IMMUNOLOGICAL ASPECTS OF *THEILERIA ANNULATA* INFECTION IN CATTLE WITH REFERENCE TO THE ROLE OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

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DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts, and has not been submitted in any previous application for a degree. The work on which it is based is my own except where stated in the text and in the acknowledgements section.

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An introduction and review of the relevant literature concerning immunological aspects of Theileria annulata infection and the bovine major histocompatibility complex is given as a background to the experimental work presented.

An immunisation trial was conducted in Morocco using T. annulata infected lymphoblastoid cell lines (LCL) and the effects of cell dose, cell line and bovine lymphocyte antigen (BoLA) compatibility between cell line and recipient were assessed. All the factors examined were shown to play a role in the response to LCL immunisation although none appeared to be of critical importance. Four schizont infected cell cultures at passage three were inoculated at four different cell doses (10^8, 10^6, 10^4 and 10^2) into pairs of calves, one of each pair being BoLA class I matched to the immunising LCL, the other BoLA class I mismatched. The recipients showed variation in the severity of disease symptoms not linearly correlated with the cell dose. The most severe disease symptoms recorded after inoculation of the LCLs were in the 10^2 cell dose group. Both BoLA class I matched and mismatched LCLs infected and immunised susceptible cattle, although in general the mismatched recipients showed a more severe reaction. This difference was most marked at the highest cell dose, 10^8. One of the four LCLs appeared to be more pathogenic than the other three.

The development and specificity of cytotoxic cells generated in vivo in cattle injected with either autologous or allogeneic LCL or sporozoite stabilate material were examined. In cattle immunised with allogeneic LCL at a dose of 10^6 cells the animals showed mild disease symptoms and the initial cytotoxic response appeared to be directed against the foreign MHC antigens of the LCL, similar to a graft rejection phenomenon. After parasite transfer from LCL to recipient cells and also after a subsequent sporozoite infection and recovery the cytotoxic response was parasite species specific and predominantly MHC restricted to autologous BoLA class I antigens. The recipients of 10^6 autologous infected cells and the recipients of the sporozoite material showed low levels of non-specific cytotoxic activity after primary immunisation coinciding with severe disease symptoms. After sporozoite challenge, to which both groups were immune, they showed a cytotoxic response similar to that observed in the allogeneic infected cell line recipients after sporozoite challenge.

A further study was conducted to examine the target cell for infection in vitro by sporozoites of T. annulata and T. parva. The results suggested that T. annulata preferentially infected cells expressing MHC class II antigens which include B cells and macrophages, whereas T. parva preferentially infected T cells. These findings, if representative of the situation in vivo, may have important implications in the differing pathogenesis of and ease of cell line immunisation against T. annulata and T. parva infections.

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COMMON ABBREVIATIONS

BoLA	bovine lymphocyte antigen
CTL	cytotoxic T lymphocyte
FACS	fluorescence activated cell sorter
GUTS	ground up tick supernatant
LCL	lymphoblastoid cell line
MAb	monoclonal antibody
mhc	major histocompatibility complex
MLR	mixed leucocyte reaction
PBM	peripheral blood mononuclear cells
w	BoLA workshop specificity

CHAPTER 1

Introduction and Literature Review

GENERAL INTRODUCTION

"In 1929 a farmer from the town of Setif in Algeria was keeping a dairy cow in isolation from all the other animals and infected stables. A wagon was brought to the town containing material from the yards of a contaminated farm at Bouphzal. Sometime after the visit of this wagon the dairy cow fell ill and the farmer found on it a single *Hyalomma detritum* tick. The cow died 20 days later and the diagnosis of theileriosis was confirmed".

E. Sergent (1945)

The above observation describes the potentially devastating effect on livestock production of the tick borne disease commonly known as tropical theileriosis or Mediterranean Coast fever. The causative agent is the protozoan parasite *Theileria annulata* which infects bovine leucocytes, inducing them to proliferate in an unregulated manner. The disease is present in most areas where the vector tick is present and occurs over a vast area stretching from Southern Portugal, Spain and Morocco in the West through the Meditteranean countries to India, Southern USSR and China in the East. A conservative estimate suggests that 250 million cattle are potentially at risk with mortality rates in susceptible cattle between 40-60%, although animals which do recover develop an effective immunity to subsequent disease challenge (Neitz, 1957).

An understanding of the mechanisms of protective immunity and their induction within the bovine host is an essential prerequisite in the development of effective methods of immunoprophylaxis.

The disposal of foreign or pathogenic material is the effective function of the immune system but this requires a sophisticated recognition system to distinguish "self" from "non-self". An essential component of this recognition system involves cell surface molecules coded for by the highly polymorphic genes of the Major Histocompatibility Complex (mhc).

The work presented in this thesis examines various aspects of the host parasite relationship involving the intracellular stage of the parasite with emphasis placed on the role of the bovine mhc. The study can be divided into three main areas:

(a) the use of parasite infected cells as immunoprophylatic agents;

(b) the use of parasite infected cells as targets for immune attack by cytotoxic T lymphocytes;

(c) the definition of the target cell for *in vitro* parasite infection.

LITERATURE REVIEW

1.1 *THEILERIOSIS*

1.1.1 Historical background and classification

The protozoan parasites of the genus *Theileria* were first recognised and recorded at the beginning of this century in German East Africa (Tanzania). It was while investigating an outbreak of redwater (*Babesia bigemina*) that Koch, in 1897, observed minute rod shaped organisms in the erythrocytes of infected cattle, which were initially assumed to be immature forms of *B. bigemina* (Koch, 1903). However following the catastrophic spread of a disease called East Coast fever from infected cattle in Tanzania to Rhodesia (Zimbabwe) and Southern Africa, these intraerythrocytic organisms were recognised to be distinct from *B. bigemina* and were named *Piroplasma parvum* (Theiler, 1904). Following the discovery of an exoerythrocytic schizont stage inside lymphocytes, (Koch, 1906) the parasites were subsequently moved to a new genus called *Theileria* (Bettencourt, Franca and Borges, 1907).

This difference in the parasite life cycle and also in its morphology when compared to that of *P. bigemina* justified its removal from the genus *Piroplasma*. The new parasite was now called *Theileria parva* (Bettencourt, Franca and Borges, 1907).

Almost simultaneous to the original identification of *Theileria parva*, a fatal disease of cattle was recorded by Dschunkowsky and Luhs (1904) in Transcaucasia (Southern Russia) and named tropical piroplasmosis. Cattle afflicted with this disease had oval or round erythrocytic parasite forms similar but not identical to the rod shaped parasites observed by Koch in East Coast fever. This new "Russian" parasite was named *Piroplasma annulata*, (annulus meaning a ring shaped structure). With further investigations and the discovery of schizont forms of the parasite in various organs from infected animals this parasite was also placed in the new genus *Theileria* and named *Theileria annulata* (Bettencourt, Franca and Borges, 1907). Subsequent investigation in Europe, North Africa and Asia revealed many observations of an undefined type of *theileriosis* (Neitz, 1957). Lack of knowledge or defined reference stock led to much speculation as to whether the infectious agent was *T. annulata*, *T. parva* or *T. another.* As a consequence many "new" *Theileria* species were put forward by various groups; Sergent and colleagues in Algeria considered the Algerian *Theileria* to be a distinct species and named it *Theileria dispar* (Sergent *et al.*, 1924), theileriosis in Turkey was thought to be caused by the protozoan *Theileria turkestanica* (Oboldoueff and Galouzo, 1928) and in East Asia the causative agent was named *Theileria sergenti* (Yakimoff and Dekhtereff, 1930). Perhaps not surprisingly a great deal of controversy and

confusion has surrounded the taxonomy of the *Theileria* species. Most of the difficulties arose because taxonomists deal with individual organisms which they attempt to arrange into groups with common relationships. As new knowledge is gained and more criteria for distinguishing between individuals becomes available the situation becomes more complex (or clarified, depending on your point of view), with species being split up recombined or shifted from one genus to another. The currently accepted classification of *Theileria* parasites is based on a recent revision of the classification of the Protozoa by Levine *et al.* (1980).

Subkingdom	Protozoa		
Phylum	Apicomplexa		
Class	Sporozoea		
Subclass	Piroplasmia		
Order	Piroplasmida		
Family	Theileriidae	Babesiidae	
Genus	*Theileria*	*Babesia*	
Species	*Theileria parva*		(Theiler, 1904)
	Theileria annulata		(Dschunkowsky and Luhs, 1904)

Species differentiation within the genus *Theileria* has recently been reviewed by Uilenberg (1981a). Apart from the two most important species illustrated above there are many different species of *Theileria* affecting domestic livestock. A summary of the more important species is given in Table 1.

Identification is based on the following defining criteria: morphological characterisation of different parasite stages; host and vector specificity; pathogenic and epidemiological characterisation; serological characterisation; and cross immunity tests. More recently new techniques have been employed, including monoclonal antibodies, parasite isoenzyme patterns and genetic probes (Irvin, 1987). Precise definition of parasite strains becomes more important when trying to dissect protective immune responses or design appropriate prophylactic measures. Taxonomic consensus is an essential prerequisite for all research activity in any group of organisms.

In this thesis two species of *Theileria* parasites are considered: *T. parva* and *T. annulata*. The major emphasis is on the latter, the former being used for comparative purposes. The following terminology, defined by Irvin and Morrison (1987), is used to describe the parasite material used in the study.

Isolate: viable organisms isolated on a single occasion from a field sample into experimental hosts or culture systems or by direct preparation of a stabilate.

TABLE 1 **Important Theileria species infecting domestic livestock**

Species	Host	Distribution
T. parva (Theiler, 1904)	cattle, buffalo	East and Central Africa
T. annulata (Dschunkowsky and Luhs, 1904)	cattle, buffalo	Southern Europe, North Africa, Middle East, India, Southern Russia, South East Asia
T. mutans (Theiler, 1906)	cattle, buffalo, sheep?	Africa
T. sergenti (Yakimoff and Dekhteroff, 1930)	cattle	Eastern Siberia, Japan, Southern Korea, China
T. taurotragi (Martin and Brocklesby, 1960)	eland, cattle	East, Central and Southern Africa
T. velifera (Uilenberg, 1964)	cattle, buffalo	Most of Africa south of the Sahara
T. hirci (Dschunkowsky and Urodschevich, 1924)	sheep, goats	South East Europe, North Africa, Near and Middle East

Stock: all the populations of a parasite derived from an isolate without any implication of homogeneity or characterisation.

Stabilate: a sample of organisms preserved alive on a single occasion.

Line: a laboratory derivative of a stock maintained under defined physical conditions *e.g.* as a culture of parasitised bovine lymphoid cells.

1.1.2 The disease

1.1.2.1 *Host range*

The pathogenic *Theileria* species have a relatively limited host range and will infect and cause disease in domestic cattle, sheep and goats. Table 1 illustrates the common hosts and disease distribution of the most important *Theileria* species.

It is thought that the natural host for the parasite was originally the buffalo or other species of wild bovid (Barnett, 1977). Domestic cattle presumably became infected by contact with ticks which had previously fed on wild buffalo. In the "abnormal" cattle host the parasite caused high mortality in comparison to the mild or inapparent infection observed in the natural host. In the case of *T. annulata* the natural host is believed to be the Asian water or swamp buffalo (*Bubalus bubalis*), whereas *T.p. parva* is thought by some to be the cattle adapted version of *T.p. lawrencei* that infects the African buffalo (*Synerus caffer*) (Schindler, Mehlitz and Matson, 1969). Once the parasite had become established and adapted to the cattle population it was maintained in the cattle-tick-cattle cycle without requiring the original host.

1.1.2.2 *Transmission vector*

Theileria parasites are transmitted trans-stadially from larva to nymph or nymph to adult by two and three host ixodid ticks. The most common vector of *T. parva* is *Rhipicephalus appendiculatus*, while *T. annulata* is transmitted by the *Hyalomma* species of tick. The transmission of *T. annulata* has recently been reviewed by Robinson (1982). In *T. annulata* infection the most important vectors are the three host tick *Hyalomma anatolicum anatolicum* and the two host tick *Hyalomma detritum* which have a wide distribution from Southern Europe and North Africa through to Asia and China (Barnett, 1977). Other *Hyalomma* species (such as *H. excavatum)* have been known to transmit the disease in experiments (Delpy, 1969) but these are unlikely to be important vectors as their immature forms normally feed on non susceptible hosts such as small mammals and birds.

It is commonly believed that transmission of the parasite to the bovine host does not occur immediately when the tick attaches for feeding and that the stimulus of

the blood meal is required to induce maturation of the parasite within the salivary glands of the tick. The parasites are released into the saliva 2 to 4 days after feeding commences (Purnell *et al.*, 1973; Samish and Pipano, 1978). However some recent research examining *T. annulata* transmission has suggested that increasing the temperature and relative humidity of infected adult ticks is effective in the production of infective parasites without the need of a blood meal (Samish, 1977). This may prove to be important when using acaricides in warmer climates.

1.1.2.3 *Disease distribution*

Figure 1 illustrates the geographical distribution of *T. annulata* and *T. parva*, the geographical limitations of each disease depending on the availability of suitable vector species, host species and various environmental factors such as temperature (Robinson, 1982).

The extent of the problem in individual countries is poorly documented and is likely to be in a state of flux depending on the changing needs of livestock production. There has been a steady increase in milk and beef consumption in many of the *Theileria* endemic areas, leading to the introduction of exotic cattle which are very susceptible to the disease (Barnett, 1977). An illustration of the magnitude of the problem in India is as follows: the contribution of livestock to the Gross National Product is around Rs.200 000 million per annum (£10 000 million) with produce from cattle and buffalo comprising 80% of this value. The major emphasis in dairy development in India has been to upgrade low producing indigenous cattle with high producing exotic breeds in various cross breeding programmes. To implement this "genetic improvement" a total of 8.38 million inseminations have been established annually to be developed further to give 12.75 million inseminations per annum (Acharya and Chatterjee, 1987). These figures imply an estimated economic loss to India as a result of *T. annulata* infection of £1000 million per annum (assuming a 40% mortality rate among these exotic or crossbred cattle having a market value of about £200 a head).

1.1.2.4 *Host susceptibility*

There are numerous reports from endemic areas that the cattle most susceptible to *Theileria* infection are newly introduced exotic breeds, the indigenous breeds having developed an effective resistance (Rafyi, Maghami and Hooshmand-Rad, 1965). The precise mechanisms involved in developing this resistance are not known, inadequate characterisation of contributory factors leading to much of the confusion. The main controversy is whether resistance is innate or acquired. When a group of 373 indigenous Algerian cattle, reared in disease free

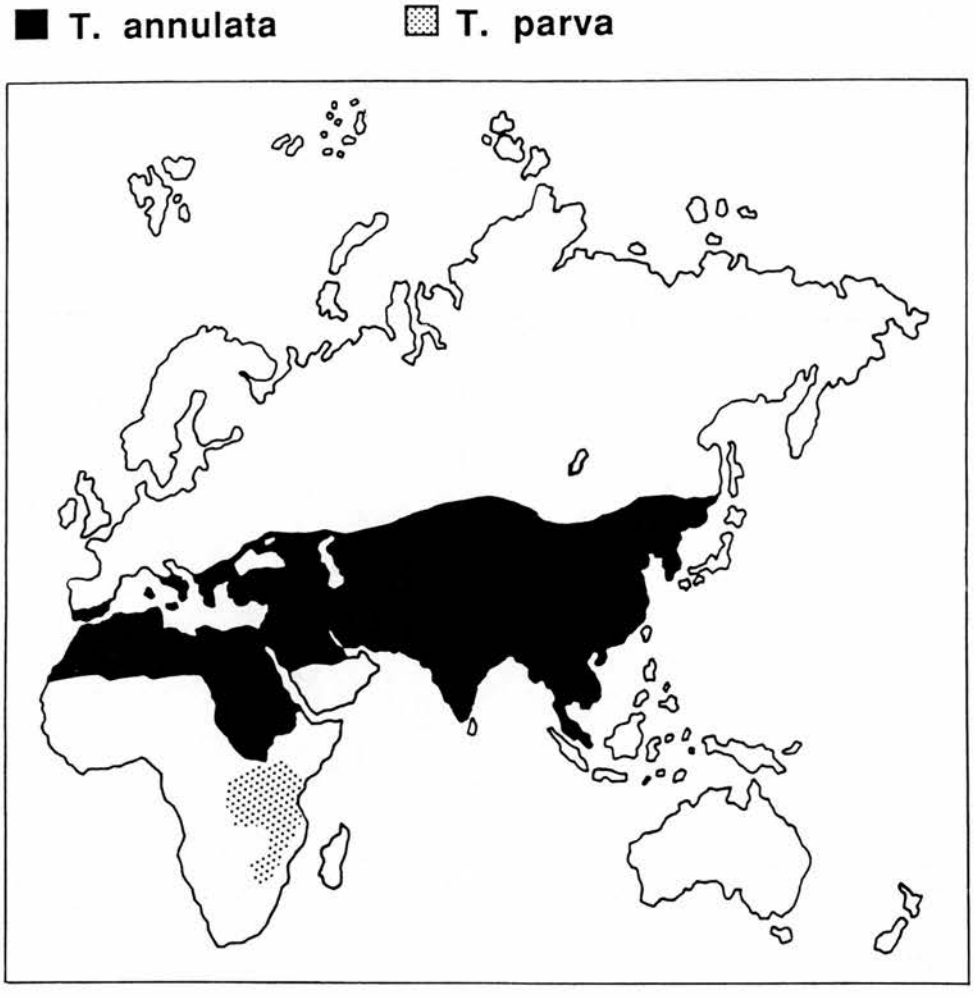


Fig. 1
The distribution of tropical theileriosis (T.annulata infection) and East Coast fever (T.parva infection).
(adapted from W.O.Neitz,1957)

conditions, were challenged alongside 842 exotic cattle (Aubrac breed, recently imported from France) the mortality rate was 23% in the indigenous stock and 12% in the exotic animals (Sergent, 1945). A similar phenomenon was also reported by Irvin and Morrison (1987); *Bos indicus* cattle (which are presumed to be more resistant) from non enzootic areas show mortality rate to East Coast fever similar to *Bos taurus* when exposed to field challenge. It appears that one of the important factors in the ability of an animal to withstand disease challenge is previous exposure to the parasite.

