	6	7	IRISH
SCATTERGUN	10^5	100	100	100	90	90	50	ND	ND
	10^4	100	80	100	75	25	5	ND	ND
	10^3	33	10	50	7	6	0	ND	ND
PANNED	$3x10^3$	100	100	80	100	60	10	100	100
	10^3	85	90	55	93	40	0	91	93
	10^2	16	68	13	45	5	0	38	66
	10^1	6	16	10	13	0	0	12	28

TABLE 5

ELISAs USING SUPERNATANTS FROM WELLS CONTAINING CLONES FROM PANNED E- POPULATION

DONOR	NUMBER OF CELLS/WELL	NUMBER BINDING TO ALL ANTIGENS	NUMBER BINDING ONLY TO MEROZOITES	NUMBER OF POSITIVES /10^6 CELLS
1	$3x10^3$	6/10	1	33.3
	10^3	4/14	1	50.0
	10^2	0/11	0	0
	10^1	1/4	0	0
2	$3x10^3$	30/30	0	0
	10^3	18/27	0	0
	10^2	13/41	0	0
	10^1	2/10	0	0
3	$3x10^3$	1/8	0	0
	10^3	2/11	1	90.9
	10^2	0/8	0	0
	10^1	0/1	0	0
4	$3x10^3$	27/30	1	11.1
	10^3	17/28	1	35.7
	10^2	3/27	0	0
	10^1	2/8	0	0
5	$3x10^3$	2/8	2	250
	10^3	2/6	0	0
	10^2	0/5	0	0
	10^1	0/0	0	0

(TABLE 5 CONTD.)

6	$3x10^3$	0/1	0	0
	10^3	0/0	0	0
	10^2	0/0	0	0
	10^1	0/0	0	0
7	$3x10^3$	45/60	3	16.6
	10^3	9/55	2	36.6
	10^2	5/44	0	0
	10^1	1/23	0	0

The reactivity of supernatants from the scattergun experiments are shown in Table 6. These give a value of 1-2 reactive cells per 10^6 E- cells, a percentage value of 0.0001-0.0002. This 10-100 fold difference in the numbers of positives demonstrates the enrichment produced by the panning procedure not only with regard to the numbers of B-cells present but also in the selection of those which recognise merozoite antigens. However, because of the larger numbers of monocytes present in this scattergun population, these numbers will be lower than expected and so again cannot be regarded as an absolute figure.

3.6.2.4 Merozoite neutralisation assay

This was carried out with only two of the supernatants which appeared to be specific for anti-merozoite antibodies. The reason for this was that the majority of the clones ceased to produce antibody before volumes of supernatant large enough for this assay could be collected. Table 7 shows the results obtained using "J" strain merozoites from human culture. Supernatants 40 and 42 had been positive for anti-merozoite activity in the ELISAs and originated from donor number 5 from wells seeded with $3x10^3$ panned B-cells. Number 35 was taken from a well containing clones from the same donor but not producing antibody, as demonstrated by ELISA, against any of the antigens tested. The control was 10% FCS as used in the maintenance of the B-cell cultures. Supernatant 40 produced a significant reduction in merozoite invasion when compared to the FCS control ($p= 0.05$) but in comparison to the control supernatant (35) the difference was not significant (Mann-Whitney U-Test). The other supernatant tested, number 42, did not produce significant inhibition.

3.7 MACROPHAGE ASSAYS

3.7.1. Release of reactive oxygen intermediates (ROI) by human blood monocytes

The purity of the adherent population with regard to contaminating lymphocytes was assessed by estimating the percentage of cells containing the black formazan deposit in the PMA positive control. These were viewed using an inverted microscope before the solubilisation procedure. From counts of 250 cells for 8 normal donors, an average of 91.7% ± 1.4 cells contained this deposit. The adherent population from the Irish donor was found to contain only 70% of cells which were activated by PMA in this way.

TABLE 6

ELISAS USING SUPERNATANTS FROM WELLS CONTAINING CLONES FROM SCATTERGUN POPULATION

DONOR	NUMBER OF CELLS/WELL	NUMBER BINDING TO ALL ANTIGENS	NUMBER BINDING ONLY TO MEROZOITES	NUMBER OF POSITIVES /10^6 CELLS
1	10^5	4/10	2	2
	10^4	1/20	0	0
	10^3	0/10	0	0
2	10^5	2/10	1	1
	10^4	0/16	0	0
	10^3	0/3	0	0
3	10^5	4/10	1	1
	10^4	1/20	0	0
	10^3	0/15	0	0
4	10^5	4/9	1	1.1
	10^4	2/15	0	0
	10^3	0/2	0	0
5	10^5	3/9	1	1.1
	10^4	0/5	0	0
	10^3	0/2	0	0
6	10^5	2/5	1	2.0
	10^4	0/1	0	0
	10^3	0/0	0	0

TABLE 7

MEROZOITE NEUTRALISATION ASSAY USING B-CELL SUPERNATANTS

SUPERNATANT	*PARASITAEMIAS (%)	MEAN PARASITAEMIAS ± SE
40	2.5, 2.5, 2.6	2.53 ± 0.05
42	2.8, 2.6, 4.8	3.40 ± 0.99
35	2.2, 4.4, 5.0	3.86 ± 1.20
10% FCS	2.9, 3.5, 4.9	3.76 ± 0.84

*Parasitaemias were counted after 40 hours

Assessment of the amount of NBT reduced by the adherent cells was carried out when the formazan had been solubilised, by optical density readings at 630nm. A calibration of this ELISA reading with the number of nmoles of NBT reduced/well is shown in appendix figure 2.

The reduction of NBT by human monocytes from unexposed donors and from the Irish patient after activation with PMA or "J" strain merozoites grown in human blood is shown in table 8. Donors 1-4 correspond with the donors numbered 1-4 in the B-cell assays and results are shown also for monocytes from the donor previously exposed to *P. falciparum*. The level of activation seen in the control wells which contained no PMA or merozoites differs widely from person to person. Therefore the reactivity to the merozoites for each donor cannot be directly compared. A non-parametric statistical test (Mann-Whitney) was used to compare absorbance between sets of wells (Siegel, 1956). In 6 out of the 10 normal donors tested (donors 5-10) and with the monocytes from the donors previously exposed to *P. falciparum* (donor 11) and *B. divergens* (Irish monocytes), the addition of merozoites to the monocytes caused a significant increase in NBT reduction, an indication that the parasites are promoting oxygen-dependent oxidase activity in the monocytes (Rook *et al*, 1985). In one case (donor 4), the level of NBT reduction seen in the control wells was significantly above that reached when merozoites were added, possibly indicating the existence of some form of suppression.

Once serum from the Irish patient became available, further experiments were carried out to assay the effects of opsonisation upon NBT reduction by "J" strain merozoites. Table 9 shows the effects on NBT reduction by monocytes when merozoites were incubated with normal or immune sera prior to addition to the monocyte cultures in comparison to merozoites not so incubated. For all but donor 1, there is a significant difference in NBT reduction in monocytes exposed to merozoites alone and to merozoites which had been preincubated with normal serum. This suggests that there is enhancement of the oxidative burst as a result of the binding of serum components to the merozoites. However, the wells in which monocytes were incubated with normal serum alone, also exhibited levels of NBT reduction well above that of the controls in some cases, so no firm conclusions can be drawn from these results.

All donors tested showed significant increases in NBT reduction by monocytes after their exposure to merozoites which had been preincubated with the Irish serum when compared to that given by monocytes exposed to

TABLE 8

NBT REDUCTION BY NORMAL HUMAN BLOOD MONOCYTES FROM 11 VOLUNTEERS

DONOR (n=number replicates)	OD_{630} (x ± SE) MEDIUM CONTROL	PMA ALONE	MEROZOITES ALONE
1 (n=5)	25.2±8.0	86.4±24.1	14.2±5.4
2 (n=4)	162.7±43.7	339.0±59.1	153.5±10.5
3 (n=5)	15.4±12.2	132.2±48.0	29.8±20.2
4 (n=4)	106.0±9.8	214.7±47.0	73.5±8.0 [1]
5 (n=8)	58.6±6.7	93.2±11.2	67.1±6.9 *(p≤0.007)
6 (n=8)	150.7±19.9	298.6±38.0	188.0±12.5 *(p≤0.007)
7 (n=4)	80.2±3.6	120.7±11.5	98.5±10.7 *(p≤0.014)
8 (n=4)	84.0±4.3	123.2±11.3	98.8±8.1 *(p≤0.014)
9 (n=4)	60.6±16.4	142.2±21.4	103.2±17.3 *(p≤0.018)
10 (n=3)	50.3±3.3	166.3±11.2	66.0+4.5 *(p≤0.018)
11 (n=3)	42.3±3.0	131.3±11.2	61.3±3.4 *(p≤0.05)
IRISH PATIENT (n=6)	2.8±2.1	14.8±2.3	10.5±2.8 *(p≤0.001)

* denotes absorbance levels significantly above those of the controls (Mann-Whitney U test)

[1] this value was significantly below that for the control (p≤0.014)

TABLE 9

EFFECTS OF PREINCUBATION OF "J" STRAIN MEROZOITES WITH SERUM ON REDUCTION OF NBT BY HUMAN MONOCYTES

DONOR	CONTROL	MEROZOITES	MEROZOITES + NORMAL SERUM	MEROZOITES + IRISH SERUM	NORMAL SERUM	IRISH SERUM
1	25.2 ±8.0	14.2 ±5.4	22.7 ±5.8	64.6 [4] ±26.0	13.5 ±6.1	33.7 ±7.3
3	15.4 ±12.2	29.8 ±20.2	71.2 [4] ±9.5	81.8 [4] ±21.7	55.0 ±20.0	40.7 ±20.1
5	58.6 ±6.7	67.1 ±6.9	95.6 [1] ±2.1	ND	29.4 ±8.3	ND
6	150.7 ±19.9	188.0 ±12.5	220.7 [2] ±14.2	ND	166.3 ±18.6	ND
9	60.6 ±16.4	103.2 ±17.3	174.5 [3] ±19.3	164.7 [3] ±11.7	114.0 ±23.2	133.0 ±20.0
10	50.3 ±3.3	66.0 ±4.5	90.6 [5] ±5.2	127.3 [5] ±16.0	129.3 ±5.5	105.3 ±7.3
11	42.3 ±3.0	61.3 ±3.4	80.5 [3] ±6.3	ND	46.9 ±12.1	ND
IRISH PATIENT	2.8 ±2.1	10.5 ±2.8	15.8 [6] ±4.5	16.0 [7] ±3.6	8.3 ±1.2	2.8 ±2.1

Levels of significance with respect to merozoites alone :-
[1] $p \leq 0.003$, [2] $p \leq 0.041$, [3] $p \leq 0.014$, [4] $p \leq 0.004$, [5] $p \leq 0.05$, [6] $p \leq 0.047$, [7] $p < 0.021$.

merozoites alone. However, as with the normal serum, Irish serum alone produced some activation and so the increase in monocyte activity as a result of merozoite opsonisation cannot be confirmed. Two out of the four normal monocyte populations tested using the Irish serum (1 and 10) showed significantly increased activity ($p=0.004$ and $p=0.05$ respectively) when the merozoites were preincubated with this as opposed to normal serum. Monocytes from the Irish patient showed a significantly increased response to the merozoites when they had been preincubated with both normal and autologous serum. Since there was no difference in the monocyte responses following incubation with these two sera, it would suggest in this case that the enhancement was not due to the presence of specific IgG in the autologous serum.

To determine whether there are any differences between merozoites raised in human and bovine blood with regard to their ability to activate human monocytes, assays were carried out using these and their respective blood controls as stimuli for normal human monocyte populations. No significant differences were found between the human and bovine raised merozoites in activation of monocytes isolated from donors 1,3,9 and 10. (Table 10).

In order to assess the role of superoxide release in the reduction of NBT, superoxide dismutase (SOD) was added to half the wells in one experiment. Unfortunately the SOD had no significant effect upon the optical densities produced and proved too expensive to repeat so the actual contribution of the superoxide anion in the phagocyte reactions is not known.

