

**CHARACTERISATION OF THE MAJOR MEROZOITE
POLYPEPTIDES OF THEILERIA ANNULATA**

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Publications included are:

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Disruption of synchrony between parasite growth and host cell division is a determinant of differentiation to the merozoite in Theileria annulata. B.Shiels, J.Kinnaird, S.McKellar, J.Dickson, L.Ben Miled, R.Melrose, D.Brown and A.Tait, Journal of Cell Science, 101, 99-107 (1992).

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ABBREVIATIONS

ATP	adenosine triphosphate
CaCl ₂	calcium chloride
Ci/mMol	Curies per millimole
DNA	deoxyribonucleic acid
<u>E.coli</u>	<u>Escherichia coli</u>
EDTA	ethylenediaminetetra-acetic acid
HCl	hydrochloric acid
Ig	Immunoglobulin
kDa	kilodalton
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
PTFE	Polytetrafluoroethylene
revs	revolutions
RNA	ribonucleic acid
SDS	sodium dodecylsulphate
SSC	standard saline solution
Tris	Tris (hydroxymethyl) aminomethane
ul	microlitre

SUMMARY

Much of the pathogenicity of the bovine disease tropical theileriosis, caused by the protozoan parasite Theileria annulata, results from the invasion of host erythrocytes by the merozoite stage of the parasite. The aim of this study was, primarily, to investigate the expression of immunodominant, and potentially protective, antigens of T.annulata merozoites. Furthermore, it was intended to characterise the antigenicity and biochemical nature of a previously identified 30 kDa merozoite surface polypeptide, to clone the gene encoding this protein, and to develop an in vitro merozoite invasion assay in order to analyse the process of invasion in detail, particularly with respect to the role of identified merozoite surface proteins.

Theileria annulata macroschizont-infected cell lines can be induced to produce merozoites by culture at an elevated temperature (41°C). This in vitro system, in conjunction with Western blotting techniques and monoclonal and polyspecific antibody reagents, enabled the identification of novel polypeptides, expressed by uncloned and cloned cell lines (Ankara stock), and associated with the production of merozoites. Two major polypeptides were identified; the 30 kDa merozoite surface polypeptide, and a 117 kDa merozoite rhoptry polypeptide. Analysis of the two types of cloned cell line (enhanced and diminished differentiators) showed that, with respect to monoclonal antibody reactivity, both of these molecules

were antigenically diverse and furthermore, the 30 kDa polypeptide was shown to be polymorphic in size, as diminished cloned cell lines possessed a 32 kDa molecule, in contrast to the 30 kDa molecule associated with the enhanced cloned cell lines. In addition, the analysis of different T.annulata stocks, isolated from diverse geographical regions, showed that the 30 kDa molecule is extensively polymorphic.

Analysis of the 30 kDa and 32 kDa molecules by 2D-PAGE, peptide mapping and amino acid sequencing showed that the molecules are closely related. Furthermore, periodate experiments showed that the molecules are glycosylated, and that the sugar residues on the molecules determine much of their antigenicity. The nature of this glycosylation could not be determined using conventional techniques of enzymatic carbohydrate cleavage or lectin binding. The possibility that the 30/32 kDa molecules have an unusual and complex carbohydrate composition, which makes them difficult to analyse conventionally, is discussed. Experiments involving extraction of the molecules with the detergent Triton X-114 confirmed that the 30/32 kDa polypeptides are integral membrane proteins. Further studies carried out to determine if the 30/32 kDa molecules are attached to the merozoite surface by a GPI-anchor were inconclusive, and experiments designed to determine if the molecules are secreted from the membrane surface, or have protease activity, indicated that the molecules did not have either of these properties.

Isolation of the gene coding for the 30 kDa protein

was attempted using two strategies; by immunoscreening a T.annulata piroplasm genomic expression library with polyspecific antiserum, and by screening a cloned cell line (D7) genomic library with two oligonucleotide pools derived from 30 kDa protein amino acid sequence data. However, neither of these approaches were successful, and the possible reasons for this are examined. The in vitro invasion of bovine erythrocytes with culture-produced merozoites was achieved to a limited extent, with the observation of piroplasm forms. However, the estimated percentage of parasitised erythrocytes was too low to allow the system to be used as a reproducible assay of invasion, and the possibility that merozoites generated in vitro differ from merozoites generated in vivo is discussed.

CHAPTER ONE
GENERAL INTRODUCTION

INTRODUCTION

1:1 Tropical Theileriosis

Tropical theileriosis is a debilitating and often fatal disease that threatens around 200 million cattle worldwide (Purnell, 1978). The organism that causes the disease is a protozoan parasite belonging to the genus Theileria in the sub-phylum Apicomplexa, the class Sporozoea, and order Piroplasmida (Levine et al., 1980). The species Theileria annulata causes tropical theileriosis in cattle and domestic buffalo, and the parasite is transmitted by tick vectors of the genus Hyalomma. The distribution of Theileria annulata stretches from areas of southern Europe, such as southern parts of Portugal, Spain, Italy, Bulgaria and Greece, to southern Russia, the Middle East, Pakistan, India and into China. In Africa, the parasite is found in northern countries such as Morocco, Algeria, Tunisia, Libya, Egypt and Sudan (Purnell, 1978; Dolan, 1992; and Figure 1.1).

Indigenous cattle (Bos indicus) in the endemic areas are relatively resistant to the effects of T.annulata infection, and mortality is only around 5% (Neitz, 1957). However, the indigenous cattle are not highly productive in meat and milk, and productivity is reduced further by infection with the parasite. Because of the expansion of urban communities in developing countries, the demand for milk and meat is increasing, and highly productive European taurine (Bos taurus) breeds have been introduced into these areas and cross-breeding programmes have been initiated

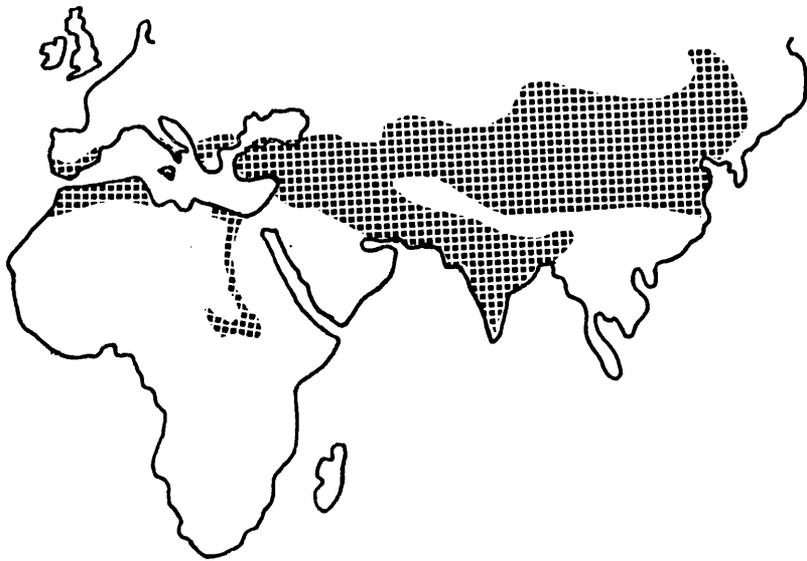


Figure 1.1:
Map showing the Distribution of the Theileria annulata Parasite

between the European breeds and local breeds. Unfortunately, the European and European cross-bred cattle are highly susceptible to tropical theileriosis, suffering mortality rates varying from 40% to 95% (Hashemi-Fesharki, 1992), and this is a major constraint on livestock improvement (Robinson, 1982). Furthermore, cattle which are not killed by the disease can suffer abortion, infertility, a reduction in milk yield and loss of body weight. Losses also arise from the extra cost of keeping and treating affected cattle. Estimates of the economic impact of tropical theileriosis on milk and meat production in affected areas are not available, but it is thought to be significant (Jongejan, 1992), and the development of a safe, effective and cheap method for the control of tropical theileriosis is of great importance for the economy of those countries affected.

1:1:1 The Theileria annulata Life-cycle

Theileria undergoes a complex life-cycle in the bovine host and the tick vector, as illustrated in Figure 1.2. The Theileria annulata parasites are transmitted by ticks of the genus Hyalomma. The ticks may feed on different cattle as larvae, nymphs and as adults (three-host ticks, for example, H.anatolicum anatolicum), or as larva and nymph on one host, and as adults on a second host (two-host ticks, for example, H.detritum) (Jongejan, 1992; Robinson, 1982). After the tick feeds on an infected animal, the disease is transmitted to another animal by the next stage of the tick (transtadial transmission). Transovarian transmission, by

stages of the next generation of ticks, does not occur. Most commonly, the larvae or nymphal stages of the ticks feed on infected cattle and then the adult ticks transmit the parasite to disease-free animals.

1:1:1:1 The Bovine Host Stages

Theileria species are transmitted by the tick vector as sporozoites, which are inoculated into the cattle with the saliva of an infected tick as it feeds. These invade a variety of leucocyte cell populations, where they differentiate to the macroschizont stage (Neitz, 1957). Spooner et al. (1988, 1989) and Glass et al. (1989) have shown that T.annulata preferentially infects major histocompatibility complex (MHC) class II-positive cells of the immune system, particularly B-cells and cells of the monocyte/macrophage lineage, and does not infect T-cells. However, these experiments were undertaken in vitro, and in vivo, it has been shown that infected cells stop expressing a number of surface markers (Spooner and Brown, 1980), including those of monocytes and B-cells (Spooner et al., 1988). Therefore, it is difficult to specifically determine the cell-type infected in vivo. The sporozoites invade the host cells rapidly (Jura, 1981; Fawcett et al., 1982i; Jura et al., 1983), most sporozoites completing penetration in 30 to 60 minutes. There are conflicting theories on the method of Theileria sporozoite invasion. Fawcett et al. (1982i) showed that invasion can occur at 4°C, probably by passive endocytosis. In contrast, Jura et al. (1983) has shown invasion to be an active, temperature-

dependent process, with an optimum at 37°C. In either case, the process of invasion begins when the membranes of the parasite and host cell come into very close apposition. The sporozoite sinks into a deepening invagination of the target host cell until the sporozoite is completely surrounded by host membrane and is interiorised (Jura et al., 1983). Mehlhorn and Schein (1984) have reported that, unlike other sporozoan parasites, such as Eimeria or Plasmodium, the Theileria sporozoites may enter in any orientation, and do not require initial attachment of the apical complex to the host cell membrane. Experiments performed by Jura et al. (1983), however, suggest that the sporozoites consistently attach to the lymphocytes by their basal end. Once intracellular, the host cell membrane surrounding the sporozoite fragments and disappears, and the parasite remains bound by a single membrane (the pellicle) during further development in the lymphocytes (Schein et al., 1978; Webster et al., 1985).

The intracellular sporozoites first develop through a transient uninucleate feeding stage, the trophozoite (Jura et al., 1983), and then undergo nuclear division to form multinucleate macroschizonts, which lie free within the host cell cytoplasm (Mehlhorn and Schein, 1984; Shaw and Tilney, 1992). The macroschizont has no obvious surface coat, and pairs of schizont mitochondria and nuclei are scattered randomly throughout the cytoplasm. The macroschizont cytoplasm also contains membrane-free ribosomes and some small clusters of polysomes but very few other organelles. For example, it contains no smooth or

rough endoplasmic reticulum or golgi apparatus (Shaw and Tilney, 1992). On being infected by the parasite, the host cells are induced to proliferate permanently, and in vitro studies indicate that the division of the parasite and host cell occurs synchronously (Hulliger et al., 1964). This has allowed the establishment, in vitro, of continuously growing macroschizont-infected cell lines (see section 1:1:4). The mechanism of the host cell immortalization is unknown. However, in vitro, it has been shown that T.annulata-infected peripheral blood lymphocytes (PBL) constitutively express interleukin 2 (IL-2) receptors (Hermann et al.,1989), and the growth of these cells can be enhanced by the addition of human recombinant IL-2 (Ahmed et al.,1991). There is also evidence that infected cells can produce a factor with IL-2-like properties, and this might be involved in an autocrine mechanism for the proliferation of the infected cells (Ahmed et al., 1992). In addition, there is evidence to suggest that Theileria-infected lymphocytes require cell-cell contact in order to proliferate indefinitely (Dobbelaere et al., 1991).

While the majority of macroschizont-infected cells continue to grow and divide, a proportion of the multinucleate schizonts are induced to differentiate to become uninucleate, extracellular merozoites. This process begins with a number of morphological changes; the chromatin of the macroschizont nuclei condenses, and the nuclei develop a distinct nuclear envelope. In the cytoplasm, both rough and smooth endoplasmic reticulum appears, and the outer surface coat of the schizont becomes

covered by a prominent outer coat (Shaw and Tilney, 1992). The schizont nuclei then migrate to the periphery of the schizont body, and rhoptries appear in the schizont cytoplasm. The internal organelles then associate with the nuclei so that they are arranged as they appear in the mature merozoite. Unicellular merozoites are then formed from the syncytial schizont and appear to bud out from the surface of the schizont, into the host cell cytoplasm, to produce the morphological form of the parasite which has been called the microschorizont (Mehlhorn and Schein, 1984). The schizont residual body is left behind, which contains a number of nuclei, rhoptries, mitochondria and some endoplasmic reticulum. The merozoites are then liberated from the host cell by the breakdown of the host cell plasma membrane (Jarret and Brocklesby, 1966; Mehlhorn and Schein, 1984; Shaw and Tilney, 1992).

In vivo, schizonts start forming merozoites 8 to 10 days after infection with T.annulata. The mature merozoites are pear-shaped and are 1-2um in length and 0.6um in diameter (Mehlhorn and Schein, 1984). They have a large nucleus, a rhoptry complex, micronemes and are surrounded by a three-layered pellicle (an outer membrane and two inner membranes). At the apical pole, subpellicular microtubules are anchored to a polar ring (Schein et al., 1978). The merozoite pellicle enables the parasite to survive extracellularly, and to penetrate the host erythrocytes. Little is known of the mechanism of erythrocyte invasion, although work on Theileria sergenti suggests that the anterior portion of the merozoite is

involved in the initial attachment to the host cell (Kawamoto et al., 1990). Within the erythrocytes, the merozoites develop into piroplasms, which have two forms; an initial trophozoite form, followed by intraerythrocytic merozoite forms (Conrad et al., 1985). The merozoites are formed by the intraerythrocytic schizogony of the trophozoites into quadruplet forms and they have the same ultrastructural features as T.annulata merozoites produced by intralymphocytic schizogony. In addition, there is some evidence that infective merozoites produced by intraerythrocytic schizogony may reinvade host erythrocytes (Conrad et al., 1985). The piroplasm stage is infective for the tick vector, which ingests the piroplasms while feeding (Sergent et al., 1945), to complete the bovine phase of the parasite life-cycle.

1:1:1:2 The Tick Vector Stages

Theileria piroplasms, contained within the erythrocytes of the infected bovine host, are ingested by feeding ticks. After ingestion, the erythrocytes are lysed and a variable proportion of the piroplasms undergo a sexual cycle (Cowdry and Ham, 1932; Mehlhorn and Schein, 1984). These piroplasms first develop into macro- and microgametes (ray-bodies) which undergo syngamy in the tick gut to form zygotes. The zygotes enter the cell lining of the gut where they differentiate into kinetes. These move through the haemolymph to the salivary glands where they penetrate salivary gland cells. This stage remains dormant until the tick moults and the next stage starts feeding (Schein et

al., 1975). The parasites are then stimulated to replicate and undergo nuclear division, forming a multinucleate syncytium from which large numbers of uninucleate sporozoites develop (Fawcett et al., 1982ii; 1985; Mehlhorn and Schein, 1984). The sporozoites are secreted in the tick saliva (Purnell and Joyner, 1968), and this is the stage infective for the mammalian host.

Finally, it is interesting to note that the process of sporogony has distinct similarities to the separate processes of merogony and piroplasm differentiation, which occur in the bovine host, because all three involve the formation of uninucleate cells from a multinucleate syncytium. Shaw and Tilney (1992) suggest that these repeating patterns in the life-cycle of Theileria are unlikely to have evolved independently, and they put forward a hypothesis that a developmental pattern of cellularisation was evolved only once, but has been used repeatedly by the parasite throughout its life-cycle.

1:1:2 Pathogenesis

The pathogenesis of tropical theileriosis is associated with the parasite-induced lymphoproliferation and subsequent lymphodestruction, and the anaemia resulting from the phagocytosis and destruction of infected erythrocytes (Neitz, 1957; Barnett, 1977). The disease is first manifest as a swelling of the lymph nodes, draining the site of inoculation of the T.annulata sporozoites. Discrete foci of macroschizont-infected leucocytes and

uninfected lymphoblastoid cells proliferate and develop throughout the animal's body, and, as the disease progresses, a marked fall in peripheral blood leucocytes is observed (Srivastava and Sharma, 1981; Eisler, 1988; Preston et al., 1992). The onset of pyrexia, usually 10 to 13 days after infection, coincides with the detection of macroschizonts in lymph node smears and with a phase of extensive destruction of parasitised and non-parasitised cells in the lymphoid tissues, which is associated with the appearance of natural killer cells in the peripheral blood (Preston et al., 1983 and see section 1:1:6). In addition, in acute infections, severe anaemia can develop due to the destruction of erythrocytes by the continuing replication of piroplasms. In susceptible animals, the disease can be fatal and death usually occurs 3 to 4 weeks after infection. Recovered animals commonly suffer relapses, and they also act as a source of infection for the vector, as they maintain a low level of parasitised erythrocytes (Neitz, 1957).

1:1:3 Diagnosis and Epidemiology

Tropical theileriosis is diagnosed by the observed clinical symptoms, or by the use of Giemsa-stained blood or tissue smears to detect either macroschizonts or piroplasms. In addition, indirect immunofluorescence antibody tests have been used to detect piroplasm, cultured schizont antigen, or sporozoite antigen (Shiels et al., 1986; 1989; Glascodine et al., 1990). This method has been used for epidemiological surveys also (for examples see Sayin et

al., 1991; Flach et al., 1991), although it is an inconvenient technique for screening large numbers of sera, and so has limited use. This problem could be solved by the use of an enzyme-linked immunosorbent assay (ELISA) (Gray et al., 1980; Kachani et al., 1992). Further to this, the technique of ELISA-detection has been tested, using T.annulata (Ankara) sporozoite, schizont and piroplasm antigens, and sera collected from animals before and after immunization with T.annulata sporozoites (Kachani et al., 1992). Wells were coated with each antigen and sera (pre-infection or immune) added. In these experiments, it was found that piroplasm antigens were most suitable for the development of a highly sensitive ELISA because they exhibited low non-specific pre-infection levels, and high post-immunisation values (Kachani et al., 1992). Alternatively, several Theileria annulata parasite genes have been cloned and characterised (Hall et al., 1990; Mason et al., 1989; Williamson et al., 1989). These could be used as DNA probes (oligonucleotides) to detect parasite DNA within samples of blood or tissues. The cloned extra-chromosomal DNA element (Hall et al., 1990) could be particularly useful in this context as it is present in all life-cycle stages, all strains of T.annulata examined, and in multiple copies per genome. DNA probes have been used in epidemiological studies of T.annulata isolates originating from Tunisia (Ben Miled et al., 1992). Two DNA probes, randomly isolated from a genomic library constructed from piroplasm DNA, were used to detect polymorphism in Southern blots of DNA from the isolates. In this study, considerable polymorphism was detected, both in macroschizont-infected

cell lines and in piroplasms, and no two isolates gave identical patterns of hybridisation with the probes.

1:1:4 In vitro cultivation of Theileria annulata

The analysis of the Theileria parasite life-cycle has been greatly assisted by the development of in vitro systems for the cultivation of certain stages of the parasite; most significantly in the establishment of macroschizont-infected lymphocyte cell lines, and in the ability to generate merozoites from these lines in vitro.

Macroschizont-infected cell line cultures can be established from infected lymphoid tissue (Malmquist et al., 1970), from peripheral blood mononuclear cells of an infected animal, or by the in vitro infection of lymphoid cells with Theileria sporozoites (Brown et al., 1973; Brown 1979; 1983; 1987). Sporozoites are usually isolated from ground-up ticks (GUT extracts) or tick salivary gland explants (Brown, 1981; Walker and McKellar, 1983). The ticks are infected by feeding on infected bovids, or by the percutaneous injection of infected blood (Jongejan et al., 1981), and sporozoite production is stimulated by feeding the ticks on cattle, goats, rabbits, or other mammals, for a period of 3 to 5 days (Samish and Pipano, 1976; Singh et al., 1979). Production can also be induced by maintaining the ticks at elevated temperatures (37°C) with high humidity (Samish, 1977), although fed ticks usually provide a higher number of sporozoites (Walker and McKellar, 1983; Reid and Bell, 1984). Peripheral blood mononuclear cells

can then be infected with the sporozoites at 37°C, and these develop into macroschizont-infected cell lines (Brown et al., 1973). The cell lines, however initiated, can be grown indefinitely as a cell line in the laboratory (Brown, 1979; 1981; 1983; 1987). The use of complex media, such as RPMI 1640 supplemented with 15-20% foetal calf serum, has replaced the initial need for feeder layers (Hulliger, 1965; Malmquist et al., 1970; Malmquist and Brown, 1974). The parasites of the infected cell lines are genetically heterogeneous, but from these, cloned cell lines can be produced (Irvin, 1987), enabling cell lines of single genotype to be studied. This development, in conjunction with monoclonal antibody technology, has been important for investigations into parasite molecular biology. For example, different cloned cell lines of T.annulata can be distinguished by monoclonal antibody reactivity (Shiels et al., 1986; 1992), and by DNA restriction fragment length polymorphism (Shiels et al. 1992).

When cultured at 37°C, macroschizont-infected cell lines undergo no significant morphological changes. In vivo, a proportion of schizonts undergo extensive nuclear division and differentiate to become merozoites (section 1:1:1:1). Various approaches have been taken to generate merozoites from macroschizont-infected cell lines in vitro. Experiments performed by Danskin and Wilde (1976) with T.parva showed that the addition of bovine lymph to 5-10%, and the increase of foetal calf serum to 20 or 30%, stimulated macroschizonts to produce merozoites in vitro. Fritch et al. (1988) attempted to induce T.annulata

merogony by using drugs to inhibit host cell division, while not inhibiting the replication of the schizont nuclei. The use of two mitosis-inhibiting drugs (colchicine and taxol) resulted in a moderate increase in the number of schizont nuclei, but merozoite formation was not seen. The most successful and reproducible method for merozoite production in vitro is the incubation of macroschizont-infected cells at an elevated temperature (Hulliger et al., 1966; Fritch et al., 1988; Glascodine et al., 1990). At the elevated temperature (41°C), cell lines of low passage number differentiate most efficiently, although, even then, only a proportion of the parasites differentiate (Glascodine et al., 1990; Shiels et al., 1992). However, clones of T.annulata (Ankara) macroschizont-infected cell lines have been isolated which undergo extensive differentiation to produce large quantities of merozoites (Shiels et al., 1992). These have been described as "enhanced" differentiating cloned cell lines, in contrast to "diminished" differentiating cloned cell lines, in which few parasites differentiate to produce merozoites at 41°C.

The mechanism of the macroschizont differentiation to the merozoite has not been fully elucidated. In vivo, the production of merozoites is thought to coincide with the onset of host pyrexia (section 1:1:2), and it was initially thought that heat-shock proteins could be involved in the induction of merogony. The gene encoding a T.annulata 70-kilodalton heat-shock protein (hsp70) has been cloned and shown to be expressed constitutively in sporozoites,

piroplasms and in a macroschizont-infected cell line, and hsp70 RNA expression from the macroschizont-infected cell line did increase following heat shock at 42°C (Mason et al., 1989). However, comparison of the expression of hsp70 RNA in enhanced and diminished differentiating cloned cell lines showed that the level of hsp70 expression was equal in the two types of cloned cell line before heat shock, and increased to the same level after heat shock (B.R.Shiels, unpublished observations). Therefore, although the increased expression of heat-shock proteins may contribute to the early stages of merogony, this is unlikely to initiate the process of differentiation. Interestingly, studies on the differentiation of Leishmania promastigotes to amastigotes have shown that the increased expression of hsp70 does not play a role on the regulation of differentiation of this parasite also (Shapira et al., 1988; Zilberstein et al., 1991). Comparison of the growth of T.annulata enhanced differentiating cloned cell lines at 37°C and 41°C has given an indication of the events which may initiate merogony in Theileria (Shiels et al., 1992). It has been shown that at 37°C, the parasite divides synchronously with the host cell, so that the parasite maintains a steady number of macroschizont nuclei, and remains a constant size. However, at 41°C, the growth of the macroschizont becomes asynchronous with the division of the host cell (Hulliger et al., 1966); the parasite nuclei divide at an accelerated rate so that the nuclear number increases, and after a certain point the host cell division, and hence parasite cell division, decreases (Shiels et al., 1992). Therefore, the parasite grows and

eventually occupies a large proportion of the host cell, and then differentiates to form merozoites. Therefore, it is postulated that this disruption of synchrony between parasite growth and host cell division determines differentiation to the merozoite in vitro, and is a direct response of the parasite and host cell to culturing at the higher temperature. It is not yet known exactly how the expression of schizont and merozoite polypeptides is regulated, but it would be interesting to pinpoint any stage-specific expression of merozoite polypeptides, and to relate this to the expression of stage-specific schizont polypeptides (Shiels et al., 1986; 1989; and see section 1:1:8). In this way, it is possible that the mechanism of the regulation of macroschizont differentiation could be elucidated.

A continuous cultivation system has not been established for the piroplasm stages of T.annulata (Conrad, 1983), and there is no reproducible system for merozoite invasion of erythrocytes in vitro. However, infected erythrocytes can be maintained for 10 to 27 days in culture at 37°C, allowing intraerythrocytic schizogony to be observed (Conrad et al., 1985). Only limited cultivation of the tick vector stages is possible also, although kinetes can be produced from zygotes in tick organ cultures (Bell, 1984).

1:1:5 Other bovine-infecting Theileria species

There are six species of Theileria which are infective for bovids: T.annulata, T.parva, T.sergenti, T.mutans, T.taurotragi and T.velifera (Uilenberg, 1981; Irvin, 1987).

Theileria parva causes East Coast Fever (ECF) in cattle (Bos taurus) and the African buffalo (Syncerus caffer) throughout East and Central Africa. It is transmitted by the three-host tick species Rhipicephalus appendiculatus and R.zambeziensis. Like T.annulata, imported European cattle and their crosses are very susceptible to T.parva infection. In addition, improved indigenous Zebu (Bos indicus) cattle which originate from non-endemic areas are severely affected by ECF. Up to 80-100% of animals of all types and of all age groups may die once infected with the parasite. ECF can be fatal even in indigenous cattle originating from endemic areas, with mortality rates as high as 50%, although this is mostly in calves (Irvin and Morrison, 1987). Because of these high mortality rates, control of the disease is of economic importance. For example, in 1989, it was estimated that ECF killed 1.1 million cattle, costing U.S. \$168 million in losses (Mukhebi et al., 1992). The greater pathogenicity of ECF compared to tropical theileriosis (section 1:1) is thought to be associated with the different cell preferences of the two types of parasite for infection. T.parva, unlike T.annulata (section 1:1:1:1), can infect T-cells but not B-cells or monocytes/macrophages. T-cells are important for the immune system effector response (Morrison et al., 1987; Innes et al., 1989ii), so once infected, T.parva may be

able to subvert the T-cell effector function, and this could lead to the greater severity of the disease compared to that of tropical theileriosis (Glass et al., 1989). Another major difference between T.annulata infection and T.parva infection is that there is limited intraerythrocytic division and no evidence of haemolytic anaemia with T.parva infection, and pronounced leucopenia is the usual cause of death in animals killed by ECF. In severe T.annulata infections, not only does leucopenia occur, but anaemia also arises from extended piroplasm replication within the red cells (section 1:1:2). In addition, the infections can be distinguished on the basis of piroplasm morphology; the T.annulata piroplasms are predominantly round and oval in shape, whereas T.parva piroplasms are comma- and rod-shaped forms (Mehlhorn and Schein, 1984).

Theileria sergenti is endemic in parts of Asia, and is transmitted by ticks of the Haemaphysalis genus. It is a parasite of domestic cattle and the theileriosis caused is generally mildly pathogenic. However, infection with T.sergenti, particularly when in conjunction with other tick-borne haemoprotozoan parasite infections, can result in mild pyrexia and anaemia, which greatly reduces productivity. For example, in Japan, the bovine theileriosis caused by T.sergenti is an economically significant disease, and is the most common protozoan disease of grazing cattle in this country (Minami et al., 1981). Taxonomically, two other benign species, which also cause anaemia rather than leucopenia, and also originate

outside of Africa, T.orientalis and T.buffeli, have previously been classified with T.sergenti as the T.sergenti/orientalis/buffeli parasite group. However, the validity of this classification has been confused (Uilenberg, 1981; Uilenberg et al., 1985), and recent phenotypic and genomic characterisation of the three species indicates that the species should be separated into two taxonomical groups, i.e. T.sergenti and T.orientalis/buffeli (Fujisaki, 1992).

The Theileria mutans species is a parasite of African buffalo (S.caffer) and domestic cattle, and is also a mildly pathogenic species. T.mutans is distributed by ticks of the Amblyomma genus (Saidu, 1982). Similarly, T.taurotragi is a mildly pathogenic species which is found in Africa, although it is distributed by ticks of the Rhipicephalus genus (Stagg et al., 1983). Theileria velifera is a parasite of African buffalo, and like T.mutans, is transmitted by ticks of the genus Amblyomma. This species is not pathogenic to cattle (Uilenberg, 1981).

1:1:6 Bovine Immune Responses to T.annulata Infection

Animals which have recovered from a primary infection of Theileria annulata are strongly immune to challenge with the same stock of parasite, and a variable degree of immunity is exhibited to challenge with heterologous stocks (Gill et al., 1981). The recovered cattle develop antibodies against all mammalian stages of the parasite, and these antibodies can be used as markers of infection in

immunological tests (Gray et al., 1980; Cowan, 1981; Irvin and Morrison, 1987; Kachani et al., 1992). In addition, the host may become resistant to tick infestation (Allen, 1973). There is evidence that this resistance is mediated by mast cells, basophils and eosinophils (Gill and Walker, 1985), but it is unknown if such an inflammatory response hinders or helps the establishment of the T.annulata parasite in the bovine host (Innes, 1992).

The T.annulata life-cycle stages which are potential targets for a protective host immune response are the extracellular and invasive sporozoite and merozoite stages, the schizont-infected cell, and the piroplasm-infected erythrocyte.

1:1:6:1 Immune Responses to the Sporozoite

The infected host clearly has an anti-sporozoite immune response as immune serum can inhibit the penetration of sporozoites into uninfected host leucocytes in vitro (Gray and Brown, 1981; Preston and Brown, 1985; Ahmed et al., 1988). However, this is usually only observed with serum derived from animals recovered from multiple challenge infections (Preston and Brown, 1985). The sporozoite is exposed to the host immune system for only a short time (Brown et al., 1978), so repeated sporozoite challenge is probably needed to boost the immune response so that the titre of anti-sporozoite antibodies is high enough to inhibit sporozoite infection. Serum from animals recovered from primary infections is more likely to simply suppress the development of the intracellular trophozoite to

schizont. (Preston and Brown, 1985). Therefore, at best, the host anti-sporozoite response probably functions to reduce sporozoite infection and enable the host immune system to control the infection.

1:1:6:2 Immune Response to the Schizont-infected Cell

By being intracellular, the schizont is protected from a significant host immune response, and there is no evidence to suggest that anti-macroschizont antibodies generated during primary infection contribute to immunity (Kachani, 1990). However, cell-mediated immune responses appear to be effective against the schizont-infected cell. Infection-associated antigens have been identified in surface labelling experiments, and by monoclonal antibody reactivity, on the surface of the parasite-infected cell (Shiels et al., 1986; 1989). It has also been shown that monocytes infected with T.annulata have high levels of MHC class II markers expressed on their surface, and it is postulated that T.annulata-infected cells may have an augmented ability to present parasite antigens in conjunction with the MHC class II markers, so that the antigens can be recognised by T-cells (Glass et al., 1991; Glass and Spooner, 1990). Preston et al. (1983) showed that cytotoxic T-cells appear in the blood and lymph nodes of calves recovering from T.annulata infection. Furthermore, when immune cattle are challenged, two peaks of cytotoxic cells are generated at approximately one and three weeks after challenge. The first peak of cytotoxic cells is found to comprise of bovine MHC-restricted cytotoxic T-

cells, while the second peak is comprised of MHC-restricted T-cells and non-MHC restricted natural killer (NK) cells. Although macroschizont peptides which are associated with class I MHC antigen and are also recognised by cytotoxic T-cells have yet to be found, in vitro studies suggest that the cytotoxic T-cells inhibit the proliferation of macroschizonts in primary infections, and lyse macroschizont-infected cells resulting from subsequent challenges (Preston et al., 1983). These cytotoxic responses are transient, but other effectors of the cellular immune response appear to be involved also. Adherent cells (presumed to be macrophages) can be isolated from the peripheral blood of immunised calves over a period of several weeks after T.annulata inoculation, and in vitro, these exhibit strong cytostatic effects on leucocytes infected with parasites from homologous or heterologous stocks (Preston and Brown, 1988). In addition, there is evidence that macrophage migration inhibition factor (MIF) is secreted by sensitised leucocytes from T.annulata-infected animals (Singh et al., 1977; Rehbein et al., 1981; Ahmed et al., 1981).

1:1:6:3 Immune Response to the Merozoite and Piroplasm

Research on the nature of host immunity to the merozoite and piroplasm stages is not as advanced as research on the sporozoite and macroschizont stages. Antibody responses are detected to piroplasm and merozoite antigens in cattle recovering from T.annulata infection (Pipano, 1974; Irvin

and Morrison, 1987), although there is no evidence that anti-merozoite or anti-piroplasm antibodies, generated during primary infections, contribute to host immunity (Irvin, 1985; Hall, 1988). In immunofluorescence assays, serum taken from an animal immune to T.annulata challenge reacts with free merozoites, but not with the surface of infected erythrocytes (Ahmed et al., 1988), and monoclonal antibody and surface labelling experiments do not detect T.annulata infection-specific erythrocyte surface antigens (Glascodine, 1989). This indicates that parasite-derived antigens are not presented on the erythrocyte surface in the same way as schizont infection-associated antigens are presented on the surface of the infected leucocyte, and therefore the piroplasm-infected erythrocyte is probably not a target for a cellular or humoral immune response. This is in contrast to malaria parasites, which incorporate parasite proteins into the erythrocyte membrane, some of which form antigenic determinants on the surface of the infected erythrocyte (Howard et al., 1988). However, the Theileria merozoite stage is extracellular and a potential target for a host immune response. The nature of such a response is unknown, but it is likely to be mainly a humoral response, similar to that against the sporozoite. Interestingly, research on T.sergenti has shown that the passive transfer of a monoclonal antibody, which detects a merozoite and piroplasm surface polypeptide, resulted in a protective effect against T.sergenti infection in calves (Tanaka et al., 1990). Like T.sergenti, the invasion of erythrocytes by the T.annulata parasites and their

subsequent intracellular division, results in host anaemia. Therefore, an effective host immune response against T.annulata merozoites is probably important for the development of successful immunity, or at least for a reduction of the pathogenicity of severe tropical theileriosis. Furthermore, immunity to merozoites and piroplasms should block the transmission potential of the parasite. For example, in Plasmodium vivax, monoclonal antibodies generated against gametocytes (an intraerythrocytic stage) have been shown to reduce the infectivity of the parasite to mosquitoes (Mendis et al., 1987; Carter et al., 1990).

1:1:7 Current Control Measures and Future Approaches

The major approaches for the control of tropical theileriosis are tick vector control, chemotherapy of the infected host, and host vaccination.

1:1:7:1 Tick Vector Control

Tick populations can be controlled by the use of acaricides. However, because of their expense, acaricides tend to be used only in areas of heavy tick infestation, and unless it is very well managed, an exclusive use of this approach rarely results in the effective control of tropical theileriosis outbreaks (Hashemi-Fesharki, 1988). Frequent acaricide dipping of cattle does not allow cattle to acquire a natural resistance to the disease, and the interruption of such programmes leaves cattle very much at risk to infection. For example, in Zimbabwe, when dipping

programmes failed at the onset of civil war, there were rapid increases in both tick numbers and outbreaks of tick-borne diseases (Sutherst and Tahori, 1981). In addition, the use of acaricides risks the contamination of milk, and ticks may become acaricide-resistant. The use of acaricides is more effective when used as part of an integrated approach, in conjunction with other methods of control, so that cattle are dipped minimally, rather than maximally, to enable the development of host resistance to ticks and immunity to disease.

1:1:7:2 Chemotherapy of the infected host

The drugs which have been most successful in the treatment of clinical theileriosis are oxytetracycline (Terramycin, Pfizer), halofuginone (Terit, Hoechst), parvaquone (Clexon, Coopers Animal Health), buparvaquone (Butalex, Coopers Pitman-Moore) and primaquin phosphate.

Oxytetracycline dihydrochloride suppresses the production of macroschizonts, by inhibiting protein synthesis, when administered soon after sporozoite inoculation. However, it has the disadvantage of not being effective against the sporozoite or piroplasm stages, so it cannot be used as a therapeutic at the height of parasitemia and fever, and it is more effective against T.parva infections (Hashemi-Fesharki, 1992; Radley, 1981). The drug halofuginone, a febrifugine compound, also destroys intralymphocytic macroschizonts only, but is effective even at low doses. In an in vitro test on

macroschizont-infected lymphocytes, 0.025 ppm of halofuginone lactate reduced the percentage of schizont-infected cells from 82% to 42% (Schein, 1986), and in trials in vivo, administration of halofuginone to Theileria annulata-infected cattle resulted in the total elimination of schizonts, four days after treatment (Schein and Voight, 1981).

The drugs parvaquone and buparvaquone, both analogues of the naphthoquinone menoctone, not only destroy intralymphocytic macroschizonts, but are also effective against piroplasms. It has been found that parvaquone is more effective against T.parva infections than T.annulata infections (McHardy et al., 1983; McHardy et al., 1985), but buparvaquone has given good results in drug trials on both T.parva and T.annulata infections. Buparvaquone is thought to act as a specific inhibitor of parasite mitochondrial electron transport (Fry et al., 1984), and its administration does not give any adverse side-effects, whereas parvaquone tends to cause some swelling at the site of injection (Hashemi-Fesharki, 1991). A summary of the results of different buparvaquone field trials has been recently reported (McHardy, 1992). These took place in 19 countries in which T.annulata was prevalent in 12, and T.parva in seven. Treatment of approximately 4,000 cattle with buparvaquone resulted in cure rates of 85-95%, whether for T.annulata or T.parva, and when buparvaquone was used to treat early or moderate cases of theileriosis, nearly 100% cure rates were obtained from just a single injection of the drug. Like parvaquone, buparvaquone is effective

against piroplasms as well as schizonts. In an Egyptian trial of buparvaquone efficacy on T.annulata-infected cattle, all the treated cattle showed elimination of the piroplasm parasitemia within a week of being treated with the drug (Michael et al., 1989). In addition, this drug is also effective against T.sergenti infection (Minami et al., 1985).

Primaquin phosphate is an effective drug used mainly in China (Zhang, 1992). The cattle are usually treated for three days, and by the end of this course of treatment, the piroplasms are totally eliminated (Zhang, 1987). Clinically, it is a very safe drug, and its efficacy rate is as high as 100% (Zhang, 1992).

These developments in the effective chemotherapy of tropical theileriosis are promising. The drugs tend to be very expensive, but the recovered animals are generally resistant to further T.annulata infection. In addition, the simultaneous administration of drug and parasite by deliberate infection ("infection and treatment" therapy), for example with attenuated schizonts (see next section), has been found to effectively immunize cattle to T.annulata challenge (McHardy, 1992; Mutugi et al., 1988; Dhar et al., 1987). Very young cattle (less than 2 months), which would not normally respond to vaccination, can be treated, and there is evidence that the use of buparvaquone, in conjunction with cell culture vaccination, protects the animal against any likely tick exposure while the vaccine induces immunity (Grewal, 1992). The mechanism of the action of the drug in this application is not understood

because the buparvaquone does not appear to interfere with the efficacy of the schizont-parasitised lymphocyte cell culture vaccine, even though buparvaquone is known to be an anti-schizont drug.

Therefore, chemotherapy, in conjunction with other methods of control, appears to be an effective route for tropical theileriosis control, although the problem remains that, for much of the rural communities of developing countries, the cost of chemotherapy is too high, and other cheaper methods of control are preferably used.

1:1:7:3 Host Vaccination

The most successful host vaccination programmes to date have involved the use of attenuated macroschizont-infected cell lines. Maintaining Theileria schizonts in culture for months or years results in a loss of parasite pathogenicity for cattle (Gill, 1976i; 1976ii), and this parasite attenuation has allowed the development of vaccination programmes using live attenuated strains of T.annulata grown in cultured lymphoid cells (Pipano, 1981). The cultured schizont-infected cells can be cryopreserved at -70°C , and approximately 4×10^6 cells are used as one dose of vaccine. Administration of the attenuated vaccine does not cause clinical symptoms of the disease, and the inoculated cattle show no adverse reaction to it. The vaccinated cattle have very good resistance to both natural infections and to homologous and heterologous parasite stock challenge (Hashemi-Fesharki, 1988), although one cross-

protection trial reported in India described an isolate (Parbhani) against which the standard vaccine stock did not protect (Grewal, 1992). It is not necessary to match the MHC class I markers of the vaccine cell line to that of the recipient (Innes et al., 1989i). This is in contrast to T.parva infected cell lines which only protect cattle if the MHC class I markers are matched (Dolan et al., 1984), and only high doses of T.parva infected cells effectively infect and immunise cattle (Brown, 1981). Therefore, it is much easier to infect and immunise animals with T.annulata-infected cell lines than with T.parva-infected cell lines. Successful immunisation against both T.annulata and T.parva appears to require the transfer of macroschizonts from the immunising cells to the host cells. The efficiency of T.parva transfer in vivo and in vitro (Brown et al., 1973) is much lower than that with T.annulata, probably because of the different specificities of infection for the parasites; T.annulata preferentially infects B cells and monocytes/macrophages, whereas T.parva preferentially infects T-cells (Spooner et al., 1989; and sections 1:1:1:1 and 1:1:5). Therefore, macroschizont transfer must depend on the leucocyte sub-types present in the immediate micro-environment, and those permissive to infection by either parasite. The attenuated T.annulata vaccine has now been produced in a growing number of countries and its use has had great success. A large number of cattle have now been immunized in this way, for example, over 1.9 million cattle in China (Zhang, 1992), more than 100,00 cattle in India (Singh, 1992), and more than 150,000 cattle in Iran (Hashemi-Fesharki, 1992). However, the immunity conferred

by these vaccines frequently does not prevent the appearance of piroplasms in the bovine blood, and this leads to the development of a host carrier state. It is thought that the piroplasms could result from new infection from feeding infected ticks, and/or from the differentiation of a very limited number of schizonts, which may have originated from the vaccine cell line used for immunisation (Pipano, 1992). Alternatively, the number of piroplasms could also be maintained by the reinvasion of erythrocytes by piroplasms (section 1:1:1:1). Therefore, the use of attenuated cell line vaccines does not result in the total eradication of tropical theileriosis, and is thus dependant on epidemiological stability to maintain the equilibrium between host immunity and parasite challenge.

The T.annulata invasion of bovine erythrocytes causes much of the pathogenesis of tropical theileriosis (section 1:1:2), so host vaccination against the merozoite or piroplasm stage may be necessary for total eradication of the disease. Much of the early research work on tropical theileriosis centred on the vaccination of cattle with blood collected from infected animals at the peak of the disease reaction (Sergent et al., 1945; Hashemi-Fesharki, 1992), and this approach has also been used to vaccinate cattle against the T.sergenti parasite (Kobayashi et al., 1987). However, the use of infected blood carries the risk of transmitting other parasites such as Babesia, Anaplasma, and viruses such as bovine leukemia virus (Pipano, 1992; Wright and Riddles, 1989). Sporozoite vaccines have been tested whereby vaccination is performed by the inoculation

of sporozoites, followed by treatment with tetracycline, parvaquone or buparvaquone (Pipano et al., 1981; Dhar et al., 1987; Bansal and Sharma, 1989). However, the cost of preparing the sporozoite vaccine and the cost of drugs for treatment is much higher than that required for producing the attenuated cell line vaccine (Pipano, 1992), so this approach is not practical for the treatment of tropical theileriosis at present.

The use of live vaccines has a number of limitations; they have a short shelf-life (1 month at 4°C), so have to be stored in liquid nitrogen; as was mentioned above, other infectious agents may be co-transmitted with the vaccine; for the attenuated culture vaccines, a long period of time is needed to produce and test the cultures, and cell lines can possibly revert to virulence. An alternative and safer approach is to identify the specific parasite antigens which are the potential targets for a protective immune response. Using recombinant DNA techniques to isolate and express the antigens, their effectiveness could be tested by in vivo vaccination and challenge experiments, and a stable, highly protective, reproducible and cheap sub-unit vaccine could be made, which also had a long shelf-life. This approach has given promising results in the development of recombinant Babasia (reviewed in Wright et al., 1992) and Plasmodium vaccines (Patarroyo et al., 1987). Furthermore, in T.sergenti, a 32 kDa merozoite/piroplasm surface antigen has been identified, and the passive transfer of monoclonal antibodies directed to this molecule in vivo has been shown to have a

protective effect (Tanaka et al., 1990). Work is currently being undertaken on the Theileria species, so that protective antigens can be identified.

