STAGE SPECIFICITY AND THE HOST RED CELL MEMBRANE IN THELLERIA ANNULATA

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Theileria annulata is an economically important protozoan parasite (Apicomplexa) which cycles between bovine and invertebrate tick hosts. Within the bovid, sporozoites invade leucocytes and develop into macroschizonts: macroschizonts divide, initially by binary fission, in synchrony with the newly transformed host cell, but later merogony takes place, producing merozoites which invade erythrocytes. These are subsequently ingested by the tick. The post-macroschizont life cycle stages are poorly defined, and this experimental project was aimed at investigating the basic biology of these stages at the molecular level.

The polypeptide complement of the infected erythrocyte membrane has been investigated. The precise location of piroplasm polypeptides in the infected cells was not determined, but the experimental results indicate that several (most notably molecules of 122kDa, 98-100kDa & 77kDa) are associated with the erythrocyte membrane.

Monoclonal antibodies have been raised against infected erythrocytes. By immunofluorescence microscopy, several antibodies recognise, mainly, either the outer perimeter of the piroplasm, vesicular structures (dots), or molecules located within the piroplasm. For the purpose of strain differentiation, a panel of these monoclonal antibodies distinguish between uncloned preparations of Ankara, Hissar and Gharb stocks. The vast majority of the monoclonal antibodies (27 cloned lines) are stage specific and do not recognise slide preparations of macroschizonts or sporozoites. Merogony has been induced in vitro, in recently transformed macroschizont infected cell lines, and four anti-piroplasm monoclonal antibodies recognise preparations of merozoites. Immunoelectron microscopy results have shown that antibody 5E1 recognises the surface of heat induced merozoites. Western blot analysis suggests that the 30kDa and 120kDa polypeptides, recognised by antibody 5E1, also elicit an antibody response in the bovine host. These antigens are preliminary candidates for part of a molecular sub-unit vaccine against the disease (Tropical theileriosis) caused by T. annulata. The T. annulata gene, which encodes the epitope determining antibody 5E1 has been cloned from a lambda gt11 genomic expression library.

A model system for investigating the molecular mechanisms of stage differentiation has been developed. Macroschizont merogony can be induced in recently infected cell lines by elevating culture temperatures. Similar heat-treatment, of the same cell line after prolonged passage, fails to result in the differentiation into merozoites. The epitope recognised by antibody 5E1 is stage specific to merozoite and piroplasm stages and preliminary Northern slot-blot analysis, with the cloned gene sequence, suggests that expression is regulated at the level of transcription.
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DECLARATION

This thesis has been composed by myself. The results presented in this thesis are my own work except for the following experiments which were performed in collaboration, and to which I made a significant contribution: FACs separations, immunoelectron microscopy, Northern and Southern hybridisations.
LIST OF ABBREVIATIONS

ATP  adenine triphosphate
Bis  N,N’-methylenebisacrylamide
cpm  counts per minute
DNA  deoxyribonucleic acid
E. Coli  Escherichia Coli
EDTA  ethylenediaminetetra-acetic acid
FITC  fluorescein isothiocyanate
HAT  hypoxanthine, aminopterin, thymidine
HEPES  N-(2-Hydroxyethyl)-piperazine-N’-2-ethansulphonic acid
HRP  horse radish peroxidase
IFA  immunofluorescence assay
Ig  immunoglobulin
IPTG  isopropyl-1-thio-b-D-galactoside
ISEMPs  infection specific erythrocyte membrane proteins
kb  kilobases
kDa  kilodaltons
Mr  molecular weight
nm  nanometer
NHS  N-hydroxysuccinamide
OD  optical density
PBMC  peripheral blood mononuclear cells
PAGE  polyacrylamide gel electrophoresis
PBSpi  phosphate buffered saline/protease inhibitors (2.1.3.)
PMSF  phenylmethyl sulphonyl fluoride (2.1.3.)
RNA  ribonucleic acid
SDS  sodium dodecyl sulphate
TCA  trichloroacetic acid
Tris  Tris (hydroxymethyl) aminomethane
Scientific research into the protozoan parasite, *Theileria annulata*, has two main aims. Firstly, its economic importance as a pathogen of cattle in endemic areas has led to approaches to generate a prophylactic for the disease. Live, cell based vaccines and a few drugs are available, which are effective in disease protection or treatment. However neither of these treatments are optimal and a molecular based sub-unit vaccine is a highly prized objective which is being investigated as an alternative means of protection. Secondly *T. annulata* is an interesting parasite; it is an excellent example of a eukaryotic protozoan with several unique properties which are not found in higher organisms. Most obviously, a digenetic life cycle which involves large changes in morphology, physiology and behaviour. Little of the molecular mechanisms which underlie and orchestrate these changes are understood, and as far as the subjects of this study are concerned, namely the piroplasm and merozoite life cycle stages, very little is known. Thus the second research interest is to understand the basic molecular biology of *T. annulata* and compare it with what is known about other organisms, for example, *Theileria parva*, *Babesia*, *Plasmodium* and higher eukaryotes. The work for this thesis was directed towards understanding the relationship between the merozoite and piroplasm life cycle stages of *T. annulata*, and how they interact with the bovine host, as this was seen as a requirement for advancing both the above aims.

The following introductory sections review the current information on *T. annulata* and related species, with particular reference to the life cycle, and the bovine immune response as it pertains to *Theileria* vaccine work.
1.1. THE LIFE CYCLE

The life cycle of *Theileria annulata*, which is illustrated diagrammatically in figure 1.1., involves an invertebrate ixodid tick vector (which appear to be infected with impunity) and a bovine host (where acute infections are frequently fatal within 2-4 weeks). The life cycle has been reviewed recently by Irvin & Morrison (1987) and by Melhorn & Schein (1984).

Thousands of uninucleate sporozoites are inoculated into the bovid when a tick host takes an inter moult blood meal. Sporozoites enter leucocytes and rapidly become established in local lymph nodes, initially as uninucleate trophozoites and then, after further differentiation and nuclear division, as macroschizonts (10-20μm in diameter) which are bound by a single plasmalemmal membrane and contain approximately eight to twenty nuclei (Jura et al. 1983b). Macroschizonts divide by binary fission and this is generally accompanied by the unprecedented division of host leucocytes, such that each daughter macroschizont is located within a daughter leucocyte (Hulliger et al. 1964; De Martini & Moulton, 1973; Schein et al. 1978b). The resulting lympho-proliferation causes many of the disease symptoms, but the mechanism of leucocyte transformation has not been elucidated. Macroschizonts may also transfer directly into non-daughter leucocytes; when macroschizont infected cells are introduced into a bovid, its leucocytes become infected and transformed (Brown et al. 1973a; Wilde, 1967). It has been postulated that this involves the fusion of infected with uninfected leucocytes, however, the precise mechanism remains obscure. Macroschizonts differentiate, through the asexual division process of merogony, into the next invasive stage which are termed merozoites. As described by Jarrett and Brocklesby (1966), macroschizont nuclei undergo a "condensing process", and then merozoites are produced by a "budding process". Fully differentiated merozoites appear to be spherical-pyriform (0.5-2μm diameter) with a large nucleus, a rhoptry complex and a single limiting plasmalemmal membrane which is underlayed by segments of double unit membranes (Conrad et al. 1985). In addition, Schein et al. (1978a) describe of a simple polar ring, to which sub-pellicular microtubules are attached.
Figure 1.1. Life cycle of *T. annulata*

- **Proliferation**
  - Macroschizont
  - Leucocyte
- **Merogony**
  - Sporozolite
  - Red blood cell
  - Piroplasm
- **Sporogony**
  - Sporoblast
- **Gametes & Zygote**
  - Kinetes
  - Gut epithelial cells
Hundreds of merozoites are formed per macroschizont, which are released from the host cell and enter red blood cells. Electron microscopy studies of *T. annulata* red blood cell stages by Conrad *et al.* (1985) and Melhorn and Schein (1984) have produced conflicting descriptions of events in the life cycle at this stage as follows:

- **Conrad *et al.* (1985)**

Within the red blood cell cytoplasm, two forms were recognised within the umbrella term of piroplasms; an initial trophozoite stage was followed by the production of intraerythrocytic (red blood cell) merozoites. The trophozoite or "feeding stage" was bound by a single plasmalemmal membrane and ranged in size from 0.6-1.0μm width and 0.9-1.5μm length. A cytostome and food vacuoles were described, as well as free ribosomes and two acristate mitochondria.

The differentiation into merozoites took place by merogonous division into four. Merozoites were spherical-pyriform (0.5-0.7μm diameter) and two or more rhoptries extended towards one of several inner double membrane segments. The observation of trophozoites undergoing merogony was found to be a rare event but the relative proportions of trophozoites and merozoites in infected blood were not determined.

- **Melhorn and Schein (1984)**

Twenty percent of *T. annulata* piroplasms were described as comma shaped precursors of a more common ovoid form (80%), neither of which had rhoptries. It was suggested that the ovoid forms were preparative stages, prior to the differentiation of gametes in the tick vector. The comma shaped piroplasms had a single plasmalemmal membrane which was underlayed by segments of double unit membranes, and these segments were absent in the ovoid form. The comma shaped forms divided by binary fission and the ovoid forms did not divide.
Clearly, further work is required to clarify several discrepancies, and from these different reports it can only be concluded that both modes of division may occur in T. annulata.

Piroplasms become patent approximately eight to thirteen days after sporozoite inoculation, and parasitaemias may rise rapidly, until the majority of red cells are multiply infected. In comparison with other related protozoa, the term merozoite, used by Conrad et al. (1985) to describe the division products of piroplasms, implies that these intraerythrocytic red blood stages might leave red blood cells and be invasive. However, reinvasion into red blood cells, invasion of leucocytes, or any other cell type has not been demonstrated. As this terminology could become confusing, macroschizont-derived merozoites are termed "merozoites", and piroplasm-derived merozoites are termed "red cell merozoites".

The life cycle is continued when a tick takes a blood meal from an infected bovid. Within the tick gut lumen, red blood cells lyse and free piroplasms differentiate into ray-bodies (Schein et al. 1975a & b) which are morphologically similar to "strahlenkorper" in Babesia (Schein, 1975c; Friedhoff & Buscher, 1976; Rudzinska et al. 1983 & 1979) and spherical forms, which are thought to represent microgamonts and macrogamonts respectively. Ray bodies divide to produce uninucleate gametes which fuse with other gametes; derived either from macrogamonts or microgamonts (it is not established yet), to produce a zygote and this stage enters tick gut epithelial cells. When ticks are engorged, twelve to thirty days later, a kinete stage differentiates from the zygote within gut epithelial cells; these are released into the tick hemolymph (Schein, 1975a) and from here kinetes invade salivary gland cells. After the tick has moulted and re-attached to a bovine host, an initial "feeding phase" is followed by a phase of intensive nuclear divisions yielding thousands of small nuclei. As this stage increases in size, cytoplasmic division occurs producing cytomeres which contain approximately twenty nuclei. The cytomeres differentiate into sporozoites which are released from salivary gland cells into the saliva of a feeding tick. The elaboration of sporozoites from cytomeres appears to be similar to that of merozoites from macroschizonts: like merozoites,
sporozoites are spherical-pyriform (1μm diameter) and possess rhoptries. Jura et al. (1983a) report that infective *T. annulata* sporozoites have a polar ring but no inner membrane segments, however, Schein et al. (1978a) suggest that these additional inner segments are present. Immunoelectron microscopy studies (Webster et al. 1985) suggest that a surface coat, which is loosely attached to the surface membrane, is shed as the sporozoite enters host cells.

1.2. **THEILERIA CLASSIFICATION**

As no formal reproductive species concept exists for Protozoa, classical *Theileria* species and sub-species designations have been made using other differentiating criteria. Traditionally, the ecological, morphological, immunological and pathological characteristics of the parasites, and the hosts and diseases involved have been compared. Recently, molecular and biochemical techniques have been applied to the study of *Theileria* species variation and strain variation, which provide the opportunity to investigate the observed diversity using more intrinsic markers.

1.2.1. Higher taxonomic status

The current scheme of protozoan classification (Levine et al. 1980; Cox, 1981) defines the higher taxonomic status of *Theileria* as follows:

- Subkingdom, Protozoa
- Phylum, Apicomplexa
- Class, Sporozoa
- Subclass, Piroplasmia
- Order, Piroplasmida
- Family, Theileridae
- Genus, *Theileria*

The Apicomplexa are defined as Protozoa possessing an apical complex in some life cycle stages and by the absence of cilia or flagella except in gamete stages. These features also define the class Sporozoa which contains the gregarine parasites of invertebrates (for example *Monocystis*), and the coccidian
parasites: either of vertebrates (for example *Eimeria* and *Toxoplasma*) or of vertebrates which also have an invertebrate vector (for example *Plasmodium*).

The subclass *Piroplasmia* is defined by *Sporozoea* which parasitise red blood cells, and which are transmitted by ticks. The order *Piroplasmida* contains two genera, namely *Theileria* and *Babesia*, which when taken together threaten the lives of cattle on every continent and are consequently of great socio-economic importance. There are several distinguishing features between *Theileria* and *Babesia*. The main one, which has caused some debate, is the presence, only in *Theileria*, of an intraleucocytic macroschizont stage. Macroschizont stages have been reported in *Babesia equi* infections (Schein et al. 1981a), and unpublished observations (reported by Melhorn & Schein, 1984) suggest intraleucocytic stages for *Babesia microti*. *Theileria sergenti* is also an exception as macroschizonts have not been found in this species (Uilenberg, 1981). However *T. sergenti* appears to be closely related to, or synonymous with, *T. orientalis* in which macroschizonts have been described (Uilenberg, 1985). Division of *Babesia* species within red blood cells is well established, and so too is the repeated cycle of release of division products (merozoites) which re-invade red blood cells. This event, which is not established in *Theileria*, is the major method of asexual proliferation within *Babesia* species and is a further differentiating characteristic of *Babesia*.

Other taxonomic differences between *Theileria* and *Babesia* are as follows. The piroplasm stages and sporozoite stages of *Babesia* species are larger than in *Theileria* species but the kinetes are smaller. In *Babesia* species sporozoites develop in several tick organs including tick oocytes, and transovarial transmission can take place. *Theileria* sporozoites develop within salivary gland cells and transmission is transstadial. There are reports of transovarial transmission in *Theileria*, which would be important in an epidemiological context; however, it is considered unlikely that natural transmissions are transovarial, or that one-host ticks (which would transmit *Theileria* transovarially) are natural field vectors (Robinson, 1982). Lastly, the gametes of *Theileria* and *Babesia* differ (Melhorn & Schein, 1984).
1.2.2. Species and sub-species status of *Theileria*

*Theileria* species are host specific, and *T. annulata* and *T. parva* belong to a small group of *Theileria* species which infect either cattle and buffalo, or sheep and goats. In relation to other *Theileria* species (Levine *et al.* 1971), this group has been well studied and practical species designations have been made on ecological, morphological, immunological and pathological features (Irvin, 1987; Irvin and Morrison, 1987). Isolates can exhibit variations in any of the above respects (Sergent *et al.* 1945; Pipano *et al.* 1974; Gill *et al.* 1980a; Irvin *et al.* 1972; Purnell, 1977). These variant forms are recorded as separate isolates or stocks, or in the case of *T. parva* at present, given a trinomial signature. The most rigorous test, which has traditionally been used to determine differences between isolates, involves immunising cattle with one isolate and challenging with a second. However, as isolates are not cloned prior to immunisation, there are interpretive problems associated with this test, which is also very expensive and time consuming (Irvin, 1987).

Six species of *Theileria* which infect bovids are recognised: *T. annulata*, *T. sergenti*, *T. parva*, *T. mutans*, *T. taurotragi* and *T. velifera* (Uilenberg, 1981; Irvin, 1987). The last three listed are not usually serious pathogens, but their distribution overlaps with that of *T. parva* which is pathogenic, and this has complicated the fields of parasite definition, epidemiology and immunology. At least five species infect sheep & goats, but only *T. hirci* is pathogenic (Uilenberg, 1981).

1) *Theileria annulata* (Dschunkowsky and Luhs, 1904)

*T. annulata* infects cattle (*Bos taurus* and *Bos indicus*) and the Indian water buffalo (*Bubalus bubalis*). It is transmitted by ixodid ticks of the genus *Hyalomma*. The principle vectors are *H. anatolicum anatolicum*, *H. asiaticum*, *H. detritum* and *H. dromedarii* (Irvin, 1987; Robinson, 1982).

*T. annulata* infections cause a disease known as Tropical or Mediterranean theileriosis, which is endemic in a geographical belt extending from Morocco to China, as shown in figure 1.2. The
Figure 1.2. Distribution of Tropical theileriosis
infection is persistent and subclinical in buffalo, but it is acute and frequently fatal in cattle. It is estimated that there are well over two hundred million cattle at risk from the disease (Purnell, 1978). All breeds of cattle seem to be susceptible to infection, but the most susceptible are dairy cattle (rather than beef cattle) imported from *Theileria* free areas (Pipano, 1976). The question of innate resistance as been raised (Rafyi et al. 1965; Irvin & Morrison, 1987), but has not been resolved. Adult cattle, reared in endemic areas, are often refractory to local *T. annulata* populations (Hashemi-Fesharki, 1988). Cattle that recover from the infection continue to carry parasites so both cattle and buffalo act as reservoirs for transmission. Calves born in endemic areas are more susceptible to infection than adults, which suggests that there is no inherent calf resistance, and no transfer to offspring of factors mediating maternal resistance.

In many areas endemic stability has been disrupted after the importation of high yielding cattle from *Theileria* free areas, through programs which were designed to improve the milk and meat yields of native livestock. The effect of these programs was very high mortality rates amongst the imported and cross-bred cattle, and an increase in transmission rates which jeopardized indigenous herds. Informed data are available on the scale of the problem from India (Gill et al. 1976; Singh 1986), Pakistan (Siddiqui, 1977), Iran (Hooshmand-Rad, 1975; Hashemi-Fersharki, 1988), Turkey (Mimioglu, 1977; Sayin, 1986), Egypt (El-Rafaii 1977), the Sudan (Jonejan, 1986), USSR (Stepanova, 1977) and Israel (Pipano, 1976).

Besides the immune status of the host, factors which determine whether infections of *T. annulata* are mild or fulminating include the stock of the parasite (Pipano, 1974), and the number of sporozoites which are inoculated (Gill et al. 1980b). Details of the pathogenesis are reviewed by Uilenberg (1981), Hooshmand-Rad (1976) and Irvin & Morrison (1987). The combined effects of lymphoproliferation and high parasitaemia generally result in death. In some acute cases, high parasitaemias (which can be well over 90% in terminally sick cattle) are observed in the absence of any patent parasitosis. Anaemia, anorexia, jaundice and secondary infection are common symptoms here. However anaemia can occur in the absence of high parasitaemias (Hooshmand-Rad, 1976).
2) **Theileria sergenti** (Yakimoff & Dekhtereff, 1930)

*T.* sergenti may be synonymous with *T.* orientalis (Yakimoff & Soudatschenkoff, 1931), placed in a *T.* sergenti/orientalis group or it may be called *T.* buffelli (Uilenberg, 1981; Uilenberg et al. 1985). Uilenberg et al. (1985) found no differences between *T.* sergenti and *T.* orientalis isolates in cross-challenge experiments but the definition of these species is clearly confused at present. *T.* sergenti infects cattle and buffalo (*Bubalus bubalis*) and is transmitted by ixodid ticks of the genus *Haemaphysalis*. Isolates of this species have been reported from USSR, Japan, Iran, Ethiopia, India and elsewhere, but definitive comparative studies have not been carried out. Reports vary, as to the pathogenicity of infection and the abundance of macroschizonts. Macroschizonts have been reported in *T.* orientalis which is considered to be non pathogenic (Uilenberg et al. 1985), but they are not observed in *T.* sergenti in Japan (Minami, 1981) where this species causes one of the most important pathogenic diseases in cattle (Yagi et al. 1987). Minami (1981) gives more details but like *T.* annulata (frequently) and *T.* mutans (predominantly) the pathology is associated with the parasitaemia rather than lymphoproliferation.

3) **Theileria parva** (Theiler, 1904)

*T.* parva infects cattle (*Bos taurus* and *Bos indicus*) and buffalo (*Syncerus caffer*) and is transmitted by the ixodid ticks *Rhipicephalus appendiculatus* and *R.* zambeziensis. *T.* parva is widespread in East and Central Africa, where cattle are not indigenous species, although certain types of *Bos taurus* have been on the continent two thousand years longer than *Bos indicus*. There are reports suggesting that *Bos indicus* is more refractory or resistant to infection than *Bos taurus* (Dolan et al. 1982). However, like *T.* annulata infections, mortality generally reflects naivety. As with other *Theileria* species, piroplasms persist in animals which recover from infection and these carriers act as reservoirs for transmission (Young, 1981). Calves are more susceptible to infection than adults (Neitz, 1957), and there is no evidence for inherent calfhood resistance as calves, born to dams
which are solidly immune to sporozoite challenge, are susceptible (Cunningham et al. 1989).

T. parva has three sub-types (Uilenberg, 1981), formerly species, which at present have putative sub-species status. T. parva parva causes a highly pathogenic disease called East Coast fever in cattle. T. parva lawrencei causes a cattle disease called Corridor disease when it is transmitted from buffalo infections. T. parva bovis causes a milder cattle disease called Zimbabwean malignant theileriosis. Approximately fifteen million cattle are thought to be at risk from East Coast fever and the related diseases. If East Coast fever-causing T. parva isolates are maintained in Buffalo and then used to infect cattle, the resulting disease is Corridor disease. Similarly, if Corridor disease-causing T. parva lawrencei isolates are maintained in cattle, the disease begins to resemble East Coast fever (ILRAD, 1987). So it appears that populations of T. parva have exclusive relationships with both hosts. T. parva bovis cannot be modified in this way (Uilenberg, 1981). The three sub-species are serologically indistinguishable (by immunofluorescence microscopy). However, there is a report that the sporozoites of T. parva and T. lawrencei can be distinguished by ultrastructural criteria (Fawcett, 1985).

As with T. annulata, the pathogenesis of the disease is strain, host and dosage dependent (Barnett & Brocklesby, 1961; Dolan et al. 1984a; Young et al. 1978). Details of the pathology of T. parva infections are reviewed by Irvin and Morrison (1987). The disease symptoms generally arise as a result of lymphoproliferation, and high parasitaemias are not a characteristic of T. parva infections.

4) Theileria mutans (Theiler, 1906)

As reviewed by Wilde (1967) and Saidu (1982), T. mutans infects cattle (Bos taurus and Bos indicus) and buffalo (S. caffer) and it is transmitted by ixodid ticks of the genus Amblyomma. The parasite is widespread in Africa and has also recently been reported in the Caribbean. Although infections are generally not pathogenic, severe infection may occur (Irvin et al. 1972). The
pathology is associated with the parasitaemia rather than lymphoproliferation.

5) **Theileria taurotragi** (Martin and Brocklesby, 1960)

*T. taurotragi* infects cattle and other bovids including primarily the eland (*Taurotragus oryx*, Stagg *et al.*, 1983). *T. taurotragi* is transmitted by ixodid ticks of the genus *Rhipicephalus*; the main vectors being *R. appendiculatus* for cattle stocks and *R. pulchellus* for eland stocks. It is widely distributed in Africa, and is normally mildly pathogenic although severe infections have been reported (Grootenhuis *et al.*, 1981). Macroschizonts and piroplasms are patent in clinical infections.

6) **Theileria velifera** (Uilenberg, 1964)

*T. velifera* infects cattle and buffalo (*S. caffer*), and is transmitted by ticks of the genus *Amblyomma*. It is present in Africa and is not pathogenic. Macroschizonts occur but the piroplasm stages predominate infections.

7) **Theileria hirci** (Dschunkowsky & Urodschevich, 1924)

*T. hirci* infects sheep and goats, and is transmitted by ticks of the genus *Hyalomma*. It is widespread in a similar geographical belt to *T. annulata*, and it is highly pathogenic but little studied. Hooshmand-Rad & Hava (1973) review the economic importance of *T. hirci* and describe the disease, called Malignant ovine theileriosis. As with *T. annulata* both the parasitaemia and lymphoproliferation can be important in causing the disease symptoms.

1.2.3. More intrinsic markers of diversity

Considering the species descriptions given above, it is clear that a wide spectrum of imprecisely defined *Theileria* parasites and associated diseases exist from an essentially lymphoproliferative *T. parva*, to *T. sergenti* where macroschizonts have not been
identified and the piroplasm stage is most pronounced. Without markers of genetic or phenotypic diversity it becomes difficult, especially in the absence of a laboratory animal model for Theileria, to accurately monitor and investigate the basis of this variety.

Monoclonal antibodies have been generated against T. parva and T. annulata macroschizonts which detect antigenic differences between stocks (Pinder & Hewett, 1980; Minami et al. 1983 with T. parva: Shiels et al. 1986b with T. annulata). Isoenzyme polymorphisms have been detected between stocks of T. annulata (Melrose et al. 1984). Stock specific T. parva DNA probes have recently been identified using oligonucleotide probes, generated against a variable region of the genome, in polymerase chain reactions (Allsopp et al. 1989). Using these probes, the diversity within isolates has been identified (Irvin, 1987). Thus the field application of these probes and the production of cloned material for future work of this nature, and for other applications, such as detection of genetic exchange in Theileria, analysis of immune responses and protein variation, is important.
1.3. AVAILABILITY OF LIFE-CYCLE STAGES FOR STUDY AND IN VITRO SYSTEMS

Studies on Theileria have been greatly assisted by work which has produced in vitro systems for certain stages of the life cycle. The history, present status and applications of these systems are reviewed by Brown (1979, 1981, 1983).

Laboratory tick colonies can be maintained outside the vector belts. Ticks are infected by feeding them on naturally or experimentally infected bovids, or by percutaneous injection of infected blood (Jongejan et al. 1981). The stimuli for the development of sporozoites can be provided by feeding ticks on laboratory rabbits or by maintaining them for periods at elevated temperatures of 37°C (Samish, 1977). Sporozoites can be isolated from ticks or salivary gland explants (Brown, 1981; Walker & McKellar, 1983), and will invade isolated, peripheral blood mononuclear cells (Brown, 1973b; Kirtti, 1981). Transformation of leucocytes occurs, and subcultures of proliferating macrogametocyte infected cells may be maintained indefinitely without the need for exogenous growth stimulants (Dobbelaere et al. 1988). T. parva parva, T. parva lawrencei, T. annulata, T. taurotragi, and T. hirvi can be cultivated in vitro. Macrogametocyte infected cell lines may also be established using infected lymphoid or spleen tissue, or with peripheral blood, from bovids exhibiting a patent parasitosis (Tsur, 1945; Tsur & Adler, 1965; Malmquist et al. 1970; Stagg, 1974; Hooshmand-Rad, 1975; Young, 1977).

Attempts to derive a laboratory model animal for Theileria have been unsuccessful. Irvin et al. (1975b) found that T. parva macrogametocyte infected cells proliferated within irradiated athymic mice to produce large tumours, but did not transfer into host cells. This characteristic of tumour formation in mice, together with the observation of continuous reproduction in culture, confirms the use of the descriptive term "transformed" for macrogametocyte infected leucocytes (aspects of transformation are reviewed by Ling & Kay, 1975). Thus, although infected leucocytes resemble leucocyte blasts, induced by mitogenic lectins or antigens, they differ as leucocyte blasts are transiently proliferative and do not induce tumour formation.
In the case of *T. annulata*, macroschizont infected cell lines are the basis of a readily produced live vaccine, which is efficient in protecting bovids against sporozoite challenge. This type of vaccine has been used in several endemic countries with successful results (Pipano, 1977a; Hashemi-Fesharki, 1988). The same approach has been tried with *T. parva*, with less success (Brown et al. 1978), and the reasons for this have not yet been clarified (see section 1.5.1.).

There are several problems associated with cell line vaccines, and one of the most basic is the risk of fatality if too virulent a stock of the parasite is used. Non-viable macroschizont infected cell lines will not immunise against homologous sporozoite challenge (Pipano et al. 1977b with *T. annulata*; Wagner et al. 1974 with *T. parva*). However, cell lines which are maintained in culture over extended periods (six months to three years) produce an attenuated infection in bovids, in which parasitosis and parasitaemias are rarely patent (Pipano & Tsur, 1966; Pipano, 1981 with *T. annulata*). Thus infected cell lines become modified in culture, such that they are more safely used as a vaccine. This has been a useful finding but the mechanism of attenuation has not been elucidated.

Sporozoite invasion and macroschizont development in *vitro* forms the basis of a convenient assay for factors which might inhibit these events *in vivo*: for example, drugs (McHardy, 1976; Melhorn & Raether, 1986; Pinder et al. 1981) and bovine sera (Preston and Brown, 1985). Furthermore, it has been possible to study cellular immune effector mechanisms (Pearson et al. 1979) as peripheral blood mononuclear cells may be isolated from a calf which has been immunised against a stock of *Theileria* and tested for their ability to kill autologous infected cells.

The availability of these culture systems has also lead to a means of producing cloned cell lines (Irvin, 1987). The valuable study of a uniform clone of macroschizonts in a uniform host cell is therefore feasible. Clones of piroplasms could be produced by infecting bovids with cloned cell lines and sporozoites could be harvested from ticks which had fed on these bovids. With the application of DNA probes, or other reliable markers of genetic diversity, the remaining problem of validating cloned
populations is also experimentally approachable. 

In vitro invasion by sporozoites, and macroschizont culture are now established: less well studied are the conditions required to trigger and sustain merozoite development from macroschizonts in vitro. The production of merozoites under raised culture temperature conditions has been demonstrated with T. parva (Hulliger et al. 1966) and recently with T. annulata (Fritsch et al. 1988). The subsequent entry of induced merozoites into red blood cells in vitro has also been observed (Danskin & Wilde, 1976; Fritsch et al. 1987). In stationary cultures of infected red blood cells, piroplasm division takes place, but there is no evidence for invasion, by the division products, into red blood cells in vitro (Conrad, 1983).

Progress in tick-stage development in vitro has been reviewed by Kirtii (1979). Primary cultures and cell lines of R. appendiculatus have been generated, but no parasites have been cultured. However, backless tick-explant culture systems have been shown to accommodate the production of kinetes from infected blood (Bell, 1980).

In summary, because of the autonomous transforming nature of Theileria macroschizonts and much innovative experimentation by those involved in developing the first macroschizont culture systems, these stages can be routinely maintained. Sporozoite availability is dependent upon infecting bovids, but these stages can infect isolated peripheral blood mononuclear cells to generate macroschizont infected cell lines. Piroplasm stages can only be obtained from infected bovids at present, but as merozoite production from macroschizonts, and invasion of red blood cells, have been demonstrated in vitro, it is reasonable that the induction of piroplasm stages is practicable. Similarly, as kinetes can be generated in vitro, the possibility of inducing sporozoites is feasible, although not yet realised.
1.4. BOVINE IMMUNE RESPONSES IN THEILERIA INFECTIONS

1.4.1. Cell-mediated responses

Following inoculation with low doses of viable sporozoites, cattle can recover from the resulting infection and may be immune to homologous challenge. In the case of *T. annulata* infections, protective immunity is also frequently raised against heterologous sporozoite challenge. Similarly, inoculation of live attenuated macroschizont infected leucocytes results in an infection and generates protection against sporozoite challenge (Brown *et al.* 1973a; Pipano *et al.* 1966 & 1977). *T. parva* differs from *T. annulata*, with respect to this second immunisation route, in that many more *T. parva* infected cells must be inoculated in order to establish infection, and this aspect is reviewed in section 1.5.1. Both immunisation routes are dependent on the establishment of macroschizont infections within host cells. The immune status is retained for approximately three years and is not dependent upon circulating anti-*Theileria* antibodies (Wagner *et al.* 1974 with *T. parva*; Bell, 1986 with *T. annulata*). Emery (1981) showed that adoptive transfer of thoracic duct leucocytes, from immune cattle to their naive chimaeric twin, conferred immunity on the recipients, which suggested that cell mediated responses were important in immunity. Using in vitro assays, Eugui & Emery (1981) showed that isolated peripheral blood leucocytes from immune bovids possessed a specific lytic activity against autologous *T. parva* macroschizont infected cells. These cytotoxic cells failed to lyse allogeneic infected cells and uninfected xenogeneic cells. Preston *et al.* (1983) showed a similar response in *T. annulata* immune bovids, and Innes *et al.* (1989a & b) have demonstrated that part of the immune response is BoLA restricted (BoLA is the bovine MHC, see footnote*), while an unrestricted cytotoxicity was also detected. Morrison *et al.* (1987), have demonstrated, with *T. parva* lysis of macroschizont infected cells by immune cytotoxic T-cells, which was BoLA restricted and parasite specific. The BoLA A locus products were matched in these experiments: this locus codes for class 1 products, therefore the cytotoxic T-cells were said to be class 1 restricted. There is some evidence that parasite specific
T-helper cells are also generated and that class II restriction may be important (Gooderis et al., 1986; Morrison et al., 1989).

As reviewed by Hall (1988), work with *T. annulata* (Preston, 1981; Preston & Brown, 1988) has demonstrated a role for adherent cells (macrophages), in protective immune responses. Cytostatic macrophages were generated by immunisation with either sporozoites or macroschizont infected leucocytes. These cells delivered an equally strong cytostatic effect against autologous and allogeneic cell lines, infected with homologous or heterologous *T. annulata* stocks, but they had no effect on uninfected peripheral leucocytes. This cytostatic activity was mediated by a soluble factor and it was suggested that this could be Tumour Necrosis Factor. Thus while MHC restricted cytotoxic mechanisms are undoubtedly important, further study may show that other responses are also important in providing immunity.

Much work has centred on identifying the infection-specific targets of the *Theileria* cytotoxic response, which the evidence suggests should be present on the surface of host leucocytes. Monoclonal antibodies have been generated which exclusively recognise *T. annulata* infection-specific epitopes on the surface of infected leucocytes (Shiels et al., 1986a). The importance of these epitopes as cellular immune targets has not been determined, and it is not known whether they are of parasite or host origin. A different approach to the same question has been to use relevant effector T cell clones to identify and isolate infection specific target molecules (Morrison et al., 1986) however, to date, none have been characterised by this method.

1.4.2. Antibody mediated immunity

Using several different assay systems (immunofluorescence, complement dependent lysis, and antibody dependent cell mediated lysis), Creemers (1982) failed to detect antibodies in *T. parva* immune bovine sera, which recognised the surface of infected leucocytes. Similarly, *T. annulata* immune sera is negative in immunofluorescence assays for the infected leucocyte membrane (Shiels et al., 1986a). Thus, there appears to be no antibody
response against the surface of infected leucocytes, despite the fact that monoclonal antibodies have been generated which specifically recognise infection-associated epitopes on the surface of infected leucocytes (Shiels et al. 1986a).

Bovine sera from immune animals show a strong reactivity against all parasite stages in indirect immunofluorescent assays as reviewed by Irvin and Morrison (1987). As reviewed by Cowan (1981) for T. parva and reported by Bell (1986) for T. annulata, there appears to be no relationship between antibody titre during immunisation and subsequent immune status. Antibody titre and parasite-stage reactivity seems to record the duration of infection, the immunisation route and reflect, in part, the intensity of response to infection. As many factors, besides from these, probably act to determine the antibody response, the interpretation of any observed trend in antibody levels is beset with difficulties. Passive transfer of serum or purified antibody, from T. parva immune bovids to naive recipients failed to confer immunity (Muhammed et al. 1975), which suggested that antibodies do not play a role in immunity. On the basis that cell-mediated immune mechanisms are essential for immunity, but antibody responses might have a limited effect on Theileria infections, a small amount of work has been performed to investigate the role of antibody in the immune response.

Although the sporozoite is not an essential target for an effective immune response, because immunity can be engendered with macroschizont infected cell lines alone, it has been postulated that an antibody response against the sporozoite may be protective. In vitro sporozoite invasion assays have been used to demonstrate that immune serum neutralises the infectivity of sporozoites for bovine leucocytes (Gray & Brown 1981 with T. annulata; Musoke et al. 1982 with T. parva). Although species specific in their activity, the sera were found to neutralise the infectivity of heterologous isolates. In further work with T. annulata (Preston & Brown, 1985) immune sera was added to leucocytes immediately after they had been infected. This had an inhibitory effect on the subsequent development of trophozoites into macroschizonts. It was suggested that this activity could be mediated by antibodies binding to sporozoite antigens which were
deposited on the surface of leucocytes during invasion.

In summary, an MHC-restricted, cytotoxic T-cell-mediated immune response, which is effective in lysing macrogenchizont infected leucocytes, and a sporozoite invasion-blocking activity of bovine immune sera have been identified. In addition, there is evidence that macrophage secreted factors have a cytostatic effect on developing macrogenschizonts. There is no evidence for antibody dependent cellular cytotoxicity against infected leucocytes or any form of cell mediated cytotoxicity against intraerythrocytic or extracellular stages.

*Footnote—the Bovine lymphocyte antigens (BoLA) are considered to be the bovine MHC, equivalent to the HLA system in man and H-2 in mice. The BoLA system has been partially defined using alloimmune sera in lymphocytotoxicity tests. These serologically defined antigens have been given workshop specificities (Spooner et al. 1979). They are analogous to polymorphic MHC class I determinants which are expressed on most nucleated cells. Class II determinants have also been identified on bovine B cells and antibodies against class II epitopes have been produced, however serological markers are not yet established (Spooner, 1986). Cell lines and recipient cattle are said to be "matched" if they share BoLA specificities; the assumption being that both parties recognise matched cells as "self". An important finding was that macrogenchizont infected cell lines retain their MHC (class I) molecules in vitro (Spooner & Brown, 1980).
Strategies for Theileria control and their implementation are not well advanced or integrated, and theileriosis is currently a major constraint on livestock production in endemic countries.

1.5.1. Live vaccines

The acquisition of immunity by establishing a mild infection seems to be similar for T. parva and T. annulata, however there are two important differences. First, protection against heterologous sporozoite challenge is not common with stocks of T. parva (Radley et al. 1975; Irvin, 1983), but is common with T. annulata stocks (Pipano, 1981; Gill et al. 1980a; Ozkoc & Pipano, 1981). Secondly, with cell line immunisations, BoLA histoincompatibility is a major barrier to establishing infection with T. parva (Dolan et al. 1984b) but does not act as an operational barrier for T. annulata infection (Innes et al. 1989a; Brown, 1981).

The failure of T. parva infected, mismatched cell lines to establish an infection as readily as T. annulata mismatched lines is not understood although there are several lines of speculation. It has been suggested that T. parva transforms helper T-cells preferentially (Lalor, 1985; Morrison et al. 1989). Other evidence suggests that T. annulata and T. parva differ because peripheral blood leucocytes, transformed by T. parva, express helper T-cell markers or cytotoxic T-cell markers or both, while T. annulata transformed cell lines are negative for both helper and cytotoxic T-cell markers (Spooner et al. 1989). It has been proposed that macroschizonts have different abilities to transfer to the new host leucocytes, depending on the original host leucocyte sub-type. If T. parva does not transfer into new host leucocytes, and instead proliferates within inoculated leucocytes, this would explain the observed BoLA barrier to immunisation. There is evidence that T. parva transfers in vitro and in vivo (Brown et al. 1973) however, the efficiency of transfer is much lower than with T. annulata.
At present, it is difficult to assess the role which host leucocyte type may play in producing this difference between T. parva and T. annulata infections. The consequence, however, is that while attenuated cell line vaccines have been used successfully in several countries against T. annulata (in Iran, Hashemi-Fersharki, 1988; in India, Singh, 1986; in Turkey, Sayin, 1986; in Israel, Pipano, 1981; in the USSR, Stepanova, 1977); alternative methods of control have been sought for T. parva infections in Africa. The most promising of these is a program of infection with sporozoite stabilate followed by drug treatment with tetracyclines (Cunningham, 1977; Uilenberg et al. 1977; Radley, 1981). This method has also been used with good results against T. annulata infections (Pipano, 1981; Gill et al. 1978). As with the macroschizont cell line vaccine, there are problems of parasite virulence and stock specific immunity associated with the sporozoite vaccine.

1.5.2. Chemotherapy and tick control

Tetracyclins suppress the production of macroschizonts, when administered during the prepatent period after sporozoite inoculation, and various formulations are used prophylactically in the infection and treatment regime described above. They act by inhibiting protein synthesis and are marginally effective in treatment of clinical theileriosis, but do not affect sporozoite or piroplasm stages. Two other classes of compound, which are effective against macroschizont infections, have been singled out for development. These are analogues of naphthoquinone derivatives (993C and 720C: Coopers Animal Health) and a febrifugine, (halofuginone lactate). 993C has been placed on the market (as Clexon: Wellcome) but it is prohibitively expensive, only works well if administered early during an infection and is not effective against piroplasm stages.

Acaricides are used widely for vector control in endemic areas and these are marginally effective in protecting herds. The method is impractical as acaricides have to be applied very frequently. There have been no reports of acaricide resistant ticks, however the possibility of future resistance has discouraged
total dependence on this form of control (Cunningham, 1981). As this is the most widely used control measure in Africa, where East Coast fever is becoming more prevalent, it is clearly not a sufficient method of control in its present state.

1.5.3. Molecular vaccines

With the recent advances in recombinant DNA technology and immunology, it may be possible to genetically engineer an effective vaccine against theileriosis. The advantages of such a method of control are manifold. Perhaps most importantly, a synthetic vaccine could be cheap and therefore widely used. Furthermore the technology might allow a flexible vaccine product, targeted against several stages or stocks of the parasite at once. Molecular vaccines exist for viral diseases, namely hepatitis, rabies and foot and mouth disease, but none have been produced for parasitic diseases. The first of many stages in rational molecular vaccine work is to identify relevant immunogenic molecules. Candidate molecules have been identified in Plasmodium (Hall et al. 1984; Herrington et al. 1987) and in Schistosoma (Balloul et al. 1987). Theileria molecular vaccine work is just beginning. Williamson et al. (1989) have used monoclonal antibodies to clone the T. annulata gene for an epitope which resides on the surface of sporozoites. Antibodies against the polypeptide inhibit invasion of sporozoites, thus the molecule is a candidate for part of a sub-unit vaccine for theileriosis.

1.6. PROJECT WORK

The preceding introductory sections show that the post-macroschizont stages of the life cycle in the bovine host are poorly understood at every level. This has probably stemmed from the idea that merozoites are inaccessible and therefore impractical to study and that the piroplasm is a dead-end stage in the bovid. By raising monoclonal antibodies against infected red blood cell material it was hoped that interesting probes would be generated which would enable a molecular characterisation and better definition of these stages. As T. annulata, T.
sergenti and T. mutans produce haemolytic diseases, basic information concerning red blood cell stages was seen as important information which might be used to rationalise strategies to define protective measures in effective long term control policies. Current theileriosis control measures do not act directly against the merozoite or piroplasm stages and the bovine immune response to these stages has not been characterised. Moreover, it is not known whether theilerial piroplasms or red cell merozoites reinvade red cells. If a cycle of reinvasion does take place this would have an important effect on the course of infections. This project set out to study these stages and define possible targets for immune attack which could be used as part of a molecular sub-unit vaccine. Initial experiments (chapter three) focussed on investigating whether infection specific erythrocyte membrane proteins (ISEMPs) are present on the surface of infected red blood cells. On the basis of the results obtained in these experiments it was determined that an infected red blood cell membrane preparation was required in order to continue this investigation and this study is presented in chapter four. A body of data generated in these studies which were formally peripheral to the aims of the experiments undertaken. This data comprised a preliminary characterisation, in terms of the molecular mass of infected erythrocytes polypeptides and it is presented in appendix form (appendix 1).

To initiate investigations into the molecular basis of differentiation events in Theileria, anti-piroplasm monoclonal antibodies were screened against sporozoite, macroschizont and in vitro produced merozoite life cycle stages in order to identify stage specific epitopes. The characterisation of the reactivity of monoclonal antibodies is presented in chapter five. Chapter six presents consequent experiments undertaken to characterise and to clone the gene coding for the epitope recognised by one of these stage specific monoclonal antibodies and describes the observations from experiments carried out to determine the basic parameters required to generate merozoites in vitro. Thus the two related long term aims outlined on page one were addressed: the generation of a subunit vaccine against theileriosis and the characterisation of stage differentiation events at the molecular level.
CHAPTER TWO
MATERIALS AND METHODS

2.1. PARASITES, CELL LINES AND SERA

Three uncloned stocks of T. annulata were used in different experiments, depending on their availability:

- T. annulata Hissar from India (Gill, 1976)
- T. annulata Ankara from Turkey (Schein, 1975a)
- T. annulata Gharb from Morocco (Ouhelli, 1985).

The terms isolate, stock, strain and clone are used in the specific manner in which they are applied to trypanosomes (WHO, 1978).

2.1.1. Collection of infected blood

Piroplasm infected blood was obtained from Mr. C.G.D. Brown at the Centre For Tropical Veterinary Medicine (CTVM) Edinburgh. Calves were infected by sub-cutaneous inoculation of potentially lethal doses of sporozoites, and then maintained in an isolation unit. 10-20 days later, when piroplasm parasitaemias were between 20-80%, calves were bled by jugular puncture and free flowing blood was collected into anticoagulant. Pre-infection blood and uninfected control blood (cow110) was collected by the same method.

When the blood was to be used for experiments involving the red blood cell membrane, it was collected into citrate-phosphate-dextrose-adenine (CPDA-1, 14.3 mM citric acid, 89.4 mM sodium citrate, 14.1 mM sodium dihydrogen orthophosphate, 193.3 mM glucose monohydrate, 3.26 mM adenine hydrochloride, pH 7.2). Fungicides and antibiotics were also required and amphotericin-B solution and gentamycin sulphate were added to give final concentrations of 75 μg/ml and 50 μg/ml respectively. Blood was diluted 1: 0.15 in CPDA-1 and it was determined that blood could be stored for up to 3 weeks at 4°C (section 4.3.1.).

In a single experiment (see 4.4.1. for details) blood was collected into CPDA-1 but stored at 4°C in RBC storage solution, (110 mM glucose, 55 mM mannitol, 25.8 mM disodium hydrogen...
orthophosphate, 2mM adenine hydrochloride, 14.7mM sodium dihydrogen orthophosphate, 17.9mM potassium citrate, 50mM ammonium chloride, pH 7.1) as described by Meryman (1986). Amphotericin-B and gentamycin sulphate were also included to give final concentrations of 75µg/ml and 50µg/ml respectively. Blood in CPDA-1 was centrifuged at 800g for 10 minutes and the pellet resuspended in an equal volume of RBC storage solution. Where blood was required for isolating piroplasms, it was collected into a final concentration of Heparin of 10 units/ml.

2.1.2. Giemsa staining

Infected red blood cells were applied to glass slides as a monolayer, air dried and fixed in methanol for 10 minutes. Macroschizont infected leucocytes and merozoites from cell line cultures were applied to glass slides by cytocentrifugation. Cell suspensions (50µl, 1-5x10⁶ cells/ml) were cytocentrifuged at 500g (2,000rpm) for 10 minutes, the slide material was air dried and fixed in methanol for 10 minutes.

Fixed slide preparations were incubated for 45 minutes in a 1:20 dilution of Giemsa staining solution (BDH) in Giemsa buffer (0.15M sodium phosphate buffer, pH 8.0). The stained slide material was rinsed in distilled water, air dried and examined under the 50x or 100x objective of a light microscope under oil immersion.