3.7.2. *In vitro* killing by non-immune white blood cells (WBCs)

3.7.2.1 Human

Three populations of PBM were used to determine their relative cytotoxicity towards the "J" strain of *B. divergens* in human erythrocyte culture : (a) the total population (b) the adherent population of PBM and (c) the remaining population after the adherent cells had been removed. Table 11 shows the parasitaemias obtained using PBM from four normal donors. (These are not the same donors as those used in the NBT reduction experiments). All of the donors tested inhibited growth of the parasites at the lowest ratio of PBM : parasites (0.1 : 1), and a further 50% of donors (Nos. 1 and 4) produced significant inhibition at a ratio of 1:1 also. There was a notable increase in parasitaemias at the highest PBM numbers in all

TABLE 10

NBT REDUCTION BY NORMAL HUMAN BLOOD MONOCYTES TO "J" STRAIN MEROZOITES RAISED IN HUMAN AND BOVINE BLOOD CULTURES

STIMULUS	DONOR			
	1 (n=5)	3 (n=5)	9 (n=4)	10 (n=3)
Control	25.2±8.0	15.4±12.2	60.6±16.4	50.3±3.3
Merozoites from human culture	14.2±5.4	29.8 ±20.2	103.2±17.3	66.0±4.5
Merozoites from bovine culture	24.8±6.0	27.2±20.4	108.5±11.7	77.3±8.0
Human blood	6.0±1.0	16.2±2.0	57.0±12.2	39.3±14.3
Bovine blood	22.3±3.0	20.0±1	68.0±17.1	47.5±4.5

TABLE 11

THE EFFECT OF NORMAL PBM ON *B. divergens in vitro*

DONOR	PBM: PARA[1]	PBM /WELL (x10^6)	% PARASITAEMIA AT 72 HOURS[2] MEAN	RANGE	%GROWTH[3]	p[4]
1	-	0	4.25	4.1-4.9	100	-
	10:1	1.0	4.90	3.3-6.4	115	NS
	5:1	0.5	2.36	1.0-4.3	55	NS
	1:1	0.1	0.98	0.9-1.0	22	0.05
	0.1:1	0.01	1.80	0.6-2.7	42	0.05
2	-	0	10.10	8.8-11.0	100	-
	10:1	1.0	16.30	15.0-18.0	161	0.028
	5:1	0.5	12.73	11.9-14.0	126	0.028
	1:1	0.1	9.00	8.7-9.5	89	NS
	0.1:1	0.01	6.60	6.0-8.0	65	0.05
3	-	0	10.10	8.8-11.0	100	-
	10:1	1.0	13.23	12.1-15.0	131	0.028
	5:1	0.5	8.96	8.1-10.3	89	NS
	1:1	0.1	8.63	6.0-10.8	85	NS
	0.1:1	0.01	7.13	4.9-8.7	71	0.05
4	-	0	16.50	15.4-18.4	100	-
	10:1	1.0	21.46	20.0-23.1	130	0.028
	5:1	0.5	17.10	16.2-17.8	104	NS
	1:1	0.1	9.66	9.0-9.9	58	0.05
	0.1:1	0.01	5.60	4.6-6.3	34	0.05

[1] Ratio of PBM: Parasites at 0 hours

[2] Minimum of 3 wells per test.

[3] % Growth : mean % parasitaemia in wells expressed as a percentage of the controls (without PBM).

[4] p value from %P in individual wells using Mann-Whitney U Test.

cases which suggested that the PBM actually had a stimulatory effect upon parasite growth at these concentrations.

The effects of PBM with the adherent population removed (PBM-MØ) and the adherent population alone (MØ) upon parasitaemias can be seen on Tables 12 and 13 respectively. The PBM-MØ populations all had a marked stimulatory effect upon parasite growth at the higher cell numbers and the presence of these cells were only inhibitory at the lowest concentration tested (10^3 cells per well) and then only in two of the four cases. This growth stimulation is more marked than for the PBM alone and can be seen in figure 34. The adherent cells clearly have an inhibitory effect upon parasite growth at the higher cell numbers since when these are removed from the PBM population the effect of the cells becomes stimulatory. In wells where parasitaemias were considerably reduced it was usually possible to see a high percentage of abnormal ("crisis") parasites in the Giemsa stained smears used to assess the parasitaemias.

3.7.2.2. Gerbil

Table 14. shows the results obtained using pooled PBM from 10 non-immune gerbils. The effects upon parasite growth are quite distinct from those found using human PBM with none of the populations causing significant inhibition at any cell concentration. All populations were stimulatory to growth, particularly the adherent cells (Fig. 35). 7.2% percent of the total gerbil PBM adhered to the gelatin coated flasks in comparison to the 2-6% found for human cell adherence which could offer one source for variation in the results.

3.7.3 Assay of *in vitro* killing by non-immune activated monocytes

3.7.3.1 Human

Table 15 shows that with the non-activated adherent cells, only one out of the four donors (No.2) produced a significant reduction in parasitaemias at the 1 : 1 cell:parasite ratio used, a similar result to that shown on Table 13. Activation of the adherent cells with LPS prior to the addition of the parasite culture caused a significant reduction of parasite growth in only one of the four donors tested (No.2) when compared to non-activated controls. However, there was some degree of enhancement in parasite killing in all cases as a result of this activation.

3.7.3.2. Gerbil

Prior treatment of the cells with LPS had a marked effect upon the monocyte activity (Table 15). Where previously these adherent cells had had

TABLE 12

THE EFFECT OF NORMAL PBM-MØ ON *B. divergens in vitro*

DONOR	PBM-MØ : PARA [1]	PBM-MØ /WELL (x10^6)	% PARASITAEMIA AT 72 HOURS[2] MEAN	RANGE	% GROWTH [3]	p [4]
1	-	0	4.25	4.1-4.9	100	-
	10:1	1.0	14.80	12.9-15.9	385	0.028
	5:1	0.5	19.30	17.7-20.2	454	0.028
	1:1	0.1	10.13	9.4-10.7	288	0.028
	0.1: 1	0.01	3.20	3.0-3.4	75	0.05
2	-	0	10.10	8.8-11.0	100	-
	10:1	1.0	15.36	14.7-16.2	152	0.028
	5:1	0.5	13.36	11.9-15.0	132	0.028
	1:1	0.1	9.56	8.9-10.0	95	NS
	0.1:1	0.01	7.43	6.6-8.6	74	0.05
3	-	0	10.10	8.8-11.0	100	-
	10:1	1.0	17.20	16.2-18.4	170	0.028
	5:1	0.5	13.20	12.9-13.6	131	0.028
	1:1	0.1	12.06	11.3-12.7	119	0.028
	0.1:1	0.01	8.16	7.7-8.8	81	NS
4	-	0	16.50	15.4-18.4	100	-
	10:1	1.0	50.00	49.0-51.1	299	0.028
	5:1	0.5	31.30	25.9-34.1	187	0.028
	1:1	0.1	20.30	19.6-21.2	121	0.028
	0.1:1	0.01	17.26	16.0-18.2	102	NS

[1] Ratio of PBM-MØ: Parasites at 0 hours.

[2] Minimum of 3 wells per test.

[3] % Growth : mean % parasitaemia in wells expressed as a percentage of the controls (without PBM-MØ).

[4] p value from %P in individual wells using Mann-Whitney U Test.

TABLE 13

THE EFFECT OF NORMAL MØ ON *B. divergens in vitro*

DONOR	MØ:PARA[1]	MØ /WELL (x10^6)	% PARASITAEMIA AT 72 HOURS[2] MEAN	RANGE	%GROWTH[3]	p[4]
1	-	0	4.25	4.1-4.9	100	-
	10:1	1.0	2.06	1.9-2.6	48	0.05
	5:1	0.5	2.56	2.0-3.4	60	0.05
	1:1	0.1	5.20	4.7-5.9	122	NS
	0.1:1	0.01	11.60	10.0-12.5	273	0.028
2	-	0	10.10	8.8-11.0	100	-
	10:1	1.0	1.16	0.9-1.4	11	0.05
	5:1	0.5	3.13	2.8-3.5	31	0.05
	1:1	0.1	7.20	4.9-8.0	71	0.05
	0.1:1	0.01	8.26	8.1-8.5	82	0.05
3	-	0	10.10	8.8-11.0	100	-
	10:1	1.0	0.60	0.5-0.8	6	0.05
	5:1	0.5	3.33	2.7-4.1	33	0.05
	1:1	0.1	7.16	5.0-9.6	71	NS
	0.1:1	0.01	7.76	7.2-8.1	77	NS
4	-	0	16.5	15.4-18.4	100	-
	10:1	1.0	11.76	10.0-14.4	71	0.05
	5:1	0.5	15.90	14.7-17.0	96	NS
	1:1	0.1	21.63	20.2-24.3	129	0.028
	0.1:1	0.01	33.76	32.0-34.8	204	0.028

[1] Ratio of MØ:Parasites at 0 hours.

[2] Minimum of 3 wells per test.

[3] % Growth : mean % parasitaemia in wells expressed as a percentage of the controls (without MØ).

[4] p value from %P in individual wells using Mann-Whitney U Test.

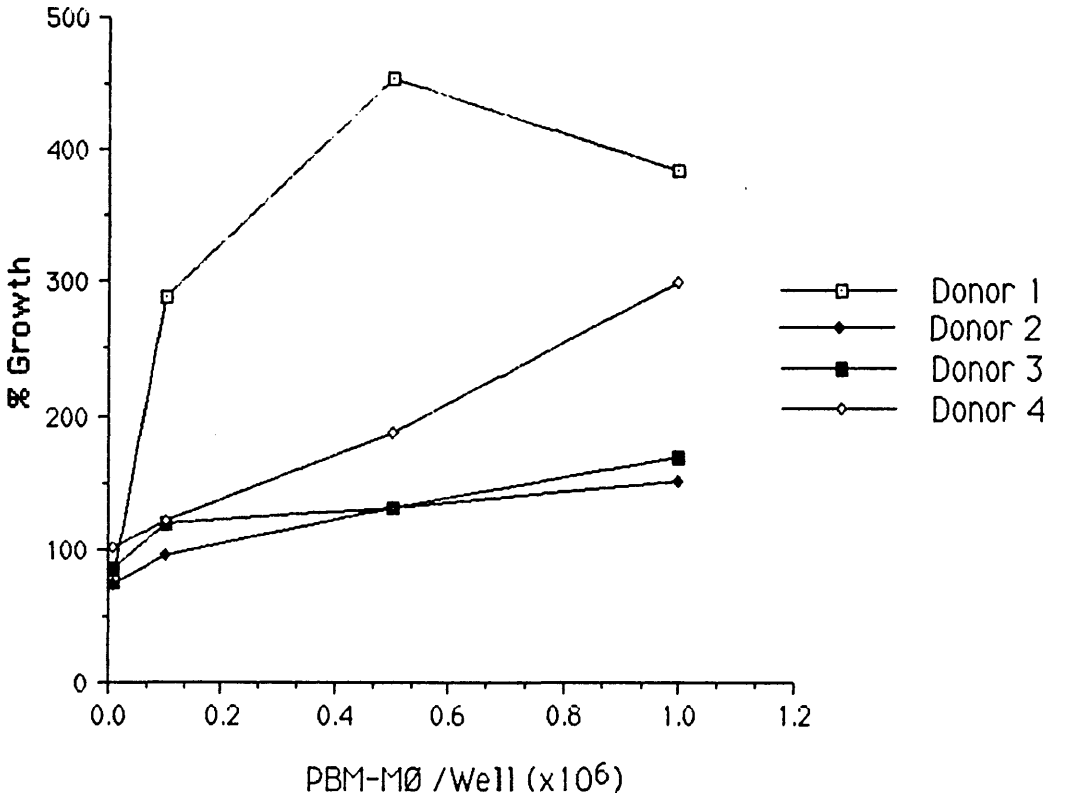

Figure 34. *In vitro* growth of the "J" strain of *B. divergens* in human erythrocytes in the presence of non-immune PBM-MØ populations. (% Growth compared with controls)

TABLE 14

THE EFFECT OF NON-IMMUNE GERBIL PBM, PBM-MØ AND MØ POPULATIONS ON *B. divergens in vitro*

CELL TYPE	WBC:PARA[1]	WBC/WELL (x10^6)	% PARASITAEMIA AT 72 HOURS[2] MEAN	RANGE	% GROWTH[3]	p[4]
NONE	-	0	16.50	14.6-18.4	100	-
PBM	10:1	1.0	29.33	28.0-31.2	177	0.028
	5:1	0.5	25.00	24.9-25.1	151	0.028
	1:1	0.1	19.93	18.4-21.8	121	NS
	0.1:1	0.01	16.63	15.8-17.8	101	NS
PBM-MØ	10:1	1.0	26.30	25.0-30.0	159	0.028
	5:1	0.5	23.20	22.1-24.6	138	0.028
	1:1	0.1	17.40	16.4-18.0	105	NS
	0.1:1	0.01	16.00	14.7-17.0	97	NS
MØ	10:1	1.0	27.83	25.3-31.0	169	0.028
	5:1	0.5	34.43	33.1-34.0	203	0.028
	1:1	0.1	22.66	17.6-26.0	137	NS
	0.1:1	0.01	16.83	16.4-17.1	102	NS

[1] Ratio of WBC population:Parasites at 0 hours.