1:1:8 Identification of protective Theileria annulata surface antigens

As discussed in section 1:1:6, the best potential targets for a protective immune response to T.annulata are likely to be the surface antigens of the sporozoite, the macroschizont-infected cell and the merozoite. Antibodies directed to the sporozoite surface are clearly protective as immune serum can inhibit sporozoite invasion (section 1:1:6:1). Furthermore, two anti-sporozoite monoclonal antibodies (1A7 and 4B11) have been isolated which identify epitopes on the sporozoite surface, and have been used to inhibit sporozoite invasion of peripheral blood mononuclear cells in vitro (Williamson, 1988). The monoclonal antibody 1A7 was used to screen a lambda gt-11 genomic expression library (Williamson, 1988), and two recombinant DNA clones were isolated which expressed the 1A7-recognised epitope, SPAG-1 (sporozoite antigen-1) (Williamson, 1988; Williamson et al., 1989). Antiserum was generated against recombinant fusion protein made from one of the positive clones, and this serum was used to block the sporozoite invasion of leucocytes in vitro. On Western blots, the serum recognised a complex of polypeptides, probably derived from the proteolytic processing of a single gene product (Williamson et al., 1989; Hall et al., 1992). The cloned DNA was used to isolate the whole Spag-1 gene, which has now been

completely sequenced (Hall et al., 1992). Northern and Southern analysis shows that the Spag-1 gene is expressed only in sporozoites, it occurs as a single copy in the genome, and there are restriction fragment length polymorphisms, both between different genotypes within a single parasite stock and between isolates from different geographical regions. Furthermore, two regions in the sequence have been identified which have close homology to the repetitive domain in bovine elastin (Hall et al., 1992). It is speculated that SPAG-1 may function as a ligand which binds to elastin receptors present on a range of cell types, including macrophages/monocytes, which are a major class of host target cells (section 1:1:1:1). Therefore, SPAG-1 may have a significant role in host cell invasion. Work is also progressing on the 4B11-detected epitope. This monoclonal has also been used to screen a genomic expression library, and from this, a clone was isolated containing a 900 base-pair insert. The nucleotide sequence of the cloned insert is distinct from the Spag-1 sequence. Rabbit immune serum has been generated against fusion protein made with the cloned DNA, and this has been shown to inhibit the invasion of sporozoites in vitro (P.Knight, unpublished data).

Surface labelling studies of Theileria annulata-infected and uninfected lymphoid cells showed that T.annulata infection results in major changes at the surface of the host cell, with the appearance and disappearance of specific polypeptides (Shiels et al., 1989). Monoclonal antibodies have been raised to the

macroschizont-infected cell and to the piroplasm stages (Shiels et al., 1986; Shiels et al., 1989; Glascodine et al., 1990). The monoclonal antibody 4H5 detects an infection-associated cell surface glycoprotein, which has a variable (100-125 kDa) molecular mass (Shiels et al., 1989), and in the presence of complement, the binding of this monoclonal has been shown to induce the lysis, and to suppress the proliferation, of T.annulata-infected cells (Preston et al., 1986). This suggests that infection-associated antigens on the host cell surface may be susceptible targets for in vivo protective immune responses. However, the role (if any) of the 4H5-detected antigen in a host immune response is not yet known.

Along with the sporozoite and macroschizont-infected cell, the merozoite is also a potential target for a protective host immune response. One merozoite surface antigen (MAG-1) has been cloned by screening a genomic expression library with the monoclonal antibody 5E1 (Glascodine, 1989). Rabbit serum raised against the recombinant fusion protein of the expressed cloned DNA detects a complex of polypeptides and, although these polypeptides are not detected by the monoclonal 5E1, they are specific to the merozoite and piroplasm stages and the serum most likely detects a surface antigen (Hussain et al., 1990). Analysis of the Mag-1 gene sequence shows that the central part of the gene is composed of a block of complex, highly conserved, repeat sequences (Hussain et al., 1990). There are three motifs; a 75 base-pair sequence (K repeat), a 72 base-pair sequence (Z repeat), and each K

or Z repeat is flanked by a 14 base-pair repeat. The Mag-1 gene is polymorphic between strains of the parasite, and there are two copies of the gene per haploid genome.

Research on the merozoite stage has been limited because an in vitro merozoite invasion assay is not available to test the inhibition of invasion by immune serum, antibodies generated against recombinant fusion proteins, or monoclonal antibodies. However, by analogy with work on related protozoan parasites such as Plasmodium (Dubois et al., 1984; Hall et al., 1984i; 1984ii; Perrin et al., 1984) and Babesia (Wright et al., 1992), and on the related parasite species T.sergenti (Tanaka et al., 1990), raises the possibility that surface components of the Theileria annulata merozoites could be used to induce protective immune responses similar to that induced against the sporozoite stage. The use of merozoite surface polypeptides to elicit a protective immune response would have certain advantages. Immunofluorescence assays have shown that, one year after infection, antibodies to schizonts fall to undetectable levels, whereas antibodies to piroplasms remain detectable, because the recovered animals become carriers of T.annulata piroplasm infection. In contrast, sporozoite-induced infection, and hence schizont production, is dependant on tick inoculation, which is seasonal (Kachani et al., 1992). In immunofluorescence assays, using anti-piroplasm monoclonal antibodies, the piroplasm stage appears to be closely related to the merozoite stage (Glascodine et al., 1990). Therefore, a protective immune response generated against

merozoite polypeptides would be constantly boosted in the carrier host. Furthermore, as there is evidence for the reinvasion of erythrocytes by piroplasms (Conrad et al., 1985), the carrier status could even be eliminated if the anti-merozoite response was effective against piroplasms, vulnerable to attack from the immune system prior to reinvasion. Hence, both sporozoite and merozoite protective antigens should be included in a recombinant sub-unit vaccine for maximum effectiveness. Not only would this approach potentially protect the host from the pathogenic effects of tropical theileriosis, but the inclusion of merozoite protective antigens would prevent animals becoming carriers for the disease, and this could lead to a reduction in the spread of the disease.

1:1:9 Aims of this Project and Experimental Approach

Much of the previous work on Theileria annulata has centred on the sporozoite stage and macroschizont-infected cell. Work on the merozoite stage has been hindered by the inability to produce sufficient quantities of merozoites in vitro, but this is no longer a limiting factor, with the development of a reproducible system for the induction of merogony from macroschizont-infected cell lines in vitro. This system, using elevated culture temperatures and cloned macroschizont-infected cell lines, produces large quantities of merozoites (section 1:1:4). By analogy with the studies undertaken with the sporozoite stage, it is possible that surface polypeptides of the merozoite could have a role in protective immunity (sections 1:1:6:3 and

1:1:8), although research on parasites such as Schistosoma (Simpson, 1990) and Babesia (Wright et al., 1992) has shown that not all surface antigens are necessarily targets for protective immunity, and not all protective antigens are located on the parasite surface. However, protective or not, parasite surface antigens are frequently stage-specifically expressed. Part of this project aimed to examine the differentiation of the macroschizont to merozoite, in order to characterise such stage-specific polypeptides. In addition, prior to this project, anti-merozoite/piroplasm monoclonal antibodies were isolated (Glascodine et al., 1990), one of which detected a major merozoite surface antigen. This project aimed to analyse the antigens recognised by these monoclonal antibodies further, and in particular, to characterise the major merozoite surface antigen in detail, using both antibody and biochemical techniques, with the ultimate aim of cloning the gene for this surface protein. Furthermore, because the parasite surface coat (or pellicle) is the only interface between the parasite and the extracellular host environment, surface antigens can have significant functions in parasite pathogenicity and/or survival, for example in mediating host cell binding or invasion, as well as providing protection against a variety of host defense mechanisms. As the T.annulata merozoite stage is invasive for host erythrocytes, ligands on the merozoite surface are likely to be involved in the initial binding to the host cell and in other processes associated with erythrocyte invasion, as is found with other parasite invasive stages

(Hyde, 1990i; Hadley et al., 1986). For example, specific Plasmodium merozoite surface antigens have been identified which are important for erythrocyte invasion, some of which are proteases (Hadley et al. 1986; Rosenthal, 1991), and many carbohydrate epitopes on the surface of schistosomes appear to have important roles in the attachment of the parasite to host cells (Simpson, 1990; Dunne, 1990). Research on the process of Theileria merozoite invasion is limited because there is no efficient in vitro system for erythrocyte invasion by merozoites, so an inhibitory effect of antibodies on merozoite invasion cannot be assessed. The ability to perform such experiments with the sporozoite stage, which quite easily invades host leucocytes in vitro (section 1:1:4), has proved invaluable in the identification of protective sporozoite antigens (section 1:1:8). Therefore, a further part of this project aimed to develop a reproducible method for merozoite invasion in vitro.

In summary, this project aimed to investigate the stage-specific expression of Theileria annulata merozoite polypeptides; to characterise the antigenicity and biochemical nature of a major merozoite surface polypeptide, and to clone the gene encoding this protein; finally, to establish a reproducible system for the invasion of merozoites in vitro. These are areas which require investigation, and in their study, it was possible that merozoite polypeptides evoking a protective immune response would be isolated.

CHAPTER TWO
MATERIALS AND METHODS

2:1:1 Piroplasm Material

Three uncloned stocks of Theileria annulata piroplasms were used; Ankara stock piroplasms, from Turkey (Schein, Buscher and Friedhoff, 1975); Hissar stock piroplasms, from India (Gill, Bhattacharyulu and Kaur, 1976); and Gharb stock piroplasms, from Morocco (Ouhelli, 1985). In addition to these, Leila Ben Miled, at the Centre for Tropical Veterinary Medicine (C.T.V.M.), Edinburgh, kindly donated piroplasm samples which originated from Tunisia, and were isolated from infected cattle in a small area (less than 10 Km radius) surrounding the Ecole National de Medecine Veterinaire in the Tunis region. These samples were given the numbers 11, 12, 13, 14C, 18A, 18C, 19 and 20. Samples 18A and 18C came from two cattle on the same farm.

2:1:2 Collection of Infected and Uninfected Blood

Piroplasm infected blood was obtained from Mr C.G.D. Brown at the C.T.V.M., Edinburgh. Calves were infected by the sub-cutaneous inoculation of a potentially lethal dose of sporozoites. Over a period of approximately two to three weeks, the degree of piroplasm parasitemia was monitored and assessed on Giemsa stained slides. When parasitemia reached between 20% and 90%, the calves were bled by jugular puncture and the blood collected into anticoagulant (10 units/ml heparin).

If required, the blood could be stored at 4°C in red blood cell storage solution (110mM glucose, 55mM mannitol, 25.8mM disodium hydrogen orthophosphate, 2mM adenine

hydrochloride, 14.7mM sodium dihydrogen orthophosphate, 17.9mM potassium citrate, 50mM ammonium chloride, pH 7.1; Meryman et al., 1986). Amphotericin-B and gentamycin sulphate were also included in the solution to give final concentrations of 75ug/ml and 50ug/ml respectively.

Uninfected blood, used as a control for infected blood samples, and in merozoite invasion assays, was also collected by jugular puncture. 10-20ml of blood was defibrinated by shaking gently with glass beads (2mm diameter) for approximately 5 minutes, at room temperature. The defibrinated blood was separated from the clotted blood by pipetting.

2:1:3 Isolation of Piroplasms

Heparinarised blood was centrifuged at 1,000g at 4°C for 15 minutes. The supernatant and the buffy layer of lymphocytes (found at the interface of the red cell pellet and the supernatant) were discarded. The cells were washed three times in Phosphate Buffered Saline (PBS; 137mM sodium chloride, 2.682mM potassium chloride, 8.1mM disodium hydrogen orthophosphate, 1.47mM potassium dihydrogen orthophosphate, pH 7.2, pre-chilled to 4°C), removing the buffy coat at each step, and resuspended in an equal volume of PBS. To lyse the cells and release the piroplasms, the red blood cell suspension was incubated at 37°C with 10 volumes of lysis buffer, composed of 9 volumes of 0.83% ammonium chloride and 1 volume of 0.17M Tris-HCl, pH 7.4. Haemolysis was observed after 3 to 5 minutes, when the red cell solution underwent a colour change from bright red to

deep red. The piroplasms were harvested by centrifugation at 1,000g, at 4°C, for 15 minutes. After several washes in PBS, the piroplasm pellet was stored at -70°C until required.

2:2:1 Maintenance and culture of Theileria annulata infected cell lines

A T.annulata (Ankara stock) macroschizont-infected cell line (TaA₂) was provided by Mr C.G.D.Brown (C.T.V.M.). It was established by the in vitro infection of peripheral blood mononuclear cells with sporozoites of the Ankara stock (Brown, 1983). Cloned macroschizont-infected cell lines were isolated from the T.annulata (Ankara stock) parental cell line by limiting dilution (Shiels et al., 1986). The cloned parasite lines were of two types; lines that produced few merozoites upon heat induction ("diminished" differentiating cell lines, D3 and E3) and those that produced large quantities of merozoites ("enhanced" differentiating cell lines, C9 and D7).

The cell lines were cultured at 37°C, with 5% CO₂ in a humidified incubator, and were diluted every 2-3 days to a cell density of approximately 1 x 10⁵ cells/ml. The cultures were diluted with Complete TBL medium, composed of RPMI-1640 (Gibco) supplemented with 20% heat-inactivated foetal calf serum (Gibco), 8ug/ml streptomycin, 8 units/ml penicillin, 0.6ug/ml amphotericin B (Fungizone, Gibco) and 0.05% sodium hydrogen carbonate.

For long term storage, cell lines were preserved in

liquid nitrogen. The cells were first centrifuged at 400g for 5 minutes and gently resuspended in 3mls complete TBL medium (for a 10 ml culture) with 10% dimethylsulphoxide (DMSO). The resuspended cells were split into two cryotubes and frozen in a pre-cooled polystyrene insulating box at -70°C. After 24 hours, the cryotubes were transferred to liquid nitrogen.

2:2:2 Induction of Differentiation in vitro

Induction of differentiation of the parasite from macroschizont to merozoite was carried out by increasing the culture temperature of the macroschizont-infected cell line from 37°C to 41°C. The cultures were diluted every 2-3 days, as given in section 2:2:1. The progress of differentiation was followed by light microscopy of cultures and by light microscopy of Giemsa-stained cytopsin preparations (see next section).

2:2:3 Giemsa Staining of Cytospins

Morphological examination of a culture could most easily and accurately be performed by the light microscopy of Giemsa-stained cytopsin preparations. A sample of culture (100ul) was spun at 1,500 revs/min (240g) for 5 minutes, onto a glass slide, in a Shandon cytopsin 2 apparatus. The preparations were dried at 37°C for 15 minutes and fixed in methanol for 30 minutes. The slide was then stained for 40 minutes with a solution of 4% Giemsa's stain (Gurr's improved R66, BDH) in distilled water. The slide was then

rinsed in cold tap water, left to dry, and visualised under oil at x 1,000 magnification, using a Leitz Wetzlar SM-Lux light microscope.

2:3:1 Preparation of Parasite Extracts

Extracts were made from isolated piroplasm preparations, from macroschizont-infected cells and from merozoites, to allow polypeptide analysis of these stages.

Piroplasms were isolated from infected bovine blood, as described in section 2:1:3. For extraction, a piroplasm pellet was thoroughly vortexed in approximately 5 volumes of PBS and lysed in an equal volume of 2x SDS-PAGE sample buffer (0.25M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.001% bromophenol blue; Laemmli, 1970). The sample was passed through a 25 gauge needle several times to shear DNA, boiled for 5 minutes, and centrifuged at 10,000g, to pellet insoluble material.

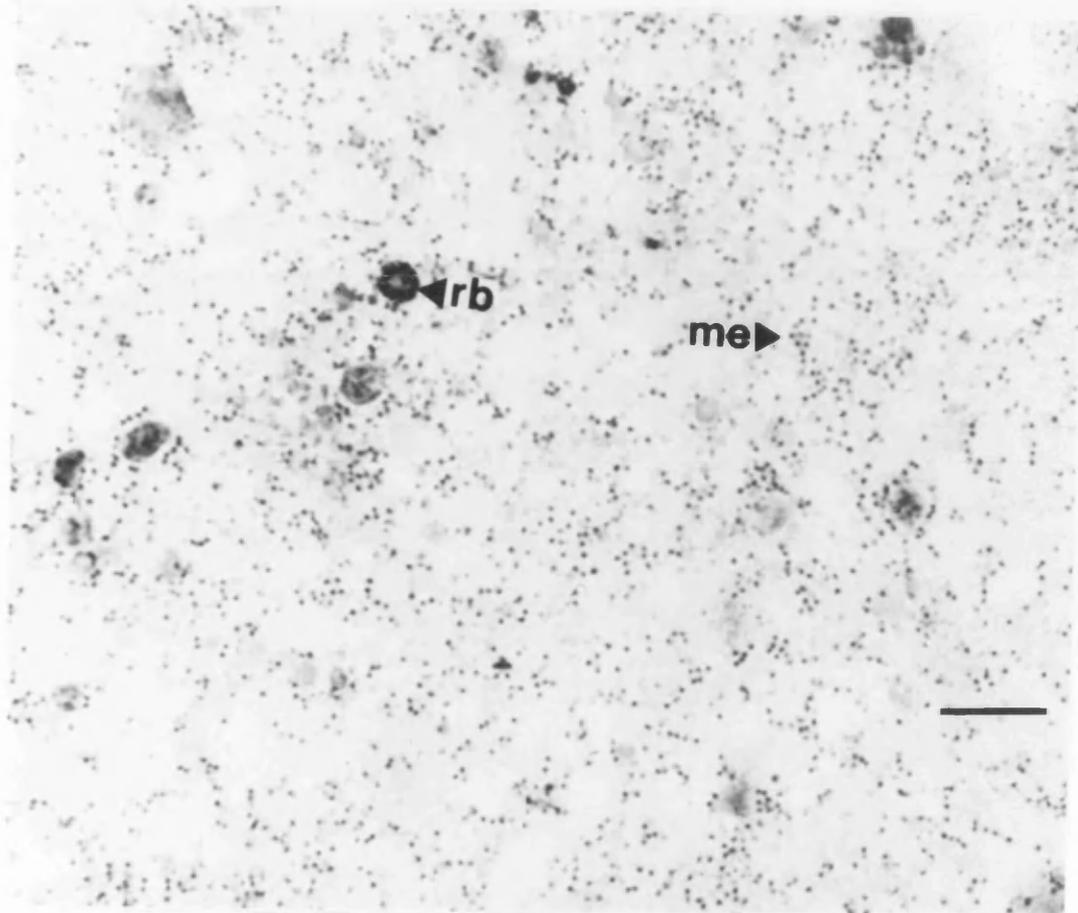
Cultured macroschizont-infected cells were pelleted by centrifugation at 300g for 5 minutes and washed three times with PBS, to remove culture medium. The cells were resuspended in 200ul of PBS (for a 10 ml culture), lysed in SDS-PAGE sample buffer, and treated as above.

Merozoites were isolated from differentiating heat-induced cultures by differential centrifugation. The cultured cells were first centrifuged at 120g for 5 minutes, to pellet undifferentiated macroschizont-infected cells. The supernatant from the first spin was centrifuged at 1000g for 15 minutes. The pellet from this second spin

Figure 2.1: Giemsa-stained cytospin preparation of partially purified cloned cell line (D7) merozoites.

me; merozoites.
rb; residual body.

Scale bar; 10um.



contained mainly differentiating macroschizont-infected cells. The supernatant from the second spin was then centrifuged at 4300g for 15 minutes to give a pellet highly enriched in merozoites. The merozoite pellet was washed three times in PBS and lysed in SDS-PAGE sample buffer, as described above. A Giemsa-stained cytopsin of the partially purified merozoites (Figure 2.1) shows that there is only minor contamination from host material and residual bodies (rb) in the merozoite preparation.

2:3:2 Protein Quantitation of SDS-Extracted Samples

Protein concentrations of samples extracted in SDS-PAGE sample buffer was estimated by a modification of the Bradford assay (Bradford, 1976). Firstly, the proteins contained in the sample were precipitated as follows; 20ul of extract was transferred to an eppendorf and incubated for 30 minutes, at room temperature, with 200ul of 10% trichloroacetic acid (TCA). The sample was spun at 10,000g for 5 minutes in a bench centrifuge, and the pellet was washed twice with 200ul 10% TCA. The precipitate was solubilised in 10ul of a saturated solution of Tris and 40ul PBS, followed by the addition of 5ml Bradford's dye binding solution (Sigma). The colour of the solution was allowed to develop for at least 5 minutes. The solution absorbance was read at 595 nm, and the concentration of protein in the sample was estimated from a set of bovine serum albumin (BSA) standards, ranging from 50ug to 1500 ug/ml.

2:4:1 Production of Antisera

2:4:1:1 Bovine Antisera

Serum was taken from an animal which had been challenged with T.annulata sporozoites (Shiels et al., 1989). The animal (calf 155) had been infected with a T.annulata (Ankara) sporozoite stabilate and treated with Parvaquone (Clexon, Coopers Animal Health), 10 days post-infection. The calf was challenged with the Ankara sporozoite stabilate 18 days later. The immune bovine serum 155 (IBS 155) was taken one week after challenge. Control pre-immune serum was taken from the animal before infection.

2:4:1:2 Rabbit Antisera

The E3d antiserum and C9m antiserum were generated against differentiating cloned cell lines E3 and C9 respectively. The E3 cell line was a diminished cell line, while the C9 cell line was an enhanced cell line (see section 2:2:1). The E3d antiserum was generated against the 1,000g pellet of an E3 differentiating culture by the subcutaneous injection of this material, mainly composed of infected cells, in Complete Freund's adjuvant, into a rabbit. The C9m antiserum was generated against the 4,300g pellet (composed of partially purified merozoites; see section 2:3:1) of a C9 differentiating culture, in the same way. After 4 weeks, the rabbits were injected with identical material in Incomplete Freund's adjuvant. Control pre-immune serum was taken from each animal before infection.

2:4:1:3 Monoclonal Antibodies

Hybridoma supernatant monoclonal antibodies 5E1, 1C2 and 1D11 were raised against T.annulata (Ankara) infected red blood cells by J.Glascodine (Glascodine, 1989; Glascodine et al., 1990).

2:5 Indirect Immunofluorescence Assay

Indirect immunofluorescence assays (IFAs) were performed on fixed cells on multispot slides. The cells were centrifuged at 1,000g, washed twice with PBS, resuspended in PBS and fixed on ice for 10 minutes in 1.8% formaldehyde. The cells were centrifuged again, as before, washed twice with PBS and resuspended in PBS (3 ml final volume, for a 10 ml culture). The cells were then spotted onto PTFE Multispot slides (C.A.Hendley, Essex) and air dried. The slides were stored at -20°C with indicator silica gel, to exclude moisture.

Multispot slides stored at -20°C were placed in a dessicator for 30 minutes at room temperature before the IFA. 20ul of test antibody was added to the appropriate well on the slide and placed in a humidified box, for 30 minutes at room temperature. Unbound antibody was removed from the wells with sequential washes in PBS for 1, 5 and 10 minutes. The slide was then dried at 37°C. 20ul fluorecein isothiocyanate (FITC) conjugated anti-IgG was then added to the wells, used at a dilution of 1:100 in PBS. The slide was again incubated in a humidified box for 30 minutes at room temperature, washed in PBS as before and

counter-stained with Evans Blue (0.1%) in PBS for 5 minutes. The slide was 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. The fluorescing cells were visualised under the x50 objective of a Leitz ortholux II transmitted light fluorescence microscope.

2:6 Immunoprecipitation

Piroplasm extracts and the monoclonal antibody 5E1 were used in immunoprecipitation experiments, and an antibody raised against the surface of the nematode Dictyocaulus (donated by J. Gilleard, W.U.M.P.) was used as a control in these experiments. Piroplasm samples were extracted with 1% SDS in 50mM Tris-HCl, pH 8.0. After shearing the DNA, the sample was boiled for 5 minutes, diluted 1 in 5 with NET buffer (0.5% Nonidet P40, 5mM EDTA, 150mM NaCl, 50mM Tris-HCl pH 8.0, 10mM sodium azide), and centrifuged at 10,000g for 5 minutes to remove insoluble material.

The method of immunoprecipitation was altered according to the amount of piroplasm extract used. A "Batch" procedure was employed for 500ul of diluted sample and a "Column" procedure used for 7mls of sample. In the Batch procedure, 500ul of piroplasm preparation was incubated overnight with 25ul of concentrated (x20) 50% ammonium sulphate precipitate of monoclonal antibody 5E1 culture supernatant or with 25ul of the control Dictyocaulus monoclonal antibody. Protein A sepharose CL-4B beads (Pharmacia, 30ul pre-swollen packed volume) were added and

the mixture incubated for 60 minutes on a rotator at room temperature. The sample was centrifuged at 10,000g for 3 minutes, and the pellet of beads plus attached immune complexes recovered. The beads were washed three times with NET buffer and once with Tris-buffered saline (TBS, 10mM Tris-HCl pH 8.0, 150mM NaCl). The beads were finally boiled in SDS-PAGE sample buffer (60ul) for 5 minutes, and centrifuged, as before, to separate the beads from the supernatant containing the immune complexes.

Using the Column procedure, 7mls of diluted extract was incubated with 200ul of monoclonal antibody overnight. The mixture was passed through a protein A sepharose bead column (1ml column bed) several times. The column was washed with 10 mls NET buffer, followed by 10 mls TBS. The immune complexes were eluted from the beads in 0.1M glycine, pH 2.9, 500ul fractions being collected into separate eppendorfs containing 50ul of 2M Tris, pH 8.0.

2:7:1 Sodium Dodecylsulphate Polyacrylamide

Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the discontinuous buffer system of Laemmli (1970), with a Bio-Rad Protean II vertical slab cell apparatus. The resolving slab gels (150mm x 150mm x 1.5mm) varied in acrylamide concentration, depending on the polypeptide resolution required. A 13% acrylamide gel was used routinely, but for better resolution a 10-20% gradient gel was used. For the resolution of peptides from protease digests (see section 2:7:5:1), a 12-22.5% gradient gel was used.

2:7:1:1 Gel Stock Solutions

All gel stock solutions were kept at 4°C. The resolving gel stock solution was composed of 30% acrylamide, 0.8% N-N Bis-methylene acrylamide solution (both NBL); 1.5M Tris-HCl pH 8.8 (resolving buffer); and 10% SDS. A 4% stacking gel was used for all resolving gels. The stacking gel stock solutions was composed of 30% acrylamide:0.8% Bis-acrylamide solution, as above; 1.0M Tris-HCl pH 6.8 (stack buffer); 10% SDS.

2:7:1:2 Working Gel Solutions

The resolving and stacking gel solutions were made up from the stock solutions, as follows. For a 13% resolving gel, the resolving gel mix was composed of 13ml 30% acrylamide solution, 7.5ml resolving buffer, 0.3ml 10% SDS, and 9ml distilled water, to give a final volume of 30ml. Polymerisation was initiated by the addition of 0.3ml 10% ammonium persulphate (freshly made) and 0.012ml N,N,N,-N-Tetra-methyl-1,2-diaminoethane (TEMED, Sigma). Once poured, the gel was overlaid with water-saturated 2-butanol and left to set for approximately 60 minutes.

For a 10-20% gradient resolving gel, a 10% acrylamide solution and a 20% acrylamide solution were prepared, containing 10ml and 20 ml acrylamide solution respectively, and adding the appropriate quantity of distilled water, so that both solutions had a final volume of 30 ml. Similarly, for a 12-22.5% gradient resolving gel, a 12% and a 22.5% acrylamide solution was prepared.

The gradient gels were made using a Bio-Rad gradient former attached to a peristaltic pump adjusted to a flow rate of 1.5mls/minute. The polymerisation steps were carried out as above.

Once the resolving gel had polymerised, the overlay was poured off and the top of the gel rinsed with a little water. The stack gel mix was then poured on top of the resolving gel. This contained, per gel, 3ml stack buffer, 1.6ml acrylamide stock solution, 6.2ml distilled water and 0.2ml 10% SDS, and polymerisation was initiated by adding 0.25ml ammonium persulphate and 0.02ml TEMED.

2:7:1:3 Sample Preparation and Running Conditions

SDS-sample buffer (see section 2:3:1) was added to the protein solutions to be electrophoresed. In addition, two sets of standard molecular weight markers were used to estimate the molecular weight of resolved polypeptides; high weight molecular markers and/or low weight molecular markers (Sigma). The high weight molecular marker set was composed of myosin (205 kDa), beta-galactosidase (116 kDa), phosphorylase-B (97.4 kDa), bovine plasma albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). The low weight molecular markers were bovine plasma albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and bovine lactalbumin (14 kDa). The test samples and molecular weight standard markers were electrophoresed overnight at 55-60 volts in SDS running buffer (50mM Tris,

384mM glycine, 2mM disodium EDTA and 0.1% SDS). Following electrophoresis, a calibration curve of \log_{10} molecular weight versus distance migrated was constructed from the standard polypeptide migration pattern and from this, the molecular weight of the sample polypeptides could be determined.

2:7:2 Coomassie Staining of SDS-PAGE Gels

After electrophoresis, the gel was fixed and stained simultaneously in a solution of 20% methanol, 10% acetic acid and 0.5% Coomassie Brilliant Blue R-250 in distilled water, for approximately 60 minutes. The gel was destained in the same solution without Coomassie dye.

2:7:3 Western Blotting and Antibody Detection of Proteins

The polypeptides of sample extracts were resolved by SDS-PAGE and transferred from the gel to nitrocellulose (Schleicher and Schuell BA85) by electroblotting (Western blotting) in a Bio-Rad transblot cell, following the manufacturers instructions. It was ensured that there was a tight connection between gel and nitrocellulose by sandwiching the gel between two pieces of filter paper (Whatman 3MM) and two pieces of Scotch-brite pad (3M Corporation) soaked in transfer buffer (25mM Tris, 192mM glycine and 20% methanol) within a manifold. Transfer was achieved at approximately 70 volts (0.25 amps) at 4°C for 3 hours. After transfer, the nitrocellulose filter was usually stained with Ponceau stain (0.3% Ponceau S in 5%

trichloroacetic acid) for 3 minutes, and destained in cold tap water to allow the polypeptide bands to be visualised.

The filters were then incubated in blocking buffer, 5% dried skimmed milk ("Marvel") and 10% heat-inactivated horse serum (Gibco) in TBS, for a minimum of two hours at room temperature on a rocking platform. The filters were then incubated for two hours, or overnight, with primary antibody at the appropriate dilution in block buffer (usually 1:400 for monoclonal antibodies; 1:200 for bovine antiserum and 1:1000 for E3d and C9m antisera) at room temperature. Unbound antibody was removed by washing the filters in TBS, 0.1% Tween-20 for 10 minutes, followed by a further two 10 minute washes in TBS alone. The filters were then incubated for 60 minutes with alkaline phosphatase-conjugated anti-species second antibody (IgG whole molecule (Sigma)) at a 1:300 dilution in block buffer. The filters were washed as before. To detect antibody binding, a bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution was used, as outlined by Harlow and Lane (1988). This generates a purple precipitate at the site of alkaline phosphatase binding. The reaction was stopped by rinsing the blots in tap water and the filters were left to air dry.

2:7:4 Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional PAGE was performed when it was necessary to separate polypeptides both by molecular weight and by net charge. The O'Farrell method (1975) was used in which the proteins were separated in the first dimension at their

isoelectric point (pI), and in the second dimension, as a function of polypeptide molecular weight.

First-dimensional separation was performed by electrofocusing in glass tube gels (125mm x 5mm outer diameter, 3mm inner diameter) mounted on a Bio-Rad 2-D gel apparatus. The tube gels were composed of 4.5% acrylamide, 0.06% bisacrylamide, 9.5M urea, 2% Triton X-100 and 0.2% carrier ampholyte mixture (ampholines, pI 3.5-10; LKB). Polymerisation was initiated by the addition of 10ul of 10% ammonium persulphate and 5ul TEMED. The gel mixture was pipetted into the gel tubes up to within 0.5cm of the top, overlaid with water-saturated 2,-butanol and allowed to set for approximately 60 minutes. After aspiration of the overlay, the gels were placed in the electrophoresis apparatus containing 10mM phosphoric acid (H_3PO_4) in the lower reservoir, connected to the positive terminal (anode), and 20mM NaOH in the upper reservoir, which was connected to the negative terminal (cathode).

Protein samples were resuspended in 2-D sample buffer (9M urea, 5% 2-mercaptoethanol, 2% NP-40, 0.1% SDS and 2% ampholines, pI 6.5-9.0). About 25ul of sample, containing approximately 20ug protein, was loaded onto each gel, except for one gel which was loaded with a solution of marker proteins (Pharmacia isoelectric focusing calibration kit for proteins in the range pI 3-10). The gels were run at 100 volts for 30 minutes, then at 200 volts for 2 hours, followed by 400 volts for 16 hours and, finally, at 800 volts for 30 minutes. The tube gels were extruded gently

with distilled water applied with a syringe. They could be stored at this point wrapped in parafilm and put at -20°C .

For the second dimensional separation, a resolving slab gel containing 13% acrylamide was prepared as described in section 2:7:1:2, but using a front bevelled plate instead of a normal front plate. The first-dimensional rod gels were placed in 2-D equilibration solution containing 3% SDS, 50mM Tris-HCl (pH 6.5) and 10% glycerol for 60 minutes. The rod gel was then laid on top of the stacking gel (noting the polarity of the gel), and sealed to the stacking gel by overlaying the tube gel with warm 1% agarose solution containing 0.1% bromophenol blue. A marker track at the extreme left-hand-side was then loaded with SDS-PAGE marker protein solution, and the gel run overnight at 60 volts. After electrophoresis, the gel could be stained or Western blotted, as described in sections 2:7:2 and 2:7:3.

2:7:5:1 Peptide Mapping by Limited Proteolysis **using SDS-PAGE**

The Cleveland method (Cleveland et al., 1977) was used for peptide mapping. First, the polypeptides of interest were located after SDS-PAGE by staining the gel in Coomassie solution for 20 minutes and destaining for not more than 60 minutes (see section 2:7:2). Selected polypeptide bands were excised from the gel with a scalpel. The gel slices could then be stored at -20°C , if necessary. Otherwise, polypeptide contained in the gel slice was digested during a second electrophoretic separation. The gel slices were

incubated in protease buffer (0.125M Tris-HCl pH 6.8, 0.1% SDS) for 60 minutes at 30°C. The slices were then inserted into the wells of a 1.5x normal thickness (2.25mm) acrylamide gel, with a 5cm stacking gel, and a 12-22.5% polyacrylamide gradient resolving gel. The slices were then overlaid with protease buffer containing 20% glycerol and then with a mixture of 10ul protease buffer/20% glycerol solution and 10ul of protease V8 (0.17mg/ml), from Staphylococcus aureus (Sigma), diluted in protease buffer, and followed by 10ul of buffer with 0.001% bromophenol blue and 20% glycerol. The gel was run at 125 volts (20 mAmps), with recirculating warm water to give a running temperature of around 37°C, until the dye front had reached two-thirds of the way down the stacking gel. At this point the power was switched off and the proteins left to digest for 30 minutes. Then the power was turned back on and the gel left to electrophorese overnight, at room temperature, as normal.

2:7:5:2 Electroblotting for Protein Sequencing

Peptides separated by SDS-PAGE were first transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting. To ensure minimum contamination of glycine from the gel in the sequencing reactions, the gel was rinsed several times in CAPS transfer buffer (10mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), pH 11.0, and 10% methanol). Transfer conditions and times were as previously described (see section 2:7:1:3) and, after transfer, the membrane was stained for 15 minutes in 0.2%

Coomassie brilliant blue R250, in a 50% methanol/10% acetic acid solution, and destained in 90% methanol/2% acetic acid for approximately 5 minutes. Following a brief wash in distilled water, the membrane was allowed to air dry at room temperature. The peptide bands of interest were cut out of the membrane and sequenced directly using a gas-phase sequencer. All sequencing was performed by Dr M. Cusack of the Geology Department, Glasgow University.

2:7:6 Periodic Acid Schiff (PAS) Staining

The technique of PAS staining was used to detect the presence of glycoproteins in a mixture of polypeptides separated by SDS-PAGE. After SDS-PAGE, the gel was immersed in 12.5% trichloroacetic acid (TCA) for 30 minutes and then rinsed briefly in distilled water. Following this, the gel was immersed in a solution of 1% periodic acid in 3% acetic acid for 50 minutes. After several washes (15 minutes each) in distilled water, the gel was immersed in Schiff's reagent (Sigma), in the dark, and left for 50 minutes. The gel was then washed three times in freshly prepared 0.5% sodium metabisulphite for 10 minutes, and finally washed in distilled water with frequent changes, on a rocking platform, until the stained bands (pink) could be seen clearly.

2:7:7 Periodate Oxidation on Nitrocellulose in conjunction with Western Blotting

Periodate oxidation, following Western blotting, was

carried out essentially as described by Woodward et al., 1985. The nitrocellulose was stained with Ponceau-S and the blot cut into strips corresponding to each sample well. The strips were rinsed in a solution of 0.02M sodium acetate pH 5.4, 0.05M NaCl, 0.005M sodium azide (NaN_3) and then incubated in the same buffer containing 0.01M sodium metaperiodate for 60 minutes. Control strips were incubated in buffer alone. The strips were then rinsed three times in distilled water, incubated in 0.15M NaCl, 20mM Tris-HCl pH 7.2, 0.2M glycerol for 20 minutes and rinsed a further three times in 0.15M NaCl, 20mM Tris-HCl pH 7.2. The strips were then exposed to primary antibody and underwent the method of immunodetection as described in section 2:7:3.

2:8:1 Enzymatic Glycoprotein Cleavage

Various approaches were taken to remove the carbohydrate linkages from T.annulata glycoproteins. N-glycosidases, O-glycosidases and an inhibitor of N-glycosylation were used for deglycosylation purposes. SDS-extracted T.annulata (Ankara) piroplasm extracts were used in these experiments. A piroplasm pellet was resuspended in 100ul distilled sterile water and SDS added to a final concentration of 1%. The sample was diluted 1:10 in water, boiled for 5 minutes and allowed to cool before Nonidet P-40 was added to a final concentration of 0.6% to avoid inactivation of the enzyme by SDS. All samples were incubated at 37°C in a total volume of 100ul, with the appropriate amount of enzyme and with protease inhibitors (20ug/ml aprotinin and 2mM phenylmethylsulfonylfluoride). All enzymes were

obtained from Boehringer Mannheim and were used according to the manufacturers instructions.

N-Glycosidase F (PNGase F) : The sample was incubated with 5 units of PNGase F in the presence of 0.25M sodium phosphate buffer, pH 7.5, 10mM EDTA and 10mM 2-mercaptoethanol. Bovine calf fetuin (Sigma), at 5mM, was used as a control for deglycosylation with the same incubation conditions as above.

Endoglycosidase F : The sample was incubated with 0.36 units of endoglycosidase F in the presence of 0.25M sodium acetate, pH 6.5, 20mM EDTA and 10mM 2-mercaptoethanol. Again, fetuin was used as a control.

Endoglycosidase H : 40mU of the enzyme was used in the presence of 50mM sodium phosphate buffer.

O-Glycosidase : The sample was incubated with 2mU of O-glycosidase in a solution of 0.2M sodium phosphate buffer, pH 6.1, 100mM EDTA and 10mM 2-mercaptoethanol. Fetuin was used as a control, as described above.

In addition to the above enzymes, which cleave glycosides at the position where the carbohydrate attaches to the peptide, a range of enzymes were used which cleave specific glycosyl residues only. These were used on SDS-extracts of piroplasms, prepared as described above, in a total volume of 100ul, and incubation was at 30°C for 18 hours. The enzymes (Sigma) are listed below, along with the amount of enzyme and the buffer used.

Neuraminidase (sialidase from Vibrio cholerae) : 0.5 units were added in 50mM sodium acetate pH 5.5, and 4mM calcium

chloride, final concentration.

Alpha-D-mannosidase (from Jack Beans) : 0.44 units were added in 3M ammonium sulphate, 0.1mM zinc acetate pH 7.5.

Beta-D-Galactosidase (from E.coli) : 6.7 units were added in 0.1M sodium phosphate buffer pH 7.3, 10mM 2-mercaptoethanol and 0.03M magnesium chloride.

Beta-D-acetyl hexosaminidase (from Jack Beans): 0.625 units were added in 2.5M ammonium sulphate pH 7.0.

Alpha-Galactosidase (from Aspergillus niger): 0.6 units were added in 3.5M ammonium sulphate, 50mM sodium acetate pH 5.5.

Alpha-L-Fucosidase (from bovine kidney): 0.025 units were added in 3.2M ammonium sulphate, 10mM sodium dihydrogen phosphate and 10mM citrate pH 6.0.

2:8:2 Inhibition of Glycosylation in Culture

Tunicamycin (Boehringer Mannheim) was added to the culture medium during heat-induction of differentiation (see section 2:2:2). It was added at day 2 of culture and, in separate experiments, at day 4 and at day 6, at a final concentration of 1ug/ml, in a total volume of 10ml culture medium. The cultures were fed every second day as normal (section 2:2:1), but the cultures which had already been tunicamycin-treated were diluted with TBL medium containing 1ug/ml tunicamycin. Protein extracts were made before tunicamycin addition and at 2 day intervals thereafter.

2:8:3 Lectin Binding

A range of biotinylated lectins were used to investigate the specificity of lectin binding to T.annulata glycoproteins. The lectins used were contained in a Lectin-Link kit (Genzyme), and a mixture of glycoprotein standards were also provided as controls for lectin binding.

Piroplasm samples and the glycoprotein standard samples were first separated on an SDS polyacrylamide slab gel, electroblotted onto nitrocellulose, and stained with Ponceau-S (section 2:7:3), so that the nitrocellulose filter could be cut into strips. Each strip was incubated with a different biotinylated lectin, and bound lectin was then complexed with avidin-alkaline phosphatase, and then developed with the NBT/BCIP staining reagent, as described in section 2:7:3. Hence, lectin binding could be visualised by the appearance of dark brown bands. The five lectins contained in the Lectin-Link kit were Concanavalin A, Ricinus communis agglutinin, Datura stramonium agglutinin, Phaseolus vulgaris erythrolectin and wheat germ agglutinin. These were used according to the manufacturers instructions.

2:9 Triton X-114 Phase Separation

Triton X-114 was employed to isolate piroplasm integral membrane proteins. The method of phase separation used was that of Bordier (1981) and Rogers et al. (1988). The Triton X-114 (Sigma) was first condensed. Neat Triton X-114 (10ml) was added to 500ml ice cold PBS and thoroughly mixed. This

was then placed in a 37°C incubator and left for 16 hours. The upper aqueous layer was discarded and a further 500ml of ice cold PBS added to the detergent layer. This was homogenised, by shaking, and left at 37°C for 16 hours. The upper layer was again discarded and the whole process repeated. After the final step, the lower phase (50ml of 11.4% Triton X-114) was placed into a 50ml screw-capped nylon tube (Nunc) and stored at 4°C until required.

A pellet of T.annulata piroplasms (approximately 50ul) was homogenised in an eppendorf by vortexing in 200ul PBS. To this, 1ml of ice cold 0.5% Triton X-114 was added, thoroughly mixed and left on ice for 60 minutes, vortexing every 10 minutes. The sample was centrifuged at 10,000g for 10 minutes at 4°C to separate out detergent-insoluble material. The supernatant was then loaded onto a 1ml 6% sucrose cushion and this was incubated in a 37°C water bath for 5 minutes. The sample was centrifuged for 5 minutes at room temperature at 10000g to give an upper aqueous phase and a lower detergent-rich phase (visible as an "oily" pellet). The phases were separated by pipetting, and the layers were re-extracted to ensure maximum purity. To the upper layer, 200ul of 11.4% Triton X-114 was added, mixed, and left at 37°C for 5 minutes. This was then centrifuged as before, and the upper aqueous layer was retained. This layer contained the hydrophilic (aqueous) polypeptides. The lower detergent phase was resuspended in 200ul of ice cold PBS, loaded again onto a 6% sucrose cushion, incubated at 37°C for 5 minutes and centrifuged as before. The detergent phase pellet was resuspended in 200ul ice cold PBS. This

phase contained hydrophobic or amphiphilic polypeptides.

2:10 GPI Anchor Detection

Many integral membrane proteins are anchored in the membrane by covalent attachment to a glycosylphosphatidylinositol (GPI) anchor. If a GPI-anchored protein is cleaved at the anchor attachment, the protein becomes no longer membrane-associated, but aqueous phase-associated, and this alteration can be monitored by Triton X-114 phase separation, as described above. Furthermore, the action of GPI- or PI-specific phospholipases may generate a carbohydrate epitope, the cross-reacting determinant (CRD), which can be detected using polyclonal antiserum (anti-CRD antiserum) raised in rabbits immunised with soluble Trypanosoma brucei variant surface glycoprotein (VSG) (Zamze et al., 1988).

Three phospholipase C (PLC) enzymes were used; PI-PLC from Bacillus thuringiensis (Oxford Glycosystems), PI-PLC from Bacillus cereus (Sigma) and GPI-PLC from T.brucei (Oxford Glycosystems). A T.annulata piroplasm sample was subjected to Triton X-114 phase separation, as described in the previous section. The detergent fraction was diluted 1:1,000 in PBS so that the Triton X-114 would not interfere with enzyme action. 40ul of diluted sample was treated with PLC (10mU of B.thuringiensis PI-PLC, 50mU of B.cereus PI-PLC and 2000U of T.brucei GPI-PLC), in a total volume of 50ul, at 30°C for 60 minutes. The membrane form of the VSG of T.brucei was used as a control substrate for the PLC enzymes at a concentration of 0.1mg/ml. Treated and

untreated samples and controls were then re-extracted with Triton X-114, and the aqueous and detergent phases separated, as described in the previous section. The various phases were then analysed by SDS-PAGE, Western blotted, and probed with anti-CRD anti-serum (Oxford Glycosystems), using the protocol described in the manufacturers instructions.