2.1.3. Removal of peripheral blood leucocytes

Blood was centrifuged at 800g for 10 minutes and the resulting plasma and buffy layer of leucocytes were discarded. The cells were washed 3x in 5-10 volumes phosphate buffered saline (PBS, 137mM sodium chloride, 2.682mM potassium chloride, 8.1mM di-sodium hydrogen orthophosphate, 1.47mM potassium di-hydrogen orthophosphate, pH 7.2, pre-chilled to 4°C) and then further depleted of leucocytes and platlets by passage through a CF11 cellulose (Whatman) column as described by Williams & Richards (1973). A CF11 column was constructed by cutting a hole in the base of a 50ml conical centrifuge tube so as to accept an eppendorf
vial. Having removed the base of the eppendorf vial with a scalpel, it was filled with glass wool and inserted into the hole in the centrifuge tube. Then 30ml dry CF11 was added to the tube and 200ml of PBS was passed through to pack and pre-wet the column. The column was loaded with 25ml packed red blood cells resuspended in four volumes of PBS. When the cell suspension had passed into the column, red blood cells were eluted with 50-100ml PBS. To check that leucocytes were absent from the eluate, 20ul of packed cells (2x10⁷ cells) were added to 80ul of 1% acetic acid to selectively lyse red blood cells. This sample was examined in a haemocytometer by light microscopy and if leucocytes were observed then the procedure was repeated with a fresh column of CF11 cellulose.

Red blood cells were washed 1x in PBS; with the exception of red blood cells which were intended for use in metabolic labelling studies, the PBS was supplemented with a mixture of protease inhibitors (PBSpi - PBS supplemented with 1mM phenylmethyl sulphonyl fluoride (PMSF) from a freshly prepared stock solution of 100mM in acetone, 0.1mM N-p-tosyl-l-lysine chloromethyl ketone (TLCK) from a stock solution of 10mM in distilled water, 0.1mM N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) from a stock solution of 10mM in distilled water, 0.25mM benzamidine hydrochloride from a stock solution of 25mM in distilled water, 100 Kallikrein Inhibitor Units (KIU)/ml aprotinin, 5mM iodoacetamide from a stock solution of 500mM in methanol)

2.1.4. Isolation of piroplasms

Piroplasms were prepared by the ammonium chloride lysis method of Martin et al. (1971). After CF11 processing (2.1.2.), washed and packed red blood cells were resuspended in 1 volume of PBS and then red blood cells were selectively lysed by the addition of 10 volumes of warm (37°C) Tris-buffered ammonium chloride (1 volume 0.17M Tris-HCl, pH 7.85 and 9 volumes 0.83% ammonium chloride, pH 7.4). The cells were incubated at 37°C until lysis was observed (after 3-5 minutes). Piroplasms were harvested by centrifugation at 300g for 10 minutes at 4°C and washed 5x with PBSpi.
2.1.5. **T. annulata** infected cell lines

The **T. annulata** macroschizont infected cell lines and uninfected lines used in experiments were originally provided by Mr. C.G.D. Brown (CTVM); these were as follows:

- **TaA4S** which is a *Theileria annulata* Ankara infected cell line, derived by *in vitro* sporozoite infection of peripheral blood mononuclear cells (PBM) from calf 46 (Brown, 1973)
- **TaH4e** which is a **T. annulata** Hissar infected line derived as above
- **TaA1** which is a **T. annulata** Ankara infected line derived (as above) by infection of PBM from calf 1 (similarly, line TaA2 from calf 2)
- **BL-20** which is an uninfected, non-viral, bovine lymphosarcoma cell line (Morzaria, 1984)
- **TaHBL20** which is a **T. annulata** Hissar infected equivalent of BL-20.

Cell lines were cultured at a cell density of between $10^5$ and $10^8$ cells/ml by thrice weekly dilution 1:5 to 1:10 in complete Ta medium (RPMI 1640 (Gibco), 25mM HEPES buffer, 8.33mM (0.07%) sodium hydrogen carbonate, 2mM L-glutamine, 10mM 2-beta-mercaptoethanol, 0.75µg/ml amphotericin-B solution (Fungizone, Gibco), 50µg/ml gentamycin sulphate or 50units/ml penicillin and 50µg/ml streptomycin, 15% mycoplasma screened, heat inactivated (56°C for 30 minutes), foetal calf serum (Gibco), pH 7.2). The cell lines were maintained, at 37°C with 5% CO$_2$ in a humidified incubator, as 10ml cultures in 25cm$^2$ flasks giving a medium depth of 4mm.

Cultured cell lines were cryopreserved in the presence of 10% dimethylsulphoxide (DMSO) as follows (Brown, 1979). 10ml cultures ($10^7$ cells) were centrifuged at 200g for 7 minutes and the pellet resuspended in 1ml ice cold complete Ta medium. An equal volume of cold freezing medium (complete Ta medium, 20% DMSO) was added dropwise and four 0.5ml aliquots (2.5x$10^8$ cells) were dispensed into freezing vials. The vials were held in a pre-cooled polystyrene insulating box at -70°C for 24 hours and then transferred to liquid nitrogen. To remove cells from liquid
nitrogen, vials were placed at 37°C and when the contents were sufficiently thawed the cells were poured into 10ml warm complete Ta medium. The cells were centrifuged at 2000g for 7 minutes, the pellet resuspended at approximately 2x10^6 cells/ml in 10ml complete Ta medium and placed at 37°C, 5% CO₂.

2.1.6. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBM) were isolated by density gradient centrifugation (Brown, 1979). 50ml of blood (cow 110) was collected into a heparinised vacutainer, transferred to a centrifuge tube and the cells were centrifuged at 2000g for 15 minutes. PBM, which formed a buffy layer on top of packed red blood cells, were aspirated in a maximum volume of 3ml and mixed with 9ml PBS. The cell suspension was layered onto 8ml of Ficoll-Paque (specific density 1.077g/cm³, Pharmacia) and centrifuged at 800g for 35 minutes at 15°C. Unlike red blood cells and granulocytes, PBM do not enter the Ficoll-Paque, so they could be collected from their banding position at the interface between the upper layer, comprising PBS, plasma and platelets, and the lower layer of Ficoll-Paque. PBM were recovered from the interface region, resuspended in 50ml PBS and centrifuged at 300g for 10 minutes. The pellet was resuspended in PBS and the washing step was repeated. As determined by haemocytometry, 50ml of blood yielded approximately 5x10^8 PBM.

2.1.7. Concanavalin A stimulated PBM

Peripheral blood mononuclear cells were treated with the T-cell mitogen, concanavalin A to induce proliferation (Bradley, 1980). PBM were resuspended in complete Ta medium (2.1.5) with 2μg/ml concanavalin A (Sigma Type 1V-S) at a cell density of 2x10^6/ml. The cells were incubated at 37°C, 5% CO₂ in a humidified incubator for 48-72 hours, by which time the culture contained a proportion of enlarged blastoid cells. These were harvested by centrifugation (300g, 5 minutes) and washed 3x in PBS.
2.1.9. Preparation of heat induced merozoites.

Macroschizont infected cell lines were maintained in culture at 37°C as described in section 2.1.5. To induce differentiation into merozoites, these cells were transferred, in closed flasks, to a 41°C incubator. The initial cell density was 1x10^6/ml, and for the first 5-9 days of incubation, cells were maintained at 1-5x10^6/ml by daily dilution (1:10) in fresh complete Ta medium. Thereafter, the rate of host cell division decreased, and cultures were maintained by daily dilution (1:5) in the same medium.

In order to monitor the progress of macroschizont differentiation into merozoites within these cultures, Giemsa stained cytocentrifuge preparations (section 2.1.2.) were prepared and examined on a daily basis. After approximately 10-15 days, the majority of host cells ceased to divide and medium was then provided by pelleting the cells by centrifugation at 2,000g for 10 minutes followed by resuspension to the original volume in fresh medium.

Whilst host leucocytes, containing presumptive merozoites, were still intact IFA slides were prepared according to the procedures detailed in section 2.3.1.1. for leucocytes. On approximately day 12 of heat induction, many merozoites were observed in the culture medium, and the IFA slide preparation protocol was altered by performing centrifugation steps at 3000g so that merozoites would be pelleted. On day 15, when many host cells were fragmented, and free merozoites were abundant, merozoites were isolated from heat induced cultures, for slide preparation or Western analysis, by differential centrifugation. For this, cultures were centrifuged at 300g for 10 minutes to remove the bulk of intact leucocytes. The supernatant was centrifuged at 4000g for 15 minutes at 4°C, and the resulting pellet was resuspended in 1.5ml PBSpi and washed x4 in PBSpi by centrifugation at 9000g for 1 minute. For IFA slides; 10ml cultures of 50% differentiated macroschizonts yielded 1-2μl merozoite pellets which were resuspended in 0.5ml PBS prior to spotting onto IFA multi-spot slides. For Western analysis: 1-2μl merozoite pellets were resuspended in 10μl PBSpi and then solubilised in 100μl SDS sample buffer. Approximately 30-60μl of clarified, extracted material was loaded per track of a 150mm SDS-PAGE gel.
2.1.8. Bovine sera

Serum 155. Calf 155 was infected (day 0) with *T. annulata* Ankara sporozoite stabilate and treated on day 10 with Parvaquone (Clexon, Coopers Animal Health). On day 28, the calf was challenged with sporozoite stabilate and it recovered. On day 69 the calf was splenectomised, it relapsed and then recovered. On day 144 it was challenged with Ankara sporozoite stabilate, subsequently exhibited high parasitaemias and was destroyed on day 174. Serum was prepared on day 170, 9 days after the second challenge.

Serum 128. Calf 128 was infected with *T. annulata* Hissar sporozoites and on day 18 the calf exhibited a 3% parasitaemia. The calf was challenged twice with sporozoites and serum was collected 28 days after the last challenge on day 100.

2.2. PRODUCTION OF MONOCLONAL ANTIBODIES

2.2.1. Immunisation protocol

Two BALB/c mice were immunised with three intra-peritoneal injections, at two weekly intervals, of $2 \times 10^7$ Ankara infected red blood cells exhibiting a 40% parasitaemia. The red blood cells (2.1.3.) were stored at -70°C over the immunising period by the method of Trager (1976). Briefly, the red blood cells were packed by centrifugation at 500g for 10 minutes and an equal volume of a solution of 28% glycerol, 3% sorbitol and 0.85% sodium chloride was added dropwise, with mixing, at room temperature. Aliquots (0.1ml) of $2 \times 10^7$ cells were added to freezing vials, held at -70°C for 24 hours and then transferred to liquid nitrogen. Cells were thawed rapidly at 37°C, made up to 0.5ml in PBS and injected. This method of storage was chosen in an attempt to keep the immunising cells, a) uniform over the immunising period, so that the immune responses to secondary injections were a true consolidation of reactions to primary injections, and b) intact because intact cells are generally highly immunogenic (Goding, 1983).
One week after the final injection, 100µl of blood was taken from the orbital sinus of each mouse. The blood was centrifuged at 300g for 10 minutes and antibodies in the supernatant were detected, by indirect immunofluorescence assay (2.3.1.), which recognised acetone fixed preparations of the immunising cells. Three days before the day of the fusion and one month after the last immunisation, the mice were boosted with an intra-venous injection of 10⁴ cells.

2.2.2. Myeloma cell line

The Mouse myeloma cell line, P3-X63/Ag8 (Kearney, 1979. Clone 653 from Flow Laboratories), was cultured in complete hybridoma medium (RPMI 1640 medium (Gibco), 25mM HEPES buffer, 2mM L-glutamine, 8.33mM (0.07%) sodium hydrogen carbonate, 10mM 2-beta-mercaptoethanol, 0.75µg/ml amphotericin-B solution (Fungizone. Gibco), 50µg/ml gentamycin sulphate or 50 units/ml penicillin and 50µg/ml streptomycin (Gibco), 15% heat-inactivated (58°C, 30 minutes), mycoplasma screened, Myoclone foetal calf serum (Gibco), pH 7.2).

Myeloma cells were removed from liquid nitrogen storage (2.2.5. and 2.2.6.) and cultured, for 10 days prior to their use, as exponentially growing cultures, at 10⁴-10⁵ cells/ml, by thrice weekly dilution 1:5 or 1:10 in complete hybridoma medium. Cell cultures containing more than 90% viable cells were used in fusion experiments and viability was assessed by Trypan Blue exclusion (Mishell 1980). For this, 100µl cell culture was added to 100µl of 0.2% (w/v) Trypan Blue in PBS and the cells were immediately examined in a haemocytometer under the x10 objective of a light microscope. The number of viable cells, which were impermeable to the dye, was expressed as a percentage of the total.

2.2.3. Fusion protocol

Polyethylene glycol fusion protocols were performed according to those outlined by Galfre and Milstein (1980) and Goding (1983). 5x10⁷ viable myeloma cells were centrifuged at 200g for 10 minutes and washed 2x in incomplete medium (complete medium detailed in
2.2.2. lacking foetal calf serum). Spleen cells from an immunised mouse were dissected into 5ml of incomplete medium in one side of a tilted petri dish. Clumps of cells were broken up and organ debris removed by gently resuspending the cells through needles of decreasing gauge (20G, 21G and 26G). The cells were then centrifuged at 200g for 7 minutes at room temperature, washed 2x in incomplete medium, and resuspended in 10ml of incomplete medium at 37°C. Cell numbers were estimated by haemocytometry, after selectively lysing red blood cells in twice the packed cell volume of 1% acetic acid.

10⁷ myeloma and 10⁶ spleen cells were mixed in a test tube, centrifuged at 200g for 7 minutes and all the supernatant was removed. 0.5ml of 41.6% polyethylene glycol with 15% dimethylsulphoxide in incomplete medium, was added gradually with mixing over a period of 1 minute; followed by 0.5ml of 25% polyethylene glycol in incomplete medium, added with mixing over 1 minute. The cell suspension was diluted by addition of 10ml complete hybridoma medium which was added gradually over 2-5 minutes with continuous mixing. Finally, the total volume was made up to 50ml with the addition of 40ml of complete hybridoma medium. The cells were plated out at 10⁶/ml/well, in 96-well microtitre tissue culture plates and placed in a humidified incubator at 37°C, 5% CO₂.

2.2.4. HAT selection

The following day, hybrid cells (i.e. myeloma-spleen cell fusion products) were selected by the addition of hypoxanthine, aminopterin and thymidine (HAT, 1xHAT medium - 10⁻⁷M hypoxanthine, 4x10⁻⁸M aminopterin, 1.6x10⁻⁹M thymidine, complete hybridoma medium, 20% peritoneal macrophage conditioned medium (2.2.6.)). The cells were incubated for 10 days, after which time each well was screened by light microscopy to detect viable fused cells. These cells were provided with fresh complete hybridoma medium and the original medium was gradually diluted by replacing half the medium in each well every 2 or 3 days.
2.2.5. Hybrid cell culture

When cells were nearly confluent, supernatants were assayed using an indirect immunofluorescence antibody test (2.3.1.) for the presence of antibodies which recognised infected red blood cells. Cells from wells which were positive in this respect were expanded into larger 24-well tissue culture plates with peritoneal macrophage conditioned medium (2.2.6.). Once growth was established, cells were transferred to 25cm² culture flasks and maintained as 10ml cultures at a cell density of 2-5x10⁵ cells/ml. Exponential growth was maintained by thrice weekly dilution 1:5 or 1:10 in complete hybridoma medium. If hybridoma lines failed to continue growing exponentially after dilution, the cells were centrifuged (200g, 5 minutes) and resuspended in 3ml of peritoneal macrophage conditioned medium in a flask which was tilted so that cells were concentrated at the bottom of the flask. Once growth was established the culture volume was made up to 10ml with complete hybridoma medium. Samples of individual cultures were cryopreserved (2.2.7.) at this early stage in an attempt to provide a back up of cells in the event of cultures becoming contaminated. Spent culture supernatant was stored at 4°C for short periods, or at -20°C for extended periods, with the addition of sodium azide at a final concentration of 10mM.

2.2.6. Preparation of peritoneal macrophages

Growth stimulants from murine peritoneal macrophages were used to overcome the problem of poor growth of hybridoma cell at low cell densities. The peritoneal cavity of a sacrificed BALB/c mouse was injected with 5ml of cold (4°C) incomplete medium (2.2.3). The abdomen of the mouse was agitated to suspend cells in the medium and then this fluid was withdrawn by syringe. The cells were centrifuged at 300g for 5 minutes, the supernatant was removed and the pellet resuspended in 10-20ml of complete hybridoma medium (2.2.2.). The cells were maintained at 37°C, 5% CO₂ for 2 days before use to ensure that bacterial and fungal contaminants were absent.
2.2.7. Cryopreservation of myeloma/hybridoma cells

10ml cultures in exponential phase of growth (3-5x10^8/ml) and more than 90% viable (2.2.1.) were centrifuged at 200g for 5 minutes. The supernatant was removed and the cells resuspended in 1ml (5x10^8 cells/ml) of ice-cold complete hybridoma medium (2.2.2.). Then 1ml of cold freezing medium, (20% dimethylsulphoxide (DMSO) in complete hybridoma medium) was added dropwise with mixing and the suspension was transferred in 0.5ml (approximately 10^8 cells) aliquots to freezing vials. The vials were placed on ice, transferred to a pre-cooled polystyrene insulating box and then held at -70°C for 24 hours before transfer to liquid nitrogen.

2.2.8. Recovery of myeloma/hybridoma cells from liquid nitrogen

Frozen vials were thawed rapidly at 37°C followed by the addition of 1ml of warm complete hybridoma medium (2.2.2.). The cell suspension was poured into 7ml of warm medium, centrifuged at 200g for 5 minutes and the pellet was resuspended in 1ml of warm medium. A sample was examined by light microscopy to assess cell viability. If the majority of cells were viable, 100μl of the cells (approximately 10^8 cells) was added to 1ml of peritoneal macrophage conditioned medium (2.2.6.) in a single well of a 24-well culture plate and incubated overnight at 37°C in 5% CO2. If growth was obviously established then cells were transferred to 25cm^2 flasks in 5ml of fresh medium. If only a minority of the cells were viable, then a series of two fold dilutions were prepared in conditioned medium in order to obtain an optimum cell density for cell growth.

2.2.9. Cloning hybridoma cells by limiting dilution

Cell density was determined by haemocytometry and 320 hybridoma cells were added to 2ml of medium. A series of 5 doubling dilutions were prepared in peritoneal macrophage conditioned medium so that the final dilution contained 5 cells/ml. Samples of each dilution were plated out at 100μl/well in 96-well tissue culture plates and placed at 37°C, 5% CO2. Five days later the wells were
screened for the presence of single cells, or single clusters of cells: such wells were noted, and once the cells were confluent, supernatants were assayed by IFA for evidence of antibody production (2.3.2.1.). Cloned hybridoma cell lines were cultured up to 10 ml cultures, the supernatants from spent cultures were stored (2.2.3) and the cells cryopreserved (2.2.5).

2.3. IMMUNOFLUORESCENCE ASSAYS

Indirect immunofluorescence assays (IFAs) were performed with fixed cells (Goddeeris, 1982; Burridge, 1971) and viable unfixed cells, using fluorescein isothiocyanate (FITC) conjugated anti-IgG second antibody. In a small number of experiments, an FITC conjugated streptavidin-biotin second antibody system was used (adapted from McLean, 1986).

2.3.1. Fixed cell indirect immunofluorescence assay

2.3.1.1. Slide preparation

Cells were applied to multispot slides (Hendley-Essex). Slide preparations were air dried at 37°C and stored at -20°C in resealable plastic bags with indicator silica gel to exclude moisture. Slide preparations of several cell types were produced as follows:

- Red blood cell slides
Blood (1 ml) was centrifuged at 800g for 10 minutes; the supernatant discarded and the cells resuspended in 10 ml PBS. After two further washes in PBS, the cells were diluted to a density of approximately 3 x 10^7/ml and spotted onto slides. The preparations were air dried at 37°C and then fixed in acetone (Analar) for 10 minutes.

- Leucocyte slides
3-5 x 10^7 cells were centrifuged at 500g for 10 minutes, washed 3x in PBS and resuspended at a cell density of 10^7/ml in PBS. An equal volume of ice cold fixative (3.7% formaldehyde in PBS) was
added dropwise with mixing to give a final concentration of 1.9% formaldehyde. After 10 minutes on ice, the cells were washed 3x in PBS, resuspended in PBS at approximately $5 \times 10^8$ /ml and applied to slides by adding 100 μl of cell suspension, followed by removal of excess liquid.

- **Sporozoite slides**
  Formaldehyde fixed sporozoite slides were obtained from Mr. C.G.D. Brown (CTVM, Edinburgh). Sporozoites were purified from ground-up infected ticks (*Hyalomma anatolicum anatolicum*) by Percoll density gradient centrifugation (Walker & McKellar, 1983). The sporozoites were washed 3x in PBS, formaldehyde fixed as described above; washed a further 3x to remove the fixative, applied to slides and air dried.

- **Piroplasm slides**
  100 μl of packed, purified piroplasms (2.1.4.) were resuspended in 1ml PBS by gentle vortexing. Piroplasms were formaldehyde fixed and slides were prepared as described above.

2.3.1.2. Fixed cell IFA protocol

Multispot slides, stored at -20° C, were placed in a dessicator at room temperature for 30 minutes. 200 μl of test antibody was added to each well on the slide which was placed in a humidified box for 30 minutes. Unbound antibody was removed by sequentially washing the slides for 1, 5 and 10 minutes in PBS. The area surrounding the wells was dried and 150 μl FITC conjugated anti-IgG (molar ratio > 3.0, Sigma) at 1:100 dilution in PBS, pre-cleared by centrifugation at 13,000g for 10 minutes, was applied to each well. The incubation and washing steps were repeated and the slides counter stained in 0.1% Evans Blue in PBS for 5 minutes. The slides were mounted with a few drops of 50% glycerol in water with 2.5% w/v 1,4-diazabicyclo(2.2.2)octane (DABCO) pH 8.0 (Johnson 1982) to retard the fading of the fluorochrome. Fluorescing cells were visualised by examination with X50 or X100 objectives of a Leitz ortholux II transmitted light fluorescence microscope fitted with a WOTAN 50W super pressure mercury lamp and a Leitz filter block 12/3.
Photographs were taken using a Leitz orthomat-w microscope camera with Kodak film EES 135-36.

For experiments using the biotin-streptavidin second antibody system, biotin anti-IgG conjugate (Sera Labs) was used at a concentration of 15μg/ml and FITC conjugated streptavidin (Sera Labs) was used at 1.5μg/ml. Incubation and washing times were exactly as described above.

2.3.2. Surface, indirect immunofluorescence assay

The reactivity for antibodies for the surface of viable red blood cells and leucocytes was assessed with this assay. Damaged, or non viable cells, are permeable to antibody and experiments were controlled for internal labelling as described in the results sections. Assays were performed in the presence of 10mM sodium azide and at 4°C in order to prevent antibody-induced capping, endocytosis or shedding of surface antigens.

a) Red blood cells

Packed red blood cells (10^7 cells) were resuspended in 1ml cold (4°C) PBS:FCS (PBS, 5% foetal calf serum, filtered through 0.22μm nitrocellulose filters). The cells were centrifuged at 9000g for 1 minute and the pellet resuspended in 100μl of an appropriate dilution of test antibody in PBS. After 40 minutes at 4°C, with occasional mixing, unbound antibody was removed by washing 4x in 1.5ml cold PBS:FCS. FITC-conjugated anti-IgG (Sigma) at 1:100 dilution in PBS was pre-cleared by centrifugation at 13,000g for 10 minutes; the pelleted cells were resuspended in 100μl of the conjugate and the incubation and washing steps were repeated as described above. The final pellet was resuspended in 50μl PBS, 10μl was applied to a microscope slide, mounted with a coverslip, and the cells were examined under the 100x objective of a fluorescence microscope (2.3.1.).

In experiments where a biotinylated second antibody probe was used; biotin conjugated anti-IgG and FITC conjugated streptavidin were used at the concentrations given in 2.3.1. Both conjugates were pre-cleared by centrifugation at 13000g for 10 minutes prior to use. This three-stage assay was performed with
the same incubation, washing and mounting procedures used in the two-stage assay described above.

b) Leucocytes

Leucocytes were washed 3x in PBS:FCS and 5x10⁵ cells were resuspended in 100μl of appropriately diluted first antibody in an eppendorf tube. All subsequent steps were performed as for surface IFAs with red blood cells (2.3.2.a).

2.3.3. Monoclonal antibody isotype

The antibody isotype of monoclonal antibodies was determined by indirect immunofluorescence assays. The procedure for this assay was as described in 2.3.1., except that FITC conjugated anti-IgG was replaced by a panel of FITC conjugated sheep anti-mouse Ig subtypes (The Binding Site, Birmingham). Each monoclonal antibody, as spent supernatant, was distributed onto 11 wells of a 12-well infected red blood cell slide and after 30 minutes unbound antibody was removed with three washes in PBS. One of each of the following FITC conjugated anti-mouse Ig subtypes was then applied to an individual slide well, after dilution according to the manufacturers instructions: anti- Ig GAM, raised against heavy and light immunoglobulin chains; anti-IgG (Fc), raised against IgG gamma heavy chain fragments; four antibodies raised against IgG subclasses 1, 2a, 2b and 3 (which differ in gamma heavy chain amino acid sequence); anti-IgA (Fc), raised against IgA alpha heavy chain fragments; anti-IgM (Fc) raised against the IgM mu heavy chain fragment; and anti-Ig kappa, raised against murine kappa light chain fragments. FITC rabbit anti-mouse IgG at 10μg/ml was included as a positive control on wells which had received first antibody, and as a negative control on wells which has not received first antibody. After incubation for 30 minutes the slides were washed 3x in PBS and examined as described in 2.3.1.
2.4. PROTEIN LABELLING

2.4.1. Metabolic labelling

The method for $^{35}$S methionine metabolic labelling *T. annulata* infected red blood cells was adopted, after experimentation (see 3.5.1.), from a method used to metabolically labelled *Plasmodium chabaudi* infected red blood cells (Dr A. Fenton, pers. comm.). 5x10^7 piroplasm infected red blood cells (2.1.3.) were resuspended in 100μl of incorporation medium (methionine-free Modified Eagles Medium (Gibco), 25mM HEPES buffer, 2mM L-glutamine, 8.33mM sodium hydrogen carbonate, 50μg/ml hypoxanthine, 500μg/ml reduced glutathione pH 7.2, 20μg/ml D-glucose, 10μg/ml para-aminobenzoic acid (PABA), 0.75μg/ml amphotericin-B, 50μg/ml gentamycin sulphate and 1% heat-inactivated (56°C for 30 minutes) foetal calf serum (Gibco)). Labelling was initiated by the addition of 300 μCi of methionine L-$^{[35}S]$ (Du Pont U.K. Ltd, NEG-008, >800Ci/mM), and carried out in one well of a 96-well tissue culture plate in a humidified incubator at 37°C with 5% CO$_2$. The reaction was terminated, 24 hours later, by the addition of RPMI-1640. Unincorporated isotope was removed by washing the cells 4x by centrifugation at 300g for 10 minutes followed by resuspension in 8mls PBS.

For SDS-PAGE analysis of total extracts, metabolically labelled cell pellets were solubilised in 200μl of SDS sample buffer for 1 hour on ice, clarified by centrifugation at 10,000g for 10 minutes and the supernatant stored at -70°C. The level of $^{35}$S incorporation into protein was determined by scintillation spectrophotometry of trichloroacetic acid (TCA) precipitated samples. Extracted samples (1μl and 5μl) were spotted onto glass microfibre filters (Whatman, GF/A) which were air dried in a sampling manifold holder (Millipore) connected to a vacuum pump. Using the same apparatus, the filters were washed with 50mls of cold 10% TCA to precipitate the protein and remove unincorporated isotope, followed by 50mls of 1:1 ethanol/ether to remove lipid. The filters were dried and then added to 10mls of scintillation fluid (BDH cocktail EX).
2.4.2. **$^{125}$I labelling**

Isolated red blood cells and piroplasms were surface iodinated by the Iodogen method (Markwell & Fox, 1978) in which Iodogen catalyses the iodination of tyrosine residues via oxidation of I$-$

1 ml of 1mg/ml 1,3,4,6-tetrachloro-3, 6-diphenyl glycoluril (IODO-GEN, Pierce) in chloroform, was added to a glass tube (7mm internal diameter), the chloroform was evaporated under vacuum in a dessicator and the tube was rinsed with PBS. Piroplasms or red blood cells (2x10$^8$ cells) were resuspended in 1ml PBS and added to the Iodogen coated tube followed by 500pCi $^{125}$I (Du Pont Ltd. specific activity 100inCi/ml, as carrier-free sodium iodide). After 10 minutes at room temperature, with occasional mixing, the labelled cells were washed 4x in PBS by centrifugation at 300g for 10 minutes and resuspending the pellet in 10ml PBS. For SDS-PAGE analysis of total extracts of iodinated cells, the cell pellets were solubilised in 200μl of SDS sample buffer for 1 hour on ice, clarified by centrifugation at 10,000g for 10 minutes and the supernatant stored at -70°C.

For immunoprecipitation experiments, iodinated cell pellets were solubilised in 0.5ml of NET buffer (0.15M sodium chloride, 0.5% Nonidet P40, 5mM EDTA, 50mM Tris-HCl, pH8.0) containing protease inhibitors, for 1 hour on ice, centrifuged at 10,000g for 10 minutes, and the supernatant stored at -70°C.

Piroplasm and red blood cell lysates were labelled by the same method. Lysates were prepared by resuspending approximately 2x10$^8$ piroplasms or 4x10$^8$ infected red blood cells in 1ml NET buffer containing protease inhibitors, followed by incubation for 30 minutes on ice. After labelling, unbound $^{125}$I was removed from the lysates by dialysis against 2-3 litres of NET buffer containing 10mM sodium azide, with 3 changes of buffer. Prior to use, dialysis tubing was boiled for 10 minutes in 500ml 2% sodium hydrogen carbonate, 1mM EDTA, followed by extensive rinsing with distilled water. The dialysed samples were centrifuged at 10,000g for 10 minutes, the supernatant removed and stored at -70°C.

To determine the level of $^{125}$I incorporation into protein, 1μl and 5μl extracted samples were precipitated with 150μl of cold 10% TCA for 30 minutes on ice. The precipitates were
centrifuged at 13,000g for 5 minutes and washed 2x in cold 10% TCA: 
gamma emission from the pellets was then determined in a gamma 
counter.

2.4.3. NHS-biotin labelling

Biotinyl-N hydroxysuccinamide (NHS-biotin) was synthesised by Dr 
A. Gray according to the procedures described by Jasiewicz (1978). 
NHS-biotin which binds to lysine residues at pH 8.0, was used to 
biotinylate the red blood cell and piroplasm surfaces. In 
addition, lysates of these cell types and red blood cell membranes 
or piroplasms which were bound to Affi-Gel beads were also 
labelled. In the latter half of the experimental work described in 
the results sections, biotin-streptavidin labelling superseded 125I 
labelling as the preferred method of extrinsic protein labelling; 
the reasons for this are given in section 4.3.5. The methods for 
biotin labelling were adapted from the procedures of Dr A. Gray 
(pers. comm.), Bayer & Wilchek (1980) and Hurley et al. (1985)

2.4.3.1. Surface labelling red cells and piroplasms

A solution of 10mg/ml NHS-biotin was made up in dimethylformamide 
and stored at 4°C in the dark. 20µl of this stock solution was 
added to 0.9ml PBS (pH 8.0) to give a final biotin concentration of 
200µg/ml. 100µl of a red blood cell or piroplasm suspension (10⁷ 
cells/ml) in PBS (pH 8.0) was added to the biotin solution and 
incubated at on a rotator for 1 hour at room temperature. Unbound 
biotin was removed by washing the cells 3x by centrifugation at 
3000g for 10 minutes and resuspending in 8ml of PBS. Biotinylated 
cell pellets were solubilised in 200µl SDS-sample buffer on ice for 
1 hour, clarified by centrifugation at 10,000g for 10 minutes and 
stored at -70°C.

2.4.3.2. Labelling red cell and piroplasm lysates

Red blood cells and piroplasms (5x10⁸ cells) were resuspended in 1ml 
of a modified RIPA buffer (12.5mM sodium phosphate buffer, pH 7.5, 
20mM EDTA, 150mM sodium chloride, 1% Nonidet P-40, 0.1% SDS, 1%
sodium deoxycholate, pH 8.0) with protease inhibitors and incubated for 30 minutes on ice. The resulting lysates were biotinylated by the addition of 1ml of PBSpi (pH 8.0) containing 100μl of 10mg/ml NHS-biotin in dimethylformamide to give a final biotin concentration of 500μg/ml. The reaction mixture was incubated at room temperature for 1 hour on a rotator and then 10μl of ethanolamine was added to quench unbound biotin. The lysates were clarified by centrifugation at 10,000g for 10 minutes and the supernatant was stored at -70°C.

2.4.3.3. Biotinylation of protein on Affi-Gel beads

The protein complement on Affi-Gel beads was analysed by biotinylating bound polypeptides in situ on the beads as follows. 100μl of an protein bound-Affi-Gel bead suspension (50μl packed volume) was washed 3x in PBS (pH 8.0), resuspended in 1ml of PBSpi containing 300μg/ml biotin and incubated for 1 hour at room temperature on a rotator. Unbound biotin was removed by washing the beads 3x by centrifugation at 9,000g for 1 minute and resuspending the beads in 1.5ml PBS. The biotinylated proteins were eluted from the beads and solubilised by the addition of 150μl SDS-sample buffer followed by incubation on ice for 1 hour. The Affi-Gel beads were pelleted by centrifugation at 9,000g for 1 minute, the supernatant was clarified by centrifugation at 12,000g for 10 minutes and the resulting supernatant was stored at -70°C.

2.5. SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was carried out using the discontinuous buffer system of Laemmli (1970) using a Bio-rad Protein-11 vertical slab cell apparatus. Resolving slab gels (150mm x150mm x1.5mm) of 3 different acrylamide concentrations were constructed to obtain different degrees of polypeptide resolution in different experiments. These were 7.5% gels, 8.6% gels and 4.5-15% exponential gradient gels. The latter were cast from the top of the gel plates using a Bio-rad gradient former according to the manufacturers instructions. The gradient former was attached to a
peristaltic pump adjusted to a flow rate of 1.5 mls/minute. A 4% acrylamide stacking gel was used with all resolving gels in order to sharpen polypeptide bands.

The molecular mass of resolved polypeptides were determined by comparison with the mobilities of internal standard molecular weight markers (Sigma. High molecular weight markers) which were: myosin (205kDa), beta-galactosidase (116kDa), phosphorylase-b (97.4kDa), bovine plasma albumin (66kDa), ovalbumin (45kDa) and carbonic anhydrase (29kDa). 15-30 µg of this protein mixture was loaded per gel track. For each gel, the equation of the line, derived by plotting relative mobility against the molecular mass of each marker was calculated, and from this line, the molecular mass of sample polypeptides was obtained.

Gels were fixed and stained with 0.3% Coomassie Brilliant Blue R-250 in 40% methanol, 10% glacial acetic acid. They were destained in the same solution without stain.

Gels which contained 35S-methionine labelled material were treated for fluorography by impregnating the gel with 1M sodium salicylic acid in 10% glycerol for 2 hours at room temperature. For autoradiography, gels were dried down on to 3MM (Whatman) paper under vacuum at 82°C. The dehydrated gels were exposed to X-ray film which was subsequently developed in an automatic film developer. Autoradiography with 35S gels was improved by pre-exposing X-ray film by electronic flash (Laskey 1975) and exposure at -70°C without intensifying screens. Autoradiography of gels or filters probed with 125I was carried out at -70°C with or without an intensifying screen.

Protein concentration was estimated by Bradford assay (Bradford, 1976). A standard curve for a given batch of Bradford reagent was constructed from known concentrations of protein in Bradford reagent against optical absorbance at 595 nm. Then the same dilutions of reagent were used to test unknown protein concentrations.

2.5.1 Stock solutions
- Acrylamide/bis: 29.2% acrylamide, 0.8% N-N Bis-methylene acrylamide (both from BDH chemicals. Electran grade).
- Gel buffer: 1.5M Tris-HCl, 6mM EDTA, 0.4% SDS, pH 8.8
- Stack buffer: 0.5M Tris-HCl, 8mM EDTA, 0.4% SDS, pH 6.8
- Electrode buffer: 50mM Tris-HCl, 384mM glycine, 2mM disodium EDTA, 0.1% SDS
- Sample buffer: A 4x stock was prepared - 0.5M Tris-HCl, 8% SDS, 8mM EDTA, 40% glycerol, 10% 2-beta-meraptoethanol, 0.008% Bromophenol Blue, pH 6.5. A mixture of protease inhibitors were added to SDS sample buffer prior to use (2.1.3.)
- Polyacrylamide: 3% polyacrylamide (Aldrich, carboxyl modified, molecular weight 200Kda).
- Ammonium persulphate and N,N,N,-N-Tetra-methyl-1,2-diaminoethane (TEMED): A fresh 10% stock solution of ammonium persulphate in distilled water was used.

2.5.2. Stack and resolving gel solutions
Stacking and resolving gel solutions were made up from the stock solutions given above, as follows:-

- 4% stack solution for 2x 1.5mm slab gels - 3.2mls bis-acrylamide, 8ml stack buffer, 10.2ml distilled water, 4.4ml polyacrylamide, 500μl ammonium persulphate, 40μl TEMED
- Resolving gel solutions for 2x 1.5mm slab gels
Exponential gradient gels were constructed with 4.5% and 15% acrylamide solutions. Linear slab gels were made with 7.6% or 8.6% acrylamide solutions:

i) 4.5% acrylamide solution: 9ml bis-acrylamide, 15ml gel buffer, 10ml polyacrylamide, 25.5 ml distilled water, 240μl ammonium persulphate, 30μl TEMED.

ii) 15% acrylamide solution: 15ml bis-acrylamide, 7.5ml gel buffer, 5.1ml polyacrylamide, 2.4 ml distilled water, 180μl ammonium persulphate, 15μl TEMED.

iii) 8.6% acrylamide solution: 19.8ml bis-acrylamide, 15ml gel buffer, 13.2ml polyacrylamide, 20.4ml distilled water, 500μl ammonium persulphate, 40μl TEMED.

iv) 7.5% acrylamide solution: 15ml bis-acrylamide, 12ml gel buffer, 9.9ml polyacrylamide, 20.4ml distilled water, 240μl ammonium persulphate, 30μl TEMED.
2.6. WESTERN BLOTTING

Sample extracts were resolved by SDS-PAGE and transferred from gels to nitrocellulose filters by Western- or electro-blotting according to established protocols (Burnett, 1981; Towbin, 1984) in a Bio-rad transblot cell using the manufacturers operating instructions. The gel, a sheet of nitrocellulose (0.45μm pore diameter) together with four sheets of Whatman 3MM paper and two Scotchbrite pads were soaked briefly in transfer buffer (25mM Tris-HCl, 192mM glycine, 0.02% SDS, 20% methanol). A sandwich was constructed with the gel and nitrocellulose held together by 2 sheets of paper and a pad on each side. A pipette was rolled over the surface to exclude air bubbles; the sandwich was inserted into a perforated plastic cassette which was slotted into the transblot tank, containing cold (4°C) transfer buffer, with the nitrocellulose towards the anode. Transfer was achieved by applying a field of 12Volts/cm between the electrodes for 2 hours at 4°C, or at 30V overnight at 4°C. The efficiency of the transfer was assessed by staining a portion of the filter with Ponceau solution (0.3% Ponceau S in 5% trichloroacetic acid) for 3 minutes followed by brief washing in warm water to allow visualisation of stained bands.

2.6.1. Immunoperoxidase staining

Filters were blocked by incubation on a rocking platform for 1 hour at room temperature in blocking buffer (5% dried skimmed milk and 0.05% Tween-20 in PBS). Monoclonal antibodies as ascitic fluid were diluted in blocking buffer and checked by IFA (2.3.1) to ensure that they gave a strong reaction against appropriate slide material. Spent hybridoma cell supernatants were also checked by IFA and were routinely used undiluted for immunoprobing. If IFA results showed a weak signal with antibody then antibodies were concentrated by ultrafiltration (Amicon, Centricon microconcentrators). Concentrated antibody solutions were diluted with PBS until they reacted by IFA to give a bright fluorescence against appropriate slide preparations. Bovine and murine sera were diluted 1:200 in blocking buffer.

The filters were incubated for 2 hours, or overnight, in
500µl/cm² (of filter) first antibody solutions at room temperature. Unbound antibody was removed by washing the filters in three changes of 500mls PBS/0.05% Tween-20 (Batteiger et al. 1982) over 1.5-2 hours. The filters were probed with horse radish peroxidase (HRP) conjugated anti-species IgG (SAPU, Scotland) at a 1:200 dilution in blocking buffer for 2-4 hours. The washing step was repeated and then the filters were developed in either 50mM Tris-HCl, pH 7.4, 0.6mg/ml 4-chloro-1-naphthol and 0.01% hydrogen peroxide or in 0.5mg/ml o-dianisidine hydrochloride, 10mM imidazole, pH 7.4 and 0.3% hydrogen peroxide (Towbin, 1984).

2.6.2. Probing biotinylated proteins with ¹²⁵I streptavidin

Biotinylated polypeptides were resolved by SDS-PAGE and transferred to nitrocellulose filters by electroblotting as described in 2.5 and 2.6. The filters were blocked in 500ml TST buffer (100mM Tris-HCl, pH 8.0, 150mM sodium chloride, 1% Tween-20) for 1 hour at room temperature on a rocking platform. ¹²⁵I-labelled streptavidin was diluted in TST/10mM magnesium sulphate, to a final concentration giving 1count/second/µl and the filter was incubated in 500µl/cm² of this solution for 2 hours at room temperature. Unbound streptavidin was removed by washing in 5 changes of 500mls of TST over 1.5 hours, or until the radioactivity in the used wash buffer was negligible. The filter was dried, exposed to X-ray film at -70°C with an intensifying screen and the film was developed after 5-72 hours in an automatic film developer.

2.7. IMMUNOPRECIPITATION

Radiolabelled red blood cell and piroplasm extracts, equivalent to 0.5x10⁸ TCA precipitable cpm were diluted to 100µl with NET buffer (2.4.2.) and incubated with 40µl (packed) pre-swollen protein A-sepharose beads (Pharmacia) for 30 minutes on a rotator at room temperature in order to pre-absorb the sample. The suspension was centrifuged at 10,000g for 1 minute, the supernatant removed to a fresh eppendorf tube and the beads were washed as described below. The protein concentration in the pre-absorbed sample was adjusted to 200µg/ml by the addition of bovine serum albumin (BSA, Sigma
Grade III. Bovine sera (20 µl) were added to the pre-absorbed samples and incubated overnight at 4°C; the resulting immune complexes were absorbed onto protein A-sepharose by incubation with 40 µl (packed volume) of beads for 30 minutes at room temperature. The suspension was centrifuged at 10,000g for 1 minute and non-specifically absorbed material was removed by washing the resulting pellet 2x in NET/0.2% SDS, 2x in NET and 1x in NET lacking Nonidet P40. The bound immune complexes were eluted from protein A-sepharose beads by boiling the sample for 3 minutes in 150 µl SDS sample buffer followed by centrifugation at 10,000g for 1 minute. The resulting supernatant was clarified by a further centrifugation at 10,000g for 10 minutes and then stored at -70°C.

2.8. FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Fluorescence activated cell sorting was performed in order to separate infected red cells from uninfected red cells, on the basis of differential fluorescence, after Acridine Orange staining of intraerythrocytic piroplasm nuclei. Infected red blood cell samples (50% parasitaemia, 2x10⁷/ml in PBS) were treated with a final concentration of 1 µg/ml Acridine Orange, resuspended in 10 ml PBS and pelleted by centrifugation at 300g for 10 minutes. The cells were resuspended to 2x10⁵/ml in PBS and introduced into a FACS IV (Becton Dickinson) for analysis and cell sorting. The cells were analysed by simultaneous forward scatter and red fluorescence using a 640 nm long pass filter and a 680 nm band pass filter for low angle scatter. Forward angle scatter signals were used to exclude debris and any leucocytes. Infected and uninfected red cells were sorted at a rate of approximately 2000/second.

2.9. IMMUNOELECTRON MICROSCOPY

Immunoelectron microscopy was carried out according to the pre-embedding method of Dobbelaere et al. (1985) with minor modifications. Heat-induced cultures (30 ml, section 6.1.2.2.) or 10⁷ macroschizont infected cells (2.1.5.) were centrifuged at 4000g for 15 minutes and washed 3x in PBS. The cells were fixed in 4%
freshly prepared formaldehyde/PBS at room temperature for 30 minutes. The cells were then washed 3x in PBS and 1x in PBS/50mM ammonium chloride. Centrifugations were carried out with a horizontal rotor in a bench microfuge at 10,000g for 3 minutes. The cells were resuspended in PBSag (PBS containing 0.2% gelatin and 10mM sodium azide), incubated for 10 minutes at room temperature and then centrifuged. The antibody fraction of hybridoma superatants or diluted ascitic fluid (0.3ml) were used to resuspend the cells, which were then incubated for 30 minutes at room temperature with rotation. Unbound antibody was removed by washing 3x in PBS and 1x in PBSag. The cells were resuspended in 0.1M phosphate buffer pH 7.0, containing 0.1% bovine serum albumin and protein A colloidal gold particles (12nm), and the suspension was incubated for 30 minutes at room temperature on a rotator. The cells were washed 3x in PBS and resuspended in fixative (2.5% gluteraldehyde in phosphate buffered sucrose (PBSU); 0.1M phosphate buffer pH7.0, 7.5% sucrose) for 1 hour at room temperature. Gluteraldehyde fixed cells were washed 3x in PBSU, then fixed in 1% osmium tetroxide in PBSU for 1 hour and washed 3x in distilled water.

The pellet was infiltrated with 1.5% agarose (Seaplaque-R, FMC Corporation. USA) in PBS/5% sucrose at 37°C and brought to 10°C to block the cells in the agarose matrix. The cells were stained en bloc with 0.5% aqueous uranyl acetate for 30 minutes and serially dehydrated in alcohols via propylene oxide to embed in Araldite resin polymerised at 60°C for 48 hours. Sections (200nm) were cut on an ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Zeiss 902) in spectrometer elastic bright field mode to enhance contrast. Images were recorded on KODAK SO281 70mm rolk film.
2.10. RECOMBINANT DNA TECHNIQUES

2.10.1. Construction of lambda gt11 expression library

A T. annulata Hissar genomic expression library was constructed in lambda gt11 by Dr. F.R. Hall (WUMP, Glasgow). The bacteriophage lambda expression vector gt11 (lac5, c1857, nin5, S100) was grown in E.Coli strain Y1090 (lac U169, proA; lon ara D139 str A; sup E; trvC22 Tn10 (pMC9)). The plasmid (pMC9) carries the gene for ampicillin and tetracyclin resistance and also the lacI gene whose product represses the lac operon. The vector has a single Eco RI cleavage site within the lacZ gene into which up to 7.2kb of foreign DNA may be inserted. Insertion of DNA into the Eco RI site inactivates beta-galactosidase; therefore recombinant phage are recognised by the property of producing colourless plaques, in pMC9-containing hosts such as E. Coli Y1090, in the presence of the lac operon inducer IPTG and the chromogenic beta-galactosidase substrate X-gal. Non recombinant phage produce blue plaques, as beta-galactosidase converts X-gal into a blue insoluble product (Huynh et al. 1985).

The library was constructed as follows: DNA was purified by standard procedures (Maniatis et al. 1982) from isolated piroplasms. The DNA was randomly sheared by 250 passages through a 25G needle, ends were made flush with T4 DNA polymerase (BCL) and then Eco RI sites were methylated with Eco RI methylase (Pharmacia). EcoRI linkers (Pharmacia) were added and the DNA was ligated to EcoRI digested lambda gt11 arms (Huynh, 1985). The DNA was packaged (Maniatis et al. 1982) and plated out on E. coli Y1090 r-m+ (Promega, Huynh et al. 1985). The library was found to contain 2x10^5 plaque forming units/ml of which 90% were recombinant as assessed by insertional inactivation (Huynh et al. 1985).