[2] Minimum of 3 wells per test.

[3] % Growth : mean % parasitaemia in individual wells expressed as a percentage of the controls (without WBC).

[4] p value from %P in individual wells using Mann-Whitney U Test.

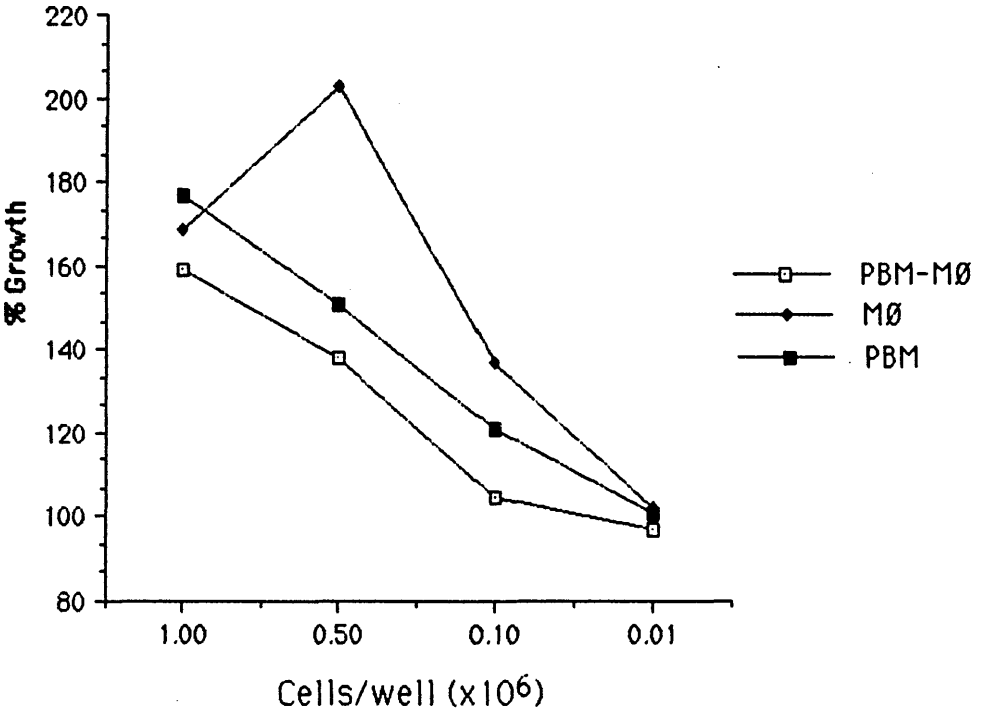

Figure 35 *In vitro* growth of the "J" strain of *B. divergens* in human erythrocytes in the presence of non-immune gerbil PBM populations. (%Growth compared with controls)

TABLE 15

THE EFFECT OF NON-IMMUNE ACTIVATED ADHERENT CELLS ON THE "J" STRAIN OF *B. divergens in vitro* (Cell : parasite ratio = 1 : 1)

DONOR	ADHERENT CELLS[1]	%PARASITAEMIA AT 48 HOURS[2] MEAN	RANGE	%GROWTH[3]	p[4]
1	-	3.70	3.2-5.1	100	-
	NON-ACTIVATED	4.87	4.8-5.3	132	NS
	LPS-ACTIVATED	4.57	3.9-5.1	123	NS
2	-	8.25	8.3-9.2	100	-
	NON-ACTIVATED	7.83	7.6-8.0	95	0.05
	* LPS-ACTIVATED	4.76	3.9-5.6	58	0.05
3	-	8.25	8.3-9.2	100	-
	NON-ACTIVATED	7.16	6.0-8.3	87	NS
	LPS-ACTIVATED	6.20	5.9-6.6	75	0.05
4	-	19.00	17.9-21.0	100	-
	NON-ACTIVATED	22.73	21.4-24.8	119	0.028
	LPS-ACTIVATED	21.57	20.0-23.2	113	0.032
GERBIL	-	19.00	17.9-21.0	100	-
	NON-ACTIVATED	21.15	19.8-22.0	111	0.016
	* LPS-ACTIVATED	13.07	12.5-14.0	69	0.014

[1] Adherent cells were incubated overnight with LPS or medium prior to addition of *B. divergens* culture.

[2] Minimum of 3 wells per test.

[3] % Growth : mean % parasitaemia in wells expressed as a percentage of the controls (without adherent cells).

[4] p value from Mann-Whitney U Test comparing % parasitaemias in wells containing adherent cells to those in control wells.

* These adherent cells caused a significant reduction in %P when compared to non-activated controls (p=0.05 for donor 2 and p=0.014 for the gerbils)

a stimulatory effect upon the parasites at this cell:parasite ratio (Tables 14 and 15), the LPS activated population produced a significant inhibition of parasite growth. These cells were also tested for their reactivity against the "J" strain of *B. divergens* in bovine culture. Here they produced a 50% reduction in parasite growth (p=0.014) but the results were not significantly different from those found for the human cultures and are not shown.

3.7.4. Effect of supernatants from activated adherent cells on *B. divergens*

3.7.4.1. Human

Supernatants taken from wells containing adherent cells which were not stimulated with LPS proved inhibitory for parasite growth in only two of the four cases and then only when used undiluted (Table 16). One notable feature, however, is the absence of any significant increase in parasitaemias as a result of the addition of adherent cell supernatants. This is in contrast to the stimulation of parasite growth found after the addition of low numbers of these cells (see Table 13).

Supernatants taken from cells which had been activated with LPS prior to addition to the cultures caused greater retardation of parasite growth, with all supernatants proving inhibitory at one or more dilutions. In all cases this effect was lost when the supernatants were diluted by more than 1 in 2. When the effects of LPS activation are compared to the results obtained using supernatants from non-activated cells, this increase in inhibition is less notable. Only in 3 cases is this inhibition significant with regard to these non-activated controls.

3.7.4.2.Gerbil

Supernatants taken from non-activated gerbil adherent cells did not prove inhibitory to parasite growth at any concentration used (Table 16). However, as in the experiments with human cells , the supernatants did not cause any significant stimulation of parasite growth as seen on Table 14, again suggesting that it is the physical presence of the cells which is required for this phenomenon.

Those supernatants taken from cells which had been pre-treated with LPS were inhibitory to parasite growth at all three dilutions tested. When compared to the supernatants from non-activated cells, this inhibition remains significant in all three cases.

TABLE 16

THE EFFECT OF SUPERNATANTS FROM NON-IMMUNE ADHERENT CELLS ON THE "J" STRAIN OF *B. divergens in vitro*

DONOR	SUPERNATANT[1]	% PARASITAEMIA AT 48 HOURS[2] MEAN	RANGE	% GROWTH[3]	p[4]
1	CONTROL	5.40	4.9-6.1	100	-
	NON-ACTIVATED (100%)	4.93	4.8-5.0	91	NS
	(50%)	5.23	4.9-5.5	97	NS
	(10%)	5.93	5.8-6.1	110	NS
	LPS-ACTIVATED (100%)	4.16	3.2-4.8	77	0.05*
	(50%)	4.66	4.4-5.0	86	0.05
	(10%)	5.80	5.2-6.2	107	NS
2	CONTROL	12.83	12.0-13.6	100	-
	NON-ACTIVATED (100%)	9.16	7.9-11.1	72	0.05
	(50%)	13.46	12.0-15.0	105	NS
	(10%)	12.93	11.1-14.8	101	NS
	LPS-ACTIVATED (100%)	9.86	8.0-11.4	77	0.05
	(50%)	8.96	6.9-10.2	70	0.05*
	(10%)	11.06	6.2-13.8	86	NS
3	CONTROL	12.83	12.0-13.6	100	-
	NON-ACTIVATED (100%)	9.66	8.8-10.2	75	0.05
	(50%)	10.80	7.4-13.0	84	NS
	(10%)	12.76	11.1-14.7	99	NS
	LPS-ACTIVATED (100%)	8.30	6.9-11.1	65	0.05
	(50%)	11.10	6.8-13.6	86	NS
	(10%)	13.00	10.8-15.4	101	NS

CONTINUED OVERLEAF

(TABLE 16 CONTINUED)

DONOR	SUPERNATANT[1]	%PARASITAEMIA AT 48 HOURS[2] MEAN	RANGE	%GROWTH[3]	p[4]
4	CONTROL	16.70	16.0-17.2	100	-
	NON-ACTIVATED (100%)	16.36	13.2-19.9	98	NS
	(50%)	16.63	15.8-17.2	100	NS
	(10%)	16.80	16.2-17.6	101	NS
	LPS-ACTIVATED (100%)	12.70	12.0-14.0	76	0.05
	(50%)	14.06	12.3-14.0	84	0.05*
	(10%)	15.23	8.9-18.8	91	NS
GERBIL	CONTROL	16.70	16.0-17.2	100	-
	NON-ACTIVATED (100%)	16.20	14.8-18.6	97	NS
	(50%)	18.23	16.3-20.0	109	NS
	(10%)	17.16	15.5-18.0	103	NS
	LPS-ACTIVATED (100%)	12.80	11.8-13.0	77	0.05*
	(50%)	13.80	13.0-16.0	83	0.05*
	(10%)	13.93	14.4-15.5	89	0.05*

[1] Pooled supernatants taken from wells containing $2x10^5$ adherent cells after 24 hours in culture with LPS or medium alone. These were used neat, at 1 in 2 and 1 in 10, being diluted in normal medium before addition to the cultures.

[2] Minimum of 3 wells per test.

[3] % Growth : mean % parasitaemia in wells expressed as a percentage of the controls (ie. wells which received normal culture medium and not supernatants).

[4] p value from Mann-Whitney U Test comparing % parasitaemias in wells receiving supernatants to those in control wells.

* These supernatants caused a significant reduction in % parasitaemia when compared to the corresponding dilutions of those from non-activated cells.

3.7.5. Assays for TNF release

Adherent cells from 6 unexposed human donors were tested for their ability to produce TNF in response to merozoites isolated from human and bovine cultures and also in response to parasite enriched erythrocyte lysis preparations (PELP) from cultures (see 2.2.2 materials and methods). None of these preparations produced TNF levels above those found for the medium only controls. Since all groups of "stimulants" to be tested were made up in medium containing Polymyxin B, levels of TNF release from the macrophages in response to these antigens must be compared with the medium control containing polymyxin B. This was carried out to prevent TNF release by any endotoxin present, which can be seen to have occurred from comparisons of the TNF levels found in the medium controls with and without Polymyxin B.

Peritoneal macrophages from gerbils were tested for their TNF response to the above antigens and also to supernatants from infected gerbil erythrocytes and the same supernatants following treatment with papain. Macrophages were pooled from 3-4 gerbils for each of 5 experiments. The response to the LPS controls was not consistent, as can be seen on Table 17, although all gerbils tested received thioglycollate broth IP three days prior to sacrifice to activate the macrophages and increase the yield. The macrophages did not produce a TNF response, above that found for the associated controls, to any of the antigens tested. Two different preparations of supernatants were tried and papain treatment had no effect. Merozoites collected from human blood cultures had no stimulatory effect with regard to TNF release and neither did those collected from bovine cultures (results not shown).

TABLE 17

ASSAYS OF TNF RELEASE FROM GERBIL PERITONEAL MACROPHAGES

STIMULUS		TNF units/ml EXPT.1	EXPT.2	EXPT.3	EXPT.4	EXPT. 5
CONTROL		133	368	400	226	160
CONTROL + POLY'M[1]		100	35	131	120	ND
LPS 1µg/ml		34048	9051	919	ND	320
0.1µg/ml		34048	9051	800	844	905
0.01µg/ml		17024	4525	800	640	183
INF.SUP.[2]	1/5	<50	35	141*	ND	ND
	1/20	<50	29	400*	ND	ND
	1/80	50	29	283*	ND	ND
CONTROL SUP.	1/5	50	29	230*	ND	ND
MEROZOITES	10/MØ	ND	ND	348	<10	<40
	1/MØ	ND	ND	151	69	56
	0.1/MØ	ND	ND	151	92	183
BLOOD CONTROL		ND	ND	131	121	<40

[1] Polymyxin B was added to all wells except those receiving LPS and the associated controls.