Cattle of both sexes are equally susceptible to infection (Irvin and Morrison, 1987). However, as is the case with other haemoprotozoan infections, pregnant and lactating cows offer least resistance (Pipano, 1978). There is some controversy as to whether young calves are more resistant than adult animals. Possible explanations for the alleged greater resistance of young animals may involve lower tick burdens on the calves or specific *Theileria* antibodies in the colostrum of immune dams (Burridge and Kimber, 1973a; Pipano, 1977).

There is, however, a paucity of conclusive data in a situation where the mechanisms of resistance are likely to be multifactorial.

Current research efforts into defining more precisely the mechanisms of resistance are concentrated on the search for relevant markers. Potential candidates are the cell surface molecules coded for by the genes of the major histocompatability complex (mhc). These molecules are known to influence an individual's resistance to disease by predetermining the quantity and quality of immune responsiveness to a given antigen (Schwartz, 1985). The bovine mhc molecules (BoLA) are discussed in section 1.2.

1.1.3 Parasite life cycle

In order to design appropriate methods of disease control it is necessary to understand the host-parasite interaction. Although the important pathogenic *Theileria* species are each transmitted by a specific genus of tick (Brocklesby, 1978), in most other respects the parasite life cycles are very similar.

Most of the literature on this subject concerns the life cycle of *T. parva*, which has been reviewed in detail (Gonder, 1910; Cowdry and Ham, 1932; Schein, Warnecke and Kirmse, 1977; Barnett, 1977; Irvin and Cunningham, 1981; Morrison *et al.*, 1986a). Reviews of the life cycle of *T. annulata* can be found in the following papers: Sergent *et al.* (1945); Neitz (1957); Schein (1975); Uilenberg (1981b). A brief account of the life cycle of the *Theileria* parasite is given below with a more detailed account of particular aspects following (section 1.1.5). Figure 2 presents a simplified diagramatic illustration of the life cycle in both the bovine and tick hosts. The life cycle of the parasite

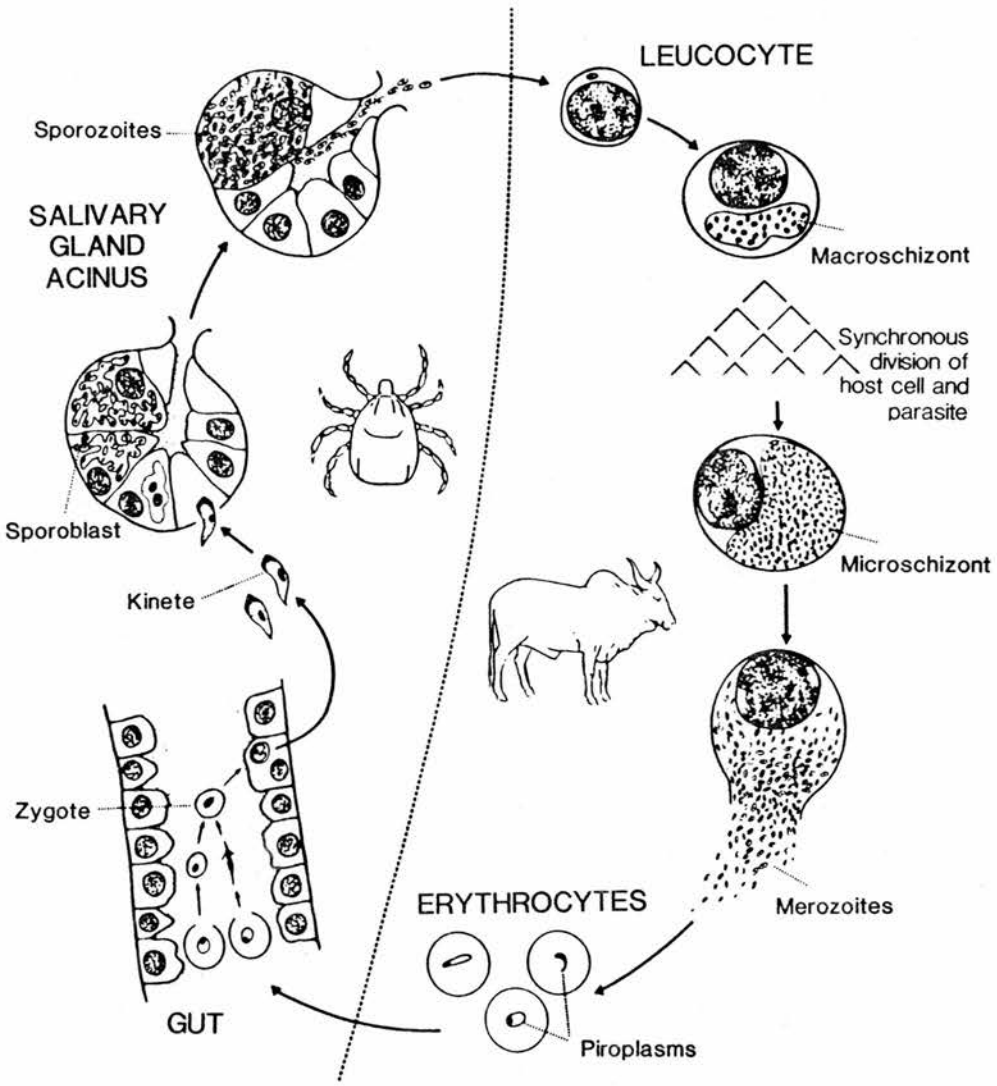


Fig. 2
Diagramatic representation of the life cycle of the Theileria parasite in the bovine and tick host.

can be roughly divided into three main stages: schizogony in the bovine host, gametogony, and sporogony in the tick host.

1.1.3.1 *The bovine host*

An infected tick transmits the parasite to the susceptible bovine host by taking a blood meal from the animal. During this process the sporozoites are activated within the salivary glands of the tick and are expelled into the cow. *In vitro* studies with *T. parva* have suggested that sporozoites rapidly become associated with and enter host target cells (Brown *et al.* 1978a; Fawcett *et al.* 1982). It is assumed that this reflects the situation *in vivo* but due to difficulties in detection of the parasite until day 4-5 after infection this has yet to be confirmed (Irvin and Cunningham, l981). Prior to detection of the parasite there is usually pronounced cellular activity and lymphoblastosis in the local draining lymph node (Sergent *et al.*, 1945; Morrison *et al.*, 1981). Figure 3 shows a macroschizont infected cell. The macroschizont infected cells disseminate quickly to secondary lymphoid tissues (documented for *T. parva* infection) including the thymus, bone marrow, gut and epitheilial tissue (De Martini and Moulton, 1973; Morrison *et al.*, 1981). In *T. annulata* infection the parasite distribution is more irregular, being concentrated predominantly in the local lymph node (C.G.D. Brown, personal communication). The intracellular parasite replicates by inducing synchronous division of the parasite and the host cell (Hulliger *et al.*, 1964). Macroschizonts may also be capable of invading uninfected cells by a mechanism that is not yet clear. This aspect of parasite replication is discussed further in section 1.1.5.

The next stage of parasite development is the microschizont which develops directly from the macroschizont by a process of nuclear multiplication and "budding" from the schizont nucleus (Jarrett and Brocklesby, 1966; Schein, Mehlhorn and Warnecke, 1978). The stimulus for the maturation of the parasite from macroschizont to microschizont is unclear. As the infection progresses *in vivo* the mean number of macroschizont nuclei per cell increases and microschizonts are observed (Jarrett, Crighton and Pirie, 1969). On disintegration of the schizont these microschizonts give rise to merozoites which upon release from the infected cell invade red blood cells to form piroplasms (Schein, Mehlhorn and Warnecke, 1978). In some recent ultrastructural studies the intracellular division of both *T. annulata* (Conrad, Kelly and Brown, 1985) and *T. parva* (Conrad, Denham and Brown, 1986) within erythrocytes was confirmed.

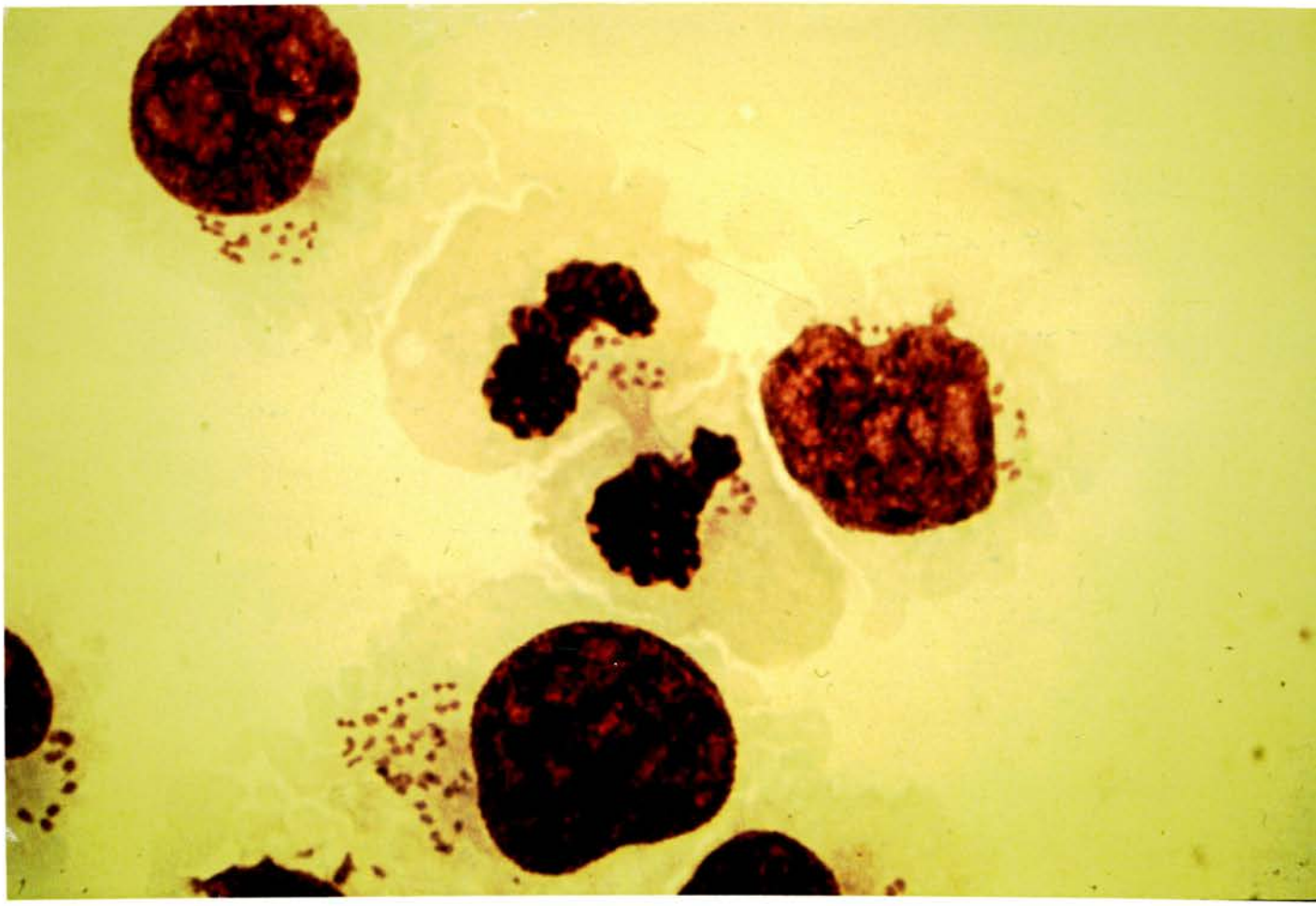


FIGURE 3. Macroschizont infected cell

1.1.3.2 *The tick host*

In addition to the asexual multiplication of *Theileria* parasites within the bovine host, sexual replication of both *T. annulata* (Sergent *et al*, 1945; Neitz, 1957; Schein, 1975) and *T. parva* (Cowdry and Ham, 1932; Mehlhorn and Schein, 1984) occurs within the gut of the tick. The process may be summarised as follows: the tick takes a blood meal from an infected animal and male and female gametes (from piroplasms) are released into the tick gut; syngamy follows, with the production of zygotes that subsequently invade the gut wall to develop into motile kinetes; these then leave the intestinal cells, moving actively within the haemolymph and finally penetrating the cells of the salivary glands. It is within the salivary glands that the kinetes undergo sporogony to produce sporozoites which are then injected into a new host animal when the tick takes its next blood meal (Mehlhorn and Schein, 1977).

1.1.4 *In vitro* culture

As mentioned above *Theileria* parasites have the ability (unique among protozoan parasites) to infect and "transform" host leucocytes. These "transformed" parasitised cells can be maintained *in vitro* for a seemingly indefinite period without the addition of exogenous growth factors (Hulliger *et al.*, 1964). Extensive coverage of this can be found in the following reviews: Brown, 1979; l983; 1987. Historical evidence and practical experience suggests that it is easier to adapt *T. annulata* infected cells to *in vitro* culture than *T. parva* (Brown, 1981).

It is not known whether this reflects intrinsic differences between the two parasites or the *in vitro* culture requirements of their particular host cells.

The first reported culture of a *Theileria* parasite was achieved by Tsur (1945) with *T. annulata*. This was achieved by culturing liver and spleen explants, taken from infected cattle, in plasma clot cultures. This discovery was followed up and developed further by Tsur and colleagues in Israel with many further reports of monolayer infected cells established either from infected tissues or blood (Tsur and Adler, 1962; Tsur and Adler, 1965). However, when the same technology was used in attempts to establish comparable *T. parva* infected cell cultures, it did not prove so successful (Brocklesby and Hawking, 1958). Despite various further attempts and claims (Hulliger, 1965) the first successful establishment of a *T. parva* infected cell line *in vitro* was achieved by Malmquist, Nyindo and Brown (1970), using spleen cells from an infected animal. Development of tissue culture techiques improved the establishment and maintenance of *T. parva* cell lines *in vitro*. However the most enlightening breakthrough in this area came with the work of Brown *et al.* (1973), in which normal lymphoid cells were infected *in vitro* with sporozoites of *T. parva*. This technology was

extended to culture other species of *Theileria* including *T. annulata* (Brown, 1979). This important development has allowed both cytological and ultrastructural studies concerning mechanisms of sporozoite invasion and the intracellular host parasite relationship. It also provides the possibility of obtaining a parasitised cell line from an uninfected animal.

On a more practical level, applying more to *T. annulata* than to *T. parva*, *Theileria* infected cell lines can be used to immunise animals against disease challenge (Pipano, 1977; Brown, 1978b; Pipano, 1981).