2.10.2. Standard reagents and host bacteria

Standard reagents were prepared according to Maniatis et al. (1982). DE52-cellulose/LB was prepared by adding DE52-cellulose (Whatman) to several volumes of 0.05N HCl and titrating the pH to 7.0 with 5N sodium hydroxide. The cellulose was washed 5x in 3
volumes of L-broth, resuspended in L-broth (1% (w/v) bacto-tryptone, 0.5% yeast extract, 1% sodium chloride) containing 200μg/ml ampicillin to give a final ampicillin concentration of 50μg/ml and stored at -20°C.

Host bacteria was prepared as follows. E. Coli Y1090 and Y1089 (Promega) from glycerol stocks were streaked out on to LB plates (L-broth, 1.5% bacto agar, 10mM magnesium sulphate, 50μg/ml ampicillin) and incubated overnight at 37°C. A single colony was picked and grown overnight at 37°C in an orbital incubator to stationary phase in 10ml L-broth supplemented with 10mM magnesium sulphate, 50μg/ml ampicillin, 0.2% maltose. 50ml of supplemented L-broth was inoculated with 200μl of this culture and the bacteria were grown at 37°C until an optical density of 0.5, measured at 600nm, was reached (2.5x10^8 cells/ml). The bacterial suspension was centrifuged at 8000g for 10 minutes, the pellet was resuspended in 5ml 10mM magnesium sulphate (2.5x10^8 cells/ml), stored at 4°C and used within 3 days.

2.10.3. Library immuno-screening

To screen the whole unamplified library, 300,000 plaque forming units in SM phage buffer (50mM Tris-HCl, pH 7.0, 10mM magnesium sulphate, 0.1M sodium chloride, 0.01% gelatin) were adsorbed to 1ml of Y1090 cells (2.10.2.) and incubated for 20 minutes at 37°C. The cells were then added to 25mls of molten (55°C) top agarose (L-broth supplemented with 10mM magnesium sulphate, 50μg/ml ampicillin, 0.7% agarose) and poured onto LB plates. The inverted plate was incubated at 42°C for 5-6 hours until plaques were visible. A sheet of nitrocellulose (140mm x140mm) was soaked for 1 hour in 10mM isopropyl-1-thio-b-D-galactoside (IPTG) in distilled water and then air dried. The filter was layed over the plaques, incubated for 2 hours at 37°C; then the LB plate was cooled to 4°C for 30 minutes before transferring the filter to blocking buffer for 1 hour or overnight (blocking buffer - 5% dried skimmed milk powder in TS buffer: 10mM Tris-HCl, pH 8.0, 150mM sodium chloride with 1mM sodium azide).

Monoclonal antibodies (as spent culture supernatant or ascitic fluid) and sera were diluted to approximately equivalent
titres (as determined by IFA against piroplasm slide preparations). Supernatants which gave a strong positive fluorescence were used undiluted and ascites fluids were diluted 1:100 in PBS such that they gave approximately the same intensity of signal by IFA as the supernatants. Blocked filters were incubated in 50-100mls of antibody solution for 5 hours or overnight on a rocking platform and unbound antibody was removed by washing the filters 3x in 500mls of TS supplemented with 0.01% Tween-20. The bound antibody was probed for 3 hours with horse radish peroxidase conjugated anti-IgG (SAPU, Scotland) at (1:200) in blocking buffer, and the washing steps were repeated. The filters were developed with o-dianisidine as described in 2.6.1. Plaques which were positive, that is those which specifically bound the first antibody, were picked by transferring them from the agar plate to 1ml of SM phage buffer containing 1% chloroform. The phage were dispersed into the SM medium by incubation on a rotator for 1 hour.

Subsequent immunoscreens were carried out with 90mm LB plates using 90µl of Y1090 host bacteria and 2.5ml top agarose. These immunoscreens had two purposes: first, in the cases where a mixture of different monoclonal antibodies had been used in the original screen, subsequent screens were carried out to determine which monoclonal antibody recognised the positive recombinants. For this, approximately 500 plaque forming units were plated out and proteins were transferred to nitrocellulose filters. These were then cut into segments and each was screened with an individual monoclonal antibody. Second, in the cases where a single antibody had been used, the immunoscreen was used to clone the phage by plating out 100-200 plaque forming units, rescreening with antibody and re-isolating presumptive clones until all the plaques on a plate were positive.

In order to amplify cloned phage stocks, cloned phage in 80µl Y1090 plating bacteria were plated out in top agarose onto 90mm LB-plates at a density which yielded confluent plaques after incubation at 42°C overnight. The plate was overlayed with 8mls of SM phage buffer with 1% chloroform and placed on a rocker for 3 hours to disperse phage into the SM medium. The SM was then collected and stored at 4°C after the addition of a further 1% chloroform to ensure lysis of host bacteria.
2.10.4. Preparation and analysis of DNA from recombinants

DNA was prepared using the Alloa method (Dr H.P. Beck, pers. commun.) which is based on the procedures of R. Taylor (NCI-FCRF), M. Graham & M. Olson (Washington University Medical School) and D. Ish-Horowiz (ICRF, London) as modified by L. Shozo Ozaki & S. Sharma (New York University, 1983).

An aliquot of amplified phage stock (1ml) was placed in an eppendorf and bacterial debris was pelleted by centrifugation at 8000g for 10 minutes. The supernatant (0.8ml) was removed to an eppendorf and treated with 0.5ml of a thawed suspension of DE52-cellulose/LB by incubating the mixture on a rotator for 15 minutes. The DE52-cellulose and bound bacterial proteins were removed by centrifugation at 9,000g for 5 minutes. The supernatant (0.8ml) was transferred to an eppendorf tube, 150μl of extraction buffer added (0.5 M Tris-HCl, pH 8.0, 0.25M EDTA, 2.5% SDS) and the proteins were solubilised over 15 minutes at 67°C. The suspension was cooled to room temperature, 200μl of 8M potassium acetate was added with mixing and the eppendorf transferred to ice for 15 minutes. The precipitated phage proteins and RNA were removed by centrifugation at 9,000g for 10 minutes. The supernatant (0.9ml) was transferred to an eppendorf, 0.6ml isopropanol was added with mixing and the solution incubated at room temperature for 10 minutes. Precipitated DNA was harvested by centrifugation at 9,000g for 10 minutes, the pellet washed 1x in 70% ethanol, dried, and then dissolved in 20-50μl Tris-EDTA buffer (TE, 10mm Tris-HCl, pH 8.0, 1mM EDTA).

A sample of the DNA (10μl) was digested with Eco R1 by adding: 3μl of 10x Eco R1 buffer (90mM Tris-HCl, pH 7.5, 50mM sodium chloride, 10mM magnesium chloride, 0.1mg/ml bovine serum albumin), 10-20 units of Eco R1 (BRL) and distilled water to give a final volume of 30μl. Digestion was carried out at 37°C for 2-3 hours and terminated by heating at 65°C for 10 minutes with 5μl of gel loading buffer (0.25% Bromophenol Blue, 40% w/v sucrose in distilled water).

The denatured sample was analysed by agarose gel electrophoresis according to the methods described by Maniatis et al. (1982). Gels were prepared with 100-150mls of 0.8% agarose in
Tris-borate buffer (TBE, 89mM Tris-HCl, 89mM boric acid, 2mM EDTA, pH 8.0), with 0.5µg/ml ethidium bromide and run in TBE with 0.5µg/ml ethidium bromide at 25V overnight or until the bromophenol blue had almost migrated the full length of the gel. An aliquot of 1 Kb markers (1µl; DNA ladder, BRL) was resuspended in 8µl of TE and 2µl of gel loading buffer, heated for 10 minutes at 65°C and this was electrophoresed in parallel with the sample and used to estimate the size of inserts. Bands were visualised by exposing the gel to long wave ultraviolet light (366nm) and photographed, where necessary, with a Polaroid camera and Polaroid Type667 film.

2.10.5. Generation of lambda gt11 recombinant lysogens in Y1089

Y1089 host cells (10⁷ cells, 2.10.1) were transferred to 3 eppendorf tubes and 10⁷, 5x10⁷ and 10⁸ plaque forming units from a titred, cloned, amplified phage stock were added such that the ratios of bacteria to phage were 1:1, 1:5 and 1:10, respectively. Infection was established by incubation at 30°C for 1 hour then bacteria were centrifuged at 9000g for 3 minutes, resuspended in 100µl of supplemented L-broth and incubated at 30°C for 30 minutes. The bacteria were centrifuged and the resulting pellet resuspended in 100µl 10mM magnesium sulphate at 30°C. To remove uninfected bacteria, 10⁷ C1− phage (C1− were provided by Dr. F.R. Hall; these phage lack the phage repressor) in 10µl SM phage buffer were added and incubated with the bacteria at 30°C for 20 minutes. The cycle was repeated by incubating the bacteria first in L-broth and then in 10mM magnesium sulphate with C1− phage.

In order to isolate lambda lysogens from the infected Y1089 bacteria the temperature sensitivity at 42°C of individual colonies was determined. Bacterial dilutions (5x10³ and 5x10² infected Y1089 bacteria/ml) were prepared in L-broth with 50µg/ml ampicillin, and 100µl of each was spread out over an LB plate and incubated overnight at 30°C to produce isolated colonies. The temperature sensitivity at 42°C of 50 individual colonies was tested by spotting cells onto two LB plates and incubating the first at 42°C and the second at 30°C overnight. Colonies which grew at 32°C but not at 42°C were assumed to be lambda lysogens, which were stored as glycerol stocks at -70°C (Maniatis et al. 1982).
2.10.6. Induction and Western blot analysis of fusion proteins

Lysogens were prepared as described above and cells were grown overnight in 10mls of L-broth with 100μg/ml ampicillin at 30°C. 200μl of this suspension was added to 800μl of fresh L-broth and incubated at 30°C. After 2 hours the cells were transferred to a 42°C waterbath, 10μl of IPTG from a stock solution of 1M IPTG in distilled water was added with mixing to give a final concentration of 10mM, and the incubation was continued at this temperature for 30 minutes with occasional mixing. The bacteria were then incubated at 37°C for 90 minutes, harvested by centrifugation at 6000g for 10 minutes and the pellet resuspended by brief vortexing. The bacteria were solubilised by the addition of 100μl SDS-sample buffer (2.5.) containing 0.1mM PMSF (2.1.3.), followed by heating at 100°C for 4 minutes. The sample lysate (50μl) was resolved by SDS-PAGE in 7.5% or 4.5-15% acrylamide gels (2.5.), transferred to nitrocellulose filters by electroblotting, and probed with antibody using the previously described immuno-screening method (2.6.1.).

2.10.7. Southern blotting

Theileria DNA was extracted from purified Hissar piroplasms according to the procedures described by Maniatis et al. (1982). 5μg DNA (Theileria DNA and bovine fibroblast DNA) was digested separately with Eco RI, Hind III and Bam HI (BRL) for 4 hours at 37°C with 10 units of enzyme (Maniatis et al. 1982). The digests were heated at 65°C for 5 minutes and then fragments were resolved by agarose gel electrophoresis (0.7% agarose) at 30V for 18 hours in TBE buffer (0.089M boric acid, 0.089M Tris-HCl, 2mM EDTA) containing ethidium bromide (0.5μg/ml final concentration). The DNA was transferred from the gel to nylon membrane filters (Nytran, Schleicher & Schuell) by electroblotting for 2 hours at 15V in 25mM phosphate buffer, pH5.5. The gel was incubated in 0.4M sodium hydroxide for 1 minute to denature the DNA, neutralised by incubation in phosphate buffer (25mM, pH5.5.) for 1 minute and then fixed under short wave ultraviolet light for 5 minutes.

The DNA probes were prepared as follows. DNA was purified from phage by the alloa method and the vector/insert DNA
was digested with Eco RI (2.10.4.). Fragments were resolved in 0.7% low melting point agarose gels, as above, inserts were excised from the gel and boiled for 7 minutes in 1.5ml distilled water/g agarose. Denatured DNA was incubated for 10 minutes at 37°C, random prime labelled (Polymeraid, P & S), and the probes (10µg) were boiled for 10 minutes.

The filters were hybridised in 25mM phosphate buffer (pH5.5) containing 5% SDS at 65°C for 18 hours. The filters were then washed 2x in 1xSSC (0.15M sodium chloride, 0.015M sodium citrate, pH7.0) with 5% SDS at 65°C for 20 minutes, rinsed in distilled water and exposed to X-ray film.

2.10.8. RNA slot blot

Total RNA was prepared by a guanidium thiocyanate/caesium chloride gradient method (Turpin & Griffith, 1986) and heated for 15 minutes at 65°C in phosphate buffer (25mM, pH5.5). 5µg RNA was slotted (BRL, hybri-slot manifold) onto membrane filters (Hybond-N, Amershain) and fixed under ultraviolet light for 3 minutes. 30ng of random primed labelled probe was prepared as described above. The Hybond filters were hybridised, washed and developed as for the Southern reactions described above (2.10.7.)
2.11. Preparation of piroplasm infected red cell membranes using Affi-Gel beads.

Affi-Gel beads (Bio-Rad, Affi-Gel 731) were washed x2 in 5 volumes of 2M ammonium chloride by resuspending the beads and then allowing them to settle. Then the beads were washed x3 with distilled water, x3 in filter sterilised sucrose phosphate buffer (SP buffer, 12mM sodium phosphate buffer, pH7.5, 250 mM sucrose) and resuspended in an equal volume of sucrose phosphate buffer.

Infected red cells were prepared as described in section 2.1., washed x5 in sucrose phosphate buffer and resuspended in an equal volume of sucrose phosphate buffer. Red cell samples were only used for membrane preparation if they appeared to contain no free piroplasms. The presence of free piroplasms was assessed as described in section 4.3.3. Red cells were centrifuged at 300g for 10 minutes, so that free piroplasms would form the bottom portion of the pelleted sample. This portion was transferred to a glass cover slip with 1ul of 1µg/ml Acridine Orange in PBS. A cover slip was applied and the cells were examined for free piroplasms under the 50x objective of a fluorescence microscope with a Leitz 12/3 filter block.

Prepared Affi-Gel beads (100µl) were transferred to an eppendorf tube and 100µl (5x10⁷ red cells) of the red cell suspension was added with gentle mixing. Unbound red cells were removed by resuspending the red cell-Affi-Gel mixture in 1.5ml sucrose phosphate buffer, and allowing the beads to settle. The supernatant was removed and this washing step was repeated x3. Lysis of bound red cells was achieved by the addition of 200µl lysis buffer supplemented with Dextran sulphate (EP lysis buffer, 12mM sodium phosphate buffer, 10mg/ml freshly prepared Dextran sulphate [Mr 5,000], 20mM EDTA, pH7.5). The bead suspension was vortexed at high speed for 20 seconds, 1ml lysis buffer was added and the beads allowed to settle. The supernatant was aspirated and the lysis and washing sequence was repeated x5. Samples were extracted in 150µl of SDS-sample buffer (2.5.1.). A flow plan of this protocol appears in table 4.5.3.2.
Recent advances in Plasmodium research have begun to elucidate some of the mechanisms by which intraerythrocytic stages acquire molecules from outside the red cell, and how infection of red cells results in functional, morphological and antigenic alterations in the red cell membrane. As reviewed by Sherman (1988), Plasmodium infected red cells are more permeable than uninfected red cells to amino acids, nucleosides, glucose and anti-malarial drugs: the associated changes are related to the developmental stage of the parasite and to the species of Plasmodium. Similarly, infection of bovine red blood cells with Babesia bovis results in the induction of novel adenosine nucleoside permeation sites which have different characteristics to normal human erythrocyte nucleoside uptake sites (Gero, 1989).

It is not yet clear to what extent, or in what way, the parasites contribute to these alterations, but there is good evidence that parasite encoded molecules are located in the membrane of Plasmodium infected erythrocytes. For example with Plasmodium falciparum, a 155kDa polypeptide (pf155 or RESA) is discharged from micronemes at the time of merozoite invasion and is transported to, and remains associated with, the cytoskeletal network of the red cell membrane in a phosphorylated state (Perlmann et al. 1984; Coppel et al. 1984). It has also been reported that parasite phospholipid is transferred to the red cell membrane, at the time of merozoite invasion, and that a concomitant rise in fluidity of the membrane takes place (Ullrich et al. 1989). The precise mechanisms of all these "transport" systems are unclear. A second infection specific phosphoprotein of 250-300kDa named MESA (Coppel et al. 1986) or pfEMP2 (Howard et al. 1987) is synthesised by trophozoites and schizonts, and is then transported to the red cell membrane. MESA has been found to bind to the red cell protein band 4.1, which shows elevated levels of phosphorylation in infected cells, and it has been postulated that MESA is responsible for phosphorylation changes in the red cell membrane (Coppel et al. 1989). Band 4.1 is thought to strengthen a
weak interaction between spectrin and actin; it also binds to membrane lipid and glycophorin C: thus it probably plays an important role in maintaining membrane integrity and elasticity, control of lateral motility of transmembrane proteins and regulation of lipid bilayer asymmetry (Gratzer, 1983). Several histidine-rich *Plasmodium* proteins have also been identified which are transported to the red cell membrane: a polypeptide of 85-105kDa (Knob-associated histidine-rich protein KAHRP or HRP-I) is associated with knobs on *P. falciparum* infected red cells (Culvenor, 1987) and a 72kDa polypeptide (pfHRP-II) which is water soluble and is transported outside the red cell (Howard *et al.* 1986).

It is possible that *Plasmodium* gene products are exposed on the surface of red cells; these may be involved in the sequestration of infected cells, which regulates the parasites ability to produce chronic infections. Leech *et al.* (1984) identified a *P. falciparum* polypeptide (260-285kDa, pfEMP-1 Howard *et al.* 1987) on the surface of infected knob bearing red cells. Aley *et al.* (1984) characterised the same molecule and showed that it was present on knob-bearing, but not knob-deficient, infected red cells. As both knob-bearing and knob-deficient infected red cells may sequestrate (Smalley *et al.* 1980; Howard, 1988; Udomsangpetch *et al.* 1989), it seems unlikely that the knob associated pfEMP-1 molecule is directly involved with sequestration. Udomsangpetch *et al.* (1989) have isolated a monoclonal antibody (33G2) which recognises the surface of infected cells. The antibody specifically inhibits cytoadherence of both knob-deficient and knob-bearing, cytoadhering infected red cells. Udomsangpetch *et al.* (1986) reported that the same monoclonal antibody inhibited merozoite invasion of red cells. Further work has demonstrated that antibody 33G2 recognises several distinct gene products which are of parasite origin (Scerf *et al.* 1988; Mattei *et al.* 1989) including: pf155-MESA described above, a 1.5 megadalton polypeptide product of a gene clone 11.1, and at least seven other molecules. Several of the structural genes have been characterised, and the predicted secondary structures (based on sequence data) reflect the observed cross-reactivity of the gene products. Some of the polypeptides in this family have been shown
to be immunogenic. On the basis of the ability of antibodies, raised against some of these polypeptides, to block merozoite invasion or inhibit cytoadherence, the polypeptides may be relevant to protective immunity. The extent to which their relationship with the red cell membrane determines their properties is unknown. Thus it is clear that considerable changes are induced in the infected red cell membrane which alter its properties in relation to the uptake of nutrients, adhesiveness and the host immune response.

Infection specific Plasmodium proteins in the red cell membrane and infection specific host modifications may perform diverse functions, but they represent an opportunity for determining likely methods of controlling the intraerythrocytic parasite stages. The work with Plasmodium raised the possibility that analogous events are occurring in Theileria infected red cells and this provided the rationale of the experimental study described in this chapter. The red cell stages of Theileria have not previously been studied at a specific molecular level. Previous work has concentrated on characterising the sequence of morphological changes which take place at this point in the life cycle, and specifically the ultrastructural changes (see section 1.1.).

At present there are no control measures for theileriosis which have an effect on the piroplasm directly: cattle can be immunised with live cell vaccines and infected cattle can be drug treated, but in both cases, recovery from parasite challenge does not involve the direct removal of piroplasm stages, which are therefore a source of fresh infection via the tick vector. Piroplasm burdens are reduced by immunisation and drugs, as a result of controlling life cycle stages which precede the piroplasm stages. Immune animals are therefore infected and infectious: the extent to which low levels of infection are debilitating, and the role that low levels of infection play in the epidemiology and maintenance of immunity is unknown. An integrated approach to controlling theileriosis would probably require the ability to control piroplasm burdens as well as other life cycle stages. Thus the definition, at a molecular level, of the alterations induced in the red cell by Theileria and the role of the parasite and host
surface proteins in the invasion of the red cell by merozoites would potentially provide the necessary basic information on which strategies aimed at eliminating this stage of the parasite could be based. The aim of the work presented in this chapter was to characterise the *T. annulata* infected red cell membrane, in terms of polypeptide alterations between infected and uninfected red cells, as an initial step towards understanding its relationship with the merozoite and piroplasm.

3.1. IMMUNOFLUORESCENCE MICROSCOPY

It has been established that sera from bôvids, immunised or infected with *T. annulata*, recognise piroplasms in indirect immunofluorescence assays (Bell, 1986), but no reactivity against the infected red cell membrane has been reported. If the surface of the red cell is modified by infection with piroplasms, it is possible that immune sera would recognise these alterations: immune sera could then be used to isolate and characterise the antigens recognised. This provided the rationale of the following experiments with immune sera. Clearly only a weak signal on the surface was expected, so the objective was to use an assay which was sufficiently sensitive to be able to assess the specificity of any reactivity.

Immunofluorescence assays were performed to find out whether the two available immune sera (serum 155 and serum 128), recognised the surface of infected red cells. The derivation of immune sera 155, which was raised against Ankara parasites, and immune serum 128, which was raised against Hissar parasites, is described in section 2.1.7. These sera were chosen because they showed a strong IFA reaction against piroplasms on acetone fixed infected red blood cell slides (Ankara and Hissar), displaying positive reactions at dilutions of up to 1/1000. No fluorescence was observed about the infected red cell membrane, however this was the expected result as a weak signal could have been obscured by strong fluorescence from the piroplasms. In order to determine whether the immune sera recognised the infected red cell surface, immunofluorescence assays were performed with unfixed infected red cells so that the serum antibodies would have access to the red
cell surface but not to intracellular antigens or the piroplasm. Surface IFAs were carried out with unfixed, infected red cells (Hissar & Ankara stocks) as described in section 2.3.1 with either FITC conjugated anti-IgG, or with biotin and FITC conjugated avidin. The surface IFAs were controlled for internal labelling as the sera recognised piroplasm determinants, however positive controls were not available. No fluorescent reaction was detected in the surface IFAs which suggested that immune sera 128 and 155 do not recognise antigens on the surface of red cells.

3.2. IMMUNOPRECIPITATION STUDIES

It was possible that a weak reaction between immune sera 155 or 128 and the red cell surface was outside the detection range of the immunofluorescence assay used above. An alternative method is immunoprecipitation with immune sera from surface labelled infected red cells; this could provide a more sensitive assessment of the reaction between sera and surface antigens. As certain membrane proteins have been reported to be insoluble in immunoprecipitation buffers containing non-ionic detergent (for example, the "SICA" red cell membrane-associated antigens of P. knowlesi are partially insoluble in Triton X-100 (Howard et al. 1983)), the experiment was carried out in conjunction with Western blotting and surface labelling analyses. If a polypeptide was shown to be on the surface of red cells by surface labelling analysis and a similar polypeptide was detected with immune sera by Western blotting analysis, but not detected in immunoprecipitation experiments, then the latter experiments would become suspect.

Immunoprecipitations were performed in suspension with ammonium sulphate fractionated immune sera and Nonidet P-40 extracts of \( ^{125}\text{I} \) surface labelled red cells and piroplasms (section 2.7). In order to assess the surface specificity of the labelling protocol, lysates of infected and uninfected red cells and of piroplasm preparations were also labelled with \( ^{125}\text{I} \). These samples were analysed by SDS-PAGE and autoradiography in parallel with surface labelled extracts. The results suggested that there was a significant restriction of the label to the surface of red cells and piroplasms as many more polypeptides were labelled in the
lysate extracts. Immune complexes were isolated with Protein A-sepharose beads which bind bovine IgG1 and IgG2 immunoglobulin subclasses (the latter is bound more efficiently and immune complexes are bound more strongly than free antibody, see Langone, 1982). Five infected red blood cell samples were used and these were: infected with one of each of the three stocks of T. annulata, exhibited different parasitaemias and were surface labelled to different specific activities. A description of each sample is given in table 3.2.

Hissar infected red cells were immunoprecipitated with immune serum 128 (see section 2.1.7. for details of the immunisation), and serum (DCS54) from a calf which died from an overwhelming infection of T. annulata Hissar. This serum reacted strongly in IFAs against acetone fixed piroplasm preparations. Ankara infected red cells were immunoprecipitated with immune serum 128 and immune serum 155. Gharb infected red cells were immunoprecipitated with immune serum M58, which was raised against Gharb parasites and which strongly recognised piroplasms in fixed cell IFAs.

Isolated piroplasms from the five infected red cell samples were surface labelled and immunoprecipitated with the same sera. Uninfected red cells (cow110) were surface labelled, and these were used as a negative control in each immunoprecipitation experiment. All the surface labelled samples were also immunoprecipitated with pre-infection sera as a further negative control. The extracted samples were pre-incubated with Protein A-sepharose beads in order to remove non-specifically binding polypeptides. The results of the immunoprecipitation experiments were as follows:

- Immune serum 128 vs Hissar infected red cells (45% parasitaemia)

Immune serum 128 recognised a polypeptide of approximately 215kDa in the Hissar infected red blood cell sample (figure 3.2.1.a. arrowed in track 1). Pre-infection serum and non-convalescent serum (DCS54) did not immunoprecipitate any labelled polypeptides, thus demonstrating that the reaction with the immune serum was
Table 3.2.

$^{125}\text{I}$ incorporation into protein after surface labelling of 15 samples. $2 \times 10^8$ red cells or piroplasms were labelled and then solubilised into 500μl NET buffer (section 2.4.2.). The parasitaemia of each infected red cell sample, and the stock of parasite used, is indicated. Piroplasms were prepared from infected red cells by an ammonium chloride lysis method (see section 2.1.4.). Control, uninfected red cells were collected from cows 110 or from calves prior to infection.
Table 3.2

Immunoprecipitation sample data

<table>
<thead>
<tr>
<th>stock</th>
<th>% parasitaemia</th>
<th>surface labelled sample</th>
<th>cpm x10^3/λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hissar</td>
<td>20</td>
<td>infected rbc</td>
<td>5</td>
</tr>
<tr>
<td>Hissar</td>
<td>45</td>
<td>infected rbc</td>
<td>5</td>
</tr>
<tr>
<td>Gharb</td>
<td>50</td>
<td>infected rbc</td>
<td>37</td>
</tr>
<tr>
<td>Ankara</td>
<td>35</td>
<td>infected rbc</td>
<td>90</td>
</tr>
<tr>
<td>Ankara</td>
<td>50</td>
<td>infected rbc</td>
<td>40</td>
</tr>
</tbody>
</table>
Figure 3.2.1.

Immunoprecipitations of Nonidet-P40 lysates of surface iodinated red cells and piroplasms. Samples (1x10^6 cpm) were immunoprecipitated using protein A-sepharose and electrophoresed on 4-15% SDS-PAGE gels.

Fig. 3.2.1.a;

Track 1 - Immune serum 128 with Hissar piroplasm infected red cells (45% parasitaemia). Track 2 - Pre-infection serum 128 with Hissar piroplasm infected cells as above. Track 3 - Pre-infection serum 128 with Hissar piroplasms prepared from red cells as above. Track 5 - Immune serum 128 with Hissar piroplasms prepared from red cells as above. Track 6 - Pre-absorbed, surface labelled piroplasm lysate polypeptides, bound to protein A-sepharose in the absence of antibody.

Fig. 3.2.1.b;

Track 1 - Non-convalescent serum 54 with Hissar infected red cells (45% parasitaemia). Track 3 - Immune serum 128 with control uninfected red cells. Track 4 - Pre-infection serum with control red cells. Track 5 - Non-convalescent serum 54 with control red cells.

The mobilities of standard polypeptides are indicated on the left of the figure. The numbers are the molecular masses of these standards in kDa. The arrow to track 1 in Fig 3.2.1.a indicates the position of a labelled polypeptide.
specific (tracks a2 and b1). These three sera failed to immunoprecipitate polypeptides from the surface labelled uninfected red cell sample which suggested that the 215kDa polypeptide was infection specific (figure 3.2.1.b, tracks 3, 4, & 5).

Immunoprecipitation of surface labelled piroplasms (figure 3.2.1.a, tracks 3, 5 & 6) failed to detect a 215kDa polypeptide. Track 6 shows the pre-absorbed, surface labelled piroplasm polypeptides which had bound to Protein A-sepharose beads in the absence of any antibody; tracks 3 and 5 show the immunoprecipitated polypeptides with pre-infection and immune serum respectively. As all the polypeptides present in tracks 3 and 5 were also non-specifically bound to the beads, this single experiment with the piroplasm extracts was unsuccessful. However, it was inferred from this data that the 215kDa polypeptide was not simply present in the immunoprecipitated red blood cell extract because of internal labelling of the infected red blood cell.

When immune serum 128 was used to immunoprecipitate extracts of Hissar infected red cells which had a 20% parasitaemia, no polypeptides were immunoprecipitated. Surface labelled piroplasm polypeptides were specifically recognised by immune serum 128 and not by pre-infection serum; four polypeptides of molecular mass 104, 100, 84 and 81kDa were detected. Similarly immune sera 128, 155 and M56 failed to immunoprecipitate any polypeptides from surface labelled infected, or uninfected red cells, but they specifically detected piroplasm surface polypeptides, of a similar molecular mass range to those detected in Hissar samples by serum 128. The details of surface labelled piroplasm polypeptides which were immunoprecipitated by immune sera 155, M56 and 128 are given in appendix Ib.
3.3. WESTERN BLOT ANALYSIS

Western blotting experiments (section 2.3.) were undertaken in an attempt to confirm that the immune sera recognised a 215kDa polypeptide. Each of the sera described above was screened against SDS extracts of Hissar, Ankara and Gharb piroplasms, their infected red blood cell equivalents and uninfected red blood cells (cow110). The results showed that many polypeptides (20-30 molecules, see appendix 1b) were specifically recognised by the immune sera, and non-convalescent serum DC554, but not pre-infection sera. Uninfected red cell extracts (cow 110) were not recognised by any of the sera. However, the 215kDa polypeptide was not unequivocally recognised. Comparisons between immuno-blots of piroplasm and infected red cell extracts were made to determine whether there was any indication that piroplasm polypeptides were either being lost during the piroplasm isolation procedure, or were present in the membrane or cytoplasm of the red cell. The results suggested that there were no abundant, immunogenic polypeptides reproducibly associated with the infected red cell extracts, which were not also represented in the piroplasm extract.

Certain experiments indicated that polypeptides were recognised by immune sera in infected red cell extracts which were absent in the derived piroplasm extracts. For example, 168kDa and 156kDa polypeptides were recognised by immune serum 128 on extracts of Ankara infected red cells and these molecules were absent in the derived piroplasm preparation. However, when the same serum was screened against different Ankara infected red cell and piroplasm extracts, these two polypeptides were recognised in both extracts. Such results were therefore irreproducible, and there was no apparent relationship between the experiments where these differences were detected, and the parasitaemia or stock of the sample involved. Thus, it was not possible to determine whether the piroplasm extracts had lost polypeptides during purification steps, or whether the infrequent detection of polypeptides, which were specific to infected red cell extracts, reflected a real difference in the location of parasite polypeptides in these instances.
Infected red blood cell samples, showing a range of parasitaemias from 15-65%, and infected with Hissar, Ankara or Gharb piroplasms, were surface labelled by the method of Iodogen catalysed iodination on numerous occasions. These sample extracts were subjected to SDS-PAGE separation, in 4.5-15% acrylamide gels (5x10⁴ TCA precipitable cpm), in parallel with surface labelled uninfected red cells (cow110 or pre-infection red blood cells). In addition, all the labelled extracts described in the previous section (3.3), were subjected to direct comparative SDS-PAGE analysis. The resulting autoradiographs were examined for the presence of infection specific polypeptides, or indications of differences in polypeptide mobility between the samples. The results were very variable, but there was no detection of infection specific polypeptides or polypeptides showing altered mobility. In several experiments, four polypeptides were detected in infected and uninfected samples of molecular mass approximately 160, 119, 95 and 48kDa. The Hissar infected and uninfected red cell extracts, which were used in the experiment described in the previous section where a 215kDa polypeptide was specifically immunoprecipitated by immune sera from the infected red cell surface, were compared. The two samples were indistinguishable and the four molecules listed above were detected.

The same number of experiments resulted in the detection of a greater range of labelled polypeptides. In addition to the four listed above, polypeptides of 218kDa, one polypeptide of 180-200kDa, three polypeptides of approximately 119kDa, two polypeptides of approximately 95kDa, one molecule of 64-71kDa, a second polypeptide of approximately 48kDa and four polypeptides of approximately 20-30kDa. Major surface labelled piroplasm polypeptides were not detected, but spectrin doublets (molecular mass 243kDa and 265kDa) and haemoglobin were nearly always labelled. Appendix Ia lists the surface labelled red cell polypeptides which were detected.
3.5. METABOLIC LABELLING STUDIES

As mature red blood cells do not synthesise protein and are thought to be the only host cell type for *T. annulata* piroplasms, metabolic labelling of infected red blood cells could enable the characterisation of piroplasm polypeptides. This approach could circumvent the problem of distinguishing between red blood cell and piroplasm polypeptides. Moreover if, pure infected red blood cell membranes could be prepared from metabolically labelled cells, it might be possible to begin to define piroplasm polypeptides which were associated with the membrane.

3.5.1. Labelling conditions

There is no established protocol for metabolically labelling *T. annulata* piroplasms, so preliminary experiments were performed to investigate labelling conditions with $^{35}$S methionine. The protocol described in 2.4.1. was used with the following variations: three different concentrations of $^{35}$S methionine were used (50μCi, 6.3x10$^{-6}$M methionine; 300μCi, 3.8x10$^{-7}$M methionine and 500μCi, 6.3x10$^{-7}$M methionine). Infected red blood cells (50% parasitaemia) were incubated in 100μl of incorporation medium at three different red cell densities (2.5x10$^8$/100μl, 2.5x10$^8$/100μl and 2.5x10$^7$/100μl incorporation medium). The time course of incorporation was determined at 0, 3, 6, 12, 19, 24 and 36 hours.

The results showed that most TCA precipitable cpm were incorporated in the samples which had the highest cell density (2.5x10$^7$/100μl). The same level of incorporation was achieved when 300μCi or 500μCi $^{35}$S were employed and this was ten fold greater than the incorporation achieved with 50μCi $^{35}$S. The TCA precipitable cpm were negligible at time 0 and greatest at 24 and 36 hours, where the incorporation was the same (80cpm/2.5x10$^4$ red cells). The sample extracts which showed the greatest incorporation were separated by SDS-PAGE (25μl was loaded per track which was equivalent to 3x10$^8$ red blood cells and 4x10$^3$cpm) and autoradiographs showed that after three weeks exposure, approximately forty polypeptides were detected. These polypeptides together with the results from several similar experiments are.
presented in appendix Ia. This number was approximately the same as the number of polypeptides which could be visualised (after staining protein with Coomassie Blue) in similar gels loaded with unlabelled purified piroplasm extracts (approximately $5 \times 10^6$ piroplasms). Therefore, this level of incorporation, and the amino acid used, were considered to be adequate for initial experiments, and the metabolic labelling protocol which is described in section 2.4.1. was adopted.

3.5.2. Control labelling experiment

The utility of the metabolic labelling technique depends upon ensuring the absence of metabolically active leucocytes in infected red blood cell samples. Leucocytes were routinely removed from blood samples, as described in section 2.1.3., by differential centrifugation and passage through a CF11-cellulose column. 100μl samples, containing $2 \times 10^7$ lysed red cells, were analysed by light microscopy to determine whether leucocytes were present. When no leucocytes per $2 \times 10^7$ red cells were observed, the sample was considered to be free from leucocytes. This was not an accurate test for leucocyte contamination when small numbers of leucocytes were involved and moreover, it was not known how many leucocytes would have to be present before they would make a detectable contribution to the metabolically labelled polypeptide profile. The following experiment was carried out to monitor whether this potential level of leucocyte contamination, would significantly contribute to $^{35}$S metabolic labelling experiments with piroplasm infected red cells.

Control uninfected blood (cow 110) was processed as described in section 2.1.3. to remove leucocytes. Red blood cells, which showed no leucocytes per $2 \times 10^7$ red blood cells, as visually assessed by light microscopy, were metabolically labelled as described in section 2.4.1. Negligible TCA precipitable counts were recorded in the sample by scintillation spectrophotometry. A quarter of the total extracted sample ($10^7$ red blood cells) was subjected to SDS-PAGE on each of four tracks of a 4-15% linear gradient gel, which was treated for fluorography, dried and exposed to x-ray film for six weeks. The resulting autoradiograph
Control $^{35}$S metabolic labelling experiment.

Uninfected blood was processed for the removal of white blood cells and platelets (section 2.1.3.). $4 \times 10^7$ red cells were treated as infected red cells for $^{35}$S metabolic labelling (section 2.4.1.). One quarter of the solubilised sample, showing negligible TCA precipitable radioactive counts by scintillation spectrophotometry, was subjected to SDS-PAGE on each of four tracks of a 4-15% acrylamide gel. The gel was treated for fluorography, dried, and exposed to x-ray film for six weeks.

Figure 3.5.2.2.

The effect of storage of infected red cells in CPDA-1 for 21 days at $4^\circ C$ monitored by metabolic and surface radiolabelling.

Infected blood (Hissar stock, 55% parasitaemia) was treated as described in section 2.1.3., to remove white blood cells. Red cells were resuspended in 10x their packed cell volume of CPDA-1 (section 2.1.1.) and stored at $4^\circ C$ in darkness.

Fig. 3.5.2.2.i shows a fluorograph of $^{35}$S methionine labelled red cell polypeptides (section 2.4.1.) prepared on days 0 (track d0), 3 (track d3), 5 (track d5) and 21 (track d21) of storage.

Fig. 3.5.2.2.ii shows an autoradiograph of surface iodinated (section 2.4.2.) red cell polypeptides prepared on days 0, 3, 5, and 21 as above. Iodinated and metabolically labelled samples were resolved electrophoretically in separate 10% SDS-PAGE gels. $10^6$ TCA precipitable cpm of iodinated extract, and $2 \times 10^4$ TCA precipitable cpm of metabolically labelled extract were loaded per track.

The position of internal molecular weight standards are shown on the left hand side of the figure, and the relative molecular masses (in kDa) of certain labelled polypeptides are indicated by arrows on the right hand side of the figure.
Figure 3.5.2.

1 kDa

2 kDa

116—

117—

130—

97—

78—

64—

54—

43—

25—

20—

d0 d3 d5 d21

d0 d3 d5 d21

a b c d

80—
(figure 3.5.2), tracks a, b, c and d are identical) showed that four polypeptides (between 65kDa and 80kDa) were labelled. Therefore, despite the fact that the TCA precipitable incorporation had not exceeded background levels, there was a significant level of contamination after six weeks autoradiography, which would have to be considered in future experiments. This is discussed in section 3.7, however, as described in section 4.3.1, the problem was circumvented by the finding that infected blood which was stored at 4°C could be used in labelling experiments. After storage periods of more than one week, any contaminating leukocytes would be non-viable, and stored blood was used in the following experiment.

3.5.3. Crude membrane preparation

Several lines of evidence suggested that piroplasms, which were prepared from infected red blood cells by the ammonium chloride lysis method (section 2.1.4.), were relatively free from red blood cell contamination. First, Coomassie Blue stained SDS-PAGE gels indicated (see Appendix 1a) only six co-migrating polypeptides of molecular mass 103, 74, 64, 46, 44 and 39kDa (in one experiment also 18kDa, 27kDa and 31kDa molecules) in piroplasm and red blood cell extracts. Apart from the 103kDa and 39kDa polypeptides, these molecules were more heavily stained in piroplasm extracts than red cell extracts. This suggested that in the piroplasm extracts these polypeptides were probably of piroplasm origin rather than red cell contaminants. Secondly, comparisons by SDS-PAGE followed by autoradiography of surface labelled piroplasm and red blood cell extracts showed that the profiles were similar, but clearly distinct: polypeptides of molecular mass 130, 118, 62, 47 and 20kDa appeared similar in both extracts (see Appendix 1a). Thirdly, a monoclonal antibody (2A3) which recognises uninfected red blood cells, failed to recognise acetone fixed piroplasm preparations in IFAs. This suggested that the red blood cell lysis step involved release of intact piroplasms from the red blood cell and consequently it was envisaged that infected red blood cell membranes in the lysis suspension would be relatively free from piroplasm contamination.
An experiment was performed to try to prepare red cell membranes from infected red blood cell lysis suspensions by differential centrifugation. Unlabelled infected red blood cells (Hissar stock, 50% parasitaemia) were lysed as described in section 2.1.4. with buffered ammonium chloride. Piroplasms and any attached red cell material were pelleted by centrifugation at a range of gravitational forces between 200g and 800g for 30 minutes. The supernatant fraction from each centrifuge step was aspirated and particulate material in this fraction was pelleted by further centrifugation at 100,000g for 1 hour at 4°C. The resulting pellets were applied to microscope slides, stained with Giemsa and examined by light microscopy for the presence of piroplasms. The membrane pellet originating from the greatest initial 800g centrifugation step was free from piroplasm nuclei, but pellets, derived from supernatants obtained after 200-600g centrifugations of lysis suspensions, contained high levels of parasite contamination.

Based on this information, membranes from metabolically labelled (2000cpm/μl packed cells) infected red blood cells were prepared as follows. 5x10⁷ metabolically labelled infected red blood cells (Hissar stock, 50% parasitaemia) were lysed in buffered ammonium chloride. Piroplasms were pelleted by centrifugation at 800g for 30 minutes and then the supernatant was transferred to a centrifuge tube containing 40ml PBSpi. After centrifugation at 100,000g for 1 hour at 4°C, the pellet was solubilised in SDS-sample buffer, a sample was removed for scintillation counting and the remainder (1000cpm TCA precipitable counts) was applied to a 4.5-20% acrylamide gradient gel. This sample was subjected to electrophoresis in parallel with an extract of metabolically labelled, purified piroplasms (1000cpm) and an extract of the intact metabolically labelled infected red blood cells (1000cpm).

The resulting autoradiograph is shown in figure 3.5.3.1: track c) is the SDS extract of intact metabolically labelled cells, track b) is the purified piroplasm fraction and track a) shows the membrane fraction. The loss of 25kDa and 38kDa polypeptides, and the gain of an approximately 215kDa polypeptide was apparent in the membrane fraction relative to the other tracks. The other
Figure 3.5.3.1.

Fluorograph showing metabolically labelled polypeptides in piroplasm infected red cell membrane fractions, derived by differential centrifugation.

5x10^7 ^35_S methionine labelled infected red cells (Hissar stock, 50% parasitaemia, 2000 cpm/μl packed cells) were lysed in buffered ammonium chloride and red cell membrane and piroplasm fractions were prepared as described in section 3.5.3. by differential centrifugation.

Red cell membrane (track a), piroplasm (track b) and intact infected red cell (track c) samples were solubilised in SDS-sample buffer and 1000 TCA precipitable cpm of each was applied to a 4.5-20% SDS-PAGE gel. After electrophoresis, the gel was treated for fluorography, dried down and exposed to x-ray film for six weeks. Molecular mass estimates (see right hand side of figure, in kDa) were derived using internal standards whose mobility is illustrated on the left hand side of the figure.
Figure 3.5.3.1.

kDa

a b c

> 205
196
160
128
118
100
88
71
66
48
38
25
20
labelled polypeptides in the membrane fraction which included twenty-two molecules between 20kDa and 128kDa were also present in either the piroplasm, or the membrane fraction. Quantitative differences were observed, in that polypeptides of molecular mass 20kDa and 128kDa were enriched in the membrane fraction.

3.6. MONOCLONAL ANTIBODIES

Two fusion experiments were carried out in order to generate monoclonal antibodies which specifically recognised infection specific epitopes in the membrane of infected red blood cells (surface ISEMPs). Monoclonal antibodies were produced as described in section 2.1. A detailed description of the screening procedure for determining the reactivity of hybridoma supernatants is given in section 5.2. which deals with anti-piroplasm monoclonal antibodies. This is because none of the hybridoma supernatants (except one which was lost) appeared to recognise infection specific epitopes in the red cell membrane, while many recognised epitopes in the piroplasm. Briefly, supernatants from the uncloned hybridoma cells were characterised by indirect immunofluorescence assays in terms of their reactivity against the following slide preparations: acetone fixed infected (40% parasitaemia) red blood cells, acetone fixed uninfected red blood cells and formaldehyde fixed, purified piroplasm preparations. One of the supernatants appeared to recognise the outer perimeter of 40% of red cells in infected red cell slide preparations, but failed to recognise control uninfected red cells. However, the hybridoma cells secreting this antibody were lost due to bacterial contamination.

Several of the anti-piroplasm monoclonal antibodies (1C2, 2D5, 1D11, 10C3 and 5G1) appeared to weakly recognise epitopes within the infected red cell cytoplasm, but failed to recognise uninfected red cells. As it was likely that a strong fluorescent reaction against the piroplasm would obscure a weak reaction against the infected red cell membrane, these monoclonal antibodies were screened on unfixed infected red cells to determine whether they recognised surface epitopes (section 2.3.2.). Monoclonal
antibody 2A3, which recognised infected and uninfected red blood cells, was included in the assay as a putative positive control. The results showed that while the antibodies reacted on acetone fixed infected red blood cell preparations, there was no reaction against unfixed cells. Therefore it appears that the epitopes recognised by these antibodies are not on the surface of infected red cells.

3.7. DISCUSSION

Probably the most broad-based of the strategies adopted in this experimental work, was to try to generate specific antibody probes against infection specific erythrocyte membrane proteins (ISEMPs). Antibodies could potentially be raised against host and parasite gene products, and a variety of post-translationally derived epitopes. Monoclonal antibodies were raised against infected red cells, but unfortunately the single antibody which appeared to recognise an ISEMP, by IFA with acetone fixed infected red cells, was lost due to bacterial contamination at an early stage in the hybridoma production process. Its brief existence, nevertheless, supports the idea that antigenic, infection specific epitopes are associated with the red cell membrane.

Five anti-piroplasm antibodies (1C2, 2D5, 1D11, 10C3 and 5G1) which appear, in fixed cell assays, to weakly recognise the red cell membrane, were tested in surface IFAs but they failed to recognise the surface of unfixed infected red cells. It is possible that the relevant epitopes are located on piroplasm polypeptides which are associated with the cytoplasmic face of the red cell membrane and the cytoskeleton.

In the absence of monoclonal antibody probes, the two polyvalent bovine immune sera, which showed high anti-piroplasm titres, were tested in unfixed IFAs to determine whether the serum antibodies recognise the surface of infected red cells. The T. annulata stock of piroplasm material tested was, in each case, the same as the stock of sporozoite material used to immunise animals and generate the immune sera, but the sera and the piroplasm infected red cells which were tested were otherwise unrelated. These sera failed to recognise ISEMPs and this result raised a
number of questions. Firstly, the particular sera used may have been inappropriate for detecting surface ISEMPs. In several respects the immune sera were not optimal, as they were derived from animals which had been immunised with two to three large doses of sporozoite stablate and which never showed high parasitaemias for prolonged periods. For the purpose of generating a polyclonal serum probe against ISEMPs, a pure infected red cell membrane preparation might be a more suitable immunogen. If parasite antigenic variation takes place in red cell surface molecules, as has been found with Plasmodial systems (McLean et al. 1986), sera would have to be tested against the parasites directly involved in eliciting the particular antibody response. Secondly, immune sera would not be an appropriate probe for ISEMPs if antibodies are not generated against them. The failure of parasite epitopes to elicit host antibody responses due to a similarity between the parasite and host epitopes is sometimes designated (rather subjectively) as molecular mimicry. It has been suggested that mimetic parasite molecules confer a selective advantage and occur due to the inability of the host to recognise them. However, explanations of the term also embrace the possibility that cross-reactive epitopes occur through mechanisms of co-evolution or conservation. Whatever the evolutionary mechanism, similarities between host and parasite gene products have been reported for several parasite systems (Damian, 1989). Certainly, the same immune sera fail to recognise the surface of infected leucocytes, while monoclonal antibody probes detect infection-associated epitopes on the surface of these cells (Shiels et al. 1986a). To determine whether there is an \textit{in vivo} antibody response to the infected red blood cell surface, a better immunisation protocol may be one which mimics host exposure to parasitaemias in endemic areas. This might involve immunisation with low doses of infected red cells or merozoites, at frequent intervals, over a long period of time relative to the life expectancy of the bovid. Thirdly it is possible that a weak reaction between serum antibodies and ISEMPs would not be detected by the surface immunofluorescence assay used. This third possibility was tested by performing immunoprecipitation experiments with surface labelled infected red cells.