[2] Supernatants collected from gerbil blood infected with *B. divergens*. Two different batches were tried in experiments 1 and 2.

*Both the infected and control supernatants tested in experiment 3 were those used in experiment 1 but treated with papain prior to use.

4. DISCUSSION

4.1 MICROAEROPHILOUS STATIONARY PHASE (MASP) CULTURES

The ability of the Weybridge and "J" strains of *B. divergens* isolated from bovine and human infections respectively to adapt to both types of host erythrocytes in MASP cultures has been demonstrated. The serum requirements for both strain were less rigid in human erythrocytes and parasitaemias of twice the magnitude of those found in bovine MASP cultures were reached. With multiple invasions of erythrocytes commonly occurring, the high incidence of maltese-cross tetrads, as seen for *B. microti*, along with the increased size of the parasite, the morphology of *B. divergens* in human erythrocytes is quite distinct from that seen in the bovine host. In addition, the parasites are seen much less frequently in the peripherally divergent form from which they received their name. This is in accordance with previous reports on alterations in typical parasite morphology occurring in abnormal host erythrocytes (Canning *et al*, 1976). *P. relictum*, a species of avian malaria, has also been shown to change its character in abnormal hosts. Its morphology is quite distinct even in two of its natural hosts, silver gulls and sparrows (Garnham, 1966) and this infact led to a separate specific status being given to the same parasite in different hosts. In human culture the parasite forms closely resemble those seen in jird (*Meriones unguiculatus*) erythrocytes, where single pyriforms are rare and there is an increased frequency of budding forms and of round and double round forms (Gray *et al*, 1985). The latter authors suggested that the increased prevalence of these forms in abnormal hosts may be related to the more rapid replication rate seen in these erythrocytes, with single pyriforms converting to round forms more promptly in such infections. With as many as twelve parasites developing in one red cell, the parasite morphology and resultant increased rate of proliferation resembles that seen for human malarial infections and goes some way to explaining the misdiagnosis of many human *Babesia* infections as malaria.

Three attempts were made to establish *B. bovis* in human culture by the direct addition of merozoites to uninfected erythrocytes and also by the gradual addition of human erythrocytes to bovine cultures of *B. bovis*. Although a few invasions were observed these parasites did not survive during further culture, suggesting that the internal RBC environment may be

of importance for *B. bovis* development. This repeated failure to produce an infection somewhat counters claims that several of the European cases of babesiosis were caused by *B. bovis* (Skrabalo and Deanovic, 1957, Calvo de Mora *et al*, 1989). Although this obviously cannot be seen as an absolute test of its *in vivo* infectivity for humans, in light of the ease with which the two strains of *B. divergens* adapted to and multiplied in human erythrocytes it would seem more likely that the infections were due to this species rather than *B. bovis*.

Since *B. divergens* infections have been shown to develop only with difficulty in mice (Adam and Blewett, 1974), these rodents would appear to possess an innate resistance to clinical infections. In order to ascertain if this resistance is due to serum or erythrocyte features, an attempt was made to infect mouse erythrocytes *in vitro* with "J" strain merozoites. As in the case of *B. bovis*, some invasions were noted but these progressed no further, suggesting an ability to invade the erythrocytes but perhaps an unsuitable intracellular environment for further development. These invasions were only seen when heat inactivated human serum was used in the culture, with no surviving parasites being observed in those wells which received autologous mouse serum. These results are consistent with the finding that normal mouse serum is toxic to *P. falciparum* at levels over 5% unless dialysed (G. Butcher, personal communication). This contrasts with the results of studies on the host specificity of *P. knowlesi* by Butcher *et al*, (1973) which suggested that the host specificity of the parasite was not related to the serum but to the erythrocyte species and lack of receptors for entry and/or an unsuitable intracellular environment. Miller *et al*, (1973) then demonstrated the importance of erythrocyte membrane components to the host range of *P. knowlesi* through studies on the effects of enzymatic treatment of human and rhesus erythrocytes. Although these authors absorbed the serum to be tested with the appropriate erythrocytes before addition to the cultures, the use of autologous serum in the experiments with *B. divergens* should abolish the need for this and so the results found implicate both serum and erythrocyte factors in the resistance of mice to infection with *B. divergens*.

Enhancement of the *in vitro* growth of *P. falciparum* upon addition of mononuclear cells was noted by Butcher and Clancy (1984), particularly where the numbers of monocytes had been reduced. This was also found to be the case in human *B. divergens* cultures using both human and gerbil

mononuclear cells. The addition of human or mouse feeder cells to MASP cultures of *P.falciparum* has been used as a mechanism for adapting poorly growing strains to culture (Phillips *et al*, 1987; Mirovsky *et al*, 1990) and increasing multiplication rates (Trenholme and Phillips, 1989). Several reasons for these effects upon parasite growth are postulated, including the reduction of oxygen levels in the culture by the presence of the mononuclear cells. The *Babesia* cultures containing the mononuclear cells were grown in 5% CO_2 in air, the same conditions as normal cultures, and in view of the observed increase in growth which occurs in human and bovine MASP cultures from the use of a gas mix containing only 3% O_2, a reduction in oxygen tension from cellular activity seems one possible explanation for the observed effect. The failure of the mononuclear cell supernatants to reproduce this improved growth suggested that it was the physical presence of the white cells which was required and not secreted factors. However, Trenholme *et al*, (1990) have been able to increase the multiplication rate of *P. falciparum* by the addition of L-cysteine to the culture medium every 12 hours. This amino acid is extremely labile and is destroyed by freezing. The supernatants used in the assays with *B. divergens* had been frozen prior to use and could therefore have failed to stimulate growth for this reason. Interestingly, cysteine is known to act as an antioxidant and so it is possible that it is in this capacity that the cysteine works to enhance growth of cultures, suggesting again that a reduction in oxygen tension could be responsible for the increased growth produced.

4.2 MEROZOITE SERUM PROTEIN ADHERENCE

The adherence of human IgG from the culture medium to merozoites occurred to such a degree that the use of ELISAs to test for reactivity against *Babesia* antigens in human sera could not be carried out unless the merozoites had been isolated from cultures supplemented with HI bovine serum. This binding was seen from the immunoblots to be only slightly reduced by extensive washing and so is unlikely to be due merely to free IgG present in the merozoite preparation. Further immunoblots carried out on merozoite preparations showed that there is no IgG present in normal human serum which is specific for merozoite antigens. Although the IgG attachment could be to a three dimensional determinant which is removed upon reduction of the merozoites it must be considered immunologically non-specific as there is negligible binding of IgG from normal human serum

to human isolates raised in HI bovine serum. IgG is known to bind non-specifically to erythrocyte antigens (Alderman *et al*, 1980), however, the large reduction in background achieved by using HI bovine serum in the cultures suggests that this is not due to inherent anti-red cell activity. While there appeared to be little binding of IgM to the merozoites in this way, this could simply reflect the relative amounts of each antibody present in human serum with IgG comprising 70-75% of the total Ig production compared to only 10% for IgM.

The merozoite stage in the life-cycle exists outside the host erythrocytes for a short period of time and consequently is fully exposed to direct immune attack. It has been suggested that the parasites' affinity for host proteins may be exploited as a means of avoiding immune mechanisms. This has been especially noted for serum albumin (Ristic and Kakoma, 1988) which they proposed could mask important parasite antigens and result in a delayed recognition of protective antigens. This is consistent with the slow development of protective immunity found in bovine babesiosis. While the immunoblot results showed that binding of albumin to merozoites occurred when the merozoites were raised in human erythrocytes, this was reduced following the rigorous washing of the merozoites carried out in an attempt to remove IgG. Thus, the IgG clearly binds to the merozoites with a greater affinity than does albumen. Whether this could be due to the presence of Fc receptors on the merozoites or merely non-specific adherence can not be ascertained from these experiments. However, since the binding of human IgG was not observed in human cultures raised using HI bovine serum the former explanation seems unlikely.

Binding of IgG to merozoites is not limited to human cultures, but has also been observed in merozoites isolated from bovine MASP cultures (C. Winger, personal communication). Although this has only been observed *in vitro*, its occurrence in both the human and bovine cultures suggests it is not responsible for the difference in susceptibility found to *B. divergens*. The binding of non-specific antibodies would in theory act to retard the development of specific antibodies with consequent inhibitory effects upon affinity maturation, again a feature of cattle babesiosis. Binding of non-specific IgG to the surface of other parasites in contact with body fluids has been reported, including species of malaria (Ristic and Kakoma, 1988), schistosomes (McLaren and Terry, 1982), *Brugia pahangi* (Premaratne *et al*, 1989) and *Taenia pisiformis* (Craig, 1988). These non-specific

antibodies were seen bound to the parasites even where high affinity specific IgG antibodies were present and it was postulated by Premartne *et al* that they are used as a means of avoiding destruction by complement fixation. Therefore, although it cannot be definitely ruled out that the binding of non-specific IgG to merozoites is an artefact of the isolation procedure, this binding has also been noted for several different parasite-host systems, including human infections with *P. falciparum* and bovine infections with *B. bovis* and *B. bigemina* (Ristic and Kakoma, 1988), where it is thought to be beneficial to the parasite through immune response evasion.

4.3 ANTIGENIC PROFILES OF MEROZOITES FROM HUMAN AND BOVINE CULTURES

Since the monoclonal antibody 13.4 was initially raised against Weybridge strain merozoites isolated from bovine MASP cultures, the finding that it did not bind to "J" strain merozoites from human blood posed the question whether this was a strain related feature or a result of the different species of host erythrocytes. The ELISA, IFAT and immunoblot results all point to this being a host related difference rather than based on the strain of *B. divergens* used. Coupled with the finding that Mab 13.4 will bind to *B. bovis* merozoites from bovine cultures (C. Winger, personal communication) the evidence indicates an alteration or decrease in the numbers of these antigens as a result of the host erythrocyte.

The extent of these antigenic differences would appear to be limited to those antigens recognised by Mab 13.4 at 26kD and around 50-60kD which have both been shown to be involved in erythrocyte entry (Winger *et al*, 1987a). Binding of the serum from the Irish patient to protein profiles of "J" strain merozoites raised in both human and bovine erythrocytes showed that there were differences in recognition at these molecular weights. At 55kD a different pattern of binding was seen and at 26kD the band is much lighter in the track containing merozoites from human cultures. The Irish patient, however, cannot have been exposed to any of the parasite antigens which are present only on the merozoites from bovine cultures. Thus the increased binding seen at 26kD to merozoites isolated from bovine cultures is more likely to reflect a difference in amount of antigen present or non-specific binding rather than an alteration in molecular structure as it is unlikely that IgG which binds with a greater affinity to slightly different antigens than those occurring on the infecting parasites would be present in

larger amounts than that specific for antigens from the infecting species. All groups of merozoites were isolated and prepared for SDS-PAGE in precisely the same way, but a difference in the amount of parasite antigen present in each track would have produced these results. The merozoites were all isolated from cultures supplemented with heat inactivated bovine serum and this produces a small amount of red cell lysis in those grown in human erythrocytes in addition to that caused by the release of parasites. Consequently, a given volume of merozoite preparation may have contained a greater proportion of red cell membranes than those parasites raised in bovine erythrocytes, and therefore less parasite material. The decreased binding to the 26kD antigen in the track containing merozoites isolated from human cultures could therefore be a result of the culture system used. With Mab 13.4, it was found that a small amount of binding occurred to a merozoite antigen at 26kD when these were isolated from human cultures. Again this could be as a result of differing amounts of antigen present but in this case the human MASP cultures from which the merozoites were isolated were supplemented with human serum and not HI bovine serum. Therefore a decrease in parasite material from this source did not occur and so these differences in binding are more likely to be a parasite derived feature. Thus from the immunoblots with the Irish serum, in spite of differences in the amount of antigen present in the tracks, it would appear that some degree of cross-reactivity exists at this 26kD antigen in human and bovine merozoites.

The control monoclonal antibody MopC 21 exhibited a small amount of reactivity to some antigens in the tracks containing merozoites. This is a mouse IgG1 ascites, as is Mab 13.4, and was used at the same dilution in all experiments where it showed a degree of non-specific stickiness to these antigens. Such non-specific binding may have occurred with Mab 13.4 to the merozoites raised in human erythrocytes although any contribution of this to the results cannot be ascertained. In the merozoite neutralisation assay using "J" and Weybridge strains raised in human erythrocytes, limited inhibition of 19.5% and 24.2% respectively was produced with Mab 13.4. This is in contrast with the 76% inhibition achived using Mab 13.4 with the bovine system. Since the activity of Mab 13.4 was expressed as a percentage of that achieved using Mab 13.5 which recognises an internal antigen, these results show the binding and consequent inhibition of entry to be specific. Therefore it is unlikely that non-specific stickiness is a major factor in the binding to these antigens in either group of merozoites.