2:11 Gelatin Gel/Protease Assay

Sample proteinases were analysed after non-reducing SDS-PAGE (12% acrylamide), by using Coomassie blue stain to detect their hydrolysis of gelatin co-polymerised in the SDS-PAGE gel (Lockwood et al., 1987). The method of SDS-PAGE is given in section 2:7:1, except 3ml of distilled water in the normal resolving gel mix was replaced with 3ml of a 3% gelatin stock solution, to give a final concentration of 0.3% gelatin in the gel. Also, the protein sample solutions, which were extracted in SDS-sample buffer (section 2:3:1), were not boiled, so that any protease activity present was not inactivated. After SDS-PAGE, the gel was soaked in 1% Triton X-100 for 60 minutes to remove SDS contained in the gel. The gel was then transferred to a 0.1M glycine solution pH 8.0, incubated at 37°C overnight, and then fixed and stained by immersing in a 0.1% solution of Coomassie Blue in 30% methanol/10% acetic acid. After destaining in 30% methanol and 10% acetic acid, areas of protease activity could be detected as clear bands on the gel where protease had digested the gelatin.

2:12 Recombinant DNA techniques

2:12:1 Theileria annulata Gene libraries

Two gene libraries were used - a T.annulata (Hissar) lambda gt11 genomic expression library made by Dr F.R.Hall (W.U.M.P., Glasgow); and a lambda-Dash genomic library, made by Dr J.Kinnaird (W.U.M.P., Glasgow), and generated from the Sau3A partial digestion of DNA of a merozoite-enriched fraction from a cloned T.annulata (Ankara) macroschizont-infected line (D7).

2:12:2 Host Bacteria

E.coli Y1090 was the host bacterium used for bacteriophage lambda gt11 and E.coli PLK17 was the host used for lambda-Dash. Y1090 cells (Promega) and PLK 17 cells (Stratagene) were stored as glycerol stocks. The bacteria were streaked out from stock onto LB plates (L-broth; 1% bactotryptone, 0.5% yeast extract, 1% sodium chloride, in 1.5% bactoagar, 10mM magnesium sulphate and, for Y1090 only, 100ug/ml ampicillin) and the plates were incubated overnight at 37°C. A single colony was picked from the plate into 10ml L-broth supplemented with 10mM magnesium sulphate, 0.2% maltose and (Y1090 only) 100ug/ml ampicillin. The bacteria were grown overnight to stationary phase at 37°C in an orbital incubator. 50ml of supplemented L-broth was inoculated with 0.5ml of the overnight culture and the bacteria grown at 37°C in the orbital incubator until the culture optical density had reached 0.5 at 600nm (2.5×10^8 cells/ml). The culture was then centrifuged at 800g for 5 minutes at 4°C, and the bacterial pellet resuspended in 5ml

10mM magnesium sulphate or 5ml SM buffer (50mM Tris-HCl pH 7.5, 10mM magnesium sulphate, 0.1M sodium chloride and 0.01% gelatin). The resuspension was stored at 4°C and used within 3 days.

2:12:3 Library Immuno-screening

To screen the T.annulata genomic lambda gt11 library, 70,000 plaque forming units, in SM buffer, were incubated with 300ul of a Y1090 cell suspension at room temperature for 15 minutes. The cells were added to 7ml of warm top agarose (L-broth with 0.7% agarose; Sigma type-1, low EEO) and poured onto a 200mm x 200mm LB plate (the agar containing 100ug/ml ampicillin). Once set, the plate was incubated at 42°C to inactivate the phage vector temperature-sensitive repressor (c1857) and allow phage growth (Huynh et al., 1985). After 4-5 hours, phage plaques became visible on the lawn of E.coli cells. Meanwhile, a square of nitrocellulose (180mm x 180mm) was soaked in a solution of 10mM isopropyl-1-thio-b-D-galactosidase (IPTG) in water and then air dried. The impregnated filter was laid over the plaques and the plate was incubated at 37°C for a further 2-4 hours, during which time vector beta-galactosidase expression was induced by the IPTG. The LB plate was chilled at 4°C and the filter removed, briefly rinsed in TBS, and transferred to blocking buffer (5% dried skimmed milk and 10% heat-inactivated horse serum in TBS) for a minimum of 2 hours.

The filter was incubated overnight with anti-E3d serum

(section 2:4:1:2), at a 1:300 dilution in blocking buffer. The filter was washed in TBS, incubated with anti-rabbit second antibody conjugated with alkaline phosphatase, and washed again, as described in section 2:7:3. Positive plaques were identified on the filters by the detection of alkaline phosphatase activity (section 2:7:3), picked, and transferred to 1ml of SM buffer containing 1% chloroform. Some negatively staining plaques were also picked as controls.

Verification of the positives was carried out by replating each pick, rescreening each with the anti-E3d serum, as before, and reisolating presumptive positive clones. Picks were also screened with pre-immune rabbit serum at a 1:200 dilution, as a control. For these subsequent screens, the phage (approximately 3,000 plaque forming units) were adsorbed onto 100ul Y1090 suspension and plated onto 90mm diameter circular LB plates in 2.5ml top agarose. Replating and rescreening was carried out until the picks were pure.

2:12:4:1 Library Oligonucleotide Screening

Two oligonucleotides (the ANG-oligonucleotide and PNN-oligonucleotide) were synthesised (Genetics Department, Glasgow University) from amino acid sequence data generated from the V8 protease digestion of the 30 kDa polypeptide (see section 2:7:5), and the oligonucleotides were synthesised to give all possible combinations of base sequence. The oligonucleotides were end-labelled with p^{32} and used to screen the D7 lambda-Dash library by

hybridisation. The amino acid sequences and their derived oligonucleotide sequences are given in the results section of Chapter 6, in Figure 6.10A.

2:12:4:2 Oligonucleotide End labelling

Approximately 200ng of each oligonucleotide was added to 1ul of x10 polynucleotide kinase buffer (PNK buffer; 500mM Tris-HCl pH 7.6, 100mM magnesium chloride, 50mM DTT, 1mM spermidine, 1mM EDTA), and 2ul (30 pmol) gamma ATP-P³² (Crude ATP 7000 Ci/mmol, ICN Biomedicals), and the total volume made up to 10ul with distilled water. Finally, 0.5ul (5-6 units) of T4 Polynucleotide Kinase (T4 PNK, Gibco BRL) was added and the reaction was incubated at 37°C for 20 minutes before an additional 0.5ul of T4 PNK was added, mixed and incubated as before. After the final incubation, 1ml of Tris-EDTA buffer (TE; 10mM Tris-HCl pH 7.4, 1mM EDTA) was added. Unincorporated gamma-P³² was removed by chromatography on DE-52 cellulose (Whatman). The mixture was loaded onto a column of DE-52 (bed volume, approximately 400ul), which had previously been equilibrated in a solution of 0.2M NaCl, 10mM Tris-HCl pH 7.4 and 1mM EDTA. After loading, the column was washed with 4x1ml aliquots of TE. Unincorporated P³² was removed from the column with 5x1ml aliquots of TE, 0.2M NaCl. Finally, the labelled oligonucleotide was recovered by elution with 4x1ml aliquots of TE, 0.5M NaCl, and used directly in hybridisation experiments.

2:12:4:3 Oligonucleotide Screening

To screen the D7 lambda-Dash library, 30,000 plaque forming units were adsorbed onto 1.5ml of the host bacterial suspension, PLK 17, by incubation at room temperature for 20 minutes. The lambda-Dash vector is known to produce small plaques, so it was desirable to retard bacterial growth, and so allow the development of larger plaques, by using BBL trypticase in the incubation media, in preference to bactotryptone and yeast extract (section 2:12:2). Top agarose (7ml of a solution of 1% BBL trypticase, 0.5% sodium chloride and 0.7% agarose) was added to the incubating bacterial mixture and poured onto a 140mm diameter circular plate containing BBL/agar (1% BBL trypticase, 0.5% sodium chloride, 1.5% Difco agar). The plate was incubated at 37°C overnight, and chilled at 4°C for 2 hours. A circle of nitrocellulose filter was laid on top of the plate and left for 1 minute. The filter was then removed and immersed (DNA side up) in a shallow tray of denaturing solution (0.5M NaOH, 1.5M NaCl) for 7 minutes, transferred twice to a tray of neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH 7.4) for 3 minutes, rinsed in 2x SSC buffer (0.15M NaCl, 0.015M sodium citrate pH 7.0), 0.5% SDS and allowed to air dry. To fix the DNA onto the nitrocellulose, the filter was baked at 80°C with a vacuum of 800 mBar for 2 hours.

The filter was hybridised with approximately 20ng p³² labelled oligonucleotide in 15ml Pipes hybridisation buffer (50mM Pipes pH 6.8, 100mM NaCl, 50mM sodium phosphate buffer, 1mM EDTA and 5% SDS) at 40°C for 18 hours. The

filters were then washed for 30 minutes at 4°C in TMA buffer (3M Tetramethylammonium chloride (TMAC, Sigma), 2mM EDTA and 50mM Tris-HCl pH 8.0) and for 30 minutes in the same buffer at room temperature. Further washes were carried out at 50°C, at 60°C and finally at 70°C, for 15 minutes each time in TMA buffer. The filters were exposed to autoradiography film (Kodak X-Omat AR) overnight at -70°C, and the film was developed by submerging the film in Kodak LX24 developing solution for 3 minutes in the dark, rinsed in 3% acetic acid for 30 seconds and fixed in Ilford Hypam fixing solution for 3 minutes. The film was washed in clear tap water and possible positive plaques identified. Picks were then taken from the original plates, and replated in order to verify the positives. In these subsequent rounds of oligonucleotide hybridisation, approximately 3000 plaque forming units were adsorbed onto 0.5 ml of PLK 17 suspension, and plated onto 90 mm diameter circular BBL/agar plates in 2.5 ml top agarose. Oligonucleotide-screening was repeated as before.

2:12:5:1 Preparation of Phage DNA

The recombinant phage sample was grown on LB plates (bacterial host Y1090) or BBL plates (host PLK 17) at 37°C overnight as described in section 2:12:2. Approximately 5,000 plaque forming units of phage were plated so that the plaques were confluent on a 90mm plate. The phage were harvested by overlaying the agar with 5ml SM buffer. A few drops of chloroform was added and the plates left for 5-6 hours. The liquid was then taken off and centrifuged at

800g and 4°C for 10 minutes. The supernatant contained amplified phage material, which could be used as a stock source.

The phage DNA was prepared using a modification of the Alloo method (Glascodine, 1989). An aliquot of the amplified phage stock (0.9ml) was transferred to an Eppendorf and centrifuged at 10,000g for 10 minutes. The supernatant (0.8ml) was transferred to another Eppendorf and treated with 0.6ml of a suspension of DEAE-cellulose (Whatman)/LB medium by inverting the mixture approximately 50 times. The Eppendorf was centrifuged again at 10,000g for 5 minutes and the supernatant was removed to an Eppendorf tube. The pellet contained the DE52-cellulose and bound bacterial proteins. To solubilise the phage, 150ul of extraction buffer was added (0.5M Tris-HCl pH 8.0, 0.25M EDTA and 2.5% SDS) to the supernatant, and the mixture incubated at 65°C for 15 minutes. The Eppendorf was cooled to room temperature and 200ul of 8M ammonium acetate added. The mixture was incubated on ice for 15 minutes so that phage proteins and RNA were precipitated. The precipitate was collected by centrifugation at 10,000g for 10 minutes, and the supernatant (0.9ml) was removed to another Eppendorf. 0.6ml isopropanol was added to the supernatant, and the mixture was incubated at room temperature for approximately 30 minutes. The precipitated DNA was harvested by centrifugation at 10,000g for 10 minutes. The pellet was washed with 1 ml 75% ethanol in TE, dried and resuspended in 40 ul TE.

2:12:5:2 Preparation of Insert DNA

Recombinant lambda gt11 DNA was digested with the restriction enzyme EcoR1 and lambda-dash DNA was digested with the enzyme Sal1. In both cases, digestion was carried out at 37°C for 2-3 hours, in a total volume of 20ul containing 2ul of x10 enzyme buffer (100mM Tris-HCl pH 7.5, 10mM magnesium chloride, 50mM NaCl for EcoR1; 6mM Tris-HCl pH 7.9, 7mM magnesium sulphate, 150mM NaCl, 6mM 2-mercaptoethanol for Sal1), 10-20 units of enzyme and 10-30 ug of DNA. Digestion was terminated by heating at 65°C for 5 minutes. The samples were chilled on ice and 2ul of gel loading buffer (100mM EDTA pH 7.5, 22% Ficoll 400, 0.05% Bromophenol blue) was added to each. The samples were then loaded onto an agarose gel and the DNA separated by gel electrophoresis, as described by Sambrook, Fritsch and Maniatis (1989). A 0.7% gel was prepared by dissolving 1.05g agarose (Sigma Ultrapure electrophoresis grade) in 150ml Tris-borate buffer (TBE, 89mM Tris-HCl, 89mM boric acid, 2mM EDTA, pH 8.0) with 0.5ug/ml ethidium bromide. Electrophoresis was at 30 volts overnight in TBE. An aliquot (1ul diluted in 17ul TE and 2ul gel loading buffer) of a ladder of DNA fragments, ranging from 12,216 base pairs to 75 base pairs (Gibco-BRL), was electrophoresed along side the DNA samples and, as standard markers, these enabled the estimation of the size of the insert which had been cut out of the vector. Bands were visualised by exposure to short wave ultraviolet light (366nm). For the isolation of insert DNA, digested DNA was separated on a gel containing 0.7% low melting point agarose (Sigma).

Following electrophoresis, the identified insert fragment was excised from the agarose gel.

2:12:6:1 Expression of Recombinant Fusion Protein

The DNA insert isolated from the recombinant lambda phage was ligated into the expression vector pGEX-1N (Pharmacia) and transfected into the bacterial host XL-Blue (Stratagene). Prior to ligation the insert DNA was purified as follows. The isolated agarose band containing the insert DNA (see previous section) was placed into a eppendorf tube. The agarose was then melted at 65°C for 5 minutes, and NaCl was added to the agarose to give a final concentration of 0.5M NaCl. The solution was then extracted with an equal volume of phenol saturated with 0.5M NaCl, shaking vigorously for 10 minutes, followed by centrifugation at 10,000g for 10 minutes. The aqueous phase was then reextracted, in the same way as above, with a 24:1 solution of chloroform:isomyalcohol. 5ug of glycogen was added to the aqueous phase, and an equal volume of TE buffer added. The DNA was then precipitated with two volumes of ethanol, and isolated by centrifugation at 10,000g for 10 minutes. The DNA pellet was washed with 1ml 75% ethanol in TE, dried and resuspended in 9ul distilled water.

The plasmid pGEX-1N is constructed to give a fusion protein with the carboxyl terminus of the Schistosoma japonicum glutathione S-transferase protein, and expression is induced with IPTG. The bacteria were plated onto an LB

plate, and grown overnight at 37°C.

Picks of transformed colonies were taken into 5ml aliquots of LB medium supplemented with 100ug/ml ampicillin and the bacteria grown at 32°C overnight. The overnight culture was centrifuged at 800g for 5 minutes and resuspended in 10ml of LB. The bacteria were grown for 2-3 hours at 32°C until the optical density at 560nm was 1.0. The culture was induced with 5mM IPTG and incubated for a further 2 hours at 32°C. The bacteria were then centrifuged at 800g for 10 minutes and resuspended in 500ul PBS and 1% Triton X100. A sample (60ul) of the resuspended material was taken, 20ul of SDS-sample buffer added and the mixture boiled for 5 minutes. This aliquot was then analysed by SDS-PAGE (section 2:7:1) so that the fusion protein expression could be assessed.

The glutathione S-transferase (GST) fusion proteins were purified from lysates of the induced and pelleted bacteria with a Glutathione Sepharose 4B (Sigma) column. Glutathione, attached to the sepharose beads, binds GST and, after washing the column, the GST fusion protein can be eluted from the column, by addition of glutathione, and recovered. To lyse the bacteria, the cells were sonicated on ice. A Soniprep 150 sonicator was used with a 6mm probe. The bacteria were sonicated at 6 microns for 30 seconds, six times, with 30 second breaks in between each burst, and then centrifuged for 10 minutes at 800g and 4°C to pellet the unlysed material. The success of cell lysis was monitored by SDS-PAGE analysis of a sample of lysate.

The column used was 200mm x 16mm (Pharmacia), and all steps involving the column were performed at 4°C. The column was filled with 4ml (packed volume) of glutathione sepharose beads, and washed with 10x bed volumes of PBS. The column was then equilibrated with 3x bed volumes of PBS, 1% Triton X100. The column was loaded with the bacterial lysate and this was allowed to run through slowly. The column was washed with approximately 20 bed volumes of PBS, or until the wash had an optical density of zero at 280nm. The bound GST-fusion protein was eluted from the column with approximately 20 ml glutathione elution buffer (5mM reduced glutathione in 50mM Tris-HCl pH 8.0). The eluate was collected in 1ml fractions and the optical density of each was taken. Elution was stopped when the optical density fell to zero at 280nm. A sample of each fraction was taken to analyse by SDS-PAGE.

2:12:6:2 Generating Antibody to the Expressed Protein

Anti-serum was made against the GST fusion protein, purified from a glutathione sepharose column, as described in the previous section. Approximately 900ng of fusion protein in Complete Freund's adjuvant was inoculated into two mice, by intraperitoneal injection. After 3 weeks, the mice were injected again, with an identical amount of fusion protein in Incomplete Freund's adjuvant. 10 days later, a tail bleed was taken from the mice. The blood was incubated at 4°C for 60 minutes and then centrifuged at 10,000g for 10 minutes. The serum was removed from the red cell pellet and retained. This material was used as primary

antibody in Western experiments at a concentration of 1:50 (section 2:7:3). The mice were injected again, 4 weeks after primary immunisation, and a tail bleed taken 10 days after. The final injection was 10 weeks after the initial inoculation and a tail bleed was taken 14 days later. The second and third bleeds were treated in the same way as the first.

2:12:7:1 Southern Blotting

DNA was extracted from T.annulata (Ankara) piroplasms and from T.annulata cloned macroshizont-infected cell lines using standard techniques (Sambrook, Fritsch and Maniatis, 1989). Approximately 5ug of DNA was digested with EcoR1 as described in section 2:12:5:2. The DNA fragments were resolved by agarose gel electrophoresis at 30 volts overnight in TBE buffer and the DNA transferred to nylon membrane filters (Hybond-N, Amersham) by capillary blotting. The gel was first washed with a solution of 0.25M HCl for 15 minutes and rinsed in distilled water twice. The gel was then denatured in a solution of 1.5M NaCl, 0.5M NaOH for 60 minutes, neutralised in 2M NaCl, 0.5M Tris-HCl (pH 7.4) for 30 minutes and equilibrated in transfer buffer (50mM phosphate buffer; 25mM NaH₂PO₄ pH 5.5-6.0 with 25mM Na₂HPO₄). The DNA was transferred to nylon by capillary action for at least 18 hours using transfer buffer. The nylon filter was removed and the DNA fixed by exposure to short wave ultra-violet light for 5 minutes.

2:12:7:2 Southern blot Hybridisation

Recombinant DNA was purified by the method given in section 2:12:5:1, the DNA was restricted as described in section 2:12:5:2, run on a 0.7% low melting point agarose gel and the insert DNA excised. Distilled water was added to give a final concentration of approximately 100ug/ml DNA. The agarose was melted by boiling for 5-10 minutes and the DNA was labelled with P³² by random priming (Boehringer Mannheim, Random Priming Kit). To 9ul of DNA (approximately 1ug), 3ul of 2mM each of three dNTPs (a mixture of dATP, dTTP and dGTP), 2ul of 10x reaction mix (500mM Tris-HCl pH 7.5, 100mM MgCl₂, 10mM dithiothreitol, 500ug/ml bovine serum albumin), and 5ul alpha-P³² dCTP (specific activity 10 mCi/ml) were added. Finally, 1ul (1 unit) of Klenow fragment enzyme was added and the reaction was incubated at 37°C for 30 minutes, after which time 80ul of TE buffer was added to stop the reaction, and the DNA was denatured by boiling the sample for 5 minutes.

Prior to hybridisation, the Southern blot (prepared as described above) was first incubated in 15 ml PIPES hybridisation buffer (section 2:12:4:3), at 65°C for 30 minutes. The buffer was drained from the blot, and the labelled DNA was then added to the Southern blot in a further 15 ml PIPES hybridisation buffer. Hybridisation was carried out at 65°C overnight. The filter was then washed three times with 2xSSC buffer, 5% SDS for 15 minutes at 65°C and then exposed to autoradiography film (Kodak) overnight at -70°C. The film was developed, as described in section 2:12:4:3.

2:12:8 RNA Slot Blot Hybridisation

RNA from piroplasms (Hissar and Ankara stocks) and from uninfected and macroschizont-infected cell lines (BL-20 and T.annulata, Ankara stock, respectively) was prepared by the method of Lamers et al. (1982). The cells were lysed in 4M guanidine thiocyanate, 0.5% sodium N-lauroylsarcosine, 20mM Pipes, pH 7.0. Caesium chloride was added, to a final concentration of 1.4M, and 9 ml of the cellular extract was layered over a 2 ml cushion of 5.7 M caesium chloride, 0.1M EDTA, pH 7.0. The mixture was centrifuged at 33,000 rpm for 18 hours at 25°C in a SW 40.1 rotor. The pelleted RNA was dissolved in 10mM Hepes, 1mM EDTA, 0.1% SDS, pH 7.5 (0.1% diethyl pyrocarbonate (DEPC)-treated), and precipitated in 2.5 volumes of ethanol. The RNA was diluted in DEPC-treated phosphate buffer (50mM, pH 5.5) to give approximately 5ug of RNA in a total volume of 50ul. The solution was heated for 15 minutes at 65°C and was bound to nylon membrane filters (Hybond-N, Amersham), which had been presoaked in phosphate buffer, under vacuum using a hybri-slot manifold (Gibco BRL 24-well filtration manifold). The RNA was fixed onto the filters by exposure to short wave ultra-violet light for 5 minutes. The filters were probed with P³²-labelled DNA, using the same method as described in the previous section for Southern blot hybridisation.

CHAPTER THREE

DIFFERENTIATION OF MACROSCHIZONT TO MEROZOITE

3:1:1 Introduction

Like many parasitic protozoa, Theileria annulata has a biphasic life-cycle that involves an arthropod vector and a mammalian host. Transition from the tick vector to the bovine host exposes the parasite to a shift in temperature, from approximately 22°C-28°C in the tick, to 38.5°C in the cow (Blood et al., 1979). Furthermore, a fever response of the host to theilerial infection can increase the temperature of the infected animal by up to 3°C, to approximately 41°C.

A temperature shift from 38.5°C to 41°C in other eukaryotes normally results in a heat-shock response and a shut-down in overall protein synthesis (Lindquist, 1986). However, parasitic protozoa generally do not go into a quiescent state when heat-shocked (Hyde, 1990 ii), but continue to proliferate. In fact, it has been postulated that the differentiation of certain parasites may be initiated by the expression of specific heat-shock proteins. For example, in Leishmania major, a temperature shift induces differentiation of the parasite in vitro, and this process is associated with changes in heat-shock gene expression (Van der Ploeg et al., 1985). Differentiation of the macroschizont stage of Theileria annulata to the merozoite stage can also be induced in vitro, by elevating the temperature of macroschizont-infected cell culture from 37°C to 41°C (see sections 1:1:4 and 2:2:2), but there is limited evidence to suggest that the increased expression of heat shock proteins is involved in the initiation of macroschizont differentiation to the merozoite (Mason et

al., 1989 and section 1:1:4).

When differentiating to another life-cycle stage, parasites often undergo radical morphological changes (Van der Ploeg et al., 1985; Hyde, 1990ii). These changes are usually accompanied by differential and/or stage-specific expression of various parasite polypeptides, particularly surface polypeptides. For example, in the parasitic nematode Trichinella spiralis, the surface antigens expressed at each stage in the life-cycle are stage-specific, indicating both positive and negative regulation of polypeptide expression (Philipp et al., 1981). The T.annulata parasite also undergoes extensive morphological changes when differentiating from the macroschizont stage to the merozoite stage of development. The merozoite has a more structured morphology than the macroschizont (Shaw and Tilney, 1992). The macroschizont is surrounded by a plasma membrane that has no obvious outer surface coat, and other than nuclei and mitochondria, it has few distinct internal organelles (see section 1:1:1:1). The merozoite, however, has a thick external coat, rhoptries, distinct nuclei with associated mitochondria, ribosomes, endoplasmic reticulum and micronemes (Melhorn and Schein, 1984; Shaw and Tilney, 1992). In previous studies, monoclonal antibodies were generated against the sporozoite, macroschizont and piroplasm stages of T.annulata (Shiels et al., 1986; 1989; Williamson et al., 1989; Glascodine et al., 1990). In IFAs, where the expression of the monoclonal antibody-specific epitopes was monitored at different life-cycle stages, it was shown that there were changes in the monoclonal

Table 3.1 Reactivity of monoclonal antibodies with different life-cycle stages of T.annulata (from Glascodine *et al.*, 1990).

Monoclonal	Macroschizont	Merozoite	Piroplasm
2E4	+	+	+
1C7	+	-	-
1C12	+	-	-
1E11	+	-	-
5E1	-	+	+
2D5	-	+	+
1D11	-	+	+
5A10	-	-	+

+ reactivity
- no reactivity

antibody reactivity during differentiation of the macroschizont to merozoite. Therefore, these results indicated that, along with the morphological changes, alterations in polypeptide profile also took place. Furthermore, the monoclonal antibody reactivity showed that the merozoite is more closely related to the piroplasm than to the macroschizont, as some antibodies detected both merozoites and piroplasms, but none detected both merozoites and macroschizonts (Glascodine et al., 1990 and see Table 3.1). These observations indicated that substantial changes in epitope expression occur during merogony and that there is both positive and negative regulation during macroschizont differentiation (Glascodine et al., 1990). This implied that merogony is a major point of differentiation in the bovine phase of the T.annulata life-cycle.

Work on the merozoite stage of T.annulata has been hindered, previously, by the inability to produce sufficient quantities of merozoite material. This problem has been overcome by two developments; the development of an in vitro system for the reproducible production of merozoites (section 1:1:4) and the isolation of cloned macroschizont-infected cell lines, of a distinct parasite genotype, which produce large quantities of merozoites (Shiels et al., 1992 and section 1:1:4). A minority of the parasites of the uncloned T.annulata (Ankara stock) cell line (TaA₂) differentiated to merozoites, whereas more than 70% of the parasites of these cloned cell lines ("enhanced differentiating cell lines") produced merozoites (Shiels et

al., 1992 and section 1:1:4). This development has allowed the preparation of larger quantities of concentrated merozoites from a single parasite culture, allowing analysis of the molecular events which occur during merogony to be undertaken.

3:1:2 Summary and Aims

The differentiation of the macroschizont to the merozoite stage (merogony) is an important point in the bovine phase of the T.annulata life-cycle. Previous studies have shown that polypeptide expression is both positively and negatively regulated during this process.

The aim of the work presented in this chapter was to follow the molecular and structural events which occur during differentiation to the merozoite. Protein analysis techniques were employed to characterise temperature-induced and stage-specific polypeptides so that their appearance or disappearance could ultimately be correlated with morphological events occurring during parasite differentiation.

3:2 Results

3:2:1 Morphological changes induced in vitro by increased culture temperature

The growth of an enhanced cloned cell line (D7) and a diminished cloned cell line (D3) was monitored by the Giemsa-stain of cytopsin samples, and parasite development at 41°C was compared to development at 37°C. The host and parasite nuclei stained densely with the Giemsa stain (Fig. 3.1A, hn and mn, respectively) and before the induction of differentiation, the two clonal types differed in the average number of schizont nuclei present. It has been estimated that, at 37°C, the enhanced cloned cell lines have, on average, 20 nuclei per schizont, whereas the diminished cloned cell lines have an average of 13 nuclei per schizont (Shiels et al., 1992). Hence, at 37°C, the D7 macroschizont was observed to occupy approximately 10% of the host cell (Fig. 3.1A), whereas the D3 macroschizont occupied a slightly smaller area (Fig. 3.1D). The host cell in the top right-hand corner of Figure 3.1A is seen to be undergoing mitosis, as indicated by the presence of condensed chromosomes, which are arranged along the host spindle. The macroschizont nuclei (mn) are arranged along this spindle, so that during host cell division, the macroschizont is divided between daughter cells. At 41°C, the D7 macroschizonts were seen to enlarge, and the number of nuclei per macroschizont greatly increased. After four days at 41°C, the number of nuclei per schizont of the enhanced cloned lines had increased so that the parasite occupied almost half of the host cell cytoplasm (Fig.

Figure 3.1: Giemsa-stained cytospin preparations of cultured T.annulata (Ankara) cloned cell lines D7 and D3.

A-C; cloned cell line D7.

D-F; cloned cell line D3.

A; at 37°C.

D; at 37°C.

B; day 4 at 41°C.

E; day 8 at 41°C.

C; day 8 at 41°C.

F; day 12 at 41°C.

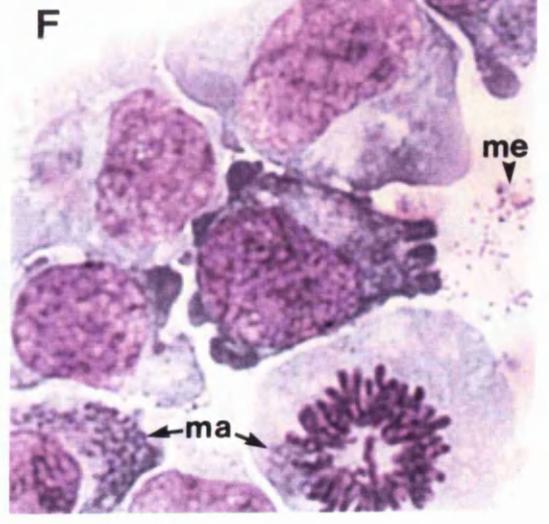
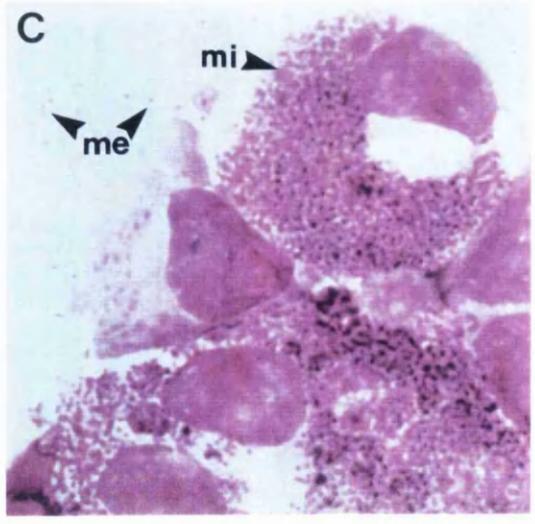
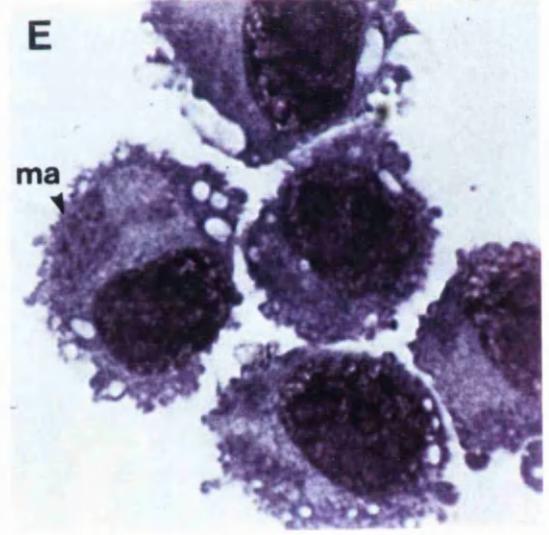
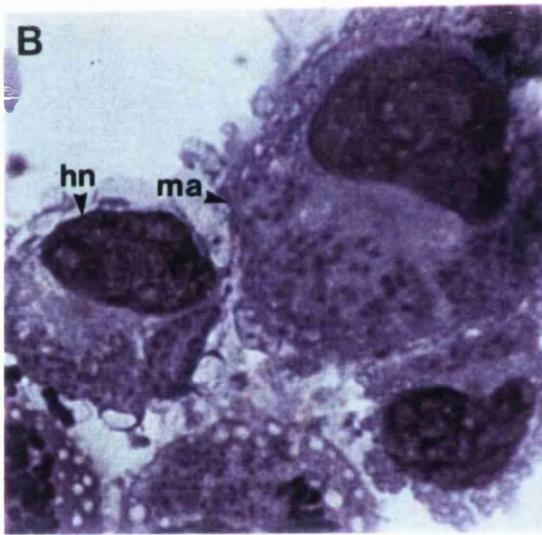
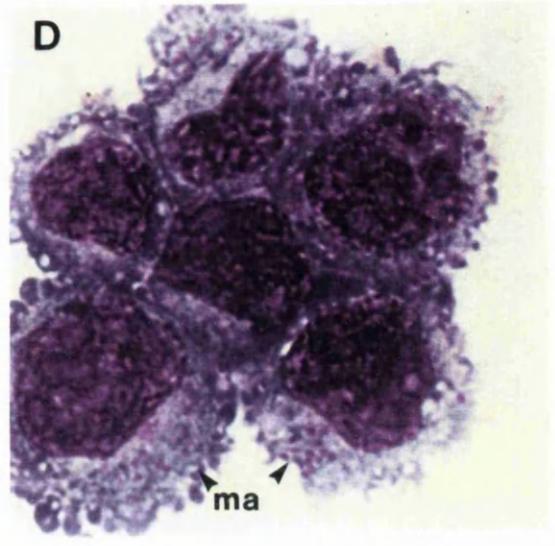
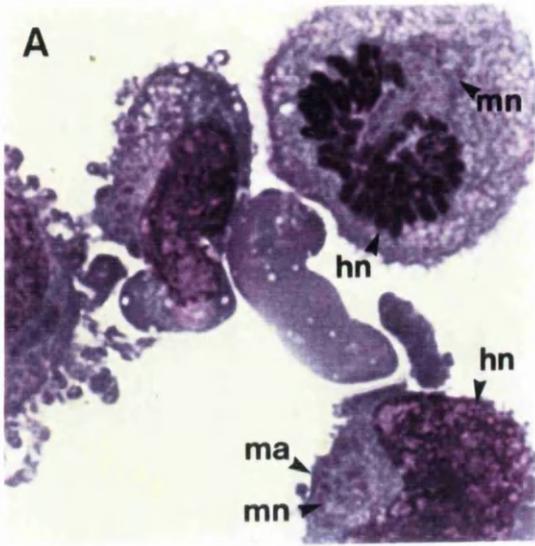
hn; host cell nucleus.

ma; macroschizont.

mn; macroschizont nuclei.

mi; microschizont nuclei.

me; merozoites.



3.1B). After this point, small densely staining particles appeared with the macroschizont nuclei (Fig. 3.1C), and were identified as microschizont nuclei (mi). The microschizonts developed into particles which appeared to burst out of the host cell (Fig. 3.1C), leaving residual schizont bodies behind. The extracellular particles each had a densely staining nucleus, and were identified as merozoites (me).

In contrast, after 8 days at 41°C, the level of differentiation of the diminished cell lines was not as extensive, and less than 10% of the infected host cells contained enlarged macroschizonts (Fig. 3.1E). At day 12, there were few merozoites released from the host cells (Fig. 3.1F). After 12 days at 41°C, the enhanced cloned cell lines were no longer viable, presumably because of extensive host cell breakdown associated with merozoite liberation. The diminished cloned cell lines survived however, and retained the morphology of macroschizont-infected cells indefinitely, with the occasional infected cell undergoing differentiation at random periods.

3:2:2 Immune bovine serum detection of merozoite antigens

Polypeptide extracts of macroschizont-infected cell lines, clone D7 merozoites and Ankara piroplasms were separated by SDS-PAGE and Western blotted. The tracks were probed with serum from an animal immune to T.annulata challenge (immune bovine serum 155, section 2:4:1:1), and separately, with pre-immune bovine serum as a negative control.

Figure 3.2: Western blot analysis of T.annulata (Ankara) antigens with immune bovine serum (155).

Panel A; Immune bovine serum detection.

Panel B; Pre-immune bovine serum detection.

Track 1; macroschizont-infected cell line (TaA₂).

Track 2; purified merozoites (D7).

Track 3; piroplasms (Ankara).

Position of molecular weight standards (kDa) indicated at left-hand-side (see section 2:7:1:3).

Position of 30 kDa molecule arrowed (panel A, track 3).

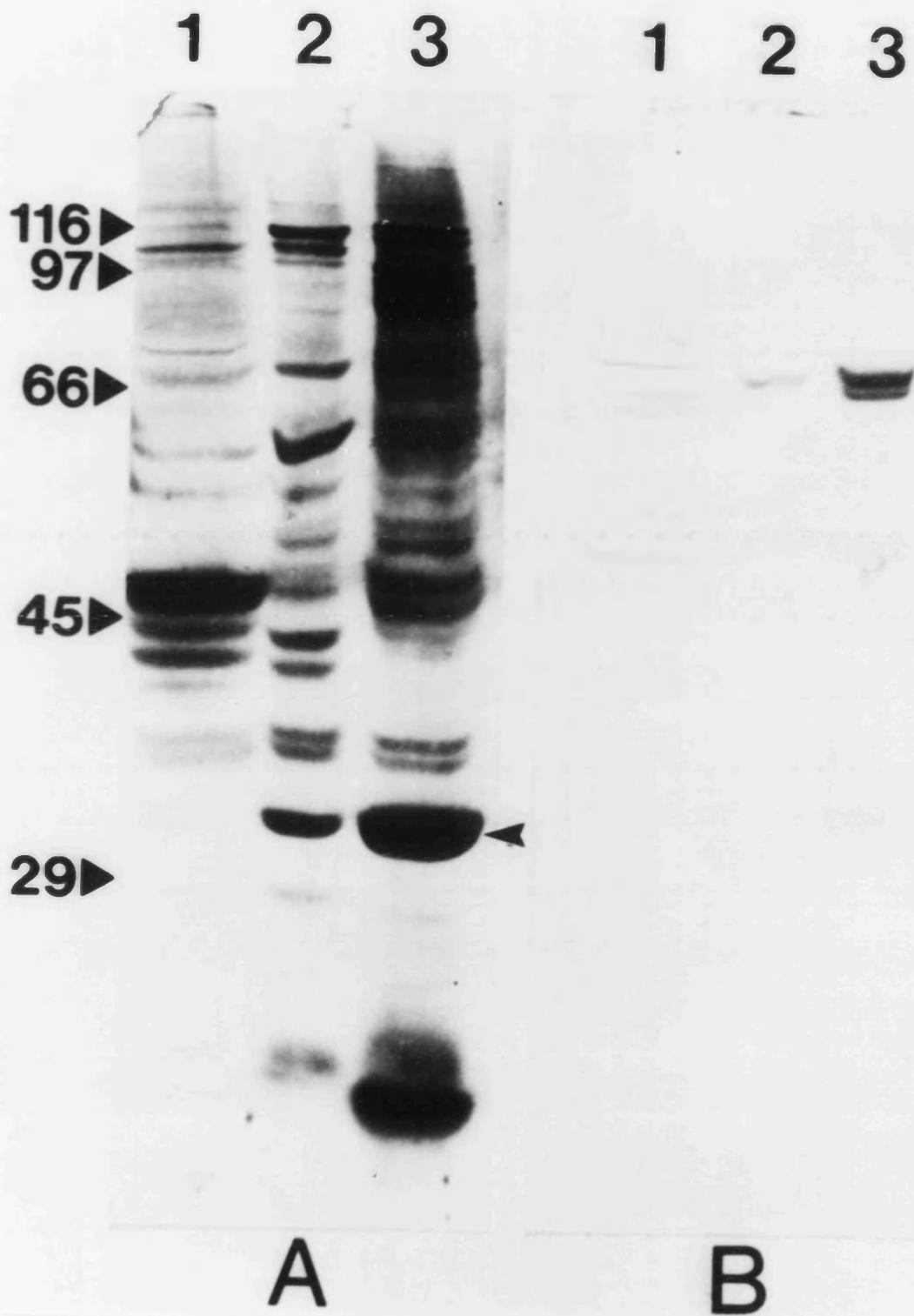


Figure 3.2 shows that many merozoite and piroplasm polypeptides were recognised by the immune serum (Fig. 3.2A, tracks 2 and 3), with reactivity at 30 kDa being especially prominent in both tracks (arrowed). This 30 kDa polypeptide was absent in the extract of macroschizont-infected cells (Fig. 3.2A, track 1). The band at approximately 15 kDa, detected in the piroplasm extract, is thought to be a product of polypeptide breakdown. The pre-immune serum recognised little (Fig. 3.2B, tracks 1 to 3), except for a polypeptide at approximately 70 kDa in the merozoite and piroplasm extracts, which was possibly the Theileria annulata 70 kDa heat-shock protein (Mason et al., 1989).

3:2:3 Detection of antigens during merogony

Polypeptide extracts of cloned cell lines were made at two day intervals during the heat induction of merogony. The protein concentration of each extract was determined by the Bradford's assay, and the SDS-PAGE gel was loaded with equal quantities of protein in each track. Westerns of the extracts were probed with immune bovine serum (155), and with monoclonal antibodies 5E1, 1D11 and 1C2.

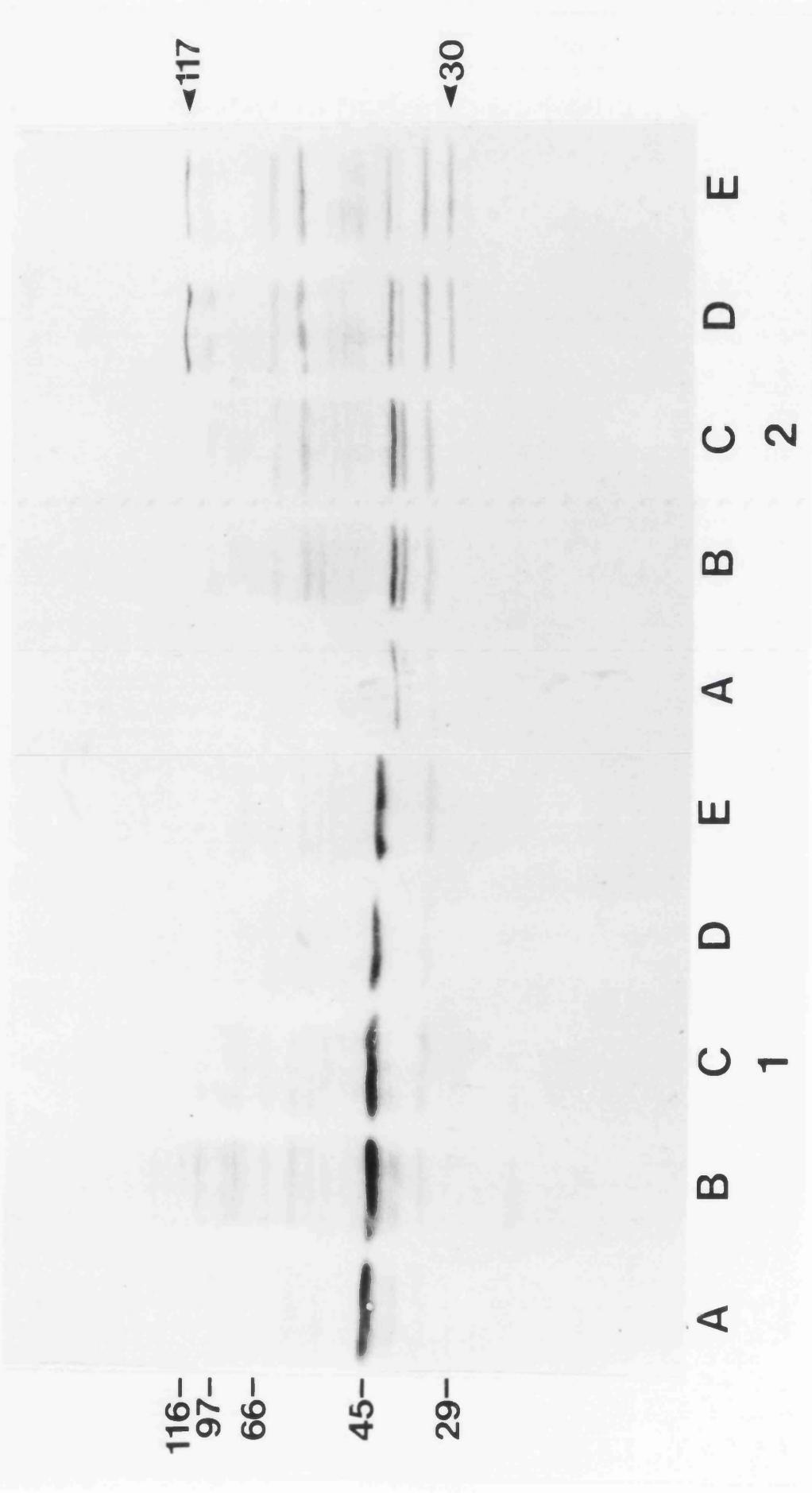
3:2:3:1 Immune bovine serum detection of antigens

Immune bovine serum was used to probe a Western blot of the differentiation time-course undergone by an enhanced cell line (D7; Fig. 3.3, panel 2) and a diminished cell line (D3; Fig. 3.3, panel 1). When compared to immune serum

Figure 3.3: Reactivity, on Western blots, of T.annulata (Ankara) cloned cell line D7 and D3 proteins with immune bovine serum during heat-induced merogony.

Panel 1; Cloned cell line D3.
Panel 2; Cloned cell line D7.

Track A; at 37°C.
Track B; day 2 at 41°C.
Track C; day 4 at 41°C.
Track D; day 6 at 41°C.
Track E; day 8 at 41°C.