The macroschizont is still the only stage of the parasite that can be grown indefinitely *in vitro* and consequently most research effort has centred around this stage. There have been occasional reports of the induction of merozoite formation *in vitro* with *T. parva* (Hulliger, Brown and Wilde, 1966; Danskin and Wilde, 1976; Nyindo *et al.*, 1978). However, none of these results has proved easily repeatable. This may be because the infected cells best adapted to the culture conditions are highly selected and only represent a minor population compared to those cells infected *in vivo* or that the cells cultured *in vitro* are not subjected to the same stresses as those *in vivo*.

Using similar techniques as those applied to the culture of *babesia* parasites limited success has been achieved with the culture of *T. annulata* and *T. parva* intraerythrocytic piroplasm stages (Pipano and Fish, 1982; Conrad, Kelly and Brown, 1985; Conrad, Denham and Brown, 1986). However, it has not been possible reproducibly to infect normal bovine erythrocytes *in vitro* mainly because of the difficulty of obtaining a suitable source of merozoites.

The infected tick

A fundamental prerequisite for most aspects of *Theileria* research is the maintainance of infected vector tick colonies. The colonisation of *T. parva* in *Rhipicephalus appendiculatus* has been reviewed by Irvin and Brocklesby (1970), and of *T. annulata* in *Hyalomma anatolicum anatolicum* by Walker *et al.* (1985). Motile kinete stages can be harvested from the backless tick culture developed by Bell (1984), but these can only be maintained for a limited period of time *in vitro*. This system, however, does provide a potential means of obtaining cloned parasite material for immunological and genetic studies.

1.1.5 Further aspects of the host parasite interaction

1.1.5.1 *Sporozoite invasion and infection of host cell*

The precise mechanism of sporozoite entry to and subsequent infection of target cells is unclear. *In vitro* studies with *T. annulata* suggest that attachment of the sporozoite to the target cell is receptor dependent as tryptic proteolysis of peripheral blood leucocytes (target cells) markedly reduces the attachment and interiorisation of the sporozoite (Jura, 1984). A similar candidate glycoprotein receptor has been described for *T. parva* sporozoites (ILRAD Annual Report, 1986). Monoclonal antibodies (MAbs) have been raised which apparently inhibit *T. parva* sporozoite invasion *in vitro*. These antibodies appear to recognise a 44000 dalton glycoprotein molecule on the surface of bovine lymphocyte populations.

There is some controversy as to whether or not sporozoite entry to the cell is energy dependent. *In vitro* studies with *T. annulata* demonstrated that sporozite entry to the cell was significantly reduced at 0°C (Jura, 1984), whereas work on *T. parva* sporozoites (Fawcett *et al.*, 1982; Fawcett and Stagg, 1986) suggested that the interiorisation of the parasite into lymphocytes or adherent cells did not depend on energy and could be achieved at temperatures as low as 2°C. Fawcett *et al.* (1982) introduced the concept of passive endocytosis to explain this phenonomenon, which refuted the widely accepted view that endocytosis does not occur in macrophages at low temperatures (Griffin *et al.*, 1975).

It appears that the sporozoite surface coat is shed during endocytosis (Webster, Dobbelaere and Fawcett, 1985), and on entering the host cell the sporozoite locates itself within the Golgi region. In order to escape destruction by the phagolysosomal system of the host cell the parasite activates its own defence system, which differs from that of other intracellular protozoan parasites such as *Toxoplasm gondi* (Jones, Yeh and Hirsch, 1972) and *Leishmania donovani* (Lewis and Peters, 1977). *Theileria* sporozoites posesses secretory granules (micronemes) in their cytoplasm, which were originally thought to be involved in the entry process to the cell. Within a few hours of sporozoite entry these micronemes disappear, coinciding with the dissolution of the host cell membrane surrounding the sporozoite. This effectively leaves no vacuole with which the lysosomes can fuse. The sporozoite is now free in the cytoplasm of the host cell (Fawcett *et al.*, 1982).

1.1.5.2 *Macroschizont development and transformation of host cell*

After sporozoite invasion the morphology of the host cell changes rapidly, becoming lymphoblastoid, with the development of a more extensive cytoplasm and a

more marked Golgi apparatus (Stagg *et al.*, 1981; Musisi *et al.*, 1981). The preschizont stage (mononuclear trophozoite) appears close to the host cell nucleus (Mugera and Munyua, 1973). After a further 24-48 hours nuclear division of the parasite occurs resulting in the appearance of typical multinucleate macroschizonts. *In vitro* studies have shown that the parasite then multiplies within the host by inducing synchronous division of parasite and host cell (Hulliger *et al.*, 1964). The precise mechanism by which the *Theileria* parasite induces synchronous division of itself and the host cell has not yet been elucidated, although observations made by Stagg *et al.* (1980) may help to explain this phenomenon. In this study electron micrographs were made of an eland monolayer cell line infected with *T. taurotragi*. The developing macroschizont appeared to be associated with the microtubules of the centriole during interphase. The macroschizont then orientates within the mitotic spindle, resulting in the passive and sometimes uneven distribution of the macroschizonts during the separation of the chromosome pairs and division of the host cell, leaving two infected daughter cells. Pulse labelling of infected cells *in vitro* with tritiated thymidine has indicated that DNA synthesis by the host cell and the parasite occurrs at different times during the host cell cycle (Irvin, Ocama and Spooner, 1982; Jura, Brown and Perry, 1985). This suggests that both *T. parva* and *T. annulata* regulate their own DNA synthesis independently of the host cell. Such an interpretation may help to explain the observation that when host cells are treated with colchicine (an inhibitor of cell mitotic division) the parasite continues to multiply within the "inhibited" host cell, resulting in a dramatic increase in the number of parasite nuclei per cell (Hulliger *et al.*, 1964).

The "optimal" culture conditions present *in vitro* presumably allow, or select for, a balance between host cell and parasite, with the number of macroschizont nuclei per host cell staying relatively constant at around 15-20. However, *in vivo* the average number of macroschizont nuclei per cell increases during the course of the disease, presumably due to stresses brought to bear on the host cell-parasite balance by toxic or other host factors which are absent *in vitro* (Jarrett, Crighton and Pirie, 1969; Barnett, 1977).

The control of lymphoproliferation by *Theileria* parasites is currently being examined at the molecular level. A recent discovery is that *T. annulata* parasite DNA contains sequences homologous to the "yes" oncogene (a retroviral transforming gene encoding a tyrosine kinase) which may activate the growth control pathway of the cell in the absence of an external stimulus (Dyer and Tait, 1987). However work with *T. parva* suggests that the *Theileria* infected cells are not classically "transformed", as the process can be reversed by removal of the parasite (Pinder *et al.*, 1981). Work with *T. parva* suggests an alternative mechanism, whereby *T. parva* infected T lymphocytes

produce a growth factor similar to IL2 (a T cell growth factor) which may contribute to the maintenance of their proliferative state (Brown and Logan, 1986).

1.1.5.3 *Schizont transfer*

The macroschizont is an obligatory intracytoplasmic stage of the *Theileria* parasite which is dependant on the host cell for its maintenance and survival. Extracellular macroschizonts have been observed in biopsy smears taken from infected animals, particularly in the later stages of the disease (Barnett, 1977). It is not known whether these extracellular schizonts have the capacity to invade and infect new cells. Uilenberg (1981b) suggested that transfer of schizont material to uninfected cells was likely to contribute to parasite multiplication within the host and that this was more likely to occur with *T. annulata* than *T. parva* infection. In a study reported by Nyindo *et al.* (1978) it was claimed that cattle inoculated with cell free schizonts and merozoites derived from *T. parva* infected cells *in vitro* were partially protected from a virulent sporozoite challenge. However there was no evidence from this study that the cell free schizonts were able to invade and infect host cells. It is possible that the schizont particles can only survive for a limited period in an extracellular environment. In the above study the authors claimed that the schizont material was viable having measured uptake of tritiated thymidine, but these results may have been due to a small proportion of contaminating viable macroschizont infected cells.

Evidence for *in vivo* schizont transfer comes from studies where animals were immunised using *T. parva* schizont infected cells (Wilde, 1967). It was found that the parasite established infection within the cells of the host; donor infected cells were distinguished from host infected cells by karyotypic analysis. Parasite transfer from the immunising cell line to host cells seems to occur at a much higher frequency with *T. annulata* cell lines (Pipano and Tsur, 1966) than with *T. parva* (Theiler, 1911; Walker and Whitworth, 1930; Brown, 1981). As infection of the host animal appears to be a prerequisite for the development of protective immunity with both *T. annulata* (Pipano *et al.*, 1977) and *T. parva* (Emery *et al.*, 1981a), the superior ability of *T. annulata* schizonts to transfer and infect host cells may explain why an infected cell line vaccine is effective in protecting cattle against *T. annulata* infection whereas the same approach is not so successful with *T. parva* infection. This is discussed more fully in section 1.1.12.

It is not known whether the infection of host cells is achieved by active invasion of macroschizonts, by phagocytosis, or by some form of cell fusion. Prior induction of anti macroschizont antibody has been implicated as a mechanism of blocking schizont transfer (Pipano *et al.*, 1977; Emery *et al.*, 1981a).

This area of study has received surprisingly little attention, but it illustrates one of the more striking differences between *T. annulata* and *T. parva*. If the mechanisms of schizont transfer from cell to cell are understood the implications for a practical tissue culture vaccine against East Coast fever (*T. parva* infection) may be very important.

1.1.5.4 *Nature of the target host cell for sporozoite infection and transformation*

The ability of both *T. parva* (Stagg *et al.*, 1983) and *T. annulata* (Steuber *et al.*, 1986) sporozoites to infect and transform leucocytes *in vitro* from a wide range of Bovidae and other mammals has been compared. It was found that *T. parva* sporozoites only attached to and infected leucocytes of cattle whereas *T. annulata* sporozoites were also able to infect cells of sheep and goats. The precise characterisation of the target cell for infection with either *T. annulata* or *T. parva* has been a matter of controversy for many years, research in this area being mainly hampered by a lack of suitable reagents with which to identify populations of potential target cells. Most of the work reported has involved *T. parva* although a few preliminary studies have been reported for *T. annulata*.

Work analysing the phenotypes of *T. parva* infected cells derived from infections both *in vivo* (Emery, 1981; Emery, MacHugh and Morrison, 1988) and *in vitro* (Duffus, Wagner and Preston, 1978; Pinder, Withey and Roelants, 1981) suggested that the cells were negative for the expression of both surface and cytoplasmic immunoglobulin (Ig). It was subsequently suggested that *T. parva* infected a distinct subpopulation of T cells, based on the observation that *T. parva* infected cell lines stained with the lectins soyabean agglutinin and peanut agglutinin, both of which bind resting T lymphocytes (Pinder, Withey and Roelants, 1981). A further report by Naessens *et al.*, (1985) described the expression of antigens on *T. parva* infected cells characteristic of a subpopulation of activated T cells.

More recently monoclonal antibodies (MAbs) have been produced which recognise specific subpopulations of bovine leucocytes (Lalor *et al.*, 1986). These have been used to identify the target cells infected *in vitro* by *T. parva*.

The results suggest that *T. parva* sporozoites infect phenotypically defined B cells and T cells, but not monocytes as defined by the monoclonal antibody P8 (Lalor, Morrison and Black, 1986). However, two separate studies have reported the infection (Fawcett and Stagg, 1986) and transformation (Moulton *et al.*, 1984) of adherent macrophage type cells by sporozoites of *T. parva*.

A more comprehensive study was recently carried out by Baldwin *et al.* (1988) using a wider range of phenotypic markers. Cell subpopulations were sorted

using cell phenotype specific monoclonal antibodies into positive and negative populations using a fluorescent activated cell sorter (FACS). These sorted cell subpopulations were then infected *in vitro* with *T. parva* sporozoites. The results confirmed the infection and transformation of B cells, T cells (both T4 and T8 subpopulations) and null cells, but not of monocytes or neutrophils. After transformation the appropriate infected cells retained expression of most of the T cell differentiation antigens but the transformed B cells lost expression of surface Ig. All the cells transformed by *T. parva*, regardless of their lineage, expressed mhc class II antigens as defined by the monoclonal antibody R1 (Lalor *et al.*, 1986). Both *T. parva* and *T. annulata* infected cell lines express mhc class I antigens the bovine leucocyte antigen (BoLA) specificities of which were unchanged from the normal lymphocytes from which they were derived (Spooner and Brown, 1980).

Little work has been done to evaluate the cell types that become infected *in vivo*. This is probably due to the logistical difficulties of simultaneously monitoring parasites and surface phenotype, and also to the unavailability of suitable monoclonals that define specific cell populations post-infection. Baldwin *et al.* (1988) observed that the majority of the *in vivo T. parva* transformed cells were of T cell origin. A subsequent study confirmed this result by showing that 99% of infected cells recovered from efferent lymphatic lymphocytes of infected cattle co-expressed the T lymphocyte markers BoT4 and BoT8 (Emery, MacHugh and Morrison, 1988). This co-expression of both T cell markers is normally characteristic of immature T cells (Reinherz and Schlossman, 1980).

In comparison to the work done on *T. parva* very little has been reported on the characterisation of *T. annulata* infected cells. Cells separated by rosetting techniques (Ahmed *et al.*, 1981a) or purified by nylon wool (Julius, Simpson and Herzenberg, 1973) were infected with *T. annulata* sporozoites. The results showed that it was possible to infect and transform cells from both the nylon adherent (B cells) and non adherent (T cells), and also from E rosetted cells (T cells) and EAC rosetted cells (B cells). It was concluded that *T. annulata* infected cell lines could be established from either T cells or B cells (Ahmed, Rehbein and Schein, 1984).

1.1.6 Pathology

Since much more is known concerning the pathology of East Coast fever (*T. parva* infection) this is often used as an example of the pathogenesis of *Theileria* infections in general. This may be misleading as there are distinct differences in the pathology of *T. annulata* and *T. parva* infections which may be pertinent in understanding the host-parasite relationship and designing methods of disease control.

Accounts of the pathology of *T. annulata* infection can be found in the reviews of Sergent *et al.* (1945), Neitz (1957), and more recently Gill, Bhattacharyulu and Kaur (1977), Uilenberg (1981b) and Ouhelli (1985). Reviews on the pathogenesis of *T. parva* infection are found in Cowdry and Danks (1933), Wilde (1967), Barnett (1977), and Irvin and Morrison (1987).

The severity of the disease depends on many factors including host susceptibility, parasite virulence and the immunological response of the host. The following is a generalised account of the principle features in the pathogenesis of *T. annulata* infection. Comparison with *T. parva* infection illustrates some of the differences between the two diseases.

The initial symptoms of infection are hyperplasia of the local lymph node. Prior to the detection of parasitosis (within the node) there is rapid transformation of resting lymphocytes to lymphoblasts. This hyperplasia spreads to other lymph nodes (in *T. parva* infection) and secondary lymphoid tissue again preceding the detection of macroschizonts in any of these tissues. An important difference between *T. annulata* and *T. parva* at this stage is the development of leukopenia in *T. parva* infection (Wilde, 1967). This usually occurs approximately 4-5 days after the initial detection of intracellular parasites, when infection levels of 5-10% are recorded in various lymph nodes. The parasitised leucocytes increase but the total cellularity of the node decreases to below resting levels (Morrison *et al.*, 1981; Emery, 1981). The mechanisms involved in the panleukopenia observed in East Coast fever are as yet unresolved. Autoimmune mechanisms have been suggested, including antibody responses (Wilde, 1967) and the generation of promiscuous cytotoxic cells capable of killing a range of both infected and uninfected target cells (Emery *et al.*, 1981b). These cytotoxic cells bear a strong resemblance to the lymphokine-activated killer (LAK) cells reported in immune responses induced against various tumours (Grimm *et al.*, 1982; Teh and Yu, 1983). Indeed *T. parva* infected cells *in vitro* have been shown to produce a growth factor similar to interleukin 2 (IL2) (Brown and Logan, 1986).

In a recent study cloned cytotoxic T cells were infected *in vitro* with *T. parva* (Baldwin and Teale, 1987). After infection the cloned T cells retained their cytotoxic function and specificity for a limited time but later became promiscuous and functionally nonspecific. These mechanisms may play a contributory role in the characteristic lymphocytosis of East Coast fever.

This panleukopenia is not so prominent in *T. annulata* infection, where the number of circulating lymphocytes actually increases during the acute phase of infection (Sergent *et al.*, 1945; Barnett, 1977). The reasons for this difference between *T. parva* and *T. annulata* are unknown, but characterisation of the blasting lymphocytes in *T.*

annulata infection may provide some clues. It is tempting to speculate that the difference might be due to the two parasites preferentially infecting different host cells.