This finding could thus reflect a decreased expression of the antigens recognised by Mab 13.4 or merely a reduced affinity as a result of alteration.

From the IFATs carried out using the mouse anti-sera it was seen that binding to merozoites from both human and bovine cultures occurred whatever group of merozoites the anti-sera was raised against. In view of the fact that the merozoite surface coat is sloughed off during erythrocyte invasion (Rudzinska *et al*, 1976), the results from the IFATs performed on fixed smears of infected culture show that sufficient cross reactivity of internal merozoite antigens occurs to produce comparable fluorescence of intracellular forms in both human and bovine erythrocytes whatever the merozoite type used for immunisation. Thus, even with the morphological differences seen between the parasites in human and bovine cultures, antigenic alterations would appear to be limited to those antigens associated with erythrocyte entry. Notably, none of the *in vitro* assays carried out comparing human, bovine or gerbil immune responses to the two groups gave any indication of differences in immunogenicity. Therefore the significance of these antigenic differences with regard to the possible influence of host susceptibility would appear to be negligible.

Propagation of erythrocyte stages *in vitro* has been associated with alterations in various features of the parasites, such as virulence (Winger *et al*, 1989), infectivity for ticks, drug resistance and immunogenicity (Freidhoff, 1988). The virulence of the "J" strain for gerbils after 18 months in continuous culture was found to be comparable with that of non-attenuated Weybridge bovine cultures although there would appear to be a slight reduction in virulence when the parasitaemias produced are compared to those found with the initial isolate by Liddel *et al* (1980). These authors found that an inoculum of around 10^7 parasites resulted in a maximum febrile response in the gerbil at day 3, whereas the "J" strain from the MASP cultures reached this stage at day 5. However, Liddel *et al* used an inoculum derived from the 96th passage in gerbils and the previous infections in gerbils were initiated from infected bovine blood, so a direct comparison cannot be made. Thus, the relevance of this to any alteration in infectivity for humans and any bearing this may have on potential *in vitro* responses clearly cannot be ascertained. Similar results were reported by Gorenflot *et al*, (1991) where human MASP cultures of *B. divergens* were maintained for two years without any reduction in virulence. These authors suggest that this feature could be related to the homologous serum-

erythrocyte system utilised, since Yunker *et al*, (1987) found a reduction in virulence of *B. bovis* parasites using a heterologous system as did Winger *et al*, (1989) with long-term cultures of *B. divergens*. However, such a system was used throughout the duration of this project and so it seems unlikely that this could be responsible for the maintenance of virulence.

Due to the similarity of the disease produced with the "J" strain and a bovine (Drumnadrochit) strain in splenectomised calves (Lewis *et al*, 1980) these authors concluded that the human "J" strain was derived directly from this bovine strain. From comparisons of the size of both of these parasites in the calf blood, Lewis *et al* found those of human origin to be significantly larger than those from the bovine infection. Such an increase in parasite size in calf blood has been reported following passage in jirds (Gray *et al*, 1985). Why such changes should result from a single passage through an abnormal host is not known but clearly the size of the host erythrocytes cannot be the only factor in operation. Although the "J" strain parasites which had been introduced into bovine MASP cultures from human erythrocytes in this laboratory were not studied in this way, they tended to be more prolific than the Weybridge strain which had originally been isolated from a bovine infection and so strain related differences were noted. It has been suggested that higher rates of parasite replication are responsible for morphological differences seen (Gray *et al*, 1985) but whether in this case such a feature is related to the prior passage through abnormal host erythrocytes cannot be ascertained without reference to the original bovine (Drumnadrochit) isolate. However, it was observed by Pudney (1984) that early *in vitro* cultures of the "J" strain in bovine erythrocytes were characterized by unusually large parasites. These were found to be replaced by parasites of more normal proportions after several sub-cultures. Therefore the implication is that such morphological alterations are transient *in vitro*.

4.4 In Vitro IMMUNE RESPONSES

4.4.1 T-cell responses

In unexposed donors, there was no antigen specific T-cell proliferation in response to *B. divergens* merozoites with the range of antigen concentration used. As the T-cells from the Irish patient failed to show any significant activation because of the overwhelming proliferating B-cell population, the absence of any other exposed donor prevented assays being carried out to determine the optimum numbers of merozoites required

for any potential T-cell response. However, the range of merozoite numbers tested was within that used for the investigation of human T-cell responses to *P. falciparum* (Good *et al*, 1987 ; Jones *et al*, 1990) and so should have covered the optimum concentration. Supernatants from infected cultures were not used in these assays because of the lack of a suitable positive control to determine whether the sub-population of merozoite antigens present in the supernatants were stimulatory.

The apparent absence of antigenic stimulation in unexposed donors negates the possibility that exposure to a more common cross-reactive pathogen could be responsible for the failure of immunocompetent humans to contract a clinical form of the disease, at least with regard to T-cell involvement/priming. Recent experiments carried out in France by Roussilhon *et al* (1990) have confirmed the absence of response in unexposed donors. These authors found antigenic responses to occur only in lymphocytes from patients who had suffered from an infection with *B. divergens*. This is in direct contrast to the situation occurring with human malaria where proliferative responses towards *P. falciparum* antigens have been found in peripheral blood lymphocytes from unexposed donors (Chizzolini and Perrin, 1986 ; Good *et al*, 1987 ; Sinigaglia *et al*, 1988 ; Jones *et al*, 1990 ; Roussilhon *et al*, 1990). Jones *et al* (1990) found the proliferative response to peak at day 6 and to consist of T-cells which were undergoing a secondary response. These results suggest some cross-reactivity exists between *P. falciparum* and other common immunogens and sheds some doubt upon previous findings (Wyler *et al*, 1979 ; Gabrielsen and Jensen, 1982) which suggested a non-specific mitogenic activity was responsible for the T-cell stimulation produced. There is evidence for *Plasmodium* spp. of shared epitopes between malaria and proteins from humans and human viruses (McLaughlin *et al*, 1987) which may have provided a source for this response. With the cross-reactivities found between *Babesia* and malaria parasites, especially between *P. falciparum* and *B. bovis* (James *et al*, 1987), such a lack of response could have some bearing upon the differences in host susceptibility. The role of T-cells in the development of immunity to *B. divergens* in immunocompetent humans is not known due to the subclinical nature of the infection. The "long-lived" T-cell responses to *B. divergens* found by Rousilhon *et al*, (1990) cannot be used as an indication of T-cell involvement in immunity due to the use of drugs in resolution of the infections. Also, the function of those T-cells proliferating *in vitro* has not been ascertained. CD8+ T-cells recognizing

malaria blood stage antigens can be isolated from patients recovering from an attack of *P. falciparum* (Sinigaglia *et al*, 1987) but this subgroup will not aid antibody production and a CD8+ T-cell clone reactive with a major *P. falciparum* surface protein has been shown to actively suppress the antibody response in mice (Pink and Suss, 1991). These results seen with *P. falciparum* must bring into question the relevance of follow up *in vitro* studies on T-cell proliferation with regard to functional immunity since it is evident that recognition and proliferation are not necessarily associated with protection.

There was no non-specific mitogenic stimulation by *B. divergens* antigens found in non-immune donors. This was also found to be the case with PBM from the exposed Irish donor although the mitogenic response to PHA was on day 7 rather than the normal peak at day 3. The difference could be due to the low proportion of T-cells present, drugs taken for the hairy-cell leukaemia or possibly a result of suppression mechanisms. The treatment for this type of leukaemia can include splenectomy, administration of steroids (Magee *et al*, 1987), pentostatin (Spiers *et al*, 1984) or alpha-interferon (Quesada *et al*, 1984) any of which could affect responses. Suppression of mitogenic responses in the acute phase of infection has been reported in *P. falciparum* infections (Troye-Blomberg *et al*, 1983a) and also in human *B. microti* infections, where it was shown to be due to factors present in the serum (Benach *et al*, 1982). However, the Irish donor had recovered from the infection for several months and with no mitogenic responses being found with autologous serum or FCS the likelihood of serum factors still being involved in any suppression of T-cell mitogenic responses is small. In view of the high proportion of B-cells present as a result of the leukaemia, the most likely reason for the observed reduced mitogenic response is the low percentage of T-cells present in the total PBM population. The number of reacting cells per well has been shown by Winger *et al*, (1977) to be an important factor in the timing of a mitogenic response and these results were confirmed in the preliminary T-cell assays.

The absence of mitogenic stimulation by *B. divergens* antigens in lymphocytes from unexposed donors, again contrasts with results found for *P. falciparum* (Wyler *et al*, 1979 ; Gabrielsen and Jensen, 1982). As already discussed however, recent experiments carried out by Jones *et al* (1990) suggest the proliferation found in unexposed donors is not due to a non-specific mitogenicity but is an antigen-specific response by T-cells

primed *in vivo* to cross-reactive antigens. The lack of non-specific stimulation of T-cells by *B. divergens* may not therefore have any bearing upon the ability of the intact human immune system to overcome *Babesia* infections. The absence of any mitogenic or antigenic stimulation in the bovine T-cell proliferation assay indicates that this does not occur in the parasites' natural host. Although more testing of bovine donors would be required before basing any firm conclusions on this finding, these results suggest that, perhaps unlike *P. falciparum*, *B. divergens* does not use non-specific stimulatory mechanisms to delay the onset of an effective immune response in its natural or human hosts and is therefore not a characteristic on which to base the differences in susceptibility to infection.

4.4.2 B-cell responses

4.4.2.1 Serum antibody responses

Background binding of normal human serum to blood and serum components in the merozoite preparations meant that the ELISAs carried out using merozoites isolated from human cultures could not show a definite lack of anti-merozoite reactivity, but no binding to these merozoite preparations was noted above control levels. The immunoblots clearly showed there to be an absence of IgG molecules in normal serum which recognise antigens of *B. divergens*. These results are in accordance with those found by Gorenflot *et al.*, (1991), where no binding of normal serum to parasite antigens occurred. There would appear to be one exception to this with a band being found at 45kD in the track containing merozoites raised in bovine blood. Why this should occur only in this group of merozoites and not in those raised in human erythrocytes is not clear but the band could represent an antigen of blood origin. It has been noted in the ELISAs that IgG from normal human serum recognises antigens in the bovine blood controls and that this binding has not been recognised in the immunoblot possibly due to insufficient amounts of red cell control being used. Clearer results may have been achieved in these experiments if the IgG in normal serum had been purified prior to use. Whatever the source of this antigen, however, in a human infection the parasite would only express antigens present in the group of merozoites grown in human erythrocytes and the presence of IgG reactive with bovine erythrocyte antigens would be irrelevant. Therefore if this antigen is of parasite origin

the ability of IgG from unexposed donors to bind to it is of questionable relevance to the immune response in human infections. These findings preclude the existence in unexposed donors of any innate or cross-reactive IgG antibodies which might act to combat an infection with *B divergens* upon initial exposure. The absence of such IgG in normal human sera strengthens the conclusion reached from the T-cell results indicating there had been no recent exposure to babesial or other cross-reactive antigens in those individuals tested. In addition, the inhibition of entry of merozoites into erythrocytes in the merozoite neutralisation assay by both the Irish and normal (unexposed) human serum, suggests that specific IgG is not involved in blocking activities. Although the molecule(s) responsible for this inhibition cannot be identified from these experiments, it is possible that IgM present in normal human serum may be involved in its capacity as a natural agglutinin. In the ELISAs, any anti-merozoite IgM activity could not be distinguished from anti-red cell activity and with an immunoblot grade anti-IgM antibody proving too expensive, the presence of IgM binding to specific *Babesia* antigens could not be investigated.

The inhibitory activity of human serum from donors unexposed to *Babesia* could thus be concluded to be a result of innate immunity to this parasite. However, other features may be in operation and these must be considered. One means by which inhibition of growth of *P. falciparum in vitro* is believed to occur is due to the presence of a dietary toxin. (Butcher *et al*, 1987). These authors found the antimalarial properties of these sera from Papua New Guinea to be lost following dialysis, suggesting this feature was not due to antibodies. Also, it is known that storage of sera may result in the generation of lipid peroxidation products (Morel *et al*, 1983) and that these are toxic to *P. falciparum* (Clark *et al*, 1986). The levels of these products will vary as a result of differences in lipid content and so genetic as well as dietary factors may contribute to the *in vitro* response. Although dialysis of the sera would not remove all such influences, the merozoite neutralisation assays with *B. divergens* should have included this step, if only to determine the involvement of antibody in the inhibition produced. In addition, comparisons with the activity of normal bovine serum in such an assay may prove of value in establishing a functional significance for these findings.