Formal proof that the immunoprecipitated 215kDa
polypeptide is of piroplasm origin might come from immunoprecipitation experiments with intact, metabolically labelled infected red cells. This experiment was not carried out, primarily because the signal from the immunoprecipitated 215kDa polypeptide was very weak (figure 3.2.1.a track 1), and it was not detected in Western blots with infected red cell extracts: this suggested that the sera were not going to be useful in detecting ISEMPs. When a crude red cell membrane fraction was prepared from metabolically labelled infected red cells, a similar 215kDa polypeptide, which was not detected in the whole cell extract (in this experiment), was detected in the membrane fraction after SDS-PAGE analysis and autoradiography (figure 3.5.3.1. track a). Thus, polypeptides in this size range are synthesised by the piroplasm and may be associated with the red cell membrane. A 215kDa polypeptide was only weakly detected in red cell surface, or metabolically, labelled extracts which suggests that the polypeptide is not abundant. However subsequent 35S metabolic labelling experiments, carried out with longer incubation times (for 48 hours rather than 24 hours), with red blood cells infected with Ankara, Gharb or Hissar piroplasms, showed that several large polypeptides, including a 215kDa polypeptide are synthesised by the piroplasm (approximately 215, 237, 247 and 266kDa polypeptides) as major products. This experiment is presented in section 4.6. and figure 4.6.1.2. track b, shows the high molecular mass polypeptides. It appears, therefore, that these polypeptides and possibly the 215kDa polypeptide detected in the immunoprecipitation experiments, are abundant after forty-eight hour synthetic labelling periods. Further work also showed that extracellular piroplasms or merozoites are sometimes numerous in infected red cell material. These would have to be removed before carrying out red cell labelling studies. It is unlikely that the detected 215kDa polypeptide was derived from surface labelled piroplasms which were free in the red cell sample as this molecule is one of the least heavily surface labelled and immunoprecipitated piroplasm polypeptides (appendix 1b).

One of five immunoprecipitation experiments produced evidence for the existence of a 215kDa infection specific polypeptide on the surface of red cells. The failure of the other
immunoprecipitation experiments to confirm this result may be due to a number of factors. It is clear that the signal from the 215kDa polypeptide was weak and it is possible that, where it was not detected, this was due to insensitivity on the part of the assay. Thus, it is possible that low levels of the 215kDa polypeptide, rather than its absence in Gharb and Ankara stocks, would account for the results obtained. Similarly, low titres of specific antibodies, rather than absence of antibodies in Gharb (M56) or Ankara (serum 155) immune serum, could also account for the results. This suggested that the immunoprecipitation experiment with immune serum 128 and Hissar infected red blood cells (45% parasitaemia) was more sensitive than the other immunoprecipitation experiments. All these experiments were performed using labelled material with approximately $0.5 \times 10^8$ TCA precipitable cpm. As the specific activity of $^{125}I$ labelled protein varied considerably (see table 3.2.) between experiments, the amount of protein which was introduced into each immunoprecipitation experiment also varied. In the two experiments with Hissar infected red blood cells, approximately $10-100$ fold greater amounts of protein extract were used than in the other experiments. In one of the experiments with Hissar infected cells, the 215kDa polypeptide was detected, and in the other, the parasitaemia was perhaps too low (20% compared to 45%) to allow detection. By using equal levels of radioactivity in all the experiments, it was imagined that the assays would all be equally sensitive, with low protein concentrations being compensated for by high specific activities. While this is probably true for the more abundant polypeptides, it may not be applicable with polypeptides which are less abundant, such as the 215kDa polypeptide. A very dilute polypeptide, which is labelled to a high specific activity, is less likely to be detected by immune sera and subsequently in autoradiographs; while a more concentrated polypeptide, labelled to a lower specific activity, is more readily detected. Also, polypeptides labelled to a high specific activity may have a reduced avidity, even for polyvalent serum antibodies, as a result of the modification of tyrosine residues by iodine.

This argument might explain why the 215kDa polypeptide was not detected in the Ankara and Gharb immunoprecipitations. It
would not explain the failure to detect a 215kDa polypeptide in the surface labelled, uninfected red cell extracts, because the labelling efficiencies of this sample and the Hissar infected red cell samples were very similar. When these two labelled extracts were subsequently subjected to direct SDS-PAGE analysis, the resulting autoradiograph showed that the labelled polypeptide profiles were indistinguishable and the 215kDa polypeptide was not detected. Therefore, not only was the total incorporation into both samples very similar, but it was likely that each polypeptide in each sample had been labelled to approximately the same relative specific activity. This provided further evidence that the 215kDa polypeptide had not simply been specifically detected due to differences in labelling efficiency.

The failure of the immune sera to recognise the 215kDa polypeptide in Western blotting experiments (section 3.3.) may reflect the fact that the polypeptide, or antibodies against it, are of relatively low abundance, but could also be due to poor electrophoretic transfer of polypeptides of this size from the gel to nitrocellulose, or possibly a reduced signal due to denaturation of conformational epitopes in SDS. When the reactivity of the immune sera on Western blots of infected red cell and the derived piroplasm extracts were compared, the reaction profiles were generally indistinguishable, or fewer polypeptides were detected in infected red cell extracts. Infrequently, polypeptides were detected in the infected red cell extracts which were not detected in the piroplasm extracts. In two experiments with immune serum 155, piroplasm (Ankara) polypeptides of molecular mass 156kDa and 168kDa were only detected in infected red cell extracts. Whilst this result may be due to differential degradation in the two samples, it is suggested that these polypeptides may be products of the piroplasm which are, at some stage, located outside the piroplasm. Interestingly, molecules of 156kDa and 168kDa are abundant in supernatants from $^{35}$S metabolic labelling experiments (section 4.7.2.) but, although present, neither are abundant in metabolically labelled piroplasm extracts.

In addition to the 215kDa polypeptide, the results of metabolic labelling experiments followed by analysis of red cell membrane preparations, suggest that polypeptides of 20kDa and...
128kDa are enriched in crude membrane fractions compared to either infected red cell extracts or purified piroplasm extracts. The membrane preparations were, however, highly contaminated with piroplasm polypeptides from the intracellular parasite. On the basis of this tentative result it was determined that it would be worthwhile deriving a pure infected red cell membrane which might provide evidence for membrane ISEMPs. At least, such a membrane preparation would provide suitable material for attempting to generate monoclonal antibodies or a polyclonal serum against ISEMPs.

The control metabolic labelling experiment, which was carried out to ensure that only piroplasm polypeptides were labelled in infected red cell samples, was informative because four labelled polypeptides (68-80kDa) were detected in uninfected red cell samples. The experiment was not a quantitative assessment of the observed contamination and a better experiment would involve labelling a known number of peripheral blood leucocytes. On the basis of the results, however, it was estimated that the observed level of contamination would not be detected if sufficient radioactive counts were loaded onto a gel track, such that all labelled piroplasm polypeptides could be relatively rapidly detected. It was predicted that the level of contamination would become significant in samples containing low amounts of radioactivity, such as in infected red cell membrane preparation. In practice, there were no interpretive problems associated with the results of this form of labelling experiment and so further experimentation was not required.

Either four-five or fifteen-twenty surface labelled red cell polypeptides were detected using the Iodogen method of surface iodination. Interpretation of the results was not aided by the finding that the labelled polypeptide profiles were very variable between different red cell samples and between different labelling experiments of similar samples. However, from numerous experiments it was concluded that each polypeptide, which was characterised by SDS-PAGE and autoradiography in the infected samples, was also represented in the uninfected samples. In addition, the data was too crude to permit the conclusion that infection specific polypeptides do not exist. The iodination method was discarded in favour of biotin-streptavidin labelling as
described in section 4.3.2., which has proved to be far more reproducible as a general labelling technique.

It is probable that if tyrosine/methionine-adequate ISEMPs are abundant in the red cell membrane, that the experimental approaches adopted here would have detected them. From this it may be inferred that such ISEMPs are not prevalent in the membrane. In several respects the results described here mirror early projects with *Plasmodium* which had similar aims. As reviewed by Sherman (1985) surface labelling strategies produced only marginal evidence for the existence of Plasmodial ISEMPs and it has taken a combination of more sophisticated approaches to demonstrate their existence.

The accumulated results suggest that the following molecules may be ISEMPs: the immunoprecipitated 215kDa polypeptide, the 20kDa and 128kDa piroplasm molecules which were enriched in metabolically labelled red cell membrane fractions, and the 168kDa and 156kDa polypeptides which were occasionally detected in infected red cell extracts but not piroplasm extracts by Western blotting with immune sera. If these molecules are located on the surface of red cells, they may be disguised by normal red cell surface polypeptides in direct SDS-PAGE/autoradiographic analyses of labelled red cell components (see appendix 1a). For example, surface labelled polypeptides of 128kDa are detected in control red cell extracts consistent with the expected mobilities of the anion transporter protein (band 3) and the glycoprotein group PAS-I. Similarly, the *Theileria* polypeptide of 215kDa co-migrates with a red cell polypeptide (possibly ankyrin) and the *Theileria* 20kDa polypeptide may have been obscured by red cell PAS-III glycoprotein (glyconnectin) which in human erythrocytes has a molecular mass of 24kDa in Laemmli SDS-PAGE buffer systems. Molecules of 160kDa were also frequently detected in both infected and uninfected, surface labelled red cell extracts.

It was concluded that a better experimental approach to the question of the existence of ISEMPs, would be to use metabolically labelled red cells in order to determine whether piroplasm encoded polypeptides reside in the red cell membrane; combined with the use and analysis of an infected red cell membrane preparation which is representative of its *in vivo* composition.
The aim of the experiments described in this chapter was to produce a method for isolating red blood cell membranes from infected red blood cells that were free from contaminating piroplasm material. The rationale of this was that such material would allow an alternative approach to be taken in the continuing study of the infected red blood cell membrane. The experimental objective was to metabolically label piroplasm polypeptides within the intact red cell and then analyse red blood cell membrane preparations by SDS-PAGE to determine whether piroplasm derived polypeptides were present. The experimental approach was to use a solid support of Affi-Gel beads which are coated with positively charged polylysine. Red blood cells, with a net negative charge, bind to Affi-Gel beads. The bound red cells could then be lysed and the membranes subjected to effective washing steps to remove any contaminating piroplasm material.

4.1. INITIAL RESULTS

Jacobson & Branton (1977) used polycationic beads to isolate plasma membranes and there are now several reports of the use of Affi-Gel beads to isolate red blood cell membrane from *Plasmodium* infected red blood cells (Gruenberg & Sherman, 1983; Haldar et al. 1986). As a starting point, three protocols were followed with infected and uninfected red blood cells and conditions were adapted from all three to give the procedure described below.

4.1.1. Affi-Gel membrane protocol I

All steps were performed at room temperature and in the presence of protease inhibitors (2.1.3.). Affi-Gel beads (Bio-Rad, Affi-Gel 731) were washed 2x in 5 volumes of 2M ammonium chloride by resuspending the beads and then allowing them to settle. Then the beads were washed 3x with distilled water, 3x in filter-sterilised sucrose phosphate buffer (SP buffer, 12mM sodium phosphate buffer, pH7.5, 250mM sucrose) and resuspended in an equal
volume of sucrose phosphate buffer. Red blood cells were prepared as described in section 2.1, washed 5x in sucrose phosphate buffer and resuspended in an equal volume of sucrose phosphate buffer. Prepared Affi-Gel beads (100μl) were transferred to an eppendorf and 100μl (5x10^7 cells) of the red blood cell suspension was added with gentle mixing. To determine the efficiency of the binding, in terms of the coverage of beads by red blood cells, 1μl of the red cell-Affi-Gel mixture was dropped into 200μl of sucrose phosphate buffer. The container was inverted to disperse unbound red cells and then a sample was examined under the x10 objective of a light microscope. If the beads were not totally covered, starting samples of red cells and Affi-Gel beads were re-washed 3x in sucrose phosphate buffer after which the binding efficiency was generally improved. Red blood cells which bound to Affi-Gel beads were not eluted during subsequent washing steps.

Unbound red blood cells were removed by resuspending the red cell-Affi-Gel mixture in 1.5ml of sucrose phosphate buffer and allowing the beads to settle. The supernatant was removed and this washing step was repeated 3x. Lysis of bound red blood cells was achieved by the addition of 200μl lysis buffer (EP lysis buffer, 12mM sodium phosphate buffer, 20mM EDTA, pH7.5). The bead suspension was vortexed at high speed for 20 seconds, 1ml lysis buffer was added and the beads allowed to settle. The supernatant was aspirated and the lysis and washing sequence repeated 5x. Membrane proteins were extracted by the addition of 15μl SDS-sample buffer and incubation for 30 minutes at room temperature. The bead suspension was centrifuged at 10,000g for 1 minute to pack the beads, the supernatant was collected, clarified by centrifugation at 10,000g for 10 minutes and the resulting supernatant stored at -70°C. Table 4.1.1. shows a flow plan of the protocol.

This protocol gave the greatest yield of red blood cell membrane proteins as determined by SDS-PAGE and staining of polypeptides with Coomassie Blue. The reported binding capacity of Affi-Gel beads is 5x10^7 human red blood cell membranes per 100μl of prepared beads (50μl packed) and is equivalent to 15μg of protein (Jacobson & Branton, 1977). Using the protocol described above 100μl (50μl packed volume) of Affi-Gel beads bound approximately
Table 4.1.1. Affi-Gel membrane protocol I

1. infected rbc washed x3 in sucrose phosphate buffer (SP buffer, 4.1.1.) 
   \((100\mu l \text{ rbc } (5\times10^7 \text{ rbc}) (100\mu l \text{ 1:1 v/v) in SP buffer})\)

2. mix with prepared beads in SP buffer

3. wash x3 in SP buffer

4. lyse rbc in phosphate-EDTA buffer (EP lysis buffer, 4.1.1.) & vortex mixture at high speed for 20 seconds

5. wash x5 in EP lysis buffer with vortexing

6. extract sample in SDS sample buffer (2.5.1.).

Table 4.2.1. Artificial contamination of beads (4.2.1.)

I. \(^{35}\)S-metabolically labelled infected rbc\((5\times10^7)\) in SP buffer

1a. mix with prepared beads in SP buffer

2a. wash x3 in SP buffer

3a. lyse rbc in EP lysis buffer and vortex at high speed for 20 seconds

4a. wash x5 in EP lysis buffer with vortexing

5a. extract samples in SDS sample buffer

II. unlabelled uninfected rbc\((5\times10^7)\) in SP buffer

1a. 

2a. 

3b. contaminate with \(2\times10^5\) \(^{35}\)S labelled piroplasms in EP lysis buffer

4a. 

5a. 
$3 \times 10^7$ bovine red cells, when fully covered and the normal yield of uninfected red blood cell membrane protein from beads was approximately $15 \mu g/100 \mu l$ SDS sample buffer/50\(\mu l\) of packed beads. A minimum of $150 \mu l$ of SDS sample buffer was required to elute the bound proteins from this volume of beads in a retrievable volume of $100 \mu l$; approximately $50 \mu l$ of buffer was always lost in the dead space surrounding the packed beads. $100 \mu l$ was also the upper limit of the volume that could be loaded into one sample well on a gel when using the available apparatus.

Infected and uninfected red cells bound equally well to Affi-Gel beads. This was determined by applying $5 \times 10^7$ red blood cells, with a 65% parasitaemia, to $100 \mu l$ Affi-Gel beads and collecting the unbound red cells from 3 washes (approximately $2 \times 10^7$ red cells). The parasitaemia of the unbound red blood cells was the same as the starting sample, as assessed by examination of Giemsa stained preparations, which implied that there was equal binding to Affi-Gel beads by infected and uninfected red cells.

4.2. OBSERVATION OF CONTAMINATION

When Affi-Gel bound membranes from infected red blood cells (40% parasitaemia) were stained with Giemsa, examined under a light microscope and several piroplasm nuclei were seen on each bead. It appeared that either nuclei or whole parasites (it was difficult to define which under these conditions) were attached directly to the beads, or to bound red cell membranes. This contamination may have been an artefact of the slide preparation (i.e. unattached piroplasms or nuclei dried down onto beads): more importantly it was not known whether this (potential) level of contamination would make a detectable contribution when the membranes were analysed by SDS-PAGE.

Membrane extracts from this preparation were separated in a 4.5-15% acrylamide gel. The Coomassie Blue stained polypeptide profile was identical to that produced with uninfected membranes. Therefore the contribution of this contamination was insignificant on the basis of the protein stained gels. However it could be significant when metabolically labelled samples were used.
4.2.1. Artificial contamination of Affi-Gel beads

In order to examine this possibility, uninfected red blood cells (cow110) were bound to beads and approximately $2 \times 10^5$ metabolically labelled piroplasms, containing $5 \times 10^4$ TCA precipitable cpm, were added with the first addition of lysis buffer during the membrane isolation procedure. In addition, metabolically labelled infected red blood cells ($5 \times 10^7$ Gharb infected red cells, 40% parasitaemia, $3 \times 10^4$ TCA precipitable cpm) were bound to beads and both samples were processed simultaneously for membrane isolation (see Table 4.2.1.). The artificially contaminated and the infected red cell membrane extract contained 20% of the original cpm. 50µl (approximately 3,000 cpm) of each membrane extract, together with a total extract of the piroplasm sample used to contaminate the Affi-Gel beads, were electrophoretically separated in parallel in a 4.5-15% acrylamide gradient gel. The control experiment, to screen for the labelling of polypeptides which are not of piroplasm origin, in which uninfected red blood cell material was metabolically labelled, has been described previously in section 3.5.2. and is discussed in section 3.7. (p<sub>372</sub>)

For the test experiment it was important that the metabolically labelled piroplasms, used to contaminate the uninfected membranes, were free from any metabolically labelled polypeptides situated in the red cell membrane. The infected red blood cells which were used to prepare infected membranes had been metabolically labelled as intact cells, but the "contaminating" material had been labelled after lysis of the red cell as follows. Red blood cells (Gharb stock, 40% parasitaemia) were lysed with Tris-buffered ammonium chloride (2.3.1.7.), the piroplasms were washed 4x in RPMI-1640, harvested by centrifugation at 300g for 10 minutes, metabolically labelled (2.1.) and then washed 3x in sucrose phosphate buffer. The reasons for lysing the red blood cells before the labelling step were two fold. First, incorporation into protein was found to be greater by this method (100x greater in this experiment) than with intact red blood cells and it was decided to bias the experiment in favour of identifying labelled proteins in the uninfected membrane preparation by using proteins labelled to a high specific activity.
This was done rather than adding more parasite protein than was present in the infected membrane preparation, as the removal of piroplasms from the beads was found to be, at least in part, a function of washing times. The second reason was to further preclude the presence of metabolically labelled peptides in the red blood cell membrane.

Inspection of the extracted polypeptides after SDS-PAGE and autoradiography (figure 4.2.1.) indicated which of the labelled polypeptides in the membrane fractions were contaminants. Track a) shows the artificially contaminated membrane preparation, track b) shows the membrane preparation from metabolically labelled infected cells and track c) shows the total SDS extract of the metabolically labelled piroplasms used to contaminate the uninfected membranes. The loading on this gel was unintentionally unequal: longer autoradiographic exposure times clearly showed that each of the polypeptides present in track c) was also present in track a). This suggested that the observation of many, if not all the polypeptides in the infected membrane preparations was due to the presence of contaminating piroplasms in the preparation. Comparisons between tracks b) (the infected membrane) and c) (the total extract of piroplasms) in figure 4.2.1. are hindered as the labelling procedure was different for the two extracts. Variations in the polypeptide profile could reflect elution or concentration of certain polypeptides during the membrane preparation, but they could also reflect the fact that the cells were labelled under different conditions. However, polypeptides of molecular mass 136, 62, 61, 48, 47kDa and four molecules of a larger molecular mass than myosin (> 205kDa) seemed to be concentrated in the infected membrane preparation (b), while 42kDa and 40kDa molecules were less abundant. The twelve major polypeptides in the infected membrane preparation (52kDa to 129kDa) were all present in the artificially contaminated uninfected red cell membrane preparation. Representatives of all the available "contaminating" labelled polypeptides were clearly not being removed from the beads which suggested the contamination was by whole piroplasms and not by a subset of piroplasm polypeptides.
Figure 4.2.1.

Analysis of contamination, by $^{35}$S methionine labelled piroplasm polypeptides, of red cell membranes bound to Affi-Gel beads.

The design of this experiment is outlined in table 4.2.1. The Affi-Gel membrane preparation protocol is described in section 4.1.1. and outlined in table 4.1.1.

Track a = "contaminating" piroplasm polypeptides. Unlabelled, uninfected (cow110) red cells were bound to Affi-Gel beads, and exposed to $^{35}$S methionine metabolically labelled freeze thawed piroplasms, at the red cell-lysis stage of Affi-Gel membrane preparation (Gharb stock, from 40% parasitaemia blood [see section 4.2.1. for piroplasm preparation protocol and rationale], 2x10$^5$ piroplasms containing 5x10$^7$ cpm). The resulting membrane extract contained 20% of the cpm originally exposed to Affi-Gel beads.

Track b = resolved extract of piroplasm-infected red cell membrane preparation. Metabolically labelled, (section 2.4.1.) infected red cells (Gharb stock, 40% parasitaemia, 5x10$^7$ red cells, 3x10$^4$ TCA precipitable counts) were processed for red cell membrane preparation with Affi-Gel beads. The extracted membrane sample contained 20% of the original cpm applied to the Affi-Gel beads.

Track c = metabolically labelled piroplasm polypeptides. The total extract of labelled piroplasms used to contaminate red cell membranes shown in track a.

For each track, approximately 3,000 cpm of the sample extract was resolved by SDS-PAGE in a 4.5-15% gel. The gel was treated for fluorography, dried down and exposed to x-ray film for six weeks. The mobility and molecular mass of internal standard polypeptides are indicated on the left hand side of the figure and the estimated molecular mass of five labelled polypeptides are indicated on the right hand side of the figure.
4.3. METHODS OF ANALYSIS

In order to test various methods for obtaining purified infected membranes, several logistical requirements had to be considered so that the necessary experiments could be undertaken. These requirements were as follows:

- A reasonably consistent supply of infected red blood cells. The normal supply of fresh infected material comprised large amounts, at infrequent intervals (once or twice every three months) and the *T. annulata* stock and parasitaemia of the material varied. Fresh infected material was routinely collected, processed and experiments initiated the same day; rather than stored, because no data were available from longitudinal studies with stored infected red blood cells. This was inefficient, and the number of variables in any experiment were enlarged when different stocks, and samples with different parasitaemias, were consistently included.

- A highly sensitive method for the detection of piroplasm and red blood cell membrane polypeptides, so that preparations could be rapidly assessed for purity.

- Methods for detecting piroplasms and their nuclei without fixation. This was to avoid artefacts generated by slide preparation, fixation and Giemsa staining.

- Development of storage methods for Affi-Gel bound red blood cells so that comparisons between different samples could be made at one time, even when the required, fresh material was not available simultaneously.

The approaches that were taken to develop methods to fulfil these requirements are described in the subsequent sections, 4.3.1. to 4.3.4.
4.3.1. Storage of red blood cells over three weeks

The British Transfusion Service stores human blood, according to standard Red Cross procedures for up to six weeks in CPDA-1 (2.1.1.). This was taken as evidence of its efficacy as an anticoagulant and as a red cell storage medium and so piroplasm infected blood was routinely collected into CPDA-1. The following experiment was carried out to monitor deterioration in infected red blood cells during a three week storage period in CPDA-1 at 4°C. It was hoped that infected blood could be maintained in its original state during storage, as this would extend the time during which experiments could be undertaken.

Infected blood (Hissar stock, with a parasitaemia of 55%) was treated, as described in 2.1.3., to remove leucocytes and plasma. Red blood cells were resuspended in ten times their packed cell volume of CPDA-1 (2.1.1.) and stored at 4°C in darkness. The surface protein profile of stored red blood cells was monitored by surface (iodogen catalysed) iodination (2.4.2.) on days 0, 3, 5 and 21 of storage. The metabolic activity of piroplasms was monitored by metabolic labelling with $^{35}S$ methionine for a 24 hour incubation period (2.4.1.) on days 0, 3, 5, and 21 of storage. The radioactivity of TCA precipitated aliquots of each sample was determined. This revealed that $^{35}S$ incorporation on days 0 and 21 of storage was identical and some 15% higher on the intervening days, which suggested that there had been no major changes in metabolic activity. The $^{125}I$ label was more varied as the TCA precipitable counts in the day 21 sample were three times greater than in the day 0 sample and 10x greater than in samples from days 3 and 5.

Iodinated and metabolically labelled extracts were resolved electrophoretically in separate 10% acrylamide SDS-PAGE gels. $10^6$ TCA precipitable cpm of iodinated extract and $2\times 10^4$ TCA precipitable cpm of metabolically labelled extract were loaded per track. The resulting autoradiographs are shown in figure 3.2.4b. Figure 3.2.4b shows the metabolically labelled profiles on days 0, 3, 5 and 21 of storage. The profiles on days 3, 5 and 7 are distorted by the presence of bovine plasma albumin (66kDa) from the foetal calf serum (1%) in the incorporation medium and in this
figure it appears that there is a unique band of 78kDa in the fresh sample which is absent from the stored samples. This is artefactual and all the polypeptides shown in the figure were present in all the samples, apart from a 159kDa polypeptide which was only observed in the day 5 preparation. Twelve polypeptides between 31kDa and 131kDa were detected and they conformed well to the molecular masses of major metabolically labelled polypeptides derived in other experiments. Three polypeptides of 159kDa to 190kDa were observed in subsequent metabolic labelling experiments, where the incubation time was extended to 48 hours, so the observation of this polypeptide was not thought to be an artefact of storage at 4°C. Figure 3.ii shows the profile of $^{125}$I surface iodinations of fresh infected red cells (do) and on days 3, 5, and 21 of storage. On the basis of previous surface iodination experiments, it was expected that the labelled polypeptides would be molecules of approximately 115, 96, 64, 43, and 20kDa. The observed profile consisted of polypeptides of 142, 130, 125, 117, 102, 76, 64, 54, 43, 25 and 20kDa. As with the metabolically labelled profiles, all the labelled polypeptides were present in each of the samples. A large degree of variation in the specific activity of labelled polypeptides was apparent between each sample, especially in the day 21 sample where the 117, 64, 43, 25 and 20kDa polypeptides were most the intensely labelled.

A visual appraisal of the stored red cells by light microscopy suggested that the red cells had retained their normal discoid shape, without forming spiky echinocytes. However, it was noted that after three weeks in storage, the red cells became echinocytic more rapidly (in PBS at room temperature) than fresh cells. After four weeks in CPDA-1 the infected and uninfected red cells lost their discoid shape and became echinocytic. This was not observed when red cells were stored for four weeks in RBC storage solution (section 2.1.1).

It was also determined that stored and fresh red cells bound equally well to Affi-Gel beads.
4.3.2. Biotin-\(^{125}\) Streptavidin labelling

The protocols described in section 2.4.3. were followed to surface label red blood cells and piroplasms and to label cell lysates and Affi-Gel bound protein with biotin. Samples were analysed by direct extraction and SDS-PAGE in 4.5-15% acrylamide gradient gels, followed by Western blotting and probing with \(^{125}\)I-streptavidin (sections 2.5. & 2.6.). Unbiotinylated samples were also probed with \(^{125}\)I-streptavidin to assess the specificity of the probe. The unbiotinylated samples did not bind detectable levels of \(^{125}\)I-streptavidin. Many more labelled polypeptides were detected in the lysate extracts than in the surface labelled extracts (data not shown) which suggested that there was some restriction of the biotin label in the surface labelling experiments to the surface of piroplasms and red cells. When surface labelled infected (40-60% parasitaemia) and uninfected red blood cell extracts were compared the separated profiles were generally indistinguishable. Either 4-5 or 15-20 surface labelled red cell polypeptides were routinely detected, which accorded with the results from direct surface iodination experiments. The labelling of red cell membranes and piroplasm polypeptides was found to give very consistent polypeptide profiles. Appendix 1 gives details of the polypeptides detected. In the following results sections 4.4 and 4.5, the biotin-\(^{125}\)I-streptavidin Western blotting system of labelling and detection was the principal method used in analysis of red cell and piroplasm polypeptides. Biotin labelled samples appeared to remain stable at -20°C: after two months storage at -20°C, labelled sample extracts generally gave the same profiles of detected polypeptides as freshly prepared extracts. The \(^{125}\)I-streptavidin probed blots were rapidly visualised after 1-4 days autoradiography.

4.3.3. Acridine Orange staining

The fluorochrome Acridine Orange was added to samples of infected red blood cells, or membranes which had been bound to Affi-Gel beads, on a microscope slide to give a final concentration of 0.1\(\mu\)g/ml in PBS. Under the x50 objective of a fluorescence
microscope, with a Leitz 12/3 filter block, membrane material could be seen fluorescing with a faintly luminous green colour and nuclei were bright yellow-green. Under the x10 objective, and without applying a coverslip, beads with attached piroplasms could be observed directly. Figure 4.3.3. a) shows the resulting fluorescent pattern when Affi-Gel bound infected red blood cells were treated with Acridine Orange and b) shows the remaining fluorescing piroplasm nuclei on Affi-Gel beads after the lysis and vortexing steps of membrane preparation (section 4.1.1.).

It was determined (see section 4.4.1.) that free piroplasms bind to Affi-Gel beads, and therefore it was important to ensure that free piroplasms were absent from red cell samples which were going to be processed for membrane isolation with beads. Certain infected red blood cell samples were found to contain high levels of free piroplasms and in these samples red blood cells were characteristically fragile. Percoll density gradient centrifugation was found to be effective in separating free piroplasms from intact red blood cells, however, the additional washing steps required to remove Percoll from the red cells generally resulted in the lysis of a proportion of these fragile red cells and in the generation of free piroplasms. The simple solution was not to use red blood cell samples which contained free piroplasms and this was routinely assessed as follows. Red blood cells were centrifuged at 300g for 10 minutes so that free piroplasms would form the bottom portion of the pelleted sample. This portion was transferred to a glass slide with 1ul of 1ug/ml Acridine Orange in PBS. A coverslip was applied and the cells observed as described above. At this concentration of Acridine Orange both piroplasms, their nuclei and red blood cell membranes fluoresced, so free piroplasms could be identified.

4.3.4 Storage of Affi-Gel bead-bound membranes at -20° C

It was not known whether red blood cells which were bound to Affi-Gel beads could be stored frozen, and then subsequently processed for membrane isolation, without generating storage artefacts. This was investigated as follows: infected and uninfected red blood
Figure 4.3.3.

Photograph of Acridine Orange stained piroplasms.

Fluorescence patterns were detected by observation through a 50x objective of a fluorescence microscope (Leitz 12/3 filter block) and photographs were taken with a microscope camera.

Fig. 4.3.3.a is a photograph of infected red cells which have been bound to Affi-Gel beads and treated with Acridine Orange (0.1 μg/ml, section 4.3.3.).

Fig. 4.3.3.b is a photograph of the same sample of Affi-Gel beads after the membrane preparation protocol outlined, in section 4.1.1, has been carried out, and after staining with Acridine Orange.
Figure 4.3.3.

a

20μm

b

20μm
cells were bound to Affi-Gel beads (4.2/1.), unbound red cells were removed and the packed mixture was stored at -20°C for five weeks. Identical fresh samples were processed for membrane isolation (4.1.2l), biotin labelled in situ on Affi-Gel beads; extracted and supernatants were stored at -70°C for five weeks. The stored red blood cell-Affi-Gel mixture was processed for membrane isolation after rapidly thawing at 37°C with the addition of warm lysis buffer. The resulting bead bound membranes were biotin labelled and extracted in SDS-sample buffer as above. The membrane extracts from stored and fresh, infected and uninfected red blood cells were subjected to SDS-PAGE, Western blotted, probed with 125I streptavidin and autoradiographed. After exposure for seven days, the developed autoradiograph indicated (data not shown) that the polypeptide composition of stored and fresh membranes was identical and that the membranes from infected red blood cells were heavily contaminated with piroplasm polypeptides.
4.4. CONTAMINATION ASSAY

It was envisaged that piroplasm contamination could come from several sources:

- from free piroplasms in the material, binding alongside red blood cells to the Affi-Gel beads

- from piroplasms released from red cells during the lysis step, which bound to unoccupied sites on the beads

- from the released piroplasms binding to the cytoplasmic face of bound red cell membranes, or from piroplasms simply not being washed away.

Free piroplasms (or merozoites, they were not readily distinguishable by light microscopy, but both could have been present) were frequently observed, either singly or in clumps of 10-20 cells, in infected red blood cell material. These were first observed in immunofluorescence microscopy assays with anti-piroplasm/merozoite monoclonal antibodies screened against infected red blood cell material. Here, counter staining with Evans Blue allowed visualisation of red blood cells. Parenthetically it seemed unlikely that free merozoites, which invade negatively charged red cells, would bear a similar, net negative surface charge.

The binding of released piroplasms might be prevented by selective washing procedures. Alternatively, this contamination might be prevented by using an effective blocking agent, to occupy areas of the beads which were not covered by red cell membrane, or compete with beads for piroplasms. The possible simultaneous binding of free piroplasms to Affi-Gel beads alongside red cells was more of a problem because any blocking agent would presumably also inhibit the binding to red cells. The conditions required to remove bound piroplasms might also elute red cell membranes, or selectively remove membrane proteins.
4.4.1. Piroplasm contamination assay

In order to determine whether piroplasm proteins would bind to Affi-Gel beads under the membrane isolation conditions, the following experiment was carried out. $5 \times 10^5$ purified piroplasms which had been stored at $-70^\circ C$ were rapidly thawed at $37^\circ C$ and then biotinylated according to the procedures for surface biotinylating piroplasms (2.4.3). Freeze-thawing rendered the piroplasms susceptible to internal labelling by this method, but the nuclear membrane was not disrupted and Giemsa stained piroplasms appeared intact. This number of piroplasms was $100 \times$ less than of the number of piroplasms which were normally applied to Affi-Gel beads as infected red blood cells. The biotinylated piroplasms were exposed to Affi-Gel beads and the red blood cell membrane-preparation procedure was followed with all lysis, vortexing and washing steps (see table 4.4.1.). The bound proteins were extracted and compared by electrophoresis of the samples in a 10% acrylamide gel with a sample obtained by direct extraction of the same biotinylated piroplasms. The samples were Western blotted and biotinylated polypeptides were probed with $^{125}$I-streptavidin.

Figure 4.4.1. shows the resulting autoradiograph, track 1) shows the polypeptides from $5 \times 10^5$ piroplasms, track 2) shows the polypeptides which were recovered from Affi-Gel beads after $5 \times 10^5$ piroplasms had been processed as for membrane preparation. All the polypeptides in the starting material were represented in the sample extracted from Affi-Gel beads, which demonstrated that piroplasm proteins and piroplasms bind to Affi-Gel beads under the conditions used. The absence of labelled spectrin polypeptides (243kDa and 265kDa), which were characteristic of biotinylated extracts from Affi-Gel red blood cell membrane preparations, suggested that the piroplasm sample was not significantly contaminated with red blood cell membrane polypeptides.

It appeared that there was quantitatively less of each polypeptide present in the material extracted from the beads (track 2) compared to the amount which was loaded on to the Affi-gel beads (equivalent to the polypeptide profile in track 1) which indicated that some of the original material was removed by the washing procedures. As there was no qualitative difference between
Table 4.4.1. Piroplasm contamination assay

1. freeze thaw purified piroplasms

2. biotin label piroplasms \( \lambda \) (section 2.4.3.1)

3. resuspend in sucrose-phosphate (SP) buffer and mix with prepared beads

4. wash x3 in SP buffer

5. wash x5 in phosphate-EDTA (EP) lysis buffer with vortexing

6. extract washed sample in SDS sample buffer

Table 4.5.1. Blocking assay I

1. freeze thaw purified piroplasms

2. wash x3 in SP buffer

3. resuspend in: 
   - dextran sulphate/SP buffer
   - or polyaspartate/SP buffer
   - or SP buffer

4. mix with prepared beads, wash x3 in SP buffer

5. wash x5 in EP lysis buffer with vortexing

6. wash x3 in PBS pH 8.0

7. biotinylate samples in situ on beads (2.4.3.3.)

8. extract samples in SDS sample buffer
SDS-PAGE analysis of piroplasm contamination of Affi-Gel beads using the membrane protocol outlined in section 4.1.1.

$5 \times 10^5$ freeze-thawed piroplasms were biotinylated (section 2.4.3.), applied to Affi-Gel beads (50µl packed volume) and the mixture was processed for red cell membrane preparation according to the protocol outlined in section 4.1.1. The final eluted polypeptides (track 2) were resolved by SDS-PAGE in a 10% gel, transferred to nitrocellulose and the Western blot was then probed with $^{125}$I-streptavidin and exposed to x-ray film (section 2.6.). Track 1 is a sample obtained by direct extraction of $5 \times 10^5$ biotinylated piroplasms. The procedure for this experiment is outlined in table 4.4.1. The mobility and molecular weight of internal polypeptide standards are indicated on the right hand side of the figure.

The procedure for this experiment is outlined in table 4.5.1. and section 4.5.1. Freeze-thawed purified piroplasms ($5 \times 10^5$ piroplasms) were applied to Affi-Gel beads (50µl packed volume) in the presence of two different possible blocking reagent. The red cell membrane preparation protocol was performed (section 4.1.1.) and then the Affi-Gel beads were biotin labelled (section 2.5.3.2.). Samples of the eluates from the Affi-Gel beads (10µl out total 100µl eluate) were subjected to SDS-PAGE in 10% gels, Western blotted, probed with $^{125}$I-streptavidin and the filters were exposed to x-ray film for one week.

Track 1 = an extract of piroplasm polypeptides bound to Affi-Gel beads in the presence of polyaspartate (10mg/ml).
Track 2 = an extract of piroplasm polypeptides bound to Affi-Gel beads in the presence of dextran sulphate (Mr 5,000, 10mg/ml).
Track 3 = an extract of piroplasm polypeptides bound to Affi-Gel beads in the absence of a blocking agent.

The mobilities of internal molecular weight markers are indicated on the left hand side of the figure.
Figure 4.4.1.
Figure 4.4.1. b Blocking assay II

Track 1 = biotinylated piroplasm polypeptides bound to Affi-Gel beads in the presence of dextran sulphate (10mg/ml).
Track 2 = biotinylated piroplasm polypeptides bound to Affi-Gel beads in the absence of dextran sulphate.

Unlabelled, uninfected red blood cells (5x10⁷ red cells) were applied to Affi-Gel beads (50µl packed volume) and red cell membranes were prepared (section 4.1.1.). The membrane coated Affi-Gel beads were then exposed to freeze-thawed biotin labelled piroplasms, either in or without the presence of dextran sulphate (10mg/ml). The experimental protocol is outlined in table 4.5.3.1. and is based on the protocol described in sections 4.1.1. and 4.5.3. The Affi-Gel membrane protocol was followed (section 4.1.1.); eluted polypeptides were subjected to SDS-PAGE, Western blotted, probed with ¹²⁵I- streptavidin and exposed to x-ray film. The molecular mass of internal molecular weight standards is indicated on the left hand side of the figure.

Figure 4.4.1. c

A comparison of the blocking ability of freshly prepared dextran sulphate and solutions which were more than 24 hours old.

Autoradiograph shows the profile of biotinylated piroplasm polypeptides which bind to Affi-Gel beads during the red cell membrane preparation protocol. The experimental procedure for this experiment was exactly as described for "blocking assay II" (see section 4.5.3.) except that a freshly prepared solution of dextran sulphate (track 2) and a solution which was more than 24 hours old (track 1) were used as possible blocking agents. The mobility and molecular mass of internal molecular weight standard are illustrated on the left hand side of the figure.
polypeptides represented in the two samples and the polypeptide profiles indicated that internal as well as surface piroplasm molecules had been biotinylated; the result suggested that the Affi-gel beads were binding most, if not all, piroplasm polypeptides and not a selected subset.

This experiment was repeated and extended to include an assessment of the binding of biotinylated piroplasms to Affi-Gel beads which had been coated with unlabelled, uninfected red blood cell membranes (cow 110) as this simulated the experiment with the intact red cells more closely. The result from the autoradiograph (data not shown) indicated that there was a quantitative reduction in the level of binding compared to the previous experiment with uncoated beads, although exactly the same profile of biotinylated polypeptides were bound and subsequently detected in autoradiographs.

4.5. AFFI-GEL MEMBRANES II

Gruenberg et al. (1983) described the use of negatively charged dextran sulphate as a blocking agent to reduce the level of Plasmodium contamination in Affi-Gel red blood cell membrane preparations. It was possible that this reagent would be an effective block against T. annulata piroplasm contamination. Polyaspartate, which is positively charged at pH 7.2-7.5, was a second possible blocking agent. In principle, if piroplasms were binding to the beads because of their own positive charge under the low ionic strength buffer conditions, then the inclusion of polyaspartate in the lysis buffer would compete with Affi-Gel beads for piroplasms and thus reduce the binding of piroplasms to the Affi-Gel.

The Affi-Gel red cell membrane protocol of Haldar et al. (1986) involved the inclusion of 0.6M sodium chloride and 3M urea in post-lysis washing buffers. Protein estimations by Bradford assay had previously suggested that this protocol yielded less membrane protein than other methods which were tested; namely those of Cohen et al. (1977) and Gruenberg et al. (1983). Consequently the protocol of Haldar had not been adopted and a less stringent sucrose-phosphate washing buffer had been used (4.1.1.).
Considering the evidence for piroplasm contamination, which was presented in sections 4.2. and 4.4., it was possible that more stringent washing conditions would produce contamination free membranes. Therefore, experiments were carried out to determine the effect on Affi-Gel bound membranes, of the presence in post-lysis washing buffers, of high salt concentrations and 3M urea, and also the blocking effects of dextran sulphate and polyaspartate.

4.5.1. Blocking assay I

Freeze thawed piroplasms were washed 3x in sucrose phosphate buffer. Approximately $5 \times 10^5$ washed piroplasms were added to Affi-Gel beads in the presence or absence of a potential blocking agent in sucrose phosphate buffer ($10 \text{mg/ml}$ dextran sulphate, Mr 5,000; $10 \text{mg/ml}$ polyaspartate). The red cell membrane preparation protocol was carried out (see table 4.5.1.) and then the beads were biotinylated (section 2.5.3.2.). A 10\% sample (10\% of the eluted volume) of the eluates from the beads was separated in a 10\% acrylamide gel by SDS-PAGE, Western blotted, probed with $^{125}$I-streptavidin and the filter autoradiographed.

Figure 4.4.1.a shows the autoradiograph after a one week period of exposure, track 3) shows the profile of piroplasm polypeptides which bound to Affi-Gel beads in the absence of a blocking agent, track 1) shows the effect of polyaspartate and track 2) shows the effect of dextran sulphate. The results suggested that although there had been some reduction in the amount of protein which had bound to the beads, in the presence of either blocking agent compared to the unblocked sample, there was still a significant level of contamination. Dextran sulphate at $10\text{mg/ml}$ was more effective than polyaspartate at $10\text{mg/ml}$. Neither blocking agent appeared to alter the effects of the lysis buffer which lysed red cells but not parasites. Giemsa stained piroplasms, which had been treated with lysis buffer containing either blocking agent, were indistinguishable from piroplasms treated with normal lysis buffer. As dextran sulphate had been moderately effective in blocking piroplasm contamination, a more comprehensive experiment was designed to monitor the effect of dextran sulphate and more stringent washing protocols on membrane composition as follows.

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4.5.2. Blocking and washing assay

The Affi-Gel membrane preparation protocol was carried out as previously described (section & table 4.1.1.) with the following modifications. Dextran sulphate (10mg/ml) was included in the lysis buffer for the initial lysis step and for the five subsequent washing cycles. After the last washing cycle, the bead bound membranes were washed 3x in 1.5ml phosphate-EDTA lysis buffer (pH7.2) supplemented with 0.6M sodium chloride, and then 3x in 1.5ml phosphate-EDTA lysis buffer supplemented with 150mM sodium chloride and 3M urea, pH 7.2.

Changes in the polypeptide composition of bound membranes, during the washing procedure, were assessed in the following experiments:

- Firstly, infected (Hissar) and uninfected red blood cells were surface biotinylated, membranes were prepared as described above and sample extracts from 50µl of bead-bound membranes were collected at three stages during the membrane isolation procedure: after the first five washes in lysis buffer, after the three high salt washes, and after the three washes in lysis buffer containing urea (see table 4.5.2.a).

- Secondly, the membrane isolation procedure was carried out with unlabelled, infected and uninfected red blood cells. Samples (50µl packed beads) of the Affi-Gel bound membranes were collected after each of the three different washing steps described above, washed 3x in PBS (pH 8.0), biotinylated and polypeptides were extracted (see table 4.5.2.b).

- Thirdly, uninfected, unlabelled red blood cell membrane preparations were contaminated with approximately 5x10^5 freeze-thawed piroplasms, which were included in the lysis buffer for the first lysis step. Samples (50µl packed beads) of Affi-Gel bound contaminated membranes were biotinylated after the three different washing steps described above, and then the polypeptides were extracted (see table 4.5.2.c).
**Table 4.5.2. Blocking and washing assay**

**A) SURFACE LABELLED PROFILE WITH DIFFERENT WASHING CONDITIONS**

I. surface biotinylate infected rbc \( q_{10}^{10} / \lambda c \) (section 2.4.3.1.), resuspend in sucrose phosphate (SP) buffer with 10mg/ml dextran sulphate (DS)

1a. mix with prepared beads, wash x3 in SP buffer with DS

2a. wash x5 in phosphate-EDTA (EP) lysis buffer/DS with vortexing
   - extract an aliquot of this sample

3a. wash x3 in EP lysis buffer + 0.6M sodium chloride
   - extract an aliquot of this sample

4a. wash x3 in EP lysis buffer + 150mM sodium chloride & 3M urea
   - extract an aliquot of this sample

II. surface biotinylate uninfected rbc \( q_{10}^{10} / \lambda c \) resuspend in SP buffer with DS

1a. mix with prepared beads

2a. wash x3 in SP buffer with DS
B) CHANGES IN POLYPEPTIDES ON BEADS AFTER DIFFERENT WASHING PROCEDURES

I. unlabelled infected rbc in SP buffer

II. unlabelled uninfected rbc in SP buffer

1a. as in A) 1a. above

2-4a. as in A) 2-4a. above except samples are biotin labelled in situ on beads (2.4.3.3.) prior to extraction

5a. extract samples in SDS sample buffer

C) CHANGES IN CONTAMINATING PIROPLASM POLYPEPTIDES ON BEADS AFTER DIFFERENT WASHING PROCEDURES

I. unlabelled uninfected rbc in SP buffer

1. mix with prepared beads and wash x3 in SP buffer

2. freeze thaw purified piroplasms and resuspend in EP lysis buffer with DS

3. Lyse rbc by addition of suspension C) 2. above

4. wash and biotin label as in B) 2-4a above

5. extract samples in SDS sample buffer.
The sample extracts (10-20μl) were subjected to electrophoresis on 4.5-15% acrylamide SDS-PAGE gels, Western blotted, probed with 125I-streptavidin and filters were autoradiographed.