Attention should also be paid to the method used to induce infection when studying pathogenesis. In the studies reported for *T. annulata* animals were infected by mechanical transmission using infected blood or leucocytes, whereas with *T. parva* sporozoites were used. It is possible that these differences in the mode of infection may contribute to the different pathological manifestations observed.

In *T. annulata* infection both the intracellular schizont stage and the intraerythrocytic piroplasm contribute to the pathogenesis (Uilenberg, 1981b), whereas with *T. parva* the main pathogenic stage appears to involve the intracellular schizont. Severe anaemia is a common symptom of *T. annulata* infection, with a reduction of erythrocyte numbers occurring during parasitaemic crisis (Sergent *et al.*, 1945; Neitz, 1957). The cause of this anaemia is not well understood. It has been speculated that infected erythrocytes are removed by phagocytosis rather than destroyed by the parasite itself (Neitz, 1957; Uilenberg, 1981b). Splenectomy tends to exacerbate symptoms or cause a resurgence of parasitaemia in *T. annulata* infection (Hooshmand-Rad, 1976) whereas the same treatment has no obvious effect in *T. parva* infection (Barnett, 1968).

Autohaemagglutinating antibodies have been implicated in the production of the anaemic state (Hooshmand-Rad, 1976). However the events leading to the induction of these autohaemagglutinin antibodies are not known. It is interesting to note that anaemia was still induced in animals that had been immunised with strains of *T. annulata* that had lost the ability to produce piroplasms (Pipano and Tsur, 1966) and that while parasitaemia levels in *T. parva* infection can be very high, anaemia is not a common symptom in East Coast fever (Wilde, 1967). This does suggest that there may be factors contributing to the anaemia other than intraerythrocytic piroplasms. It has been observed that mice infected with *P. vinkei* malaria are very sensitive to Tumour Necrosis Factor (TNF), inoculation of which exacerbates symptoms of malaria and induces pathological changes characteristic of the terminal stages of the disease. Such pathological changes include erythrocytosis and dyserythropoeisis (Clark, 1987). TNF is produced by activated macrophages (Old, 1988) which are likely to be present in *T. annulata* infection (Preston, 1981), so it is possible that TNF may contribute not only to the induction of anaemia but also to the characteristic weight loss in *T. annulata* infected animals (Uilenberg, 1981b).

1.1.6.1 *Clinical symptoms*

Similar clinical symptoms are recorded in both *T. annulata* and *T. parva* infection. Death usually occurs between one and two weeks after the onset of clinical signs, although in very acute cases infected animals may die sooner. The mortality rate in susceptible adult cattle is between 40-60% with *T. annulata* infection (Uilenberg, 1981b) and 85-90% with *T. parva* (Brocklesby *et al.*, 1961). The first symptoms include high fever and swelling of regional and superficial lymph glands. As the disease progresses general symptoms include listlessness, accelerated respiration rate, swollen eyelids and lachrymation. Diarrhoea and loss of appetite are common (Uilenberg, 1981b).

At *post mortem* typical lesions in East Coast fever include abundant frothy fluid in the bronchi and trachea due to massive pulmonary oedema (usually the cause of death) (Irvin and Morrison, 1987). This lesion is also seen in *T. annulata* infection although anaemia is thought to be the main cause of death (Barnett, 1977). In both diseases haemorrhages are common on many organs including the heart, kidney, peritoneum and the gastro- intestinal tract, where ulceration often occurs. In *T. annulata* infection the liver and spleen are often enlarged.

It is believed that recovery from *T. annulata* results in the induction of a lifelong carrier state, whereas with *T. parva* it is thought that the existence of a carrier state is less common. This point remains controversial (Young, Leitch and Newson, 1981), especially when examining field cases of a subsequent resurgence of infection which may in fact be a result of waning immunity followed by reinfection.

1.1.7 Immunology

The main problem areas in designing effective vaccines are:

1) the identification of relevant antigens;

2) identification of relevant immune responses and methods to induce and maintain this responsivenes;

3) the identification of individuals likely to respond suboptimally to the vaccine.

In any host-parasite relationship it is unlikely that any one anti-parasite effector mechanism operates in isolation to effect host resistance. The net effect of any one component of the spectrum of immune responses induced by the various parasite life cycle stages will either be host protective, parasite protective, irrelevant or harmful to the host. The immune responses induced by a vaccine need not necessarily cover the whole spectrum of potential immune responses; the effects of a few "key" responses

may tip the balance in favour of the host. Identification of important immune responses is therefore essential for the identification of relevant antigens to use as candidate vaccines.

The *Theileria* parasites are particularly interesting because they infect and live within the cells of the immune system. Vaccination is however a real possibility with this disease since animals which do recover from primary infection become immune to subsequent homologous challenge.

The following section reviews the current state of knowledge concerning immune responses involved in *T. annulata* infection at each stage of the parasite life cycle. Comparison is made with the more extensively studied species *T. parva* and some other intracellular pathogens. The immunology of *Theileria* infections has recently been reviewed by Neitz (1957), Pipano (1974), and Irvin and Morrison (1987).

The main pathogenic stages in *T. annulata* infection appear to be the macroschizont infected cell and the piroplasm infected erythrocyte, although the former is considered to be the more important.

1.1.8 Immune response to the tick

The normal host response to tick bite injury involves a local inflammatory reaction, the extent and complexity of which depends on the species of tick and on whether or not the host is sensitised (Allen, 1973). A study of the cellular responses induced by *Hyalomma anatolicum anatolicum* (a tick vector for *T. annulata* infection) was recently reported by Gill and Walker (1985). Neutrophils, mononuclear cells, eosinophils and basophils were present, the main effectors of resistance to the tick being mediators released by mast cells, basophils and eosinophils. However, as regards *T. annulata* infection, it is not known whether or not this inflammatory response is beneficial or detrimental to the host in that it facilitates or hinders establishment of the parasite in its target host cell. A recent article in *The Guardian* newspaper (4/10/1987) reported that scientists in Australia have identified and characterised a tick gut protein which elicits deterrent antibodies in cattle. The gene producing this protein has been cloned and there is optimism that an anti-tick vaccine may be produced in the near future (Lackie, 1988).

1.1.9 Immune response to the sporozoite

It is known that immune sera from mice reduce the infectivity of *P. berghei* sporozoites although passive immunisation has failed to establish a protective role for humoral in malaria. Specific antibodies produced by immunised mice react *in vitro* with

the malaria sporozoite membrane, as shown by the circumsporozoite precipitation (CSP) reaction and by indirect immunofluorescence (Cochrane *et al.*, 1976).

A similar approach has been used to examine immunity to the *Theileria* sporozoite. Although the invasive sporozoite stage of the parasite is only exposed to the host immune system for a very short time (Brown *et al.*, 1978a) it is possible to generate *in vivo* immune serum that inhibits the penetration of sporozoites into uninfected host leukocytes *in vitro* (Gray and Brown, 1981; Preston and Brown, 1985; Ahmed *et al.*, 1988).

The "humoral factor" found in the serum of calves recently recovered from *T. annulata* infection was equally effective against different geographical isolates of *T. annulata* (Gray and Brown, 1981). However, antisera raised in *T. parva* infected animals did not neutralise the infectivity of *T. annulata* sporozoites *in vitro*, implying that the serum factor is parasite species specific. This humoral factor was investigated further by Preston and Brown (1985). A dual functional role was proposed. The serum could inhibit the actual invasion of the sporozoite into the target cell and/or suppress the intracellular development to macroschizont of the trophozoite stage of the parasite, depending on whether the serum was derived from animals recovered from primary or multiple challenge infections. Serum from the multiple challenge group was more likely to inhibit sporozoite invasion.

In field challenge situations, where the animals are presumably constantly exposed to infection, immunity against the sporozoite may play a protective role by reducing the number of host cells that become infected.

Evidence from electron microscopic observations on *T. parva* infection of host cells shows that the sporozoite surface coat is left on the outside of the cell after sporozoite entry (Webster, Dobbelaere and Fawcett, 1985). It is not known how long these sporozoite surface coat antigens remain on the host cell surface, but they may provide an important source of antigen with which to prime the immune response or alternatively act as targets for immune attack. Preliminary investigations into the identification of the protective antigens on the surface of *T. annulata* sporozoites have recently been reported (Williamson, 1986). Two surface staining monoclonal antibodies (MAbs) were found to neutralise 75-92% of sporozoite infectivity for normal peripheral blood lymphocytes (PBL) *in vitro*. The gene coding for one of these proteins has been identified and the resultant recombinant protein has been used to immunise cattle, where it was found to induce an antibody response which would neutralise sporozoite infection of lymphocytes *in vitro* (S. Williamson, personal communication). This antigen is therefore a strong candidate for a sporozoite vaccine.

Similar sporozoite neutralizing humoral immune responses have been recorded for *T. parva* from sera derived from repeated infections *in vivo* (Musoke *et al.*, 1984) and by monoclonal antibodies (Dobbelaere *et al.*, 1984). As is the case with *T. annulata* the neutralising factor appears to be mediated by antibodies to determinants common to different *T. parva* sporozoite stocks.

However, any immune response generated against the sporozoite stage of the parasite would have to be extremely efficient to completely prevent infection of host cells. The macroschizont stage of the parasite is believed not to be susceptible to attack by anti-sporozoite antibody (I. Morrison, personal communication). A more realistic role for the anti-sporozoite response is to reduce the initial level of infection sufficiently to allow the host immune system to control the subsequent infection.

1.1.10 Immune response to the macroschizont infected cell

The main pathogenic stage in both *T. annulata* and *T. parva* infection involves the macroschizont infected cell. The parasite, by choosing an intracellular location, resembles a biological Trojan horse and is effectively protected from the humoral arm of the immune response. Cell mediated immune mechanisms are more likely to be effective against this stage, as is the case with other intracellular pathogens (Mims, 1977). During intracellular replication of the pathogen various antigens derived from the pathogen appear on the surface of the infected host cell which can be recognised by T lymphocytes. The action of the T lymphocytes may be categorised into "direct" and "indirect" effector mechanisms. Direct effector mechanisms involve cytolysis of the infected cell by the appropriate T cell as occurs in many viral infections (Townsend and McMichael, 1985). Indirect effector mechanisms involve the activation of T cells to produce various lymphokines which in turn activate other cells of the immune system (macrophages) known to be effective against various intracellular parasites such as *Mycobacterium tuberculosis*, *M. Leprae* (Hahn and Kaufmann, I98l) and the *Leishmania* species (Preston, 1987).

Various cell mediated effector mechanisms directed against or induced by the macroschizont infected cell have been reported in *Theileria* infections (Irvin and Morrison, 1987).

The study of cell mediated, or indeed any other, immune responses against the intracellular stage of the *Theileria* parasite has been greatly facilitated by the development of techniques which allow the *in vitro* infection of normal bovine PBL with sporozoites (Brown *et al.*, I973). Autologous cells taken from animals prior to *in vivo* infection can be infected *in vitro* and used as targets in *in vitro* assays which measure immune responses in animals undergoing various regimens of infection or immunisation.

1.1.10.1 *Cell proliferation responses*

The first meaningful report that cell mediated immune mechanisms could be generated against macroschizont infected cells was made by Pearson *et al.* (1979) for *T. parva*. In this study it was demonstrated that irradiated *T. parva* infected cells could induce proliferative responses in autologous peripheral blood mononuclear cells (PBM) irrespective of the immune status of the responder animal. However the generation of cytolytic cells was only achieved with cultures using immune responder PBM. This suggested that as a consequence of parasite infection "new" antigenic determinants on the surface of the infected cells were capable of inducing cell-mediated immunity. A similar transformation of bovine lymphocytes in culture with autologous *T. annulata* infected cell lines has been reported (Preston and Brown, 1981). This co-culture of PBM with irradiated autologous *Theileria* infected cells and its resultant proliferative response is known as the bovine autologous *Theileria* mixed leucocyte reaction (MLR) (Goddeeris and Morrison, 1987). The magnitude of the proliferation is dependent on the presence of monocytes in the responder population and on the phenotype of the parasitised stimulator cell. It is likely, as is the case in the autologous MLR described in man (Opelz *et al.*, 1975), that some of the proliferation will be induced by mhc class II antigens. *T. parva* infected cell lines are known to express high levels of class II molecules (Lalor, Morrison and Black, 1986) so the proliferative response observed may not be directly related to the presence of the parasite within the stimulator cell but rather to the amount of class II antigen present (Unanue, 1984).

In a subsequent study by Emery and Morrison (1980) it was demonstrated that proliferation would occur if an autologous *Theileria* MLR was set up using cryopreserved PBM (collected prior to infection) and *T. parva* infected cells obtained *in vivo*. The authors put this forward as evidence that the proliferative response was not merely directed against antigens acquired by the infected cells in culture (*c.f.* Thomas and Edwards, 1973).

An important question arising from these observations is: how relevant is the autologous *Theileria* MLR in the induction of a parasite-specific immune response *in vivo*?

Pearson *et al.* (1979; 1982) demonstrated that cytotoxic cells could be generated *in vitro* using PBM from immune animals in an autologous *Theileria* MLR. These cytotoxic cells would preferentially lyse the autologous *T. parva* infected cell targets but would also lyse both allogeneic infected target cells and uninfected target cells. This indicates that cytotoxic cells generated in this sytem were not necessarily parasite specific or genetically restricted.

1.1.10.2 *In vivo generation of cytotoxic cells*

The induction of cytotoxic cells *in vivo* has been investigated in cattle undergoing various immunisation regimes with *T. parva*. The effector function of PBM from these animals was examined directly on various target cells without additional stimulation *in vitro* in a *Theileria* autologous MLR (Eugui and Emery, 1981; Emery *et al.*, 1981b).

Animals immunised by infection with *T. parva* sporozoites and treated with tetracycline developed cytotoxic cells at the time of remission of infection, which would only lyse the autologous *Theileria* infected cell line, whereas animals undergoing a lethal infection with *T. parva* developed more promiscuous cytotoxic cells which would lyse both allogeneic infected cell lines and a mouse tumour cell line (YAK) with known susceptibility to lysis by murine Natural Killer (NK) cells. On further challenges to recovered animals the cytotoxic response was always directed against the autologous infected target cell, and the effector cell in this response was in the T lymphocyte population (Emery, Tenywa and Jack, 1981).

The first study on cytotoxic responses in animals infected with *T. annulata* sporozoites was reported by Preston, Brown and Spooner (1983). Recovery from infection was accompanied by the appearance of cytotoxic cells which were not manifest in animals undergoing a fatal reaction. During recovery from primary infection two peaks of cytotoxicity were observed. The first appeared to be BoLA (bovine mhc) restricted; the second was not genetically restricted. After challenge the majority of the cytotoxic cells were directed against the autologous *T. annulata* infected cell line.

The results of these initial studies in both *T. parva* and *T. annulata* infection, although not directly comparable because of different experimental procedures, suggest a role for genetically restricted cytotoxic cells in protective immunity. Cytotoxic cells recognise some form of parasite induced antigenic changes on the surface of the infected cell in conjunction with self mhc antigens. Such mhc restricted cytotoxic cells have been well documented in the recognition of virally infected cells (Zinkernagel and Doherty, 1979), and have been reported in connection with other intracellular pathogens such as *Listeria monocytogenes* (Kaufmann, Hug and De Libero, 1986), *Mycobacterium tuberculosis* and *M. bovis* (Kaufmann, 1988), *M. leprae* (Chiplunkar, De Libero and Kaufmann, 1986), *Rickettsia typhi* (Rollwagen, Dasch and Jerrells, 1986), and malaria (Schofield *et al.*, 1987).

These results also stress the necessity for the establishment of the parasite in host cells before the induction of specific immune responses which recognise the correct host mhc determinants in association with parasite induced antigens on the surface of the infected cell.

Recent work with *T. parva* has provided convincing evidence that bovine mhc class I antigens act as restricting elements in the recognition of parasite cells by cytotoxic T cells (Morrison *et al.*, 1986b). In this report cytotoxic cells generated in immune cattle following challenge with sporozoites were assayed on panels of parasitised target cells. A close correlation was found between target cell susceptibility to lysis and the sharing of bovine mhc class I specificities with the effector CTL: target cells that were mhc class I matched or half matched to the effector CTL were lysed whilst those that were not mhc matched were left intact. The cytotoxic response could be inhibited by monoclonal antibodies (MAbs) to class I mhc molecules and to a limited extent by MAb to the T cell subset marker BoT8 (Ellis *et al.*, 1986), implying that at least part of the cytotoxic response was mediated by BoT8 lymphocytes. BoT8 is thought to be the bovine equivalent of CD8 in man (Ellis *et al.*, 1986).