4.4.2.2 In vitro B-cell responses

The increase in precursor cell frequency found with those B-cells

which were panned prior to exposure to EBV indicates the existence of B-cells in the peripheral blood with receptors which recognise merozoite antigens. However, the percentage of such B-cells in the blood of unexposed donors cannot be estimated from these results as the cells used were not a true representation of the population due to the panning procedure. The value of between 0.0001%-0.0002% found from the scattergun experiments should give a closer representation of that found *in vivo*. However, there are further sources affecting this figure which need to be taken into consideration. In those wells containing the higher numbers of cells there was generally more than one clone present and so the polyclonal supernatant produced may have appeared non-specific because any clones present producing specific antibodies would have been masked. Therefore artificially low numbers of specific reactive cells would have been recorded, especially in view of the fact that it was in those wells with high cell numbers that the majority of the positives were found. The frequency of transformation was found to vary from person to person as is consistent with published findings which estimate that only 10-30% of B-cells are susceptible to transformation (Bird *et al*, 1981). In addition, it is thought that only 5% of these B-cells are induced to secrete Ig after EBV infection and that only half as many again are eventually transformed (Tosato *et al*, 1985). Therefore this mechanism for determining the existence of a potential anti-merozoite B-cell response and assessment of its frequency is extremely limited, especially from the recent finding that EBV preferentially induces proliferation of primed B-cells (Crain *et al*, 1989). Since the ELISAs and immunoblots carried out with normal human serum failed to find any evidence of recent exposure to *Babesia* or any cross-reacting antigens, any primed B-cells present in the preparation would not be reactive with these. Thus the failure to produce clones secreting anti-merozoite antibody in some donors is by no means an indication that that individual could not produce such antibody *in vivo*, nor can the frequency of such clones in others be taken as a measure of the total potential capacity for IgM production.

As a means of arbitrary assessment of B-cell capability, these assays have shown the existence, in many normal donors, of B-cell precursors with receptors capable of recognising merozoite antigens and the ability of a few of these cells to go on and produce the appropriate IgM. No clones were found which produced IgG reactive against merozoites or against the blood and serum controls. There is evidence that IgG production

may be induced by T-cell independent (Tind) antigens on the CS protein of *Plasmodium* sporozoites (Schofield and Uadia, 1990). Tind antigens are believed to occur on *B. bovis* (James, 1984) although the presence of these repetitive antigens on *Babesia divergens* has not been investigated. The potential production of IgG from a primary exposure, albeit *in vitro*, must therefore be considered a possibility, although with the small percentage of B-cells actually transformed in each assay, the likelihood of picking up such an occurrence is extremely remote. Therefore, it cannot be ascertained whether this absence of IgG production was due to the insensitivity of the system or a lack of previous exposure to *Babesia*. A report by Gray and Skarvall (1988) which provides evidence of a short-lived B-cell memory in the recirculating lymphocyte pool in the absence of antigen, indicates that the absence of a secondary response may not reflect a lack of previous exposure. However, the T-cell results suggest a lack of exposure to *Babesia* or any cross-reactive antigens, a finding strengthened by the follow up studies on T-cell proliferation in exposed individuals by Roussilhon *et al* (1990) which indicate the existence of an extremely long-lived T-cell response. Therefore it would seem likely that the constraints of the system are not responsible for the apparent lack of IgG secreting clones.

Because of the low numbers of B-cells which produced IgM reactive against merozoite antigens and the even smaller fraction of these which did so for a sufficient period of time to collect enough supernatant, merozoite neutralisation assays were only carried out on a small number of supernatants. None of the supernatants tested inhibited merozoite entry into erythrocytes but, as for the above results, this lack of neutralisation cannot be taken to indicate that antibodies cannot be produced *in vitro* or indeed *in vivo*.

4.4.3 Macrophage responses

4.4.3.1 ROI release

These experiments revealed large variations between donors with regard to the capacity of their adherent cell populations to reduce NBT. The levels of reduction produced in the unstimulated controls and in those exposed to PMA or merozoites within each individual experiment differed for each donor. Since the method of collection appeared to enrich those adherent cell populations capable of NBT reduction at a relatively constant rate between donors (with the exception of the Irish donor), it is unlikely

that differences in cell numbers are reponsible for this. Variations in the ratios of sub-populations could, however, produce these results. It has been demonstrated that malaria infection alters the frequency of macrophage sub-populations in the spleens of mice, as does the presence of thymic lymphoma (Roubin *et al*, 1981). Therefore various pathologic stimuli can lead to an altered frequency of macrophage subpopulations. It is possible that endotoxin may have been present in the culture medium and in view of the extreme sensitivity of human macrophages to endotoxin (Clark, 1982), this could have proved a source for the differences in the levels of activation seen between donors. However, cells from two donors were generally processed at the same time, for example donors 1 and 2 together and donors 3 and 4 together. These groups of cells were all cultured in identical media and yet each produced quite different levels of reduction in the controls so this cannot be the only possible explanation.

Protection of mice from infection with *B. microti* and *B. rodhaini* and from the high parasitaemias and death caused by *P. vinckei* infections has been generated by prior injection with BCG (Clark *et al*, 1976 and Clark *et al*, 1977). Since this was shown not to be due to the presence of cross-reactive antigens or other specific mechanisms, these results led to the suggestion that non-specific activation of the reticuloendothelial system was responsible. The improved immunity to *Babesia* produced as a result of prior or concurrent infections with other protozoa has also been suggested to occur by similar mechanisms to those operating in the experiments with BCG (Cox, 1978 and Millot and Cox 1985). Alterations in the level of reticuloendothelial activation in normal human donors could easily be produced as a result of other infections, particularly when it is noted that protection of mice from *Babesia* can also be achieved by agents such as *Salmonella*, *Listeria* (Clark, 1979,) as well as by muramyl dipeptide and mycobacterial cell walls (Cox, 1980). In man, interferon-gamma is produced spontaneously by 1% of blood mononuclear cells without *in vitro* culture or stimulation by antigens or mitogens (Martinez-Maza *et al*, 1984) and this will in turn affect the activation of monocytes and macrophages. There is an increase in the plasma levels of interferon in humans throughout the day (Bocci *et al*, 1985) so variations will occur depending upon when the blood is taken. Nutritional status, diet, environmental conditions, stress and hormonal changes are further factors which can influence the normal production of interferon (Bocci, 1988). Therefore what may be essentially a variation in the immune status of individuals blood monocytes could neatly

account for these differences seen between donors but additionally presents problems in analysing the results found and in drawing definite conclusions from them.

In most cases, merozoites alone were found to stimulate ROI release at levels significantly above the controls. This is in accordance with the results found for *P. falciparum*, where similar activation was attributed to the presence of endotoxin-like substances in the parasite preparations (Kharazmi *et al*, 1987). Also, the findings of Smith and Alexander (1986), from experiments on *P. yoellii*, indicate that only those parasites which invade macrophages, ie sporozoites, fail to induce the production of free radicals. In this context, since neither *B. divergens* sporozoites nor merozoites actively invade macrophages such activation of the respiratory burst is not surpising. This point raises further considerations, as to the potential anti-babesial activity of macrophages from different organs. In this study, only those present in the blood were tested but it is known that differences in the level of activation and the susceptibility to activating agents occurs between, for example, liver Kupffer cells and splenic macrophages (Filice, 1988).

The adherence of IgG from the culture medium to the merozoites, discussed previously, may be involved in the phagocyte activation but as the IgG sub-class and the mechanism of antibody adherence to the merozoites (ie via Fc or Fab regions) is not known the possible contribution of IgG adherence cannot be assessed. In one donor, the level of NBT reduction in the control wells was significantly above that reached when merozoites were added, possibly indicating the existence of some form of suppression. This is suggested to occur following overload of macrophages with *P. knowlsei* (wyler *et al*, 1979), However, as this was produced with the phagocytes from one donor only and it was not possible to repeat the experiment, no firm conclusions can be drawn about the cause of the low level of activation by merozoites. The levels of activation produced with merozoites were increased following pre-incubation with the Irish serum and also, to a lesser extent, after pre-incubation with normal serum. Although the immune serum alone occasionally stimulated ROI release, these findings suggest some form of opsonization is in operation. Since it has already been ascertained that the serum from the Irish patient contains IgG which recognises merozoite antigens and normal serum does not, it is possible that the difference in levels of activation occurs as a result of opsonisation by this specific IgG. This enhancement of the adherent cell

response could be occurring via their Fc receptors. However, the expression of Fc and complement receptors are increased according to the state of macrophage activation, therefore introducing a potential source for variation between donors. In addition, for opsonization to be occurring by this route the reactive IgG molecules must be of the subclass 1 or 3 and whether this is the case is not known. Thus no definite conclusion can be reached in the context of these experiments as to the involvement of specific IgG.

Since the pre-incubation of the merozoites with normal serum also produced enhancement of NBT reduction, mechanisms other than the specific one discussed above must be in operation. Opsonization by complement components may be responsible for the enhancement, although again the contribution of this could well have been dictated by the numbers of CR1 and CR3 receptors present on the phagocytes which in turn is related to their state of activation. For *Plasmodium* spp., there is evidence for the involvement of both classical and alternative pathways in the activation of the respiratory burst (Salmon *et al*, 1986 ; Kharazmi *et al*, 1987). This may well be the case for *B. divergens*. However, as the Irish patient was essentially cured of the babesial infection by drug administration it is questionable whether the immune responses seen have a protective role. Since it was not possible to detect the presence of IgM specific for merozoite antigens in normal human serum due to the background binding to blood antigens, any role for this in opsonization for phagocytes cannot be assessed. The enhanced phagocyte activation seen following preincubation of the merozoites with normal human serum therefore could be due to the involvement of antibody (IgM) and/or direct complement binding ie both alternate and classical pathways could be involved in opsonization. *In vitro* cytotoxicity assays comparing inhibition of growth of *B. bovis* in bovine erythrocytes have shown no significant difference to occur between the inhibition produced by monocytes preincubated with *B. bovis* exoantigens or immune complexes of these antigens with immune serum (Montealegre *et al*, 1985). Thus it is possible that both mechanisms are of importance.

The role of oxygen intermediates in the killing of protozoa is well documented (Babior, 1978 ; Wozencraft *et al*, 1984 ; Clark and Hunt, 1983 ; Nathan, 1983). However, there is some evidence for *Babesia* and *Plasmodium* spp. in mice that the generation of intermediates is not correlated with the observed inhibition of parasite growth (Millot and Cox,

1985 ; Cavacini *et al*, 1989). Furthermore, analogous experiments in gerbils infected with *B. divergens* (Langley and Gray, 1989) failed to correlate inhibition of parasite growth with the induction of oxidative radicals. In view of the recent discoveries relating to the anti-microbial activities of nitric oxide, it may be worth looking into this line of defence in future experiments with *B. divergens* since nitric oxide derivatives have been found to be toxic for *P. falciparum in vitro* (Rockett *et al*, 1991). However, it is worth noting that, to date, release of NO from human macrophages has not been detected.

In humans, therefore, experiments investigating mechanisms of non-specific immunity cannot easily provide definitive answers in view of the inconsistent levels of macrophage activation seen in different individuals. Although the actual mechanisms employed in parasite killing are not clear, assays determining the effects of peripheral blood mononuclear cells upon cultures of *B. divergens* have provided a clearer functional assessment of the activity of these cells *in vitro*

4.4.3.2 In vitro cytotoxicity assays

Although as in the previous assays the levels of PBM activity differed from person to person, the experiments investigating the contribution of the different cell populations of PBM to cytotoxicity produced clear results. Whole populations of human PBM caused both increased and decreased growth of the parasite depending upon the ratio of cells to the number of parasites initially present. The apparently conflicting observations of growth inhibition at the lower cell numbers and its absence or indeed reversal at the higher cell concentrations is a feature noted by Butcher and Clancy, (1984) in similar experiments with *P. falciparum*. The reason(s) for this are not known but these authors suggested the possible involvement of factors such as *in vitro* cell-cell interactions, for example the suppression or stimulation of monocyte function by lymphocytes of different sub-groups ie CD8+ and CD4+ (T_H1 subset) , or simply variations in the state of activation of the adherent cells present. That inhibition of parasite growth only became noticable at lower cell numbers could be attributed to a counteracting stimulatory effect produced by the non-adherent cells present (94-98% of the PBM). Although the ratio of adherent : non-adherent cells was the same for each group, there would be increased cellular activity in those wells containing large numbers of cells and so enhancement of parasite growth may have occurred as a result of the

reduction in oxygen concentrations already discussed.