116-
97-
66-
45-
29-

117
30

A B C D E
A B C D E
1 2

reactivity with the polypeptides of the D7 day 0 culture (maintained at 37°C; Fig. 3.3, track 2A), it could be seen that the immune serum did not detect any novel polypeptides in the D7 extracts derived from the day 2 and day 4 cultures (Fig. 3.3, tracks 2B and 2C). However, at day 6 of D7 culture at 41°C (Fig. 3.3, track 2D), two novel polypeptide bands were clearly detected by the immune serum at approximately 117 kDa and 30 kDa (arrowed). These proteins were detected in the D7 extract at day 8 also (Fig. 3.3, track 2E). The immune bovine serum did not detect novel polypeptides in any of the D3 cloned cell line extracts (Fig. 3.3, 1A-E). There were negligible differences between the pattern of polypeptides recognised in the 37°C D3 culture extract (Fig. 3.3, track 1A), and the recognition pattern of the immune serum with the D3 culture extracts at 41°C (Fig. 3.3, tracks 1B-1E). A major band, however, was detected in every extract of both the D7 and D3 time-courses; at 43 kDa in the D7 extracts and at 45 kDa in the D3 extracts. This band is most likely schizont polypeptide material, as it is absent in extracts of uninfected bovine lymphoma cells (BL-20 cells) and piroplasms (data not shown), but is present in the extracts of macroschizont-infected cells at 37°C. It was interesting to note that the intensity of the D7 43 kDa band increased after day 2 at 41°C (Fig. 3.3, 2B), and continued to increase until day 4 (Fig. 3.3, 2C). By day 6 and 8 however (Fig. 3.3, 2D and 2E), the intensity of this band decreased in the D7 line. In contrast, the intensity of the D3 45 kDa band remained constant throughout the D3 culture time-course.

Figure 3.4: Western blot analysis of cloned cell line D3 and D7 differentiation time-courses with monoclonal antibody 5E1.

Panel 1; Cloned cell line D3.
Panel 2; Cloned cell line D7.

Track A; at 37°C.
Track B; day 4 at 41°C.
Track C; day 6 at 41°C.
Track D; day 8 at 41°C.

Figure 3.5: Western blot analysis of cloned cell lines D3 and D7 time-courses with monoclonal antibody 1D11.

Panel 1; Cloned cell line D7.
Panel 2; Cloned cell line D3.

Track A; at 37°C.
Track B; day 2 at 41°C.
Track C; day 4 at 41°C.
Track D; day 6 at 41°C.
Track E; day 8 at 41°C.

66-

45-

36-

29-

24-

A B C D A B C D
1 2



116-
97-

66-

45-

29-

A B C D E A B C D E
1 2



3:2:3:2 5E1 detection of antigens

The D3 and D7 extracts, made at days 0, 4, 6 and 8, were probed with the monoclonal antibody 5E1. It can be seen from Figure 3.4 that the monoclonal did not react with the diminished cell line, D3, at any time-point tested (Fig. 3.4, tracks 1A-1D). However, when the extracts from the D7 cell line time-course was probed with the monoclonal 5E1, a polypeptide was clearly detected at day 6 and day 8 (Fig. 3.4, tracks 2C and 2D). This reactivity was observed to be with a protein of 30 kDa, and the relative molecular weight of this molecule was assessed to be identical to the polypeptide previously detected by this monoclonal antibody in extracts of merozoites and piroplasms (Glascodine et al., 1990).

3:2:3:3 1D11 detection of antigens

Figure 3.5 shows the reactivity of the monoclonal antibody 1D11 on a Western blot of extracts from the differentiation time-courses of the D7 and D3 cloned cell lines (Fig. 3.5, panels 1 and 2, respectively). The monoclonal antibody did not react with the extracts from the D3 cloned cell line at any of the time-points (Fig. 3.5, panel 2), but, as with the monoclonal 5E1, reactivity was strong with the D7 cloned cell line at days 6 and 8 (Fig. 3.5, 1D and 1E). The detected protein was estimated to be 117 kDa molecular weight. Some weak reactivity against several smaller proteins was also detected in the day 8 extract (Fig. 3.5, track 1E), which were thought to be breakdown products from the 117 kDa molecule.

Figure 3.6: Western blot analysis of D3 and D7 cloned cell line merozoites with monoclonal antibody 1C2.

Track 1; D3 merozoite extract.

Track 2; D7 merozoite extract.

Track 3; Piroplasm (Ankara) extract.

116-

97-

66-

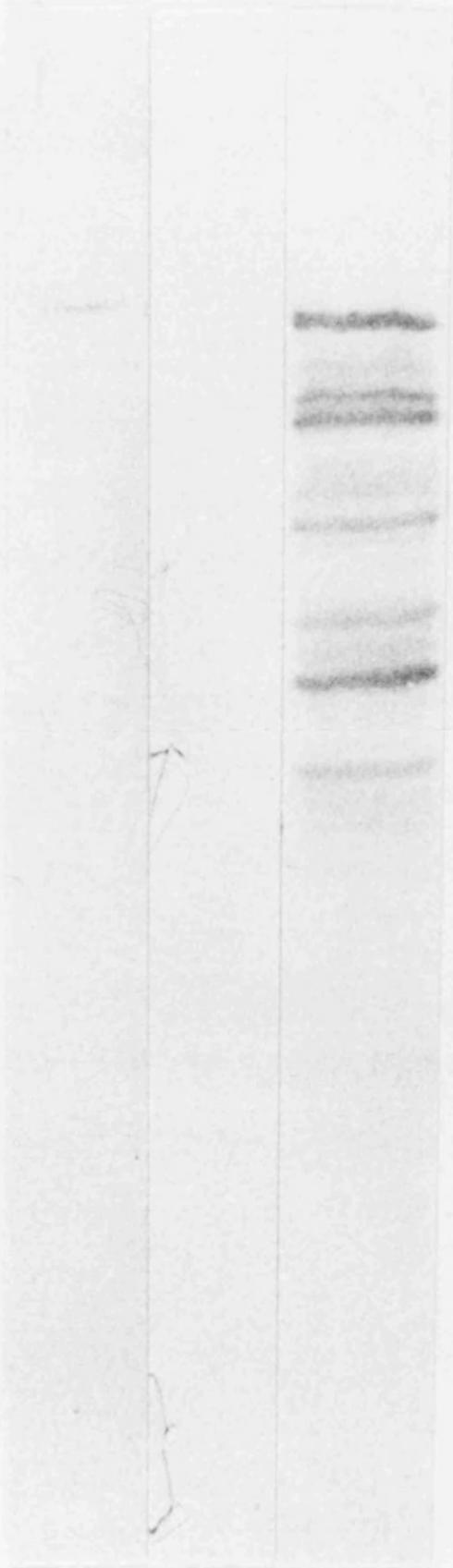
45-

29-

1

2

3



3:2:3:4 1C2 detection of antigens

When the monoclonal antibody 1C2 was used to probe Western blot time-courses of the D3 and D7 cell line culture extracts, no proteins were recognised in the tracks of either type of cloned cell line (data not shown). However, because this monoclonal was known to react in IFAs of differentiating diminished cell lines (see section 3:2:5), it was thought that the lack of reactivity on Western blots may have been due to the low level of D3 parasite antigen present in the extracts. To test for this, Western blots of extracts of isolated D3 and D7 cloned cell line merozoites were probed with the monoclonal 1C2. Figure 3.6 shows that the monoclonal recognised a 117 kDa polypeptide (arrowed) of the D3 merozoites (track 1), but it failed to detect any polypeptides in the D7 merozoite extract (track 2). The 1C2 monoclonal detected a 117 kDa polypeptide in the piroplasm (Ankara) extract also, and a number of smaller polypeptides (track 3). The monoclonal antibody 1D11 was also found to detect a 117 kDa polypeptide in Western blots of both diminished cell line and enhanced cell line merozoite extracts (data not shown).

3:2:4 Immunoprecipitation of antigen with the monoclonal antibody 5E1

Results presented in sections 3:2:3:1 and 3:2:3:2 showed that a 30 kDa protein was recognised by immune bovine serum, and by monoclonal antibody 5E1, at approximately the same time-point (Figs. 3.3 and 3.4). To determine if the same protein was recognised by both antibody reagents, an

Figure 3.7: Coomassie-stained SDS-PAGE gel of T.annulata (Ankara) piroplasm polypeptides immunoprecipitated with monoclonal antibody 5E1, and eluted from a protein A sepharose column.

Track 1; Piroplasm extract/antibody mixture before separation on column.

Tracks 2-6; Fractions eluted from column.

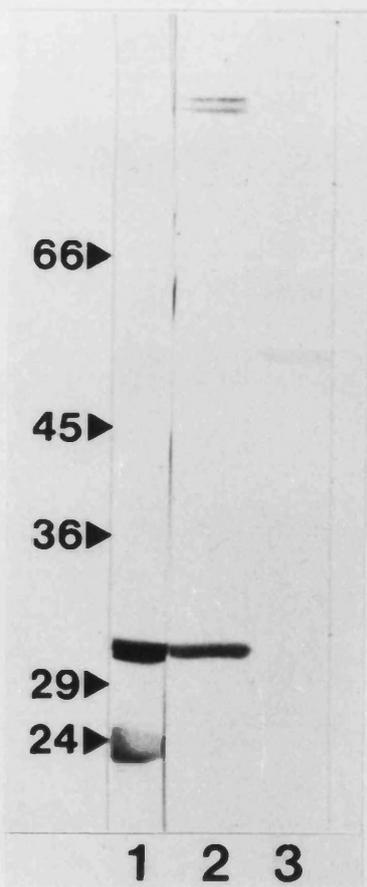
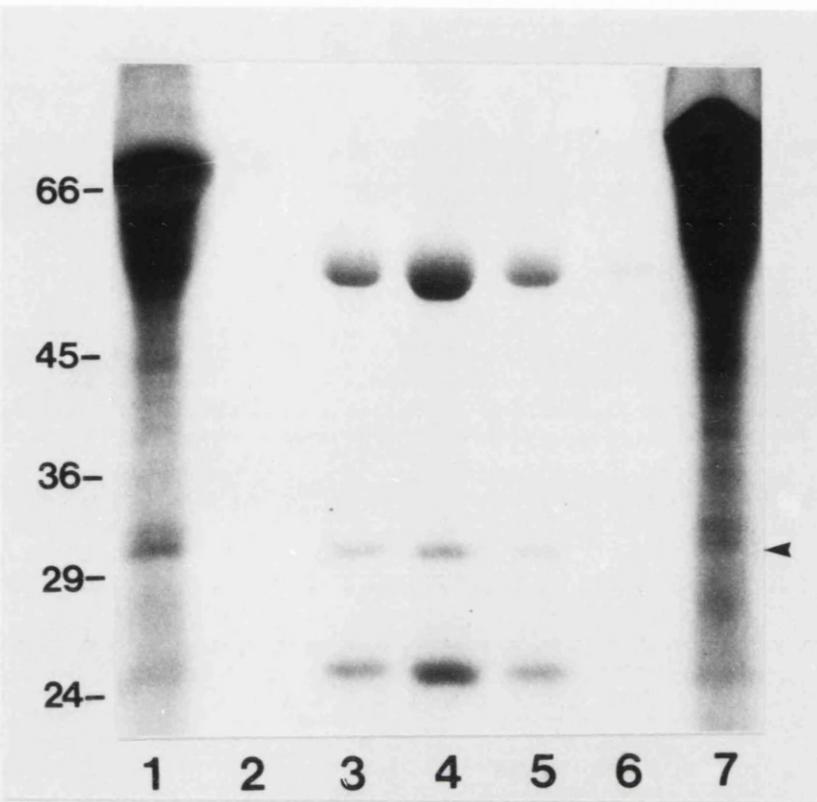
Track 7; Piroplasm extract/antibody mixture after passing through column.

Figure 3.8: Western blot of monoclonal 5E1-immunoprecipitated piroplasm polypeptides probed with monoclonal 5E1 and immune bovine serum (155).

Track 1; 5E1-immunoprecipitated polypeptides probed with monoclonal 5E1.

Track 2; 5E1-immunoprecipitated polypeptides probed with immune bovine serum.

Track 3; Polypeptides immunoprecipitated by control anti-Dictyocaulus monoclonal antibody and probed with immune bovine serum.



immunoprecipitation experiment was carried out. In this experiment, extracts of piroplasms were incubated with the monoclonal antibody 5E1, and the antibody/antigen complexes were bound to a protein A sepharose column, as described in section 2:6. The bound material was then eluted from the column, samples of the eluted fractions were subjected to SDS-PAGE, and the gel was Coomassie-stained (see Figure 3.7). Most of the 30 kDa protein (arrowed) was eluted in the second, third and fourth fractions (Fig. 3.7, tracks 3-5) and the monoclonal was successful in binding most of the 30 kDa protein contained in the piroplasm extract (Fig. 3.7, compare tracks 1 and 7; piroplasm extract before and after loading onto the column). The bands at approximately 55 kDa and 25 kDa represent the heavy and light IgG chains, respectively. Not all of the 30 kDa protein was extracted from the piroplasm sample, probably because of the large quantity of 30 kDa protein in the extract and/or because the SDS, used for polypeptide extraction, interfered with antibody binding.

The eluted immunoprecipitated material was run on a SDS-PAGE gel and Western blotted. The 5E1-precipitated protein was probed with 5E1 and, separately, with immune bovine serum (Figure 3.8, tracks 1 and 2, respectively). The result demonstrated that the 30 kDa polypeptide, affinity purified by monoclonal 5E1, was also recognised by the immune bovine serum. This precipitation was specific, as a control anti-Dictyocaulus monoclonal antibody did not bind 30 kDa material recognised by immune bovine serum (Fig. 3.8, track 3). The high molecular weight doublet

Figure 3.9: Indirect immunofluorescence assay using monoclonal antibodies 5E1, 1C2 and 1D11 against fixed slide preparations of T.annulata (Ankara) cloned cell lines C9 and E3 after 8 days of culture at 41°C.

A,C,E; Cloned cell line C9.

B,D,F; Cloned cell line E3.

A,B; Monoclonal antibody 5E1 reactivity.

C,D; Monoclonal antibody 1C2 reactivity.

E,F; Monoclonal antibody 1D11 reactivity.

Scale bar; 10um.

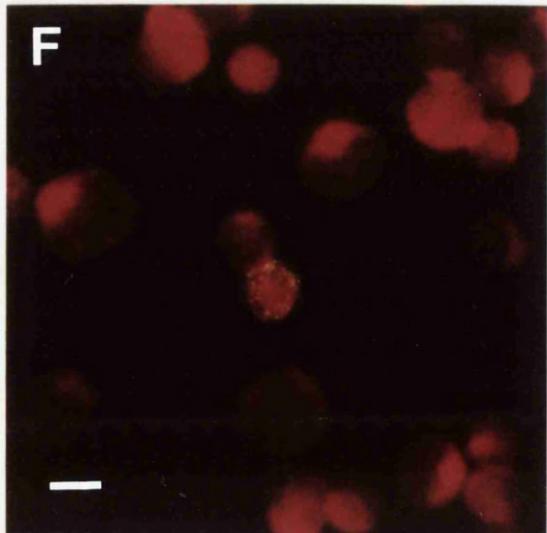
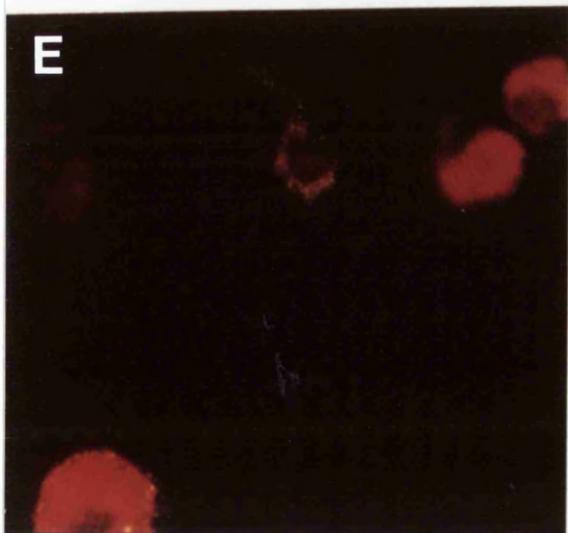
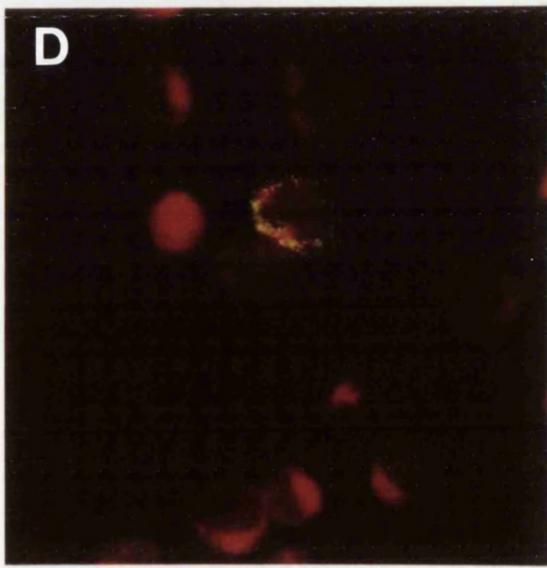
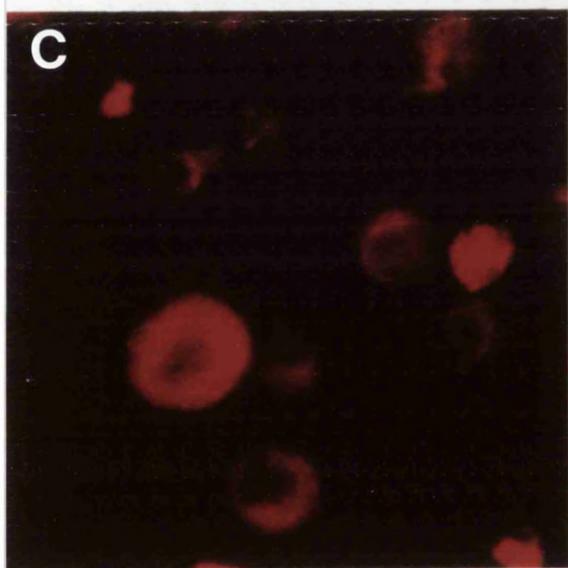
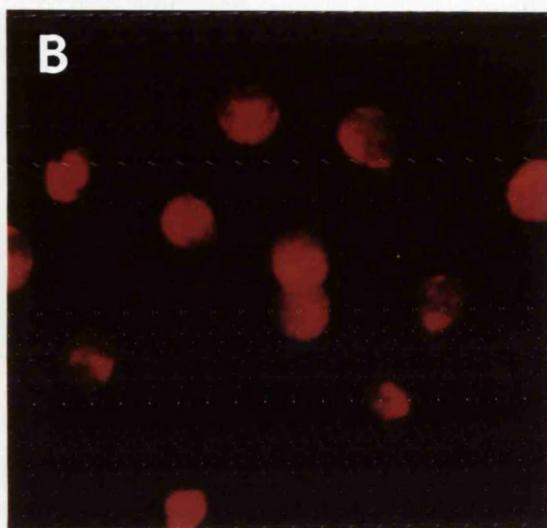
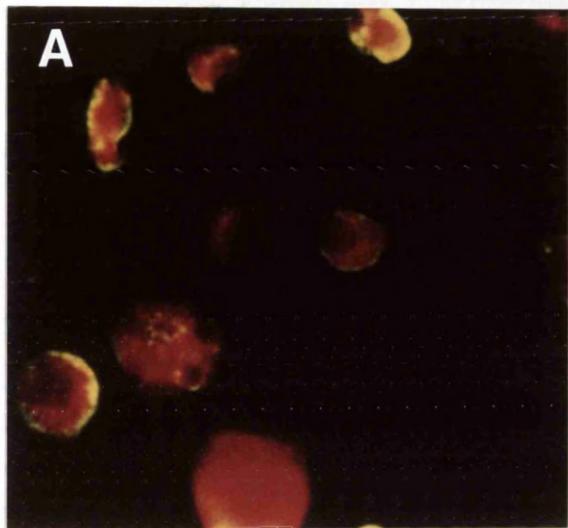


Table 3.2 Summary of immunofluorescence assay reactivity of monoclonal antibodies 5E1, 1C2 and 1D11 with cloned cell lines and pattern of reactivity observed.

	Enhanced (D7/C9)	Diminished (D3/E3)	Pattern
5E1	+	-	Halo
1C2	-	+	Dot
1D11	+	+	Dot

Assessment of reactivity was carried out by analysis of random microscope fields.

- + reactivity
- no reactivity

detected by the immune serum in the 5E1-precipitated material (Fig. 3.8, track 2) is most likely non-specific, as these bands are also present in the control track (Fig. 3.8, track 3).

3:2:5 Detection of merozoite antigens by indirect IFA

The monoclonal antibodies 5E1, 1C2 and 1D11 were used in indirect immunofluorescence assays (IFAs) against the differentiating macroschizonts of cloned cell lines, after 8 days of culture at 41°C. Most of the cells (approximately 75%) of the enhanced cell line (C9) reacted with the monoclonal 5E1 (Fig. 3.9A), whereas the cells of the diminished cell line (E3) did not react (Fig. 3.9B). However, it was estimated that 2% of the infected cells of the diminished cell line reacted with the monoclonal 1C2 (Fig. 3.9D), and an identical percentage of the cells reacted with the 1D11 monoclonal (Fig. 3.9F). The enhanced cell line did not react with the 1C2 antibody (Fig. 3.9C), but the monoclonal 1D11 reacted with approximately 25% of the enhanced line infected cells (Fig. 3.9E). Furthermore, it was observed that the monoclonals differed in the pattern of reactivity seen in the IFAs; the 5E1 monoclonal gave a halo-type pattern of fluorescence, whereas the 1C2 and 1D11 monoclonals gave a dot pattern. The specificity of the monoclonal antibodies, and their pattern of reactivity, is summarised in Table 3.2.

3:3 Discussion

The experiments detailed in this chapter defined the changes which occur in the polypeptide repertoire during the heat-induced differentiation of macroschizonts to merozoites in vitro. In Western blot experiments, two novel stage-specific polypeptides were identified at 30 kDa and 117 kDa, which were detected by immune bovine serum in enhanced cloned cell line extracts after four days culturing at 41°C (section 3:2:3:1). The molecules were also detected by two monoclonal antibodies raised against piroplasm-infected cells (Glascodine et al., 1990); monoclonal antibody 5E1 detected the 30 kDa molecule (section 3:2:3:2), while monoclonal 1D11 detected the 117 kDa polypeptide (section 3:2:3:3). In some Western blot experiments, it was noted that the monoclonal antibody 5E1 faintly detected 30 kDa antigen before the day 6 time-point, at day 4, and in IFAs, weak 5E1 reactivity was often seen with day 4 culture samples, whereas 1D11 reactivity was never observed at this earlier time-point. This indicated that the polypeptide recognised by 5E1 is expressed before that recognised by the monoclonal 1D11. Furthermore, the 5E1 and 1D11 patterns of fluorescence was seen to differ in the IFAs, i.e., the 5E1 monoclonal gave a doughnut-shaped pattern of fluorescence on the merozoites, and a ring-like appearance on the differentiating macroschizont, while the 1D11 and 1C2 monoclonals gave a stippled pattern (Fig. 3.9). These results indicated that the antigens detected by these monoclonals had different cellular locations, and this has been confirmed by immuno-

electron microscopy, which has shown that the 5E1 monoclonal detects a merozoite surface molecule, which is also present on the surface of the differentiating macroschizont, and the 1D11 and 1C2 monoclonals detect an internal antigen, located to the rhoptry organelle (Glascodine et al., 1990; B.R.Shiels and L.Tetley, unpublished observations). Therefore, the earlier expression of the 5E1-detected epitope, compared to the 1D11-detected epitope, shows that the merozoite surface coat, probably derived from a modification of the macroschizont surface membrane, begins to develop before the formation of certain internal merozoite organelles, such as the rhoptries, which is in agreement with the electron microscopy studies carried out by Shaw and Tilney (1992).

Examination of molecular expression of the enhanced cloned cell lines showed that the merozoite 30 kDa and 117 kDa antigens were strongly expressed after 4-6 days culturing at 41°C. In addition, the molecular analysis showed that the quantity of a 43 kDa band, representing an expressed schizont polypeptide, increased up to day 4, but after this time-point, the intensity of the schizont band significantly decreased (section 3:2:3:1). These changes in the expression of schizont polypeptide were detected on Western blots by immune bovine serum, and can also be detected in IFAs by a schizont-specific monoclonal antibody, 1C12 (B.R.Shiels, unpublished observations). In these experiments, at four days culturing at 41°C, 97% of the cells of an enhanced cell line react with the 1C12 monoclonal antibody, whereas by six days, only 72% of the

infected cells react. It is unknown if this monoclonal recognises the same schizont polypeptides detected by immune bovine serum because 1C12 reactivity cannot be detected in Western blot experiments, possibly because the epitope recognised by this monoclonal is not stable to denaturation (Johansson, 1988). However, these results show that up to four days culturing at 41°C, the expression of certain schizont polypeptides increases, and this probably reflects the faster growth of the parasite when cultured at 41°C, rather than 37°C (Shiels et al., 1992). After this point, the expression of schizont polypeptides is down-regulated. Interestingly, as the enhanced cell line schizont polypeptide expression is down-regulated, merozoite polypeptide expression is seen to be up-regulated, with the novel expression of the predominant 30 kDa and 117 kDa polypeptides, implying that the down-regulation of schizont antigen expression and the up-regulation of merozoite antigen expression could be mechanistically linked. Also, by day 6, major morphological alterations are observed in the enhanced cell lines (section 3:2:1), as the majority of macroschizonts of enhanced cell lines have differentiated into microsizont forms and have begun to produce extracellular merozoites. Studies carried out by Shiels et al. (1992) have shown that macrosizont differentiation is reversible up to four days culturing at 41°C, but after four days, parasites which have begun to observably differentiate, and are expressing the 5E1 and 1D11-detected epitopes, will continue to differentiate, even if they are returned to culturing at 37°C. Therefore, merogony appears to be a two-step process,

consisting of an initial reversible phase followed by an irreversible phase, when the parasite is committed to differentiation, and once the parasite reaches the committed phase of merogony, major alterations are observed in both morphology, and in the parasite molecular profile. In contrast, the expression of the 45 kDa schizont polypeptide of the diminished cell lines was seen to remain steady throughout the Western blot time-course at 41°C, and furthermore, novel merozoite polypeptides were not detected by immune bovine serum (section 3:2:3:1). This result reflected the low differentiation rate of the diminished cell line parasite (see section 3:2:1), which made it difficult to detect any alterations in polypeptide expression during the induction of differentiation of these cloned cell lines at 41°C.

The enhanced and diminished cloned cell lines have been shown to have distinct genotypes (Shiels et al., 1992; B.R.Shiels and S.McKellar, unpublished data), indicating that theilerial parasites of different genotype can have differing abilities to differentiate. In addition, the expression of certain schizont and merozoite polypeptides, as detected by immune bovine serum on Western blots, was seen to differ between the cloned cell line types (Fig. 3.3), and furthermore, Western blot and IFA experiments showed that the enhanced and diminished cell lines differed in their reactivity with the monoclonal antibodies 5E1, 1D11 and 1C2. Figure 3.9 showed that in IFAs, the enhanced cell lines react with the monoclonals 5E1 and 1D11, but not with 1C2, and the diminished cell lines react with

monoclonals 1D11 and 1C2 only. This result was reflected in Western blot experiments also (Figures 3.4, 3.5 and 3.6). As there was no evidence to suggest that this clonal expression changed over time (i.e. cloned cell lines maintained at 37°C for long periods of time would not alter in molecular expression once cultured at 41°C), the 30 kDa and 117 kDa molecules appeared to be antigenically divergent proteins, rather than antigenically variant, as in, for example, variant surface antigen switching in trypanosomes (Boothroyd, 1985). Hence, the parasite-infected cell line stock, from which the cloned cell lines were derived, was shown to be a heterogeneous mixture of clonal types, both in genotype and in antigen expression. This is by no means unique, as cloned parasite cell lines often differ from each other and from the uncloned parental cell line. For example, cloned lines of Babesia bovis differ in the expression of a number of polypeptides and antigens, and furthermore, the cell lines differ in virulence also (Gill et al., 1987). Heterogeneity within stocks of Theileria annulata has been reported previously. A study by Shiels et al. (1986), with monoclonal antibodies, showed that certain macroschizont antigens were antigenically diverse. In T.parva, examination of the macroschizont antigens revealed antigenic diversity within and between parasite stocks (Pinder and Hewett, 1980; Minami et al., 1983), and a polymorphic immunodominant molecule (PIM) has been characterised in T.parva sporozoites and schizonts (Toye et al., 1991).

The 30 kDa polypeptide is detected by bovine immune

serum (Figure 3.3) at the same time-point (day 6) as it is detected by the monoclonal 5E1 (Figure 3.4), and the immunoprecipitation experiment (section 3:2:4) showed that the molecules detected by both antibody reagents are identical. Furthermore, the location of the 30 kDa polypeptide on the surface of the merozoite, along with the antigenicity of the molecule, suggested that the 30 kDa polypeptide may be important for the development of protective host immunity, and may be a suitable candidate for inclusion in a sub-unit vaccine (section 1:1:8). This possibility is supported by studies on T.sergenti in which monoclonal antibodies which recognise a 32 kDa merozoite surface polypeptide (Kobayashi et al., 1987; Shirakata et al., 1989) were shown to elicit a protective effect against T.sergenti infection in calves by passive transfer (Tanaka et al., 1990). However, as the T.annulata 30 kDa molecule was found to be antigenically divergent within a single parasite stock, it was necessary to evaluate this aspect further, in order to determine the nature of this divergence, and to determine the extent of the molecule's heterogeneity within and between different T.annulata stocks. The presence of epitopes which are common to different stocks of T.annulata would be important if the 30 kDa polypeptide was to be considered as a possible vaccine candidate.

CHAPTER FOUR

ANTIGENIC DIVERSITY OF A

MAJOR MEROZOITE SURFACE POLYPEPTIDE

4:1:1 Introduction

During the in vitro differentiation of the Theileria annulata (Ankara) macroschizont to the merozoite, the expression of a 30 kDa surface polypeptide and a 117 kDa rhoptry polypeptide was positively regulated, and it was concluded that the onset of the expression of these polypeptides (detectable by antibody reaction) coincided with the irreversible phase of merogony, when the parasite is committed to differentiation (section 3.3). Furthermore, IFAs on the two types of cloned cell lines (enhanced and diminished) revealed clonal differences in monoclonal antibody reactivity, leading to the conclusion that the 30 kDa and 117 kDa molecules exhibit antigenic diversity.

The 30 kDa and 117 kDa polypeptides were highly immunogenic to the cow, and gave rise to a strong antibody response in the host (section 3:2:2). The aim of a successful vaccine is to induce a host immune response to the parasite, which will protect the animal against subsequent infection, and protective immunity to tropical theileriosis may be directed against surface antigens associated with sporozoites, merozoites and the infected host lymphoblastoid cell (see section 1:1:6). As the 30 kDa polypeptide is both located to the surface and it elicits a strong host immune response, this molecule could be important for inclusion in a sub-unit vaccine. The ability of merozoite surface proteins to elicit a protective immune response has been shown with Plasmodium falciparum. For example, affinity-purified P.falciparum major merozoite surface antigen MSA1 can induce complete protection against

a lethal challenge in immunized monkeys (Siddiqui et al., 1987). As the T.annulata 30 kDa merozoite surface protein was shown to be antigenically diverse within a single parasite stock, it would be important to show that the molecule had limited antigenic diversity between different T.annulata stocks, so that it could elicit a protective response in the host which was effective against both homologous and heterologous parasite stock challenge. It was likely that the 30 kDa protein, and/or its polymorphic form(s), existed in other T.annulata stocks because the 30 kDa molecule was shown to be an abundant protein of T.annulata Ankara stock piroplasm extracts (Figure 3.2), and in a previous study, a protein of approximately 30 kDa, which was also detected by the 5E1 monoclonal antibody, was shown to exist in a piroplasm sample of the Hissar stock of T.annulata (Glascodine et al., 1990). Therefore, widespread expression of this molecule in different T.annulata stocks would mean that the molecule had potential uses, not only as part of a sub-unit vaccine, but also as a stock marker, and could possibly be used as a tool for the serodiagnosis of infected animals in enzyme-linked immunosorbant assays (ELISAs).

4:1:2 Summary and Aims

The reactivity of differentiating macroschizont-infected cloned cell lines with a panel of monoclonal antibodies revealed polymorphism of a 30 kDa surface polypeptide and a 117 kDa rhoptry polypeptide in the Ankara stock of T.annulata.

The aim of this section was to investigate the 30 kDa polymorphism at a molecular level, by using the monoclonal antibody 5E1 and the polyspecific sera, anti-E3d and anti-C9m (section 2:4:1:2), generated against cloned cell line merozoites which were known to be genotypically distinct (Shiels et al., 1992).

As well as the examination of 30 kDa polymorphism within a single T.annulata stock, this section also aimed to study 30 kDa polymorphism between different T.annulata stocks; to firstly determine if a molecule analogous to the 30 kDa molecule existed in T.annulata stocks other than Ankara, and, if this was the case, to monitor the extent of 30 kDa antigenic diversity and size polymorphism within a single geographical region.

4:2 Results

4:2:1 Polypeptide profile of T.annulata (Ankara stock) piroplasms and merozoites

Merozoites were obtained from a differentiating enhanced cloned cell line (D7) by differential centrifugation (section 2:3:1). The polypeptides of the merozoite extract were separated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. The polypeptide profile of the merozoites was compared to that of polypeptide extracts from a macroschizont-infected cell line, piroplasms (Ankara stock), and bovine erythrocytes (Figure 4.1).

The polypeptide profile of the merozoites and piroplasms was similar (Fig. 4.1, tracks 2 and 3). Some high molecular weight proteins (above 100 kDa) were present in the piroplasm extract, but absent in the merozoite extract. One of the major polypeptides in both merozoite and piroplasm extracts was a 30 kDa polypeptide (arrowed), and a 32 kDa protein was also present in the piroplasm extract. The 30 kDa and 32 kDa polypeptides appeared to be absent in the macroschizont-infected cell line and bovine erythrocyte extracts (Fig. 4.1, tracks 1 and 4, respectively). Overall, the polypeptide profiles of the macroschizont-infected cell line and the erythrocytes were quite distinct from those of the merozoites and piroplasms, indicating that contamination of the merozoite and piroplasm extracts with host lymphocyte or erythrocyte material was minimal.

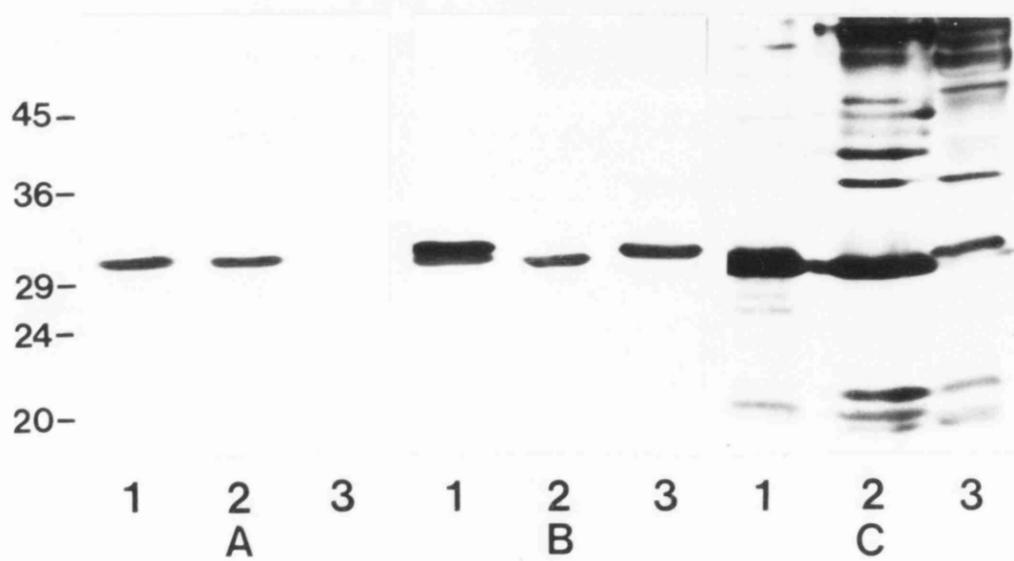
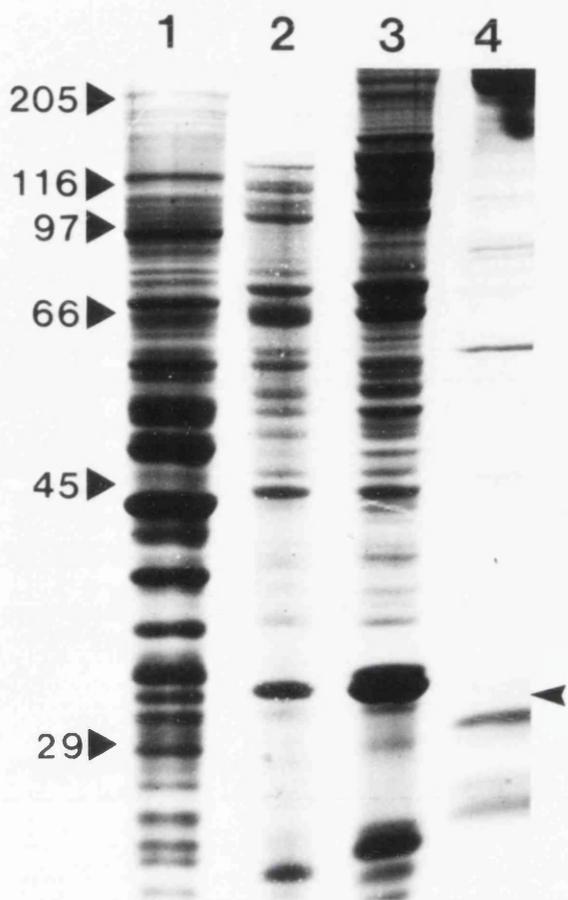
Figure 4.1: Characterisation of T.annulata (Ankara) merozoite and piroplasm polypeptides by SDS-PAGE and Coomassie-staining.

Track 1; Macroschizont-infected cells (TaA₂ cell line).
Track 2; Cloned cell line (D7) merozoites.
Track 3; Piroplasms (Ankara).
Track 4; Bovine erythrocytes.

Figure 4.2: Western blot analysis of T.annulata (Ankara) cloned cell line (D7 and E3) polypeptides probed with monoclonal antibody 5E1, anti-E3d serum and anti-C9m serum.

Panel A; Monoclonal antibody 5E1.
Panel B; Anti-E3d serum.
Panel C; Anti-C9m serum.

Track 1; Piroplasms (Ankara).
Track 2; D7 merozoites.
Track 3; E3 merozoites.



4:2:2 Molecular and antigenic diversity of the 30 kDa polypeptide, detected in Western blots

The monoclonal antibody 5E1 and the polyspecific sera, anti-E3d and anti-C9m, were tested for reactivity on a Western blot of extracts of T.annulata piroplasms (Ankara), and merozoites of the cloned cell lines D7 and E3. Figure 4.2, panel A, shows 5E1 monoclonal antibody reactivity with the extracts and, as in section 3:2:3:2, the monoclonal detected a 30 kDa polypeptide of piroplasms and D7 merozoites (Fig. 4.2A, tracks 1 and 2), but failed to detect a polypeptide in the E3 merozoite extract (Fig. 4.2A, track 3).

The 30 kDa molecule was also recognised by the anti-E3d serum in the piroplasm and D7 merozoite extracts (Fig. 4.2B, track 1 and 2). However, in addition to the 30 kDa band, the anti-E3d serum also recognised a prominent 32 kDa polypeptide in the piroplasm extract (Fig. 4.2B, track 1) and, when reacted against E3 merozoites, a single polypeptide of 32 kDa was detected (Fig. 4.2B, track 3).

A pattern similar to the anti-E3d reactivity was seen with the anti-C9m serum (Fig. 4.2C). Again, a 30/32 kDa doublet was recognised in the piroplasm extract (Fig. 4.2C, track 1), a 30 kDa polypeptide only in the D7 merozoites (Fig. 4.2C, track 2) and a 32 kDa polypeptide was detected in the E3 merozoite extract (Fig. 4.2C, track 3). The anti-E3d and anti-C9m reactivity differed in that the former gave a stronger reaction with the 32 kDa polypeptide than it did with the 30 kDa of the piroplasm extract (Fig. 4.2B,

track 1), even though there was a significantly lower amount of the 32 kDa polypeptide, as assessed by staining with Coomassie brilliant blue (Fig. 4.1, track 3). With the anti-C9m serum, the reverse was seen. This serum reacted mainly with the 30 kDa polypeptide and reflected the predominance, in quantity, of the 30 kDa over the 32 kDa polypeptide in an Ankara piroplasm extract (Fig. 4.1, track 3).

4:2:3 Molecular analysis of the 30/32 kDa antigens in other T.annulata stocks

The expression of the 30 kDa in other T.annulata piroplasm stocks was examined in stocks originating from different geographical regions, and in isolates originating from an area within a single geographical region.

4:2:3:1 Stocks originating from different regions

Piroplasm extracts of stocks originating from Turkey (Ankara), India (Hissar) and Morocco (Gharb) were analysed by SDS-PAGE. A Coomassie-stain of the gel showed that all three stocks had predominant polypeptides in the region of 30 kDa to 32 kDa (Fig. 4.3, position of the 30 kDa molecule arrowed). As defined in the previous section, the Ankara stock piroplasm extract had both 30 kDa and 32 kDa polypeptides (Fig. 4.3, track 1), with the 30 kDa predominating. The Gharb stock appeared to lack the 30 kDa polypeptide, and had a 32 kDa polypeptide only (Fig. 4.3, track 3), while the Hissar stock had a predominant polypeptide that appeared to be between 30 kDa and 32 kDa

Figure 4.3: Analysis of Ankara, Hissar and Gharb stocks of T.annulata piroplasms by SDS-PAGE and Coomassie-staining.

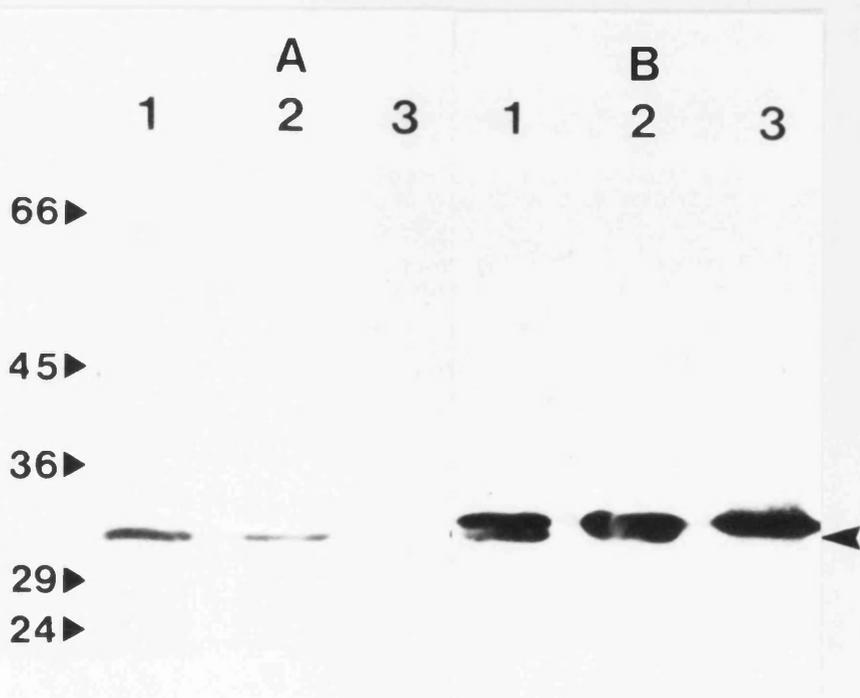
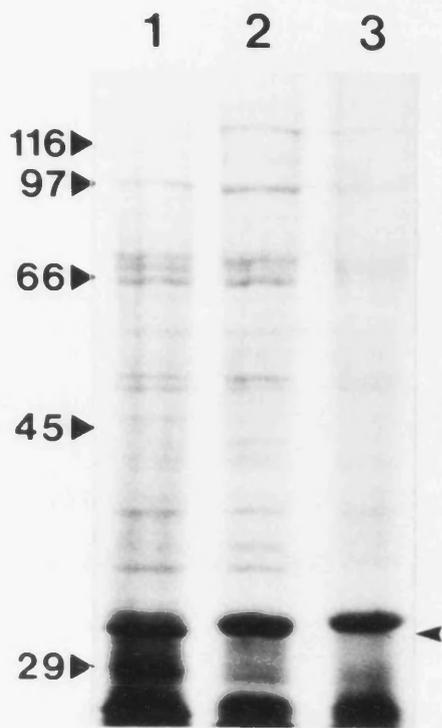
Track 1; Ankara piroplasms.
Track 2; Hissar piroplasms.
Track 3; Gharb piroplasms.

Position of 30 kDa polypeptide arrowed.

Figure 4.4: Western blot of Ankara, Hissar and Gharb stocks of T.annulata piroplasms probed with monoclonal antibody 5E1 and anti-E3d serum.

Panel A; Monoclonal antibody 5E1.
Panel B; Anti-E3d serum.

Track 1; Ankara piroplasms.
Track 2; Hissar piroplasms.
Track 3; Gharb piroplasms.



(Fig. 4.3, track 2).