1.1.10.3 *In vitro generation of cytotoxic cells*

As genetically restricted cytotoxic cells are only detected transiently *in vivo* considerable effort has gone into exploiting the autologous *Theileria* MLR to provide an *in vitro* assay to mimic the *in vivo* response and allow a more detailed study of its induction.

Several different studies have provided contradictory and confusing evidence regarding the specificity of CTL generated *in vitro*. The main controversy concerns the use of PBM from immune or naive cattle as responders and the subsequent specificity of the cytotoxic cells generated.

In the initial studies by Pearson *et al.* (1979; 1982), cytotoxic cells were generated *in vitro* using PBM from immune but not naive animals. These CTL preferentially killed autologous infected cell lines but were also cytocidal for both allogeneic infected and uninfected target cells.

Eugui and Emery (1981) reported that low levels of autologous restricted cytotoxic cells could be generated from immune cattle by restimulation *in vitro* only if the PBM were taken between 7 and 24 days after challenge. In a more comprehensive study Emery and Kar (1983) found that cytyotoxic cells generated *in vitro* in the autologous *Theileria* MLR using PBM from either immune or naive cattle would kill a range of infected and uninfected target cells, exhibiting no genetic restriction. Cytotoxic cells generated *in vivo* would lyse only the autologous *Theileria* infected cells. Futher comparison of cytotoxic cells generated *in vitro* and *in vivo* revealed that the *in vitro* generated cytotoxic T cells were composed of a heterogenous, polyspecific population whose lytic activity was not inhibited by pretreatment of target cells with either tunicamycin or 2-deoxy D-glucose (inhibitors of carbohydrate synthesis), whereas the

in vivo generated CTL were not able to kill target cells pretreated with the above compounds, suggesting that the two populations of cytotoxic cells were recognising different target antigens.

The controversy surrounding cytotoxic cells generated *in vitro* relates to a number of different factors concerning the source of both responder and stimulator cells in the autologous *Theileria* MLR. The autologous infected cell lines used as stimulators are comprised of heterogeneous cell populations and variation between the ability of phenotypically different cell lines to stimulate proliferation *in vitro* and to induce cytotoxic cells has been observed (Goddeeris, Lalor and Morrison, 1986; Lalor, Morrison and Black, 1986). The most pertinent phenotypic difference is likely to be the amount of mhc class II expression on the stimulator cell line, as mentioned previously. Other contributory factors may include differences in the methods used to immunise animals and in the timing of the collection of responder cells following immunisation. It appears that there is both a parasite specific and a nonspecific component generated in the autologous *Theileria* MLR response. In general there is wide variation between individuals as to the level and specificity of the CTL generated and, unlike the effectors generated from naive animals, those from immune animals are restricted to the autologous *Theileria* infected target (Goddeeris, Lalor and Morrison, 1986).

The method of immunisation may also be pertinent to the generation of specific cytotoxic cells *in vitro*. Current evidence suggests that in contrast to animals immunized with sporozoites those immunised with autologous infected cultured cells may also make a response to some form of culture acquired antigen (Morrison *et al.*, 1986a). Therefore although responder PBM from these cell culture immunised animals appears to elicit autologous restricted cytotoxicity, like that observed in the sporozoite immunised animals, the former effector cells may in fact be recognising culture antigen (in association with self mhc) while the latter recognise parasite antigen (in association with self mhc). This may explain the report by Morrison *et al.* (1986a) that cattle immunised with irradiated (and therefore non-infective) autologous macroschizont infected cells, in which mhc restricted cytotoxic responses were generated against the autologous infected cell *in vitro*, were not immune to a subsequent challenge. In the studies described by Pearson *et al.* (1979; 1982), in which "non specific" cytotoxicity was observed, most of their results were based on the responses of one animal which had been inoculated several times with autologous infected cells.

However, despite some of the problems outlined above concerning the specificity of cytotoxic cells generated *in vitro*, recent work has demonstrated that by repeated stimulation of PBL from *T. parva* sporozoite infected cattle in the autologous *Theileria* MLR it is possible to enrich for the parasite specific component of this

response. Using this technique T cell lines and clones have been produced that are specific for parasite infected target cells, are mhc class I restricted, and are also parasite strain specific (Goddeeris, Morrison and Teale, 1986; Goddeeris *et al.*, 1986).

Although most of the work described above concerns *T. parva* it does seem evident that infection of host cells with other *Theileria* parasites results in the induction of antigenic changes on the surface of infected cells that can be recognized by immune T cells. Perhaps not surprisingly, attempts to identify such parasite specific antigens using immune sera have proved unsuccessful (Creemers, 1982). Some success has been achieved by raising MAbs specific for the surface of both *T.p. lawrencei* infected cells (Newson *et al.*, 1986) and *T. annulata* infected cells (Shiels *et al.*, 1986a) but it is not yet known whether the molecules recognized by these MAbs are the same molecules recognised by the CTL. In studies with viral antigens it was not possible to block cytotoxic responses using virus specific antisera, although antibodies specific for the class I restricting molecules could block lysis very effectively (Sherman, Vitello and Klinman, 1983). An antibody may fail to block because it recognises a determinant on the antigenic molecule which differs from that recognised by the T cell. This discrepancy between the specificities of the T cell receptor and the antibody is a reflection of the fact that the two are different genetic and functional systems, which recognise antigen in different ways (Klein, 1986).

1.1.10.4 *Other cell mediated immune responses*

Several investigators have examined the possible role of delayed type hypersensitivity (DTH) responses by measuring *in vitro* the effect of various lymphokines produced by sensitised T cells. Lymphokines have several functions but their main purpose is to activate macrophages, attracting them to the site of antigen challenge to amplify the local response. These responses may be collectively termed the "indirect" T cell effector mechanisms as mentioned earlier.

Studies reported for *T. annulata* (Singh, Jagdish and Gautam, 1977; Rehbein *et al.*, 1981; Ahmed *et al.*, 1981) and *T. parva* infection (Muhammed, Wagner and Lauerman, 1974) emphasise the presence of leucocyte migration inhibition factor (MIF) when using PBM from immunised animals incubated *in vitro* with either schizont or piroplasm antigen. The protective role of MIF in *Theileria* infections has not yet been clarified. This response may be used to indicate the presence of sensitised T lymphocytes in immune or infected animals.

In a further study both adherent and nonadherent cells taken from *T. annulata* infected calves during recovery and convalescence were found to inhibit the growth of both autologous and allogeneic *T. annulata* transformed cell lines *in vitro*.

Adherent cells from *T. annulata* recovered calves were also found to have an immunosuppressive effect on the proliferation of primed lymphocytes in a *Theileria* autologous MLR *in vitro* (Preston, 1981).

A similar suppressive effect on proliferative responses in the *Theileria* autologous MLR was also reported for *T. parva* (Goddeeris and Morrison, I987) using adherent cells taken from animals I-2 months after immunisation.

It seems likely that adherent cells (presumably macrophages) will play some form of immunoregulatory role during *Theileria* infection, either immunopotentiating or immunosuppressive. Further studies are needed to elucidate the induction of these responses *in vivo*, and their potential host protective or host damaging roles.

1.1.10.5 *Humoral responses*

This review has concentrated on cell mediated immune responses against the macroschizont infected cell, as these are likely to be the most important. However, several studies have examined antibody responses to this stage of the parasite.

Circulating antibody has been detected, against macroschizont antigen, in cattle infected with *T. annulata* using complement fixation, haemagglutination and immunofluorescence techniques. These reports are reviewed by Pipano (1974). In most cases the antibody is detected during the acute stages of the disease, with peak titres normally observed after recovery. Antibody may persist for several months in animals, although the levels will drop if reinfection does not occur. The protective role of this response, if any, has not been resolved and it has not been possible to correlate serological titres to the degree of protective immunity (Pipano, 1981).

In most of the studies reported the results are difficult to interpret and to compare due to differences in the immunisation procedures used and to the assumed heterogeneity of test antigens, although the antibody produced in infected animals appears consistently to recognise the schizont antigen *per se* (within the cell) and not any antigen on the surface of the infected cell. In a recent study by Ahmed *et al.* (1988) it was demonstrated that serum taken from cattle immune to *T. annulata* was not opsonic for parasite infected cells and would not lyse such cells in the presence of complement. A similar finding was reported by Duffus, Wagner and Preston (1978) demonstrating that *T. parva* immune serum would not lyse infected cells in an antibody dependent cell cytotoxicity assay (ADCC).

However, as mentioned previously, monoclonal antibodies (MAbs) have been prepared which recognise some form of parasite induced antigen on the surface of *T. annulata* infected cells (Shiels *et al.*, 1986a). One of these MAbs (4H5) was able to lyse infected cells in the presence of complement (Preston *et al.*, 1986). Therefore it

would appear that this parasite induced cell surface molecule, although not serologically immunodominant in the cow, can be recognised when introduced into the "abnormal" mouse host.

There is no compelling evidence that the production of antimacroschizont antibody has any protective role in either *T. annulata* (Pipano, 1977) or *T. parva* infection (Wagner *et al.*, 1975; Muhammed, Lauerman and Johnson, 1975), although it can provide a useful diagnostic aid.

A possible role for this antibody might be to opsonise schizont particles released from infected cells thereby rendering the parasite schizonts susceptible to attack by phagocytes or complement. It is conceivable that this antimacroschizont antibody may prevent further infection of other cells by the released schizont.

1.1.11 Immune response to the merozoite and piroplasm

As the erythrocytic stage of *Theileria* occurs relatively late in the course of the disease it is generally considered of limited importance in the induction of protective immunity, and hence there is very little published work in this area. However, immunity induced against this stage may be important in blocking transmission of infection from cow to tick. Workers in malaria have attempted to identify target antigens on gametes of *Plasmodium falciparum* in order to induce transmission blocking immunity (Carter and Chen, 1976; Rener *et al.*, 1983).

Antibody responses to piroplasm can be detected in cattle recovering from infection or undergoing immunisation with either *T. annulata* (Pipano, 1974) or *T. parva* (Burridge and Kimber, 1973b).

Ahmed *et al.* (1988), using a chemiluminescence technique, showed that immune serum from *T. annulata* infected animals would react with free merozoites (produced by lysis of infected erythrocytes) but not with the surface of infected erythrocytes. Monoclonal antibodies to *T. annulata* piroplasm antigens have recently been raised, and it is found that they cross- react with free merozoites (J. Glascodine, personal communication).

In summary therefore it would appear that the humoral immune response is effective in eliminating the extracellular stages of *Theileria* parasites within the bovine host (sporozoite, extracellular schizont? and merozoite), whereas cell mediated immune mechanisms, in particular activated T cells, are effective against the intracellular macroschizont stage.

1.1.12 Immunoprophylaxis

It has been known for many years that cattle which recover from both *T. annulata* and *T. parva* infection become immune to subsequent challenge. Early work with *T. annulata* demonstrated a method for immunising cattle using blood taken from cows infected with mild strains isolated from field cases of tropical theileriosis and maintained in the laboratory by serial passage through calves. Defibrinated or citrated blood was collected at a time when schizonts were detected in the lymph nodes of the donor animal, and a subcutaneously inoculated dose of 5-10mls was commonly used (Sergent *et al.*, 1924). A mortality rate of 0.6-3% resulting from this method of immunisation was reported by various authors (Adler and Ellenbogen, 1935; Sergent *et al.*, 1945; Tsur, 1949). Early work with *T. parva* demonstrated that East Coast fever could be transmitted to cattle by the injection of emulsified spleen or lymphoid tissues from infected cows (Meyer, 1909). Although the reactions of the recipients were very variable, animals which did recover were solidly immune to subsequent challenge (Theiler, 1911; Spreull, 1914).

Various criteria presumed to be involved in the induction of immunity may be deduced from the early work with *T. annulata* and *T. parva*. Protective immunity could only be effectively induced if live, and therefore potentially lethal, parasite material was used. The strength and duration of immunity did not appear to depend on the severity of the initial reaction or the continued presence of detectable parasites. However, as is often the case when using live material for vaccination purposes, the main difficulty is to control the level of infection such that immunity is induced but severe or fatal reactions are prevented.

Currently two methods of vaccination effective against theileriosis are applied. One involves live sporozoites inoculation with concurrent chemotherapy; the other utilises macroschizont infected cell lines. Interestingly, although the inoculation of macroschizont infected cells is the favoured and successful method of vaccination against *T. annulata* it is not so effective in prophylaxis against *T. parva* infection, which relies on sporozoite infection and treatment. This dichotomy between the two diseases is one of the fundamental differences between *T. annulata* and *T. parva*.

As vaccination was appreciably more difficult to achieve with *T. parva* (Brown, 1981), intensive effort was employed to understand the mechanisms by which induction of protective immunity could be achieved by vaccination. In the majority of cases *T. parva* was studied in isolation without comparison to *T. annulata*, where the main emphasis of the work was on the development and field testing of the macroschizont cell line vaccine. Presumably because *T. annulata* vaccine was relatively

successful the impetus to understand the mechanisms involved in the induction of immunity was not immediate.

It may be informative to compare studies between *T. parva* and *T. annulata* to identify any differences between the two which may be exploited to improve vaccination against *T. parva*.

1.1.12.1 *Sporozoite infection and chemotherapy*

This method, known as the "infection and treatment" method, is more commonly used to control *T. parva*. The development and current "state of the art" of this method can be found in the following reviews: Brocklesby and Bailey (1965); Cunningham (1977); Radley (1981); and Brown (1985). A similar technique has been used to immunise against *T. annulata* infection (Gill, Bhattacharyulu and Kaur, 1976; Jagdish *et al.*, 1979; Pipano, 1981).

In general terms the current infection and treatment method involves injection of a stabilate of *Theileria* sporozoites together with a long acting oxytetracycline. The sporozoite stabilate material is prepared by grinding up infected ticks, the resultant sporozoite suspension remaining infective even after storage in liquid nitrogen for several years (Cunningham *et al.*, 1973). Using this technique it is possible to quantify sporozoite doses and to obtain more reproducible disease reactions (in similar cattle given the same dose of stabilate) than was previously possible using unpredictably infected ticks.

When sporozoites and long acting oxytetracycline are inoculated simultaneously the effects of the drug persist for approximately 5 days, which is sufficient to contain the infection and allow the development of immunity (Radley, 1981). The way in which tetracyclines exert their effect is not yet clear. They are mildly parasiticidal (Brown *et al.*, 1977) compared with drugs such as parvaquone (an analogue of menoctone, a quinone derivative). If menoctone is administered during the preclinical stages of the disease it is effective in killing the *Theileria* parasites but animals treated in this manner may be subsequently susceptible to challenge, presumably because there was insufficient time for the induction of protective immunity before the parasite was destroyed (Dolan and McHardy, 1978). However, if parvaquone is administered during the early clinical stages of the infection it is effective in controlling the disease and the animal subsequently becomes immune to further challenge (Dolan *et al.*, 1984a).

The action of oxytetracycline appears to be directed against the proliferation or expansion of the parasite infected cells. Tetracyclines have been shown to inhibit the mitogen induced proliferation of human lymphocytes, probably by inhibiting protein

synthesis (Thong and Ferrante, 1979), so the drug may simply "slow down" the expansion of the parasitised cells sufficiently to allow the immune response to develop.

This method has been used with considerable success on an experimental basis to immunise cattle against *T. parva* infection (Cunningham, 1977), but before it can be applied in any large-scale field trial a few limitations have to be taken into consideration. Some parasite stocks require higher levels of tetracycline in order to control the infection than others (Radley, 1981). A further problem is the immunological heterogeneity of parasite stocks: immunisation against one stock of *T. parva* may not necessarily provide protection against others. Immunisation with a single *T. parva* stock or a mixture of three stocks (the Muguga cocktail) did not protect very well against a heterologous *T.p. lawrencei* challenge (Radley *et al.*, 1975). In a more recent study (Irvin *et al.*, 1983), *T. parva* stocks characterised by antimacroschizont MAbs were used to immunise and challenge animals. There appeared to be a good correlation between cross resistance *in vivo* and parasite differences detected by the MAbs *in vitro*. When cattle were immunised and subsequently challenged with parasite stocks of differing MAb profiles less comprehensive cross protection was observed.