The addition of PBM depleted of adherent cells to *B. divergens* cultures caused significant increases in parasite growth at almost all cell concentrations. These results implicate the involvement of adherent cells in the growth inhibition produced by the whole PBM population. The reason(s) for the significant inhibition of parasite growth produced by the cells from two donors at the lowest concentration is not known. This may be due to the presence of an inhibitory cell type other than those in the adherent cell population, the effects of which were being masked by the stimulation of parasite growth produced at higher cell concentrations. Alternatively, these effects could have been due to the incomplete removal of adherent cells from these preparations. At the higher cell numbers when the adherent cell population was removed the stimulatory effect of the remaining lymphocytes became more apparent In contrast, the adherent cell population had a definite inhibitory effect upon parasite growth which was directly proportional to cell numbers, with crisis forms of the parasite being observed in the erythrocytes. These results confirm that this is the subpopulation of PBM responsible for the growth retardation seen with the whole population.

Separation of adherent cells from PBM populations or from E-populations by virtue of their adherence to gelatin coated flasks has been shown to produce a monocyte yield of greater than 90% by using the binding of a monoclonal antibody against human monocytes (Leu-M_3) (Hassan *et al.*, 1986). Polymorphs should have been removed prior to this by the centrifugation on Lymphoprep. Therefore, although a role for contaminating lymphocytes cannot be discounted, these findings implicate the monocyte as the main cell type responsible for inhibition of parasite growth. Similar inhibition has been found following the addition of monocytes to cultures of *P. falciparum* (Butcher , 1990), with a 10:1 ratio of monocytes to parasite producing a range of inhibitions from 0 to 75%. The corresponding range of inhibitions in the growth of *B. divergens* was 29-94%. Variations between individuals may well reflect the relative state of monocyte activation, possibly from the presence of contaminating endotoxin, as well as a variation in the numbers of any other cell population present.

Gerbil PBM and the subpopulations of these tested showed a marked difference in activity compared to the human cells. None of the gerbil PBM poulations retarded the growth of *B. divergens* at any of the cell : parasite ratios tested. Since it was found that 7.2% of the total gerbil PBM adhered

to the gelatin coated flasks in comparison to the 2-6% found for human cell adherence, this could offer one source for variation in the results. This gerbil adherent cell population failed to cause a significant inhibition in parasite growth in human blood, instead producing a stimulation in parasite growth directly proportional to the numbers of these cells present. This is a direct contrast to the results found using human adherent cells.

Barring possible contamination with endotoxin, neither the human nor the gerbil PBM had been activated before these assays were carried out. Humans, however, are extremely sensitive to endotoxin with only 0.02-0.04µg/kg being required for severe illness (Saunter and Wolfensberger, 1980). For human malaria, often only 100 parasites per µl blood are required to produce fever (Kitchen, 1949) and likewise, humans infected with *B. microti* start to feel sick at very low parasite densities (Healy and Ruebush, 1980). This is in comparison to an LD_{100} value of 25-60mg/kg for mice (Berczi, 1966). Mouse adherent cells have been shown to require priming from a *B. microti* or *P. vinckei* infection or from BCG administration before releasing monokines in response to LPS (Wood and Clark, 1984). Stimulation with LPS alone did not suffice. This was the case found in the experiments carried out on gerbil adherent cells which had been activated by IP administration of thioglycollate broth three days prior to cell harvesting. These cells produced erratic results with regard to TNF release in response to LPS, often with a level of release occurring which was no more than control values. The relative inactivity of rodent adherent cell populations with regard to the inhibition of parasite growth has in fact been exploited for cultures of *P. falciparum*. Non-activated PBM and peritoneal wash cells have been used to adapt isolates of *P. falciparum* to continuous culture (Phillips *et al*, 1987) and in further experiments it was found that it was the adherent cell population which were responsible for promoting growth (Trenholme and Phillips, 1989). Notably, the gerbil PBM were found to enhance the growth of *B. divergens* to a greater extent before the adherent cells were removed, which implicates the involvement of these cells in the growth stimulation seen.

For gerbils, which lack of an innate inhibitory monocyte response to *Babesia* coupled with the requirement for a high degree of prior activation before *in vitro* inhibitory activity is produced, the effects of any endotoxin present in the cultures upon the results would be minimal. Because of the extremely low tolerance of humans to endotoxin, its presence would be much more likely to influence the results produced in

the cytotoxicity assays using human PBM. However, such degrees of sensitivity to endotoxin also exist in humans and gerbils *in vivo* and with the barrage of microorganisms and antigens which normal individuals are exposed to, variations in the level of activation seen are as likely to occur from *in vivo* priming as they are from *in vitro* sources. Furthermore, it has been reported that *P. yoelii* and *P. berghei* possess endotoxin-like antigens (Bate *et al.*, 1989) so *in vitro* activation may also be produced by the parasite itself offering a further source of variation in monocyte activation. Whether endotoxin could have originated from the culture media or from the parasites themselves, the consequences of its presence depend upon the susceptibility of the monocyte species to endotoxin. Therefore, these results can to some extent offer a comparative study of the actions of human PBM versus gerbil PBM in representing the first response of these cells and their subpopulations to a multiplying parasite population, albeit *in vitro*.

Pre-activation of monocyte populations prior to the addition of parasite cultures produced a significant increase in the cytotoxicity of the gerbil cells towards the parasites. Parasite growth was slightly retarded in the presence of LPS-activated monocytes from half the human donors when compared with those wells of *B. divergens* culture which received no monocytes. However, significant retardation was only produced by LPS-activated cells from one donor with respect to that achieved by the non-activated monocyte population. Similar results were found using the supernatants from these monocyte populations. Some degree of inhibition was noted using supernatants from all activated and 2/5 non-activated human monocyte cultures while supernatants from the LPS-activated gerbil monocytes significantly inhibited parasite growth. Similar experiments by Montealegre *et al.*, (1985) revealed the production of an inhibitory factor in supernatants from bovine adherent cells following stimulation with *B. bovis* exoantigens. Since uninfected erythrocytes incubated with supernatants from these monocytes subsequently supported only poor growth of *B. bovis*, these authors suggested that the factors may act upon the erythrocyte rather than on the parasite, by restricting the passage of nutrients into the red cell. "Crisis" forms of *B. bovis* were noted by these authors in cultures where growth inhibition was present. In the cultures of *B. divergens* in which inhibition occurred, "crisis" forms of the parasites within the erythrocytes were also observed suggesting the existence of such soluble factors in this system. Erythrocytes are known to become more susceptible

to free radicals with age, therefore the use of outdated blood in the human *B. divergens* cultures may have had some influence upon the results found in these cytotoxicity assays. If these molecules were contributing to the cytotoxicity exhibited by the monocyte populations, then these cells would appear more toxic than they actually were. However, while this must be taken into consideration when quantitatively examining the human monocyte response, the gerbil monocytes clearly do not benefit from any increased susceptibility to free radicals.

As the supernatants from monocyte cultures derived from several human donors proved inhibitory to parasite growth even without prior activation, the actual growth retardation as a result of LPS activation was much less obvious than for the gerbil cells. Since some of the inhibition was transferable using the cell supernatants, a role for a non-dialysable factor originating from the monocytes is implicated in the cytotoxicity seen. The efficiency of the dialysis in replenishment of nutrients in the medium is of course important in ascertaining the contribution of such a factor. Comparisons between the effects on parasite growth of supernatants from non-activated monocyte cultures and fresh medium revealed a slight difference in parasite growth in two cases but only where the supernatant was used undiluted. This observation might imply that there had been inadequate dialysis, but, as it was not noted in the other three donors, this is less likely to be the case. A control should have been included to test the effect of LPS upon *B. divergens*; however, there is no evidence for *P. falciparum* of any alteration in growth due to LPS (G. Butcher, personal communication). The retardation could also have been produced by the secretion of a non-dialysable factor from "non-activated" monocytes. The assays discussed previously showed the efficiency of non-activated human monocytes in inhibiting parasite growth and so a similar effect produced with some supernatants from this group of cells cannot be considered unusual. However, similar *in vitro* cytotoxicity assays carried out using *P. falciparum* failed to consistently produce these inhibitory factors and they have not been identified (G. Butcher, personal communication). Although fresh serum was added to all the monocyte supernatants following dialysis, depletion of nutrients from the medium resulting from an increase in cellular activity from LPS treatment may not have been adequately replenished by the dialysis. The contribution of this to subsequent retardation of parasite growth cannot be accounted for in the context of these experiments and so no definite conclusions can be drawn

regarding the means of growth retardation produced as a result of LPS treatment of human monocytes.

As with the previous assays using human monocytes, the differences seen between the relative cytotoxicities of control monocytes and their supernatants may reflect variations in levels of activation between individuals. Since the gerbil cells originated from a pooled population no comparisons can be made between individual gerbils, but these experiments, using monocytes and supernatants activated with LPS prior to addition to parasite culture, have further highlighted the differences in baseline "resting" levels of activation found in human and gerbil monocytes. However, the differences in such "resting" levels of monocyte activation are not the only factors which could potentially contribute to disease susceptibility. The sensitivity of the host species to endotoxin is believed to be strongly related to the parasitaemias reached in an infection (Clark, 1982). Mice infected with *Plasmodium* sp. or *Babesia* sp. appear to be in normal health until their parasitaemias exceed 50 or 60% (Clark, 1982) and this correlates with their high tolerance to endotoxin (LD_{100} = 25-60mg/kg (Berczi *et al*, 1966)). Endotoxin-like molecules have been found to be released by *Plasmodium falciparum* (Jakobsen *et al*, 1988), a finding which adds weight to this connection. In the gerbil, infections with *B. divergens* can result in parasitaemias of up to 90%, (C. Winger personal communication). High endotoxin tolerance is also found in rats and although no LD_{100} figures are available for gerbils, it seems likely that these rodents will also exhibit a level of tolerance above that of man, especially in view of the high parasitaemias reached before illness. Cattle, like humans, are relatively sensitive to endotoxin, with an LD_{100} of 0.025mg/kg (Berczi, 1966). In accordance with this, bovine hosts become ill from infections with *B. divergens* at very low parasitaemias. Experiments investigating the relative cytotoxicity of bovine monocytes for *B. divergens* could prove enlightening, as these hosts clearly do not have the innate immunity to *B. divergens* exhibited by immunocompetent humans but, unlike gerbils, they have a low tolerence to endotoxin. In contrast, mice are naturally immune to this parasite and have a high endotoxin tolerance, such that non-activated adherent cells can be used as feeders for a growing *P. falciparum* population, where the production of endotoxin-like exoantigens has been shown to occur (Jakobsen *et al*, 1988). However, mouse red cells appear unsuitable for parasite growth and so the significance of high endotoxin tolerance with regard to murine immunity to

B. divergens is questionable.

Unlike the cytotoxicity assays, both human and gerbil monocytes require pre-activation before TNF responses can be assessed (J. Taverne, personal communication). Those experiments investigating TNF release from human monocytes failed to reveal any stimulation by *B. divergens* above that found in control groups. Peritoneal exudate cells are known to show various chemical and morphological changes from non-induced cells and it was found that the level of activation achieved, as assessed by the levels of TNF produced in response to LPS, varied for each experiment. Indomethacin was added to the monocyte cultures to ensure that no inhibition occurred in response to the presence of steroids. Polymixin B was used to prevent activation of the monocytes by endotoxin which might have been present in the cultures. This was used in all wells except those assaying the response to LPS. In the presence of these agents in the cultures, monocyte stimulation would have been due to parasite antigens. Although no firm conclusions can be drawn from the results, it would appear that the observed TNF release from monocytes produced by *P. falciparum* exoantigens (Taverne *et al*, 1990) does not occur with *B. divergens* antigens (culture supernatants, merozoites or PELP). Papain treatment was suggested by the above authors as a means of removing any proteins in the preparation which could mask or prevent expression of stimulatory antigens, as had sometimes been found with *P. falciparum*. However, this treatment appeared to have no effect in the assays with *B. divergens*. With the implications that the release of TNF may be partly responsible for the pathology seen in infections with *Plasmodium* spp. (Grau *et al*, 1989 ; Playfair *et al*, 1990) as well as being involved in cytokine mediated killing, the absence of a TNF response towards *B. divergens* antigens need not be surprising. In view of the differences in disease characteristics seen between *B. divergens* and *P. falciparum*, at least in man, the lack of TNF induction may be one means by which humans avoid clinical expression of an infection with the former parasite. *B. bovis* presents far more similar disease characteristics in terms of immunopathology and perhaps an investigation into the effects of these exo-antigens on TNF release may provide a better analogy.