Figures 4.5.2.1. and 4.5.2.2. show the resulting autoradiographs. Figure 4.5.2.1. tracks a) and b) show the total extracts of surface biotinylated, infected and uninfected red blood cells respectively. The autoradiograph is slightly under developed and, in addition to the 180, 160, 122, 115, 84 and 64kDa polypeptides, further exposure time revealed 43kDa and 26kDa polypeptides. Comparison between infected and uninfected extracts suggested that there were no differences between the two surface labelled profiles. Tracks 1-3 show the uninfected red blood cell membranes, but the infected membrane preparations were identical (data not shown). Tracks 1-3 show the extracts of surface biotinylated, uninfected membranes after five washes in lysis buffer (track 1= first wash), after a further three washes in lysis buffer/0.6M sodium chloride (track 2= second wash) and after a further three washes in lysis buffer/3M urea/150mM sodium chloride (track 3= third wash). The results suggested that the three different washing steps had no qualitative effect on the surface labelled polypeptide composition on the beads. The polypeptide profile did not conform well to the expected surface labelled profile derived from other surface labelling experiments with biotin-streptavidin and it suggested that there was some internal labelling of polypeptides. However, as the infected and uninfected extracts were indistinguishable it appears that all the labelled molecules were of red cell origin.

Figure 4.5.2.2. shows the results of the experiment where samples were biotin labelled in situ on Affi-Gel beads during membrane isolation. Tracks a-c show uninfected red cell membranes: track a= the sample biotinylated after the first washing steps, track b= the sample biotinylated after the second washing steps, and track c= the sample biotinylated after the third washing steps. Tracks d-f show the piroplasm-contaminated uninfected membranes: track d= the sample biotinylated after the third wash, track e= the second wash, and track f= the first wash. Tracks g-i show infected membrane preparations: track g= the sample biotinylated after the third wash, track h= the second wash and
Figure 4.5.2.1.

Experiment to determine the effect of different washing conditions on the profile of surface labelled red cell (infected or uninfected) polypeptides bound to Affi-Gel beads.

Autoradiograph of Western blot of biotinylated polypeptides probed with $^{125}$I-streptavidin. The experimental protocol is described in section 4.5.2. and table 4.5.2. A. Note that the modified Affi-Gel membrane preparation protocol was followed, ie. including the presence of dextran sulphate as a blocking agent.

Track a = total extract of surface biotinylated infected red cells (Hissar stock, $5 \times 10^4$ red cell equivalents were loaded).
Track b = as above but using uninfected red cells.
Track 1-3 = extracts of surface biotinylated, uninfected red cell membranes from Affi-Gel beads after five washes in lysis buffer (track 1), after a further three washes in lysis buffer/0.6M sodium chloride (track 2), and after a further three washes in lysis buffer/3M urea/150mM sodium chloride (track 3).

Material from approximately $5 \times 10^4$ red cells (applied to 50ul packed volume of Affi-Gel beads) was loaded per track. The relative mobility and molecular mass of internal molecular weight standards are illustrated on the left hand side of the figure.

Figure 4.5.2.3.

Photograph of FACS analysis of Acridine Orange stained infected red cells showing differential low angle forward scatter verses relative fluorescence intensity between infected and uninfected red cells.

Acridine Orange stained red cells (see section 2.8) were resuspended to $2 \times 10^5$ cells/ml and introduced into a FACS IV (Becton Dickinson). The cells were analysed by simultaneous low angle scatter and red fluorescence using a 640nm long pass filter and a 600nm band pass filter. The rectangle labelled a, contains infected red blood cells; rectangle b, contains uninfected red cells.
Figure 4.5.2.1.

Figure 4.5.2.3.

relative fluorescence

low angle scatter

k Da

205—

116—

97—

66—

45—

29—
Experiment to determine the effect, on infected and uninfected red cell membranes bound to Affi-Gel beads, of different washing conditions.

Autoradiograph shows a Western blot of biotinylated polypeptides after probing with $^{125}$I-streptavidin. Membrane bound Affi-Gel beads were biotin labelled and sample extracts (10-20μl) were subjected to electrophoresis in 4.5-15% SDS-PAGE gels. The experimental protocol is described in section 4.5.2 and outlined in tables 4.5.2, B & C.

Tracks a-c = uninfected red cell membranes (see table 4.5.2.BII). Samples of Affi-Gel bound membranes (50μl packed beads, originally coated with $3\times10^7$ red cells) were collected after each of the three washing steps described. Track a, after the first washing step (lysis buffer); track b, after the second washing step (lysis buffer/0.6M sodium chloride); and track c, after the third washing step (lysis buffer/3M urea/150mM sodium chloride).

Tracks g-i = infected (Hissar) red cell membranes (table 4.5.2.BII). As above; track g, sample biotin labelled after first wash; track h, after second wash; track i, after third wash.

Tracks d-f = uninfected red cell membranes contaminated with freeze thawed piroplasms (see table 4.5.2. C). As in tracks a-c above, except that each 50μl sample of Affi-Gel beads, coated with uninfected membranes was contaminated with $1.7\times10^5$ freeze thawed piroplasms, which were included in the lysis buffer in the first lysis step. Track d, sample biotin labelled after the first wash; track e, sample biotin labelled after the second wash; track f, sample biotin labelled after the third wash. The relative mobility and molecular mass of internal molecular weight standard are illustrated of the right hand side of the figure (molecular mass next to black arrows). The white arrows indicate the position of putative infection specific polypeptides in the infected membrane samples.
track i= the first wash.

The effect of the more stringent washing protocols appeared to be a small, quantitative decrease in all polypeptides, which confirmed an earlier finding of lower protein yields in membranes prepared by this method. However, a 31kDa red cell polypeptide was largely depleted after the high salt wash which suggested a relative peripheral attachment of this red cell polypeptide to the membrane. Nineteen polypeptides were detected in all the samples; two additional, mutually exclusive, polypeptides were only detected in the infected membrane preparations: a 40kDa polypeptide in track i, and a 36kDa polypeptide in track g, thus these molecules appeared to be infection specific. No differences were detected between equivalently washed samples of uninfected and contaminated membranes, which suggested that all the polypeptides observed in tracks a-f were of red cell origin. The evidence suggested that, in this experiment, dextran sulphate had been effective in blocking contamination by piroplasms, and therefore that the two polypeptides detected in the infected red cell membrane preparation were genuine infection specific polypeptides. The conclusion, that dextran sulphate had apparently removed the problem of piroplasm contamination, was only tentative because the control experiment in which dextran sulphate would have been absent had not been performed. Therefore, the following experiment was carried out.

4.5.3. Blocking assay II

A blocking assay was performed, similar to the assay described in 4.5.1., except that beads were coated with unlabelled, uninfected red blood cell membranes and then (potentially) contaminated with freeze thawed biotinylated piroplasms, with and without the presence of 10mg/ml dextran sulphate (see table 4.5.3). Considering the results presented in section 4.5.2, it was expected that dextran sulphate would show a significant blocking effect in this assay. Figure 4.4.1.b. shows the resulting autoradiograph, track 1) shows the profile of biotinylated piroplasm polypeptides which bound to unlabelled red cell membrane coated Affi-Gel beads in the presence of 10mg/ml dextran sulphate, track 2) shows the
prepare uninfected rbc and beads, mix
and wash x3 in SF buffer $5 \times 10^7$ rbc in 100 $\mu$L
plus 100 $\mu$L beads

lyse rbc by addition of EP buffer with vortexing

wash x5 in EP buffer

biotin label freeze thawed purified piroplasm
and resuspend in SP buffer with and without
the presence of 10mg/ml dextran sulphate

mix samples from 4. with samples from 3. above

wash x3 in SP buffer and x5 in EP buffer with
vortexing, then extract samples in SDS sample buffer

Table 4.5.3.1. Blocking assay II

1. mix prepared beads with prepared infected rbc
   (section 4.1.1.) containing no free piroplasms
   (section 4.3.3.)

2. wash x3 in sucrose phosphate (SP) buffer (4.1.1.)

4. lyse rbc in phosphate-EDTA (EP) buffer (4.1.1.)
   containing 10mg/ml freshly prepared dextran sulphate
   (DS) and vortex at high speed for 20 seconds

5. wash x5 in EP buffer/DS with vortexing

6. remove an aliquot and stain with Acridine Orange
   (section 4.3.3.) to check that parasite nuclei are
   absent on beads. Perform further wash/vortex cycles in
   SP buffer if necessary until Acridine Orange stained
   beads are free from fluorescing nuclei

7. extract sample in SDS sample buffer (2.5.1.)

Table 4.5.3.2. Affi-Gel membrane protocol II

mix prepared beads with prepared infected rbc
(section 4.1.1.) containing no free piroplasms
(section 4.3.3.)

1. mix prepared beads with prepared infected rbc

2. wash x3 in sucrose phosphate (SP) buffer (4.1.1.)

4. lyse rbc in phosphate-EDTA (EP) buffer (4.1.1.)
   containing 10mg/ml freshly prepared dextran sulphate
   (DS) and vortex at high speed for 20 seconds

5. wash x5 in EP buffer/DS with vortexing

6. remove an aliquot and stain with Acridine Orange
   (section 4.3.3.) to check that parasite nuclei are
   absent on beads. Perform further wash/vortex cycles in
   SP buffer if necessary until Acridine Orange stained
   beads are free from fluorescing nuclei

7. extract sample in SDS sample buffer (2.5.1.)
control experiment in which dextran sulphate was absent (the other tracks are merely different amounts of the same samples). The results suggested that, even with the presence of red cell membranes on Affi-Gel beads, there was only a small blocking effect with dextran sulphate. This interpretation was difficult to reconcile with the results of the previous experiment (4.5.2.).

One difference between these two sets of experiments was that experiments in which dextran sulphate had not proved to be an efficient blocking agent had been performed with a dextran sulphate solution which was more than twenty hours old, whereas in the previous experiment (4.5.2.) a freshly prepared solution had been used. Furthermore, it was known (Dr P. Beck, pers. comm.) that solutions of dextran sulphate become more viscous over storage periods of less than twenty-four hours.

Accordingly, an experiment was set up to determine whether this difference would account for the observed discrepancies. The experimental procedure was exactly as described above except that fresh dextran sulphate (10mg/ml) and a solution which was more than twenty-four hours old were used as blocking agents. Figure 4.4.1.c shows the resulting autoradiograph, track 1 shows the biotinylated piroplasm polypeptides which bound to membrane bound Affi-Gel beads in the presence of 10mg/ml old dextran sulphate and track 2) shows the effect of 10mg/ml fresh dextran sulphate; no polypeptides were detected in this extract. Several piroplasm polypeptides contaminated Affi-Gel beads in the presence of old dextran sulphate (>24 hours) while a fresh solution at the same concentration had been completely effective in blocking contamination.

Extrapolating from this result, it was expected that membranes prepared from infected red blood cell material, which did not contain free piroplasms, using the revised protocol with freshly prepared 10mg/ml dextran sulphate, would not be contaminated with piroplasm proteins (see table 4.5.3.2. for flow plan of this method).
4.6. PREPARATION OF CONTAMINATION FREE MEMBRANES

Red blood cells (10^8 cells, Gharb stock, 35% parasitaemia) were metabolically labelled to give precipitable counts of 7x10^4 cpm. The labelled red cells were applied to Affi-Gel beads and unbound cells from four washes of the beads in sucrose phosphate buffer were collected, pelleted by centrifugation at 300g and the number of red cells was calculated by haemocytometry. As 4x10^7 red cells had bound to 150μl of a 50% suspension of Affi-Gel beads, the starting radioactive counts of the bound cells was 2.8x10^4 cpm. Membranes were prepared according to the revised protocol with fresh dextran sulphate (table 4.5.3.2.). The extracted membrane sample (10^3 TCA precipitable cpm) and an extract of the initial labelled infected red cell material (2x10^3 cpm) were resolved in a 4-15% acrylamide SDS-PAGE gel which was treated for flurograghy, dried down and exposed to X-ray film for six weeks.

Figures 4.6.1.1. and 4.6.1.2. show the results of two similar experiments. Tracks a) show the membrane extract; no polypeptides were detected although 10^3 cpm had been loaded onto each gel track, tracks b) show the total labelled extract. Tracks c) show an extract of the excess infected red cells, which were introduced to Affi-Gel beads, but were collected during the initial washing steps. Tracks d) show a crude supernatant fraction, collected from incorporation medium, after metabolic incubation labelling periods, by centrifugation at 19,000g for 10 minutes and after the addition of protease inhibitors. These results demonstrated that the revised protocol for membrane preparation with Affi-Gel beads was efficient at generating membranes that were free from significant piroplasm contamination but they were also free from 35S labelled ISEMPs. The parasitaemia of the red cells used in this experiment had been 35% which meant that 10 fold more piroplasms had been present compared to the contamintion and blocking assays described above. Therefore, the absence of piroplasm contamination here was not due to the low parasitaemia of the starting material. It was possible that labelled ISEMPs had not been detected because of the low parasitaemia and therefore attempts were made to repeat the experiment with high parasitaemia red blood cells.
Figures 4.6.1.1. and 2.

Preparation of contamination free membranes

Autoradiographs show $^{35}S$-methionine labelled polypeptides eluted off Affi-Gel beads after labelled, infected red cells had been processed for membrane preparation (Affi-Gel membrane protocol II, see table 4.5.3.2.). Extracted samples ($1-2\times10^9$ TCA precipitable cpm) were resolved in 4-15% SDS-PAGE gels which were treated for fluorography, dried down and exposed to x-ray film.

Figures 4.6.1.1. and 4.6.1.2. show the results of very similar experiments. Infected red cells (Gharb stock, 35% parasitaemia) were metabolically labelled (section 2.4.1.). Tracks b are the total extract of labelled infected red cells prior to addition of Affi-Gel beads. Tracks c show an extract of the excess red blood cells, which were added to Affi-Gel beads but were collected during the initial washing steps. Red cell membranes were prepared (table 4.5.3.2.) and tracks a, show the membrane extracts (no radioactive bands are visible here). Tracks d, show a crude supernatant fraction (see section 4.6). The mobility and molecular mass of internal molecular weight standards are illustrated on the right hand side of the figures.
Figure 4.6.1.
4.7. FACS SEPARATION & MEMBRANE PREPARATION

Experiments were performed to try to separate infected from uninfected red cells. This was found not to be possible by Percoll density centrifugation (in my hands) because the differential between different (presumably ages) populations of uninfected red cells was at least as great as that between infected and uninfected red cells. High parasitaemia blood tended to have high levels of free piroplasms and to be fragile. This material was therefore inappropriate in the context of the experimental approach adopted here. As the fluorochrome, Acridine Orange, stained piroplasm nuclei within intact red cells, this suggested that cells might be separated by a fluorescence activated cell sorter (FACS).

4.7.1. FACS separation

The experimental procedure for FACS separations is described in section 2.8. The FACS analysed the differential forward scatter and relative fluorescence intensity between Acridine Orange treated red cells, containing strongly fluorescing (at 640nm) piroplasm nuclei, and uninfected red cells, which fluoresced weakly (uninfected red blood cells were first analysed as a control). It then separated the two populations of red cells into different containers. The two populations of cells which were sorted are represented in the dot-plot shown in figure 4.5.2.3. The rectangle labelled "a" contains infected red blood cells and rectangle "b" indicates the characteristics of uninfected cell populations which were collected. Infected and uninfected cells from infected material (Ankara stock, 2\times10^5 cells) were separated and inspection of a sample of the material under a fluorescence microscope confirmed that the two sorted populations were 100% infected or uninfected.

It was determined that Acridine Orange treated piroplasms could be metabolically labelled with $^{35}$S methionine and produced the same profile of labelled polypeptides as untreated piroplasms. Figure 4.7.1.2. illustrates this, tracks d and e show the polypeptides from Acridine Orange treated and untreated, metabolically labelled infected red blood cells respectively.
Figure 4.7.1.2.

Membrane preparation from 100% infected red cells.

Autoradiograph shows $^{35}$S-methionine labelled polypeptides from labelled infected red cells at various stages of membrane preparation.

Sample extracts were resolved in 4.5-15% SDS-PAGE gels, prepared for fluorography, dried down and exposed to x-ray film. Infected red cells (Ankara stock) were separated from uninfected red cells by FACS separation of Acridine Orange treated cells (section 4.7.).

Track a = a crude supernatant fraction, collected from incorporation medium after metabolic labelling. The medium was centrifuged at 19,000g for 10 minutes and the supernatant was extracted and loaded.

Tracks b and c = total extracts of the excess red cells which were added to Affi-Gel beads, but did not bind, and were collected during initial washing steps.

Tracks d and e = total extracts of metabolically labelled infected cells after Acridine Orange staining (track d) and without Acridine Orange treatment (track e).

Track f = extract of red cell membrane preparation.

Track g = total extract (2x10^3 TCA precipitable cpm) of metabolically labelled, 100% infected red cells.

The mobility and molecular mass of internal molecular weight standards are indicated on the right hand side of the figure. Arrowed numbers show the estimated molecular mass of three polypeptides which appeared to be present in the membrane fraction.

Figure 4.7.2.2.

Autoradiograph showing the results of a very similar experiment to that described above (fig. 4.7.1.2.).

Tracks a, b, and c = membrane fractions
Track d = total infected red cell extract
Track e = supernatant fraction extract.
4.7.2. Membrane preparation from 100% parasitaemia red cells

Infected red blood cells were isolated from fresh red cells exhibiting a 50% parasitaemia (Ankara stock) by FACS separation as described above. The Affi-Gel membrane protocol II (table 4.5.3.2.) was followed with metabolically labelled, infected red cells. The membranes were resolved in 4.5-15% acrylamide SDS-PAGE gels, prepared for fluorography, dried down and exposed to X-ray film for five weeks.

Figure 4.7.1.2. shows the membrane preparation in track f and the total extract of labelled red cells in track g. Polypeptides of molecular mass 122, 91 and 76kDa were apparent in the membrane preparation and these were also major polypeptides in the total extract (track g). Tracks d and e are the control experiment described above (4.7.1.) with Acridine Orange treated and untreated cells respectively. Tracks c and b are extracts of the excess red blood cells which were introduced but did not bind to Affi-Gel beads and were collected during the initial washing steps. Track a shows a crude supernatant fraction collected from the incorporation medium, after metabolic labelling incubation periods. This was the supernatant fraction after centrifugation of the used incorporation medium at 19,000g for 10 minutes, after the addition of protease inhibitors and removal of intact red cells. The "cloud formation" at the base of figure 4.7.1.2. is probably due to incomplete impregnation of salicylic acid (for fluorography) in the thicker, base portion of the gradient gel. Figure 4.7.2.2. shows the total red blood cell extract in track d, a supernatant fraction (prepared as above) in track e and membrane fractions in tracks a, b, and c. A weakly detected membrane associated polypeptide of 76kDa was apparent in tracks a, b and c.
4.8. DISCUSSION

4.8.1. Methods of analysis

The expectation in the 4°C storage experiment (section 4.3.1.) had been that the metabolic activity of parasites would decline over the three week storage period. However, there was no decline in \(^{35}S\) methionine incorporation, and the synthesised polypeptide profile remained constant. The iodinated red cell surface polypeptide profiles were also constant, although there was a large degree of variation in the specific activity of labelled polypeptides between samples. This variation was found to be a characteristic of the labelling method (in my hands), and the labelled profile from red cells, after three weeks storage, was the predominant profile from many surface iodination experiments with fresh infected red cells. Despite the internal variation in iodination experiments, the results suggest that stored blood can be used to study the surface and red cell membrane of infected red blood cells. The ability of stored and fresh infected red blood cells to bind to Affi-Gel beads is the same, which implies that the negatively charged sialic acid residues on the red cell surface are not significantly affected by storage.

It was observed that red cells which were stored in RBC storage solution for four weeks retained their discoid appearance, whereas blood which was stored in CPDA-1 became echinocytic after approximately three weeks. As different blood samples were used for the two storage experiments, definitive comparative statements cannot be made. However, this result suggests that RBC storage solution may be a superior storage solution compared to CPDA-1. RBC storage solution (Meryman, 1986) is a hypotonic citrate-phosphate buffer supplemented with adenine, ammonium chloride and glucose. Meryman demonstrated that human red cells which were stored in the solution for four weeks showed elevated ATP levels, averaging 165% of initial values, and improved in vivo survival after transfusion. Labelling experiments were not performed with T. annulata infected red cells stored in RBC storage solution, so it is not known what effect this solution has on the profiles of metabolically labelled or surface iodinated material. ATP is
thought to maintain the red cell cytoskeleton in a phosphorylated state, and discoid red cells, which are treated with 1mM iodoacetamide to rapidly deplete ATP levels, become echinocytic after a few hours (Rangachari et al. 1989). However the mechanical properties of ATP depleted red cells are apparently unaltered.

The finding that infected red cells can be stored at 4°C may allow experimentation to determine whether re-invasion of red cells occurs in the life cycle in vivo. Similar storage experiments could be performed with macroschizont infected cells and possibly heat induced merozoites to determine their longevity at 4°C. If these stages do not survive storage then it might be possible to introduce stored, viable infected red cells into bovids which are not contaminated with viable macroschizonts or macroschizont derived merozoites.

The results of the storage study potentially extended the scope and number of experiments which could be performed with any one batch of infected blood, however some technical problems remained. The experiments in this chapter were focussed on deriving a pure infected red cell membrane preparation from metabolically labelled red cells. Preliminary experiments suggested that the membranes which were prepared using Affi-Gel beads were contaminated with piroplasm polypeptides (figure 4.2.1. track a). Therefore, further studies were required to determine a better protocol for Affi-Gel membrane preparation. It was found that autoradiographic exposure periods of at least three weeks were necessary for the detection of metabolically labelled polypeptides in extracts of electrophoretically separated membrane preparations. A more rapid but equally sensitive method of labelling and detecting polypeptides was therefore required in order to capitalise on the findings of the 4°C storage experiment and perform the necessary experiments.

One approach was to use a biotin-125I-streptavidin system of labelling and detection. The biotin derivative, biotinyl-N-hydroxysuccinimide ester (NHS-biotin), has been used to label surface membrane polypeptides of several cell types (Bayer & Wilchek, 1980). NHS-biotin reacts specifically with lysine residues at pH 8.0 and very few bound biotin molecules are required to allow detection with 125I streptavidin. Only a two fold molar
excess of biotin to lysine residues is used to label sufficient lysine residues; higher ratios may lead to denaturation of the protein and to internal labelling. Avidin and biotin are characterised by one of the strongest known binding constants. Streptavidin has four biotin binding sites per molecule while avidin has only a single site, making it an even more sensitive probe than avidin.

Control experiments revealed that $^{125}$I-streptavidin fails to bind to unbiotinylated infected red cell extracts under the conditions used. This showed that the probe was specific and that infected red cell extracts do not contain significant levels of endogenous biotin. Streptavidin was iodinated using the Iodogen method which specifically introduces $^{125}$I into tyrosine residues. Tyrosine is important in the binding of biotin by avidin and loss of sensitivity and specificity have been reported with avidin labelled to a high specific activity. The apparent specificity and sensitivity of the $^{125}$I streptavidin used here may therefore be due to the fact that the Iodogen catalysed iodinations did not label enough tyrosine residues in the streptavidin to significantly modify its behaviour. When cell lysates were labelled, many more polypeptides were subsequently detected than when intact cells were labelled with biotin. This implied that there was some restriction of the label to the surface of cells in surface labelling experiments. Better evidence for surface specificity comes from the finding that the profiles of surface labelled infected and uninfected red cells were generally indistinguishable which demonstrated that piroplasm polypeptides were not labelled: also that surface ISEMPs were not detectable using this system of analysis. It is possible that if greater resolution of labelled polypeptides could be achieved, for example in two dimensional acrylamide gels, that differences would become apparent. The results of biotin-$^{125}$I-streptavidin surface red cell labelling experiments were similar to those derived from surface Iodogen-catalysed iodination experiments presented in chapter three. Either four-five or fifteen-twenty labelled polypeptides were detected. It is concluded that neither of the labelling methods are appropriate for detecting surface ISEMPs. However, the advantage of the biotin method was that it gave consistent results: biotin
labelled samples were stable during storage at -20°C for two months, the procedure was rapid (4-5 days) and the probed profile of biotin labelled piroplasm extract polypeptides was very similar to that produced in 35S metabolic labelling experiments. Thus this labelling and detection system was rapid and sensitive enough to be used to derive a better Affi-Gel membrane preparation protocol.

The Acridine orange staining method was an improvement on Giemsa staining of Affi-Gel bound material, not only because it was faster, which allowed the presence of piroplasms on beads to be monitored frequently whilst membranes were being prepared. It also precluded the possibility of spurious observations of piroplasms bound to beads as a consequence of the slide preparation and fixation. Hence this provided a rapid and effective staining procedure for monitoring both membranes and their contamination by piroplasms and nuclei.

Using the biotin-streptavidin system of labelling, it was found that infected or uninfected red blood cell membranes, which were prepared from Affi-Gel-bound red blood cells that had been stored at -20°C for five weeks, are identical to those produced with fresh preparations. Moreover, that storage at -20°C does not alter the strength of the association between Affi-Gel beads and red blood cells, as judged under the washing conditions used. This result, together with the results of storage experiments with infected blood at 4°C in CPDA-1 storage solution, effectively extended the availability of infected material. It also meant that comparisons between different samples could be made at one time, even though the fresh material was not available simultaneously.

In conclusion, the results obtained here allowed sequential experimental studies to be carried out to test various methods for removing piroplasm contamination.
4.8.2. Membrane preparation and analysis

The Affi-Gel bead protocol for preparing infected red cell membranes was investigated because it appeared to offer an appropriate and flexible system for removing the problem of piroplasm contamination which was found (section 3.5.3.) in membranes prepared by differential centrifugation. The possibility that the washing conditions in the protocol might be too stringent and ISEMP's therefore lost, was considered and in initial experiments low stringency washes were performed (section 4.1.1.). The yield of membrane protein was similar to that reported for Plasmodium infected red cell systems, but control experiments showed that the membranes were contaminated with piroplasm polypeptides. The evidence for this came from three different experiments. Firstly, the observation of fluorescing parasite nuclei on Acidine Orange stained Affi-Gel beads, together with the finding that the phosphate-EDTA lysis buffer which was used did not disrupt the piroplasm outer membrane, suggested that intact piroplasms were contaminating the preparations. Secondly, unlabelled, uninfected red cell membranes were coated onto beads and then metabolically labelled piroplasms were added to the beads (to simulate the number of piroplasms which would normally be present in infected blood); after following the membrane preparation protocol, the extracts were contaminated with $^{35}$S labelled polypeptides (section 4.2.). Thirdly, infected red cell membrane preparations, which were biotinylated in situ on Affi-Gel beads, showed high levels of polypeptides which were characteristic of piroplasm polypeptides (section 4.3.4).

It was known that some samples of infected blood contained abundant free piroplasms or merozoites. Therefore, in devising contamination blocking or washing strategies, it was envisaged that if piroplasms bound to Affi-Gel beads it would be important to ensure that free piroplasms were removed. A contamination assay (section 4.4.) was therefore performed to determine whether piroplasms bind to beads (under the conditions used), and the results demonstrated that they do. The only practical solution to this was to ensure that free parasites were absent from the starting samples and therefore material was routinely monitored.
with Acridine Orange.

Piroplasm contamination was detected even when starting samples appeared to contain no free parasites, as assessed by Acridine Orange staining (section 4.3.3.). A second possible source of piroplasm contamination was from the intracellular piroplasm, which could have been binding either to free sites on Affi-Gel beads or to the cytoplasmic face of bound red cell membranes. Dextran sulphate proved to be only marginally effective (polyaspartate was less effective) in preventing the binding of piroplasm to beads, or to beads which were coated with red cell membranes (section 4.5.1.). In order to investigate this system more fully and to test more stringent washing conditions, a blocking (with dextran sulphate) and washing assay was set up (section 4.5.2.). The results suggested that the additional washing cycles with lysis buffer supplemented with a) high salt and then b) 3M urea/150mM NaCl depleted a 31kDa red cell polypeptide. The high salt wash also depleted a 40kDa infection specific polypeptide. A further effect was a quantitative decrease in signal from all polypeptides. On the basis of these results the two extra washing procedures were not adopted. Importantly, there was no evidence for piroplasm contamination in either the infected red cell samples or the contaminated control samples. Also, an infection specific polypeptide was detected in infected membranes of 40kDa after sucrose phosphate buffer washes and of 36kDa after the final washing step in lysis buffer/3M urea/150mM NaCl. The relationship between these two polypeptides is unclear; the 36kDa molecule may be the product of the 40kDa polypeptide after high salt and urea washes or the 40kDa polypeptide may have been lost during the high salt wash and be unrelated to the 36kDa polypeptide which was detected for the first time after the urea containing washes. It is possible that the removal of certain molecules from the beads, for example the 31kDa molecule, by the more stringent washing conditions allowed the 36kDa polypeptide to be labelled and detected. Piroplasm polypeptides of 36kDa but not 40kDa were commonly detected in metabolically labelled extracts after SDS-PAGE and autoradiography (appendix 1a).

The results suggested that dextran sulphate had been effective in blocking contamination, which contradicted the results
of the piroplasm contamination assay. This problem was solved by the finding that fresh solutions of dextran sulphate are effective in blocking contamination, while solutions which are more than twenty-four hours old are viscous and ineffective at blocking contamination (section 4.5.3.). The effects of lysis buffer containing dextran sulphate, and of unsupplemented lysis buffer on piroplasms was assessed by examining Giemsa stained slide preparations of treated piroplasms. The piroplasm samples were indistinguishable and the piroplasm outer membrane was not disrupted, therefore the blocking effects of dextran sulphate does not involve the lysis of piroplasms.

The final test to determine whether contamination had been effectively prevented was to prepare membranes from metabolically labelled infected red cells (section 4.6.). The results demonstrated that piroplasm contamination had been prevented, as no polypeptides were detected in the membrane extracts, and the assay was considered to be a more sensitive test for contamination than the previous assays. This experiment had been performed with infected red cells with a parasitaemia of 35% and more piroplasms were present than had been used in the contamination and blocking assays. The relationship between biotin labelled and metabolic labelled profiles had been assessed. Thus it was known that comparable polypeptide profiles were obtained when SDS-PAGE gels of metabolically labelled material (3000 cpm), exposed to film for six weeks, and Western blots of biotin labelled extracts (5x10^6 cpm/10 μl) which were probed with 125I-streptavidin (1 cpm/μl) and exposed for three days were compared.

As it was possible that membrane preparations from 100% parasitaemia blood would provide evidence for ISEMPs, experiments were undertaken to separate infected from uninfected red cells. This was achieved by the FACS separation of Acridine orange stained material (section 4.7.). It was determined that Acridine orange stained cells could be metabolically labelled to give polypeptide profiles which were indistinguishable from unstained, labelled cells (figure 4.7.1.2. tracks d & e). Affi-Gel membrane preparations from metabolically labelled infected red cells (100% parasitaemia) showed putative ISEMPs in two out of two similar experiments. The results from these experiments failed to
consolidate putative ISEMPs derived from previous experiments. In
the first experiment, three ISEMPs of 122kDa, 91kDa and 76kDa were
detected (figure 4.7.1.2. track f). In the second experiment, a
76kDa putative ISEM was detected (figure 4.7.2.2. tracks a, b & c). These three polypeptides were also major polypeptides in the
labelled infected red blood cell extracts. For this reason, and
because the infected red cells had been isolated on a FACS which
involved the selection of red blood cell-sized particles, it is
considered most unlikely that the detected polypeptides were of
contaminating leucocyte origin. It appears that high
parasitaemia red cell samples are required before membrane
preparations contained sufficient labelled counts for ISEMPs to be
detected. However, this result may be aleatory as it is
conceivable that only a proportion of a population of infected red
cells carry ISEMPs at any one time and that this proportion varies
between different samples. Red cell samples were uncloned and not
synchronised, and the production and presence of ISEMPS may be a
transient event during differentiation. Furthermore, because
infected red cell material may be heterogeneous, no inference can
be made regarding the level of expression of the putative ISEMPS.

In order to test the validity of these findings, external
control experiments would have to be undertaken, for example by
testing antibodies raised against the ISEMPS in immunoelectron
microscopic studies.
It has been shown that the life cycle of *T. annulata* involves a complex series of ultrastructural and physiological changes (section 1.1). As with other Apicomplexa, the life cycle involves the invasion of target host cells by transient stages, which punctuate a longer intracellular period, during which large changes in structure occur; reproduction often takes place, and finally invasive stages are produced once more. There is some uncertainty about the life cycle, as a cycle of development may occur in the red blood cell: from merozoite, to piroplasm, to merozoite (Conrad et al. 1985). Alternatively the division products of the piroplasm may be an end point in the bovine host, and continue the progression of life cycle towards the tick stages.

The extent of protein variation between stages, and the molecular mechanisms which orchestrate development, have not been studied in detail in *Theileria*. In order to extend the study of the merozoite/red blood cell/piroplasm stage of the life cycle, monoclonal antibodies were raised against infected red blood cells. At the outset, it was hoped that antibodies would be generated which would: a) recognise infection specific epitopes on the surface of infected red blood cells, b) highlight antigenic differences, between differentiating types, within the intraerythrocytic stage, c) recognise the piroplasm and not the sporozoite or macroschizont, d) recognise merozoites derived from macroschizonts and e) for the purposes of strain differentiation, distinguish Ankara, Hissar and Gharb stocks. These antibodies could then be used as probes to characterise the polypeptides and epitopes recognised, and thus begin to define how these various stages of the parasite differ at the molecular level.
5.1. INDIRECT IMMUNOFLUORESCENT ASSAYS WITH PIROPLASMS

Monoclonal antibodies were generated as described in section 2.2. Supernatants from hybridoma cultures were screened by immunofluorescence microscopy (IFA, section 2.3.) for their reactivity against red blood cells infected with piroplasms of the Ankara stock. This technique discriminated between positive and negative supernatants; it also allowed some localisation of reacting epitopes to a limited, but useful extent. For this assay, uncloned supernatants were tested against acetone fixed preparations of: a) the infected red blood cells which were used to immunise mice for the fusions (from cow41), b) isolated piroplasms and c) uninfected red blood cells (cow110).

It was not known, at this stage, whether antibodies had been generated against piroplasm or red blood cell epitopes. The use of uninfected red blood cells in the IFA screen provided a control to test for the production of antibodies against normal red blood cell components. As the uninfected and infected red blood cells were from different animals it was possible that antibodies would be raised against certain polymorphic red blood cell antigens (e.g. ABO) in the infected material, which were absent in the uninfected cells. However, such antibodies would recognise all the red blood cells in infected material, in which only 50% of red cells were parasitised. These could, therefore, be distinguished from antibodies which specifically recognised infected red cells on the basis of the proportion of cells which were recognised.

The primary screen detected forty-six supernatants which recognised piroplasms. In addition, one of the supernatants (2A3) recognised infected and uninfected red blood cells. Two supernatants (5A10 and 5A12) appeared to recognise the uninfected red blood cell preparations, and only piroplasms in infected red cells. One supernatant recognised the infected but not the uninfected red blood cell membrane, which made it a candidate probe for infection specific epitopes in, or on, the red blood cell membrane. However the cells producing this supernatant were lost at an early stage.

The IFAs showed that the supernatants which detected piroplasms produced different profiles of reactivities. These
profiles are described as varying between supernatants in the following three ways. The intensity of the fluorescence staining ranged from very weak (orange-red) to very strong (apple-green). Secondly, some supernatants recognised a larger proportion of the available number of piroplasms on a slide than others. Thirdly, several supernatants reacted to produce characteristic designs of fluorescence, such that all or part of each piroplasm fluoresced. These categories had overlapping definitions. For example, with one supernatant (1C3), IFA results with concentrated antibody (by ultrafiltration), showed a much larger area of the piroplasm fluorescing compared to dilute supernatant. In contrast, concentration of antibody (39), resulted in an apple-green rather than the previous orange-red fluorescence, but the small portion of the piroplasm which was stained remained the same.

Twenty-eight hybridoma lines were cloned by limiting dilution, and IFAs demonstrated that the monoclonal antibodies retained the reactivity profiles of parental lines. Even in the case of antibody supernatant 5A10, which recognised uninfected red blood cells and piroplasms, there was no segregation of reactivity profiles after cloning. The isotype of certain antibodies from cloned hybridoma lines was determined (2.3.3.) and these are listed in table 5.1.1.

The indirect fluorescent assays were repeated using several T. annulata Ankara infected red blood cell preparations from different calves showing parasitaemias of between 30-90%, and serial dilutions of monoclonal culture supernatants. Using the data from these assays the monoclonal antibodies could be grouped into three types (table 5.1.2). Type 1 gave a strong reaction against all parasites in infected red blood cells (this was assessed by determining the parasitaemia from Giemsa stained preparations of infected red blood cells) and recognised the outer perimeter of the piroplasm (figure 5.1.1.t1). Type 2 were characterised by their recognition of small circular elements (dots), occurring singly or in clusters. These dots were observed within weakly fluorescing piroplasms, within the cytoplasm of red blood cells or apparently free outside red blood cells. In addition to the fluorescing dots, these monoclonal antibodies also produced a heterogeneous fluorescence pattern in infected red blood cell
Table 5.1.1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D5</td>
<td>IgG 1</td>
</tr>
<tr>
<td>1D11</td>
<td>II</td>
</tr>
<tr>
<td>10E6</td>
<td>II</td>
</tr>
<tr>
<td>5G1</td>
<td>II</td>
</tr>
<tr>
<td>1C2</td>
<td>II</td>
</tr>
<tr>
<td>5E1</td>
<td>IgG 2a</td>
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<td>IgG 2b</td>
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<td>39</td>
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Table 5.1.1.

Results of monoclonal antibody isotyping experiment, performed as described in section 2.3.3. Each monoclonal antibody was tested for its ability to bind anti-mouse Ig subtypes as follows; anti-IgG, anti-IgG (Fc), anti-IgG 1, anti-IgG 2a, anti-IgG 2b, anti-IgG 3, anti-IgA (Fc), anti-IgM (Fc) and anti-Ig Kappa.
Table 5.1.2.

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Table 5.1.2.

Monoclonal antibodies were grouped into three types on the basis of IFA results using infected red cell slide preparations.

- Type 1 antibodies gave a strong reaction against all parasites on the slide and recognised the outer perimeter of the piroplasm.
- Type 2 antibodies recognised small circular elements (dots) and produced a heterogeneous fluorescence pattern.
- Type 3 antibodies recognised parasites, but never appeared to recognise the outer perimeter of parasites (type 1) or dots (type 2).

NB the groupings were not absolute, and some type 2 antibodies also showed type 1 and type 3 fluorescence reactivities.
Photographs of the results of IFAs, viewed through the 100x objective of a fluorescence microscope, taken to illustrate the basis for grouping monoclonal antibodies into types 1-3. Individual monoclonal antibodies were tested against acetone fixed slide preparations of infected red cells.

\[ t_1 = \text{type 1 reactivity profile} \]
\[ t_2 = \text{type 2 reactivity profile} \]
\[ t_3 = \text{type 3 reactivity profile} \]
\[ t_4 = \text{illustrating all three types of reactivity by one antibody.} \]

Photographs of IFA results viewed through a fluorescence microscope, showing the reactivity of monoclonal antibody 39.

- \( rbc \) = the reactivity of antibody 39 on acetone fixed uninfected red blood cell slide preparations
- \( BL-20 \) = monoclonal antibody 39 on formalin fixed uninfected BL-20 cell slide preparations
- \( piro \) = monoclonal antibody 39 on acetone fixed piroplasm infected red cell slide preparations
- \( TaA \) = monoclonal antibody 39 on formalin fixed, macroschizont infected (Ankara stock) leucocyte slide preparations.
Figure 5.1.1.

A

t1  t2

10μm  10μm

t3  t4

10μm  10μm

B

rbc  BL-20

10μm  10μm

piro  TaA

10μm  10μm
preparations. This involved bright staining of a proportion of piroplasms, and weak, diffuse staining of the cytoplasm of a small number of the available red blood cells. The brightly fluorescing dots, were only very occasionally observed within these weakly stained red blood cells. With this form of fluorescence reactivity it was difficult to determine whether all parasites in a preparation were reacting (figure 5.1.1.t2). Type 3 antibodies (figure 5.1.1.t3) recognised piroplasms with differing intensities and different portions of the piroplasm appeared to fluoresce, but they never produced a bright halo of fluorescence around the piroplasm (type 1) or the specific pattern of dots (type 2). Some monoclonal antibodies showed all three types of reactivity together on the same slide preparation. Monoclonal antibodies 1C2 and 5A10 demonstrated all three types on the same slide preparation (see figure 5.1.1.t4).

A further level of variation was observed with type 3 monoclonal antibodies, in the proportion of piroplasms on a slide which fluoresced. For example monoclonal antibody 10A8 only recognised 50% of the total number of piroplasms on a slide preparation (data not shown). Antibodies 1H4, 1E9, 10D12, 109 and 5B9 also appeared to show a subsetting reactivity.

By screening antibodies 5A10 and 5A12 against several infected red blood cell and piroplasm preparations, derived from different animals, more information concerning their reactivities became available. Both antibodies recognised isolated piroplasms and gave a strong reaction on uninfected red blood cells (cow110). Antibody 5A12 recognised all the red blood cells in all the infected red blood cell preparations, to give a blanket of fluorescence which obscured the piroplasm within these cells. Antibody 5A10 showed a different reactivity, in that it reacted like antibody 5A12 on three infected red blood cell preparations, but on two others, only the piroplasm was recognised. This suggested that the two monoclonal antibodies recognised different epitopes, which was confirmed later by the observation that 5A10 recognised three stocks of the parasite while 5A12 only recognised Ankara piroplasms. The reactivity of antibody 5A10 against three different, infected red blood cell preparations infected with Ankara, Hissar or Gharb stocks is illustrated in figure. 5.4.1.
5.2. INDIRECT FLUORESCENCE ASSAYS: STAGE SPECIFICITY

5.2.1. Sporozoites and macroschizonts

The monoclonal antibodies which recognised the piroplasm stage were characterised further by analysing their specificity for other available life cycle stages of Theileria. Formaldehyde fixed slides of macroschizont infected leucocytes (Ankara cell line TaA48) and sporozoites (Ankara) were screened: at this time merozites were not available. Monoclonal antibody 2E4, which was generated by Shiels et al. (1986a), reacts strongly with Ankara macroschizonts and sporozoites, and it was used here as a positive control. Acetone fixed slides of piroplasm infected red blood cells (Ankara) were included in the screen as a further positive control.

The assays showed (table 5.2.1.) that one monoclonal antibody, (antibody 39), of the twenty-eight tested, recognised infected leucocytes, but not sporozoites. All the other monoclonal antibodies failed to react with these stages, showing that the vast majority were stage specific. The reactivity of monoclonal antibody 39 was remarkable because it appeared to recognise the infected leucocyte membrane, as well as the piroplasm (see figure 5.1.1.9: rbc= control uninfected red blood cells, BL-20= uninfected lymphoblastoid cell line, piro= infected red blood cells, TaA= infected lymphoblastoid cells). It was not possible, from this assay, to determine whether the macroschizont was also recognised. The results from further experiments with this monoclonal antibody are presented in section 5.3.

5.2.2. Merozoites

Merozoites were generated, in vitro, by raising the culture temperature of macroschizont infected cell lines as described in section 6.1.2.2. A restricted range of the monoclonal antibodies (2D5, 1D11, 5E1, 1C2, 5A10) were tested, by indirect immunofluorescent assay, for their reactivity against formaldehyde fixed merozoite preparations (6.4.1.). The antibodies were also
Table 5.2.1.

Summary table, compiling IFA results using all the anti-piroplasm monoclonal antibodies which were generated.

Monoclonal; antibodies were tested against the following slide preparations:

- **rbc c** = control uninfected red blood cells (cow 110) (section 2.3.1.1.)
- **piroplasm (Ankara)** = acetone fixed, Ankara-piroplasm infected red cells (section 2.3.1.1.)
- **piroplasm (Hissar)** = as above but Hissar stock
- **piroplasm (Gharb)** = as above but Gharb stock
- **merozoite** = formalin fixed heat induced merozoites from day 15 cultures (section 2.1.9.)
- **macroschizont** = formaldehyde fixed Ankara macroschizont infected leucocytes (2.3.1.1.)
- **sporozoite** = formaldehyde fixed purified Ankara sporozoites (2.3.1.1.)
- **BL20** = formaldehyde fixed uninfected lymphoblastoid cells (see leucocytes, section 2.3.1.1.)
Table 5.2.1.

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<td>weak positive</td>
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<td>□</td>
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</table>

133
screened against piroplasm infected red blood cells (Ankara), preparations of formaldehyde fixed sporozoites (Ankara) and macroschizont infected leucocytes (TaMe). Monoclonal antibody 2B4 was used as a positive control for sporozoites and macroschizonts, and monoclonal antibody 1C7 was used as a negative control on piroplasms.

The results, which are summarised in table 5.2.2., demonstrated that four of the five monoclonal antibodies tested, recognised merozoite, as well as piroplasm stages. Monoclonal antibody 5A10, which appeared to recognise piroplasms, failed to recognise merozoites; while monoclonal antibodies 5E1, 2D5, 1D11 and 1C2 all recognised merozoites with a strong fluorescence pattern. The fluorescent patterns on merozoites were similar to the fluorescent patterns observed with the monoclonal antibodies on piroplasms. Monoclonal antibody 5E1 appeared to recognise the outer perimeter of the merozoites and piroplasms, whilst monoclonal antibodies 2D5 and 1D11 appeared to react with internal determinants. Monoclonal antibody 1C2 gave an intermediate staining pattern, in that it appeared to recognise the outer perimeter of some merozoites, and internal determinants in others.

From these assays it was not possible to determine whether the fluorescing dots, which were characteristic of the reactivities of monoclonal antibodies 2D5, 1D11 and 1C2 against infected red blood cell preparations, were present in merozoite preparations. This was primarily because of the small size of this stage of the parasite, but comparative statements were also impossible due to the different way in which piroplasm and merozoite material had been prepared and fixed.

In order to determine whether antibody 5E1 recognised an epitope on the surface of merozoites, indirect fluorescent assays were performed with unfixed isolated merozoites, according to the method described in section 2.3.4. Four antibodies were screened in these assays: 5E1, 2D5 and 1D11 which recognised fixed merozoites, and a control antibody, 1C7, which had failed to recognise fixed merozoites. The results were identical to the results obtained with fixed material, which suggested that the unfixed merozoites were permeable to antibody and might have been damaged during the isolation procedure.
Table 5.2.2.
Tabulated results of IFAs to determine the stage specificity of five monoclonal antibodies.

Antibodies 5E1, 1D11, 2D5, 1C2 and 5A10 were tested by IFA against the following slide preparations of *T. annulata* Ankara;

- **sporozoite** = formaldehyde fixed purified sporozoites (section 2.3.1.1.)
- **macroschizont** = formaldehyde fixed macroschizont infected leucocytes (section 2.3.1.1.)
- **merozoite** = formaldehyde fixed merozoites
  (see section 6.4.1. for details of preparation)
- **piroplasm** = acetone fixed, piroplasm infected red blood cells (section 2.3.1.1.)

Antibodies 2E4 and 1C7 were added as positive and negative controls.
Table 5.2.2.