This does not seem to be such a problem with *T. annulata*, where parasite stocks isolated from different geographical areas show reasonably good cross protection (Gill *et al.*, 1980). However, in many of the *T. annulata* studies the parasite stocks were poorly characterised or quantified, making the situation difficult to interpret.

Recent work has examined the potential application of monoclonal antibodies raised against both *T. parva* (Pinder and Hewett, 1980; Minami *et al.*, 1983) and *T. annulata* schizonts (Shiels *et al.*, 1986b) in parasite strain identification. However, MAb profiles do not necessarily identify the antigens involved in protective immunity; they merely define sets of distinct antigenic determinants expressed on schizonts of different parasite stocks. A more relevant method, as regards predicting immunity and cross protection *in vivo* between different parasite stocks, may be the development of an *in vitro* cytotoxicity assay for parasite strain characterisation. The work of Goddeeris *et al.* (1986), in which *T. parva* specific T cell clones were developed which were both mhc class I restricted and parasite strain restricted, shows that this approach may be feasible at some time in the future.

The area of characterisation of species and strains of *Theileria* parasites has recently been reviewed by Irvin (1987).

1.1.12.2 *Macroschizont cell line immunisation*

The most widely used method of immunoprophylaxis in *T. annulata* infection involves immunisation of susceptible cattle with macroschizont infected cell lines.

Development of this vaccine is largely due to the pioneering work of a team of researchers in Israel and has recently been reviewed by Pipano (1977; 1981).

Up until the 1960's reasonable success had been achieved by inoculation of blood taken from a *T. annulata* infected animal at a time when schizonts were detectable. The "Kouba" (Donatien and Lestoquard, 1938) and other stocks of low virulence were used extensively to immunise cattle, particularly young animals in both North Africa and Israel. These mild stocks, which were analogous to a "natural" vaccine, were maintained by serial passages in cattle. A problem with this type of vaccine was that the infected blood was often derived from different animals at different passage levels and therefore was often of indeterminant virulence. There was also the danger of transmitting other parasitic protozoa such as *Babesia* with the infected blood (Sergent *et al.*, 1945).

Following the original report of the *in vitro* culture of *T. annulata* infected cells by Tsur (1945), further studies followed which described larger scale cultivation of macroschizont infected cells in monolayers (Tsur and Adler, 1962) and in suspension (Hooshmand-Rad, 1975). The use of such infected cell lines as potential vaccines was explored. *T. annulata* infected cell lines were isolated from infected cattle and established in culture (Tsur and Adler, 1962). It proved difficult to use such infected cell lines as vaccines because of the difficulty of inducing predictable levels of infection to protect the animal without killing it (Pipano, 1974).

It was noted by Pipano and Tsur (1966) that cultivation of *T. annulata* schizonts in tissue culture for a prolonged period resulted in attenuation of virulence. This phenomenon has also been reported in other protozoan parasites cultured *in vitro*, for example liver explants infected with *Plasmodium berghei* (Weiss and De Giusti, 1966). The mechanisms of attenuation are poorly understood but it appears that the period of cultivation required to reach complete attenuation will vary between different isolates and can range from several months to over two years.

Attenuation is achieved when inoculated schizonts no longer produce clinical symptoms in cattle and no piroplasm stages of the parasite are detected (Pipano and Tsur, 1966; Pipano, Kloppfer and Cohen, 1973). Most importantly, the cultures are infective and so will protect cattle against reinfection by homologous or heterologous virulent schizonts, although the latter can occasionally produce clinical symptoms (Hashemi-Fesharki and Shad-Del, 1973). If schizont immunised animals are challenged with infected ticks (*i.e.* the sporozoite stage of the parasite) there is occasionally a severe reaction, especially amongst adult Friesian cows, but generally the vaccinated cattle survive an otherwise lethal tick challenge (Pipano, 1978). When vaccinating the most susceptible and expensive animals (adult Friesians), immunity induced by the

attenuated schizonts is often reinforced by a second immunisation using infected ticks (Pipano, 1981).

The cell line vaccine is used either fresh or after cryopreservation. Fresh vaccine is commonly used at 3-5x10^6 infected cells per dose and has a four day shelf life at 4°C. Frozen vaccine is inoculated within 30 minutes of thawing at approximately 10^7 infected cells per dose (Pipano, 1981).

Cell line vaccines have been effectively used in many countries where *T. annulata* is endemic, including Israel (Pipano, 1977; 1981), Turkey (Ozkoc and Pipano, 1981), Iran, (Hashemi-Fesharki, 1978), Iraq (Hooshmand-Rad, 1973), India (Gill *et al.*, 1976), Russia (Stepanova *et al.*, 1977), and China (Gansu Provincial Institute of Veterinary Medicine, 1975).

As mentioned previously, parasite stock differences do occur with *T. annulata*, but in most cases signficant cross protection occurs between isolates from different geographical areas (Gill *et al.*, 1980). Therefore the situation is not as critical as that which occurs with *T. parva*.

The duration of immunity in the absence of reinfection has not yet been fully investigated. In one report cattle that had been experimentally immunised with attenuated schizonts did not show any clinical symptoms when challenged 18 months later (Pipano, 1977).

The advantages of the cell culture vaccine are: that it can be inexpensively produced using conventional cell culture techniques; it appears to be safe for all breeds and ages of cattle; and it will protect against tick transmitted infection in the field, although a secondary inoculation of virulent schizonts may be required to fully protect some of the more sensitive exotic dairy breeds.

As the *T. annulata* "team" had been so successful in their endeavours to produce an effective vaccine against tropical theileriosis less emphasis was directed towards understanding the mechanisms by which the vaccine extended its effect.

The development of a tissue culture vaccine against *T. parva* proved more difficult to achieve, and as a consequence the mechanisms of induction of immunity were more intensively investigated. A discussion of the important points follows.

Early observations indicated that animals inoculated with schizont infected cells derived from the tissues of *T. parva* infected animals gave an irregular response ranging from death to non-infection (Spreull, 1914). Later studies showed that at least 10^8-10^9 infected cells were required to induce immunity (Pirie, Jarrett and Crighton, 1970). This is 10,000-100,000 fold higher than is required for infection with *T. annulata*.

Further work established that an important prerequisite for effective immunisation appears to be the establishment of the parasite within the cells of the host (Wilde, Hulliger and Brown, 1966; Pipano *et al.*, 1977; Brown *et al.*, 1978a; 1978b; Emery *et al.*, 1982). Using karyotypic analysis it was observed that *T. annulata* infected cells would transfer and infect the cells of the recipient animal within hours of inoculation (Wilde, 1967) whereas *T. parva* schizont transfer was very inefficient and could take many days (Brown *et al.*, 1971).

Following up these observations, investigations were carried out to examine the effect of histocompatability differences between cell line and recipient. It was recently shown by Morrison *et al.* (1981) that cattle could be infected with and immunised against *T. parva* using as few as 10^2 autologous infected cells but that 10^7 or 10^8 allogeneic infected cells were needed to produce the same effect. This phenomenon has not been reported for *T. annulata*, presumably because no difficulty is encountered in infecting recipient cells with inoculated allogeneic infected cell lines. Both *T. annulata* and *T. parva* infected cell lines have been shown to express BoLA (bovine mhc) class I histocompatibility antigens on their surfaces and in recent studies by Teale (1983) and Dolan *et al.* (1984b) it was demonstrated that cattle inoculated with 10^3 or 10^5 *T. parva* infected cells mhc class I matched with respect to the recipient became immune to subsequent sporozoite challenge. It was not clear whether this immunity was a result of a more efficient parasite transfer into recipient cells (because of prolonged survival of the mhc matched inoculated cells) or whether the inoculated cells themselves were capable of inducing protective immunity. It is interesting that this histocompatibility barrier does not appear to jeopardise immunisation using *T. annulata* infected cell lines although the mhc relationship between cell line and recipient has never been examined in this context.

Following the breakthrough by Malmquist, Nyindo and Brown (1970), which reported the successful culture of *T. parva* infected cells *in vitro*, extensive effort went into experimental immunisation trials using a single *T. parva* infected cell line. This work has been reviewed by Brown (1981). It appeared that in order to reduce the virulence of the cell line sufficiently to prevent severe reactions, or death in a proportion of the recipients, it had to be attenuated by at least 50 subcultures *in vitro*. However, after the cells had been subcultured fifty times or more it became necessary to give larger numbers of cells (10^8) to achieve successful immunisation. It appears that culture derived attenuation is more difficult to achieve and maintain with *T. parva* than it is with *T. annulata*.

1.1.12.3 *Other methods of immunisation*

All the work published to date on immunisation with either *T. annulata* or *T. parva* stipulates that effective immunisation requires infective parasite material. If a killed vaccine could be developed it would have the advantages of stability and safety and a long shelf life.

Attempts have been made to immunise animals using uninfective antigen preparations of *T. annulata* (Pipano *et al.*, 1977). In this study cattle were inoculated with both particulate and soluble fractions of schizonts, with or without Freund's adjuvant. High antibody titres developed in all the animals receiving antigens plus adjuvant although no correlation was observed between antibody titre and protection against challenge. It was interesting to note in this study that animals having received antigen and adjuvant appeared to be immune to challenge with (presumably) homologous schizont infected cells, but succumbed to a subsequent sporozoite challenge. Another interpretation of this phenomenon may be that the immunity generated by the primary inoculation of killed antigen and adjuvant effectively "blocked" infection of the host cells by the second challenge of live schizont infected cells, as suggested by Brown (1981). Therefore, as the animal had not actually become infected by *T. annulata*, it was fully susceptible to sporozoite challenge. It is unclear whether the antibody response generated in the primary inoculation was directed against a parasite or donor cell component, or how it may effect the "blocking" of infection. In a subsequent study with *T. parva* (Emery *et al.*, 1981a) it was suggested that transfer of schizonts from donor to host cells could be prevented by the prior induction of anti-schizont antibody.

Attempts to induce protective immunity against *T. parva* infection using inactivated parasite material have not met with success (Wagner, Duffus and Burridge, 1974; Emery *et al.*, 1981a), although in a recent study reported by Emery, Morrison and Jack (1986) animals were protected against a lethal challenge of *T. parva* by prior inoculation with plasma membrane preparations derived from autologous infected lymphocytes. By contrast, animals vaccinated with irradiated autologous infected membranes were not protected. These rather contradictory results lead to speculation as to whether there was some contaminating schizont material in the "live" membrane preparation which may have established infection within the recipient animal, thus inducing protective immunity.

Another approach which has received some attention has been the use of irradiation to effectively "attenuate" the parasite, thus allowing limited parasite replication and the induction of protective immunity. In most of the studies carried out with both *T. annulata* (Srivastava and Sharma, 1977; Singh, Jagdish and Gautam, 1979;

Samantaray, Bhattacharyulu and Gill, 1980) and *T. parva* (Cunningham *et al.*, 1973b; Purnell *et al.*, 1974; Purnell, 1977) it appeared that increasing doses of irradiation would simply kill the parasites, resulting in a simple dilution, as opposed to the more desirable attenuation effect. Although some of the studies provided indications that attenuation could be achieved (Samantaray, Bhattacharyulu and Gill, 1980), the correct irradiation dose level was very variable. Possible contributory factors leading to attenuation include the sporozoite concentration and the maturity of the sporozoite preparation (Brown, 1981).

Despite the difficulties encountered so far in the reproducibility of achieving parasite attenuation by irradiation, it is still an attractive option. If a suitable irradiation protocol could be established an attenuated parasite vaccine would have the advantage of increased safety compared to live vaccination, and would induce protective immunity as a result of limited replication within the host, unlike an inactivated or killed vaccine preparation.

The failure of inactivated vaccines against *Theileria* infection is perhaps not surprising, as it is generally the case that non-replicative organisms are unable to induce effective cell mediated immune responses (Liew, 1985) such as the cytotoxic T cells or macrophage activating T cells believed to be important in immunity to *Theileria*. It is likely that intracellular replication of the infective organism results in a more efficient formation of antigen-mhc complex, resulting in a higher efficiency of antigen presentation than would the passive association of non replicative antigens.

1.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX

1.2.1 Introduction

"I shall always regard the differentiation between self and nonself as crucial to all immunological theory"

F.M. Burnett.

The wide range of cell surface structures encoded by the highly polymorphic genes in the major histocompatibility complex were first detected through their effect on transplant rejection. The pioneering work of the late Peter Medawar and his colleagues Leslie Brent and Rupert Billingham, first on skin grafting (Medawar, 1944) and then on tolerance (Billingham, Brent and Medawar, 1953) revolutionised immunological thinking by proposing that cell mediated immunity was a qualitatively different kind of immune response from humoral immunity. This paved the way to the

intensive study of the central role of both the T lymphocyte and the mhc molecules in immune responses.

In his autobiography *Memoir of a Thinking Radish* (1986), Peter Medawar described how the study of cattle contributed to our present understanding of immunological recognition.

A meeting at the International Congress of Genetics in Stockholm in 1948 between Peter Medawar and Dr. Hugh Donald (then head of the AFRC Animal Breeding Research Institute in Edinburgh) led to experimental skin grafting between sets of cattle twins in an attempt to distinguish between identical and fraternal twins. The unexpected finding was that none of the grafts were rejected. The answer to this phenomenon came from the work of American geneticist Ray Owen who had discovered that, as cattle twins share the same placenta, the twin foetuses are transfused with each other's blood prior to birth (Owen, 1945). This somehow made each twin "tolerant" to a skin graft from the other twin, but not from an unrelated animal. This exciting result confirmed previous observations that rejection of a graft from a genetically non-identical donor was due to an immunological response (Medawar, 1944) and led to the important realisation that the ability to distinguish between "self" and "non self" is an acquired characteristic of the immune system.

The work of Peter Gorer, inspired by Landsteiner, led to the identification of a group of antigens in mice which when matched between donor and recipient animal improved graft survival (Gorer, 1938). However, it took the foresight of George Snell to produce inbred and congenic strains of mice which could be used to study the effects of individual genes before the major histocompatibility complex (mhc) was formally identified (Snell, 1948).

Analogous major histocompatibility systems have been found in all mammalian species studied so far, with striking interspecies homology in both genetic organisation and gene products (Gotze, 1977; Klein, 1986). Collectively these genes encode cell surface glycoproteins which act as markers or guides by which cells of the immune system can recognise and communicate with each other and distinguish all cells in the body (self) from foreign invaders such as viruses, bacteria and parasites (non self). To carry out these functions two different sets of mhc coded cell surface molecules are required: class I antigens, which are generally expressed by all nucleated cells; and class II antigens characteristic of certain cells of the immune system.

As the work presented in this thesis is mainly concerned with the functional aspects of the mhc, that will be the emphasis of this review. However, as there is no function without structure and no structure without genes, a brief review is given of the organisation of the genes within this complex and of the structure of the proteins they

encode. As cattle are a relatively outbred species much of the work done so far to unravel the bovine mhc has been based on the human HLA system, which is used here as a comparative illustration.

1.2.2 mhc genes

1.2.2.1 *Human*

The human major histocompatibility complex is generally thought of as three major linked gene clusters. The class I and II regions encode polymorphic cell surface glycoprotein molecules involved in immune recognition, and the class III region contains the genes for the complement proteins C2, C4 and factor B.

The A, B and C loci encode the expressed class I heavy chain molecules (Figure 4). The class II genes are located in the D region of the HLA system and are arranged in three subregions DR, DQ and DP, with each region containing at least one alpha/beta pair of genes. The DR subregion consists of a single alpha gene and three beta genes, whereas the DQ and DP regions consist of two alpha and two beta chains each. Unlike the class I molecules, the class II molecules consist of two polypeptide chains (alpha and beta) (Kaufman and Strominger, 1979) and it is thought that alpha chains primarily associate with beta chains encoded within each genetic subregion. Three class II loci are expressed in man and allowing for combinatorial association, heterozygous individuals may express eight or more different class II molecules (Mengle-Gaw and McDevitt, 1985). Two to three class I loci are expressed, with a resultant four to six different class I molecules (Bevan, 1981).

1.2.2.2 *Bovine*

Current evidence conclusively demonstrates the presence of at least one class I locus in the bovine mhc, the BoLA A locus (Spooner *et al.*, 1979; Oliver *et al.*, 1981). However, extensive searching has led to the accumulation of evidence for a second BoLA class I locus. In a recent study by Bensaid *et al.* (1988a) it was found that following extensive immunoprecipitation of both BoLA A locus products from PBM lysates of a heterozygous animal it was still possible to precipitate molecules using a non-polymorphic class I specific monoclonal antibody. This implied that there were other bovine class I products which were not necessarily encoded by the BoLA A locus.