A Western blot of the three stocks was probed with the monoclonal antibody 5E1 and with the anti-E3d serum (Fig. 4.4, 30 kDa position arrowed). The 5E1 antibody clearly reacted with the 30 kDa polypeptide of the Ankara piroplasm extract (Fig. 4.4A, track 1) and, to a lesser extent, with a Hissar 30 kDa polypeptide (Fig. 4.4A, track 2), whereas 5E1 did not detect any polypeptides in the Gharb extract (Fig. 4.4A, track 3). The anti-E3d serum reacted with all three stock extracts and recognised a 30/32 kDa doublet in the Ankara stock (Fig. 4.4B, track 1), a polypeptide with mobility of between 30 kDa and 32 kDa in the Hissar stock (Fig. 4.4B, track 2), and with a 32 kDa polypeptide in the Gharb stock (Fig. 4.4B, track 3).

4:2:3:2 Isolates from a single geographical region

Piroplasm samples were taken from a small area of the Tunis region in Tunisia (section 2:1:1) and were analysed by SDS-PAGE. A gel of the samples was Coomassie-stained for protein analysis (Fig. 4.5), and other gels were Western blotted and probed with the monoclonal 5E1 (Fig. 4.6), anti-C9m serum (Fig. 4.7) and anti-E3d serum (Fig. 4.8).

Figure 4.5 shows that all samples analysed had polypeptides in the region of 30-32 kDa molecular weight (30 kDa position arrowed). The Western blot of the samples probed with the monoclonal 5E1 showed that only three of the eight Tunisian piroplasm samples tested reacted (Fig. 4.6, tracks 3, 6, 7), and it was interesting to note that

Figure 4.5: Coomassie-stained SDS-PAGE gel of Tunisian T.annulata piroplasm samples. Tracks 2-9 Tunisian piroplasm samples

Track 1; Ankara.	Track 6; Sample 18A.
Track 2; Sample 11.	Track 7; Sample 18C.
Track 3; Sample 12.	Track 8; Sample 19.
Track 4; Sample 13.	Track 9; Sample 20.
Track 5; Sample 14C.	Track 10; Gharb.

Figure 4.6: Western blot of Tunisian piroplasm samples probed with monoclonal antibody 5E1. Tracks as above.

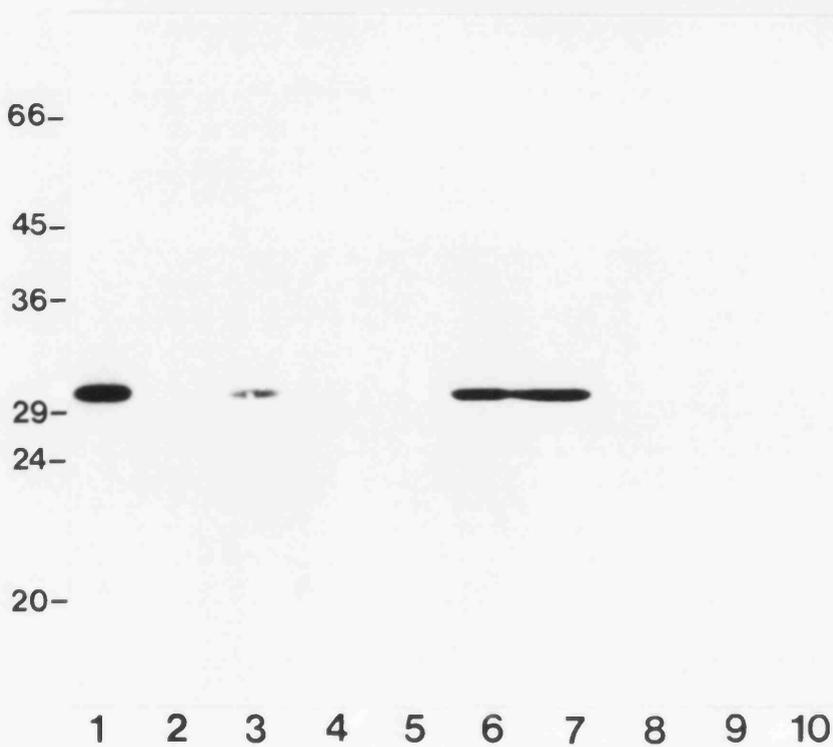
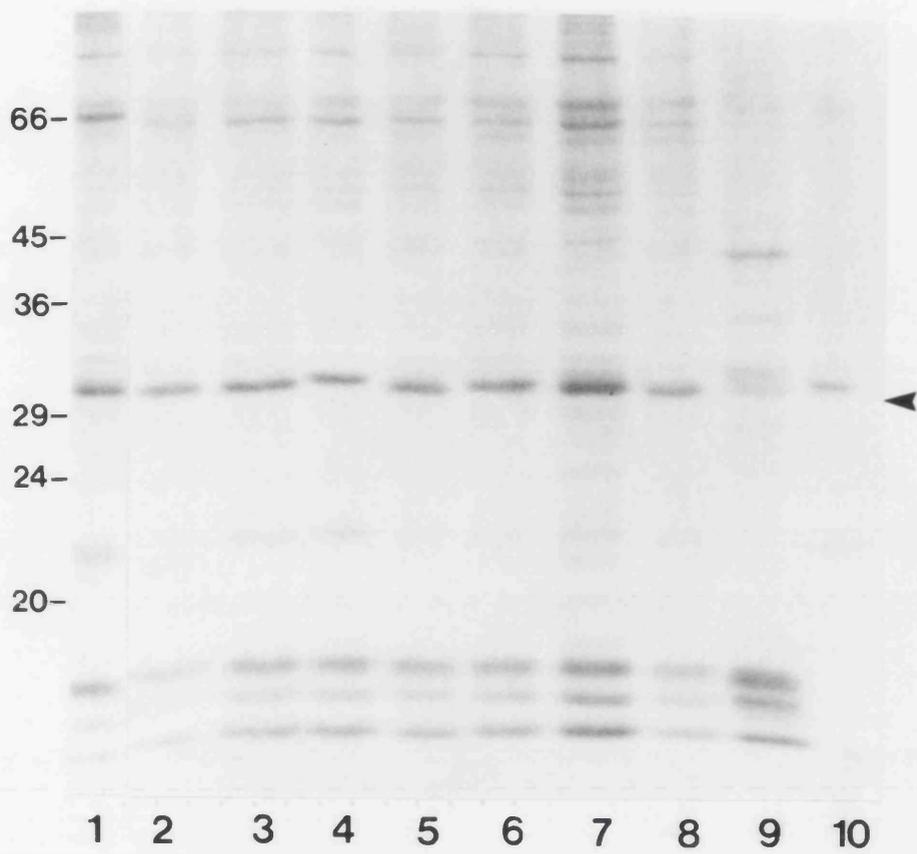
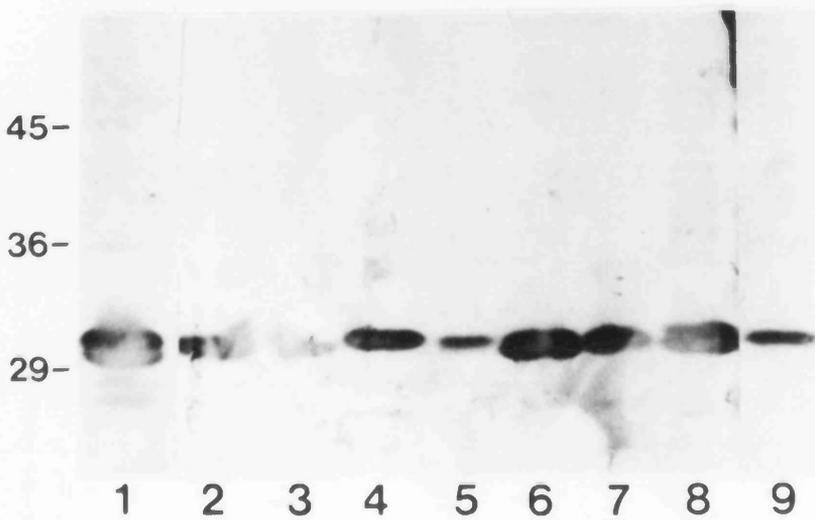
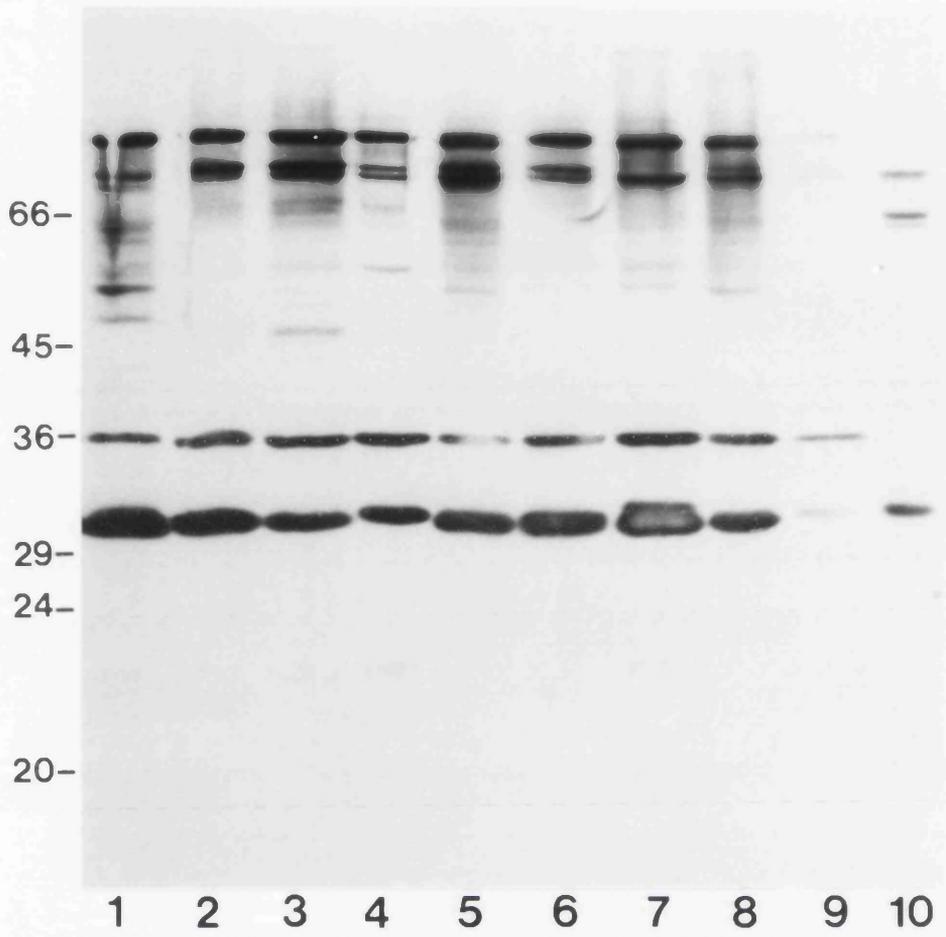


Figure 4.7: Western blot of Tunisian piroplasm samples probed with anti-C9m serum. Tracks as in Figure 4.5.

Figure 4.8: Western blot of Tunisian piroplasm samples probed with anti-E3d serum. Tracks 2-8 Tunisian piroplasm samples

Track 1;	Ankara.	Track 6;	Sample 14C.
Track 2;	Sample 11.	Track 7;	Sample 18A.
Track 3;	Sample 12.	Track 8;	Sample 18C.
Track 4;	Sample 13.	Track 9;	Gharb.
Track 5;	Sample 10.		



four other samples, which clearly had a 30 kDa polypeptide in the Coomassie-stained gel, did not react with the monoclonal antibody (compare Figs. 4.5 and 4.6, tracks 2, 5, 8 and 9). However, the 30 kDa molecule of these samples was detected by the anti-C9m and anti-E3d serum (Fig. 4.7, tracks 2, 5, 8 and 9; Fig. 4.8, tracks 2, 6; samples 19 and 20 probed by anti-E3d serum not shown). In fact, the 30-32 kDa molecules of all samples reacted with the anti-C9m and anti-E3d serum (Fig. 4.7 and 4.8). The reactivity of the anti-C9m serum with the Tunisian extracts, and with the Ankara and Gharb piroplasm extracts, corresponded approximately to the quantity of 30-32 kDa polypeptide present (compare Figs. 4.5 and 4.7). The anti-E3d serum reactivity on the Ankara piroplasm extract was as seen in Figure 4.2, i.e. the anti-E3d serum strongly detected the 32 kDa molecule, and this reactivity did not reflect the quantity of 30/32 kDa polypeptide present (Figs. 4.5 and 4.8 track 1). However, with some of the Tunisian extracts, the anti-E3d serum did not detect the 32 kDa molecule as strongly. For example, comparison of the piroplasm sample 14C extract to the Ankara piroplasm extract by Coomassie staining (Fig. 4.5, tracks 5 and 1, respectively) showed that these extracts had very similar polypeptide profiles. However, the Western blot of these extracts probed with the anti-E3d serum showed that the 30 kDa and 32 kDa molecules of the 14C extract were detected approximately equally (Fig. 4.8, track 6), whereas in the Ankara extract, the 32 kDa molecule was detected with much greater intensity by the anti-E3d serum (Fig. 4.8, track 1). The 18C sample

Table 4.1 Detection of 30kDa, or 32kDa molecules in Tunisian piroplasm samples by Coomassie-staining or antibody reactivity (monoclonal antibody 5E1, anti-C9m serum and anti-E3d serum).

Tunisian Piroplasm Samples								
Detection	11	12	13	14C	18A	18C	19	20
Coomassie	30	30	32	30+32	30	30+32	30	30
5E1	-	30	-	-	30	30	-	-
Anti-C9m	30	30	32	30+32	30	30+32	30	30
Anti-E3d	30	30	32	30+32	30	30+32	n.d.	30

- no reactivity
n.d., test not done

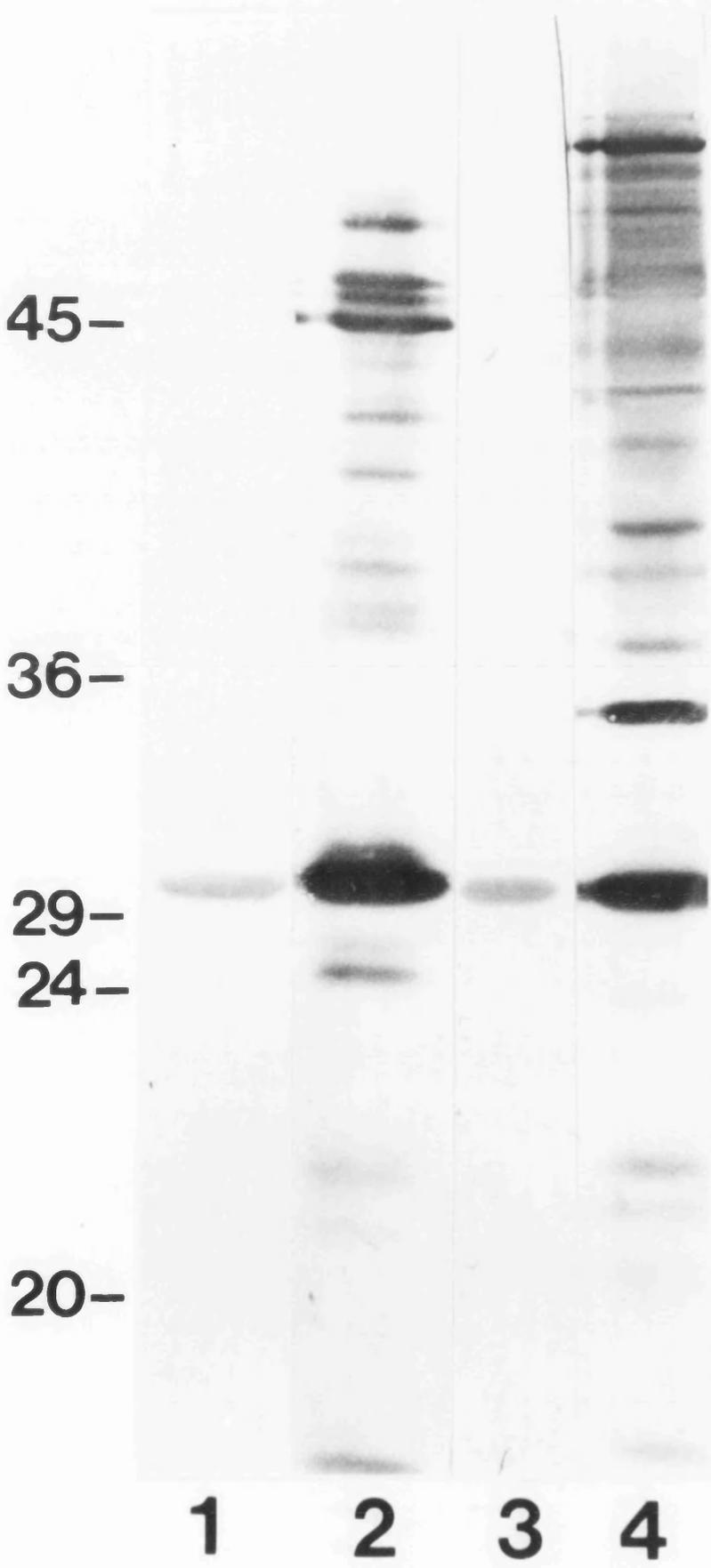
extract reactivity was similar to the 14C extract reactivity with the anti-E3d serum (Fig. 4.5, track 7 and Fig. 4.8, track 8). In conclusion, these results showed that the 30 kDa molecule is polymorphic in size and is antigenically divergent within this single geographical region. In fact, there appeared to be as much antigenic diversity within this region as there was between different regions (see previous sub-section). The observed pattern of protein stain for each sample, and the corresponding antibody reactivity with the 30-32 kDa molecules, is summarised in Table 4.1.

4:2:4 Titration of the 30 kDa polypeptide in Ankara piroplasms and merozoites

The results presented in the previous section showed that there was at least two forms of 30 kDa molecule; one with the 5E1 epitope, and another without the 5E1 epitope. It was possible, therefore, that more than one polypeptide, with a mobility of 30 kDa, existed in an Ankara piroplasm extract. The cloned cell lines have single genotypes (see section 3:3), so it was decided to titre an extract of merozoites from an enhanced cell line (D7) against an extract of Ankara piroplasms, so that there was an equal quantity of 30 kDa polypeptide in both extracts, as assessed by protein stain intensity on an SDS-PAGE gel. A Western blot of these samples was probed with the monoclonal 5E1 and with anti-C9m serum. It was expected that the presence of additional, non-reacting 30 kDa molecule in the piroplasm extract would be noted by

Figure 4.9: Western blot of T.annulata (Ankara) piroplasm and merozoite extracts, titrated by dilution and probed with monoclonal antibody 5E1 and anti-C9m serum.

- Track 1; D7 merozoites probed with 5E1.
- Track 2; Piroplasms probed with anti-C9m serum.
- Track 3; Piroplasms probed with 5E1.
- Track 4; D7 merozoites probed with anti-C9m serum.



decreased reactivity of the piroplasm extracts with monoclonal 5E1 when compared to the reactivity with the merozoite extracts. However, the result indicated that antibody reactivity was equal between the piroplasms and merozoites, as the intensity of 5E1 reactivity with the piroplasms (Fig. 4.9, track 1) was identical to the intensity of merozoite reactivity (Fig. 4.9, track 3), and similarly, the anti-C9m reactivity was identical for both piroplasm and merozoite extracts (Fig. 4.9, tracks 2 and 4). Therefore, the result of this experiment indicated that there was the same quantity of 5E1-detected 30 kDa molecule in the Ankara piroplasm sample as there was in the D7 merozoite sample, which was of single genotype.

4:3 Discussion

The results obtained from the comparative Western blot analysis of the cloned macroschizont-infected cell lines, of a single T.annulata (Ankara) stock, revealed that a 30 kDa major merozoite surface molecule was antigenically diverse and had a variable molecular mass (30 kDa or 32 kDa) in different clonal populations of the parasite (section 4:2:2). The 30 kDa molecule was associated with enhanced differentiating cloned cell lines, and the 32 kDa molecule was associated with diminished cloned cell lines, and the 30/32 kDa protein polymorphism was detected on Western blots by the anti-C9m and anti-E3d polyspecific sera. The anti-E3d and anti-C9m sera were generated against differentiating cells of a diminished cloned cell line and an enhanced cloned cell line, respectively. The reactivity of the antisera showed that the two forms of the molecule, from each cloned line, had epitopes in common, as both antisera reacted with both molecules (Figure 4.2), but the antisera reactivity also showed that the molecules had epitopes that differed. In particular, the reactivity of the anti-E3d serum with an Ankara piroplasm extract did not reflect the actual quantity of 30/32 kDa molecule present, because the anti-E3d serum gave more intense reactivity with the 32 kDa polypeptide than with the 30 kDa polypeptide (Figure 4.2B), even though the 30 kDa molecule was present in much greater quantity (Figure 4.1). This pattern was reversed when the piroplasm extract was probed with anti-C9m serum (Figure 4.2C). Therefore, there were epitopes on the 32 kDa molecule which were recognised by

the anti-E3d serum, but which were not present on the 30 kDa molecule, and the 30 kDa polypeptide had an epitope which was not present on the 32 kDa molecule, because only the 30 kDa molecule was detected by the monoclonal 5E1 (Figure 4.2A).

Comparison of the different stocks of T.annulata revealed that there was antigenic diversity of the 30 kDa molecule between parasite stocks, as well as within the single Ankara stock examined (section 4:2:3). The Western blot analysis showed that all the piroplasm stocks examined had antigen(s) in the region of 30 to 32 kDa, and these were related to the Ankara stock 30 kDa polypeptide as they were detected by the monoclonal antibody 5E1 and/or by the anti-E3d and anti-C9m sera, which were generated against Ankara stock parasites. The polypeptide profile of the Ankara stock piroplasms, which originated in Turkey, had 30 kDa and 32 kDa polypeptides, with the 30 kDa polypeptide predominating, and examination of the extracts from the Hissar stock, from India, showed that this stock had a predominant polypeptide with a molecular weight of between 30 and 32 kDa, and also a less abundant 30 kDa polypeptide detected by the monoclonal 5E1 (Figure 4.4). The Gharb stock extracts, from Morocco, were seen to possess the 32 kDa polypeptide only. The variability of the 30 kDa protein expression between the different stocks indicated that the 30 kDa and 32 kDa protein expression, which was associated with the Ankara stock enhanced and diminished cloned cell lines respectively, was not always associated with these differentiation phenotypes. For

example, it was unlikely that the observed Gharb stock polypeptide repertoire originated from only diminished type cell line merozoites possessing the 32 kDa molecule, although in this case, it is possible that Gharb merozoites could be derived from enhanced and diminished cell lines, both of which possess the 32 kDa molecule. The different forms of the 30 kDa molecule could not, therefore, be used as markers for ability to differentiate, and also, it could be postulated that the gene encoding the 30 kDa molecule is unlikely to be associated with the regulation of macroschizont differentiation.

It was assumed that if the polypeptide profiles of the piroplasm stocks examined were characteristic of the population of parasites in the geographical region from which the stocks originated, there would be sufficient 30 kDa antigenic diversity and polymorphism for the molecule to be useful as a marker for these different stocks. Major merozoite/piroplasm proteins have been identified in other theilerial species in a molecular weight range similar to the T.annulata 30 to 32 kDa polypeptides. A 33 kDa polypeptide of T.sergenti, and 34 kDa polypeptides of T.buffeli and T.orientalis, were detected as immunodominant surface proteins of the piroplasms of these species, and furthermore, these molecules have been reported to be potential markers to distinguish these Theileria species (Sugimoto et al., 1991). In T.mutans, a 32 kDa protein has been identified as a species-specific, immunodominant surface molecule of piroplasms (Katende et al., 1990), and monoclonal antibodies directed to this molecule do not

cross-react with other Theileria species, such as T.bovis, T.parva and T.annulata. However, when T.annulata piroplasm isolates were examined, which originated from different parts of a single geographical region, it was clear that the 30 kDa molecule exhibited as much antigenic diversity within a single region as was previously observed between different regions. These results therefore suggest that this molecule is not suitable as a marker for different T.annulata stocks. For example, Tunisian samples 18A and 18C came from two cows on the same farm, and sample 18A had 30 kDa polypeptide only, while 18C had both 30 kDa and 32 kDa polypeptides. It was also interesting to note that three of the Tunisian extracts (11, 14C and 19) had 30 kDa polypeptide which did not react with 5E1 monoclonal antibody. This had not been seen previously and the result showed that antigenic diversity of the 30 kDa-associated 5E1-recognised epitope existed without an associated change in 30 kDa molecular weight. However, 5E1 reactivity with a polypeptide of a molecular weight other than 30 kDa has yet to be observed. In addition, because of the observation of more than one type of 30 kDa molecule (with and without the 5E1-detected epitope) in the Tunisian samples, a titration experiment was carried out to determine if there was 30 kDa polypeptide present in Ankara piroplasm extracts which was not present in D7 cloned cell line merozoite extracts (section 4:2:4). The result of this experiment suggested that this was not the case and only 30 kDa polypeptide possessing the 5E1 epitope was present in the Ankara piroplasm extracts.

Similar studies to determine the extent of antigenic diversity of the 33/34 kDa molecules on the T.sergenti/buffeli/orientalis group of parasites and the 32 kDa molecule of T.mutans have not yet been reported. However, in the study of Katende et al. (1990), two types of ELISAs were used to identify the presence of the T.mutans 32 kDa antigen, or the presence of antibodies binding specifically to it, in sera taken from field studies. Two monoclonal antibodies, detecting different epitopes on the 32 kDa protein, were employed to detect the antigen, and purified 32 kDa protein was used to detect 32 kDa-specific antibodies. The 32 kDa protein was purified from a Tanzanian stock of the parasite and the monoclonal antibodies were raised against piroplasms of the same stock of parasite. Field sera were taken from Kenya and Tanzania. Using the ELISAs, it was found that the sera from Tanzania had very high titres of 32 kDa antigen and 32 kDa-specific antibodies, but interestingly, analysis of the sera from Kenya showed that 90% of the sera were positive for antibodies, but only 32% were positive for antigen. Katende et al. suggest that the observed discrepancy between the percentage of Kenyan sera positive for anti-32 kDa polypeptide antibodies, and the percentage of sera positive for monoclonal antibody-detected 32 kDa antigen arises because many of the animals tested were carriers for T.mutans infection. However, if the T.mutans 32 kDa polypeptide was as polymorphic as the T.annulata 30 kDa molecule, the difference in percentages could be explained if the monoclonal antibodies employed detected divergent epitopes. If this was the case, 68% of the Kenyan sera may

have had antigen that was undetectable by the monoclonal antibodies used. However, until the extent of antigenic diversity of the 32 kDa molecule of T.mutans is studied, this theory is speculative.

Stock antigenic diversity is widespread in parasitic protozoa, and antigenic diversity has been particularly well studied in Plasmodium falciparum, because of the large number of isolates available (Anders et al., 1989). Techniques such as isoenzyme typing, determination of drug resistance, two-dimensional gel electrophoresis and the study of antigens using monoclonal antibodies have shown that the species Plasmodium falciparum consists of heterogeneous populations made up of different parasite genotypes and phenotypes (McBride et al., 1982; Tait, 1981). For example, studies on the P.falciparum major merozoite surface antigen precursor, MSA 1, have shown that the molecule has a variable molecular mass of 185-200 kDa, depending on the strain studied (Hall et al., 1983; McBride et al., 1982), and the molecule contains both variable and constant antigenic epitopes (Hall et al., 1984; McBride et al., 1984; 1985; Pirson and Perkins, 1985; Lyon et al., 1987). Another P.falciparum merozoite surface antigen, MSA 2, also exhibits extensive antigenic diversity, and divergent forms of the molecule (35-48 kDa) exist in different populations, distinguishable by differential reactivity with monoclonal antibodies and by two-dimensional electrophoresis (Fenton et al., 1989; Smythe et al., 1990).

Antigenic diversity has probably evolved in extracellular parasites, such as the merozoite stages of the Theileria and Plasmodium parasites, to avoid the effects of a host antibody response. Diversity can arise because different forms of a molecule have partially different peptide sequence or differential secondary structure. As the structure of the T.annulata 30 kDa polypeptide was not known at this point, the mechanism of antigenic diversity could not be elucidated. In T.mutans, the binding of certain monoclonal antibodies to the 32 kDa polypeptide was found to be periodate sensitive (Katende et al., 1990), indicating that the molecule is glycosylated. Therefore, it was possible that differential glycosylation contributed to the observed antigenic diversity in T.annulata.

In conclusion, the results presented in this Chapter show that the 30 kDa molecule is an unsuitable candidate as a T.annulata parasite stock marker. However, being immunodominant and surface located (sections 3:2:2 and 3:2:5), the 30 kDa molecule still had properties to suggest that it could form part of a sub-unit vaccine. Ideally, a vaccine candidate should be widely distributed throughout the genus, with only minor variation, and in the T.annulata stocks examined, although the 30 kDa protein was polymorphic, the antibody studies showed there was a limited repertoire of antigenic diversity with respect to this protein. Other Theileria species have similar merozoite surface proteins, but the extent of the antigenic diversity of these proteins within a species is

unknown. In Plasmodium falciparum, classification of the MSA 1 protein of 37 strains of the parasite from different geographical regions showed that the strains could be put into seven serological groups (McBride et al., 1985), and if the polymorphism of the 30 kDa molecule was similarly limited in the theilerial species, a multivalent recombinant construct or synthetic peptide vaccine, based on defined antigenic determinants of this molecule, could be feasible.

CHAPTER FIVE

BIOCHEMICAL CHARACTERISATION OF THE 30/32 kDa MOLECULES

5:1:1 Introduction

Immunoblotting experiments, presented in the preceding Chapters, demonstrated that variant forms of the major merozoite antigen were present in extracts derived from the different cloned cell lines. These two forms were polymorphic in size and were antigenically diverse, and although the reactivity of the molecules with the antibody reagents indicated that the molecules were related, the extent of this, and the nature of the determinants which generate the antigenic diversity, remained to be elucidated.

Immunofluorescence assays and immuno-EM studies indicated that the 30 kDa molecule is located on the parasite surface (Glascodine et al., 1990). Many parasitic surface antigens are either glycoconjugates, for example, the Trypanosoma cruzi 72 kDa glycoprotein (Snary, 1985) and the Schistosoma mansoni 200 kDa, 38 kDa and 17 kDa surface antigens (Omer-Ali et al., 1986; Payares and Simpson, 1985), or proteins with repeating internal epitopes. Antigens containing repetitive peptide sequences have been described for many protozoa, including those of Plasmodium (Mendis et al., 1991; Arnot et al., 1988; Tanabe et al., 1987), Trypanosoma brucei (Roditi et al., 1987) and T.cruzi (Peterson et al., 1986). Therefore, an alteration in secondary structure, or an alteration in peptide sequence, frequently generates the antigenic diversity of surface proteins.

For example, analysis of nucleotide sequence diversity in the Plasmodium falciparum merozoite surface antigen MSA 1 has shown that intragenic crossover is the major mechanism generating antigenic diversity in this molecule. Additional diversity arises from point mutations in certain regions of the MSA 1 gene (Tanabe et al., 1987; Holder, 1988; Peterson et al., 1988i and 1988ii). In contrast, differences in glycosylation have generated antigenic diversity in Toxocara, and monoclonal antibodies generated against carbohydrates on the surface of the ascarid give differing profiles of reactivity with the two Toxocara species, T.canis and T.cati (Kennedy et al., 1987). Similarly, interspecies variability of the lipophosphoglycan (LPG) molecule on the cell surface of Leishmania promastigotes arises because of differences in the repeating oligosaccharide units (McConville et al., 1987; Sacks et al., 1990), and carbohydrate additions generate protein size and antigenic heterogeneity of the Leishmania surface protease, Gp63 (Chang et al., 1986; Russell and Wilhelm, 1986). To similarly determine the nature of the antigenic diversity in Theileria annulata, more information was needed on the biochemical structure of the 30 kDa polypeptide, so that the specificity (to peptide and/or secondary structure), of the antibodies which detect 30 kDa antigenic diversity, could be determined.

Generally, surface proteins are either integral

membrane proteins or peripheral proteins (Findlay, 1990). Integral membrane proteins, unlike peripheral membrane proteins, form hydrophobic interactions with the lipid core of membranes, providing a near permanent anchor in the membrane and making the proteins difficult to solubilise (Tanford and Reynolds, 1976; Findlay, 1990). Prior to immunoprecipitation (section 3:2:4), it was necessary to solubilise and extract the 30 kDa polypeptide from the surface membrane, in a structurally conserved form, so that the molecule could freely bind antibody. Extraction of the 30 kDa polypeptide with non-ionic detergents such as Triton X-100 and Nonidet P-40, which do not break up protein-protein interactions, was not successful, but the 30 kDa molecule was extractable in 1% SDS, a detergent which does break up protein-protein interactions. This property suggested that the 30 kDa molecule was a transmembrane protein, or integral membrane protein (Hommel and Semoff, 1988). Most eukaryotic integral membrane proteins are anchored in the membrane by a hydrophobic stretch of amino acids (Findlay, 1990). However, in single-cell protozoa, the majority of cell surface proteins are anchored to the membrane by a glycosylphosphatidylinositol (GPI) attachment (Ferguson and Williams, 1988). It is thought that the lipid attachment to the membrane may allow tight packing of the surface coat proteins, and this may contribute to the protective role of the membrane (Ferguson and Williams, 1988; Ferguson et al., 1992).

5:1:2 Summary and Aims

Immunoanalysis showed that the 30 kDa polypeptide of a Theileria annulata (Ankara) cell line exhibited antigenic diversity and molecular weight polymorphism to a 32 kDa polypeptide. In addition, IFA and immuno-EM studies have shown that the 30 kDa is located on the parasite surface, and detergent extraction experiments indicated that the 30 kDa polypeptide is an integral membrane protein.

The aim of the experiments recorded in this section was firstly, to determine how closely related the 30 and 32 kDa polypeptides were; secondly, to elucidate the biochemical nature of the 30/32 kDa antigenic diversity and size polymorphism; finally, to determine if the molecule was attached to the parasite membrane by a GPI anchor.

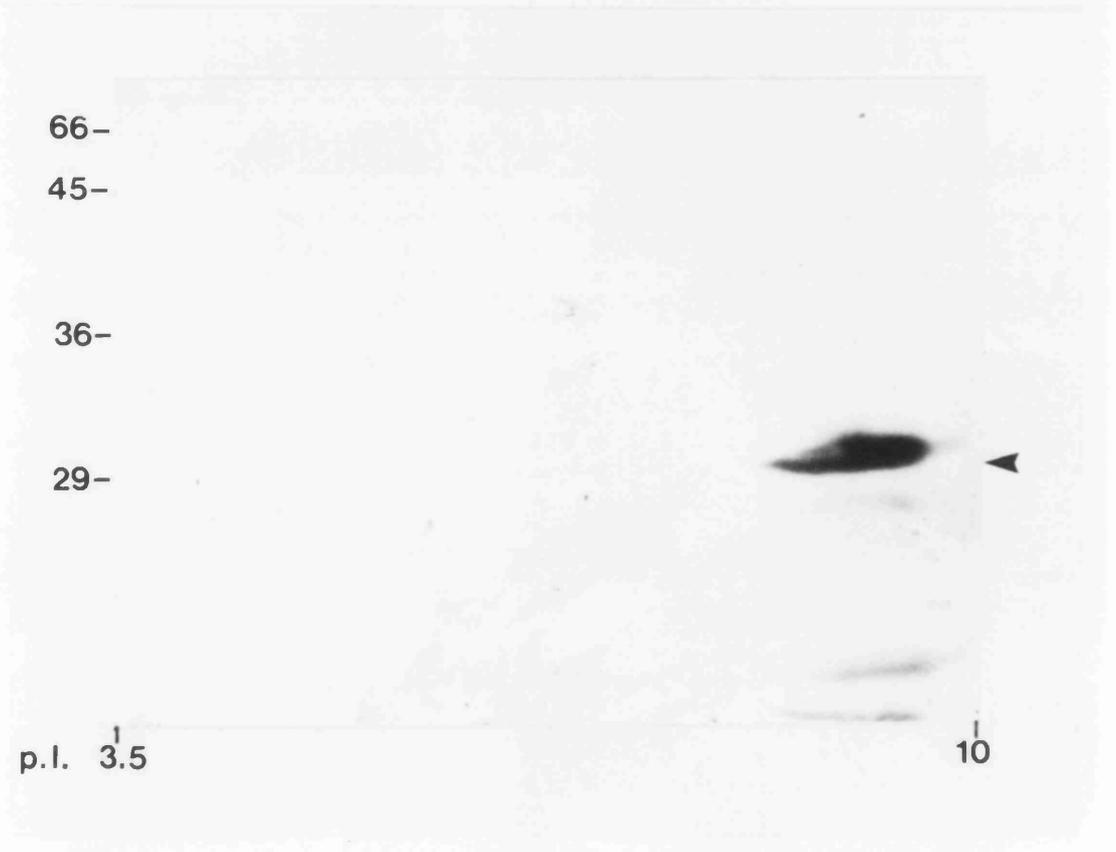
Figure 5.1: 2D-PAGE analysis of T.annulata (Ankara) piroplasm polypeptides probed with monoclonal antibody 5E1.

p.i.; Isoelectric point.

Figure 5.2: 2D-PAGE analysis of T.annulata (Ankara) piroplasm polypeptides probed with anti-E3d serum.

p.i.; Isoelectric point.

Position of 30 kDa polypeptide arrowed.



5:2 Results

5:2:1 Analysis of the 30/32 kDa polypeptides by 2D-PAGE

An Ankara stock piroplasm extract was separated by 2D-PAGE and Western blotted. The blots were probed with the monoclonal antibody 5E1 and with anti-E3d serum. The result showed that the 30 kDa molecule had the same isoelectric point (pI) when detected by 5E1 (Fig. 5.1) or by anti-E3d serum (Fig. 5.2, arrowed). The 30 kDa molecule also had the same pI as the 32 kDa molecule, detected by the anti-E3d serum alone (Fig. 5.2). Comparison of the mobility of these polypeptides to the mobility of standard polypeptides indicated that the 30/32 kDa doublet had a pI value of 8.5, showing that the polypeptides were very basic. The 32 kDa molecule of the Gharb stock of piroplasms also had a pI of 8.5, when analysed by 2D-PAGE (data not shown).

5:2:2:1 Peptide mapping the 30 kDa and 32 kDa molecules

Peptide mapping was carried out on the 30 kDa and the 32 kDa polypeptides, to get an indication of how closely related the two molecules were. The 30 kDa polypeptide was first immunoprecipitated from an Ankara piroplasm extract, as described in section 2:6, using monoclonal antibody 5E1 and a protein A sepharose column. The immunoprecipitated 30 kDa band was excised from an SDS-PAGE gel; along with the 32 kDa bands from Gharb stock piroplasm extracts. The 30 kDa and 32 kDa polypeptides were digested with V8 protease (Staphylococcus aureus) during a second SDS-PAGE separation, and the gel was Coomassie-stained. Figure 5.3

Figure 5.3: V8 protease-generated peptide maps of T.annulata 30 kDa (track 1) and 32 kDa (track 2) molecules.

Position of 16 kDa peptide band arrowed.
S1 and S2; amino acid-sequenced peptide fragments.

1

2

24▶

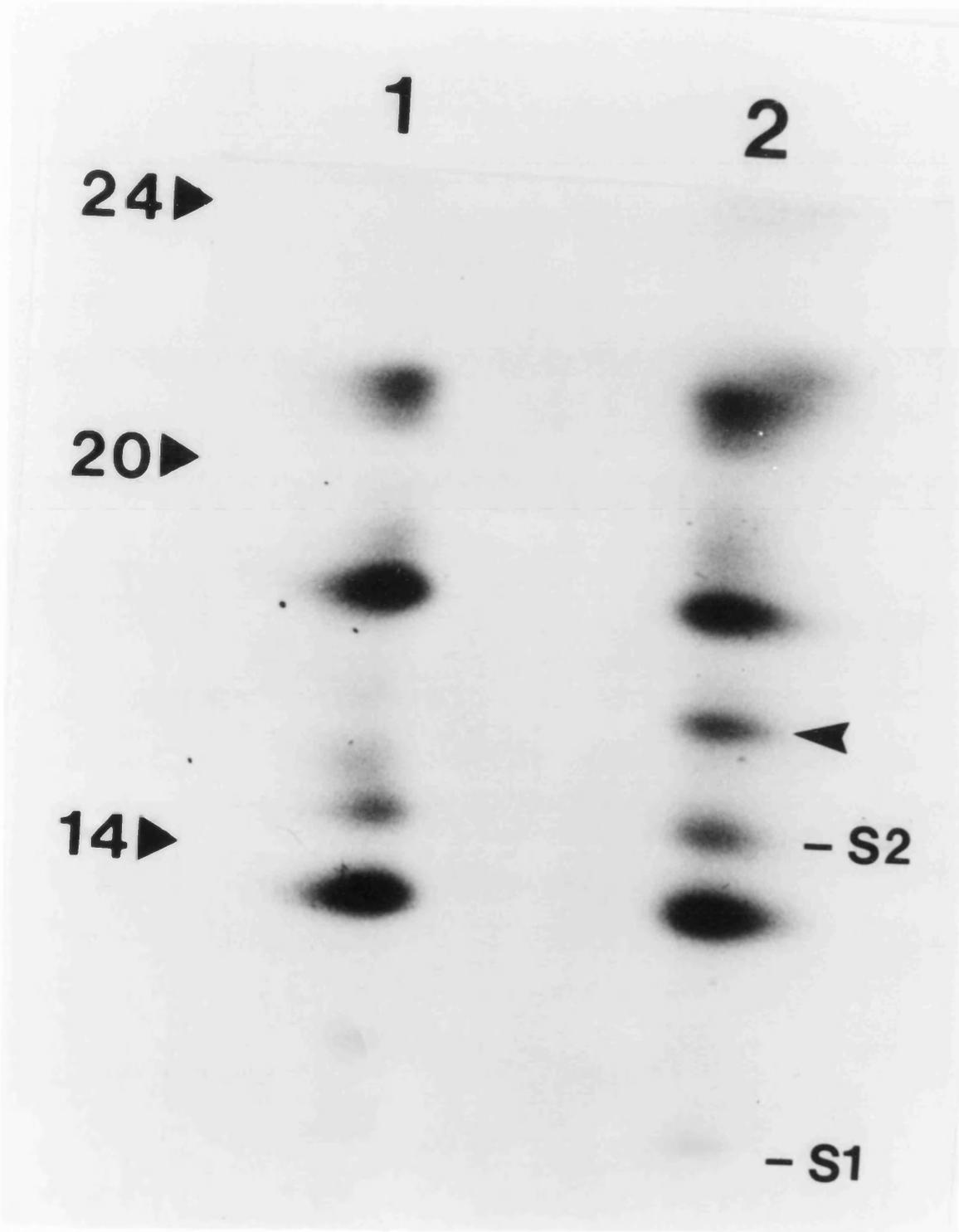
20▶

14▶



- S2

- S1



shows that partial proteolysis was obtained for both molecules, with the peptide map for the 30 kDa molecule (track 1) and the 32 kDa molecule (track 2) being very similar, indicating close relatedness of the polypeptides. Minor differences were seen, most notably the presence of a peptide of approximately 16 kDa in the 32 kDa digestion track (arrowed in Fig. 5.3, track 2). This peptide profile was repeatedly obtained, for both proteins, using the conditions described in section 2:7:5:1. This showed that, although the proteolysis was partial, the V8 protease digestion pattern did not change, indicating that some parts of the polypeptide structures were consistently less vulnerable to V8 protease digestion than other parts.

5:2:2:2 Amino acid sequencing of peptide fragments

Two peptide fragments from the 30 kDa polypeptide, estimated to be 10 kDa and 15 kDa molecular weight (marked in Fig. 5.3 as S1 and S2), were sequenced (section 2:7:5:2). The amino acid sequence obtained is presented in Figure 5.4A (also see Appendix). The 32 kDa peptide fragment of the same size as the 30 kDa "S1" peptide fragment was also sequenced, so that the two peptide fragments could be compared in detail. The amino acid sequence of the S1 peptide fragments of the 30 kDa and 32 kDa molecules was found to be identical data. The S1 and S2 amino acid sequences were also compared to the amino acid sequence of the T.sergenti 33 kDa merozoite surface protein (Kawazu et al., 1992iii). Figure 5.4B (top panel) shows that the S1 amino acid sequence (S1) had 61% identity

Figure 5.4A: Amino acid sequences of the "S1" and "S2" peptide fragments.

1. "S1" peptide fragment

Leucine-Threonine-Valine-Alanine-Asparagine-Glycine-Tyrosine-Arginine-Phenylalanine-Lysine-Threonine-Leucine-Lysine-Valine-Glycine-Glutamine-Lysine-Threonine

2. "S2" peptide fragment

Valine-Isoleucine-Leucine-Proline-Asparagine-Asparagine-Aspartic acid-Arginine-Histidine-Glutamine-Isoleucine-Threonine-Aspartic acid-Threonine-Arginine-Asparagine-Glycine-Histidine-Tyrosine-Alanine

Figure 5.4B: Comparison of the S1 and S2 amino acid sequences to the amino acid sequence of the T.sergenti 33 kDa molecule.

```

S1  1 .....ltvangyrfktlkvggkt..... 18
      :|..:| | :| | | | | | :|.
ts  51 vdasnamdvvftaeegyriktlkvgdknlytvdtskftptvahrlkhadd 100

|  exact match
:  closely related
.  related
  
```

Percent Similarity: 83.333
Percent Identity : 61.111

```

S2  1 .....vilpnndrhqitdtrnghya..... 20
      |:::..:..|.. :|.
ts  51 vdasnamdvvftaeegyriktlkvgdknlytvdtskftptvahrlkhadd 100
  
```

Percent Similarity: 40.000
Percent Identity : 15.000

and, taking conservative substitutions into account (Sambrook et al., 1989), it was found that there was 83.333% similarity to a stretch of the T.sergenti amino acid sequence (ts), while the S2 amino acid sequence (S2) had 15% identity and 40% similarity (Fig. 5.4B, lower panel).