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Key:  □ negative  ■ positive  c external control monoclonal
5.3. FURTHER STUDIES ON THE REACTIVITY OF ANTIBODY 39

It has been demonstrated (Shiels et al. 1986a) using monoclonal antibody probes, that infection-associated molecules are retained on the surface of macroschizont infected leucocytes. For this reason, and because immune bovine cells specifically recognise infected leucocytes, it is probable that infection specific surface molecules mediate cytotoxic responses in the bovine host (section 1.4). At present, however, there is no formal evidence that these molecules are of parasite origin. Preliminary IFA results (5.2.1.) suggested that monoclonal antibody 39 was interesting as a candidate for a probe which recognised both the parasite and possibly the surface of infected leucocytes; which might provide evidence for the existence of parasite encoded polypeptides on the surface of infected leucocytes.

5.3.1. Fluorescence assays with monoclonal antibody 39

The cloned cell line, which secreted monoclonal antibody 39 was re-cloned three times, however there was no segregation of the reactivity profile and the supernatants from cloned cell lines recognised piroplasms and infected leucocytes. Isotype analysis (section 2.3.3. for method and table 5.1.1. for results), showed that the supernatants contained only one immunoglobulin class (IgM). Thus, by this criterion, it seems unlikely that the reactivity profile was due to a mixture of hybridomas.

In order to determine whether the epitope, recognised by antibody 39, was located on the surface of macroschizont infected leucocytes, IFAs were performed with unfixed cells (section 2.3.). Antibody 39, as diluted ascitic fluid at 1:200, gave a very strong fluorescent reaction on formaldehyde fixed cells but a very faint reaction on viable cells. This low level of fluorescence intensity was also seen with normal mouse serum, while an intense, speckled, surface reaction was observed with a positive control monoclonal antibody 3D7 (Lesan, 1986). The results (shown in table 5.2.1.) suggested that monoclonal antibody 39 does not recognise the surface of the infected leucocytes.

Antibody 39 was screened by IFA against the following
cells: infected and uninfected red blood cells, a bovine lymphosarcoma cell line (BL-20), its infected counterpart (TaH-BL20), a macroschizont infected leucocyte cell line (TaH48), peripheral blood monocytes (PBM) and PBM which had been stimulated to undergo blastoid development with the following mitogens, concanavalin A (ConA, stimulates T-cells), phytohaemagglutinin (PHA, stimulates T-cells), pokeweed mitogen (PWM, a T- and B-cell activator) and lipopolysaccharide (LPS, stimulates B-cells). The mitogen stimulated cells were included in the screen as an additional indication of whether the epitope recognised by antibody 39 was involved with, or produced as a consequence of, the proliferation of infected leucocytes.

According with the previous results, monoclonal antibody 39 reacted with infected, but not uninfected, red blood cells, and infected leucocytes (TaH48 and TaH-BL20) but not uninfected BL-20 cells. In contrast, with the exception of LPS, mitogen stimulated PBM reacted with antibody 39 and showed a very similar fluorescence reaction to the one given with macroschizont infected cells. Unstimulated PBM and LPS-treated PBM failed to react with antibody 39. This suggested that the epitope recognised by antibody 39 was not exclusive to the parasite.

5.4. INDIRECT IMMUNOFLUORESCENCE ASSAYS: STOCK SPECIFICITY

The stock specificity of the monoclonal antibodies, which all recognised Ankara piroplasms was assessed by IFA, with acetone fixed infected red blood cells, on three uncloned stocks of T. annulata piroplasms. The stocks were: a) T. annulata Ankara (from Turkey), b) Hissar (from India) and c) Gharb (from Morocco). Initially, supernatants were tested against Ankara infected red blood cells to ensure that they were clearly positive relative to controls. Each, appropriately diluted, monoclonal antibody was tested against three slide preparations of each stock in which the parasitaemias varied between 40-90%.

The results, which are tabulated in table 5.4.1 suggested that the monoclonal antibodies could be used to distinguish between the three stocks at the epitope level. Three profiles were very
distinct and this is illustrated in figure 5.4.1. with the following examples: a) monoclonal antibody 5A10 reacted brightly with all three stocks, b) antibody 2D5 reacted brightly with Ankara and Hissar stocks, but failed to react with Gharb (monoclonal antibody 5E1 was similar, however it was noteworthy that approximately 0.5% of Gharb piroplasms were recognised by this antibody), and c) antibody 5G1 reacted brightly with Ankara but failed to recognise either Hissar or Gharb stocks.

Variation in the strength of monoclonal reaction, as judged by the observed intensity of fluorescence, was also detected between stocks (data not shown). For example, monoclonal antibody 1C2 reacted to produce a weaker fluorescent reaction on Gharb piroplasms than on Hissar or Ankara piroplasms on each of three different preparations of the three stocks.
Table 5.4.1.

Table illustrating IFA results performed to determine the stock specificity of 28 cloned monoclonal antibodies.

Each monoclonal antibody solution was tested, by IFA, against slide preparations of Ankara piroplasm infected red cells as a positive control. Antibody solutions which were positive in this respect were then tested against similar preparations of Hissar and Gharb piroplasm infected red cells. As shown previously (table 5.2.1.), with the exception of antibodies 5A10 and 5A12, none of the antibodies recognised uninfected red cells.
### Table 5.4.1.

**T. a. piroplasm stock (uncloned)**

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**Key:**
- ■ bright
- □ positive
- □ weak positive
- □ negative
Figure 5.4.1.

Photographs, of IFA results viewed through a fluorescence microscope, presented to illustrate the stock specific reactivity of the monoclonal antibodies with three examples.

Reading from left to right, the top panel of three photographs shows the positive reaction of monoclonal antibody 5A10 against acetone fixed piroplasm infected red cell slide preparations of the Ankara stock, then the Hissar stock and then the Gharb stock.

The middle panel shows a positive reaction by monoclonal antibody 2D5 against Hissar as well as Ankara, but no reaction against Gharb piroplasms.

In the bottom panel, monoclonal antibody 5G1 shows a positive reaction against Ankara piroplasms but no reaction against Hissar or Gharb piroplasms.
<table>
<thead>
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<th>Gharb</th>
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10 μm
5.5. WESTERN BLOT ANALYSIS OF MONOCLONAL ANTIBODIES

A set of twelve monoclonal antibodies (39, 5A10, 5E1, 1C2, 1C3, 5G1, 1H6, 1E9, 10A8, 5A12, 1D11, 2D5) were chosen to be representative of each major form of variation which had been observed in the IFA studies described in sections 5.1. to 5.4. Collectively, these were monoclonal antibodies which showed type 1, type 2 or type 3 reactivity profiles (section 5.1.), distinguished between Ankara, Hissar and Gharb stocks (section 5.4), appeared to recognise subsets of parasites on a slide (5.1) and recognised other stages in the life cycle which were examined (section 5.2.) as follows. Monoclonal antibody 39 was the only antibody of the twenty-eight tested by IFA, which recognised either sporozoites or macroschizont infected leucocyte life cycle stages. Antibodies 1D11, 2D5, 5A10, 1C2 & 5E1 had been screened against heat induced merozoite preparations, and all except antibody 5A10 were found to recognise this stage.

The objective of this experimental study was to attempt to define the molecular mass of the polypeptides recognised by the antibodies by Western blotting, so as to further characterise and establish any relationships between the epitopes recognised.

5.5.1. Preliminary results

Monoclonal antibodies (1D11, 2D5, 5E1, 5A10, 39, 1C2, 5G1, 1C3, 10A8, 1H6, 1E9, & 5A12) were screened against blots of uninfected red blood cell (cow110) and isolated Ankara piroplasm extracts. Stage specific, as assessed by IFA (Shiels et al. 1986b), anti-macroschizont monoclonal antibodies (1E11, 2G2, 4A4, 1F2, 1C12, 2G2, 2A6, 1C7, 1F12) were employed as possible negative first antibody controls. A further anti-macroschizont monoclonal antibody, 2E4, which detects piroplasms by IFA, was used as a putative positive control. When ascitic fluid was the source of antibody, normal mouse serum was included as a negative control. All the anti-macroschizont infected leucocyte antibodies were used as diluted ascitic fluid at 1:200 to 1:500. Red blood cell and piroplasm blots were also probed with second antibody (horse radish peroxidase conjugated anti-IgG) in the absence of first antibody to
detect any direct binding of this probe to blots.

Initial experiments demonstrated that blotting protocols that were less stringent than those described in section 2.6. in terms of antibody concentrations, incubation or washing protocols, resulted in high levels of non-specific reaction. When the Western blotting conditions which are described in section 2.6. were used, the macroschizont stage specific monoclonal antibodies, normal mouse serum and the second antibody alone failed to produce any positive reaction on blots of piroplasm extracts. The positive control antibody (2E4) reacted on piroplasm blots, recognising a single 38kDa polypeptide, and failed to recognise uninfected red blood cells. Western blots were performed with monoclonal antibodies 4A4 and 1E11 against macroschizont infected leucocyte extracts. Using the same protocol as for the piroplasm blots, specific polypeptides (data not shown) were recognised, suggesting that these particular epitopes are not destroyed under the strongly denaturing conditions used.

The twelve test monoclonal antibodies recognised polypeptides on piroplasm blots which appeared to be infection specific (figure 5.5.1.1.), as uninfected red blood cell blots failed to react (data not shown). Figure 5.5.1.1. a) shows the reactivity of the monoclonals as antibody supernatants, and figure 5.5.1.1. b) tracks 13-16 illustrate the reactivities of 2D5, 5E1, 2E4 and 1D11 as diluted ascitic fluid. The results of the control probes were as expected (see figure 5.5.1.1. b, tracks 17-20): second antibody alone failed to bind to blots and normal mouse sera (NM) also failed to react, or produced a weak non-specific staining pattern on all the major polypeptides present (as assessed by protein staining of blots with Ponceau S). The negative control, stage specific, anti-macroschizont antibody (1C7) failed to react, and the positive control, anti-macroschizont antibody (2E4) recognised a 38kDa polypeptide.

Antibodies 1C3, 5A10, 1H6, 1E9, 10A8 and 5A12 all appeared to recognise mainly the same polypeptides, although with varying intensities, and these polypeptides were also recognised by antibodies 2D5, 1C2, 1D11 and 5E1. Monoclonal antibodies 5G1 and 39 (figure 5.5.1.1.a, tracks 4 & 7) reacted with polypeptides of 36kDa and 53kDa respectively and monoclonal antibody 5E1 recognised
Figure 5.5.1.1.

Preliminary Western blot results showing apparent reactivity of 12 monoclonal antibodies against Westerns of isolated Ankara piroplasm extracts.

NB – in the case of monoclonal antibodies 1C3, 5A10, 1H6, 1E9, 10A8, and 5A12, these results are inconclusive and unrepeated (by this I do not mean "unrepeatable"). This is the only Western data that I have on these antibodies and I feel that this information might be useful in the future.

Undiluted monoclonal antibody supernatants (A, tracks 1-12) and ascites solutions (1:100 dilution, B, tracks 13-17) which all showed a strong positive fluorescence by IFA were tested against Western blots of Ankara piroplasm extracts. The monoclonal antibodies which were used are indicated at the top of the figure. Western blotting was performed, and antibody binding was detected using Horse radish peroxidase, as described in section 2.8.

Track 18 shows the reactivity of a positive control monoclonal antibody (antibody 2E4) and track 19 shows the reaction of a negative control antibody (anti-macrocohizont monoclonal antibody 1C7). Track 20 shows the second antibody control reaction. The numbers down the side of the figure indicate the molecular mass and mobility of internal molecular weight standard markers.
**Figure 5.5.1.1.**

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**1C7**

**2E4**

**NM**

**2nd Ab**

kDa

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**B**
two polypeptides of molecular masses 30kDa & 20kDa (tracks 3 & 14). In addition, these three monoclonal antibodies and the other nine (either as diluted ascitic fluid or as culture supernatants) appeared to recognise two groups of polypeptides of distinct molecular mass. The first group comprised polypeptides between 90kDa and 128kDa. This group was recognised by all the test monoclonal antibodies but to differing degrees and polypeptide staining was most intense with antibodies 1D11, 2D5 and 1C2 (tracks 2, 10, 12, 13). Only the largest (120kDa) polypeptide of the group was strongly recognised by antibody 5G1 (track 4) and the reaction varied between weak to intense with the other antibodies. The second group consisted of polypeptides between 42kDa and 80kDa which were recognised most strongly by antibody 1C2 (as antibody supernatant) but also by antibodies 1D11, 2D5 and 5E1; consistently, as ascitic fluid, and inconsistently between blotting experiments, by hybridoma supernatant. Antibody 5A10 was subsequently used to probe a range of different piroplasm extracts from infected red cells (Ankara) of different parasitaemias, but no reactivity was detected while antibody 5E1, as a positive control, consistently recognised a 30kDa polypeptide and occasionally 20kDa, 10kDa and 120kDa molecules.

5.5.2. Further Western blot analyses

Further Western blotting studies were carried out to consolidate and extend some of the preliminary results presented above. These studies were restricted to four of the monoclonal antibodies: 2D5, 1D11, 1C2 and 5E1. These antibodies reacted in Western blots which suggested that they did not recognise conformational epitopes and showed interesting profiles of reactivity by IFAs. All four recognised merozoites as well as piroplasms, but not sporozoites or macroschizonts by IFA. Antibodies 2D5 and 1D11 were very similar and showed the type 2 "dot" reactivity on piroplasms, 5E1 (type 1) appeared to recognise the outer perimeter of the parasite, and 1C2 showed type 1, type 2 and type 3 (internal epitopes recognised) classes of reactivity on any one slide preparation.

The hybridoma lines were re-cloned, and their IFA pattern of reactivity was checked, and found to be stable prior to
their use in Western blots. These antibodies, as spent culture supernatants, were screened against blots of isolated piroplasms (Ankara, Hissar & Gharb stocks), infected and uninfected red blood cells and also against heat induced merozoites. The inclusion of infected red blood cell extracts as well as isolated piroplasm extracts was important for two reasons. Firstly, the procedure for piroplasm isolation involved a fairly lengthy series of operations during which time proteolytic activity might affect polypeptide composition. A range of protease inhibitors were included in buffer solutions, however, it was not determined how effective these were. The procedure for isolating infected red blood cells was relatively rapid and non invasive (Martin et al. 1971), and it was less likely that these extracts would be artificially modified by proteases during their isolation. Secondly, red blood cell extracts might contain parasite molecules or small parasites (possibly merozoites) which could or would be lost during piroplasm purification procedures. The results are described in the following sections.

5.5.2.1 Monoclonal antibody 5E1

Antibody 5E1 failed to recognise uninfected red blood cells (cow120 figure 5.5.2.1. A, track 6), but reacted strongly with a 30kDa polypeptide in Ankara piroplasm and infected red blood cell blots (tracks 1 & 2). According with previous IFA results, antibody 5E1 recognised Hissar piroplasms but not Gharb piroplasms (tracks 3, 4, 5 & 6). Protein staining of the blots with Ponceau S suggested that the 30kDa polypeptide was also present in extracts of Gharb piroplasms. The molecules recognised in Hissar and Ankara extracts were indistinguishable. Three other polypeptides were also recognised by antibody 5E1: two smaller, 20kDa and 10kDa, polypeptides were recognised in piroplasm blots in several but not all experiments with different Ankara and Hissar extracts. The 10kDa molecule was less frequently detected, and it is absent in the illustrated blot, except possibly weakly in track 4. The 20kDa polypeptide can readily be seen in tracks 1 & 3. A larger polypeptide of 120kDa was weakly and occasionally detected in either Ankara piroplasm or infected red blood cell extracts (see
Further Western blot analysis of monoclonal antibodies 5E1, 2D5, 1D11 and 1C2.

Figure 5.5.2.1. A

Tracks 1-7 illustrate the reactivity of monoclonal antibody 5E1, as undiluted culture supernatant, against the following extracts (see section 5.5.2.); track 1, piro A = Ankara piroplasm extract (see section 2.1.4. for piroplasm preparation); track 2, rbc A = Ankara piroplasm infected red cell extract (see section 2.1.3.); track 3, piro H = Hissar piroplasm extract; track 4, rbc H = Hissar piroplasm infected red cell extract; track 5, piro G = Gharb piroplasm extract; track 6, rbc c = control uninfected red cell extract; track 7, mero = Ankara merozoite extract (see section 2.1.9. for merozoite preparation).

The molecular mass and mobility of internal molecular weight markers are illustrated on the left hand side of the figure.

Figure 5.5.2.1. B

Tracks 1-4 show the reactivity of a mixture (1:1) of monoclonal antibodies 2D5 and 1D11 as culture supernatants, against the following extracts; track 1, piro 1 = an Ankara piroplasm extract; track 2, piro 2 = a second Ankara piroplasm extract; track 3, rbc c = control uninfected red cell extract; track 4, mero = an Ankara merozoite extract.

Tracks 5-10 show the reactivity of monoclonal antibodies 2D5, (tracks 5-7) and 1D11 (tracks 8-9) against the following extracts; piro 1 = Ankara piroplasm extract (tracks 5 & 6); piro 2 = a second Ankara piroplasm extract (tracks 6 & 9); and mero = an Ankara merozoite extract (tracks 7 & 10).

Tracks 11-15 show the reactivity of monoclonal antibody 1C2 against the following extracts; track 11, piro 1 = Ankara piroplasm extract; track 12, rbc i = Ankara piroplasm infected red cell extract; track 13, piro 2 = a second Ankara piroplasm extract; track 14, mero = Ankara merozoite extract; track 15, rbc c = control uninfected red blood cell extract.
Figure 5.5.2.1.
track 2, an infected red blood cell extract). Its appearance was not apparently related to the presence of the smaller, 20kDa and 10kDa polypeptides or to the parasitaemia of the blood sample. However, its presence seemed to be related to the batch of parasite material as certain extracts of infected blood from different calves, whether probed as isolated piroplasm or infected red blood cell extracts, showed a stained polypeptide of 120kDa.

Antibody 5E1 was also screened against extracts of heat induced merozoites and a single 30kDa polypeptide was recognised. Therefore, this polypeptide did not appear to be modified in molecular mass between merozoite and piroplasm stages.

5.5.2.2. Antibodies 1D11 and 2D5

Antibodies 1D11 and 2D5 failed to recognise any polypeptides in blots of uninfected red blood cell extracts (figure 5.5.2.1. B track 3, probed with a 1:1 mixture of 1D11 and 2D5) but they recognised the same molecular mass polypeptides in blots of isolated Ankara piroplasms. This is illustrated in figure 5.5.2.1. B, tracks 1, 2, 5, 6, 8 and 9. Here, two different Ankara piroplasm extracts were probed with antibody 1D11 (tracks 8 & 9), 2D5 (tracks 5 & 6) and a 1:1 mixture of the two antibodies (tracks 1 & 2). A polypeptide of 120kDa was recognised in both piroplasm extracts. A smaller doublet, (98kDa and 100kDa), was weakly detected in one piroplasm extract (tracks 1, 5 & 8) and strongly stained in the second piroplasm extract (tracks 2, 6 & 9). In addition a smaller polypeptide (77kDa) was also weakly recognised in the second piroplasm extract. The 98-100kDa doublet and the 77kDa polypeptide were not always detected in blots of piroplasm extracts and they were not detected in blots of infected red blood cell extracts (data not shown). In contrast, the 120kDa (the molecular mass range, calculated from four experiments, was 115-130kDa) was always recognised in infected red blood cell (data not shown) and isolated piroplasm blots.

Antibodies 1D11 and 2D5 were screened against isolated piroplasm extracts of Ankara, Hissar and Gharb stocks. The results showed (data not shown) that the antibodies reproducibly recognised a 120kDa polypeptide in Ankara and Hissar extracts but failed to
recognise Gharb piroplasms, thereby consolidating the IFA results.

Antibodies 1D11 and 2D5 were screened against heat induced merozoite extracts in Western blots. Figure 5.5.2.1.B, tracks 4, 7 & 10 show the results of one such experiment (the polypeptide band in the track 7 is too weak but several experiments confirmed its positive reactivity). A single 120kDa polypeptide was recognised, by both antibodies, which suggested that a similar polypeptide was recognised in merozoite and piroplasm stages. The smaller molecules which were frequently recognised in piroplasm extracts were never observed in heat induced merozoite preparations.

5.5.2.3. Antibody 1C2

Preliminary Western blots (5.5.1.) had indicated that antibody 1C2 recognised a cluster of polypeptides which ranged between 90kDa and 128kDa, and a lower molecular mass group consisting of polypeptides between 42kDa and 80kDa. The monoclonal antibody was re-cloned and a Western blotting experiment was performed using spent culture supernatant as antibody probes. Antibody 1C2 was screened against extracts of two different Ankara piroplasm preparations, Ankara piroplasm infected red blood cells, uninfected red blood cells and Ankara heat induced merozoites. Figure 5.5.2.1.B, shows the developed profiles of reactivity. Uninfected red blood cells failed to react (track 15), and antibody 1C2 recognised 120kDa and 30kDa polypeptides in piroplasm (tracks 11 & 13), infected red blood cell (track 12) and merozoite extracts (track 14). Additional molecules were detected in the piroplasm extracts. In one extract, (track 11) molecules of 98, 100, 113, 77, 53 and 47kDa were recognised. In the second extract (track 13), antibody 1C2 recognised the 98kDa and 100kDa doublet and also a polypeptide of 77kDa.
The hybridoma experiments generated forty-six monoclonal antibodies which, by IFA, recognise piroplasms but not red blood cells. Morphologically defined differentiation events in the intraerythrocytic stages of the life cycle are poorly understood and the immunising material may have contained an unknown proportion of merozoites (defined by the possession of rhoptries), derived either from macroschizonts or from red blood cell stages. It is therefore possible that antibodies were raised against these stages, as well as against piroplasm stages (probably best defined at present, by default, as intraerythrocytic stages without rhoptries). Twelve of the cloned monoclonal antibodies were used to probe Western blots of piroplasm extracts. Five of these (antibodies 5G1, 39, 2D5/1D11, 1C2 & 5E1) showed unique profiles of reactivity in Western blots. The polypeptides recognised by the remainder were not confirmed, and the epitopes determining these antibodies may be irreversibly denatured under the strongly reducing conditions used. All of the cloned monoclonal antibodies (28 cloned lines) fail to recognise formaldehyde fixed sporozoites and only antibody 39 recognises formaldehyde fixed macroschizont infected cells. Thus the vast majority of the antibodies are stage specific. This information may be added to existing data on the stage specificity of \textit{T. annulata} epitopes and polypeptides (Shiels \textit{et al.} 1986a and 1989; Williamson \textit{et al.} 1989). The finding that four (antibodies 5E1, 2D5/1D11, 1C2), of the five (not antibody 5A10) antibodies tested, recognise heat induced merozoites and piroplasms, might suggest that the piroplasm stages are more closely related to merozoites than to either the sporozoite or the macroschizont. However, as only four monoclonal antibodies (and possibly only two polypeptides) were considered, this cannot be a significant measure of the overall level of difference between these two stages.

In considering the life cycle stages in the bovine host, antibody probes have been generated (antibodies 5E1, 2D5/1D11 and 1C2) which recognise epitopes which appear, by IFA analysis, to be differentially expressed in the consecutive heat induced merozoite and intraerythrocytic life cycle stages.
It was hoped that antibodies would be raised against surface ISEMPs, but this aim has not been realised. As previously mentioned (section 3.7.), it has not been determined whether any of the antibodies recognise ISEMPs which are not exposed on the surface of the red cell, but which are present in the red cell membrane. This question might be experimentally approached by performing IFAs with variously fixed preparations of infected red cells or by immunoelectron microscopy.

The patterns of fluorescence produced in IFAs with the monoclonal antibodies on Ankara infected red blood cell and piroplasms have been delineated. The fluorescence intensity, the proportion of available parasites on a slide which fluoresce, and the portion of each parasite which fluoresces varies between the monoclonal antibodies. This form of diversity, together with the finding that the antibodies show apparently qualitative differences in their reactivity against Ankara, Hissar and Gharb stocks, suggests that many of the antibodies are different, and may recognise different epitopes.

The reactivity profiles of the monoclonal antibodies could be grouped. Three main types of fluorescence pattern on infected red cell preparations suggest that different antibodies recognise the following areas of the piroplasm (table 5.1.2. and figure 5.1.1.): the outer perimeter of the piroplasm (classified as type 1 antibody reactivity), a very prominent reactivity against small circular elements within the piroplasm (dot reactivity, type 2), and internal epitopes but not the dot structures (internal epitopes, type 3). Some antibodies, for example antibodies 5A10 and 1C2, show all three reactivities on the same slide preparations. Therefore, although the reactivity of individual monoclonal antibodies on infected red cell preparations is distinctive, the IFA data suggest that there is a relationship between the polypeptides located in the three areas defined within the descriptions of type 1-3 reactivities. Antibodies 2D5 and 1D11 are indistinguishable on the basis of the IFA results, also by antibody isotype (table 5.1.1.) and by Western blot analyses (figure 5.5.2.1.b). They could be the same antibody and may be considered together as antibody 2D5/1D11. Antibodies 2D5/1D11, 5E1 and 1C2 also recognise formaldehyde fixed, heat induced merozoites.
Their IFA reactivity patterns are consistent between merozoite and piroplasm stages: antibody 5E1 recognises the outer perimeter of merozoites, antibodies 2D5 and 1D11 appear to recognise internal epitopes (and the fluorescence pattern is similar to the dot reactivity seen in piroplasm preparations), and antibody 1C2 shows both of these reactivity patterns.

The polypeptides recognised by antibodies 5E1 (type 1), 2D5 & 1D11 (type 2) and 1C2 (all three types) have been characterised by Western blot analysis (figure 5.5.2.1.b). Antibody 5E1 consistently and strongly recognises a 30kDa polypeptide in piroplasm or infected red cell extracts and also recognises polypeptides of 120kDa, 20kDa and 10kDa; the reaction with the 120kDa polypeptide is weak. Recognition of a 120kDa polypeptide predominates the reactivity profiles of antibodies 2D5 and 1D11 in piroplasm or infected red cell extracts. The main reactivity of antibody 1C2 is against polypeptides of 120kDa and 30kDa. Antibodies 1C2 and 2D5/1D11 also inconsistently recognise a polypeptide doublet of 98-100kDa and a 77kDa molecule in piroplasm extracts but not in infected red cell extracts. The doublet was also detected on a single experiment when antibody 5E1 was used to probe piroplasm extracts and this result has not been confirmed.

The absence of the 98-100kDa and 77kDa polypeptides in infected red blood cell extracts might be due to a number of factors. Firstly, they may be present in infected red cell extracts but the assay may not be sensitive enough to detect it. Parasite protein is diluted when infected red cell extracts are analysed compared to purified piroplasm extracts and this difference between the two extracts might explain the results. Secondly differences in sample preparation may lead to the detection of the polypeptides in piroplasm extracts due to proteolytic damage that does not take place in the faster and less invasive preparation of infected red cell samples.

Thus antibodies 5E1, 2D5/1D11 and 1C2 mainly recognise 120kDa molecules, and 1C2 and 5E1 recognise 30kDa molecules. Similar polypeptide reactivity profiles are also detected in extracts of heat induced merozoites (Ankara) but the 98-100kDa doublet is not recognised. Heat induced merozoite specific markers have not, apparently been defined. Extrapolating from these
results, it is likely that the polypeptides recognised by these antibodies in heat induced merozoite extracts have the same molecular mass after differentiation into piroplasms. An alternative interpretation of the Western blot results revolves around the possibility that infected red cell material contains an unknown proportion of merozoites. If merozoite polypeptides contribute significantly to the parasite extracts from infected red cells and "purified piroplasm preparations" then it could be inferred that the 97-100kDa and 77kDa polypeptides are piroplasm specific while the 120kDa and 30kDa polypeptides are produced in merozoites and piroplasms. This interpretation, (or variations along similar) lines is tenable, although it fails to corroborate the conflicting electron microscopical data of Conrad et al. (1985) and Melhorn & Schein (1984) and clearly requires further investigation.

It is possible that the 30kDa polypeptide is a processed product of a 120kDa parent molecule. However the two polypeptides may be expressed from different genes which code for the common or cross-reactive epitopes recognised by the three different antibodies. Additionally, the co-identity of the polypeptides of same molecular mass which are recognised by the antibodies has not been formally demonstrated.

The monoclonal antibodies were tested by IFA against piroplasm and infected red blood cell material which was uncloned, asynchronous and, as mentioned above, possibly contained an unknown proportion of merozoites and red blood cell merozoites. Therefore, the observation of fluorescence on a subset of the available parasites, and the heterogeneous reactivity of the same antibody within single (or between different) slide preparations may reflect any of these characteristics. The possibility that the subsetting reactivity (antibodies 1H4, 1E9, 1D12, 1G9, 5B9 & 10A8) is the result of genetically diverse populations of piroplasms within the uncloned stock, might be tested using series of cloned piroplasm material generated by infecting bovids with cloned macroschizont infected cell lines. If the monoclonal antibodies retain their subsetting reactivity on cloned populations, then it is likely that they are recognising different developmental stages of the life cycle or possibly cell...
cycle specific epitopes. Further characterisation of the subsets or stages which are potentially involved in producing the subsetting reactivity might be achieved if parasite differentiation could be synchronised to some definable extent in samples. The different stages might be isolated for study (probably on the basis of differential size, density and reactivity with subsetting antibodies). The subsetting monoclonal antibodies were not tested against heat induced merozoites, and this experiment might help to clarify the situation. Moreover it is envisaged that cloned merozoites could be quite readily produced in vitro from cloned cell lines. Conrad (1983) maintained piroplasm infected red cells in stationary culture and found that many piroplasms divided to produce red blood cell merozoites, which unlike piroplasms, possessed rhoptries. The antibodies could be used to probe preparations of these culture forms, which may undergo differentiation events which correspond to the in vivo life cycle.

The differentiation events in the red cell stages probably involves the modification or loss of expression of a wide range of existing epitopes. The antibodies which recognise the outer perimeter of the parasite material (type 1) recognise all the available parasites on a slide, thus, if merozoites or a range of differentiating types are present then these are also recognised. It was not determined whether any of the type 2 antibodies (dot reactivity) recognised subsets of parasites because their reactivity patterns were too heterogeneous. Thus only type 3 monoclonals, which appear to recognise internal determinants, were identified as exhibiting a subsetting reactivity.

The identity of the dot structure recognised by type 2 antibodies is an intriguing question. The dots are uniform in size, appear very precisely circular and occur singly or in symmetrical clusters of two to four dots. In addition to the dot structure, type 2 antibodies react with other areas of the piroplasm and in some IFA assays the whole of the red cell, in a small proportion of infected cells, showed a diffuse fluorescence. The type 2 antibodies 2D5, 1D11 and 1C2 also recognise heat induced merozoites, 5A10 does not recognise heat induced merozoites and the remaining type 2 antibodies (antibodies 10C3 and 5C5) were not tested against these culture forms.
It is tempting to speculate that the dot structures are merozoites. If red blood cell merozoites are generated by division of piroplasms into four, and if the antibodies recognise red cell merozoites in infected red cell preparations: then one would expect to see groups of four merozoites highlighted to some extent by the monoclonal antibodies. As symmetrical clusters of four dot structures are observed this provides tentative circumstantial evidence that piroplasm division products are recognised. The dot structures might, alternatively, be subcellular organelles, for example rhoptries or nuclei, in which case however, the implications are similar. Most importantly, this interpretation would imply that division of piroplasms into four is a common event and therefore that merozoites (with rhoptries) are generally highly represented in infected red cell samples. A number of results would have to be carefully evaluated and further work performed in order to investigate this theoretical possibility. For example, antibody 5E1 recognises heat induced merozoites, but it is not classified as a type 2 antibody as it does not show the characteristic dot reactivity on piroplasm preparations. However, antibody 5E1 recognises small parasites or parts of parasites in piroplasm preparations which could be the same forms recognised by the type 2 antibodies. The difference between the reactivity of antibody 5E1 and the type 2 antibodies, and the reason for the different classification, may be that the former recognises the surface of merozoites and piroplasms while the type 2 reactivity is produced by recognition of internal epitopes. A second problem arises with this interpretation in that antibody 5A10 recognises dots in piroplasm preparations but fails to recognise heat induced merozoites. If 5A10 recognises red blood cell merozoites but not macroschizont derived merozoites then this would explain the result. Again, this might be investigated by testing the antibody on infected blood preparations which have been maintained in stationary culture and which exhibit increased levels of quadruplet, piroplasm division products.

The reactivity of some antibodies (type 2 antibodies and antibody 5G1) in producing a diffuse fluorescence within a small proportion of the red cells in infected red cell preparations was noted. The basis for this form of reactivity is unclear; it
may, for example, highlight the occurrence of trafficking events. Alternatively it could be the result of the partial destruction of the intracellular parasite; possibly a true reflection of in vivo events, or an artefact perhaps of incomplete fixation of the slide material preparations.

Antibodies 5A10 and 5A12 appear to recognise parasites in slide preparations of the original immunising material, but also recognise uninfected red cells from cow 110. When they were tested against infected red cell samples from a further five infected bovids, antibody 5A10 recognised the red blood cell membrane of all red cells (infected or uninfected) in three of the samples, and only piroplasms in the two other samples. Antibody 5A12 recognised red cells in all five samples. The antibodies recognised isolated piroplasms, but these may have been contaminated with red cell membranes. One explanation for the reactivity profile is that the two antibodies recognise different piroplasm epitopes that are related to certain polymorphic red cell products, which were not in the immunising material. Unfortunately, antibody 5A10 failed to react in Western blots of infected or uninfected red cell extracts and this cross reactivity has not been substantiated. Anti-bovine blood group antibodies are now available and it would be possible to type infected red cell samples, in order to investigate this possibility using defined material.

Only one monoclonal (antibody 2A3) was generated which recognised only red cell material, which was quite surprising, but may reflect the similarity between bovine and murine red cells or be a consequence of the immunising protocol which was (retrospectively) clearly not ideal for generating antibodies against the surface of red cells. A better immunising protocol might involve immunisation with pure infected red cell membrane preparations.

The experiments with monoclonal antibody 39 were initiated because it was possible that the antibody recognised a parasite encoded epitope on the surface of infected leucocytes. Surface IFAs suggested that the epitope is not located on the surface of infected leucocytes. The reactivity pattern of antibody 39 on fixed cells suggests a recognition of the host cell membrane (figure 5.1.1.b, T-BL). Fixed cell IFAs with mitogen stimulated,
uninfected leucocytes suggested that the epitope was present in these cells and, therefore, it is not parasite specific. It is not known whether the macroschizont is recognised by antibody 39 or whether the macroschizont contributes (presumably together with the proliferating leucocyte) to the production of the epitope recognised by antibody 39 in the membrane of infected leucocytes. Finally, preliminary Western blotting data suggest that the same 53kDa polypeptide is recognised by antibody 39 in piroplasms, macroschizont infected cells and concanavalin A treated cells. It is therefore conceivable that the epitopes recognised by antibody 39 are related by residing on the same polypeptide synthesised by piroplasms, proliferating leucocytes and possibly macroschizonts.

The stock specificity study revealed that a panel of the monoclonal antibodies could currently be used to distinguish between Ankara, Hissar and Gharb stocks of *T. annulata* (table 5.4.1.). In addition to the immunising Ankara stock, four antibodies recognise Gharb and Hissar stocks, and eleven antibodies recognise Hissar but not Gharb stocks. Thirteen antibodies are restricted in their reactivity to Ankara parasites. As shown in appendix la, the polypeptide profiles of the three stocks are very similar when extracts are separated by SDS-PAGE and stained with Coomassie Blue. The most clear-cut stock specific polypeptide was a molecule of 19kDa which was only detected in Hissar extracts.

Western blot analysis of Ankara, Hissar and Gharb piroplasm extracts, probed with antibodies 5E1 and 2D5/1D11, consolidated the stock specificity differences found with these antibodies by IFA. Gharb extracts are not recognised by either antibody. The 30kDa and 120kDa polypeptides recognised in Ankara piroplasm extracts are also recognised in Hissar extracts. General protein staining data (Coomassie Blue and Ponceau S) suggest that 120kDa and 30kDa polypeptides are present in Gharb piroplasm extracts (see appendix la). Immune bovine sera recognise a Gharb polypeptide of 30kDa in Western blots a 120kDa polypeptide was not apparently recognised. It is likely, however, that the stock variation detected with antibodies 5E1 and 2D5/1D11 takes place at the epitope level.
CHAPTER SIX
STAGE DIFFERENTIATION

The relationship between the macroschizont and host leucocyte, including the mechanisms of leucocyte transformation (see Dyer & Tait, 1987) and merogony, are poorly understood. Macroschizont binary cell and nuclear division can take place in the absence of host cell division. However, host nuclear and cell division is driven by the presence of the parasite, via unknown mechanisms (Pinder et al. 1981). The sequence of proliferative events in cultured macroschizont infected cell lines involves the simultaneous cell division of macroschizonts with host mitosis. As host cell division progresses, daughter macroschizonts are observed within each daughter host cell (Hulliger et al. 1984). In established cell line cultures, the number of macroschizont nuclei remains approximately constant, therefore, it appears that the rate of macroschizont nuclear division is about the same as the rate of host cell division. Jura et al. (1983a) reported that macroschizont nuclei undergo closed mitosis at the pro-metaphase of host mitosis, but there is no evidence that macroschizont nuclei divide simultaneously and unequal numbers of nuclei are reported.

The onset of merozoite production and fever in infected bovids occurs at about the same time. Hulliger et al. (1986) reported that T. parva merozoites are induced in vitro by elevating the temperature of macroschizont infected leucocyte cell line cultures. Danskin & Wilde (1976) reported the same result using bovine lymph as the stimulus and also observed merozoite invasion of red cells. Despite these promising results, research in this area has not been followed up, although the merozoite stage initiates the high parasitaemia which contributes to the death of bovids, is potentially important in vaccine work as an extracellular target for immune responses, and is technically difficult to obtain from infected bovids. By analogy with work on the sporozoite stage, where antibodies from immune bovids block invasion of target leucocytes (Musoke et al., 1982; Gray & Brown, 1981; Preston & Brown, 1985), antibodies against the merozoite may prevent invasion of red cells. Electron microscopy studies on
merozoites, harvested from bovine lymphnodes 8-14 days after infection (Meihorn and Schein, 1984), have revealed the ultrastructural changes which take place during intraleucocytic merogony in *T. annulata* and *T. parva*.

The aim of the work presented in this chapter was to study stage specific differentiation at a molecular level. This was approached by using monoclonal antibodies to define stage common and stage specific epitopes and by screening a lambda gt11 expression library to clone genes coding for stage specific epitopes. As previously described (chapter 5) monoclonal antibodies were generated against piroplasms which appeared to be stage specific in that they failed to recognise sporozoites or macroschizonts. In order to test the monoclonal antibodies against the merozoite stage, an attempt was made to reproduce the work of Hulliger *et al.* (1984) and induce merozoites in *vitro*. The rationale for this approach was that an *in vitro* induction system would provide a controlled and accessible method for studying differentiation from the macroschizont to the merozoite. Moreover if induced merozoites enter red cells and differentiate into piroplasms, then the study could be extended to this stage. Clearly this system has not been extensively studied and most of the basic questions regarding the mechanisms involved are unanswered. As a first step, the experiments presented in this chapter were performed to investigate the practical parameters involved in developing a reproducible *in vitro* system of merozoite induction. Merozoites were generated in these experiments, and as previously described, they were recognised by four of the anti-piroplasm monoclonal antibodies (antibodies 5E1, 2D5/1D11 & 1C2). Monoclonal antibody probes were therefore available which recognise epitopes that appear to be expressed, for the first time in the life cycle, during the differentiation into merozoites.
6.1. IN VITRO INDUCTION OF MEROZOITES

In order to try to generate merozoites, two approaches were taken: firstly to use drugs to inhibit host cell division, and secondly, to elevate macroschizont culture temperatures as described by Hulliger et al. (1966). Hulliger et al. (1964) and Jura et al. (1983a) observed that the host spindle apparatus is involved in the process of macroschizont cell division and it has been postulated that by preventing host division, macroschizont cell division might be prevented and merozoite development stimulated. If this hypothesis is correct, it suggests that the state of proliferation in the leucocyte (initially generated as a result of infection) inhibits merogony.

6.1.1. Drug treatment of TaH4e

The following drugs, which are inhibitors of cell division, were screened against macroschizont infected leucocytes: aminopterin which inhibits folic acid reductase, colchicine which inhibits spindle formation (tubulins), emetine which inhibits cytoplasmic protein synthesis, 5-azacytidine which inhibits methyltransferases, 8-azaguanine which inhibits hypoxanthine-guanine-phosphoribosyl transferase (HGPRTase) and chloramphenicol which inhibits mitochondrial protein synthesis.

Macroschizont infected cells (TaH4e) were cultured as described in section 2.1.5. at 5x10^5-8 cells/ml in 24-well plates at 37°C for 24 hours in the presence of a range of concentrations of each drug (see table 6.1.1.a). A visual assessment, by phase contrast light microscopy, was made of the rate of growth and condition of the cells relative to untreated control cells after 24 hours (table 6.1.1.a). All the drugs were found to inhibit cell growth apart from chloramphenicol and this drug was not used in further studies. The lowest drug concentration at which host cell division was inhibited was the maximum concentration used in the next experiment, together with a further series of dilutions. TaH4e cells (5x10^5 cells/ml) were incubated with this range of dilutions of each drug for 48 hours. Half the medium was then replaced with fresh complete Ta medium without drugs and the cells were incubated.
Table 6.1.1.

In vitro induction of merozoites.

Tabulated summary of results of drug treatment experiments.

Table 6.1.1. A

Macroschizont infected cells (TaH48) were cultured (section 2.1.5.) at 5x10^5 cells/ml in 24-well plates at 37°C for 24 hours in the presence of one of six drugs over a range of concentrations (expressed in µg/ml down the left hand side of the figure). After 24 hours a visual assessment, by phase contrast microscopy, was made of the reaction of the cells to each drug treatment. The results were scored as follows (see key); (-) = no growth, (+) = normal growth relative to control untreated cells, (rg) = reduced growth relative to untreated control cells.

Table 6.1.1. B

Based on the results shown above, a longer experiment, using a more appropriate range of drug concentrations was set up to try to generate merozoites. Cells, as above, were incubated for 48 hours in a range of drug concentrations (see beneath each abbreviated drug name, in µg/ml). Half the medium was then replaced with fresh complete Ta medium without drugs, and the cells were incubated for a further 24 hours. The assessment system was as described above and the results are shown to the right hand side and adjacent to each drug concentration.
Table 6.1.1.

**A  24 hour growth**

<table>
<thead>
<tr>
<th>µg/ml</th>
<th>EM</th>
<th>COL</th>
<th>AZG</th>
<th>AZC</th>
<th>CAP</th>
<th>AM</th>
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<tbody>
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<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>22</td>
<td>.</td>
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</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>.</td>
</tr>
<tr>
<td>4.4</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
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<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>.</td>
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<tr>
<td>0.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>.</td>
</tr>
<tr>
<td>0.01</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>.</td>
</tr>
</tbody>
</table>

**B  72 hour assay**

<table>
<thead>
<tr>
<th>EM</th>
<th>COL</th>
<th>AZG</th>
<th>AZC</th>
<th>AM</th>
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</thead>
<tbody>
<tr>
<td>0.1 rg</td>
<td>1</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>0.05 +</td>
<td>0.1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>0.01 +</td>
<td>0.05</td>
<td>-</td>
<td>0.1 rg</td>
<td>0.01</td>
</tr>
<tr>
<td>0.005 +</td>
<td>0.01</td>
<td>-</td>
<td>0.01</td>
<td>+</td>
</tr>
<tr>
<td>0.001 +</td>
<td>0.005 rg</td>
<td>0.002</td>
<td>+</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**KEY:**
- EM emetine (µg/ml)
- COL colchicine
- AZG 8-azaguanine
- AZC 5-azacytidine
- CAP chloramphenicol
- AM aminopterin
- - no growth
- + normal growth
- rg reduced growth
- . not done
for a further 24 hours. The cells were assessed by light microscopy for signs of altered morphology and cell division, before harvesting and examination of Giemsa stained cytocentrifuged slides.

These treatments resulted in various alterations to the macroschizonts but merozoites were not produced. At higher drug concentrations (aminopterin at 0.22μg/ml, colchicine at 0.01μg/ml, 5-azacytidine & 8-azaguanine at 1μg/ml and emetine at 1μg/ml) host cells fragmented during the slide preparation, macroschizonts were vacuolated and there was no evidence that macroschizont nuclear division or cell division had taken place in the absence of host division. This suggested that at these concentrations the major effect of the drugs had been a toxic one which affected the host cell and the macroschizont.

At intermediate drug concentrations (aminopterin at 0.044μg/ml, colchicine at 0.005μg/ml, 5-azacytidine & 8-azaguanine at 0.1μg/ml and emetine at 0.1μg/ml), limited host cell growth had been restored after the 24 hour period at half the original drug concentration and drug-specific alterations in the macroschizonts were observed. Many host cells fragmented during the slide preparation and the observation of free parasites may have been as a consequence of this, or of some other release mechanism. (It has been shown (Pinder et al. 1981) that treatment of T. parva macroschizont infected cells with a naphthoquinone derivative, (993C) results in expulsion of macroschizonts from host cells, as well as the death of macroschizonts). The morphological alterations in macroschizonts which are described below occurred in intra-leucocytic and free macroschizonts.

Colchicine, 5-azacytidine, 8-azaguanine and aminopterin inhibited macroschizont cell division, while emetine did not. Colchicine and aminopterin permitted apparently normal macroschizont nuclear division and, in the absence of cell division, the number of nuclei per macroschizont was consistent with normal rates of nuclear division: that is approximately every 24 hours, yielding 100-200 nuclei after 72 hours. 5-azacytidine and 8-azaguanine partially inhibited macroschizont nuclear division as no more than one doubling of the number of nuclei per macroschizont was detected, also the macroschizont
nuclei became very heterogeneous in size. Emetine treatment resulted in the production of a small proportion of macroschizonts which contained smaller than normal nuclei (less than 1 μm) but at a higher than normal number (approximately 300). These multinucleated macroschizonts were not observed in the process of division while the majority of macroschizonts were observed in multiply infected host cells, which implied that macroschizont cell division had not been inhibited. It was possible that this form of multinucleated macroschizont, and the forms generated with 8-azaguanine and 5-azacytidine, represented macroschizonts in the process of differentiating into merozoites however further differentiation into merozoites was not observed.

At drug concentrations which were lower than those listed above, host cell division occurred at the same rate as in untreated control cultures. There was no evidence of a specific effect on macroschizonts, as normal nuclear and cell division appeared uninhibited.

6.1.2. Elevated culture temperature

6.1.2.1. Heat treatment of TaH48

Macroschizont infected cell cultures (TaH48) at 5x10⁶ cells/ml were incubated at 41°C for two weeks with thrice weekly passage 1:10 in complete Ta medium. Giemsa stained samples were prepared and examined at intervals of 2-3 days. For the first 7 days at 41°C, Giemsa stained macroschizonts were indistinguishable from macroschizonts cultured at 37°C. Thereafter, a small proportion of atypical macroschizonts (approximately 2-3%) were observed. These macroschizonts were enlarged, containing large numbers (20-45) of nuclei and occurred in two forms: 50% of these macroschizonts had apparently normal nuclei and in the remainder, nuclei were very heterogeneous in size. This small proportion of atypical macroschizonts did not increase over the experimental period, and these forms did not develop any further. The first form (with normal nuclei) were similar to the macroschizonts observed after aminopterin and colchicine treatment (6.1.1.) and it appeared that the rate of macroschizont nuclear division had
exceeded the rate of macroschizont cell division. The morphology of the second form (with heterogeneous nuclei) was similar to that observed after 8-azaguanine and 5-azacytidine treatment. This conformed to the expected sequence of initial events in merogony (Meihorn & Schein, 1984) thus the larger nuclei may have been producing the smaller nuclei which were presumptive merozoite nuclei.