Another important achievement in this area is the recent isolation and characterisation of a bovine class I cDNA sequence from a bovine liver cDNA library (Brown, Spooner and Clark, 1988). The high degree of similarity between the protein sequence of this clone and other class I proteins is evidence that the cloned gene may

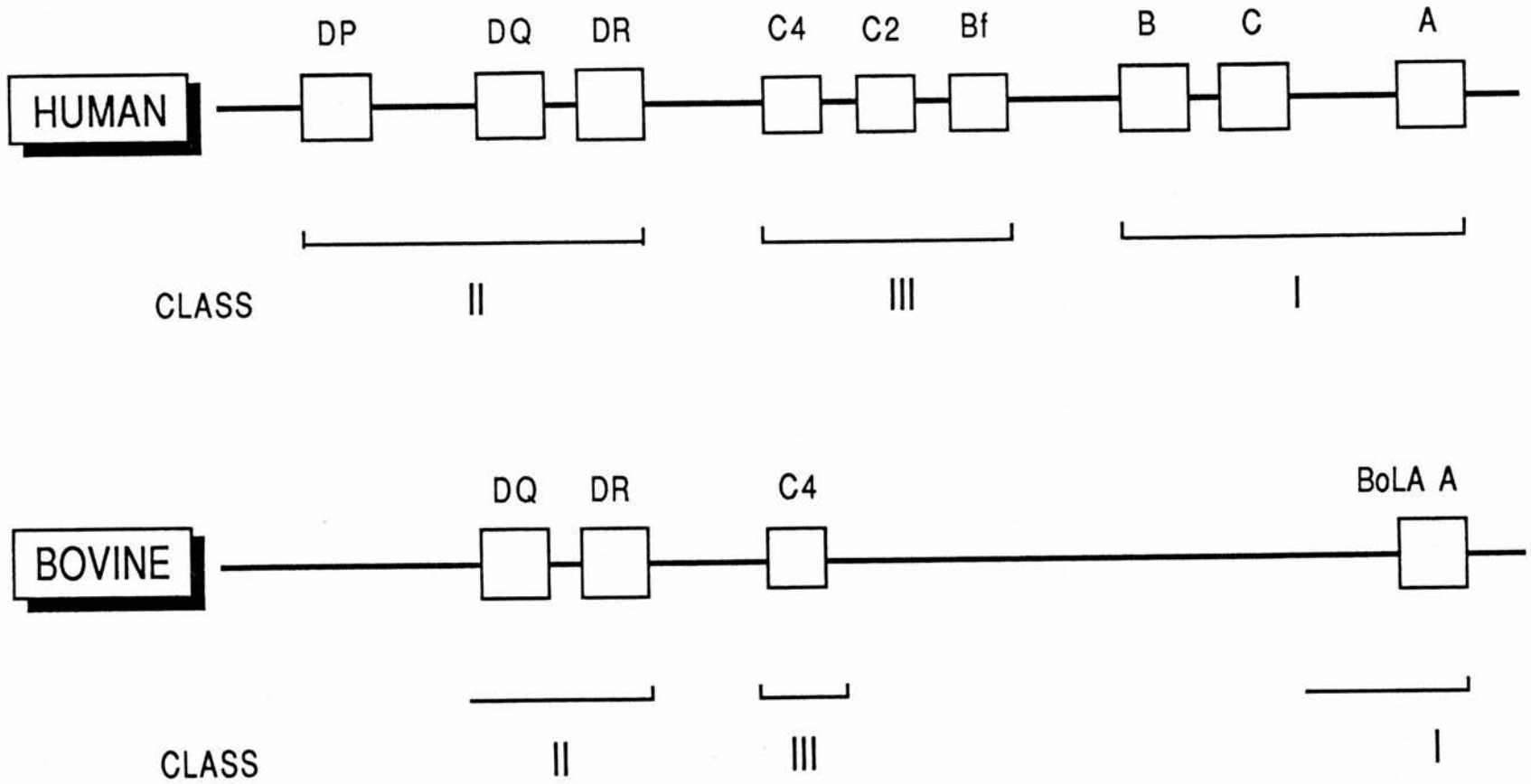


FIGURE 4

Organisation of the human and bovine MHC genetic regions

encode a functional molecule. It is hoped that this homologous molecular probe will help to elucidate the remaining mysteries of the bovine mhc class I region.

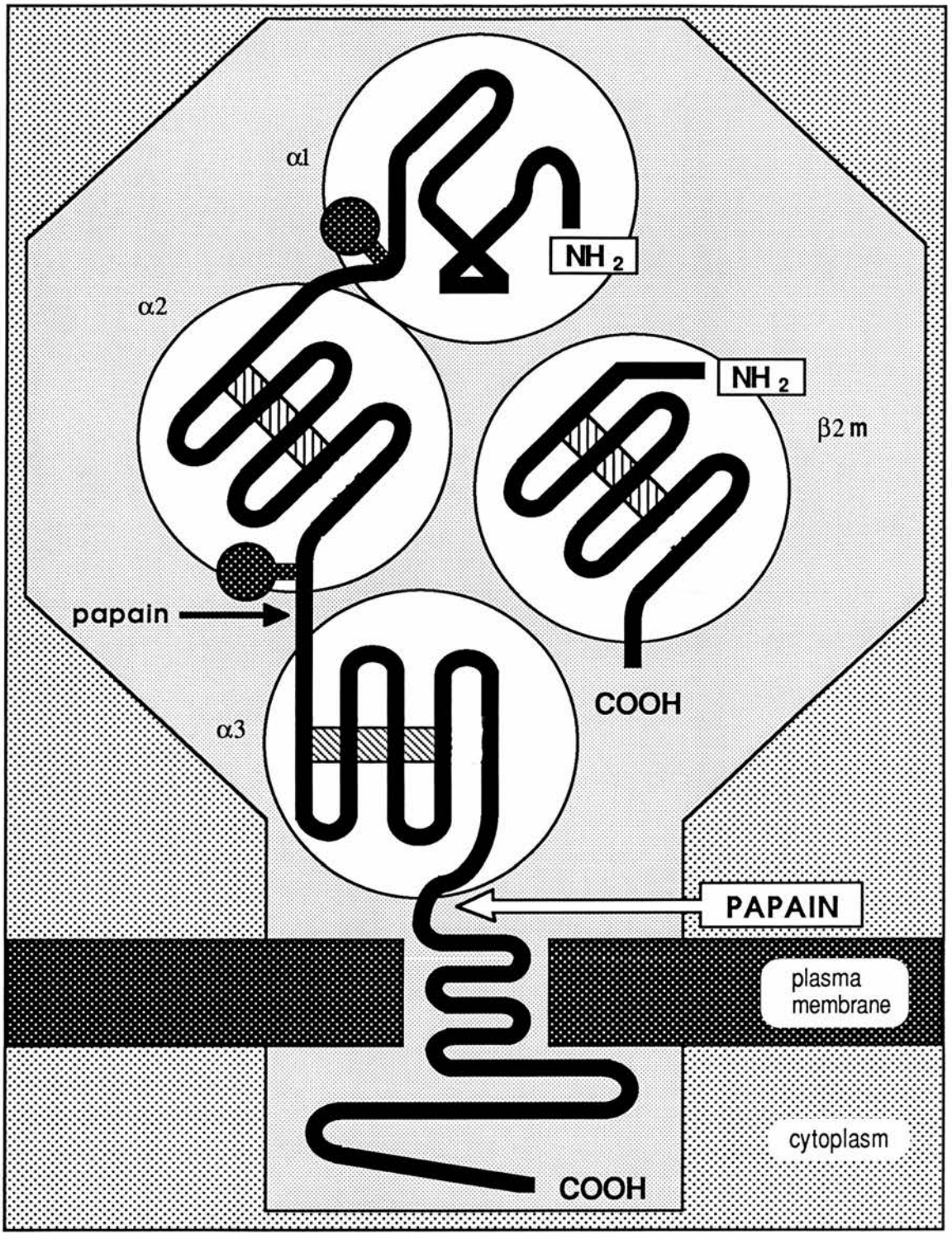
Although the existence of a putative bovine class II region has been reported (Usinger, Curie-Cohen and Stone, 1977) the study of bovine class II genes and the molecules which they encode is still at a preliminary stage. Bovine homologues of human DQ and DR mhc class II genes have been identified using human probes in a Southern Blot analysis (Andersson *et al.*, 1986a; 1986b). Human class II cDNA probes were also used to analyse the genomic organisation of the bovine mhc using field inversion gel electrophoresis (which allows the separation of high molecular weight fragments of DNA) (Bensaid *et al.*, 1988). The results of this study suggest that the bovine mhc spans over 3 million base pairs (mbp), which is similar to the HLA complex, which has at least one mbp for each of the class I and class II regions. The class II region is composed of closely linked bovine homologues of DR alpha and DR beta subregions and a more distinct DQ subregion. These studies provide the basis for the speculative gene map of the bovine mhc shown Figure 4.

1.2.3 mhc gene products

1.2.3.1 *Human*

HLA class I molecules are found on the cell membranes of nearly all nucleated cells, although the level of expression does vary. The highest levels of class I antigens are found on the cells of the immune system, whereas expression is low on most endocrine cells and practically non-existent in central nervous system neurons. The molecules are cell surface glycoproteins consisting of a mhc encoded heavy chain (molecular weight 44000 daltons) and a 12000 dalton light chain, beta-2-microglobulin (Owen and Crumpton, 1980) (Figure 5a). The mhc heavy chain which penetrates the cell membrane is composed of three globular domains. The characteristic polymorphic sites (carrying determinants specific to each individual) occur on the alpha 1 and alpha 2 domains (Orr *et al.*, 1979). Tissue typing sera are presumably directed against these allogeneic sites.

In a recent study the X-ray crystallographic structure of the HLA A2 human class I molecule was determined (Bjorkman *et al.*, 1987a). This work suggested that most of the polymorphic amino acids are clustered on top of the molecule between the alpha 1 and alpha 2 domains, identified as the recognition site for processed foreign antigens. Both serological epitopes and residues critical for T cell recognition are located in this site (Bjorkman *et al.*, 1987b), although the specific determinants recognised by B and T cells are distinct and only slightly overlap.



Structure of MHC class I molecules

Fig. 5a
A diagram of the general features of class I MHC molecules based on the murine H-2 molecule. The heavy chain is divided into three extracellular domains (α 1, α 2 and α 3), a hydrophobic segment and a hydrophobic cytoplasmic domain. The β 2 microglobulin light chain associates non-covalently with the heavy chain. **(Male, Champion and Cooke 1987).**

In contrast to the HLA class I molecules the class II molecules are mainly found on cells of the immune system, macrophages and other antigen presenting cells, B cells and activated T cells (Winchester and Kunkel, 1979). These molecules consist of two polypeptide chains (34000 dalton and 28000 dalton) which are non covalently bound together (Kaufman and Strominger, 1979). As shown in Figure 5b both chains have two globular domains.

1.2.3.2 *Bovine*

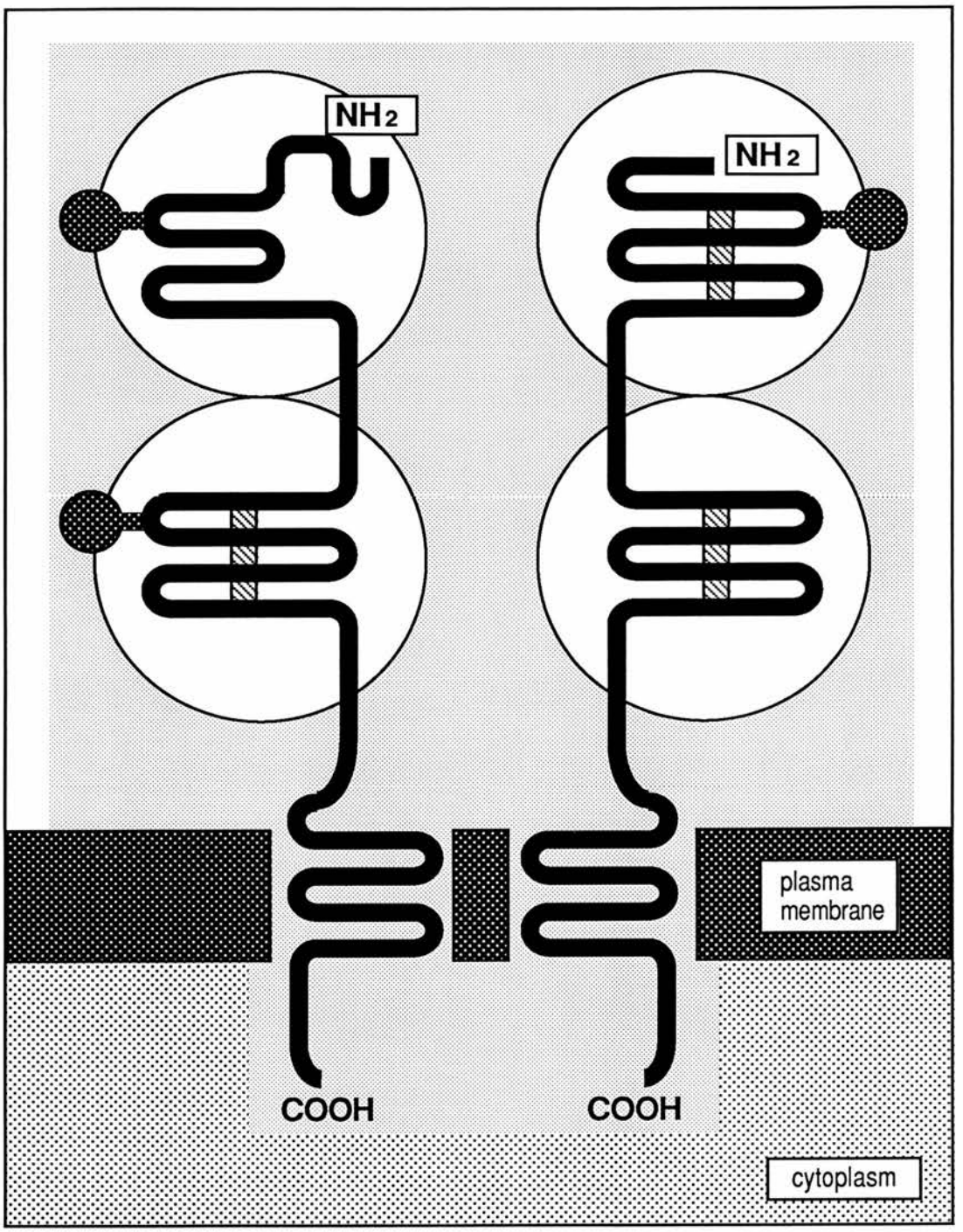
The bovine class I mhc molecules, as in other species, consist of heavy chains (44000 daltons) associated with beta-2-microglobulin (12000 daltons) (Hoang-Xuan *et al.*, 1982a). Bovine class II mhc molecules, immunoprecipitated with antibodies reacting with either murine Ia or human HLA DR molecules, share similar biochemical characteristics to those documented in mouse and man (Hoang-Xuan *et al.*, 1982b). The bovine class II antigens consist of two non covalently associated, membrane bound glycoproteins of approximately 32000-35000 daltons (alpha chain) and 25000-28000 daltons (beta chain). This result has recently been confirmed by immunoprecipitation using bovine anti class II monoclonal antibodies (Emery *et al.*, In press).

1.2.4 Detection of bovine mhc gene products

1.2.4.1 *Class I antigens*

It seems appropriate at this point to mention how the bovine mhc gene products have been characterised. Much of the work has involved the detection of class I antigens using alloantisera. This area has recently been reviewed by Spooner (1986). An important difference between the alloantisera used in BoLA typing and those used in HLA typing is that the bovine alloantisera are raised by alloimmunisation using lymphocytes or skin grafts. By a process of selective absorption or careful titre selection these typing sera can be rendered operationally monospecific (Spooner, Morgan and Oliver, 1980), whereas most of the serological HLA typing has relied on the use of sera from multiparous donors (Van Rood *et al.*, 1958).