5:2:3 Periodic acid-Schiff stain of the 30 kDa and 32 kDa polypeptides

To test for the presence of glycoproteins, an SDS-PAGE gel of Ankara, Hissar and Gharb piroplasm extracts was stained with periodic acid-Schiff (PAS) stain. The most intense staining was observed in the region of the 30 to 32 kDa bands of all three piroplasm stocks (Fig. 5.5), indicating that the 30 kDa and 32 kDa polypeptides of the Ankara stock (Fig. 5.5, track 1), the 31 kDa polypeptide of the Hissar stock (Fig. 5.5, track 2) and the 32 kDa polypeptide of the Gharb stock (Fig. 5.5, track 3) are glycosylated.

5:2:4:1 Periodate treatment of piroplasm and merozoite extracts

To determine if the antibody-detected 30/32 kDa epitopes were sodium metaperiodate-sensitive, a Western blot of piroplasm (Ankara) and merozoite (D7) extracts was periodate treated and then probed with antibody. The Western blot was first Ponceau-stained and cut into strips. Half of the strips were periodate treated, to disrupt glycosyl antigenic determinants, and the other half were left as untreated controls, as described in section 2:7:7.

Figure 5.5: Periodic acid-Schiff stain of SDS-PAGE gel of Ankara, Hissar and Gharb stock T.annulata piroplasm polypeptide extracts.

Track 1; Ankara piroplasms.
Track 2; Hissar piroplasms.
Track 3; Gharb piroplasms.

Figure 5.6: Immunoblot analysis of periodate-treated piroplasm and merozoite polypeptides.

Track 1-4,6,8; Ankara piroplasms
Track 5 and 7; D7 merozoites

Track 1 and 2; monoclonal antibody 1D11 reactivity
Track 3 and 4; anti-E3d serum reactivity
Track 5,6,7,8; monoclonal antibody 5E1 reactivity

+ treated with sodium metaperiodate
- untreated

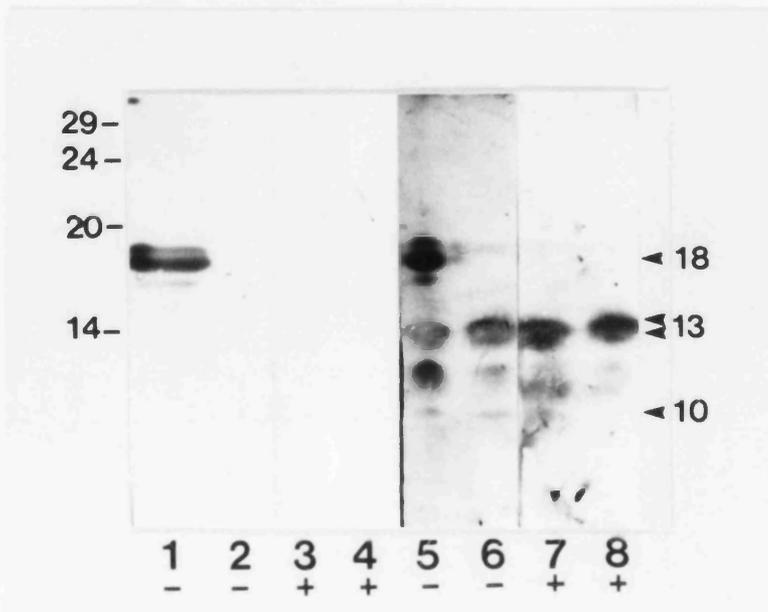
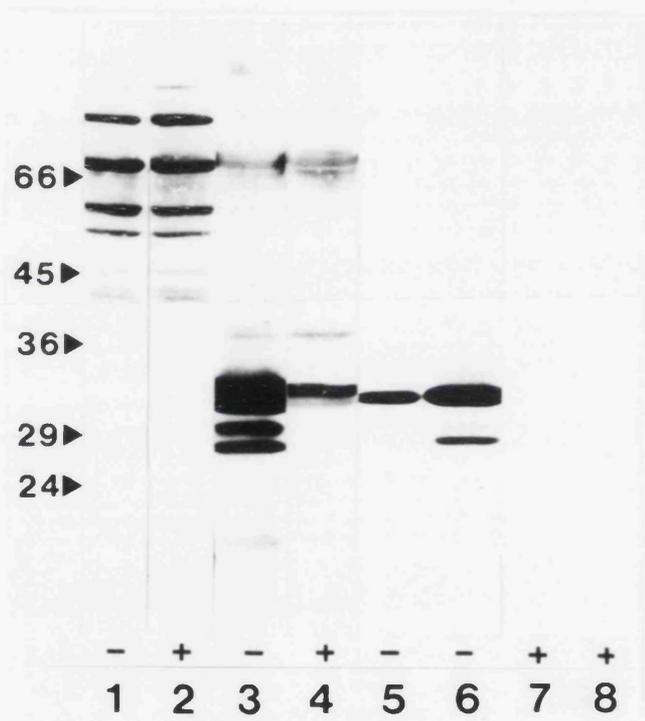
Figure 5.7: Western blot analysis of periodate-treated V8 digested 30 and 32 kDa molecules.

Track 1,3,5 and 7; V8-digested 30 kDa molecule.
Track 2,4,6 and 8; V8-digested 32 kDa molecule.

Track 1-4; monoclonal antibody 5E1 reactivity.
Track 5-8; anti-C9m serum reactivity.

+ treated with sodium metaperiodate
- untreated

Position of periodic acid Schiff-stained peptide bands arrowed.



The strips were probed pairwise (a treated strip and an untreated strip) with the monoclonals 1D11 and 5E1, and with the anti-E3d serum.

The result showed that periodate treatment had an effect on antibody reactivity with the 30/32 kDa doublet. The effect was most extreme on 5E1 reactivity, as the reactivity of this monoclonal, on the 30 kDa of the merozoite and piroplasm extracts, was abolished by periodate treatment (Fig. 5.6, compare untreated (-) tracks 5 (merozoites) and 6 (piroplasms) to treated (+) tracks 7 (merozoites) and 8 (piroplasms)). The reactivity of the anti-E3d serum was not abolished, but was significantly reduced against the 30/32 kDa doublet of the piroplasms (Fig. 5.6, tracks 3 and 4). In addition, it was noted that the reactivity bias of the serum for the 32 kDa polypeptide was not altered. Similarly, the reactivity of the anti-C9m serum was also reduced by periodate treatment, but the 30 kDa reactivity bias of the serum was not abolished (data not shown).

The reactivity of the 1D11 monoclonal with piroplasm polypeptides did not change after periodate treatment (Fig. 5.6, tracks 1 and 2), indicating that this monoclonal does not detect carbohydrate residues, which was expected as the 1D11 monoclonal recognises a 117 kDa polypeptide (section 3:2:3:3) which did not react with PAS stain (section 5:2:3). The absence of change in 1D11 reactivity also showed that periodate treatment had not adversely affected the polypeptide structure of the 1D11-detected proteins,

and was unlikely to have affected the primary structure of other polypeptides.

5:2:4:2 Periodate treatment of peptide digests

The 30 kDa and 32 kDa polypeptides were digested with V8 protease (see section 5:2:2) and Western blotted. The blot was cut into strips, and periodate treated, as described in the previous section. The strips were probed with the monoclonal 5E1 and with anti-C9m serum. The 5E1 monoclonal detected an 18 kDa peptide fragment of the digested 30 kDa molecule, and this reactivity was abolished by periodate treatment (Fig. 5.7, compare tracks 1 and 3). None of the 32 kDa peptide fragments were detected either before or after periodate treatment by the 5E1 monoclonal (Fig. 5.7, tracks 2 and 4). The anti-C9m serum also detected the 18 kDa 5E1-detected peptide of the digested 30 kDa molecule (arrowed in Fig. 5.7, track 5), which was not detected in the 32 kDa polypeptide digest track (Fig. 5.7, track 6). In addition, the anti-C9m serum detected a number of smaller peptide fragments, which were of identical molecular weight between the 30 and 32 kDa polypeptide digest tracks (Fig. 5.7, tracks 5 and 6). Periodate treatment abolished the anti-C9m serum reactivity with a 10 kDa fragment (arrowed in Fig. 5.7, compare tracks 5 and 6 to tracks 7 and 8), and appeared to reduce reactivity with fragments of approximately 12 kDa. The reactivity of a peptide doublet at 13 kDa (arrowed) was not affected by the periodate treatment. An additional strip of the 30 kDa and 32 kDa polypeptide digests was treated with PAS stain, so that the

Figure 5.8: Western blot analysis of neuraminidase-treated piroplasm (Ankara) extracts.

Track 1; untreated.

Track 2; Treated piroplasm and fetuin control.

Track 3; Untreated piroplasm and fetuin control.

Figure 5.9: Analysis of lectin binding to Western blots of piroplasm (Ankara) extracts.

Tracks A; Piroplasm (Ankara).

Tracks B; Control glycoprotein mixture.

Panel 1; Concanavalin A

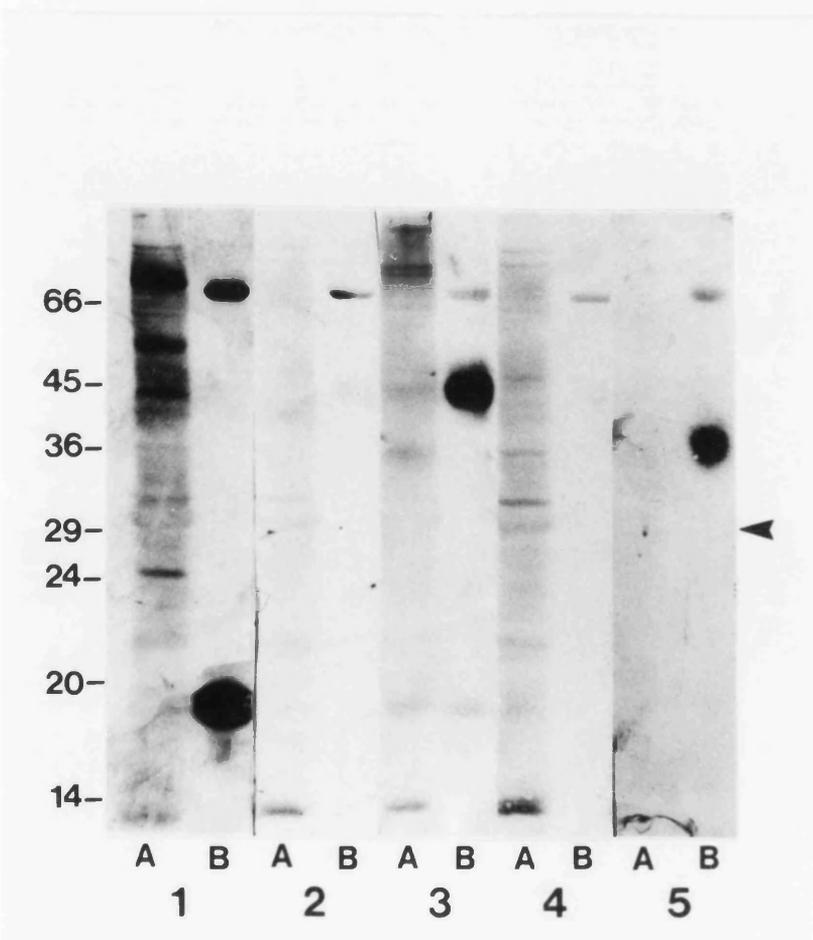
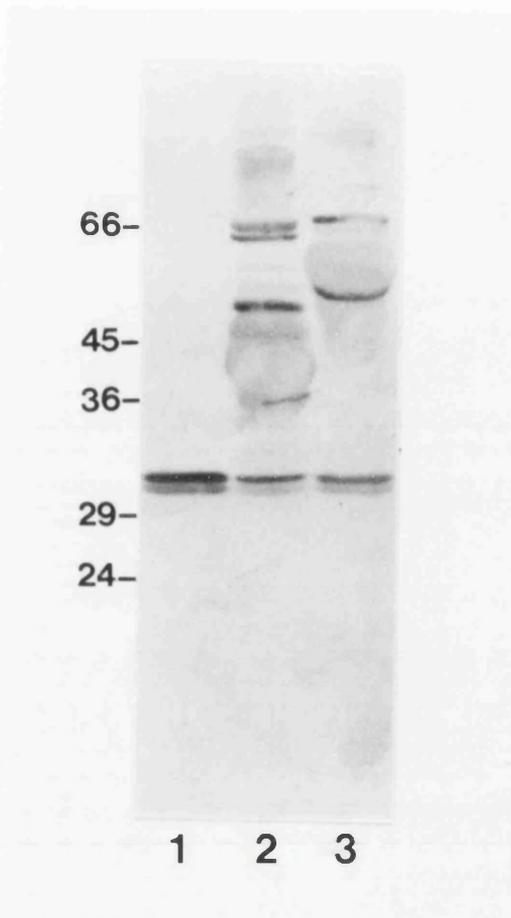
Panel 2; Ricinus communis agglutinin

Panel 3; Datura stramonium agglutinin

Panel 4; Phaseolus vulgaris erythrolectin

Panel 5; Wheat germ agglutinin

Position of 30 kDa polypeptide arrowed.



position of glycosylated peptides could be located. The positions of the PAS-stained bands (at 18 kDa, a doublet at 13 kDa, and at 10 kDa) are indicated in Figure 5.7 as arrows. As expected, the upper 18 kDa band, which was detected by the monoclonal 5E1, but the detection of which was abolished after periodate treatment, stained with PAS stain, as did the periodate-sensitive 10 kDa band detected by the anti-C9m serum. However, the doublet at approximately 13 kDa, which was detected by the anti-C9m serum, and this detection was not affected by periodate treatment, also stained with the PAS stain. This result suggested that either the anti-C9m serum was detecting non-carbohydrate residues of this 13 kDa peptide doublet, and/or the anti-C9m serum detected carbohydrate residues, but these were resistant to periodate-treatment.

5:2:5 Deglycosylation of the 30/32 kDa polypeptides

Deglycosylation of the 30 kDa and the 32 kDa polypeptides was attempted with a variety of enzymes. Calf fetuin, which is a glycoprotein with both N-linked and O-linked sugar residues, was used as a positive control for enzymatic cleavage of glycosylated residues. Figure 5.8 shows the result of neuraminidase (Vibrio cholerae) treatment of piroplasm extracts. The treated and untreated extracts were Western blotted and probed with anti-E3d serum. Comparison of antiserum reactivity with the untreated and the treated piroplasm extracts (Fig. 5.8, untreated, track 1 without fetuin, and track 3 with fetuin; treated, track 2 with fetuin) suggested that there was a slight reduction in the

Table 5.1 Enzymes used to remove the carbohydrate linkages from the 30kDa and 32kDa glycoproteins (see section 2:8:1 also).

Enzyme	Cleavage Specificity
N-Glycopeptidase F (PNGase F)	N-linkages Removes whole glycans
Endoglycosidase F	N-linkages Leaves single N-acetylgalactosamine residue linked to polypeptide
Endoglycosidase H	Same as above Does not cleave complex oligosaccharides
O-Glycosidase	O-linkages
Neuraminidase (sialidase)	Removes sialic acid from N- or O- linked glycoconjugates
α - D-mannosidase	Self-explanatory
β - D-acetyl hexosamidase	"
α - galactosidase	"
β - D-galactosidase	"
α - L-fucosidase	"

reactivity of the anti-E3d serum with the 30 kDa polypeptide after neuraminidase treatment (compare tracks 2 and 3). The estimated shift in the molecular weight of the fetuin control was from 64 kDa to 45 kDa after neuraminidase treatment (Fig. 5.8, tracks 3 and 2), indicating that sialic acid residues had been cleaved from the glycoprotein, and that the enzyme was functional in the incubation conditions used.

Different enzymes were tested for the deglycosylation of the 30/32 kDa polypeptides, to either remove N-linked or O-linked carbohydrate groups, or to remove specific sugar residues. No 30/32 kDa molecular weight size shift or reduction in antibody reactivity (except for the slight reduction in anti-E3d serum reactivity, observed after neuraminidase treatment, and described above) was detected under any of the conditions used. The enzymes tested, and their specificity, are given in Table 5.1.

In addition, tunicamycin, an inhibitor of polypeptide N-glycosylation, was tested for effect on the synthesis of the 30 kDa glycoprotein during the heat-induced differentiation of the cloned cell line D7. Polypeptide extracts were made from the differentiating cultures, with and without tunicamycin, every two days. A Western blot of the extracts was probed with anti-E3d serum and monoclonal antibody 5E1. There was no difference in antibody reactivity between the extracts from cultures with and without tunicamycin, indicating that the inhibitor had no effect on the glycosylation of the 30 kDa protein (data not shown).

Table 5.2 The specificity of binding of the lectins used (see section 2:8:3 also).

Lectin	Specificity
Concanavalin A (Con A)	Alpha-linked Mannose + Glucose
Ricinus Communis Agglutinin (RCA)	Galactose + N-acetylgalactosamine
Datura Stramonium Agglutinin (DSA)	N-acetyl glucosamine oligomers N-acetyllactosamine
Phaseolus Vulgaris Erythrolectin (PHA-E)	Complex oligosaccharides containing outer galactose residues
Wheat Germ Agglutinin (WGA)	N-acetylglucosamine N-acetylneuraminic acid (sialic acid)

5:2:6 Lectin binding to piroplasm polypeptides

A range of biotinylated lectins, which were known to bind to N-glycosylated molecules (Nicolson, 1974), were reacted with Western blots of Ankara piroplasm extracts. The lectins tested, and their binding specificities, are given in Table 5.2. The result showed that several high molecular weight piroplasm polypeptides bound two of the lectins; concanavalin A (Fig. 5.9, track 1A) and Phaseolus vulgaris erythrolectin (Fig. 5.9, track 4A), and four of the five lectins bound a 14 kDa polypeptide (Fig. 5.9, tracks 1A, 2A, 3A and 4A). However, none of the lectins gave strong reproducible binding to the 30/32 kDa doublet (position shown by an arrow), indicating that the 30 kDa and 32 kDa polypeptides did not possess the carbohydrate residues normally detected by the lectins used.

5:2:7 Triton X-114 phase separation of piroplasms

Integral membrane proteins can usually be identified by the ease with which they partition into the detergent-rich phase of Triton X-114 (Bordier, 1981). This detergent remains in solution at 4°C, but at 20°C, the detergent partitions from the aqueous phase so that integral membrane proteins can be separated, at temperatures above 20°C, from other (hydrophilic) proteins, which are retained in the aqueous phase (Bordier, 1981). Ankara stock piroplasms were subjected to a Triton X-114 phase separation, as described in section 2:9. The separate phases were run on an SDS-PAGE gel, and the gel was stained with Coomassie brilliant blue. The result showed that the 30/32 kDa doublet separated

Figure 5.10: Triton X-114 phase separation of piroplasm (Ankara) polypeptides.

Track 1; Unseparated piroplasm extract.

Track 2; Detergent fraction of Triton X114-separated piroplasm polypeptides.

Track 3; Aqueous fraction.

Figure 5.11: Detection of T.annulata (Ankara) piroplasm GPI-anchored proteins on a Western blot of separated Triton X114 detergent and aqueous fractions probed with anti-CRD antiserum.

Panel A: Piroplasm extracts

Track 1 and 2; Untreated piroplasm, TX 114 aqueous and detergent fractions, respectively.

Track 3 and 4; GPI-PLC-treated piroplasm, TX 114 aqueous and detergent fractions, respectively.

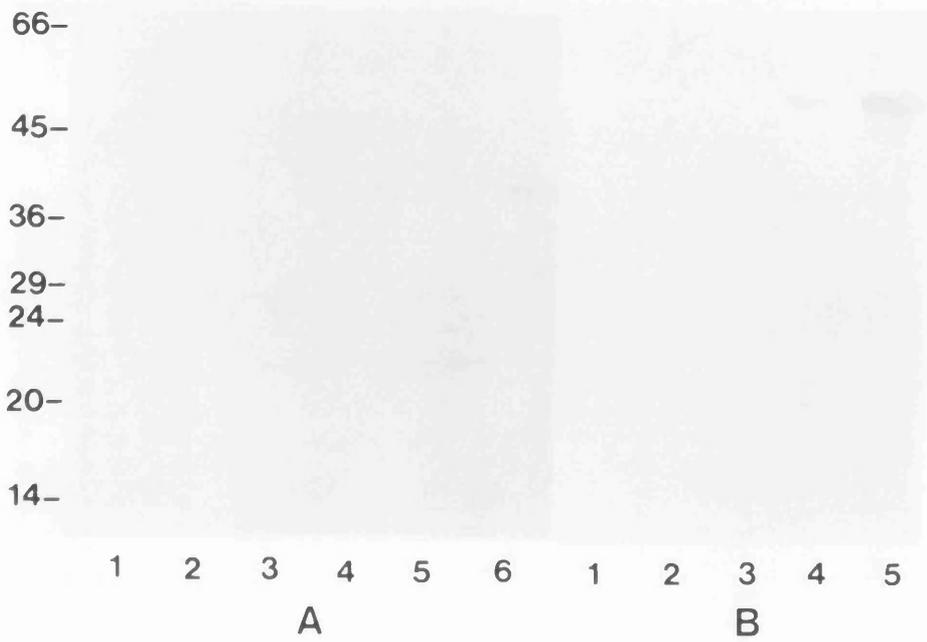
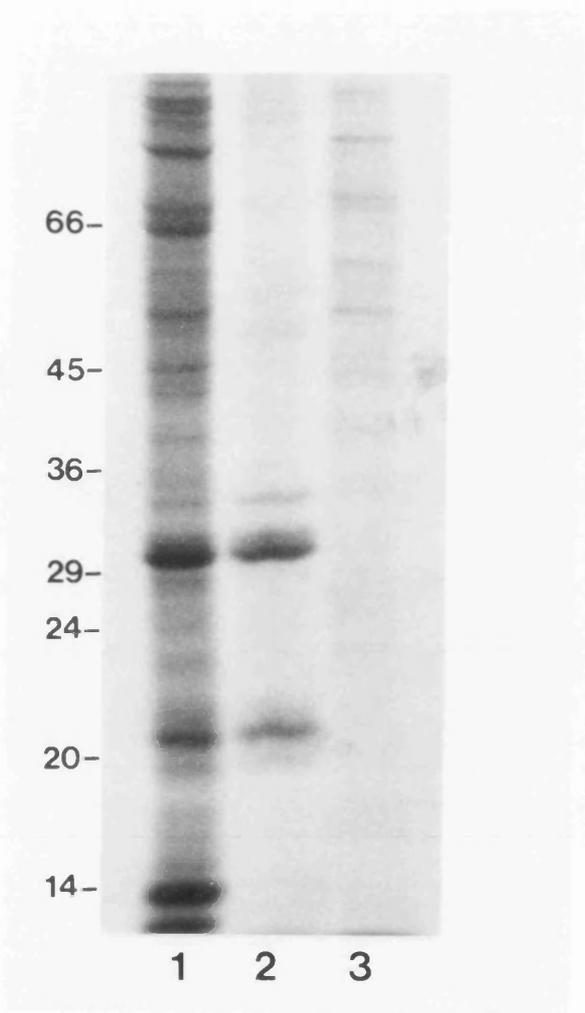
Track 5 and 6; PI-PLC-treated piroplasm, TX 114 aqueous and detergent fractions, respectively.

Panel B: Control tracks

Track 1 and 2; Untreated membrane form VSG, TX 114 detergent and aqueous fractions respectively.

Track 3 and 4; GPI-PLC-treated membrane form VSG, TX 114 detergent and aqueous fractions, respectively.

Track 5; Soluble form VSG.



exclusively into the hydrophobic detergent phase (Fig. 5.10, track 2). Other significant proteins in this phase were a polypeptide of approximately 35 kDa and a polypeptide of approximately 21 kDa. The aqueous phase (Fig. 5.10, track 3) contained a range of polypeptides, mainly over 40 kDa molecular weight, and including the 117 kDa rhoptry protein. This result indicated that the 30/32 kDa polypeptides were amphiphilic or hydrophobic, and so were likely to be integral membrane proteins.

5:2:8 Detection of GPI-anchored polypeptides

Some preliminary experiments were carried out to allow the detection of piroplasm polypeptides, which were GPI-anchored to the parasite surface membrane. A major feature of the GPI-anchored proteins, which distinguishes them from other membrane proteins, is the ability of most of them to be released from membranes by the action of specific bacterial phospholipases (Zamze et al., 1988). By this cleavage, the previously hydrophobic protein becomes hydrophilic (Low, 1990). This change, from being hydrophobic to being hydrophilic, can be monitored by the Triton X-114 method of phase separation. Three phosphoinositol phospholipase C (PI-PLC) enzymes were employed; PI-PLC from Bacillus thuringiensis, PI-PLC from Bacillus cereus and GPI-PLC from Trypanosoma brucei (Fox et al., 1986). These enzymes cleave at the same site within the variant surface glycoprotein (VSG) GPI anchor of Trypanosoma brucei. The membrane form (GPI-anchored) and the soluble (membrane-cleaved) form of the T. brucei VSG

were used as controls for enzyme cleavage.

The detergent phase of a Triton X-114 phase separation of piroplasms (see previous section) was treated with enzyme or, as a control, left untreated. Treated and untreated piroplasm samples were subjected to a second Triton X-114 phase separation, and the aqueous and detergent fractions were run on an SDS-PAGE gel, Western blotted and probed with anti-CRD antibody. This antibody detects a carbohydrate epitope, the cross-reacting determinant (CRD), which is sometimes generated by the action of the phospholipases on GPI anchors. The result of piroplasm treatment with each of the three enzymes was identical. Figure 5.11 shows the result of cleavage with two of the three enzymes, *T. brucei* GPI-PLC and *B.thuringiensis* PI-PLC. The anti-CRD antibody reacted with the aqueous fraction of the enzyme-treated membrane form VSG control (molecular weight, 58 kDa), indicating successful enzyme cleavage of the membrane form GPI anchor (Fig. 5.11B, track 4), and with the soluble form VSG control (Fig. 5.11B, track 5). The antibody did not react with the untreated VSG fractions, as expected (Fig. 5.11B tracks 1 and 2). However, in addition, the antibody did not react with the aqueous or the detergent fractions of either the treated or untreated piroplasm samples (Fig. 5.11A, tracks 1-6), showing that a CRD epitope was not generated by the phospholipase cleavage. Furthermore, Coomassie-staining of the SDS-PAGE gel of the detergent and aqueous fractions showed that none of the polypeptides contained in the detergent fraction of the first Triton X-114 phase

separation, before enzyme treatment, became hydrophilic polypeptides in the aqueous fraction of the second Triton X-114 phase separation, after enzyme treatment (data not shown). Therefore, as there was no difference between the detergent phase and aqueous phase tracks before and after enzyme treatment, it was concluded that either T.annulata (Ankara) piroplasms do not have GPI-anchored polypeptides, or the T.annulata GPI-anchored polypeptides were resistant to digestion by the three enzymes employed.

5:3 Discussion

The 2D-PAGE and the peptide mapping analyses revealed that the 30 kDa and 32 kDa molecules of Theileria annulata are closely related. The 2D-PAGE analysis showed that both molecules were basic (pI 8.5), and differed only in molecular weight on the second dimension gel (Figure 5.2). Interestingly, the 33 kDa protein of T. sergenti and the 34 kDa proteins of T. buffeli and T. orientalis are also seen as basic proteins on 2D-PAGE gels (Sugimoto et al., 1991; Kawazu et al., 1992i).

However, similarities detected by 2D-PAGE analysis can sometimes be misleading. For example, proteins may co-migrate because they are bound tightly together, or they may have an identical charge but are dissimilar in all other respects (Rickwood et al., 1990). The peptide mapping technique consists of breaking down a protein into a number of peptide fragments in a specific and controlled manner, followed by separation of the peptides on an SDS-PAGE gel to give a map, which is characteristic for each protein substrate and the proteolytic enzyme used. Therefore, peptide mapping is a stringent method of determining protein relatedness. For example, in one study of Plasmodium falciparum, two monoclonal antibodies, which detected proteins of identical molecular weight, were used to immunoprecipitate the proteins which were then treated with V8 protease, and the resultant peptide maps showed that the monoclonals recognised identical proteins, i.e. MSA 1 (Hall et al., 1984i). The peptide maps of the 30 kDa and 32 kDa molecules of T. annulata were very similar, with

minor differences between them (Figure 5.3), showing that the 30 kDa and 32 kDa proteins are closely related. In addition, the amino acid sequences of the "S1" peptide fragments of both molecules were identical (section 5:2:2:2). The 33 kDa and 34 kDa piroplasm surface proteins of T.sergenti and T.buffeli also appear to be related, although this has not been shown by peptide mapping. The genes for both proteins have been cloned and sequenced (Kawazu et al., 1992ii), and the predicted amino acid sequence showed 82% similarity between the two molecules (Kawazu et al., 1992iii). In addition, comparison of the amino acid sequences of the two peptide fragments sequenced from the T.annulata 30 kDa molecule with the T.sergenti 33 kDa protein amino acid sequence showed that one of the T.annulata peptide sequences (S1) has, taking conservative substitutions into account, over 83% similarity to a stretch of the T.sergenti 33 kDa sequence (Figure 5.4B).

Partial proteolysis was consistently obtained for the T.annulata 30 kDa and 32 kDa proteins under the peptide mapping conditions used, and the peptide map patterns obtained were consistent, as if specific regions of the polypeptides were more vulnerable to proteolysis. As the proteins were shown, by the PAS stain of SDS-PAGE gels, to be glycosylated, diminished proteolytic susceptibility could have occurred where stretches of polypeptide had attached carbohydrate, as has been found for other glycoproteins (Flannery et al., 1989). In fact, it was found that some 30 kDa peptide fragments could not be amino acid sequenced, probably because specific amino acid

residues of these fragments had attached carbohydrate and these amino acids could not be identified and chemically cleaved during the Edman degradation sequencing process (M.Cusack, personal communication).

The 30 kDa polypeptide epitope recognised by the monoclonal antibody 5E1 was shown to be periodate-sensitive. Sodium metaperiodate treatment oxidizes the glycol group of sugar moieties (Honegman, 1948) and, at the concentration used in these experiments (10 mM), does not adversely affect peptide sequence (Woodward et al., 1985). This was confirmed by the 1D11 monoclonal antibody reactivity, which was not affected by periodate treatment (Figure 5.6). The binding of the anti-E3d and anti-C9m sera to the 30/32 kDa doublet was reduced significantly by periodate treatment, but it was interesting to note that the reactivity bias of the sera normally seen, for the 30 kDa or the 32 kDa molecule (see section 4:2:1), was not affected by the treatment. This inferred that either all the carbohydrate epitopes had been destroyed by the treatment and the two antisera reacted with peptide sequence only, indicating that the 30 kDa or 32 kDa reactivity bias observed reflected differences in peptide sequence between the two molecules; or, only a proportion of carbohydrate epitopes were destroyed by the periodate, and certain residues, which differed between the two molecules, were resistant to the periodate treatment (Woodward et al., 1985). In addition, periodate treatment of the peptide digests showed that treatment affected anti-C9m reactivity with some fragments, but not with others. In

particular, a specific peptide doublet was shown to be glycosylated by PAS stain, but anti-C9m reactivity with this doublet was unaffected by periodate treatment.

Carbohydrate moieties of glycoproteins have been shown to exert a strong influence on antigenicity and, frequently, the removal of sugars results in significantly reduced antigenicity, as has been demonstrated in Plasmodium falciparum (Ramasamy and Reese, 1986), Schistosoma mansoni (Omer-Ali et al., 1986), and in Trypanosoma cruzi (Snary, 1985). Hence, it is unlikely that the anti-C9m serum reacted with only non-sugar residues of the peptide doublet, and the known antigenicity of carbohydrate moieties in other protozoan systems suggests that the carbohydrate residues of the peptide doublet were detected by the antiserum, but were resistant to periodate treatment. Furthermore, the differences in the peptide maps of the 30 kDa and 32 kDa polypeptides may be due to differences in the accessibility of the V8 protease to various peptide residues, caused by differential glycosylation along the length of the 30 kDa and 32 kDa amino acid sequences. Similarly, the 30 kDa or 32 kDa antiserum bias, before and after periodate treatment, could be due to differences in carbohydrate residues, in quantity and/or in the specific sugar residues present. The difference in the 30 kDa and 32 kDa glycosylation may not be extensive. A single sugar residue change in a glycoprotein can change its structure and biological activity, and carbohydrate structures can be altered by changing the position of sugar linkages (Olechno et al.,

1989). In fact, most glycoproteins have oligosaccharide chains at different peptide loci and often an array of carbohydrate structures is present at each of these glycosylated sites (Slomiany et al., 1984). However, single amino acid substitutions can also lead to a change in the structure and antigenicity of a molecule (Guttinger et al., 1988), so the existence of differences in amino acid sequences of the two molecules cannot be ruled out.

Surface antigens are frequently glycoconjugates (Sher, 1988), and the cell surface glycoconjugates of parasitic protozoa have key functions in, for example, host cell invasion and in the survival of these organisms (Ferguson and Homans, 1988). In the Theileria species, as well as the 30 kDa and 32 kDa molecules of T.annulata, the immunodominant 32 kDa piroplasm surface molecule of T.mutans has also been identified as a glycoprotein (Katende et al., 1990), and the predicted amino acid sequences of the 33 kDa and 34 kDa proteins of T.sergenti and T.buffeli has been reported to contain sites of potential glycosylation which differ between the two proteins (Kawazu et al., 1992i; 1992ii; 1992iii). To find out more about the biochemical nature of the T.annulata 30 kDa and 32 kDa glycoproteins, a range of lectins were tested for binding to the doublet, and deglycosylation of the polypeptides was attempted using various enzymes. None of the lectins used bound to the 30/32 kDa doublet, and as those used were usually (although not always) specific for sugar residues linked to the protein backbone by a N-(asparagine) linkage, as opposed to a O-(serine/threonine)

linkage, it was assumed that the 30/32 kDa glycosylation was probably O-linked. However, when both N-glycanase and O-glycanase enzymes were used for deglycosylation, neither treatment resulted in a size shift and/or significant change in antibody reactivity, as would have been expected if the carbohydrate portion of the glycoproteins had been removed. In addition, different enzymes were used to remove specific sugar residues, as it was found in other protozoa that some residues were more antigenic than others (Ramasamy and Reese, 1986). It was hoped that, with this approach, it could be determined which sugar residues were present in the molecules, and if these were responsible for the antigenicity of the 30/32 kDa proteins. However, this approach proved unsuccessful.

It was obvious that the 30/32 kDa glycoproteins of T.annulata were unusual, as they appeared to be resistant to every deglycosylation treatment tried. However, the biosynthesis pathway of glycoproteins and the structures of some glycoproteins of protozoa have been found to deviate significantly from those found in mammalian cells, and unusual monosaccharide configurations are often found which are not representative throughout the eukaryotes (Ferguson and Homans, 1988). For example, the GP72 glycoprotein of Trypanosoma cruzi, which is involved in the control of morphogenesis of the parasite (Snary, 1985), has unusual N-linked and O-linked protein-carbohydrate structures as it contains high levels of phosphate and atypical sugars, such as rhamnose, which are not normally found in higher eukaryote glycoproteins (Ferguson et al.,

1983; Snary et al., 1987). The Leishmania donovani major cell surface glycoconjugate, lipophosphoglycan (LPG), also has an unusual glycan structure, for example, it has 4-substituted mannose and galactofuranose, which are highly unusual constituents of eukaryotic glycoconjugates, and it contains phosphodiester linkages between sugars, as well as glycosidic bonds (Turco et al., 1987; 1989). Furthermore, the carbohydrate chains of these glycoconjugates are highly immunogenic (Snary, 1985; Handman and Goding, 1985; Handman et al., 1987), and their immunogenicity is a function of their unusual chemical structure, as compared with mammalian glycans (Ferguson and Homans, 1988). Because of the diversity in protozoan parasite glycoconjugate structure, no assumptions can be made about the structure of a parasite glycan. Therefore, the commercially available carbohydrate-cleaving enzymes and lectins, which have been mostly developed for mammalian systems, are sometimes inappropriate for experiments on protozoan parasite-specific glycoconjugates (Ferguson and Homans, 1988). Furthermore, proteins with a high degree of glycosylation are often extremely resistant to enzymatic cleavage (Paxton et al., 1987). It could prove that, in order to deglycosylate the 30/32 kDa doublet, chemical, rather than enzymatic, methods would have to be employed, such as the anhydrous hydrazinolysis of N (asparagine)-linked glycans (Takaski et al., 1982; Ashford et al., 1987) and the release of O (serine/threonine)-linked glycans by alkali in a beta-elimination reaction (Ogata and Lloyd, 1982). These procedures would necessitate the generation of large (100-

200 micrograms), and relatively pure, quantities of the glycoprotein. As the extraction of the 30/32 kDa molecules was only possible with 1% SDS, a detergent which can interfere with biological activity (section 5:1:1), the 30/32 kDa proteins would have to be recovered from the SDS-containing solution by, for example, ion exchange chromatography (Hager and Burgess, 1980), or high performance liquid chromatography (HPLC; Simpson et al., 1987), before the glycan portion of the glycoprotein could be isolated and chemical analysis of the glycan could begin. Alternatively, the monoclonal antibody 5E1 could be used to purify the 30 kDa molecule from an SDS-extract of piroplasm material in an immunoprecipitation experiment, as described in section 2:6, but using water to remove detergent from the immobilized antibody/antigen complexes before elution of the 30 kDa molecule with 1M propionic acid (Guther et al., 1992), so that the molecule is in a sufficiently pure and stable form for chemical analysis. Such carbohydrate structural analysis may involve techniques such as the sequential removal of monosaccharides by specific exoglycosidases (Yamashita et al., 1982; Kobata, 1984), gas chromatography-mass spectrometry, to provide a linkage composition of the sugars present (Aspinall, 1982), and nuclear magnetic resonance (NMR), to determine glycan primary structure, and sometimes three-dimensional structure (Ferguson and Homans, 1988).

The 30/32 kDa doublet was found to be readily extractable by the detergent Triton X-114, however, and the

polypeptides separated into the detergent phase, rather than the aqueous phase, showing that the molecules were amphiphilic or hydrophobic and so were likely to be integral membrane proteins. The membrane proteins of protozoa are frequently attached to the membrane by GPI-anchors, which give a stable association of the proteins with the plasma membrane (Ferguson and Williams, 1988). For example, the Trypanosoma brucei VSGs (Low, 1987), the lipopeptidophosphoglycan (LPPG) and the 1G7-antigen of T.cruzi (Previato et al., 1990; Lederkramer et al., 1991; Guther et al., 1992), the merozoite surface antigens of Plasmodium falciparum, MSA 1 and MSA 2 (Smythe et al., 1988; Ramasamy, 1987; Haldar et al., 1985), and the Gp63 surface protease, the lipophosphoglycan (LPG) and the glycoinositol phospholipids of Leishmania (Bordier et al., 1986; Button and McMaster, 1988, 1990; Turco et al., 1989; McConville et al., 1990i, 1990ii; McConville and Blackwell, 1991). The GPI membrane anchors of T.brucei, T.cruzi, Leishmania and Plasmodium glycoproteins are sensitive to degradation with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), releasing the protein from the cell surface and exposing a cross-reactive determinant (CRD), an epitope unique to phospholipase cleaved GPI anchors (Zamze et al., 1988). Preliminary experiments, carried out to determine if the 30/32 kDa proteins were attached to the parasite membrane by GPI anchor, provided no evidence that they were; CRD epitopes were not generated and amphiphilic/hydrophobic proteins did not become hydrophilic by phospholipase action. In addition, differentiating T.annulata (D7) cultures were incubated

with tritium-labelled myristic acid and ethanolamine, common components of GPI-anchors (Ferguson and Williams, 1988). In autoradiographs of SDS-PAGE gels of extracts from the cultures, the 30 kDa polypeptide did not appear to incorporate the compounds (data not shown). However, the structure of different protozoan GPI-anchors can vary significantly between parasite species (Ferguson and Williams, 1988) and T.annulata GPI-anchored proteins may exist, but may not be cleaved by the PI-PLC enzymes used. Alternatively, the proteins may be released chemically by nitrous acid deamination and/or dephosphorylation with cold aqueous hydrofluoride (Ferguson et al., 1988). However, the 30 kDa and 32 kDa proteins could be anchored in the parasite membrane by another type of hydrophobic interaction (Findlay, 1990). The predicted amino acid sequences of the T.sergenti and T.buffeli 33 kDa and 34 kDa proteins contains a hydrophobic region at the C-terminus and it has been suggested that this region could be anchor sequence (Kawazu et al., 1992iii). Once the structure of the T.annulata 30/32 kDa proteins has been elucidated by either chemical analysis or by cloning the 30/32 kDa protein gene, the method of the attachment of these proteins in the parasite membrane could be fully determined.

CHAPTER SIX

ISOLATION OF THE 30 kDa POLYPEPTIDE GENE

6:1:1 Introduction

The results of the previous Chapters have shown that merogony represents a major point of differentiation during the mammalian phase of the life-cycle of Theileria annulata. It was shown in Chapter 3 that, coinciding with significant morphological changes, there are changes in the parasite polypeptide profile, including a decrease in the levels of schizont polypeptides and an increase in the abundance of merozoite-associated polypeptides. This developmental switch presumably reflects the expression of genes involved in regulating macroschizont differentiation, and those necessary for the survival of the merozoite as an extracellular parasite and for subsequent invasion of host erythrocytes. The 30 kDa glycoprotein was shown to be a major stage-specific surface antigen of Theileria annulata (Ankara) merozoites and piroplasms, and the expression of the 30 kDa molecule was positively regulated during merogony, specifically during the irreversible and committed phase of merogony. However, it was not known at what level of gene expression the 30 kDa protein was being regulated, and how this related to the mechanism employed to trigger differentiation.

Knowledge of the structure of the 30 kDa protein gene would allow more to be known about the biochemical structure of the protein it encodes. The 30 kDa glycoprotein was found to exhibit antigenic diversity and size polymorphism (Chapter 4), and the mechanism of antigenic diversity may possibly be determined from analysis of the DNA sequence of the antigen-encoding gene.

The predicted amino acid sequence of the variant forms of a specific protein can be examined for variable repeat sequences or differential sites of glycosylation (for examples, see Introduction and Discussion, Chapter 5). For example, the 33 kDa and 34 kDa protein gene sequences of Theileria sergenti and T. buffeli do not contain significant stretches of nucleotide repeats and it has been suggested that antigenic diversity between these two molecules comes from differential sites of glycosylation along the length of the predicted peptide sequence (Kawazu et al., 1992iii). Attempts to study the T. annulata 30 kDa glycoprotein structure and polymorphism with standard biochemical techniques gave largely inconclusive results (Chapter 5), although sodium metaperiodate experiments (section 5:2:4) indicated that the 30 kDa and 32 kDa molecules were likely to differ in the nature and/or quantity of glycosylated residues. Therefore, it became increasingly desirable to clone and sequence the 30 kDa protein gene to get a more accurate indication of the protein's structure from a predicted amino acid sequence. In addition, surface glycoconjugates have been shown to have significant functions in other parasitic protozoa (Hyde, 1990i), and isolation of the 30 kDa gene could allow the protein function to be elucidated by studying base sequence and amino acid sequence homology to other sequenced proteins.

In order to clone the 30 kDa protein gene, there were various approaches which could be followed. The most common strategies used to clone parasite genes include heterologous probing, antibody screening and

oligonucleotide probing. Heterologous probing is usually adopted for ubiquitous genes encoding, for example, structural genes such as actin or tubulin. However, other than knowing that it was a glycoprotein on the T.annulata merozoite surface, there was little information on the nature of the 30 kDa protein, so heterologous probing with DNA from other organisms was not feasible. Many parasite genes have been isolated by screening expression libraries with antibody probes (Hyde, 1990iii). Antibody screening was used, previous to this project, to attempt to clone the 30 kDa protein gene (Glascodine, 1989). A Theileria annulata (Hissar) piroplasm genomic expression library was screened with the 30 kDa-specific monoclonal antibody 5E1. This resulted in the isolation of a clone which reacted with monoclonal 5E1. The cloned DNA was expressed as fusion protein, and polyspecific serum was generated against it. In immunofluorescence assays, the serum appeared to react stage-specifically against cultures which were producing merozoites, and not against macroschizont-infected cells, but in Western blot experiments, the antiserum reacted with piroplasm and merozoite polypeptides ranging from 40 kDa to approximately 130 kDa and did not react with the 30 kDa or 32 kDa polypeptides. However, screening an expression library with a monoclonal antibody can be problematic if the single epitope the monoclonal recognises is not encoded by the gene fragments expressed in the library, or false positives can occur if epitopes, closely resembling the one on the desired target, are detected by the antibody reagent. These problems can be reduced by the use of

polyclonal antibodies, which increases the chance of detecting the relevant recombinant phage (Hyde, 1990iii).

Oligonucleotide probing can be employed if part of the protein amino acid sequence has been determined. This information can then be used to design synthetic oligonucleotides. Because of the redundancy of the genetic code, the oligonucleotides are usually synthesised to include all possible base sequences, and are therefore degenerate, in that they contain sequence that is not identical to the sequence of the target gene. In some protozoa, for example Plasmodium falciparum, codon usage is highly biased, so oligonucleotides can be constructed using selected codons to reduce degeneracy (Hyde et al., 1989). Oligonucleotide probing has also been used to successfully isolate parasite genes with less biased codon usage, for example the RNA polymerase II gene (Evers et al., 1989) and a cysteine proteinase gene (Mottram et al., 1989) of Trypanosoma brucei.

In conclusion, isolation of the 30 kDa protein gene would not only allow examination of gene regulation, but would give further insight into the molecular structure of the polypeptide and its antigenic diversity. In addition, sequence homology analysis could possibly give some indication of the function of the protein.