6.1.2.2. Heat treatment of TaA₁

The cell line (TaH₄B) which had been used in the previous experiment and in the drug treatment experiment (6.1.1.) was one which had been maintained in culture for an extended period of time. This period was much longer than the culture life of the cell lines which Hulliger et al. (1966) has used for their experiments where merozoites had been formed.

A low passage number, Ankara macroschizont infected cell line (TaA₁) was derived and maintained at 37°C as described in section 2.1.5. To heat treat cells, cultures were incubated at 41°C and the cell density was maintained at 1-5×10⁶ cells/ml. For the first 5-9 days exponential growth was maintained by daily dilution 1:10 in fresh complete Ta medium. Thereafter the rate of host cell division decreased and cultures were diluted daily (1:5) in the same medium. After approximately 10-15 days the majority of host cells ceased to divide and medium was then provided by pelleting the cells by centrifugation at 2,000g for 10 minutes followed by resuspension to the original volume with fresh medium. Alterations in the morphology of cultured cells were monitored daily by light microscopic examination of Giemsa stained cytocentrifuge preparations (section 2.1.2.).

In this way it was possible to place morphological alternations in a chronological sequence. For ease of presentation, observations on the changes taking place in the differentiation from macroschizonts to merozoites are described separately (a) from observations on the infected cultures as a whole (b). The morphological alterations which were detected in the 41°C cultures were not observed in control 37°C cultures. Macroschizonts incubated at 37°C contained between 13 and 20
nuclei and macroschizont cell division occurred at approximately the same rate as host cell division such that most host cells contained only one macroschizont. Figure 6.1.2.2. a) shows a host leucocyte (HL) in the final stages of division with two macroschizonts (MS) positioned either side of the nucleus (HN).

a) Observations on heat treated macroschizonts

Following the elevation in culture temperature the number of nuclei per macroschizont increased to between 30 and 150 (figure 6.1.2.2. b and c). These smaller macroschizonts with 30 nuclei (b) were precursors of the larger macroschizonts with many more nuclei (c) but it appeared that merozoites were subsequently produced from both forms. It was possible that the small macroschizonts which were subsequently observed undergoing merogony were actually produced from enlarged macroschizonts containing hundreds of nuclei.

Individual extracellular merozoites were observed after approximately 9 days of culture. Giemsa stained slide preparations showed merozoites as pale blue circles of cytoplasm, 1-2μm in diameter, containing dark purple, spherical nuclei with a diameter of approximately 0.5-1μm (see figure 6.1.2.2. c, d, and e, arrowed as MER in d). It was possible to trace the production of free merozoites from two morphological types of macroschizont, which were observed in slide preparations either inside or outwith host cells. In enlarged or normal sized macroschizonts, clusters of intensely stained nuclei were observed (arrowed as AG in figure 6.1.2.2. c) and d) and these were often surrounded by merozoite nuclei. Merozoite nuclei were small (0.6-1μm), spherical and were stained dark purple with Giemsa stain. It appeared that merozoite nuclei were generated from these clusters and possibly merozoites formed, within the parent macroschizont. It was not possible to determine whether the intracellular merozoite nuclei were actually fully formed merozoites (i.e. defined here by the observation of a nucleus and surrounding cytoplasm by light microscopy) or whether merozoite formation took place at a later stage. Data from subsequent IFAs with monoclonal antibodies or bovine immune sera suggested that fully formed heat-induced merozoites were present at
Light micrographs of \textit{Giemsa} stained cytocentrifuge preparations of heat treated macroschizonts showing developmental changes over 16 days of heat treatment.

The macroschizont infected cell line, TaA1, was incubated at 41\textdegree C as described in section 6.1.2.2.

Photograph a, shows an infected leucocyte (HL) in the process of dividing on day 0 of treatment. Two macroschizonts (HS) and the host cell nucleus (HN) are apparent. Photographs b & c, illustrate changes in macroschizont morphology apparent by days 6-12 of heat treatment (see also section 6.1.2.2. a). Macroschizonts (HS) are enlarged and contain supernumerary nuclei. In enlarged and normal sized macroschizonts, clusters of intensely stained nuclei are observed (see photographs c & d, labelled AG). Individual extracellular merozoites were observed after approximately 9 days in culture, and one of these is labelled in photograph d (HER). Merozoites were also observed, apparently attached to the surface of enlarged and normal sized macroschizonts containing 2-300 merozoite nuclei. These are labelled (BM) in photographs d & e.
Figure 6.1.2.2.

(a) MS

(b) MS

(c) MS

(d) MS

(e) MS

HN

HL

AG

BM

MER

day 0

day 6

days 12–16

10μm
this stage.

The second pathway of merogony did not involve clusters of nuclei. Instead, enlarged and normal sized macroschizonts were observed containing from 2-300 putative merozoite nuclei with merozoites apparently attached to the outside of the macroschizont membrane (figure 6.1.2.2. d) and e) (BM)) suggesting that merozoites were budded off from the surface of the macroschizont membrane. The extent to which merogony took place within viable host cells was difficult to assess from observations of Giemsa stained slides. Generally, macroschizonts undergoing merogony were seen in the vicinity of fragmented host cells, but it was not known whether the final disintegration of the host cell membrane was the result of slide preparation or merogony.

b) Observations on heat treated cultures

The experiment was repeated several times over a period of four months, and merozoites were produced on each occasion. The TaA1 cell line was maintained at 37°C between induction experiments. The final yield of merozoites after 10-15 days of heat induction varied and was dependent upon the culturing regime. For the first 5-7 days at elevated temperatures, host cells continued to grow exponentially if they were passaged daily 1:10 with fresh medium. The first observation of macroschizonts containing densely stained putative merozoite nuclei and the first free merozoites were observed in very small numbers after 4-9 days at elevated temperatures. This appeared to be the length of time that an infected cell had to be exposed to elevated temperatures (41°C) before merozoite production took place. These forms were committed to merogony which proceeded even if the cultures were then incubated at 37°C. Also in exponentially growing cultures, an increasing proportion of host cells were observed containing more than one macroschizont. Therefore at this early stage, host cell doubling times were not diminished, but macroschizont cell division was taking place at a faster rate than host cell division. If host cells were not initially maintained as exponentially growing cultures by daily dilution 1:10 in fresh medium, the typical small
proportion of multinucleated macroschizonts, which subsequently developed into merozoites, was produced after 4-9 days. However for reasons which were not apparent the final proportion of macroschizonts observed differentiating into merozoites after 10-15 days was always much lower, that is 10% rather than 60-70%.

The next phase in culture, between days 7 and 15, occurred when the rate of host cell growth was diminished and cells were passaged daily 1:5. Host cells containing more than one macroschizont were observed in the process of division but cells containing enlarged macroschizonts with supernumerary nuclei were never observed undergoing nuclear or cell division.

The final phase in culture occurred when the majority of host cells had ceased to divide and fresh medium was provided without diluting cells, by replacement. Host cells were vacuolated, crenellated and were frequently fragmented in slide preparation. They harboured macroschizonts in various stages of merogony and culture supernatants were found to contain many free merozoites. At this stage the majority of macroschizonts went on to produce merozoites over a 48 hour period. The final culturing regime effectively inhibited the exponential growth of the small proportion of normal macroschizont infected cells. The cycle of merozoite production could then be re-initiated by resuspending the remaining host cells containing normal macroschizonts in fresh medium and culturing at 41°C for 10-15 days as described.

6.1.2.3. Heat treatment of TaA1 after four months in culture

After approximately four months in culture at 37°C the cell line TaA1 failed to produce merozoites at 41°C. Morphological alterations, which conformed to the expected, initial sequence of events in merogony were detected. Macroschizonts were observed with large numbers of normal nuclei and others with heterogeneous nuclei. A different low passage cell line, TaA2, was removed from liquid nitrogen and once exponential growth at 37°C was sustained, the cell line was heat treated at 41°C. This line produced merozoites after 8 days at elevated culture temperatures and large numbers of merozoites were produced after 15-20 days. The
experiment was repeated and the loss of ability to produce merozoites, over time in culture, was found to be a reproducible result. These results demonstrated that ability to produce merozoites could be diminished or lost after prolonged culture periods at 37°C.

6.1.3. Merozoite invasion assay

Preliminary experiments were carried out to determine whether heat induced merozoites would invade red blood cells in vitro. Merozoites were induced from macroschizont infected cells (TaA1) by cultivation at 41°C for ten days as described above (6.1.2.2.). Giemsa stained slide material showed that a full cross-section of developmental forms, from macroschizont to merozoite, was present in the cultures after this period: 40% of parasites were normal macroschizonts and the remainder were asynchronously undergoing merogony. Defibrinated bovine blood at a final cell density of $10^8$/ml in complete Ta medium was added to these merozoite cultures which were then maintained at 37°C and 41°C, and to control 37°C macroschizont cultures which were maintained at 37°C. The cultures were incubated for five days with daily medium changes and Giemsa stained red blood cell smears (five for each sample) were prepared after six hours and daily thereafter. As complement has been shown to be involved in the invasion of red cells by Babesia rodhaini (Ward & Jack, 1981) the experiment was performed both in the presence of foetal calf serum which was heat inactivated (and therefore complement free), and with normal foetal calf serum. The culture depth was 6mm and after four-five days, the settled layer of red cells darkened as haemoglobin became deoxygenated. After three days, the cultures at 41°C, but not those at lower incubation temperatures, showed signs of haemolysis.

None of the samples provided convincing evidence that merozoites had entered red cells. However, extracellular merozoites were observed positioned closely to red cells, which was suggestive of some form of specific attachment. It was possible that this apparent attachment was an artefact of the slide preparation. Suspensions of the cultures were stained with
Acridine Orange (0.1μg/ml) and observed under the x100 objective of a fluorescence microscope (Leitz filter block 12/3). Extracellular merozoites were observed apparently attached to red cells which confirmed the previous result, but no intra-erythrocytic fluorescence was observed, which would have been indicative of the presence of piroplasms.

6.2. IMMUNOELECTRON MICROSCOPY

As described in section 5.2.2., four anti-piroplasm monoclonal antibodies had been generated which recognised formaldehyde fixed, heat induced merozoite preparations by IFA (see section 6.4.1. for slide preparation). Antibodies 2D5 and 1D11 appeared to recognise internal epitopes, 5E1 recognised the outer perimeter of merozoites and 1C2 combined both reactivity patterns. In order to determine whether antibody 5E1 recognised the surface of merozoites, surface IFAs were performed (2.3.2.) however, this experiment was inconclusive as it was clear that control antibodies had access to internal epitopes. The results of an immunoelectron microscopy study, with antibody 5E1 and heat induced merozoites are presented here. This study had a dual purpose: firstly, to determine whether antibody 5E1 recognised the surface of heat induced merozoite and secondly, to determine whether heat induced merozoites had the expected ultrastructural features of normal macroschizont derived merozoites.

Heat induced merozoites were generated using the protocol described in section 6.1.2.2. and then prepared for pre-embedding immunoelectron microscopy as described in section 2.9. Fixed merozoites were incubated with an antibody fraction of antibody 5E1 supernatant which had been dialysed against blocking buffer (PBS supplemented with 0.2% gelatin). Control merozoites were incubated with an irrelevant anti-Trypanosoma brucei antibody as diluted (1:500) ascitic fluid in blocking buffer.

Figure 6.2.1. illustrates the resulting images after processing of heat induced merozoites with antibody 5E1 and protein A colloidal gold. Two merozoites are depicted, the lower one is apparently damaged while the upper merozoite (m) seems
Figure 6.2.1.

Immunoelectron micrograph of heat induced merozoites.

Scale bar = 200 nm
Figure 6.2.1.

key:

n  nucleus
r  rhoptry
m  heat induced merozoite
intact. These stages were spherical, approximately 0.7μm in diameter and the two most prominent structures were a large nucleus (n) and two rhoptry forms (r). The observation of rhoptry forms within these stages demonstrated that they possessed the main ultrastructural characteristic of normal merozoites. The merozoites were bound by a unit membrane and membranous bodies or swirls of membrane were observed, possibly within the merozoite. The protein A gold particles clearly labelled the outside of the merozoite, while the control merozoites, which had been incubated with an irrelevant antibody, were not labelled (data not shown). Thus it appears that antibody 5E1 specifically recognises the surface of heat induced merozoites which possess the ultrastructural characteristics of in vivo theilerial merozoites.

6.3. GENE CLONING

In this section the results of experiments to isolate and confirm the identity of the gene which codes for the epitope recognised by monoclonal antibody 5E1 are presented.

6.3.1. Library screening

Three monoclonal antibodies (5E1, 2D5, 1D11) were pooled and used to screen 300,000 plaque forming units of an unamplified, T. annulata (Ankara) lambda gt11 genomic library. The screening protocols and details of the library construction are described in sections 2.8.1. to 2.8.3. The reactivity of the individual monoclonal antibodies against piroplasms was checked, by IFA, prior to their use. Initial immuno-screening results showed that four plaques had reacted with the antibody mixture. These positive plaques were picked and the phage were re-screened with the antibody mixture. Two of the original clones produced 10% of plaques which were recognised by the antibody mixture, while control experiments with second antibody alone failed to detect any plaques. These two phage clones were isolated to homogeneity by five rounds of plaque purification as described in section 2.8.2. When the phage were approximately 20% pure an experiment was
Figure 6.3.1.

Results of immunoscreens of *T. annulata* lambda gt11 expression library with three pooled monoclonal antibodies which recognise epitopes which are expressed in merozoite and piroplasm life cycle stages.

Figure 6.3.1. a

Experiment to determine which of three pooled monoclonal antibodies recognises an epitope produced by clone PSI.

Isolate PSI, which was isolated using pooled monoclonal antibodies, and which was found to be 20% pure, was plated out and expressed protein was transferred to nitrocellulose filters (section 2.10.3.). The filter was cut up and individual segments were probed with individual monoclonal antibodies (5E1, 2D5 or 1D11). A positive reaction was only detected with antibody 5E1 and this segment is labelled 5E1.

Figure 6.3.1. b

Isolate PS2, which like PS1, was only recognised by antibody 5E1, was isolated to homogeneity. The large filter shows the positive reaction of antibody 5E1 on PS1 plaques and demonstrated that the isolate was 100% pure. The smaller filter shows the reaction of second antibody alone on the same cloned isolate.
Figure 6.3.1.

McAbs 5E1, 2D5 & 1D11

4th rescreen

McAb 5E1

5th screen

2nd antibody control
performed to determine which of the pooled monoclonal antibodies were reacting with the plaques. Impure phage were re-plated and re-screened with individual monoclonal antibodies and it was determined that antibody 5E1 recognised both phage clones (PS1 and PS2) while antibodies 1D11 and 2D5 failed to react. Figure 6.3.1. shows the results of two screens: a) shows the fourth re-screen where PS1, which was 20% pure as judged by its reactivity with the pooled antibody mixture, was rescreened with individual monoclonal antibodies. Here one of the three filters which was probed with 5E1 (labelled 5E1) shows a positive reaction on 20% of plaques while the other filters were negative with 2D5 and 1D11. Figure 6.3.1. b) illustrates the reactivity of monoclonal antibody 5E1 on PS2; all the plaques were positive which demonstrated that the phage had been successfully cloned. In this way two recombinant phage clones were isolated which appeared to express the *T. annulata* epitope recognised by antibody 5E1.

6.3.2. Detection of inserts

The presence and size of DNA inserts in the cloned phage were determined by isolating DNA and resolving EcoRI digests electrophoretically in 0.8% agarose gels as described in section 2.8.3. Both clones showed inserts giving fragments of 1.6kb and 3kb as shown in figure 6.3.2. Track a) shows the resolved kb ladder and track b) the two stained restriction fragments; uncut DNA at the origin and vector arms also formed stained bands. The presence of two recombinant fragments suggested that the original insert contained an internal EcoRI site. On this basis it appeared (surprisingly, since the library was made from randomly sheared DNA) that the two recombinant clones (PS1 and PS2) were identical.

6.3.3. Hybridisation of insert to *Theileria* DNA

In order to determine whether the cloned inserts were derived from the *T. annulata* or the bovine genome, the two recombinant fragments were radiolabelled and used to probe a Southern blot of isolated piroplasm genomic DNA and bovine fibroblast DNA, each of
Figure 6.3.2.

Preparation of cloned DNA from clone PS1.

DNA was extracted from phage clone PS1 by the Alloa method (section 2.10.4.), digested with EcoR1 and separated in a 0.8% agarose gel. DNA fragments were visualised by ethidium bromide staining and exposure to long wave ultraviolet light. Track a shows a 1-kb ladder and track b shows 1.6kb and 3kb insert fragments together with bands of uncut DNA at the origin and vector arms.

Figure 6.3.3.1.

Hybridisation of recombinant DNA to uncloned *Theileria* DNA.

Southern blot of bovine fibroblast DNA (bovine) and *Theileria* genomic DNA (parasite) digested separately with EcoR1 (E), Bam H1 (B), or Hind III (H) (section 2.10.7), probed with radiolabelled cloned fragments from PS1 (figure 6.3.2.). The sizes of fragments are indicated on the right hand side of the figure.

Figure 6.3.3.2.

Hybridisation of recombinant DNA to cloned *Theileria* DNA

Southern blot of EcoR1 digests of *Theileria* genomic DNA isolated from a range of cloned macroschizont infected cell lines (clones 1-3), the parental line and from uncloned DNA isolated from piroplasms. Blots were probed with radiolabelled 3kb fragments from clone PS1 (section 2.10.7.). The sizes (in kb) of DNA fragments are indicated on the left hand side of the figure.
which had separately been digested with Eco R1, Bam H1 or Hind III (see section 2.10.7). The resulting autoradiograph (figure 6.3.3.1.) shows a strong hybridisation with T. annulata DNA and no hybridisation with bovine fibroblast DNA, from which it was inferred that the cloned insert was T. annulata DNA. At least 3 major bands (larger than 4kb) were detected in the Eco R1 digested piroplasm DNA which suggested that the cloned sequence might detect more than one gene. As the Southern blot had been performed with uncloned DNA the experiment was repeated using the 3kb probe on Eco R1 digests of DNA isolated from a range of cloned (by limiting dilution) macroschizont infected cell lines. The resulting autoradiograph (figure 6.3.3.2.) showed that two of the bands segregated, and it was inferred that the T. annulata DNA comprised a mixture of genomes. However the cloned insert hybridised to two bands in each clone which suggests that the sequence may detect two genes within the T. annulata genome.

6.3.4. Lysogens in lambda gt11

Lysogens were prepared for Western analysis in order to determine the molecular mass of the fusion protein detected by antibody 5E1. As the cloned insert was 4.6kb, the maximum expected size of the beta-galactosidase fusion polypeptide was approximately 254kDa. Lambda lysogens were prepared in E. coli Y1089 using the protocol described in section 2.8.4. SDS-sample buffer extracts of induced recombinant lysogens were resolved electrophoretically in 7.5% or 4.5-15% acrylamide gels, Western blotted and screened with monoclonal antibody 5E1 as described in section 2.8.6. Negative controls were included in the screen as follows: an extract of an induced wild type lambda lysogen, an irrelevant induced recombinant lysogen extract and an extract of host E. coli Y1089. As a positive control, antibody 5E1 was screened against extracts of Ankara piroplasms in the same blot. In addition, a duplicate gel was stained for protein with Coomassie blue.

Monoclonal antibody 5E1 failed to recognise the negative control polypeptides. Figure 6.3.4. b) illustrates the reactivity of monoclonal antibody 5E1 on the wild type lysogen (WTL) and the irrelevant lysogen (Lr). Figure 6.3.4. c)
Figure 6.3.4.

Western analysis of the reactivity of monoclonal antibody 5E1 against induced lambda lysogens of clone PS1.

Figure 6.3.4. a

Time course experiment to determine optimum IPTG-induction time.

Western blot of two lambda lysogens (La and Lb) induced with IPTG over a time course of 20, 30, 60, 90, and 120 minutes (section 2.10.6.), probed with monoclonal antibody 5E1. The molecular mass of polypeptides are indicated on the right hand side of the figure.

Figure 6.3.4. b

Western blot illustrating the reactivity of monoclonal antibody 5E1 against the following extracts; Lr = an irrelevant control induced lambda lysogen extract; WTL = a wild type induced lysogen; La = induced lysogen from recombinant clone PS1; Lb = a second induced recombinant lysogen from clone PS2; piro = Ankara piroplasm extract. The molecular mass of polypeptides are indicated on the right hand side of the figure.

Figure 6.3.4. c

A very similar experiment to Western blot shown in fig. 6.3.4. b, above but with the additional inclusion of an extract of the host bacteria, Y1089 (track 089) as a further control.
Figure 6.3.4.

a

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b

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- 30
illustrates a non-specific reaction on Y1089 (Ø89) and the irrelevant induced recombinant lysogen (Lr). Antibody 5E1 recognised 30kDa and 20kDa polypeptides in one piroplasm extract (figure 6.3.4. b, piro) and 30kDa and 120kDa polypeptides in a second piroplasm extract (figure 6.3.4. c) piro). The reactivity of monoclonal antibody 5E1 on two induced lambda lysogens (La and Lb) prepared from the recombinant clones PS1 and PS2 is shown in figure 6.3.4. a) and b). The antibody reacted weakly on both extracts and several molecules were specifically recognised. The Coomassie Blue stained profile of polypeptides suggested that a predominant fusion protein had not been induced.

The time course of the induction of fusion protein was determined between 20 minutes and 120 minutes after addition of IPTG at 42°C. The first polypeptides were detected by antibody 5E1 in extracts prepared 60 minutes after induction, and the profile of detected polypeptides was constant thereafter (figure 6.3.4. a, La and Lb). Antibody 5E1 recognised polypeptides of molecular mass 138, 126, 124, 107 and 101kDa in extracts of lysogen La while polypeptides of 107kDa and 101kDa were detected in lysogen Lb.

6.4. STAGE SPECIFIC EXPRESSION

6.4.1. Immunofluorescence assay - induction time course

As described in section (5.2.2.), monoclonal antibody 5E1 reacted in IFAs against formalin fixed, heat induced merozoite preparations as well as acetone fixed piroplasm preparations. By Western blot analysis (5.5.2.1.) the epitope recognised by antibody 5E1 was shown to reside on the same sized polypeptides (30kDa and 120kDa) in piroplasm and merozoite extracts with the exception that 20kDa and 15kDa polypeptides, recognised in piroplasm extracts, were not detected in merozoite extracts. It appeared that the polypeptides were stable between merozoite and piroplasm stages but it was possible that the 120kDa was a precursor of the 30kDa polypeptide or that the two molecules were separate gene products with cross-reacting determinants.

A preliminary time course experiment was carried out in
order to determine at what point, during the process of heat-induced merogony, the epitope recognised by 5E1 was synthesised. As merogony took place asynchronously in heat induced cultures this experiment was approached simply by assaying for the presence of the epitope over a two week period of induction by indirect immunofluorescent assay in conjunction with parallel data from Giemsa stained slide material.

A low passage, Ankara infected cell line (TaA1) was cultured for 15 days at elevated temperatures to induce merogony as described in section 6.1.2.2. The uninfected lymphosarcoma cell line (BL-20) and its Hissar infected equivalent (TaH-BL20), both of which were cell lines with high passage numbers, were heat treated in the same way. Giemsa stained and IFA slide material were prepared: prior to elevating culture temperatures, 2 hours after transfer to 41°C and 1, 3, 6, 9, 12 and 15 days thereafter. For the first 9 days, slides were prepared for IFA analysis according to the method for leucocytes described in section 2.3.1.1.

On day 12 when many merozoites were free in the culture medium (as assessed from Giemsa stained slides) the IFA slide preparation protocol was altered by performing centrifugation steps at 3000g so that merozoites would be pelleted. On day 15 when many host cells were fragmented and free merozoites were abundant, merozoites were isolated from heat induced cultures for slide preparation by differential centrifugation. Induced cultures were centrifuged at 300g for 10 minutes to remove the bulk of intact leucocytes. The supernatant was centrifuged at 4000g for 15 minutes at 4°C and the resulting pellet was resuspended in 1.5ml PBSp. The merozoites were washed 4x in PBSp by centrifugation at 9000g for 1 minute and resuspension in 1.5ml PBSp. After the final wash, a sample of the resuspended pellet was removed and examined after Acridine Orange staining and found to contain intact isolated merozoites and a small amount of cell debris. The merozoites were formaldehyde fixed and applied to multispot slides (10 slides/10ml original culture) according to the method described in section 2.3.1.1. for leucocytes except that the pellet of merozoites (1-2µl from a 10ml culture) was resuspended in 0.5ml PBS. Indirect immunofluorescent assays were performed as described.
in section 2.3.1.2.

Each of the prepared IFA slides (days 0-15) was screened with the following monoclonal antibodies which had previously been characterised in terms of their reactivity against sporozoites, macroschizonts, merozoites and piroplasms (section 5.2.): 5E1, 2D5, 1C2, 5A10 and 1C7. As further positive and negative controls the following slide material was also included in the screen: acetone fixed slide preparations of piroplasms and formalin fixed slides of macroschizont infected cells prepared from TaH4a cells.

Examination of Giemsa stained slide preparations of TaA1 cells from the time course showed that the expected sequence of changes, culminating in the production of merozoites had taken place as described in section 6.1.2.2. The uninfected cell line BL-20 showed no changes in morphology during the culture period at 41°C and no alteration in growth rate was detected. Infected BL-20 cells (TaH-BL20) behaved like the TaH4a cell line as described in section 6.1.2.1. and did not produce merozoites. Briefly, on day 6 a small proportion of atypical macroschizonts were detected for the first time. These macroschizonts contained supernumerary nuclei, half these macroschizonts had apparently normal nuclei and the remainder contained heterogeneously sized nuclei. The formation of nuclei in the latter macroschizont type conformed to changes observed during the initial stages of heat induced merogony in macroschizonts which subsequently proceeded to produce merozoites: however, merozoites were not observed.

The main IFA reactions are illustrated in figure 6.4.1: piroplasm = infected red blood cells, schizont = macroschizont infected lymphoblastoid cells (TaA1), merozoite P1 = heat induced merozoites from macroschizont cultures (TaA1) from day 12 of heat induction and merozoite P2 = a crude merozoite preparation from heat induced cultures (day15).

Monoclonal antibodies 5E1, 1C2, 2D5 and 5A10 recognised piroplasms while the negative control (anti-macroschizont) antibody 1C7 failed to react. All the antibodies failed to recognise BL-20 time course slide preparations. Antibody 1C7 recognised normal and enlarged macroschizonts in the TaH-BL20 series, antibody 5A10 failed to react and antibodies 2D5, 1C2, 5E1 specifically recognised small specks in the day 15 preparations of this series.
Figure 6.4.1.

Photomicrographs of IFA results showing the stage specific reactivity of monoclonal antibodies 5A10, 2D5, 1C2, and 5E1 and illustrating their reactivity against merozoite preparations.

The following slide preparations were tested by IFA (section 2.3.); - piroplasm = acetone fixed piroplasm infected red cells, - schizont = macroschizont infected lymphoblastoid cells, - merozoite P1 = heat induced merozoites from macroschizont cultures prepared on day 12 of induction, - merozoite P2 = crude merozoite preparation, by differential centrifugation, taken on day 15 of heat induction (see section 2.1.9.). Monoclonal antibody 1C7 was used as a negative control antibody on piroplasm preparations and as a positive control antibody on macroschizont preparations. Slides were observed and photographed with a x50 objective. Magnification = x 625.
which may have been merozoites but no reaction was detected prior to this. The proportion of differentiating macroschizonts which showed this minor speckled fluorescent reactivity was low.

Antibody 5E1 recognised differentiating macroschizonts and merozoites in the heat treated TaA1 series, beginning on day 9 with a diffuse fluorescent pattern over the macroschizont. Antibodies 2D5, 1C2 started reacting on day 12, recognising small specks or dots thought to be presumptive merozoites. Antibody 5A10 failed to react on the TaA1 time course slides and antibody 1C7 recognised normal and enlarged macroschizonts but did not appear to recognise merozoites.

6.4.2. Western blot analysis

Antibody 5E1 was probed against Western blots of the following T. a. Ankara stage extracts: sporozoites, macroschizont infected leucocytes (cell line TaA1), merozoites and piroplasms. In addition, antibody 5E1 was screened against uninfected lymphoblastoid cells (BL-20) and its infected equivalent (TaH-BL20). The stage common antibody, antibody 2E4, was used as a positive control. As shown in figure 6.4.2., antibody 2E4 recognised a 38kDa polypeptide in sporozoite, merozoite and macroschizont infected cell line extracts, but failed to recognise uninfected BL-20 extracts. Antibody 5E1 recognised a 30kDa polypeptide in piroplasm and merozoite preparations; in the piroplasm extract, additional 20kDa and 10kDa polypeptides were recognised. Sporozoite extracts and uninfected or infected cell line extracts failed to react with antibody 5E1. The results of this experiment consolidated the IFA data which suggest that the epitope determining monoclonal antibody 5E1 is stage specific and appears to be restricted to piroplasm and merozoite life cycle stages in the bovid.
Figure 6.4.2.

Western blot analysis of the reactivity of monoclonal antibody 5E1 (fig. 6.4.2. a), and monoclonal antibody 2E4 (fig. 6.4.2. b) on the following protein extracts:-

a = sporozoites
b = BL-20, uninfected lymphoblastoid cells
c = TaH BL-20, macroschizont infected BL-20
d = TaA1, macroschizont infected leucocyte cells
e = uninfected red cell
f = merozoites
g = piroplasms

The molecular mass and mobility of internal molecular weight standards are illustrated on the right hand side of the figure.

Figure 6.4.3.

Slot-blot Northern hybridisation of 3kb recombinant fragment (5B1) isolated from a genomic library with monoclonal antibody 5E1, to total RNA isolated from piroplasms, merozoites and macroschizonts (section 2.10.8.). A control probe (4A4) was included to assess any RNA loading differences between the three extracts.
6.4.3. RNA analysis

In order to determine whether the cloned fragment shows stage specific expression at the RNA level, slot-blot Northern analyses were undertaken (2.10.8). The two recombinant fragments (3kb and 1.6kb) were isolated (2.10.4.), radiolabelled separately and used to probe slot-blot Northern preparations of total RNA from the following uncloned life cycle stages: purified piroplasms (2.1.4.), heat induced merozoite preparations (6.4.1.) and macroschizont infected cell lines (2.17.5). A control probe (4A4) was used to monitor loading differences between Theileria RNA species from the different stages. The 1.6kb probe failed to hybridise to any of the RNA samples. The 3kb fragment probe hybridised strongly to piroplasm RNA, hybridised to merozoite RNA and there was a very weak hybridisation to macroschizont RNA preparations. The results with the 3kb probe are shown in figure 6.4.3. The fairly uniform hybridisation of the 4A4 probe suggested that the RNA loading had been approximately equal between samples from which it was inferred that the RNA species recognised by the 3kb probe showed elevated levels of expression in piroplasm and merozoite life cycle stages relative to the macroschizont stage.
The results of this study suggest that *T. annulata* merozoites can be reproducibly generated from macroschizonts *in vitro* at elevated temperatures. *T. annulata*, therefore, behaves like *T. parva* in this respect (Hulliger et al. 1966). Heat induction of *T. annulata* merozoites has also recently been demonstrated by Fritsch et al. (1988), and they reported that heat induced merozoites are ultrastructurally similar to merozoites from infected bovids. The merozoites generated in this study possessed rhoptry forms, as judged from electron micrographs (figure 6.3.1.), which are the major ultrastructural characteristic of merozoites. This provides evidence, at least at a morphological level, that these induced forms are merozoites.

The induced merozoites are specified as culture heat-induced merozoites, to formally distinguish them from merozoites produced from macroschizonts *in vivo*. One measure of similarity between culture heat induced merozoites and *in vivo* merozoites would be provided if culture forms showed the behavioural characteristic of *in vivo* merozoites and invaded erythrocytes. An erythrocyte invasion assay was performed (6.1.3.), which essentially involved the conditions described by Levy & Ristic (1983) for the microaerophilous stationary phase (MASP) culture of *Babesia bovis*. Two, possibly important, differences between the assay conditions used, and standard MASP culture conditions were as follows: firstly that foetal calf serum (20%) rather than adult bovine serum (40%) was used, and secondly, the base medium was RPMI-1640 rather than Medium 199 in Hank's balanced salt solution which, unlike the latter, does not contain purine bases and nucleosides. Invasion was not demonstrated but a small proportion of red cells were observed with merozoites apparently attached to their surface. It was estimated that approximately 3x10^7 merozoites would be produced over a three days incubation period. If all these merozoites invaded red blood cells, the resultant parasitaemia of 3% would have been readily detected. A more realistic invasion efficiency might be 1%, producing parasitaemias of 0.03%. This was approximately the percentage of red cells which were observed with single, attached merozoites after three days.
Therefore, although the assay may have been too insensitive to detect intraerythrocytic merozoites or piroplasms, because of the large numbers of red blood cells which were added, the results suggest that merozoites did not enter red blood cells. Given the lack of positive controls for this invasion assay, it was not inferred that the merozoites were not viable. Instead, it is hoped that technical reasons will explain the results.

Subsequently a monoclonal antibody (5A10) has been characterised which appears to recognise piroplasms, but not merozoites or macroschizont infected leucocytes (figure 6.4.1.). This reactivity profile could be useful in assays of merozoite invasion, as the piroplasm epitope determining antibody 5A10 may be synthesised, for the first time in the life cycle, after the entry of merozoites into red cells.

An immediate parallel may be drawn between the heat induction of merozoites, and sporogony in tick salivary glands, which is inducible by elevating temperatures to 37°C (Samish, 1977). Sporogony appears to be a similar process to merogony, with nuclear division followed by the formation of merozoite anlagen and the compartmentalised production of uninucleate daughter stages. A similar reproductive process, therefore, involves the same stimulus of elevated temperature (although at 37°C not 41°C) but goes on to produce antigenically distinct stages representing different stages in the life cycle. It would be interesting to determine whether the merogonous division of piroplasms within red blood cells, described by Conrad et al. (1985), could also be placed under temperature control.

The use of predictable environmental changes (for example, seasonal changes) as developmental triggers is recognised in many biological systems. So too is the synthesis of a set of highly conserved heat shock proteins (hsp70), in a survival oriented response to environmental stress. Much speculation surrounds the precise function and role of these proteins, but as reviewed by Pelham (1988), a function of one of these heat shock proteins (hsp70) has been recently demonstrated. Chirico et al. (1988) and Deshaies et al. (1988) have demonstrated that hsp70 from yeast acts as a cytostolic factor, involved in the translocation of proteins across membranes. In a classical heat shock response, hsp
genes are rapidly expressed and there is a concomitant down regulation of normal synthetic activity (Lindquist, 1986). In addition to this rapid response, constitutive expression of hsps also occurs and some hsp genes have been shown to be developmentally regulated (Kurtz et al. 1986; Hensold & Housman, 1988). Regarding parasite systems, the potential relationship between environmental fluctuations, the production of hsps and differentiation events is poorly defined. As reviewed by Newport et al. (1988), constitutive and heat inducible synthesis of hsps have been reported in several protozoa, for example Leishmania (van Der Ploeg et al. 1985) and Trypanosoma (Glass et al. 1987), and also trematodes i.e. Schistosoma (Blanton et al. 1987) and nematodes i.e. Brugia (Selkirk et al. 1987). It is suggested that elevated levels of hsps may act as triggers for the initiation of differentiation events, or that they play a role in the mechanisms of differentiation. However, there is no evidence for the direct involvement of hsps in differentiation. The gene coding for hsp70 in T. annulata has recently been isolated and characterised (Mason et al. 1989). The gene seems to be constitutively expressed in sporozoites, macroschizonts and piroplasms, and it is heat inducible.

In the successful heat induction experiments (6.1.2.2.), which were carried out at 41°C, observations suggested indirectly that a commitment to merogony occurred before the cessation in host cell division. The precise timing is impossible to assess without defined markers for merogony, and this interpretation conflicted with the results of heat induction experiments reported by Hulliger et al. (1966) where host cells stopped dividing as soon as they were placed at elevated temperatures. In the report of Hulliger, the temperature of induction had been between 40.2°C-42.4°C, and it is possible that this accounts for the discrepancy as it was noted that the TaA1 cell line stopped growing almost immediately after transfer to 42°C.

It has been postulated (6.1.) that by inhibiting host division, macroschizont division might be inhibited and merogony induced. At 41°C, cell lines which did not proceed to produce merozoites (6.1.2.3.), continued to divide. Therefore it is possible that the reduced growth rate, which was observed in
cultures which proceeded to produce merozoites, was a consequence of merogony not of elevated temperatures. As the cell lines were uncloned, however, it is not known whether the inducible and non-inducible cell lines are comparable in this way. For example, host cells from non-inducible cultures may be less able to mount a response to elevated temperatures involving the cessation of growth. Hulliger et al. (1966) showed that culture conditions of deficient medium, lowered temperature and reduced partial oxygen pressures resulted in reduced host cell growth rate but did not result in the production of merozoites. It is not known whether this happens in *T. annulata*, but it may be that elevated temperature is a specific trigger rather than one of several environmental changes which might stimulate merogony in, for example, a more general stress response. This form of data is not conclusive, it is possible for example that the alternative induction conditions tested constituted a stress for the host cell, but not for the intracellular macroschizont: that a threshold must be passed before the macroschizont recognises stressful stimuli by differentiating into merozoites. It is envisaged that if specific drug treatments and bovine lymph also induce merozoite formation, that these operate by circumventing the requirement for elevated temperatures and induce the same responses in the macroschizont which culminate in merogony. The problem of distinguishing between the part played by the host leucocyte and the macroschizont, in initiating merogony at elevated temperatures, might be addressed by generating temperature-sensitive mutants (see Pfefferkorn & Pfefferkorn, 1976; Inselburg, 1985). Clearly this is a complex question to address experimentally, and without defined, cloned
populations of host and macroschizont cells, interpretive problems build up.

It was observed that after prolonged periods in culture, cell lines lost the ability to produce merozoites (6.1.2.3.). The cell line, TaH48, which was cultured for an extended period (> 1 year) prior to use in the drug treatment (6.1.1.), and initial heat induction (6.1.2.1.) experiments, failed to generate merozoites. Two, low-passage cell lines produced merozoites in heat induction experiments which were carried out using a similar protocol to the heat induction experiment with TaH48. After four months in culture at 37°C, one of these cell lines (TaA1) could no longer be heat induced. It was not determined whether this change took place more rapidly at 41°C than at 37°C.

Merozoites were not generated by inhibiting host cell division with a range of drugs (6.1.1.), but the cell line which had been used (TaH48), was not heat-inducible, thus, a positive control for the drug treatment experiment was negative. On this basis, the results of the drug treatment experiment had to be re-evaluated. The experiments were not repeated with heat inducible cell lines. However, as macroschizonts, which were treated with 8-azaguanine, 5-azacytidine and emetine, showed what was possibly the early stages of merogony, it is speculated that further differentiation and full merogony might be observed with inducible cells. Interestingly, it has been reported that T. parva infected cells have reduced HGPRTase activity and that macroschizonts may have none (Irvin & Stagg, 1977). If 8-azaguanine (at 0.1µg/ml) has no direct effect on the macroschizont, then it may be inferred that the alterations, which were observed with the non-inducible cell line, were the result of alterations in the host cell.

An important technical point, is that a drug concentration was not found at which host cell division was inhibited, but cells did not fragment after three-four days. Consequently it was not possible to observe differentiation events after this time. A longer treatment period may be required before merozoites are observed, certainly Hulliger et al. (1984) reported that merozoite development occurred after six-nine days at elevated temperatures. Recently Fritsch et al. (1988) have reported the results of similar drug treatment experiments and their findings.
with colchicine were similar to those presented here, as macroschizonts were observed with supernumerary nuclei. They screened other drugs, not included in the experiment presented here, which appeared to have a similar (obioactin, TLA-144, Taxol) or no effect (sodium azide, dinitrophenol). Hulliger et al. (1964) drug treated T. parva macroschizont infected cell lines with colchicine and reported the appearance of macroschizonts with supernumerary nuclei.

The results from the drug treatment experiments (6.1.1.) may be summarized. Firstly, continued macroschizont nuclear division in the absence of host or macroschizont cell division (colchicine & aminopterin) did not result in the production of merozoites after three days. Secondly, 8-azaguanine and 5-azacytidine partially inhibited macroschizont nuclear division, and nuclei became very heterogeneous. Thirdly, where host division was inhibited but macroschizont cell division continued (emetine treatment), a small proportion of macroschizonts did not divide, the number of nuclei increased and nuclear division resulted in nuclei which were smaller than normal and the same size as merozoite nuclei. It was possible that two distinct events had been observed: a) the continued division of macroschizont nuclei in the absence of host and macroschizont cell division and b) the stimulus for a form of merogony, which was flawed, and did not result in the production of merozoites after three days. Thus there was no evidence to suggest that inhibition of host division resulted in the inhibition of macroschizont division and the consequent stimulation of merogony. It was equally probable that merogony repressed macroschizont division and consequently host division.

In the merozoite induction experiments, the observation of merozoites was the only accurate criterion available, for assessment of whether merogony had taken place. Any morphological alterations which took place in macroschizonts in cultures which did not proceed to produce heat induced merozoites, either by drug treatment or at elevated temperatures, may or may not have been preliminary stages of merogony. It may be worthwhile recounting that macroschizonts with supernumerary nuclei are occasionally observed in normal 37°C cultures if passage intervals
are increased. Similar forms were also detected in cell lines which had been recently removed from liquid nitrogen storage in medium containing dimethylsulphoxide. Work with *T. parva* suggests that macroschizonts with supernumerary nuclei are observed after irradiation (Irvin et al. 1975), during growth in irradiated mice (Irvin et al., 1976) and in heterokaryons, derived from macroschizont infected cells and Ehrlich ascites tumour cells (Smith et al., 1976). Reading between the lines, it appears that free merozoites were not observed, and thus merogony was either not initiated or not completed in these experiments with *T. parva*.

It would be useful to determine whether these morphological alterations in the macroschizont represent an early stage of merogony. Particularly as similar forms appear to be generated in vitro by a variety of stimuli. This might be approached using anti-merozoite monoclonal antibodies to determine when merozoite epitopes are synthesised. In the absence of this type of data no firm conclusion can be drawn regarding the morphological alterations which take place in drug treated or heat treated cell lines which did not proceed to produce merozoites. As merogony is the prerogative of the macroschizont it is not totally implausible that the morphological alterations in the non inducible cell lines were of a flawed form of merogony.

The anti-piroplasm/heat induced merozoite antibodies, (5E1, 2D5/1D11 and 1C2), which do not recognise inducible macroschizont infected cells, were tested by IFA on uninducible heat treated cell lines (TaH-BL20, section 6.4.1.). Preliminary results suggest that the epitopes recognised by these antibodies were synthesised, but only after prolonged periods of time, and the specific fluorescence reactions were only detected in a small proportion of the treated cells. Giemsa stained preparations of this material suggested that merozoites were not produced. Therefore it is possible that only a very small proportion of the infected cells underwent normal merogony and these were detected in the IFA assays. Alternatively, the synthesis of the epitopes recognised by antibodies 5E1, 2D5/1D11 and 1C2 was induced to a limited and varying extent but merogony was not completed in any of the cells.

The observation that cell lines can lose their ability to
produce merozoites when heat treated might explain why the technique has been considered irreproducible. In addition, this observation may describe the basis for the phenomenon of attenuated cell line vaccines (section 1.3.). As uncloned cell lines were used it is not possible to distinguish between the possibility that the cells were modified in culture, such that merogony was inhibited, or that the composition of the cell line changed such that a small population of non-inducible cells were present which outgrew the population of inducible cells. This could be investigated using cloned cell lines to determine whether certain clones in a heat inducible cell line are uninducible at elevated temperatures. Similarly it would be important to determine the leucocyte sub-types infected as these could be involved in determining heat inducibility. It has been shown that T. annulata invades and becomes established in a broad range of host leucocyte sub-types in vitro (Spooner et al. 1989), and it is possible that merogony may take place in a restricted range of leucocytes.

The relevance of attenuated cell lines is obvious when considering vaccine work (section 1.5.1.) but it is possible that attenuation plays a role in the life cycle of the parasite. The question of the relative roles of the host cell and the macroschizont in differentiation is raised again. One experiment might be to to infect defined populations of leucocytes with macroschizonts from non inducible cell line clones to determine whether the macroschizont retains an inability to be heat induced. In theory, this experiment is the in vitro equivalent of what happens each time an attenuated cell line vaccine is injected into a susceptible bovid, thus it is predicted that the macroschizont carries the inability to differentiate into merozoites.

Clearly, temperature is one factor which is important in the induction of merogony and further experimentatation is required to characterise the function of temperature elevation in induction. As cell lines (and potentially, cloned and defined cell lines and parasites) which are heat inducible and others which are not inducible, are available, this in vitro system provides a very good model for studying merogony, and for determining how the differential expression of certain genes or subsequent regulatory
events are involved. Further temperature sensitive mutants might be generated, for example with defective heat-shock responses in order to study the potential role of hsp60 in merogony. As discussed above, it may be that the epitopes determined by antibodies 2D5/1D11, 1C2 and 5E1 are only expressed in heat treated cell lines which are inducible. In which case these antibodies could provide markers for attenuation, as well as probes for use in the delineation of the molecular events involved in inducing, performing and restricting merogony.

The gene/s encoding one of these epitopes has been cloned and preliminary data from Northern slot-blot assays (6.4.3.) suggest that part of the cloned sequence is expressed and that there is some regulation at the RNA level. The lambda gt11 phage clone, which was isolated with monoclonal antibody 5E1 (6.3.1.), contained two DNA fragments of 1.6kb and 3kb (figure 6.3.2.). Southern blot analysis showed that the cloned fragment was of parasite origin as it hybridised to T. annulata genomic DNA, but not to bovine fibroblast DNA, under conditions of high stringency (6.3.3.). Three major bands (> 4kb) were detected in uncloned piroplasm DNA which had been digested with Eco RI (figure 6.3.3.1.). When this experiment was repeated using the 3kb fragment with DNA from cloned cell lines, the bands segregated into two distinct patterns. Two bands were detected in each sample of cloned material (figure 6.3.3.2.) which suggests that two genes are recognised by the cloned fragment. As discussed in section 5.6, Western blot analysis has shown that monoclonal antibody 5E1 recognises mainly a polypeptide of 30kDa in piroplasm and merozoite extracts, but also detects a 120kDa polypeptide weakly. Molecules of 20kDa and 10kDa are also occasionally detected in piroplasm extracts and these may be specific products of the 30kDa molecule. These results suggest that two gene products might be recognised by antibody 5E1, and this is consistent with the finding that the cloned sequence hybridises to two genes.

When monoclonal antibody 5E1 was screened against two induced lambda lysogens, a weak positive signal was obtained and the reactivity on the two clones appeared to differ (figure 4.3.4.1.a & b). The basis for this difference was not investigated and it was concluded that the epitope recognised by 5E1 was present
in both lysogen extracts. However, on the basis of the sizes of the polypeptides recognised it appeared that breakdown products were detected. Two of the detected polypeptides (138, 126, 122, 107 & 103kDa) were smaller than beta-galactosidase (116kDa) thus they could not be complete fusion products. The insert size of 4.6kb (section 6.3.2) was relatively large and could have a coding capacity of 138kDa, thus the maximum expected size of the beta-galactosidase fusion protein was 254kDa. Such a large fusion protein might be intrinsically unstable and appear in Westerns as breakdown products which would explain the results.

The data suggest that a gene for a T. annulata epitope recognised by antibody 5E1 has been cloned, however independent evidence for the identity of the gene has not been presented. This could be obtained by raising antibodies against the fusion protein followed by Western blotting analysis to determine whether the antibodies recognise the same polypeptides detected by monoclonal antibody 5E1. Alternatively, an antibody select method could be used (Hall et al. 1984) in which antibodies from bovine immune sera are selected by absorption to, and then elution from, filters of recombinant phage. These antibodies could then be tested in Western blots against piroplasm or merozoite extracts. The 120kDa piroplasm and merozoite polypeptide which is recognised by antibody 5E1 might be detected, exclusively or together with the predominant 30kDa polypeptide.