4.4.4. Implications for the role of the spleen

In view of the differences found in innate immunity to *B. divergens* in humans and gerbils, notably with regard to the monocyte response and the

apparent irrelevance of an intact spleen to the final outcome of an infection in gerbils, the immune mechanisms employed in each host are clearly different. Considering the high tolerance of gerbils to endotoxin, the absence of endotoxin-like exoantigens in *B. divergens*, which can be deduced from the TNF experiments, may enable the parasites to reach high parasitaemias before sufficient reticuloendothelial stimulation can occur to counter the infection. This would be consistent with the rapid course of the disease in gerbils, and goes some way to accounting for the low splenic profile at least with regard to the role played by splenic macrophages. Clearly gerbils cannot be regarded as a suitable model for human babesiosis which relies heavily for its occurrence upon the absence of a spleen. However, from comparisons of the contributions made by gerbil and human blood cell populations to *in vitro* cytotoxicity, these experiments can offer a means for determining the role played and perhaps therefore the relative importance of these cells in an infection.

The investigations into the immune responses of intact immunocompetent individuals to *B. divergens* have suggested that the individuals tested had had no previous exposure to *B. divergens*. Thus it is unlikely that subsequent splenectomy would remove memory cells produced as a result of previous exposure or contact with more common cross-reactive antigens. The effects that splenectomy might have upon these immune responses cannot be predicted from the experiments performed in these studies and would be rendered more uncertain in many cases, as the condition necessitating the splenectomy could also affect these responses. This was clear with the results obtained using cells isolated from the blood of the Irish patient with hairy-cell leukaemia. Alterations in parasite virulence as a result of asplenia in the mammalian host could also be factors influencing the outcome of an infection in asplenic individuals. Unfortunately gerbils cannot be used to study the effects of splenectomy on the infectivity of *B. divergens* as splenectomy was not found to render gerbils any more susceptible to infection (Lewis and Williams, 1979).

In areas of France where exposure to *B. divergens* is known to have occurred it was not specifically associated with clinical illness. This is consistent with a predominant contribution from non-specific immune mechanisms in protection against the parasite. This view is strengthened from the finding that blood monocytes are toxic to *B. divergens in vitro* and the failure to detect a specific antibody or T-cell response in unexposed volunteers. *B. divergens* merozoites were found to stimulate the production

of ROI's and this could be increased by the addition of serum but further experiments investigating the nature of this cytotoxicity are required to determine which molecules are directly responsible.

Although it cannot be assumed that the cytotoxicity of blood monocytes for *B. divergens* is also exhibited by splenic macrophages these results certainly implicate the involvement of innate, non-specific mechanisms in dealing with these parasites. Humans without a spleen are suceptible to infection with *B. divergens* and this implies that an important contribution to immunity is made by splenic non-specific immune mechanisms. Upon transfer from the tick, *B. divergens* sporozoites directly invade erythrocytes and are thus immediately in the blood stream and pass through the spleen. The physical removal of infected erythrocytes from the circulation can occur by "pitting" and free parasites are then exposed to the anti-microbial activities of the splenic macrophages. This is in direct contrast to the initial multiplication stages of malarial parasites which occurs in the liver and of *B. microti* which occurs in lymphocytes. Larger numbers of merozoites are thus released into the blood than were present in the initial inoculum. Considering the ease with which those strains of *B. divergens* tested were found to adapt to human erythrocytes *in vitro*, such a distinction is likely to be of importance in the relative effectiveness of innate splenic mechanisms in combating the initial inoculum.

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APPENDIX

MATERIALS AND METHODS

1. Phosphate buffered saline

Na Cl	8 g/l
KCl	0.2 g/l
$Na_2 HPO_4$	1.15 g/l
$KH_2 PO_4$	0.2 g/l

2. Uranyl Acetate

Make a saturated solution (>8%) of uranyl acetate in 50% ethanol prior to use, or store in a darkened bottle and discard if cloudy or brown.

3. Reynolds Lead citrate

Lead nitrate	1.33g
Sodium citrate	1.76g
Distilled water (boiled)	30 ml

Stir on a magnetic stirrer or shake for 30 min. A milky suspension is produced. Add 8ml 1 N NaOH and dilute to 50ml. The solution should now be clear. Can store in a tightly capped bottle for up to 6 months. Do not use if cloudy.

4. Bicarbonate Buffer

Na_2CO_3	1.59 g/l
$NaHCO_3$	2.93 g/l
NaN_3	0.2 g/l
1M $MgCl_2$	100 µl/l

5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Laemmli sample buffer (reducing)

Tris-HCl	0.01M
EDTA	0.001M
2-mercaptoerthanol	5%

Tank buffer

Tris	0.025M
Glycine	0.192M
SDS	0.1%

pH 8.2

Separating gel buffer stock

Tris-HCl pH 8.8 1.5mM

Dilute 1:4 in Milli-Q (Millipore) water for use.

Stacking gel buffer stock

Tris-HCl pH 6.8 0.5mm

Dilute 1:4 in Milli-Q water for use.

Acrylamide stock

Acrylamide	75g
Bis-acrylamide	2g
MBI ion-exchange resin	2g

Make up to 250ml with Milli-Q water and filter through Whatman No.1 paper. Store in dark at 4°C.

Mix for 10% gel

Acrylamide stock	16.6 ml
Separating gel buffer stock	12.5 ml

Make up to 50 ml with Milli-Q water, degas and then add :

SDS	50 mg
TEMED	50 µl
AMPS (10% in Milli-Q water)	500 µl

Mix for 5% stacking gel

Acrylamide stock	8.0 ml
Stacking gel buffer stock	12.5 ml

Make up to 49.5 ml with Milli-Q water, degas and then add :

SDS	50 mg
TEMED	50 µl
AMPS (10%)	500 µl

6. Coomassie Blue stain

Stock :

Coomassie Blue R250	2.5 g
Methanol	454 ml

Mix these together and filter through a Whatman No. 1 filter.

Staining mixture :

Coomassie Blue stock	138 ml
Double distilled water	138 ml
Glacial acetic acid	30 ml

7. Western transfer and Immunoblotting

Western tank buffer

Glycine	14.3 g/l
Tris	3 g/l
Methanol	200 ml/l

Tris-buffered saline (TBS)

Tris	2.42 g/l
NaCl	29.24 g/l

Adjust pH to 7.5

8. Trypan Blue

Trypan blue (0.2% solution in double distilled water, filtered through Whatman No. 1 filter paper)

Salt solution (4.5% solution in double distilled water)

Working solution is 1 :4 to give a final salt concentration of 0.9%. Dilute 1 : 1 with cell suspension and count viable cells on a haemocytomter. Make up daily as required.

9. A.E.T treated sheep red blood cells (sRBC)

(a) Make up 402 mg A.E.T./ 10 ml Milli-Q, fresh each time.

(b) Adjust pH to 9 with 5M NaOH added dropwise. It is important not to overshoot pH 9 as pH should not be readjusted with Hcl.

(c) Filter through a 0.2µm filter.

(d) Wash sRBC x3 in RPMI-1640 at 800 x g for 5 min at R.T.

(e) Add 4 volumes A.E.T. to 1 volume of packed sRBC and mix.

(f) Incubate at 37°C for 30 min, mixing occasionally.

(g) Make up to 4% v/v in RPMI-1640 with 10% FCS.

A.E.T. sRBC will last up to 7 days at 4°C but wash before use.

10. Percoll preparation

(a) Mix 9 volumes Percoll and 1 volume x10 PBS

(b) Weigh density bottle and distilled water, carefully dry and re-weigh. Weigh density bottle and Percoll-PBS. Clean and dry density bottle and re-weigh.

Weight of Percoll-PBS = (weight of Percoll-PBS + bottle) - bottle alone

Weight of distilled water = (weight of water + bottle) - bottle alone

$$\text{Specific gravity (density) of Percoll-PBS} = \frac{\text{weight of Percoll-PBS}}{\text{weight of distilled water}}$$

(c) Weigh density bottle. Weigh density bottle and RPMI-1640 with 10% FCS. Clean and dry density bottle and re-weigh.

$$\text{Specific gravity (density) of medium} = \frac{\text{weight of RPMI-1640 with 5\% FCS}}{\text{weight of distilled water}}$$

(d) Calculate the density required (1.08 g/ml) according to the formula ;

$$x = \frac{V\,(S-d)}{D-d}$$

Where D = density of Percoll-PBS

d = density of medium

S = density required (1.08)

V = volume required

x = volume of percoll-PBS

The proportion of Percoll-PBS to medium should always be around 60 : 40

11. Geys solution

Solution A

NH_4Cl	35.0 g
KCl	1.85 g
$Na_2HPO_4 . 12 H_2O$	1.5 g
KH_2PO_4	0.119 g
Glucose	5 g
Gelatin	25 g
Phenol red (1% solution)	1.5 ml

Solution B

$MgCl_2 . 6 H_2O$	4.2 g
$MgSO_4 . 7 H_2O$	1.4 g
$CaCl_2$	3.4 g

Solution C

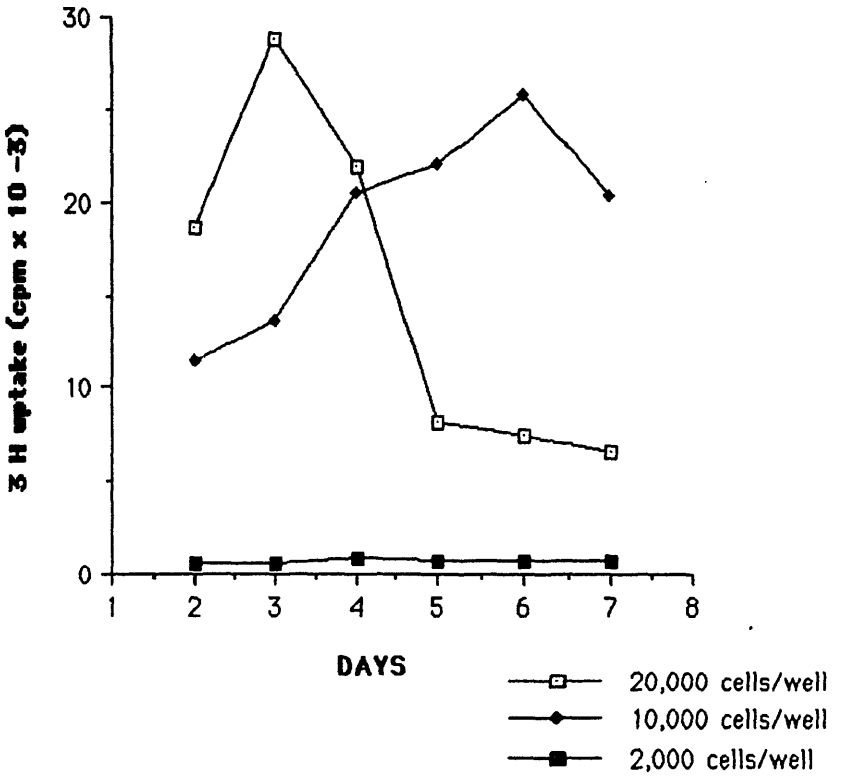
$NaHCO_5$	22.5 g

Make up each solution to 1L with distilled water. For use mix 20ml A, 5ml B, 5ml C and 70ml distilled water. Sterilize each solution separately by autoclave.

APPENDIX TABLE 1

EFFECT OF SERUM CONCENTRATION UPON PARASITAEMIAS IN MASP HUMAN CULTURES

SERUM LEVEL	PARASITAEMIAS (%)				
	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4
5%	1	3.7 ± 0.2	7.5 ± 0.6	14.8 ± 1.1	15.3 ± 0.5
10%	1	5.1 ± 0.6	11.9 ± 1.4	16.8 ± 0.8	20.2 ± 1.6
20%	1	4.6 ± 0.5	10.2 ± 0.9	21.2 ±0.6	23.2 ± 0.7
40%	1	5.0 ± 0.9	11.9 ± 1.4	18.2 ± 1.1	22.4 ± 0.6
80%	1	4.1 ± 0.2	9.8 ± 0.4	15.3 ± 0.7	22.4 ± 0.2



Appendix figure 1. Effects of cell numbers upon T-cell proliferation to PHA.