6:1:2 Summary and Aims

Differentiation of the macroschizont to the merozoite involves the positive and negative regulation of the expression of various polypeptides. The 30 kDa polypeptide expression is positively regulated during merogony and it became desirable to clone the gene for this protein in order to study its expression, and the molecular structure of the protein. As previous screening with the 30 kDa-specific monoclonal antibody 5E1 had failed, alternative methods of screening were required.

The aim of the work presented in this Chapter was to clone the 30 kDa gene. Two cloning strategies were undertaken in separate attempts to isolate the 30 kDa gene. The first was a polyclonal antibody screen and the second, an oligonucleotide screen.

6:2 Results

6:2:1 Immunoscreen of the lambda gt11 genomic library

A total of 7×10^4 recombinant phage from the Theileria annulata (Hissar) lambda gt11 genomic library were screened using the rabbit anti-E3d serum, generated against a cloned T.annulata (Ankara) differentiating macroschizont-infected cell line (E3), which recognises the 32 kDa polypeptide strongly and cross-reacts with the 30 kDa polypeptide in Western blotting experiments (section 4:2:1). As a control, the plaques were also screened with normal rabbit serum. From the screen, three immunopositive clones (C4, C6 and C10) were obtained and purified to homogeneity. Figure 6.1 shows the result of immunoscreening plaques expressing fusion protein from clone C6 with the anti-E3d serum (Fig. 6.1, side B) and with normal rabbit serum (Fig. 6.1, side A). It can be seen that the plaques from the phage clone C6 were detected strongly by the anti-E3d serum, and the positive antiserum reactivity can be seen to give a "halo" pattern on the plaques. The normal rabbit serum detected the plaques weakly, and it was assumed that this serum detected bacterial proteins, rather than expressed recombinant phage proteins.

6:2:2 DNA slot blot analysis with cloned DNA

It was essential to determine if the isolated immunopositive phage clones contained either bovine or parasite DNA sequences. Phage DNA was prepared from the three phage clones (C4, C6 and C10), which were detected

Figure 6.1: Plaque lift of lambda phage clone C6 screened with anti-E3d serum and normal rabbit serum.

Side A; Screened with normal rabbit serum

Side B; Screened with anti-E3d serum

Figure 6.2: Slot-blot analysis of genomic DNA

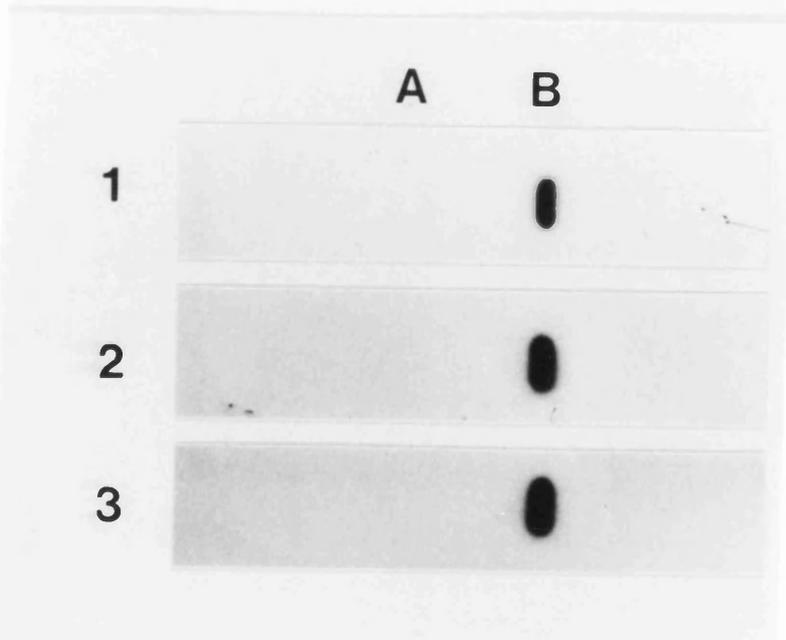
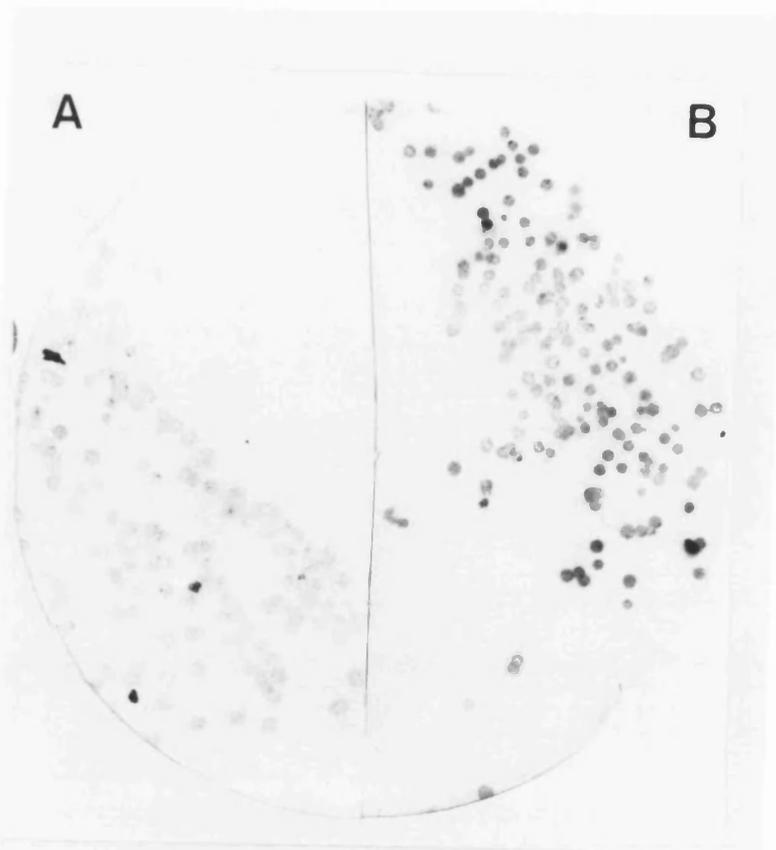
Slot A; BL20 DNA.

Slot B; T.annulata (Ankara) piroplasm DNA.

Filter 1; Hybridised with clone C4 DNA.

Filter 2; Hybridised with clone C6 DNA.

Filter 3; Hybridised with clone C10 DNA.



strongly by the anti-E3d serum during the immunoscreening process. The DNA was restriction digested with the enzyme EcoRI, so that the insert DNA could be isolated by excision from a low melting point agarose gel. The insert DNA was then denatured by boiling, and radiolabelled with P^{32} by random priming. Theileria annulata (Ankara) piroplasm DNA, and DNA isolated from a bovine lymphoma cell line (BL-20) were slot-blotted and fixed onto nylon membrane filters, as described for RNA slot-blots in section 2:12:8, and the P^{32} -labelled cloned DNA was hybridised to the DNA slot-blots overnight. After washing, the hybridised filters were exposed to autoradiography film overnight. Figure 6.2 shows that the DNA from all three clones did not hybridise to the bovine (BL-20) DNA (Fig. 6.2, tracks 1A, 2A and 3A), but hybridised strongly with the piroplasm DNA (Fig. 6.2, tracks 1B, 2B and 3B). This result therefore showed that the cloned DNA from all three phage clones was of parasite origin, and did not originate from the bovine host. The P^{32} -labelled cloned DNA was also used to hybridise to a Southern blot of EcoRI-digested piroplasm (Ankara) DNA. The washed blot was exposed to autoradiography film overnight, and it was estimated from the developed film that the labelled DNA from the phage clone C4 hybridised to a single piroplasm DNA fragment of 9.0 kilobase (kb); the clone C6 DNA hybridised to a range of DNA fragments, but most strongly to three fragments of sizes 15.0 kb, 9.4 kb and 2.0 kb; and the clone C10 DNA hybridised to DNA fragments of sizes 13.0 kb, 9.0 kb and 4.3 kb (B.R. Shiels, unpublished data).

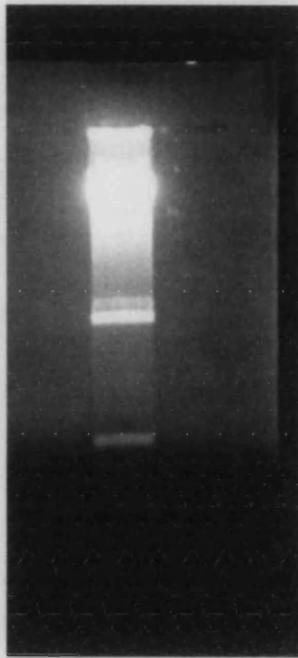
Figure 6.3: Agarose electrophoresis analysis of clone C6 DNA following digestion with restriction enzyme EcoR1.

Figures at left-hand-side indicate position, in kilobases (kb), of DNA markers.

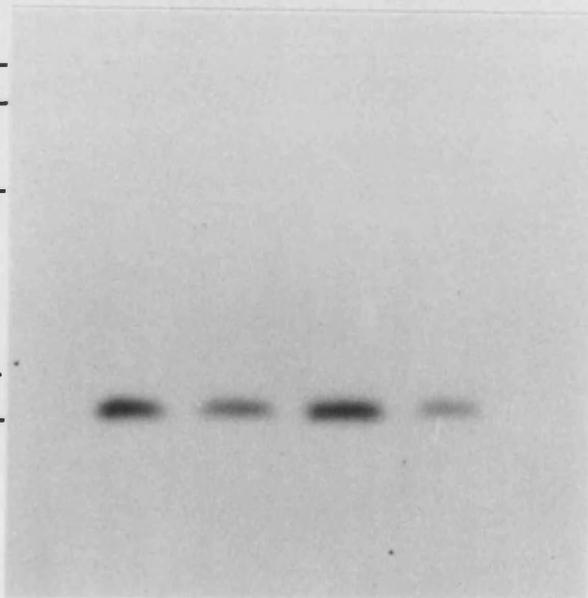
Figure 6.4: Southern blot analysis of EcoR1 restriction-digested genomic DNA isolated from cloned cell lines. The blot was hybridised with the 0.3 kb DNA fragment from clone C6.

Track 1; C9 cloned cell line.
Track 2; D7 cloned cell line.
Track 3; D3 cloned cell line.
Track 4; E3 cloned cell line.

23.1-
6.6-
2.0-
0.6-



2.0-
1.6-
1.0-
0.5-
0.4-



1 2 3 4

6:2:3 Detection of clone C6 insert DNA

The DNA from the phage clones C4, C6 and C10 was hybridised to piroplasm (Ankara) RNA in an RNA slot blot experiment. The result of this analysis indicated that DNA from the clone C6 gave the strongest hybridisation (data not shown), so it was decided to examine the DNA from this phage clone further. The C6 clone phage DNA was isolated and digested with the restriction enzyme EcoR1. The digested DNA was separated on a 0.7% agarose gel. Two recombinant fragments were detected on the gel, which were estimated to be 2.0 kb and 0.3 kb in size (Fig. 6.3). Therefore, the C6 clone appeared to have a 2.3 kb insert, which contained an internal EcoR1 site.

6:2:4 Southern analysis with the clone C6 insert DNA

The two EcoR1-generated fragments of the clone C6 insert DNA were isolated and radiolabelled separately with P³², as above. The labelled DNA was then hybridised overnight to Southern blots of EcoR1-digested genomic DNA isolated from the four T.annulata cloned macroschizont-infected cell lines (C9, D7, D3 and E3). Hybridisation of the Southern blot with the P³²-labelled 0.3 kb fragment of the clone C6 insert, and exposure of the washed blot to autoradiography film for 72 hours, showed that the 0.3 kb insert fragment hybridised to a single band of approximately 0.4 kb, which was detected in all four tracks (Fig. 6.4, tracks 1 to 4). As no differences were detected between the hybridised tracks, it was concluded that this probe did not detect

Figure 6.5: Southern blot analysis of EcoR1 restriction-digested genomic DNA isolated from cloned cell lines. The blot was hybridised with the 2.0 kb DNA fragment from clone C6.

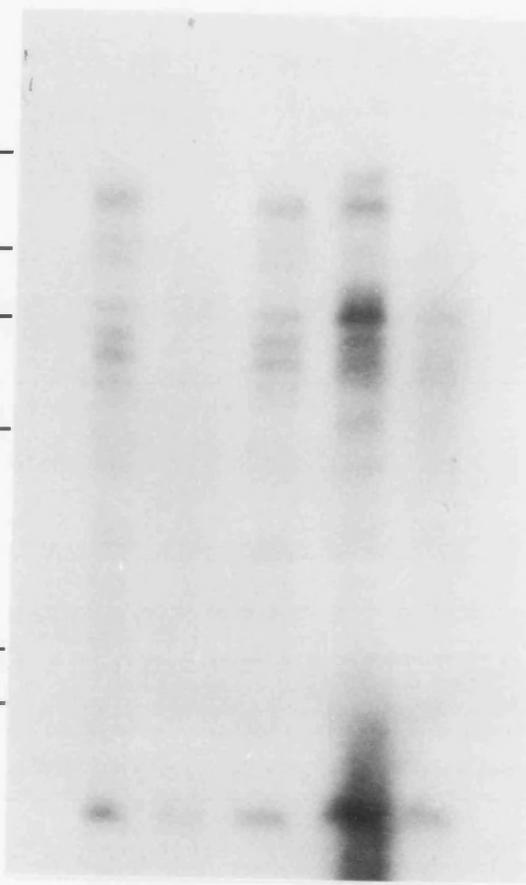
Track 1; C9 cloned cell line.
Track 2; D3 cloned cell line.
Track 3; D7 cloned cell line.
Track 4; E3 cloned cell line.
Track 5; TaA₂ uncloned cell line.

Figure 6.6: RNA slot-blot hybridised with clone C6 insert DNA.

Slot 1; BL20 RNA.
Slot 2; Piroplasm (Hissar) RNA.

Filter A; C6 0.3 kb fragment hybridisation.
Filter B; C6 2.0 kb fragment hybridisation.

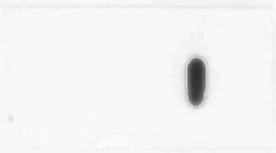
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-



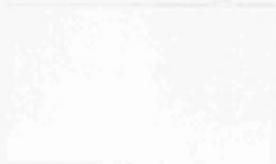
1 2 3 4 5

1 2

A



B



clonal differences between the DNA of the enhanced differentiating cell lines (Fig. 6.4, tracks 1 and 2) and diminished cell lines (Fig. 6.4, tracks 3 and 4).

Hybridisation of a Southern blot of EcoRI-digested cloned cell line DNA and EcoRI-digested uncloned cell line (TaA₂) DNA with the radiolabelled 2.0 kb fragment did not reveal clonal differences either (Fig. 6.5, enhanced cell line DNA, tracks 1 and 3; diminished cell line DNA, tracks 2 and 4; TaA₂ cell line DNA, track 5). In addition, in contrast to the hybridisation pattern observed with the 0.3 kb fragment, the 2.0 kb fragment hybridised with multiple bands. This result indicated that the 2.0 kb fragment of the insert hybridised to a DNA sequence which was frequently repeated in the T.annulata genome, whereas the 0.3 kb fragment hybridised to DNA sequence which was specific to one genomic DNA fragment.

6:2:5 RNA slot blot analysis with the C6 insert DNA

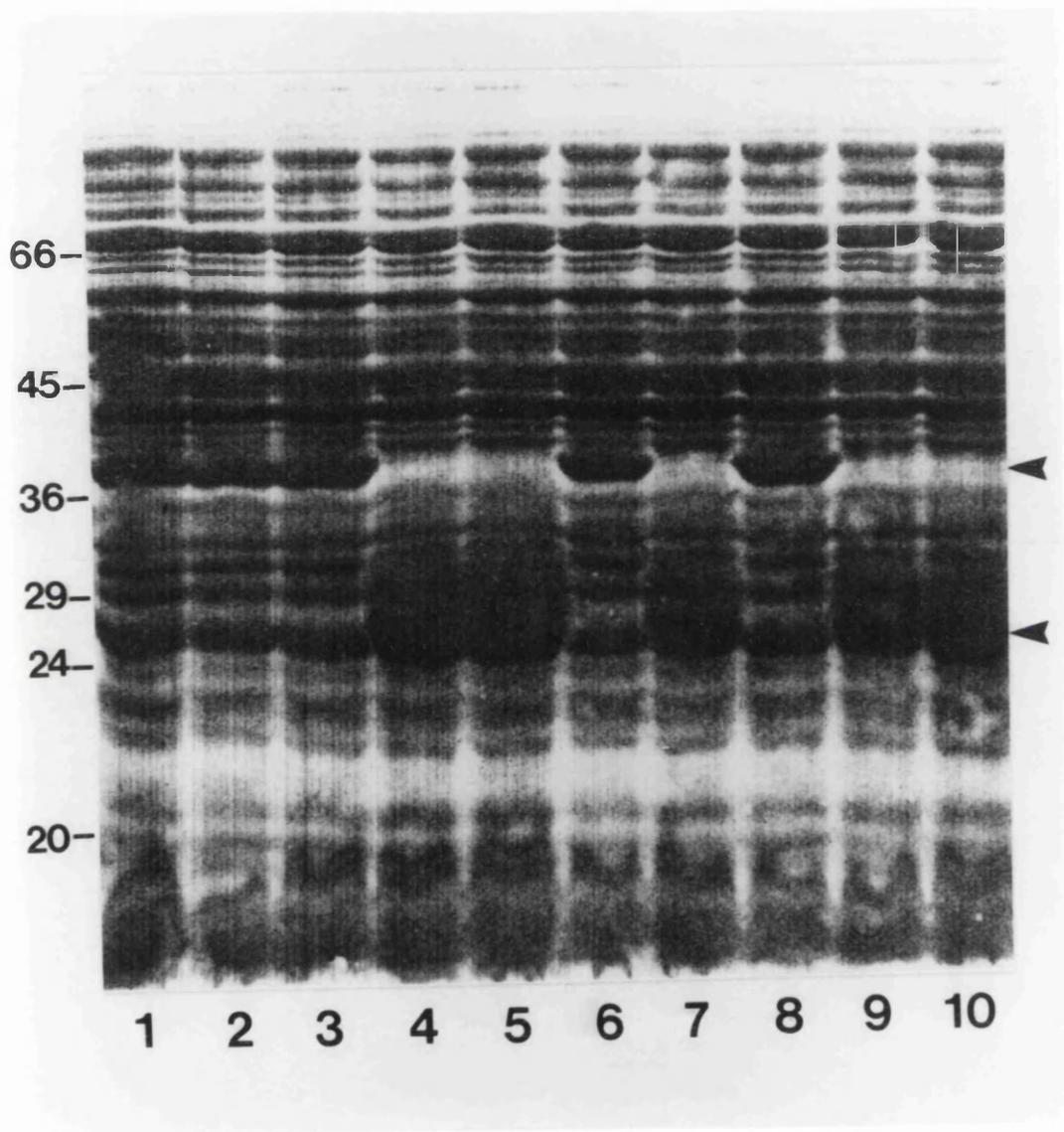
The two insert DNA fragments (2.0 kb and 0.3 kb) were labelled with p³² and hybridised to slot-blotted RNA isolated from T.annulata piroplasms (Hissar stock). The insert DNA was also hybridised to RNA from the bovine lymphoma cell line, BL-20. Figure 6.6 shows that the 0.3 kb insert fragment hybridised to the piroplasm RNA (Fig. 6.6A, track 2), but not to the BL-20 RNA (Fig. 6.6A, track 1). The 2.0 kb insert fragment did not hybridise to the BL-20 RNA (Fig. 6.6B, track 1), and hybridised only weakly with the piroplasm RNA (Fig. 6.6B, track 2). These results indicated that, when compared to the expression of the 2.0

Figure 6.7: SDS-PAGE analysis of GST fusion protein.

Tracks 1-10 Ten transformed bacterial cultures induced to express the GST fusion protein.

Upper arrow indicates position of the 37 kDa GST fusion protein.

Lower arrow indicates position of the 26 kDa unfused GST protein.



kb DNA fragment, the 0.3 kb DNA fragment of the insert was expressed in the piroplasm stage at a much higher level. Furthermore, restriction mapping analysis indicated that the 0.3 kb fragment was 5' to the 2.0 kb fragment (B.R.Shiels and S.McKellar, unpublished data), and the 0.3 kb fragment was sequenced and found to contain a complete open reading frame (B.R.Shiels and S.McKellar). However, no sequence homology was found within the gene database screened.

6:2:6 Sub-cloning the 0.3 kb fragment into pGex-1N

The DNA and RNA analysis experiments indicated that the 0.3 kb DNA fragment of the C6 lambda gt11 phage clone was uniquely and highly expressed in the T.annulata genome (sections 6:2:4 and 6:2:5). Therefore, it was decided to sub-clone this fragment into an expression vector. The 0.3 kb DNA fragment was sub-cloned into the expression vector pGex-1N, and transfected into the bacterial host XL-Blue. Ten transformed bacterial colonies were picked and grown in culture. The pGex-1N vector expresses recombinant DNA as a glutathione S-transferase (GST) fusion protein, upon induction with IPTG (section 2:12:6:1), and in these experiments, expression of the GST-fusion protein was induced with 5 mM IPTG. Figure 6.7 shows that five of the ten bacterial cultures expressed a 37 kDa GST-fusion protein (arrowed in Fig. 6.7, tracks 1, 2, 3, 6 and 8), while the other five induced cultures expressed the 26 kDa GST protein (also arrowed, tracks 4, 5, 7, 9 and 10). The observed 50% fusion protein expression was as expected, as

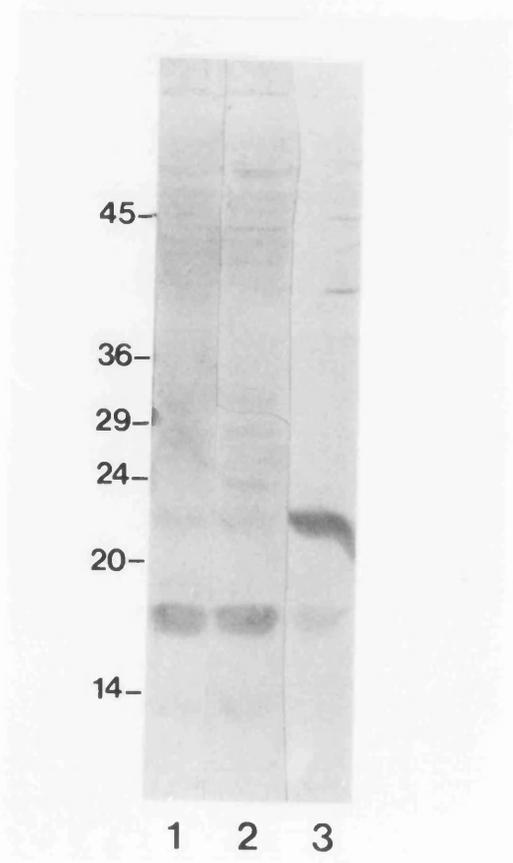
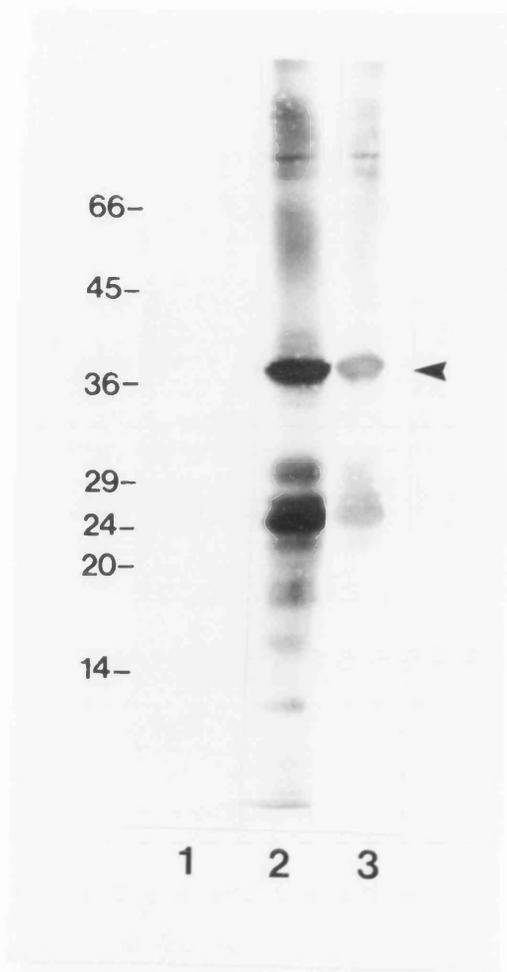
Figure 6.8: Western blot of bacterial lysate expressing the 37 kDa GST fusion protein probed with normal mouse and anti-fusion protein serum.

Track 1; normal mouse serum
Track 2,3; anti-fusion protein serum

Position of 37 kDa GST fusion protein arrowed.

Figure 6.9: Western blot analysis of piroplasm (Ankara) extracts, probed with anti-fusion protein serum and normal mouse serum.

Track 1,2; Reactivity with anti-fusion protein sera.
Track 3; Reactivity with normal mouse serum.



there was a 50% chance that the expression vector would have the insert DNA in the correct orientation for expression. To show that all ten bacterial cultures had vector DNA and the 0.3 kb insert present, DNA was prepared from the bacterial cultures, before induction with IPTG, and digested with EcoRI. Analysis of the DNA after gel electrophoresis indicated that the recombinant plasmid was present in all 10 cultures (data not shown).

6:2:7 Generation of antiserum to the GST-fusion protein

The 37 kDa GST-fusion protein was purified from the culture medium on a glutathione sepharose 4B column, as described in section 2:12:6:1. The fusion protein was eluted from the column, and two mice were injected with approximately 900ng each of fusion protein in Complete Freund's adjuvant. The mice were injected with an identical quantity of fusion protein at 3 weeks, 5 weeks and 10 weeks after the initial inoculation, and tail bleeds were taken 10 to 14 days after each inoculation. The antisera to the fusion protein was isolated from the bleeds, and control serum was also isolated from a normal mouse (section 2:12:6:2). The fusion protein antisera and normal mouse sera were then used in Western blot experiments. In these experiments, it was found that the antisera to the fusion protein did not have a high antibody titre, as the antisera had to be used at a 1:50 dilution for the detection of antigens on a Western blot, and this antibody titre did not improve with repeat inoculations. Figure 6.8 shows the antiserum reactivity when used to probe a Western blot of extracts of bacterial

lysates expressing the 37 kDa GST-fusion protein. The fusion protein antisera clearly reacted with the fusion protein (arrowed in Fig. 6.8, tracks 2 and 3), and one of the mouse sera (Fig. 6.8, track 2) gave stronger reactivity than the other. It was noted that there was also fusion protein serum reactivity with a polypeptide of approximately 26 kDa, which was possibly a degradation product from the 37 kDa GST-fusion protein. The serum from the normal mouse did not react with the bacterial lysate extracts (Fig. 6.8, track 1).

The antisera were also used to probe a Western blot of Ankara piroplasms. Figure 6.9 shows that a polypeptide band of approximately 17 kDa was detected by the fusion protein antisera (Fig. 6.9, tracks 1 and 2). The normal mouse antiserum did not detect this molecule, but detected a polypeptide band of just over 20 kDa (Fig. 6.9, track 3). A 17 kDa polypeptide band was also detected by the fusion protein antisera in a Western blot of differentiating and undifferentiated macroschizont-infected cloned cell lines (data not shown). These results indicated that the serum generated against the GST-0.3 kb fusion protein did not detect a 30 kDa polypeptide, and furthermore, did not recognise a polypeptide which was stage-specifically expressed and associated with merogony, or which was clonally expressed. This result was also supported by IFA experiments in which the fusion protein antiserum was seen to react with macroschizonts, merozoites and piroplasms with equal intensity (data not shown).

Fig. 6.10A: The nucleotide sequences of the ANG-and PNN-oligonucleotides

ANG-oligonucleotide

Amino acid sequence: Alanine - Asparagine - Glycine - Tyrosine - Arginine - Phenylalanine - Lysine - Threonine.

Oligonucleotide sequence: GC[GATC]-AA[TC]-GG[GATC]-TA[TC][CA]-G[GATC]-TT[TC]-AA[AG]-AC;

where A is the nucleoside base adenine, T is thymidine, G is guanine and C is cytosine. Bases given in brackets are all the possible bases at this position.

PNN-oligonucleotide

Amino acid sequence: Proline - Asparagine - Asparagine - Aspartic acid- Arginine- Histidine- Glutamine- Isoleucine.

Oligonucleotide sequence: AT[TC]-TG[AG]-TG[GATC]-C[GT][AG]-TC[AG]-TT[AG]-TT[GATC]-GG

Fig. 6.10B: Estimation of the oligonucleotide melting temperature of hybridisation (T_m)

$T_m = 2x$ number of A/T base pairs + $4x$ G/C base pairs
(Itakura et al., 1984)

For both oligonucleotides, assuming pairing bases at degenerate positions are G or C: $T_m = (2x9) + (4x14) = 74$

Assuming pairing bases at degenerate positions are A or

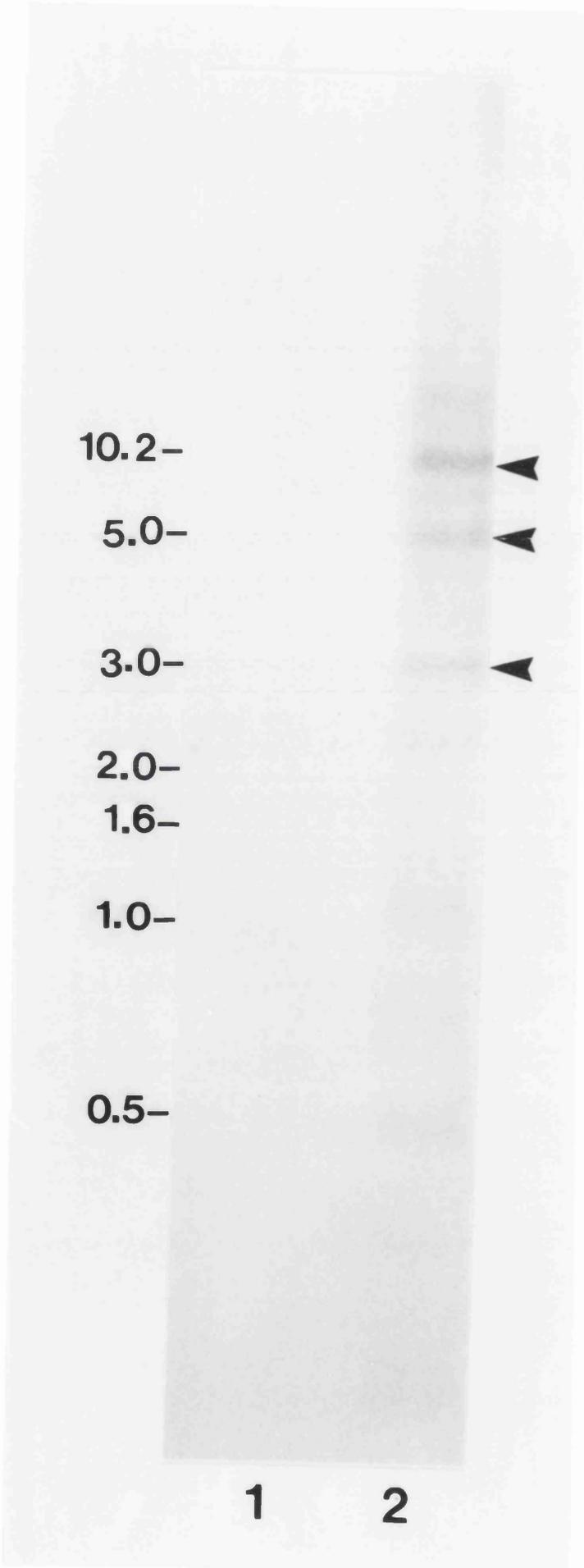
T: $T_m = (2x17) + (4x6) = 58$

Therefore, the estimated T_m for both oligonucleotides is between 58°C and 74°C .

6:2:8 Oligonucleotide screening the D7 lambda-Dash genomic library

Two degenerate DNA oligonucleotides (ANG- and PNN-oligonucleotides) were designed from amino acid sequence data generated from the Theileria annulata (Ankara) piroplasm 30 kDa polypeptide (see Figure 6.10A). Because the base sequences of the oligonucleotides contained codons with either twofold or fourfold ambiguity, it was calculated that a comprehensive pool of nucleotides containing all possible ways to code for the ANG-amino acid sequence would contain 2048 ($4 \times 2 \times 4 \times 2 \times 2 \times 4 \times 2 \times 2$) related oligonucleotides, and a comprehensive pool coding for the PNN-amino acid sequence would contain 1024 ($2 \times 2 \times 4 \times 2 \times 2 \times 2 \times 2 \times 4$) related oligonucleotides. Furthermore, because pools of oligonucleotides were being used, whose members had varying contents of G + C, it was impossible to estimate a consensus melting temperature of hybridisation (T_m) for the oligonucleotides. However, using the formula given by Itakura et al. (1984), for determining the T_m of perfectly matched oligonucleotide and target sequence hybrids, and assuming that the pairing base at the degenerate position was either A/T or G/C, the lowest possible and the highest possible T_m for both oligonucleotides was estimated to be 58°C and 74°C, respectively (see Figure 6.10B). Because of this large temperature range, it was decided to perform the hybridisations in the presence of 3M tetramethyl ammonium chloride (TMAC, Jacobs et al., 1985; Wood et al., 1985), in which the stability of hybrids is dependent only on

Figures 6.11: Southern blot analysis of EcoR1 restriction-digested piroplasm (Ankara) genomic DNA probed with the ANG (track 1) and PNN (track 2) oligonucleotides. Arrows indicate the strongest hybridising bands.



oligonucleotide length, and not on the content of G+C (Sambrook et al., 1989). Therefore, for oligonucleotides over 14 nucleotides in length, hybrids melt over a smaller range of temperature in solutions containing TMAC, than in solvents containing sodium salts (Jacobs et al., 1988). The recommended hybridisation temperature for 23-mer oligonucleotide pools in 3M TMAC is 69-73°C (Sambrook et al., 1989).

In order to determine the optimum conditions for hybridisation of the oligonucleotides in a library screen, Southern blots of EcoRI-cut T.annulata (Ankara) piroplasm genomic DNA were hybridised with each oligonucleotide separately, to find the appropriate hybridising conditions where the oligonucleotides bound to a minimum number of DNA bands and, ideally, to identical bands. The oligonucleotides were hybridised to the blots in Pipes hybridisation solution, at 40°C overnight. The filters were then washed at 4°C in TMAC buffer, and again at room temperature, to remove the sodium chloride and increase the stability of the A-T base pairs with the presence of TMAC. Further washes were carried out at 40°C and 50°C, following which the filters were exposed to autoradiography film. It was found that the ANG-oligonucleotide did not hybridise to the Southern blotted DNA under these conditions, but the PNN-oligonucleotide hybridised strongly to a range of DNA bands. Further washes at 60°C and 70°C, followed by film exposure, showed that the PNN-oligonucleotide hybridised to several piroplasm DNA bands especially, and to other DNA bands weakly (Fig. 6.11). It was decided to screen the

T.annulata D7 genomic DNA library in lambda-Dash with the oligonucleotides, using these conditions at either low stringency (final wash at room temperature), or at higher stringencies (final wash at 50°C, 60°C or 70°C). 3×10^4 recombinant phage were screened, and repeat lifts were taken so that plaques could be identified which hybridised to both probes. However, such plaques were not found. Plaques which hybridised with either one of the two probes were picked as positives, but once rescreened, the plaques from these picks did not consistently hybridise to the same probe, suggesting that it was extremely difficult to repeat the optimum conditions required for hybridisation of the probes.

6:3 Discussion

This Chapter presents the results of two cloning strategies undertaken to isolate the T.annulata 30 kDa gene. The first strategy employed was a polyclonal antibody (anti-E3d serum) screen of a T.annulata genomic expression library. Three immunopositive clones were isolated to purity, and one, clone C6, was selected for further analysis. This clone was found to contain a 2.3 kb insert with an internal EcoR1 site, to give DNA fragments of sizes 2.0 kb and 0.3 kb. Southern blot and RNA slot blot analysis results suggested that, unlike the 2.0 kb fragment, the 0.3 kb fragment was a single copy gene and was expressed in the piroplasm stage of the parasite (sections 6:2:4 and 6:2:5). The 0.3 kb fragment was subcloned into an expression vector, and expressed as a GST-fusion protein. However, antiserum generated against the recombinant fusion protein did not detect a stage-specific protein on Western blots, and did not detect a 30 kDa or 32 kDa molecule (sections 6:2:6 and 6:2:7).

An immunoscreening approach was used in a previous attempt to clone the 30 kDa polypeptide gene, employing the monoclonal antibody 5E1 to screen the T.annulata genomic expression library (Glascodine, 1989). This approach was also unsuccessful, indicating that there was a problem (technical or otherwise) in using antibodies to isolate the 30 kDa protein gene. The probable reason for this lack of success became apparent once there was more information on the biochemical structure of the 30 kDa molecule, i.e., it was shown that the 30 kDa polypeptide is glycosylated and

furthermore, that the epitope detected by the monoclonal antibody 5E1 is determined by glycosylation (section 5:2:4). Post-translational modifications (such as glycosylation) of proteins does not occur in bacterial systems such as E.coli, therefore the 5E1-recognised epitope was not likely to be expressed by the cloned gene fragments in the bacterial host (Y1090). Similarly, on Western blots, much of the anti-E3d serum reactivity was found to be with sugar residues, and so it was possible that the anti-E3d serum detection of peptide sequence (if any) may have been too weak to allow isolation of the 30 kDa protein gene.

Therefore, it appeared that library immunoscreening, using reagents generated against the modified proteins, was an inappropriate method for the isolation of the T.annulata 30 kDa protein gene. Interestingly, in T.mutans, the 32 kDa glycoprotein gene could not be isolated in this way either (S. Mozavia, personal communication). However, the T.sergenti and T.buffeli 33 kDa and 34 kDa surface piroplasm proteins were cloned by immunoscreening a T.sergenti or T.buffeli cDNA library, constructed in lambda gt11, with polyspecific sera generated against T.sergenti or T.buffeli piroplasms (Kawazu et al., 1992ii). In this case, the immunoscreening approach may have been successful because the antisera employed detected mainly (if not totally) peptide sequence, as opposed to secondary structure. Also, the antisera used for screening the T.sergenti/buffeli cDNA libraries were generated against piroplasms rather than differentiating macroschizont-

infected cells, and hence could have had an enhanced affinity for the 33/34 kDa proteins. Furthermore, screening a cDNA piroplasm gene library, rather than a genomic DNA library, may have been more appropriate if the RNA was post-transcriptionally modified, and as the 33 kDa and 34 kDa molecules are expressed at a high level, the use of a cDNA library for screening probably enhanced the likelihood of isolating the T.sergenti/buffeli 33/34 kDa protein gene.

Since all the available antibody reagents to the T.annulata 30 kDa protein detected mainly sugar residues, an alternative approach was to use yeast or higher mammalian cell expression systems to generate T.annulata proteins, complete with post-translational modifications (Beardsell and Howell, 1987). However, this method of gene expression does not allow the production of complex carbohydrates, particularly "unusual" eukaryotic glycoconjugates, such as those associated with the protozoan parasites (see discussion, Chapter 5). Therefore, because deglycosylation and lectin binding studies (sections 5:2:5 and 5:2:6) indicated that the T.annulata 30 kDa glycoprotein may have an unusual glycan structure, eukaryotic expression systems may not be appropriate for 30 kDa glycoprotein expression. Another possible approach was to produce a molecular mimic of the structure of the carbohydrate molecule (Ferguson and Homans, 1988), as there is evidence that peptides can mimic carbohydrate epitopes (Olsson, 1987). For example, monoclonal antibodies specific to an epitope can be used as antigens to raise further monoclonal antibodies, and because these anti-idiotypic

monoclonal antibodies have a structural profile that mimics that of the original epitope, they can be used in protection assays. For example, in Schistosoma mansoni, an anti-idiotypic antibody, raised against a monoclonal antibody which detects carbohydrate epitopes on a 38 kDa molecule, can stimulate protective immunity in the same way that the monoclonal antibody can (Boswell et al., 1987; Caulfield et al., 1988 and Omer-Ali et al., 1988). Furthermore, a novel system is currently being developed using filamentous fungi to generate peptide mimics of carbohydrate epitopes (M.A.J.Ferguson, personal communication). These peptides would be screened with antibody, such as the 5E1 monoclonal antibody, which detected carbohydrate epitope(s) and the isolated peptides, which had the same conformation as the carbohydrate epitope, could be used in host protection assays.

However, because part of the 30 kDa molecule had already been amino acid-sequenced (section 5:2:2:2), the second strategy employed to clone the 30 kDa protein gene was an oligonucleotide screen of a cloned macroschizont-infected cell line (D7) genomic library. The ANG- and PNN-oligonucleotides used in the screen contained degenerate base sequence, due to the redundancy of the genetic code. Each alternative base means a doubling of the number of oligonucleotides in the mixture required to cover all possible base sequences (Sambrook et al. 1989). Therefore, the PNN and ANG oligonucleotide mixtures contained a large number of base sequences which would not be an exact match to the DNA target sequence; the PNN-oligonucleotide pool

contained 1024 related oligonucleotides, and the ANG-oligonucleotide pool contained 2048 related oligonucleotides. Furthermore, estimation of the optimum conditions for probe hybridisation indicated that the desired temperature for hybridisation was likely to be within a large temperature range (between 58°C and 74°C), thus making the oligonucleotides difficult to work with. For example, if the perfectly matched oligonucleotide had a T_m in the lowest part of the temperature range, there would be little chance that its specific hybridisation would be retained when all non-specifically hybridised probe had been washed off at higher temperatures. Thus, 3M TMAC was employed in the washing solutions so that the stability of hybrids depended only on oligonucleotide length (Jacobs et al., 1988). However, under these conditions, whereas the PNN-oligonucleotide hybridised to a range of piroplasm DNA bands, the ANG-oligonucleotide, which was considerably more degenerate, did not hybridise to Southern blots of piroplasm DNA, and subsequent screening of the genomic library did not result in the identification of convincingly positive hybridising plaques.

This second strategy may not have been successful because the oligonucleotide pools contained too many nucleotide variants, and hence, the true hybridising sequence may have been diluted out to such an extent that the likelihood that the probe mixtures would give a detectable signal of hybridisation to the 30 kDa protein gene sequence became negligible. Comparison of the available amino acid sequence data from the T.annulata 30

kDa molecule to the predicted T.sergenti amino acid sequence (Kawazu et al., 1992iii) showed that the amino acid sequence of the peptide, from which the ANG oligonucleotide was made, was very similar (greater than 83% similarity) to a stretch of the T.sergenti amino acid sequence (section 5:2:2:2). Therefore, it was likely that the amino acid sequence, from which the oligonucleotide was made, encoded part of the T.annulata 30 kDa molecule, but the oligonucleotide DNA sequence was too degenerate to allow observable hybridisation. The chance of isolating the 30 kDa protein gene with oligonucleotides would be enhanced with less degenerate oligonucleotides and/or with a larger number of oligonucleotides encoding different regions of the protein. From available sequence information, the Theileria annulata genome, like the Plasmodium falciparum genome (Hyde et al., 1989), is A/T rich; 55-60% of the coding sequence bases are A and T, and 75-80% of bases in non-coding regions are A and T. Therefore, oligonucleotides containing only A or T bases at degenerate base positions could be employed to reduce degeneracy. The degeneracy of a probe can also be reduced by using inosine at the third position of four-codon choices (for example, GCI for alanine), which base-pairs to the four nucleotide bases with similar fidelity.

At this point, the future possible approaches for isolating the 30 kDa gene included isolation by homology, using the available cloned nucleotide sequences from the T.sergenti/buffeli 33 kDa and 34 kDa molecules, and/or from the T.mutans 32 kDa molecule; or alternatively, the

synthesis of novel oligonucleotides derived from the amino acid sequence of the T.annulata 30 kDa molecule, which were less degenerate than the ANG or PNN-oligonucleotides. Also, if the filamentous fungus carbohydrate epitope-mimic system is successfully established in the future, it is possible that this method could be used to bypass the recombinant DNA techniques. However, the 30 kDa protein gene was recently isolated by heterologous probing, using a cDNA clone encoding the T.buffeli 34 kDa molecule (B.R.Shiels, unpublished data). This is discussed further in Chapter 8 (General Discussion).

CHAPTER SEVEN

FUNCTIONAL STUDIES ON THE 30 kDa POLYPEPTIDE

7:1:1 Introduction

The 30 kDa polypeptide was found to be a major polymorphic surface antigen of Theileria annulata which is expressed on the surface of the merozoite and differentiating macroschizont. Furthermore, the molecule was shown to be glycosylated (Chapter 5). Studies on other protozoa, such as Leishmania (Nolan and Farrell, 1985) and Trypanosoma (Snary, 1985), indicate a connection between protein glycosylation and various cellular functions, for example growth, morphology and host-cell penetration. Surface glycoproteins are usually intimately involved in the initial interaction with host cells (Hyde, 1990i), and therefore, it was possible that the 30 kDa antigen was involved in such a role during the merozoite invasion of erythrocytes.

The invasion of bovine host lymphocytes with Theileria annulata sporozoites can be relatively easily performed in vitro (see section 1:1:4). However, invasion of host erythrocytes with the merozoite stage in vitro has had limited success. A system for the invasion of erythrocytes with in vitro-derived merozoites would provide many benefits, including the ability to examine erythrocyte invasion closely, and in a controlled fashion. For example, the role (if any) of the 30 kDa polypeptide during invasion could be assayed by antibody-blocking experiments. An erythrocyte invasion system would also allow the generation of piroplasms in vitro, and if the resultant yield of piroplasms was high enough, it is possible that the present need for infecting an animal to obtain this stage of the

parasite could be avoided.