As presented in chapter five, monoclonal antibody 5E1 shows a type 1 fluorescent reactivity pattern in IFAs, apparently recognising the outer perimeter of piroplasms and merozoites. It has not been determined whether antibody 5E1 recognises the surface of piroplasms, however, immunoelectron microscopy experiments (section 6.2.) have demonstrated that the epitope is located on the surface of culture heat induced merozoites (figure 6.2.1.). It would be interesting to determine whether the 30kDa or 120kDa polypeptides which are recognised by antibody 5E1 are involved, at some stage, in the invasion of erythrocytes. If external evidence can be obtained that the cloned gene sequence, codes for the parasite epitope recognised by 5E1, then potentially large amounts of definable recombinant protein could also be made available for study. If monoclonal antibody 5E1 or polyvalent
serum antibodies raised against the recombinant fusion protein, specifically block merozoite invasion, then the sequence would become a candidate subject in vaccine oriented studies. Circumstantial evidence suggests that the piroplasm polypeptides recognised by antibody 5E1 are immunogenic; Western blot analysis of the reactivity of bovine immune sera 155 and 128 on Ankara and Hissar piroplasm extracts shows that a 30kDa (appendix 1b) polypeptide is strongly recognised, and a 120kDa polypeptide is also specifically and consistently recognised. The data given in appendix 1a suggest that 30kDa and 120kDa molecules are not major labelled polypeptides in $^{125}$I or biotin, surface labelled piroplasm extracts; this might explain why these molecules were not immunoprecipitated from the surface of labelled piroplasm preparations. Thus evidence was not obtained here that either molecule is located on the surface of piroplasms. However, other similar surface labelling studies (Shiels et al. 1989) suggest that 31kDa and 122kDa polypeptides are exposed on the surface and further study may demonstrate that these molecules are recognised by antibody 5E1.
CHAPTER SEVEN
GENERAL DISCUSSION

The relationship between the macroschizont, culture heat induced merozoites and the intraerythrocytic stages (piroplasms) has been investigated, to begin to elucidate the mechanisms of stage differentiation at a molecular level. In addition, the infected red cell membrane has been studied with the aim of determining whether infection specific erythrocyte membrane polypeptides (ISEMPs) occur. During the course of this work, the polypeptide profiles of the intraerythrocytic stages, and the red cell membrane have been characterised (appendix 1a & b).

Several, putative, infection specific erythrocyte membrane polypeptides have been identified, using a range of different experimental approaches, but in each case, further work is required to obtain formal proof of their identity. These putative ISEMPs were detected after $^{35}$S metabolic labelling, $^{125}$I or biotin labelling; thus the analysis was restricted to the identification of polypeptides which are sufficiently rich in methionine, tyrosine or lysine residues. One exception, was the infrequent detection by bovine immune serum 128 of 156kDa and 168kDa polypeptides in Western blots of infected red blood cell extracts, which were not detected in the derived piroplasm preparation. As discussed in section 3.7., these molecules may therefore be located outside the piroplasm at some stage (section 3.3. data not shown).

Monoclonal antibodies were raised against infected red cells but, with the exception of one antibody, they failed to detect the surface of the infected red cell by immunofluorescent microscopy assay. The brief existence of one antibody which specifically recognised the infected membrane, suggests that ISEMPs are present, in or on the red cell membrane. The experimental work presented here (chapter four) has shown that infected red cell membranes can be prepared using an Affi-Gel bead system, which are free from contaminating piroplasm polypeptides. This material might provide a good immunogen in experiments to generate either monoclonal or polyspecific antibodies against ISEMPs.
Bovine immune sera 128 and 155, which were raised in calves which exhibited brief parasitaemias, but which had high anti-parasite titres by IFA, failed to recognise the surface of infected red cells, by IFA. However, immune serum 155 specifically immunoprecipitated a 215kDa iodinated polypeptide from the surface of Ankara infected red cells (figure 3.2.1. track a). This single experimental result was not consolidated by further immunoprecipitation experiments with different combinations of immune sera and infected red cell material (table 3.2.).

Infected red cells were metabolically labelled, with $^{35}$S-methionine, during incubation periods of 24-48 hours. Crude supernatant fractions contained large polypeptides of 156kDa and 168kDa (figure 4.7.1.2. track a), which were not as abundant in the labelled red blood cell extracts. These molecules (which are possibly the same molecules detected by immune serum 128 in infected red blood cell, but not derived piroplasm extracts, see above) are interesting as they may be part of trafficking, excretory or secretory events, which might, presumably, involve the infected red cell membrane at some stage. Crude membrane preparations (using differential centrifugation, section 3.5.3.; or Affi-Gel membrane protocol I., table 4.1.1.) of metabolically labelled red cells were heavily contaminated with piroplasm polypeptides. The assays for contamination were also, in some respects, assays for the binding of piroplasm polypeptides to the cytoplasmic face of red blood cells. However, as most of the available, labelled piroplasm polypeptide were bound, it was clear that the majority of the detected polypeptides were contaminants. In membrane preparation experiments, using Hissar infected red cells, piroplasm polypeptides of 20kDa and 128kDa were enriched in the membrane fractions (figure 3.5.3.1. track a). In a similar assay using Affi-Gel beads, metabolically labelled Gharb polypeptides of 136kDa, 82kDa, 81kDa, 48kDa 47kDa and four molecules larger than 205kDa, that is, approximately 215kDa, 237kDa, 247kDa and 266kDa were enriched in membrane preparations (figure 4.2.1. track a). Biotin labelled polypeptides of 36kDa and 40kDa also appeared to be infection specific and to be associated with the infected red cell membrane (figure 4.5.2.2. tracks g & i respectively). In the final experiments, to test for piroplasm
erythrocyte membrane polypeptides, 100% infected, metabolically labelled, red cells were treated for membrane preparation with Affi-Gel beads (section 4.7.). The results suggested that Ankara piroplasm polypeptides of 122kDa, 91kDa and 76kDa are associated with the membrane and are also abundant in the infected red cell (figure 4.7.1.2. track f and figure 4.7.2.2. tracks a, b & c).

Within the limits of the experimental controls (see results sections), these labelled polypeptides appear to be valid ISEMPs, which are also detected in infected red cell and piroplasm extracts. However, the possibility that they are contaminating piroplasm polypeptides must be considered, and the fact that they are also abundant piroplasm polypeptides makes this possibility more likely. From the available data, it is not possible to conclude that ISEMPs do not exist. However, it is inferred that methionine-, tyrosine- and lysis-adequate piroplasm polypeptides are not abundant in the infected red cell membrane. The failure of initial experiments to unequivocally detect ISEMPs (chapter 3), initiated experimental strategies to try to increase the sensitivity of the assay for piroplasm erythrocyte membrane polypeptides. This aim appears to have been realised in the revised Affi-Gel membrane protocol (protocol II, table 4.5.3.2.) with metabolically labelled cells which are 100% infected (section 4.7.). As parasite populations were asynchronous and uncloned in the available infected material, the possibility remains that only a small proportion of infected red cells carry ISEMPs at any one time. The finding that merozoites can be reproducibly induced in vitro from macroschizont infected cell lines (chapter 6), raises the opportunity, if invasion of erythrocytes can be achieved in vitro, for obtaining cloned and synchronous piroplasm populations. Preliminary results suggest that one marker for the piroplasm stage has been identified, as antibody 5A10 fails to recognise heat induced merozoites but recognises piroplasms by IFA (figure 6.4.1.).

Although none of the anti-piroplasm monoclonal antibodies specifically recognise the surface of infected red cells, it has not been fully determined whether or not any of them recognise infection specific epitopes within the infected red cell membrane. Collated data on monoclonal antibodies 2D5/1D11 and 1C2, and the
putative 122kDa, 91kDa and 76kDa piroplasm ISEMPs, suggest that there may be a relationship between the IFA pattern of reactivity, the polypeptides recognised in Western blots, and the putative ISEMPs. Antibodies 2D5/1D11 and 1C2 are type 2 monoclonal antibodies (figure 5.1.1. t2), which recognise the dot structure in infected red cells by IFA, but also show a diffuse fluorescence within the whole of a small proportion of infected red cells. It was suggested (section 5.6.) that this reactivity might reflect a reaction with the infected red cell membrane. Western analysis suggests that antibody 2D5/1D11 mainly recognises piroplasm and merozoite polypeptides of 120kDa; a doublet of 98-100kDa and occasionally a molecule of 77kDa are detected in piroplasm extracts, but not infected red cell or merozoite extracts. It was suggested that the 98-100kDa and 77kDa were possibly products of a parent 120kDa molecule (section 5.6.). Antibody 1C2 recognises the same 120kDa, 98-100kDa and 77kDa molecules, and in addition, may recognise a 30kDa polypeptide (which is also recognised by antibody 5E1). The 122kDa, 91kDa and 76kDa putative ISEMPs are, therefore, similar to the polypeptides recognised by monoclonal antibodies 2D5/1D11 and 1C2. Thus, antibodies 2D5/1D11 and 1C2 may recognise the putative ISEMPs, and there is some evidence to suggest that the epitopes recognised by these antibodies, and the polypeptides identified as ISEMPs, are associated with the red cell membrane as well as being located within the piroplasm.

Three stage specific monoclonal antibodies have been characterised (2D5/1D11, 1C2 and 5E1), which recognise mainly 30kDa and 120kDa polypeptides in piroplasm (Ankara and Hissar, but not Gharb stocks) and culture heat induced merozoite extracts. These antibodies do not recognise macroschizont infected cells (TaHæ) or sporozoites by IFA, and antibody 5E1 does not recognise macroschizonts or sporozoites in Western blotting experiments. Antibody 5E1 recognises mainly a 30kDa polypeptide and the evidence suggests that this polypeptide is immunogenic, as bovine immune sera strongly recognise a polypeptide of this molecular mass. Immunoelectron microscopy studies have shown that antibody 5E1 recognises the surface of culture heat induce merozoites. Thus, the antigen is of interest as candidate molecule in a sub-unit vaccine for theileriosis control. The T. annulata gene which codes
for the epitope determining antibody 5E1 has been cloned from a lambda gt11 genomic expression library so potentially large amounts of recombinant protein are available for study. It is envisaged that an effective immune response against the merozoite stage might prevent the high parasitaemias, which cause much of the pathology of the disease, and also assist as a transmission blocking step. Preliminary experiments to determine whether culture heat induced merozoite will invade red cells were unsuccessful, however the merozoites appeared to form an attachment to red cells, which was an encouraging indication that some form of initial recognition and binding sequence of events had occurred. If the conditions required to obtain invasion of red cells can be found, then an invasion assay could be used to determine whether bovine immune serum antibodies, antibody 5E1, and polyspecific sera generated against the fusion protein, are effective at blocking invasion.

Experimental studies on the post-macroschizont stages of the life cycle in Theileria have been held back by the finding that merozoites could not be reproducibly produced in vitro, despite the successful results of heat induction experiments by Hulliger et al. (1966). The results presented in chapter six demonstrated that merozoites can be reproducibly produced in vitro at elevated culture temperatures, providing that the particular cell line treated is inducible. It was observed that low passage cell lines were inducible, but lines which had been maintained in culture for extended periods became uninducible. It is suggested that the inability of high passage cell lines to produce merozoites describes the basis for the phenomenon of attenuation in Theileria macroschizont infected cell lines (section 1.3.).

The morphological alterations which were observed in Giemsa stained preparations of heat treated macroschizonts, over the fifteen day period of merozoite induction, accord with the descriptions of the events in meiosis reported by Melhorn and Schein (1984). IFA data suggest that, in inducible cell lines, the production of the epitope determined by antibody 5E1 precedes the production of the epitope determined by antibodies 2D5/1D11 and 1C2, and that the epitopes are not produced at an early stage in the differentiation (days 0-4/9). When these antibodies are screened against heat treated, uninducible cell lines, only a
limited fluorescence is observed. This data is preliminary, but it suggests that the relevant epitopes may not be produced in macroschizonts which do not differentiate into merozoites under heat treatment. If this proves correct, then it could be inferred that the production of these epitopes is indicative of a commitment to merogony. This could be a useful reactivity in experiments to assess the production of merozoites under various conditions, for example, in drug treatment experiments.

The availability of inducible and uninducible cell lines, and of an in vitro system of induction of merogony under the simple conditions of elevated temperature, appear to provide a good model system for studying differentiation events at the molecular level. Preliminary studies, presented in chapter six, have begun to investigate the regulation of gene expression during this process. If invasion of red cells can be achieved in vitro and differentiation into piroplasm stages established, then this study can be extended to the piroplasm stages of the life cycle. In addition, if cloned, synchronous red cell stages can be generated, then this material, together possibly with anti-piroplasm monoclonal antibodies, would provide a means of categorising the diversity of stages and events within the red cell.
Appendix la.

Representation of piroplasm and red cell polypeptides, summarising the results of SDS-PAGE analyses.

Track a - results of red cell $^{125}$I, and biotin-$^{125}$I-streptavidin surface labelling studies. Red cells (infected or uninfected) were surface labelled by direct iodination or with biotin (section 2.4.) and sample extracts were resolved in 4.5-20% SDS-PAGE gels. Western blots of biotinylated polypeptides were probed with $^{125}$I-streptavidin (section 2.6.).

Track b - surface labelled piroplasm preparations. Piroplasms were prepared as described in section 2.1.4., and whole piroplasms, and sample lysates, were iodinated (section 2.4.2.). Sample extracts were resolved in 4.5-20% SDS-PAGE gels, which were dried down and exposed to x-ray film (section 2.5.).

Track c - piroplasm preparations (section 2.1.4.) were metabolically labelled as described in section 2.4.1. and sample extracts were treated to SDS-PAGE in 5-20% gels which were treated for fluorography, dried down and exposed to x-ray film.

Track d - uninfected red blood cells were prepared as described in section 2.1., sample extracts were treated to SDS-PAGE and polypeptides were stained with Coomassie Blue (section 2.5.).

Track e - red blood cells (uninfected cells are shown, but infected red cells gave identical results) were prepared as described in section 2.1. and red cell membranes were prepared using Affi-Gel beads (section 2.11). Sample extracts were treated to SDS-PAGE and polypeptides were stained with Coomassie Blue.

Tracks f-h - piroplasms (stocks Ankara, Hissar and Gharb) were prepared (section 2.1.4.), and sample extracts were treated to SDS-PAGE in 4.5-20% gels and polypeptides were stained with Coomassie Blue.
**Appendix 1a**

**MAJOR Piroplasm AND RBC POLYPEPTIDES**

**KEY:**

- **a** = RBC surface
- **b** = piroplasm surface
- **c** = \(^{35}S\) labelled piroplasm
- **CB** = Coomassie Blue stained profile
- **d** = uninfected RBC (CB, cow110)
- **e** = Affi-Gel RBC membranes (CB, cow110)
- **f** = Ankara piroplasms (CB)
- **g** = Hissar piroplasms (CB)
- **h** = Gharb piroplasms (CB)

Molecular mass estimates are modal values from > 5 experiments.

Values for **a)** are from \(^{125}I\) and biotin-streptavidin surface labelling experiments. Boxes show limits of molecular masses for polypeptides observed in a given area of a gel. Numbers therein indicate the maximum number of polypeptides observed in any one gel. The white line indicates modal values from results of more consistent biotin-streptavidin labelling experiments.
Appendix 1b.

Representation of immunoprecipitated, surface iodinated piroplasm polypeptides, and piroplasm polypeptides detected by immune sera in Western blots, summarising the results of these analyses.

Tracks a-c (1.) immunoprecipitation data: -
- piroplasms were surface iodinated (section 2.4.2.) and extracts were immunoprecipitated with Protein A according to the protocol described in section 2.7. Sample extracts were treated to SDS-PAGE and dried gels were exposed to x-ray film. Ankara piroplasm extracts were immunoprecipitated with immune serum 155 which was generated as described in section 2.1.8. (track a). Hissar piroplasm extracts were immunoprecipitated with immune serum 128 (track b) and Gharb piroplasm extracts were immunoprecipitated with immune serum M56 (track c). As controls, pre-infection sera, surface labelled uninfected red cell extracts, and labelled cell-lysate extracts were included in all experiments (data not illustrated).

Tracks d-g (2.) Western blot data: -
- piroplasms were prepared (section 2.1.4.) and extracts were treated to SDS-PAGE, Western blotted and probed with antibody as described in section 2.8. Gharb piroplasm extracts were probed with immune serum M56 which was generated against Gharb piroplasms (track d). Hissar piroplasm extracts were probed in immune serum 128 (track e) and immune serum 155 (track f). Ankara piroplasm extracts were probed with immune serum 155 (track g). See section 2.1.8. for details of immune sera 155 and 128. As controls, all blots were probed with pre-infection sera, and immune sera were tested against Western blots of uninfected red cells (data not illustrated).

The black lines represent detected polypeptides. These are aligned with molecular mass estimates on the left hand side of the figure. These were derived by determining the modal estimated molecular mass, from several repeated experiments, of each polypeptide observed in a given area of the gel/blot.
Appendix 1b

1. SURFACE LABELLED PIROPLASM POLYPEPTIDES IMMUNOPRECIPITATED BY IMMUNE SERA

2. IMMUNE SERA PROBED ONTO WESTERN BLOTS OF PIROPLASM EXTRACTS

**KEY:**

1. a-c = Immunoprecipitated surface polypeptides
   
   a = serum 155 vs Ankara piroplasms
   b = serum 128 vs Hissar piroplasms
   c = serum M56 vs Gharb piroplasms

2. d-g = Western blot analyses
   
   d = serum M56 vs Gharb piroplasm extracts
   e = serum 128 vs Hissar piroplasm extracts
   f = serum 155 vs Hissar piroplasm extracts
   g = serum 155 vs Ankara piroplasm extracts
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Characterization of surface polypeptides on different life-cycle stages of *Theileria annulata*

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We describe the characterisation of polypeptides located on the surface of *Theileria annulata* sporozoites, macroschizonts, piroplasms and infected lymphoblastoid cells using surface iodination techniques. The sporozoite stage exhibited a complex profile of surface polypeptides. However, using data from experiments with defined monoclonal antibodies, the sporozoite surface appeared to be composed of several distinct groups of related polypeptides. Analysis of the macroschizont detected seven surface polypeptides, while eight polypeptides were identified for the piroplasm stage. On the basis of molecular weight comparisons, one of the surface polypeptides appeared to be common to the sporozoite, macroschizont and piroplasm. Stage cross-reactive monoclonals failed to immunoprecipitate a surface-radiolabelled polypeptide, and this prohibited the characterisation of a stage common surface antigen.

From the surface labelling studies of *Theileria*-infected and uninfected lymphoblastoid cell lines, we concluded that infection results in major changes at the surface of the host cell, including both the appearance and loss of specific polypeptides. By employing monoclonal antibodies which detect infection-associated determinants, and a polyclonal antiserum raised against a glycoprotein fraction of an infected cell lysate, surface-labelled polypeptides were specifically immunoprecipitated from extracts of infected cells. The polypeptide detected by monoclonal antibody 4H5 was characterised as an infection-associated glycoprotein which varies in molecular mass when immunoprecipitated from different infected cell lines. The identification of infection-associated glycoproteins on the surface of the lymphoblastoid cell suggests that these molecules may be recognised by the cytotoxic T cells of immune animals.

Key words: *Theileria annulata*; Surface polypeptide; Monoclonal antibody; Infection-associated glycoprotein

Introduction

*Theileria annulata* is a tick-borne parasite which causes tropical theileriosis, a disease which im-poses a significant constraint on livestock production in areas of North Africa, Asia and Europe [1]. The parasite life cycle is initiated in the mammalian host by inoculation of sporozoites during tick feeding. Sporozoites rapidly invade lymphocytes [2], where they mature into macroschizonts and induce rapid proliferation of the host cell. Development of macroschizonts into microschizonts results in the release of merozoites, which invade erythrocytes and subsequently mature into piroplasms. Piroplasm-infected erythrocytes are taken into the tick upon feeding, and the parasites develop through a series of stages to produce infective sporozoites in the tick salivary glands.

The major effective target of the immune re-
spiration against tropical theileriosis, and East Coast Fever caused by *Theileria parva*, is thought to be the macroschizont-infected lymphoblastoid cell [3]. The response is primarily mediated via cytotoxic cells [4–7], and it has been postulated that the cytotoxic cells recognise parasite-associated neoantigens on the surface of the infected lymphocyte [4,5,7,8]. More recently, monoclonal antibodies have been isolated which detect associated determinants on the surface of *T. annulata* infected lymphoblastoid cells [9,10]. Taken together, these studies imply that the infection of the lymphocyte by the parasite induces alterations in the molecular profile of the host cell surface. One aim of the work undertaken in this study was to characterise cell surface alterations associated with infection by *T. annulata*.

Other than the infected lymphocyte, the most likely targets for a protective immune response are the two invasive stages of the parasite, the sporozoite and merozoite, and possibly the surface of the infected erythrocyte. The merozoite stage is technically difficult to study, but an antigen which is common to sporozoites, macroschizonts and piroplasms may also be present on merozoites. Monoclonal antibodies have been raised which react across the different stages [9]. Therefore, in addition to studying the lymphoblastoid cell surface we carried out experiments to characterise the surface polypeptides of the sporozoite, macroschizont and piroplasm, and used the cross-reacting monoclonal antibodies to try and identify stage common surface antigens.

**Materials and Methods**

**Cell lines.** Three *Theileria*-infected cell lines and an uninfected bovine lymphoblastoid line were maintained in culture with RPMI-1640 (Gibco) supplemented with 15% foetal calf serum (Gibco). 2 mM glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin and buffered with 2 mM Hepes, pH 7.2. The three infected lines were a *T. annulata* Hissar [11] infected cell line (TaH46), derived by in vitro infection of bovine peripheral blood mononuclear cells [12]; a lymphosarcoma line similarly infected with *T. annulata* Hissar (TaHBL20) and the cell line *T. annulata* Razi (TaR), derived from an infected animal in Iran [13]. The uninfected cell line (BL20) was derived from a non-viral bovine lymphosarcoma [14].

**Isolation of parasite stages.** Sporozoites from the parasite stock, Ankara [15], were purified from infected ticks (*Hyalomma anatolicum anatolicum*) using a Percoll discontinuous gradient [16], and resuspended in phosphate-buffered saline (PBS), pH 7.2. Macroschizonts were isolated by lysis of the infected lymphoblastoid cells using a modification of an established method [17]: 100 ml of cultured cells were centrifuged at 300 × g for 10 min, washed 2 × in PBS and resuspended in 0.1 ml Tris-buffered saline (TBS: 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgSO₄, 2 mM CaCl₂). An equal volume of 4% Tween 40 was added and the cells lysed by rapid stirring for 60 min at 4°C. Lysis was checked by phase-contrast microscopy, and completed by homogenisation when necessary. The lymphoblastoid cell lysate was then overlayed onto a 45% Percoll gradient (prespun at 36 000 × g for 15 min) and centrifuged at 800 × g for 15 min. Fractions containing macroschizonts, as assessed by phase-contrast microscopy and Western blotting experiments, were collected, diluted with TBS and centrifuged at 1300 × g for 15 min. The resulting pellet was resuspended in up to 500 µl of TBS.

Piroplasms were purified from 100 ml of infected bovine blood by the ammonium chloride lysis method [18]. To separate leukocytes from the erythrocytes, the buffy coat was removed after centrifugation at 1300 × g for 5 min, and the erythrocyte fraction was passed through CF11 columns. The eluted erythrocytes were lysed by diluting 1 vol in 10 vols. of an 0.83% ammonium chloride solution, and the piroplasms collected by centrifugation at 2300 × g. The isolated piroplasms were then washed 2 × in PBS, pH 7.2, and resuspended in 0.5 ml PBS.

**Cell surface iodination.** 100 ml of cultured lymphoblastoid cells were concentrated by centrifugation at 300 × g for 10 min, washed and iodinated by the Iodogen method [19]. The labelled cells were washed 4 × with PBS to remove non-covalently bound ¹²⁵I, and the viability of the cells assessed using 1% trypan blue in PBS. Labelled preparations showing greater than 95% viability
were used for subsequent experiments.

Purified parasite stages were resuspended in PBS (sporozoites and piroplasms) or TBS (mac
croschizont) and iodinated by the Iodogen method
as described above. Unbound iodine was re-
moved by dialysis against NET buffer (0.5%
Nonidet P40 (NP-40), 5 mM EDTA, 0.15 M
NaCl, 10 mM sodium azide, 50 mM Tris-HCl, pH
8.0), and the dialysed samples freeze-thawed and
clarified by centrifugation at 10000 \times g. The
radioactivity incorporated was determined by tri-
chloroacetic acid (TCA) precipitation.

SDS-polyacrylamide gel electrophoresis. Iodi-
nated lymphoblastoid cells (5 \times 10^7) were solu-
bilised in 200 \mu l of lysis buffer (9.5 M urea, 2%
NP-40, 5% mercaptoethanol, 1 mM phenylme-
thyl sulphonyl fluoride (PMSF)) [20] on ice for 30
min, followed by freeze-thawing. The resulting
lysates were clarified by centrifugation at 10000
\times g for 10 min and the supernatant stored at
\(-70^\circ C\). The radioactivity incorporated was deter-
mined by TCA precipitation. An equal volume of
SDS sample buffer [21] was added to each super-
natant and the mixture heated at 100^\circ C for 3 min,
followed by centrifugation at 10000 \times g for 5 min.
The resulting supernatants were loaded onto SDS
linear gradient (5–20%) polyacrylamide gels
[21,22] and subjected to electrophoresis. Gels
were stained with 0.3% Coomassie Blue R250 in
40% methanol/10% acetic acid, destained in the
same solution (without added stain), dried down
onto 3 MM filter paper and then exposed to X-
ray film at \(-70^\circ C\). Molecular weights of the la-
belled polypeptides were estimated by reference
to the mobilities of standard polypeptides (High-
molecular weight markers, Sigma).

Production of monoclonal antibodies and poly-
clonal anti-Theileria sera. Monoclonal antibodies
were raised against sporozoites of \textit{T. annulata}
(Ankara) by the same protocol used to produce
antibodies against the infected lymphoblastoid
cells [9], the mice being immunised intraperito-
neally with 0.35 ml of a sporozoite suspension de-
derived from ground-up infected ticks [12]. Hybrids
and clones were screened by an indirect immu-
nofluorescence assay (IFA).

Ascites fluid was prepared from hybridoma cell
lines by injecting 5 \times 10^6 cells intraperitoneally
into individual mice which had been injected three
days previously with pristane. Immunoglobulin
fractions were prepared from pooled ascites by the
addition of ammonium sulphate to a final saturation of 50% at
room temperature. The precipitate was re-
covered by centrifugation at 20000 \times g for 20 min,
redissolved in a minimum volume of distilled
water, dialysed extensively against NET buffer and
titrated by IFA.

Mouse immune sera raised against sporozoites
were obtained by retro-orbital bleeding of the
mice immunised for the production of mono-
clonal antibodies. The bovine immune serum was
obtained from a calf immunised and challenged
with sporozoites according to the following pro-
tocol. The calf was infected with sporozoites of
\textit{T. annulata} (Ankara), treated with 20 mg/kg
(i.m.) pavaquone (Clexon, Coopers Animal
Health) ten days post-infection and then chal-
lenged with a further dose of \textit{T. annulata} (An-
kara) sporozoites eighteen days later. Serum was
collected by standard procedures one week later.

Immunoprecipitation. Radiolabelled lymphoblast-
toid cell and parasite surface polypeptides were
immunoprecipitated as follows: 1 \times 10^8 surface-
iodinated cells were solubilized on ice for 1 h in
1 ml of NET buffer containing 1 mM PMSF, cen-
trifuged at 3000 \times g for 15 min and the super-
natant centrifuged for 10 min at 10000 \times g. Approp-
riate volumes of lymphoblastoid and parasite
lysates (equivalent to 1.5–5 \times 10^7 cpm) were di-
luted to 100 \mu l with NET buffer and incubated
with 40 \mu l of packed, preswollen protein A se-
pharose 4B beads (Pharmacia) on a rotator for 30
min at 31^\circ C. The suspension was centrifuged at
10000 \times g for 2 min and the protein concen-
tration of the resulting pre-absorbed supernatant
adjusted to 200 \mu g/ml by the addition of BSA.
Aliquots (12 \mu l) of monoclonal antibodies or po-
lyclonal antisera were added to the precleared la-
belled lysates, and the samples incubated over-
night at 4^\circ C. In experiments with macroscopic
lysates, 50 \mu l of an unlabelled BL20/NP-40 ex-
tract (NET buffer) was added to the reaction. This
was to remove non-specific binding of labelled
polypeptides to the control and test antibodies.
The resulting immune complexes were absorbed onto protein A Sepharose beads by incubation with 40 µl of beads for 30 min at 31°C. The bound immune complexes were removed by centrifugation at 10,000 × g for 2 min and non-specifically absorbed material removed by washing the resulting pellet twice with 1.5 ml of NET/0.2% SDS, twice with NET and once with NET lacking NP-40. The bound immune complexes were eluted from the beads by boiling in SDS sample buffer, followed by centrifugation at 10,000 × g for 5 min. The resulting supernatants were then analysed by SDS-PAGE and autoradiography, as described above. Immunoprecipitation of lymphoblastoid cell surface glycoproteins was carried out using a rabbit antiserum raised against a glycoprotein fraction isolated from an NP-40 extract of Ta-HBL20 cells. The extract was fractionated by chromatography on a lentil lectin (Lens culinaris) Sepharose 4B column, as described by the manufacturer (Pharmacia). For immunoprecipitation, infected and uninfected BL20 cells were surface-labelled with biotin [23]. The labelled cells were then washed in PBS and extracted with PBS containing 1% NP-40. 50 µl of extract was mixed with 50 µl of NET buffer and Triton X-100 to 0.5%. The extracts were then precleared by the addition of 80 µl of 10% (v/v) *Staphylococcus aureus* (Calbiochem), previously washed in NET buffer, followed by incubation for 30 min at RT. The bacteria were removed by centrifugation (10,000 × g), and 15 µl of the anti-glycoprotein serum and 25 µl of NET containing 20 mg/ml bovine serum albumin (NET/BSA) was added to the resulting supernatant. Incubation was carried out overnight at 4°C. 80 µl of the 10% *S. aureus* suspension was then added and the incubation continued for 60 min at RT. The *S. aureus*-bound immunoprecipitate was pelleted by centrifugation (10,000 × g) washed 2× in NET/BSA, 1× in NET/1 M NaCl and 1× in NET. Elution from the bacteria was performed with 20 µl 9 M urea and 10 µl 20% SDS, prior to separation of the samples by SDS-PAGE. The polypeptides were then Western blotted onto nitrocellulose as described below, and the filter blocked with wash buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl) containing 1% Tween 20 for 1 h. The blocked filter was incubated with 125I-labelled streptavidin for 2 h at RT, washed 4× in wash buffer containing 0.05% Tween 20 and 2× in wash buffer, and then autoradiographed.

Western blotting. Western blotting was carried out following established protocols [24,25]. Polypeptides were transferred onto nitrocellulose filters by electroblotting using 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% methanol, and blotting was carried out for 3 h at 60 V, 0.22 A. The filter was blocked in 5% ovalbumin, 0.05% Tween 20 and 2% normal rabbit serum for 2 h at 42°C. After rinsing briefly in this blocking buffer, the filter was incubated with test or control antibody (diluted in blocking buffer) for 6 h at room temperature, and then washed 3× over 1 h. The filter was then incubated with a horseradish peroxidase-conjugated second antibody (Sigma) for 4 h at room temperature, washed extensively and developed with o-dianisidine and H₂O₂ [24].

**Results**

To study their surface polypeptides, three life-cycle stages of *T. annulata* (sporozoites, macroschizonts and piroplasms) were isolated. The individual parasite stages were surface-iodinated and extracted with NET buffer. Because the parasite preparations were only partially purified, an antiserum from mice injected with sporozoites, a bovine antiserum derived from an immune animal (which detects all three parasite stages) and monoclonals raised against different parasite stages were used to immunoprecipitate radiolabelled molecules from the extracts. The immunoprecipitates were denatured and then analysed by SDS-PAGE and autoradiography.

**Sporozoite surface polypeptides.** The pattern of radiolabelled bands of an NP-40 extract of surface iodinated sporozoites, immunoprecipitated with mouse anti-sporozoite serum, is shown in Fig. 1, lane 3. The serum, which showed a strong reaction with the surface of sporozoites by IFA, precipitated eight major polypeptides ranging in molecular mass from 52 to 190 kDa. No polypeptides were precipitated by normal mouse serum (Fig. 1, lane 4). To study specific sporozoite molecules, we raised monoclonals against *T. annu-
Fig. 1. Immunoprecipitation of NP-40 lysates of surface-iodinated Percoll-purified *T. annulata* (Ankara) sporozoites with monoclonal antibodies and mouse anti-sporozoite serum. Lane 1, immunoprecipitation with monoclonal 4E5; lane 2, monoclonal 5H2; lane 3, mouse anti-sporozoite serum; lane 4, normal mouse serum. The mobilities of standard polypeptides are indicated on the left of the figure. The numbers give the molecular mass of the standards in kDa.

*lata* (Ankara) sporozoites, and used these antibodies in immunoprecipitation experiments. Lanes 1 and 2 of Fig. 1 show the result of immunoprecipitation reactions using monoclonal antibodies which react against the surface of the sporozoite, as assessed by IFA using viable sporozoites (Shiels and Tait, unpublished observations). One of the monoclonals, 4E5 (lane 1), precipitated four radiolabelled polypeptides of 190, 127, 98 and 69 kDa. These molecules were the same size as polypeptides precipitated by the mouse anti-serum. The 69-kDa polypeptide was also precipitated by the second monoclonal (5H2) and by a control monoclonal which had no reactivity against the sporozoite: in addition the control monoclonal precipitated a polypeptide of 140 kDa. Thus, it is probable that the 69- and 140-kDa polypeptides were not specifically immunoprecipitated by the test antibodies. The 190-, 127- and 98-kDa polypeptides, however, were specifically immunoprecipitated by both monoclonal 4E5 and the mouse antiserum. We conclude that these molecules are located on the surface of *T. annulata* sporozoites, and are related to each other by possession of the epitope recognised by monoclonal antibody 4E5.

**Macroschizont surface polypeptides.** Using the bovine antiserum, a series of radiolabelled polypeptides was specifically immunoprecipitated from surface-labelled macroschizont extracts (as shown by Fig. 2A). The profile of the radiolabelled bands differed from the pattern detected, by the bovine anti-serum, on Western blots of macroschizont extracts (Fig. 2B). This result indicates that the immunoprecipitated radiolabelled polypeptides are a subset of the total polypeptides recognised by the serum, and provides evidence that the labelled molecules are located on the macroschizont surface. Therefore, the surface of *T. annulata* macroschizonts contains at least seven

![Fig. 2. Analysis of *T. annulata* (Hissar) macroschizont polypeptides by immunoprecipitation and Western blotting. (A) Immunoprecipitation of NP-40 lysates of surface-radiolabelled macroschizonts. Lane 1, immune bovine serum (IBS); lane 2, normal bovine serum (NBS). (B) Western blot of *T. annulata* (Hissar) macroschizont extracts. Lane 1, IBS; lane 2, NBS.](image-url)
polypeptides, which range in estimated molecular mass from 36 to 142 kDa (see Fig. 4).

**Piroplasm surface polypeptides.** The bovine antisera to characterize the surface polypeptides of the macroschizont was also used to immunoprecipitate radiolabelled molecules from an extract of surface-iodinated piroplasms. Fig. 3 shows that eight radiolabelled polypeptides were specifically immunoprecipitated, with the 100-kDa polypeptide being significantly more intense than the other seven. Western blots of piroplasm extracts showed a different polypeptide profile (Fig. 3B, lane 1), implying that the radiolabelling of the piroplasm was confined to its surface. We infer that the eight polypeptides detected by immunoprecipitation are located on the surface of the piroplasm.

A comparison of the data obtained from the analysis of the sporozoite, macroschizont and piroplasm surface polypeptides is summarised in Fig. 4. It should be noted that the comparison between the sporozoite and the other two stages is not direct as they are derived from different stocks of *T. annulata* (Ankara and Hissar). The sporozoite appears to be the least complex stage, six surface polypeptides being specifically immunoprecipitated, but the surface of the macroschizont and piroplasm are only marginally more complex. The polypeptides S5, M4 and P5 have approximately the same molecular mass, and could represent a surface molecule which is common to the sporozoite, macroschizont and piroplasm. To identify stage common surface polypeptides, we carried out immunoprecipitation experiments with monoclonal antibodies, IF2 and 2E4, which have been shown by IFA to cross-react against different stages of *T. annulata* [9]. These antibodies failed to immunoprecipitate a radiolabelled polypeptide from extracts of sporozoites, macroschizonts or piroplasms (data not shown). Monoclonal 2E4 did detect a polypeptide on Western blots of macroschizont (data not shown) and piroplasm extracts (Fig. 3B, lane 3). The molecular mass of this molecule did not match with the estimated mass of any of the detected surface polypeptides, suggesting that the molecule recognised by monoclonal 2E4 is located internally.
Infection-associated lymphoblastoid cell surface polypeptides. Characterisation of the surface polypeptide profiles of Theileria-infected lymphoblastoid cells was carried out by iodination of viable cells and subsequent analysis of the radiolabelled extracts by SDS-PAGE. To delineate lymphoblastoid cell surface polypeptides associated with Theileria infection, comparisons were made between extracts derived from (a) an uninfected lymphosarcoma line BL20; (b) its infected counterpart (TaHBL20) and (c) the infected cell line (TaH46), which had previously been used to generate a series of infection-associated monoclonal antibodies. Fig. 5B shows the profile of radiolabelled bands obtained for each of the cell lines, and Fig. 5C demonstrates that the general profiles were reproducible. These patterns were clearly distinguishable from the profile of total polypeptides identified by Coomassie Blue staining of the gel (Fig. 5A). This observation, coupled with the high viability of the cells following iodination (> 95%), indicates that specific labelling of cell surface polypeptides was achieved.

Eleven to fifteen major polypeptides were identified, which ranged in molecular mass from 15 to 210 kDa.

It is clear that the BL20 and TaHBL20 lines differ in the profile of their surface polypeptides. The changes induced by infection of BL20 were most notable in the 47-kDa region (band (a), Fig. 5B and C) where an intensely labelled doublet is observed in the infected TaHBL 20, and at 58 kDa (band (b) and 99 kDa (band (c)). In contrast, the intense band at 180 kDa (band (d)), detected in BL20, was not found in extracts of the infected TaHBL20 line. The surface of a second infected cell line (TaH46) was analysed to look for surface polypeptides common to cells transformed by T. annulata. Although it was possible to detect bands specific to the individual infected cell lines, we could not unambiguously identify an infection-associated polypeptide common to both the TaHBL20 and TaH46 lines (Fig. 5B, lanes 2 and 3). We surmised that this was due to insufficient resolution of the complex patterns. To remove these limitations of the analysis, and to de-

Fig. 5. Analysis of surface-labelled lymphoblastoid cells by SDS-PAGE and autoradiography. (A) Coomassie blue staining of surface radiolabelled extracts separated by SDS-PAGE. Lane 1, BL20 extract; lane 2, TaHBL20 extract; lane 3, TaH46 extract. (B) Autoradiography of the gel shown in (A). (C) Autoradiograph of second gel of radiolabelled lymphoblastoid cells. Lanes are designated as in (A).
fine the alterations more specifically, studies were undertaken using monoclonal antibodies and a polyspecific antiserum raised against a glycoprotein fraction of infected cells.

**Immunoprecipitation of infection-associated cell surface polypeptides.** Three monoclonal antibodies have been raised which react against infection-associated determinants on the surface of *Theileria annulata* lymphoblastoid cells. We used these monoclonals to immunoprecipitate surface radiolabelled molecules from NP-40 extracts of the infected cell lines. Two of the monoclonals (2A6 and 1E12) failed to precipitate radiolabelled polypeptides. The third monoclonal (4H5) immunoprecipitated a radiolabelled surface polypeptide of 125 kDa from an extract of the infected TaH46 line; (Fig. 6, lane 5). This band was specific to the 4H5 antibody as a control monoclonal (2G2), directed against a macroschizont antigen, gave no signal (Fig. 6, lane 6). In addition, the 4H5 antibody did not immunoprecipitate a radiolabelled molecule from the uninfected cell line BL20 (Fig. 6, lane 2) but, did precipitate molecules from extracts of two other *Theileria*-infected lines (TaHBL20 and TaR; Fig. 6, lanes 3 and 4). Interestingly, the observed molecular mass of the precipitated polypeptide varied between the infected cell lines, being estimated as 99 kDa (TaHBL20) 116 kDa (TaR) and 125 kDa (TaH46). Thus, monoclonal antibody 4H5 recognises an infection-associated determinant of a lymphoblastoid cell surface polypeptide which has variable molecular mass.

The anti-glycoprotein serum strongly precipitated two infection-associated surface-labelled molecules (a and b in Fig. 7, lane 2) with respective molecular masses of 100 kDa and 80 kDa. These bands were not precipitated from the uninfected BL20 lysate (Fig. 7, lane 1). Two other major polypeptides (c and d in Fig. 7, lanes 1 and 2) were precipitated from both the TaHBL20 and BL20 extracts. From their apparent molecular mass and their abundant levels, these bands most probably correspond to the heavy and B2 microglobulin chains of the bovine class 1 (BoLa) antigen. Thus, the antiserum raised against a gly-
coprotein fraction detects both common and infection-associated cell surface polypeptides.

Discussion

The ability of polyclonal sera to block sporozoite invasion of host cells in vitro [26,27] suggests that the sporozoite is a potential target for a protective immune response, and provides the rationale for defining the surface polypeptides of this stage. The mouse anti-sporozoite serum specifically immunoprecipitated six radiolabelled polypeptides, demonstrating that the surface of the sporozoite is complex. Although Percoll-purified sporozoites were used in these experiments, it is possible that some of the polypeptides specifically immunoprecipitated by the mouse antiserum were derived from tick salivary gland. Three of these polypeptides, however, were also immunoprecipitated by monoclonal 4E5. Furthermore, in a subsequent study a second anti-surface sporozoite monoclonal (1A7) has been shown to detect (Williams et al., in preparation) polypeptides which are approximately the same size as the three other polypeptides immunoprecipitated by the mouse antiserum. Thus, it appears that all of the molecules immunoprecipitated by the mouse antiserum have been identified by two monoclonal antibodies, suggesting that the surface of the sporozoite is composed of a limited number of sets of immunologically related molecules.

The precise relationship between the polypeptides recognised by the monoclonal 4E5 is not known. One possibility is that they are the products of a single gene, the different forms being the result of polypeptide cleavage. Alternatively, the polypeptides could be expressed from different genes which code for the common epitope recognised by the monoclonal antibody. A similar observation has been made with a sporozoite surface monoclonal raised against T. parva [28]. Clearly, further immunochemical and molecular studies are required to determine the origin of these multiple surface polypeptides.

The macroschizont and piroplasm stages showed a more complex profile of surface polypeptides than the sporozoite (see Fig. 4). On comparing these profiles one polypeptide was found to be similar in molecular mass (S5, M4 and P5, Fig. 4). Such a molecule may represent a surface antigen common to all three stages, which by inference could also be present on the merozoite stage. The stage cross-reactive monoclonal antibodies, however, failed to immunoprecipitate radiolabelled polypeptides from any of the stages, and so it was not possible to characterise a surface polypeptide as a stage common antigen. Indeed the profiles, overall, were distinct for each of the parasite stages, implying that the surface antigens are essentially stage-specific. Nevertheless, the existence of a stage common surface antigen should not be ruled out; in Plasmodium it has been demonstrated that polypeptides which share an antigenic determinant have different molecular masses when isolated from different stages [29].

The cell-mediated destruction of infected lymphoblastoid cells indicates, indirectly, that infection of leukocytes with T. annulata produces antigenic alterations at the host cell membrane [6]. In this study we have directly demonstrated that the cell surface polypeptide profiles of infected and uninfected lymphoblastoid cells are different. We conclude that the infection of the BL20 line with Theileria has altered the molecular profile of the host cell surface. Differences in the surface polypeptides of the infected and uninfected lines were characterised further by the immunoprecipitation experiments using monoclonal antibodies and the anti-glycoprotein serum. The monoclonal antibody 4H5 immunoprecipitated a surface-labelled polypeptide from an extract of the TaHBL20 line but not from the uninfected BL20 extract, indicating that the infection of the BL20 line with Theileria alters the expression of this polypeptide at the cell surface. Furthermore, the immunoprecipitation of surface polypeptides from extracts of the TaH46 and TaR lines demonstrated that the alteration was common to the infected lines tested.

The immunoprecipitation experiments with the anti-glycoprotein serum detected two surface-labelled molecules specific to the Theileria-infected BL20 extract, and as the antiserum was polyclonal, this suggests that novel expression of complete glycoproteins occurs at the surface of the infected cell. In addition, the result confirms the
findings of a study (Taracha, E., M.Sc. Thesis, University of Birmingham) which characterised differences between glycoprotein fractions from infected and uninfected cells. One of the infection-associated TaH46 glycoproteins defined by Taracha has a molecular mass (120-kDa) close to the value of the molecule immunoprecipitated by monoclonal 4H5 (125 kDa, Fig. 6). Moreover, the immunoprecipitation of a TaH46 extract with 4H5 prior to analysis by lectin affinity chromatography resulted in loss of the 120-kDa glycoprotein. From this result, and the fact that monoclonal 4H5 and the anti-glycoprotein serum immunoprecipitate polypeptides of similar molecular mass from extracts of TaHBL20 cells (99 kDa compared to 100 kDa), we have concluded that the molecule detected by 4H5 is a cell surface glycoprotein. The identification of the molecule as a glycoprotein may explain its size variability as the size difference of an antigen found on T and B lymphoblastoid cell lines has been shown to be due to glycosylation [30]. Glycoproteins comprise a major proportion of molecules found at the cell surface [31]. Therefore, the characterisation of infection-associated lymphoblastoid cell surface molecules as glycoproteins is not surprising. It is of relevance, however, as it has been shown with T. parva that pretreatment of infected lymphoblastoid cells with inhibitors of glycosylation blocked their specific lysis by cytotoxic T lymphocytes [5]. Thus, our results support the hypothesis that the antigens recognised by the cytotoxic T cells of immune animals are infection-associated lymphoblastoid cell surface glycoproteins [5].

It is now evident that the infection and transformation of bovine cells with Theileria annulata induces alterations at the infected cell surface. The mechanism by which these changes occur is unknown and may be pertinent to the rationale for designing a synthetic vaccine. Three simplistic mechanisms can be envisaged. Firstly, the molecules may be encoded by the parasite genome and be transported to and expressed upon the host cell membrane, as has been shown for other protozoan parasites [32,33]. The inability of antisera from immune animals, which recognise the parasite, to react with the infected lymphoblastoid cell surface (Ref. 34, and Shiels, unpublished observation) argues against this mechanism, but does not totally discount it. Secondly, host molecules could be directly modified by the activity of, or the association with, a parasite polypeptide. The third mechanism postulates an indirect involvement of the parasite via the transformation of the host cell. Studies have shown that differentiation [35], transformation [36] or activation [37] of cells result in changes of cellular glycoproteins, and it has been reported that the infection with T. parva can induce the expression of a specific T cell marker [38]. It is possible, therefore, that some of the infection-specific cell surface molecules detected in this study are associated with the transformed properties of the lymphoblastoid cell. As discussed above, the result with the anti-glycoprotein serum indicates novel expression of a surface polypeptide, supporting mechanism 1 or 3. Further studies using defined antibodies and recombinant DNA technology should allow the origin of the cell surface alterations to be defined.

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