In IFAs, it was observed that the pattern of reactivity of the 30 kDa-specific monoclonal antibody 5E1 was not always restricted to the parasite membrane, and the pattern of immunofluorescence extended to the host cell cytoplasm and the extracellular medium, thus giving the impression was that the 30 kDa antigen was being secreted into the host cell cytoplasm. One possibility was that the 30 kDa was being secreted as a protease. Proteases are known to be involved in various parasitic developmental processes, and appear to be essential for the host-parasite relationship, and for pathogenesis (North, 1991). For example, the major surface antigen of Leishmania is a metalloproteinase (Jongenell et al., 1989; Bouvier et al., 1990), and Plasmodium proteases have been identified which are involved with merozoite maturation and their subsequent release from the host cell, and with merozoite invasion of erythrocytes (Rosenthal, 1991). The Triton X-114 phase separation of the T.annulata piroplasm proteins (section 5:2:7) showed that the 30 kDa and 32 kDa proteins were integral membrane proteins, and were not secreted from the piroplasm surface as soluble (aqueous) proteins. However, it was possible that secretion could result from a stage-specific cleavage of the membrane-bound forms of the proteins. This is known to occur in Plasmodium falciparum. A 76 kDa GPI-anchored serine protease of P.falciparum schizonts and merozoites is activated and solubilised by a phosphatidylinositol-specific phospholipase C during the merozoite stage, and before erythrocyte invasion (Braun-

Breton et al., 1988). This protein can be subsequently detected in culture supernatants, and it was expected that, if the 30 kDa and 32 kDa polypeptides of T.annulata were also secreted, these proteins would be detectable in supernatants of differentiating macroschizont cultures, and also in serum from T.annulata-infected bovine blood.

7:1:2 Summary and Aims

The 30 kDa glycoprotein is located on the T.annulata merozoite surface and was possibly involved in host erythrocyte invasion.

The aim of the experiments presented in this Chapter was to develop a system for erythrocyte invasion by merozoites in vitro. Preliminary experiments were also carried out to determine if the 30 kDa polypeptide had any protease activity, and/or is secreted during differentiation.

7:2 Results

7:2:1 Merozoite Invasion assay

A range of culture conditions were tested so that the optimal conditions for the invasion of cow erythrocytes by merozoites, generated in vitro, could be determined. Initial experiments carried out incorporated the culture conditions used by C.G.D. Brown (C.T.V.M., Edinburgh) to successfully establish a "semi-in vitro" merozoite invasion assay. These experiments differed from those reported below because the merozoites used were generated in vivo, rather than by in vitro culture, and were isolated, by centrifugation, from a fresh lymph node biopsy taken from an animal eleven to thirteen days after T.annulata infection. The isolated merozoites were added to cow red blood cells (2×10^8 per ml) in B medium (TBL medium, containing 10%, instead of 20%, foetal calf serum), and incubated at 37°C for up to twelve days. Of eight experiments performed, the invasion rate observed (estimated from the number of piroplasms present) ranged from 1.0% to 23.8%. The condition of the lymph material and of the red blood cells appeared to be crucial for good erythrocyte invasion, and the 23.8% invasion rate was observed five days after mixing freshly isolated merozoites with fresh red blood cells (C.G.D. Brown, personal communication).

In order to develop an invasion assay using in vitro-generated merozoites, the method used was as follows. The red blood cells were prepared from a 20 ml bleed from a

cow. The blood was defibrinated with glass beads, as described in section 2:1:2, and centrifuged to separate the red cells from the serum. The red blood cells were diluted in RPMI medium (or B medium), to a final cell concentration of 3×10^5 cells per ml, as estimated by the use of a haemocytometer. The blood was then added to either differentiating macroschizont-infected cells (from an enhanced cloned cell line, D7, or an uncloned cell line, TaA₂), or to merozoites, purified by differential centrifugation (section 2:3:1). In either case, the cells were added to give a final concentration of approximately 3×10^6 merozoites per ml. The mixture of cells was incubated at either 37°C or 41°C, with 5% CO₂ in a humidified incubator, in a 10 ml culture flask or a 2 ml well of a multiwell culture plate. Attachment to and invasion of the erythrocytes (the latter detected by the observation of piroplasm forms) was constantly monitored by viewing Giemsa-stained cytopins of culture samples.

A summary of the conditions used, and the percentage of attachment or invasion of merozoites observed, are given in Table 7.1, and Figures 7.1 and 7.2 show examples of apparent merozoite attachment (am) to erythrocytes (r), merozoites which appear to be in the process of invasion (im), and observed piroplasm forms (p). It was found that of the conditions used, the incubation of differentiating TaA₂ macroschizont-infected cells in complete TBL medium, or B medium, with bovine erythrocytes in a total volume of 2mls gave the highest rate of invasion, 0.6%, estimated by counting the numbers of parasitised erythrocytes and non-

Table 7.1: Invasion of bovine erythrocytes by cultured merozoites in vitro.

Culture Conditions	% Merozoite attachment	% Invasion
1ml TaA ₂ +1ml rbc;TBL	10	0.6
5ml TaA ₂ +5ml rbc;TBL	7	0.3
1ml TaA ₂ +1ml rbc;B	9	0.5
1ml TaA ₂ +0.9ml rbc+0.1ml abs;TBL	8	0.5
As above, except h.i. abs	9	0.5
1ml D7+1ml rbc;TBL	9	0.4
As above, except D7 merozoites	4	0.0
1ml D7+1ml rbc;B	9	0.5
1ml D7+1ml rbc+0.2mM ATP;TBL	7	0.4
1ml D7+1ml rbc+0.1 mM CaCl ₂ ;TBL	4	0.0

Notes:

a. Culture conditions: TaA₂ or D7 macroschizont-infected cell lines were used at day 10 or day 6 (respectively) of incubation at 41°C, and added to the red blood cells (final concentration approximately 3x10⁵ cells/ml) to give a final concentration of 1x10⁵ infected cells/ml or 3x10⁶ merozoites/ml. Mixture of red blood cells and merozoites incubated at 37°C in a total volume of 2 ml, in a well of a multiwell culture dish, or in a total volume of 10 ml, in a 10ml culture flask.

rbc red blood cells abs adult bovine serum
TBL Complete TBL medium h.i. heat-inactivated
B B medium

b. Percentage merozoite attachment: Estimated percentage of erythrocytes seen with merozoites apparently attached, in 10 fields of view. These cells had not been centrifuged in a Ficoll gradient.

c. Percentage invasion: Estimated as the percentage of erythrocytes containing piroplasm forms in 10 fields of view.

parasitised erythrocytes in ten fields of view, and recorded at 24-48 hours of incubation. The incubation of D7 macroschizont-infected cells in identical culture conditions gave a similar, but slightly lower, percentage of invasion (0.4-0.5%). The purification of D7 merozoites prior to the assay did not enhance invasion, possibly because of cell damage occurring during the centrifugation steps of merozoite purification. In Babesia infections, activation of complement, via the alternate pathway, has been shown to facilitate the invasion of red blood cells by the parasite (Ward and Jack, 1981). Therefore, experiments were performed in the presence of heat-inactivated adult bovine serum (complement-free; incubated at 56°C for 30 minutes) or in the presence of normal adult bovine serum. However, the addition of either serum did not enhance merozoite invasion. Also, there was no observed difference in the rate of invasion whether the cells were incubated in the presence of 20% foetal calf serum (contained in TBL medium) or in the presence of 10% foetal calf serum (contained in B medium). In the malarial invasion of erythrocytes, the presence of ATP and calcium has been found to be important for this process (Olson and Kilejian, 1982; Johnson et al., 1980), but again, addition of these compounds (0.2mM ATP or 0.1mM calcium chloride) did not appear to affect T.annulata merozoite invasion. However, it was found that piroplasms could be detected in cultures maintained at 37°C, but not in cultures maintained at 41°C, probably because the bovine cells were seen to degenerate more quickly at the higher temperature.

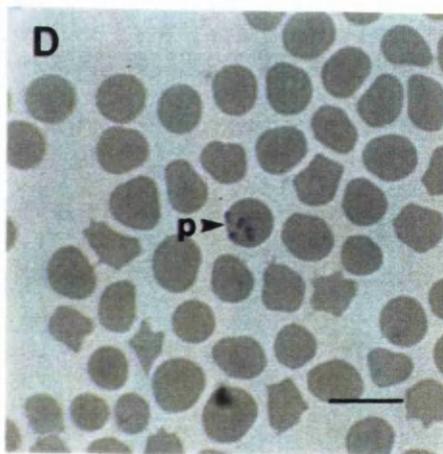
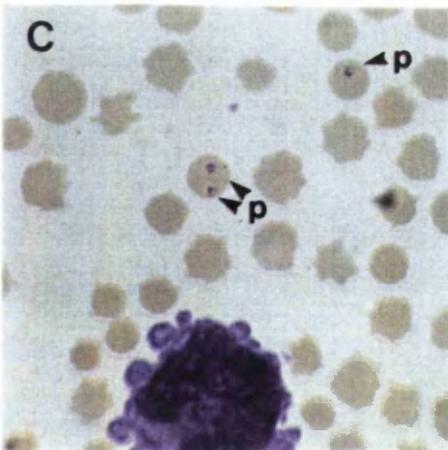
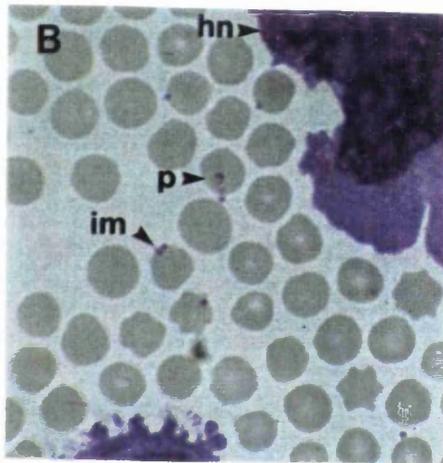
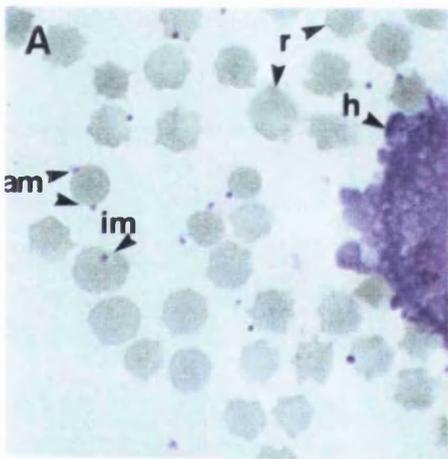
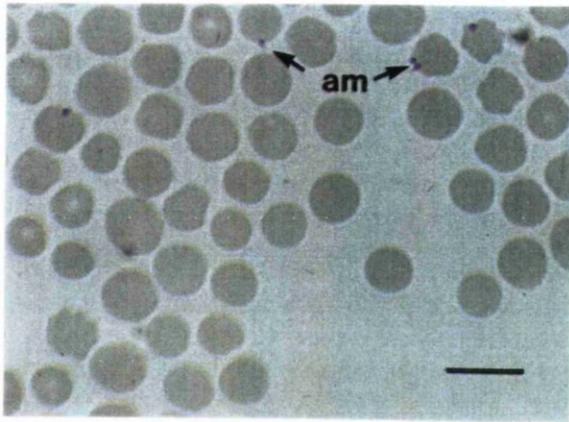
Figure 7.1: Giemsa-stained cytospin samples of bovine red blood cells and merozoites after Ficoll separation.

am; attached merozoites.
Scale bar; 10um.

Figure 7.2: Giemsa-stained cytospin samples of bovine red blood cells and merozoites after incubation at 37°C.

r; red blood cells.
h; host lymphocyte.
hn; host lymphocyte nucleus.
am; attached merozoites.
im; invading merozoites.
p; piroplasms.

Scale bar; 10um.



In addition, to test if apparent merozoite attachment to erythrocytes was specific, culture samples were loaded onto a Ficoll 400 density gradient column and centrifuged at 10,000g for 20 minutes. The band containing erythrocytes was isolated and a sample was examined by light microscopy of a Giemsa-stained slide (see Fig. 7.1; am, attached merozoites). This was compared to a slide of sample taken before centrifugation. It was found that 10% of erythrocytes (estimated as an average of the 10 fields of view counted) had merozoites which appeared to be attached to the erythrocyte surface before centrifugation. This percentage was found to decrease to approximately 3% after centrifugation. It was therefore assumed that, of the merozoites apparently attached to the erythrocyte surface before centrifugation, approximately a third of these were probably physically attached to the erythrocyte surface, and therefore likely to be in the process of invasion.

Figure 7.2A shows morphology observed after adding 5ml (approximately 5×10^5 infected cells, to give 1.5×10^7 merozoites) of a differentiating TaA₂ culture to 5 ml (approximately 1.5×10^6 cells) of bovine red blood cell suspension, and after incubation at 37°C for 48 hours. Merozoites (am) can be seen which appear to be attached to erythrocytes (r). One specific merozoite (im) seems to be in the process of invading the host cell, which is seen to be slightly invaginated at the apparent position of merozoite entry. Figure 7.2B shows the morphology observed 48 hours after the incubation of 1ml (approximately 1×10^5 cells) of TaA₂ culture to 1ml (3×10^5 cells) bovine

erythrocyte suspension. In addition to merozoites which appear to be invading the host cells, a ring-form piroplasm (p) can be seen. Similarly, piroplasms can be seen in Figure 7.2C, in which 1ml of a differentiating enhanced cloned cell line (D7) was incubated with 1ml bovine erythrocyte suspension. Piroplasms could be seen in this culture between 24 and 36 hours of incubation. Finally, Figure 7.2D shows the result of incubating 1ml TaA₂ differentiating culture with 1ml blood suspension, the cells being incubated in B-medium, rather than Complete TBL medium (section 2:2:1). The main difference between the two media is that TBL medium contains 20% foetal calf serum, while B-medium contains 10% foetal calf serum. In this Figure, four piroplasm forms can be seen within a single bovine red blood cell.

7:2:2 Gelatin gel/Protease assay

Lysates of T.annulata-infected cells were tested for protease activity using gelatin substrate SDS-PAGE. Macroschizont-infected cell extracts were electrophoresed in SDS-PAGE gels polymerised in the presence of 0.3% gelatin. After electrophoresis, the gels were incubated in 1% Triton X-100 to remove SDS, and then incubated overnight at 37°C in a 0.1M glycine solution, to allow localised proteolysis of the gelatin to occur. Protease activity was visualised by staining the gel in Coomassie blue, and areas of digestion were seen as non-stained bands. It was found that protease activity was seen only when 0.5mM calcium chloride was added to the overnight incubation solution,

Figure 7.3: Analysis of protease activity of T.annulata-infected cell extracts using gelatin substrate SDS-PAGE.

Track 1; Cloned cell line D7 extract.

Track 2; Cloned cell line D3 extract.

Track 3; Uncloned cell line TaA₂ extract.

"a" arrows indicate areas of D7 and D3 protease activity.

"b" arrows indicate areas of TaA₂ protease activity.

116-

97-

66-

45-

29-

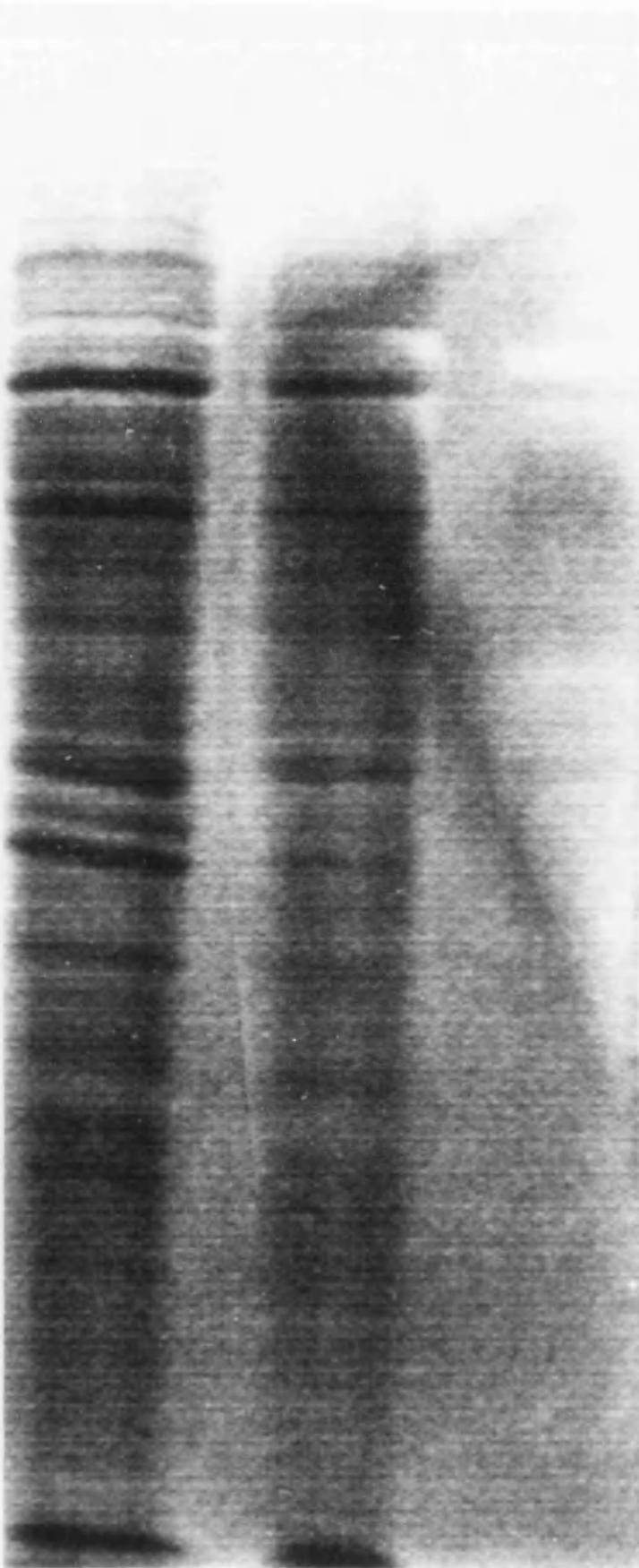
▲ a
▲ a b
▲ a

▲ b

1

2

3



and was optimal at pH 8.0. Figure 7.3 shows that there were two clear regions in the cloned macroschizont-infected cell line (D7 and D3) extracts at approximately 115 kDa and 96 kDa ("a" arrows in Fig. 7.3, tracks 1 and 2), and a clear region in the uncloned cell line (TaA₂) extract at approximately 113 kDa and 60 kDa ("b" arrows in Fig. 7.3, track 3). This protease activity was not associated with heat-induced merogony; the D3 extract was made from a 37°C culture, whereas the D7 extract was made from a culture which had been at 41°C for six days and was producing merozoites. The banding pattern was identical for both tracks, and this indicated that the degree of protease activity was identical for both extracts.

7:2:3 Assay of secretion of the 30 kDa molecule

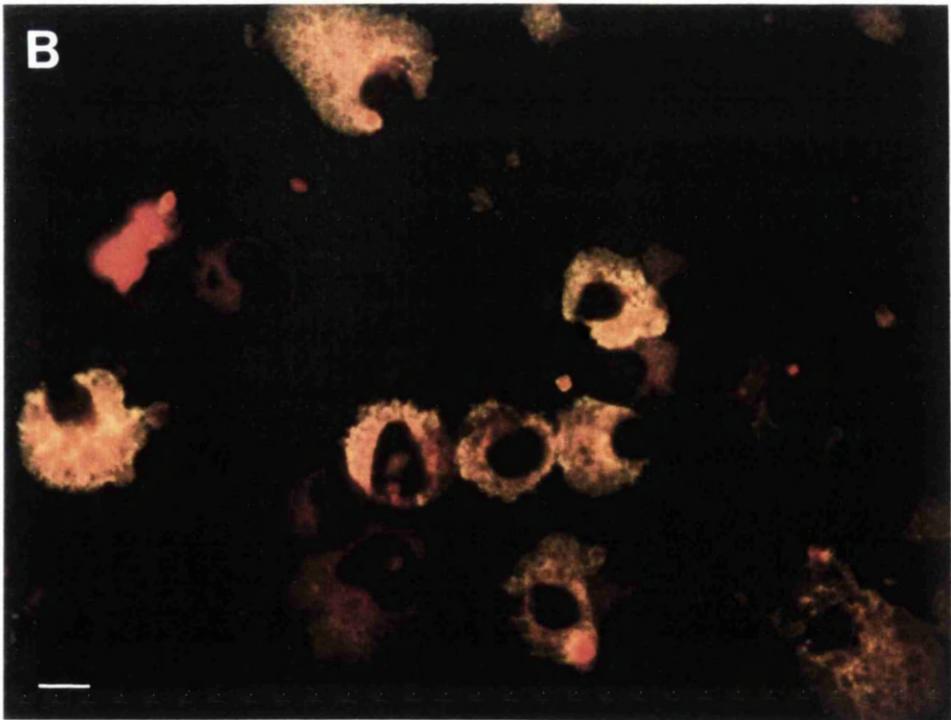
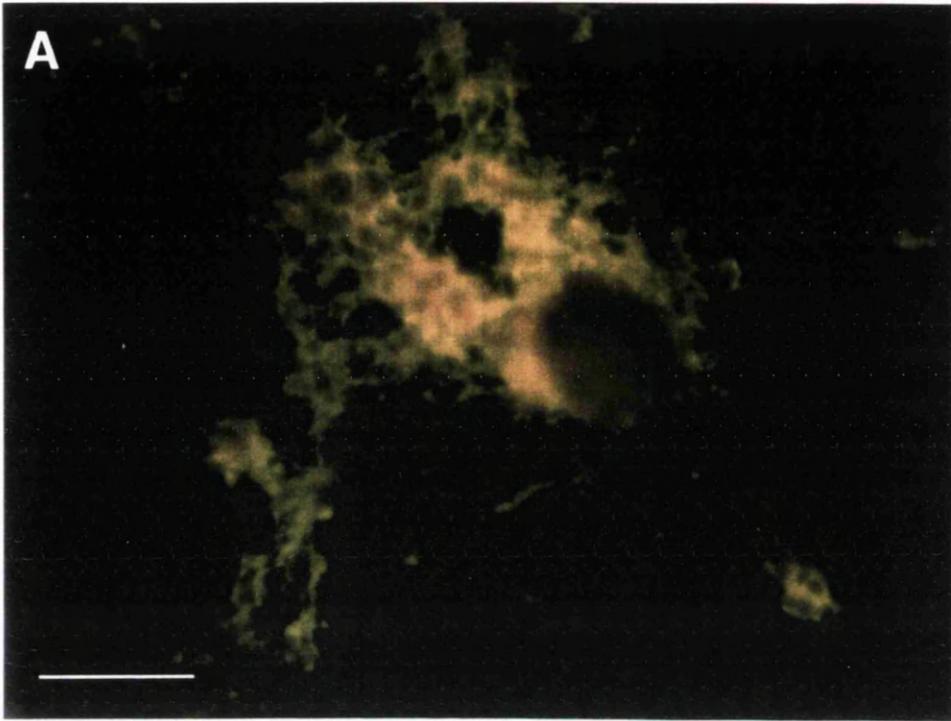
In IFAs, the monoclonal antibody 5E1 reactivity with differentiating macroschizont-infected cells was observed to be with the parasite surface (section 3:2:6). However, the pattern of fluorescence often gave the impression that the 5E1-detected epitope was being secreted from the differentiating macroschizont/merozoite surface (see Fig. 7.4A). To test this hypothesis, extracts were made from the culture supernatant of a differentiating enhanced cloned cell line (D7), and of serum from blood derived from a T.annulata-infected cow. As a control, an extract was made from normal cow serum. The extract polypeptides were separated by SDS-PAGE, Western blotted and probed with the monoclonal 5E1, immune bovine serum and anti-C9m serum. The result showed that the 5E1 monoclonal did not react with

Figure 7.4: IFA analysis of T.annulata-infected cells using monoclonal antibody 5E1.

Panel A; Formaldehyde-fixed cells probed with monoclonal antibody 5E1

Panel B; Acetone-fixed cytospin samples.

Scale bar; 10um



any of the extracts. The immune bovine serum and the anti-C9m serum detected identical polypeptide bands in the infected and uninfected bovine sera, and the most significant band recognised in these serum extracts and in the D7 culture supernatant was the albumin band at approximately 66 kDa. No bands were detected at molecular weights 30 kDa or 32 kDa (data not shown). In addition, when the method of fixing the cells prior to IFA was altered, by substituting the method of formaldehyde fixation in solution (section 2:5) with the acetone fixation of cytospin samples on glass slides, 5E1 monoclonal antibody reactivity was seen to be conserved to the parasite membrane (see Fig. 7.4B). Therefore, prior observation of possible 30 kDa secretion was thought to be an artefact of the cell fixation technique used in those earlier experiments.

7:3 Discussion

Much of the results contained in this Chapter record experiments carried out to establish an in vitro system for the merozoite invasion of erythrocytes. Previous experiments, carried out using in vivo-generated merozoites, showed that it was possible to observe significant merozoite invasion in vitro (up to 24% erythrocyte invasion). Therefore, it was decided to take this assay a step further by attempting to use merozoites generated by differentiating macroschizont-infected cell lines in vitro to invade bovine erythrocytes in vitro. Various culture conditions were employed, and some erythrocyte invasion was achieved, with the observation of intra-erythrocyte piroplasm forms. However, the best invasion rate observed in these assays was approximately 0.6% erythrocyte invasion. Interestingly, it was also estimated that 3% of bovine erythrocytes had merozoites apparently physically attached to their membrane surface. This observed difference between the percentage of merozoites attached to the erythrocyte surface and the percentage of observed piroplasms suggested that a significant number of merozoites reached an initial stage of invasion, but did not go beyond this point.

The establishment of an in vitro system for merozoite invasion has been attempted previously (Glascodine, 1989), and in this study it was thought that erythrocyte invasion was not seen because the ratio of erythrocytes to merozoites (approximately 10:1) was too high. In the experiments recorded in this Chapter, it was attempted to

reverse this ratio so that there were more merozoites than red blood cells and so the chance of a merozoite attaching to and invading an erythrocyte was increased. If all the merozoites generated were competent for invasion, it could be expected that a parasitemia of at least 10% would be detected. However, at best, less than 1% parasitemia was observed, and the rate of erythrocyte invasion was much lower than that seen with the semi-in vitro system using in vivo-generated merozoites. Therefore, it is possible that merozoites generated in vitro may not be as viable, or their viability may be shorter lived, when compared to merozoites generated in vivo. Additional factors may also have hampered invasion, such as the number of merozoites produced during the incubation period with the erythrocytes may have been too small, or the culture conditions, upon erythrocyte addition, may have been detrimental to the merozoites' survival or production.

Experiments were also carried out to determine if the T.annulata 30 kDa molecule had proteolytic activity, and if the molecule was secreted from the parasite surface. A cysteine protease gene, with homology to a Plasmodium falciparum cysteine protease gene, has been cloned from Theileria parva (Nene et al., 1990), so it was very likely that T.annulata possesses proteolytic enzymes also. Using the gelatin substrate/SDS-PAGE method, such protease activity was identified in extracts of T.annulata macroschizont-infected cells. This activity was not in association with the 30/32 kDa proteins, but a greater range of incubation conditions would have to be tested in

order to rule out the existence of 30/32 kDa protease activity. The observed protease activity was found to be optimal at pH 8.0, and was enhanced by the presence of calcium ions. It is thought that calcium may function to stabilise enzyme activity by binding to specific negatively-charged sites, and thus prevent enzyme inhibition (Yudhin and Offord, 1975). The proteolytic activity appeared to be expressed constitutively at both the macroschizont and merozoite stages. These proteases, and other proteases, have been identified in T.annulata schizonts by Baylis et al. (1992). The proteases have been reported to have molecular weights ranging from 50 kDa to 250 kDa, and, because their activity is inhibited by EDTA and 1,10-phenanthroline, the proteases are thought to be metalloproteases. Interestingly, long-term culture results in a decrease of this protease activity, and this can be correlated with parasite attenuation, or the decreased schizont ability to produce merozoites in vitro. In addition, two of the proteases have been shown to be secreted from infected cells, and the gene has been cloned for one of these secreted proteases (H.A.Baylis, personal communication).

The observed pattern of fluorescence in IFAs of differentiating macroschizont-infected cells, with the monoclonal antibody 5E1, suggested that the 5E1-recognised molecule could be secreted from the merozoite surface. However, subsequent examination of parasite culture supernatants, and of serum from a T.annulata-infected cow, indicated an absence of 30 kDa molecule in these

substrates, leading to the conclusion that the 30 kDa molecule was unlikely to be secreted, or if it was secreted, it was degraded quickly in the extracellular medium. Furthermore, IFAs of slides prepared using an alternative method of cell fixation (using acetone instead of the formerly used formaldehyde) gave a very distinct pattern of fluorescence, with monoclonal 5E1, across the surface of the parasite. This suggested that former observations of possible 30 kDa polypeptide secretion were artefactual, and probably due to poor fixation of the cells and diffusion of the antigenic material.

In conclusion, although there was no direct evidence to suggest that the 30 kDa polypeptide is secreted and/or has protease activity, it was likely, however, that being a major merozoite surface glycoprotein, the 30 kDa molecule has a significant function for the T.annulata parasite. Studies of various eukaryotic cell-surface glycoproteins have shown that these molecules can be important for intercellular recognition and communication (Ashwell and Morell, 1977). For example, the attachment of the influenza virus to the erythrocytic membrane is dependent on the structure of certain erythrocyte glycoproteins (Ashwell and Morell, 1977), and similarly, parasitic protozoa use cell surface molecules for host cell recognition and invasion (Mirelman, 1988). The invasion of erythrocytes by malaria merozoites involves attachment between lectin-like receptors on merozoites and ligands on erythrocytes (for examples see Miller et al., 1977; Perkins, 1981), and two lectins have been isolated from Entamoeba histolytica

(Kobiler and Mirelman, 1980; 1981) which are involved in the adherence of the parasite to erythrocytes and epithelial cells. Therefore, it is possible that the T.annulata 30 kDa glycoprotein may have a function in binding to host erythrocytes. If a reproducible merozoite invasion assay was available, this theory could be tested by using antibody reagents, such as the monoclonal antibody 5E1, to block merozoite invasion.

Glycoproteins have also been found which are important for parasite growth and morphology. The Gp72 cell surface protein of Trypanosoma cruzi has been shown to be involved in the control of differentiation of the parasite (Snary, 1985), and monoclonal antibodies to the major merozoite surface antigen precursor of Plasmodium yoelii, P.chabaudi, P.knowlesi and P.falciparum inhibit parasite growth (Holder et al., 1987; Howard et al., 1986; Schwarz et al., 1986 and Perrin et al., 1981). However, it is unlikely that the 30 kDa glycoprotein of T.annulata controls parasite differentiation or growth, as antibody detection of the molecule occurred after the reversible phase of merogony when the parasite was already committed to differentiation (section 3:3). It is probable that the protein has a more structural or mechanical role for the parasite membrane, but it may be involved in erythrocyte invasion also.

CHAPTER EIGHT
GENERAL DISCUSSION

The work and the results described in the previous Chapters demonstrated that the molecular expression of a 117 kDa rhoptry protein and a 30 kDa surface protein is induced during Theileria annulata (Ankara) macroschizont differentiation to the merozoite stage in vitro. The antiserum-detected expression of the 30 kDa polypeptide was seen to begin before the expression of the 117 kDa polypeptide, and after the irreversible phase of merozoite differentiation had begun. Furthermore, the increased expression of the 30 kDa molecule was seen to coincide with the decreased expression of schizont polypeptides, and it would be interesting to determine if these two events (the up-regulation of merozoite polypeptide expression and the down-regulation of schizont polypeptide expression) are mechanistically linked.

The 30 kDa protein was found to exhibit molecular polymorphism and antigenic diversity within and between different T.annulata stocks. Within the T.annulata Ankara stock, the molecule was found to occur in two polymorphic forms (30 kDa and 32 kDa molecules). It was found that the 30 kDa and 32 kDa molecules were closely related, in that they had similar peptide maps and shared antigenic epitopes, but were also divergent because the molecules were shown to have minor differences in antigenic epitopes also. It was also found that both molecules were glycosylated, and much of the reactivity of available antibody reagents was shown to be directed to periodate-sensitive sugar residues.

The results of experiments carried out to determine the

structure of the glycosylation of the 30/32 kDa polypeptides were inconclusive because the molecules appeared to be resistant to digestion by carbohydrate-specific enzymes, and did not bind a range of lectins. However, because various protozoan glycoproteins have now been identified having structures deviating significantly from those of mammalian glycoproteins (Ferguson and Homans, 1988), by analogy, it is thought that the T.annulata 30/32 kDa glycoproteins may also have an unusual and complex structure, making them difficult to examine using conventional techniques. Examination of the 30/32 kDa glycoproteins, using chemical, rather than enzymatic, methods of analysis should allow the glycosylated structure to be elucidated, and from this, it could be determined if differential glycosylation contributes to the observed antigenic diversity of the 30 kDa molecule.

The presence of sugar residues on the 30 kDa molecule caused problems, not only for enzymatic analysis, but also for isolation of the 30 kDa protein gene, by antibody-screening a T.annulata genomic expression library. The anti-E3d serum used in these experiments did not successfully isolate the 30 kDa polypeptide gene, and later it was found that much of the reactivity of the anti-E3d serum was directed to sugar residues on the molecule. Therefore, it is likely that the 30 kDa protein gene was not isolated by immunoscreening because the anti-E3d serum did not detect 30 kDa peptide sequence with a strong enough affinity. Similarly, the monoclonal antibody 5E1 was found to detect only sugar residues, so it is now understandable

why a previous immunoscreen (Glascodine, 1989), using this monoclonal antibody, was not successful.

Another approach used to clone the 30kDa protein gene employed two oligonucleotides, the sequence of which was based on the amino acid analysis of peptides from the 30 kDa molecule. The oligonucleotides used contained degenerate sequence, and considerable difficulties were encountered in establishing the ideal hybridisation conditions. Although no detailed studies have been done to determine the maximal acceptable number of different oligonucleotides in a probe mixture, it is accepted that probe complexity should be minimized (Wallace and Miyada, 1987). Therefore, it was likely that the oligonucleotide pools used in these experiments were too large (over 1000 oligonucleotides in each) to allow the 30 kDa protein gene to be detected, and it was concluded that the likelihood of using this technique successfully would be enhanced if less degenerate oligonucleotides were employed.

The T.annulata 30 kDa protein gene has recently been cloned by homology, using cDNA sequence encoding the T.buffeli 34 kDa piroplasm protein (B.R.Shiels and S.McKellar, unpublished data; Kawazu et al., 1992iii). An enhanced cloned cell line (D7) cDNA library (made by Dr J.Kinnaird, W.U.M.P.) was screened, and a full length cDNA clone was isolated and sequenced. Comparison of this sequence to the T.sergenti and T.buffeli 33/34 kDa protein gene sequences indicates 68% similarity and 52% identity. Furthermore, Southern blot analysis, using this clone as

probe, has shown the presence of restriction fragment length polymorphisms between the enhanced and diminished cloned cell lines, which suggests that there may be amino acid differences between the 30 kDa molecule of enhanced cell lines and the 32 kDa molecule associated with the diminished cell lines. The 30 kDa polypeptide gene is a single copy gene, with a 1.4 kilobase RNA message, and there are at least three alleles present in T.annulata (Ankara) piroplasm DNA. In uncloned cell line (TaA₂) DNA, the allele associated with the enhanced cloned cell lines predominates, and this reflects the results of previous Western blot experiments which show a higher level of expression of the 30 kDa molecule, also associated with the enhanced cell lines.

In addition, four other merozoite genes have recently been cloned (J.Kinnaird and B.R.Shiels, unpublished), which were isolated by screening a lambda-Dash D7 genomic expression library with the anti-C9m serum. Two of these have been found to express different merozoite surface polypeptides, while the other two genes encode antigen located to the merozoite rhoptry (J.Kinnaird and B.R.Shiels, unpublished data). Antibody select experiments, in conjunction with Western blotting, indicate that one of the cloned merozoite surface proteins is of 69-70 kDa molecular weight, and the other is thought to be over 200 kDa in size, as it has been shown to have a 12 kilobase RNA message. One of the cloned rhoptry antigens has also been characterised by antibody select, and has been shown to be 117 kDa in size, and is thought to be identical to the

molecule detected by the monoclonal antibody 1D11 (see section 3:2:3:3). RNA studies have shown that both the 30 kDa protein gene and the 117 kDa protein gene are expressed at high levels in differentiating cell lines and in piroplasms. Therefore, these genes are ideal candidates for future studies investigating the mechanism of the up-regulation of expression of merozoite proteins.

As yet, it is not known if any of the cloned T.annulata merozoite antigens are important in the development of a protective host immune response and, furthermore, nothing is known about the function of these polypeptides. An in vitro merozoite invasion assay would allow the preliminary study of these aspects because antisera generated against recombinant proteins of the cloned gene sequences could be tested for a blocking effect on merozoite invasion. In the experiments carried out to establish such an in vitro merozoite invasion assay, some invasion was achieved, with the observation of intraerythrocytic piroplasms, although the percentage of erythrocyte invasion was low (0.6%). It is possible that at 41°C, the in vitro-generated merozoites quickly lose the ability to invade erythrocytes. Recent experiments have shown that enhanced differentiating macroschizont-infected cell lines can produce merozoites at 37°C if they have been incubated first at 41°C for four days (Shiels et al., 1992), and with T.parva-infected lymphocytes (Danskin and Wilde, 1976), merogony can be induced by the addition of bovine lymph to the culture medium. It would be interesting to determine if T.annulata merozoites, produced by either of these methods, have a

greater invasive ability compared to those produced at 41°C. Also, as it was estimated that two-thirds of the merozoites which attach to red blood cells do not progress to invade the cells, an assay could be developed to study the binding of merozoites to red blood cells. This could be developed as a substitute for an invasion assay.

In conclusion, this project has shown that differentiating T.annulata (Ankara) macroschizont-infected cell lines express a 30 kDa merozoite surface glycoprotein, and comparison of cloned cell lines showed that this protein varies in molecular weight, as diminished cloned cell lines express a 32 kDa glycoprotein. It was also shown that the expression of a 117 kDa rhoptry protein is antigenically divergent between cloned cell lines. Furthermore, polypeptide comparisons of a range of different T.annulata isolates showed that the molecular weight and antibody reactivity of the 30 kDa molecule is highly variable. The cause of this variability could be due to differential glycosylation and/or to differences in amino acid sequence, and this has yet to be determined. Nothing is known of the nature of the sugar residues present on the molecules, but the glycosylation is thought to be unusual, and possibly protozoa-specific and complex. This project showed that it is possible to achieve merozoite invasion in vitro, but the rate of invasion observed would have to be improved for the assay to be useful in studying erythrocyte invasion in close detail. Finally, it was shown that the 30/32 kDa molecules are not likely to be secreted, but are integral membrane proteins

of merozoites and piroplasms, and so are most likely to have a protective and structural role for the merozoite and piroplasm surface, although a role in erythrocyte recognition and invasion cannot be ruled out.

APPENDIX

The amino acid sequences of the S1 (10 kDa) and S2 (15 kDa) peptide fragments generated by the V8 digestion of the 30 kDa molecule (see section 5:2:2) are presented. All sequencing was carried out by Dr. M. Cusack of the Geology Department, Glasgow University. An automated gas-phase sequenator was used for the Edman degradation reactions. The amino acids generated during each sequencing cycle were injected onto a HPLC column, and the elution time (retention time) of the amino acids, as detected by UV absorbance at 269nm, allowed amino acid identification.

The protein sequencer chromatogram report is presented for each fragment.

Abbreviations:

AAcid; amino acid
ID; identity
R time; retention time
C time; cycle time

(a) S1 Peptide Fragment

- Applied Biosystems 477H Protein Sequence Report -

SAMPLE : 210192JD.1
[initiated 21 Jan 1992 9:47am]

Sample Amount: 100 pmol

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	L	25.25	25.20	168.52	155.78	155.78	175.70	LEU
2	T	8.00	8.07	62.02	62.38	62.38	181.02	THR
3	V	20.07	20.03	134.38	118.73	120.63	63.02	VAL
4	A	12.12	12.15	106.93	96.88	100.97	105.13	ALA
5	N	18.97	18.92	15.03	8.10	7.36	16.98	PROASN
6	G	8.40	8.48	102.96	69.77	77.20	34.68	GLY
7	Y	15.57	15.55	49.83	45.96	52.58	122.05	TYR
8	R	6.95	7.08	69.34	35.95	35.00	6.43	SERARG
9	F	23.78	23.72	65.08	60.18	68.39	176.05	PHE
10	K	24.83	24.73	27.78	25.94	27.90	118.22	LYS
11	T	8.00	8.07	21.42	18.22	20.44	59.31	THR
12	L	25.30	25.20	47.09	40.31	51.08	57.61	LEU
13	K	24.75	24.73	15.81	13.91	16.06	68.03	LYS
14	V	20.07	20.03	49.69	41.61	56.04	29.28	VAL
15	G	8.42	8.48	50.36	39.68	51.13	22.97	GLY
16	Q?	7.22	7.45	48.11	12.28	15.85	6.62	GLN
17	K	24.83	24.73	9.96	8.15	8.47	35.91	LYS
18	T	7.98	8.07	16.57	14.26		41.38	THR

REPETITIVE YIELD ANALYSIS:

	Rep.Yield	Variance	
L: 1, 12	88.44 %	1.000	:LEU
T: 2, 11, 18	91.00 %	0.915	:THR
V: 3, 14	90.91 %	1.000	:VAL
G: 6, 15	93.92 %	1.000	:GLY
F: 10, 13, 17	84.90 %	0.984	:LYS

Average AA Repetitive Yield: 89.30 %

Combined AA Repetitive Yield: 89.22 % 0.467

Theoretical Initial Yield: 100.00 pmol (100.00 %)

Summary of S1 peptide fragment chromatogram report

Amino acid number	Three letter symbol	Amino acid
1	Leu	Leucine
2	Thr	Threonine
3	Val	Valine
4	Ala	Alanine
5	Asn	Asparagine+
6	Gly	Glycine
7	Tyr	Tyrosine
8	Arg	Arginine+
9	Phe	Phenylalanine
10	Lys	Lysine
11	Thr	Threonine
12	Leu	Leucine
13	Lys	Lysine
14	Val	Valine
15	Gly	Glycine
16	Gln	Glutamine*
17	Lys	Lysine
18	Thr	Threonine

+ Amino acid identity predicted by Applied Biosystems sequence analysis software altered when amino acid chromatogram examined.

* Unclear on chromatogram. Predicted as glutamine by sequence analysis software.

(b) S2 peptide fragment

- Applied Biosystems 4770 Protein Sequence Report -

SAMPLE : 190292JD.14K
 [initiated 19 Feb 1992 10:00am]

Sample Amount: 100 pmol

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	V	20.08	20.05	518.70	521.58	565.25	186.53	VAL
2	I	24.47	24.38	489.62	479.24	541.73	132.96	ILE
3	L	25.28	25.22	526.57	514.04	541.62	929.19	LEU
4	P	19.02	18.98	355.61	341.67	460.97	400.22	PRO
5	N	6.07	6.13	227.28	199.32	269.90	129.09	ASN
6	N	6.08	6.13	302.64	271.47	289.13	138.29	ASN
7	D	5.87	5.90	171.94	137.00	179.63	37.04	ASP
8	T R	8.07	8.12	20.63	10.84	14.34	13.44	THR ARG
9	H	12.40	12.63	147.64	148.37	231.30	83.63	HIS
10	Q	7.48	7.53	187.73	182.30	293.44	423.73	GLN
11	I	24.48	24.38	222.02	205.12	328.56	80.64	ILE
12	T	8.08	8.12	120.53	106.83	173.37	162.41	THR
13	R D	16.85	17.03	8.74	6.62	9.72	27.58	ARG ASP
14	T	8.08	8.12	122.00	106.89	169.86	159.12	THR
15	R ?	16.97	17.03	7.42	4.34	2.96	8.40	ARG ?
16	N	6.10	6.13	61.66	15.18	22.86	10.94	ASN
17	G	8.50	8.53	85.55	36.95	51.31	18.22	GLY
18	F H	23.83	23.77	20.46	4.64	9.55	9.67	PHE HIS
19	Y	15.60	15.58	52.41	44.33	76.10	184.73	TYR
20	L A	25.28	25.22	27.96	10.23		12.62	LEU ALA

 REPETITIVE YIELD ANALYSIS:

	Rep.Yield	Variance	
I: 2, 11	91.00 %	1.000	: ILE
L: 3, 20	79.42 %	1.000	: LEU
N: 5, 6, 16	77.41 %	0.968	: ASN
T: 8, 12, 14	150.47 %	0.893	: THR
R: 13, 15	80.98 %	1.000	: ARG

Average AA Repetitive Yield: 101.02 %

Combined AA Repetitive Yield: 80.69 % 0.596

Theoretical Initial Yield: 664.13 pmol (664.13 %)

Summary of S2 peptide fragment chromatogram report

Amino acid number	Three letter symbol	Amino acid
1	Val	Valine
2	Ile	Isoleucine
3	Leu	Leucine
4	Pro	Proline
5	Asn	Asparagine
6	Asn	Asparagine
7	Asp	Aspartic acid
8	Arg	Arginine+
9	His	Histidine
10	Gln	Glutamine
11	Ile	Isoleucine
12	Thr	Threonine
13	Asp	Aspartic acid+
14	Thr	Threonine
15	Arg	Arginine*
16	Asn	Asparagine
17	Gly	Glycine
18	His	Histidine+
19	Tyr	Tyrosine
20	Ala	Alanine+

+ Amino acid identity predicted by Applied Biosystems sequence analysis software altered when amino acid chromatogram examined.

* Unclear on chromatogram. Arginine predicted by sequencing software.

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