Using such alloantisera, characterisation of the bovine mhc began and at the three international comparison tests to date (Spooner *et al.*, 1979; Anon, 1982; Bull *et al.*, 1989) serum clusters detecting BoLA class I products were identified and assigned workshop specificities. Family studies indicate that the class I antigens detected are inherited in a simple Mendelian fashion and appear to be encoded at a single locus. The heterozygote expresses determinants controlled by both alleles (Oliver *et al.*, 1981). Thirty detectable bovine class I specificities, believed to be the products of individual class I genes, have been detected. This contrasts with over 70



Structure of MHC class II molecules

Fig. 5b
A diagramatic representation of the general features of class II MHC molecules with prototype (heavy) and (light) glycoprotein chains. Each chain is divided into two extracellular regions, a hydrophobic transmembrane region and a hydrophilic cytoplasmic region. **(Male, Champion and Cooke 1987).**

detectable class I specificities in the HLA system, the products of three class I loci (Klein, 1986).

It was reported by Amorena and Stone (1978) that skin grafts exchanged between cattle matched at the BoLA A locus survived longer than mismatched grafts, which supports the concept that the antigens detected were classical mhc products. The frequencies of particular BoLA class I specificities are found to vary between different cattle breeds (Spooner, 1986).

An additional method of mhc characterisation is the use of alloreactive T cells, which may not recognise the same determinants as those detected serologically (Zinkernagel and Doherty, 1979; Biddison *et al.*, 1980). This may become important in mhc restriction studies, where it is believed that the determinants recognised by classically restricted cytotoxic T cells (CTL) are equivalent to those recognised by alloreactive CTL (Allen *et al.*, 1984). The generation and functional activity of bovine alloreactive CTL has recently been reviewed by Teale *et al.* (1986) and it appears that the alloreactive CTL recognise determinants coded for by the BoLA A locus. Heterogeneity within any of the serologically defined BoLA class I specificities examined so far has not been detected using alloreactive CTL (Spooner *et al.*, 1987).

Further characterisation of class I bovine lymphocyte antigens has recently been reported using a one dimensional isoelectric focussing (IEF) technique which aims to characterise mhc polymorphism at the protein level.

Bovine mhc products are immunoprecipitated from detergent extracts of biosynthetically radiolabelled PBL using MAb w6/32, which cross-reacts with a monomorphic determinant on bovine class I molecules (Brodsky, Stone and Parham, 1981). These products are analysed by one dimensional IEF (Joosten *et al.*, 1988). Using this technique it is possible to assign IEF haplotypes which correlate with known serotypes. If a second monoclonal antibody is subsequently used different banding patterns are observed, which may provide further evidence for a second mhc class I locus in cattle. The enigma remains.

1.2.4.2 *Class II antigens*

In comparison to what is known about the BoLA class I antigens our understanding of the bovine mhc class II gene products is limited. Problems have arisen in attempts to make suitable alloantisera for class II antigen characterisation, firstly in developing simple methods for the removal of any anti class I activity from the alloantisera and secondly because only a certain proportion of bovine PBM express class II antigens.

The classical method of typing for class II antigens in man exploits the fact that T cells are stimulated to grow in the presence of cells carrying foreign histocompatibility class II antigens. This reaction is called the mixed lymphocyte reaction (MLR), and HLA specificities determined by this technique are called D region specificities. It is also possible to detect HLA D region antigens serologically, and these are known as DR specificities (Bodmer, 1978). Mixed lymphocyte reactions have been used to identify bovine class II antigens in preliminary studies (Curie-Cohen, Usinger and Stone, 1978; Usinger *et al.*, 1981). This work suggests that bovine class II antigens are polymorphic; it is possible to detect the products of more than one locus and in full sib families it appears that the genes controlling the MLR responses are linked to the class I genes. Evidence from IEF studies (Henson, 1988) on the other hand suggests that, in the general cattle population, possession of a particular class I haplotype does not necessarily indicate a particular class II haplotype. Polymorphism in the bovine class II region has also been detected using class II specific alloreactive T cell clones (BoT4) (Teale and Kemp, 1987). However, as these T cell clones are difficult to produce and maintain their use as typing reagents is limited.

At a recent workshop held in Nairobi, Kenya (1987) the current methods, limitations and future possibilities for characterisation of the bovine class II region were explored and discussed (Anon, 1988). The use of techniques such as restriction fragment length polymorphism (RFLP) analysis using heterologous DNA probes (Andersson *et al.*, 1986a; 1986b) and biochemical phenotyping using ID IEF techniques as discussed above for class I gene products (Joosten *et al.*, 1988) was felt to be very promising. However there was a body of opinion at this meeting that the "serologists should persist", perhaps taking advantage of the knowledge gained from biochemical approaches to characterise new reagents. The attainment of good serological anti class II reagents, such as had been achieved in the study of HLA class II specificities, would have the considerable advantages of simplicity and reproducibility at relatively low cost.

1.2.5 Function of the mhc

The function of the mhc genes may be simply described as "coding for molecules that provide the context for the recognition of foreign antigens by T lymphocytes" (Klein, 1986).

The specific functions attributed to mhc molecules are all variations on this central theme. Several non-immune functions of the mhc have been speculated upon, including a possible role in embryonic differentiation (Artzt and Bennett, 1975), cell contact (adhesion/inhibition) (Bartlett and Edidin, 1978), and in mating behaviour (Beauchamp, Yamazaki and Bayse, 1985).

For the purposes of this review I shall consider the role of the mhc under three general headings: mhc restriction; alloreactivity; and immune responsiveness.

1.2.5.1 *mhc restriction*

mhc restriction describes the mechanism by which a T cell recognises its target antigen. The fact that the T cell will only take notice of an antigen if it is presented along with an mhc molecule restricts its specificity. This phenomenon was first described by Zinkernagel and Doherty (1974). In this paper they described the intracerebral infection of various mouse strains with lymphocytic choriomeningitis virus (LCMV) and the subsequent testing of their spleen cells for cytotoxic activity against virus infected target cells from a particular strain of mouse (H-2k). They observed that T cells from all the H-2k strain mice could lyse the targets whereas T cells from other strains could not. Their novel interpretation of these results as a demonstration of the need for self recognition in order for T cells to be able to recognise extrinsic antigen finally suggested to immunologists a physiological role for mhc antigens.

In subsequent experiments mhc class I molecules were identified as the restricting elements in the cytotoxic T cell response (Zinkernagal and Doherty, 1975).

Such mhc restriction in CTL responses was subsequently reported in response to haptens (Shearer, Rehn and Garbarino, 1975), minor histocompatibility antigens (Bevan, 1975) and many viral infections (Moss *et al.*, 1981; Townsend and McMichael, 1985).

Class I restricted CTL seem appropriate for defence against viral or other intracellular pathogens since the range of host cells that can be infected by such pathogens is large, so the mhc restriction element required would have to have a similarly wide distribution. mhc class I restricted CTL have recently been described for other intracellular parasites, *Listeria monocytogenes* (Kaufmann, Hug and De Libero, 1986), *Mycobacterium leprae* (Chiplunkar, De Libero and Kaufmann, 1986) and, as described earlier, *T. parva* parasites (Goddeeris, Morrison and Teale, 1986).

The mhc restriction phenomenon is found to apply equally well to the class II associated recognition of antigens by T cells (Fathman and Fitch, 1982). As a general rule, human T cells expressing the CD4 molecule will recognise antigen in association with class II molecules, whereas CD8 positive T cells recognise antigen in the context of mhc class I molecules.

In functional terms both these T cell subsets possess similar potential: CD4 T cells exhibit cytotoxic activity against class II positive infected cells (Braakman *et al.*, 1987) as well as performing their more recognised role in the release of lymphokines; T cells from the CD8 subpopulation (known to be cytotoxic) have also been reported

to produce gamma interferon (Sonnenfeld, Mandel and Merigan, 1979) which activates macrophages.

1.2.5.1.1 Antigen presentation

In contrast to antibodies which bind free antigen in a direct manner, T cells can only recognise foreign antigen in association with self mhc class I or class II molecules, and it is generally assumed that two modes of presentation are required. Antigen association and hence presentation with mhc class I molecules is thought to be restricted to endogenous antigens newly synthesised within the cell, whereas exogenous antigens taken up by endocytosis (such as bacteria or protozoan parasites within the phagolysosome of an infected cell) get processed into antigenic peptides which are transported and presented on the cell surface by class II molecules acting as a "peptide shuttle" (Germain, 1986). However, the increasing reports of mhc class I restricted CTL playing an important role in many bacterial and protozoal intracellular infections (as reviewed by Kaufman, 1988), challenge the viewpoint that antigen association with class I molecules is a feature only of endogenous antigens and also that infectious agents are required to stimulate class I restricted CD8 CTL whilst non infectious agents tend to stimulate class II restricted CD4 T cells.

Several recent reports have demonstrated the induction of mhc class I restricted CTL using non-infectious viral antigen preparations (Yamada *et al.*, 1985; Braciale *et al.*, 1987) and ovalbumin antigen (Staerz, Karasuyama and Garner, 1987). Further studies have shown that CTL will recognise antigenic peptides of the influenza nucleoprotein (Townsend *et al.*, 1986). These findings, taken together with the recent elucidation of the three dimensional structure of the class I molecule (Bjorkman *et al.*, 1987a), suggest that CTL recognise foreign antigens in the form of a peptide bound to the class I molecule.

Although no direct role for denaturing or degradation of class I associated antigen has been established it is likely that some form of intracellular processing of proteins will occur prior to their association with class I molecules. Antigen processing by antigen presenting cells for recognition by class II restricted T cells is well established (Unanue, 1984).

It appears that both mhc class I and class II molecules function by presenting peptide fragments of protein antigens to the T cell receptor, although recent evidence suggests that different and distinct "antigens" are presented by the different classes of mhc molecules. It was reported by Morrison *et al.* (1986c) that influenza haemagglutinin synthesised endogenously was only seen by T cells in conjunction with class I molecules whereas antigen taken up by the cell from the culture medium would

selectively stimulate class II restricted T cells. This suggest two distinct mechanisms in antigen processing for presentation with either class I or class II molecules in which the mode of antigen entry to the cell plays an important role. An alternative explanation is that specialised antigen presenting cells exist, with the unique capacity to process and associate exogenous antigen with class I molecules (Bevan, 1987). Possible candidates are the bone marrow macrophages suggested by Kaufmann *et al.* (1988); these express class I antigen but not class II and also have good phagocytic function.

1.2.5.1.2 T cell recognition of antigens

As has been mentioned above, the T cell recognises a complex composed of foreign antigen and the polymorphic region of either mhc class I (alpha 1 and alpha 2 domains) or class II (alpha 1 and beta 1 domains) molecules. Current evidence is now strongly in favour of the "one receptor" view of T cell recognition put forward by Matzinger (1981). It appears that mhc molecules act as peptide carriers or receptors and it is the physical complex of protein antigen and mhc molecule (rather like a peptide sandwich) that is recognised by the single alpha-beta T cell receptor (Buus, Sette and Grey, 1987; Allen, Babbit and Unanue, 1987).

Further evidence for this concept of T cell recognition comes from the first crystallographic structure of a human class I molecule which reveals a putative peptide binding site immediately surrounded by aminoacid sites known to be involved in T cell recognition (Figure 6)(Bjorkmann *et al.*, 1987b).

A point for consideration is: how can a single putative binding site per mhc molecule (assuming only a limited number of polymorphic mhc molecules are expressed in each individual) bind peptides of so many different antigens to produce different and distinct combinations of self-plus-peptide to be recognised by the extensive repertoire of T cell clones in each individual? If it is possible that many different peptide ligands can occupy the binding site of a single mhc molecule then it might be expected that the peptides would share some structural homology. Some evidence for this comes from a comparison of sets of peptides known to bind to defined mhc class II molecules (Babbit *et al.*, 1985); this showed that different sets of homologous peptides could attach to a single binding site (in several different overlapping positions within the site) provided they were small enough and had a suitable conformation.

A recent study by Morrison *et al.* (1987) reports that cytotoxic cells taken from *T. parva* immune animals heterozygous at the BoLA A locus showed a bias in their mhc restriction towards one or other of the BoLA products. A major component of the cytotoxic response in each animal was restricted by determinants on a single mhc class I product. This apparent hierarchy in dominance among BoLA class I molecules in the

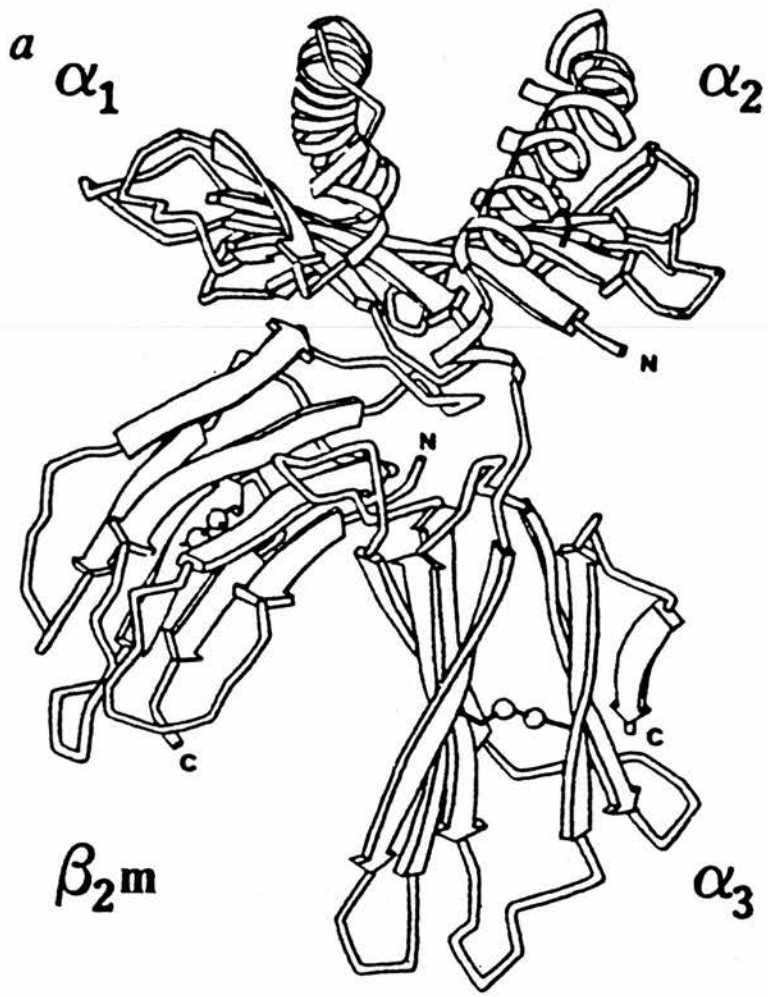


Fig. 6

A schematic representation of the HLA-A2 class I molecule. The molecule is shown with the "immunoglobulin like" domains (α_3 and β_2m) at the bottom with the polymorphic domains (α_1 and α_2) at the top. The antigen recognition site is located on the top surface of the molecule in a groove created between the α_1 and α_2 domains.

(Bjorkman,P.J. et.al. 1987)

restriction of *T. parva* specific CTL may reflect differences in the ability of a peptide antigen to associate with particular mhc class I specificities. This may have implications in the selection of relevant genetic markers for disease resistance.

1.2.5.2 Alloreactivity

In addition to recognising foreign antigen in the context of self mhc molecules T cells are capable of recognising allogeneic or mhc mismatched cells. T cell responses to mhc alloantigens *in vitro*, mixed lymphocyte responses (class II antigens), and alloreactive cytotoxicity (class I) are unique in that they can be obtained in primary culture without prior *in vivo* stimulation of the responder animal (Simpson, 1983). If one views alloreactivity as a "side-effect" of the normal response of T cells it becomes less of an inexplicable phenomenon apparently designed to frustrate transplant surgeons.

Indeed it appears that to the T cell the complex of "self mhc molecule and foreign antigen" is not fundamentally different from the recognition of allo mhc antigen. The simplest explanation being is one of cross reactivity (self plus antigen equals allo mhc molecule) (Bevan, 1977; Simpson, 1983). Several examples of cross reactivity of antigen specific class I restricted CTL with allogeneic mhc molecules have been cited in the literature (Finberg *et al.*, 1978; Braciale, Andrew and Braciale, 1981; Gaston, Rickinson and Epstein, 1983). The mechanism of this allorecognition by T cells is not fully understood but it is likely to be similar to the mechanisms involved in the recognition of foreign antigen and self. The T cell could recognise either the polymorphic residues of a foreign mhc molecule or the molecule complexed with a peptide. It was recently reported that murine CTL responses against allogeneic human class I molecules involve processed fragments of the allo HLA molecules, which bind to murine class I molecules (Parham, 1988). However, although it is conceivable than the amino acid residues on an allogeneic mhc molecule are recognised on a peptide derived from itself, the 3-D structure of the mhc class I molecule (Bjorkman *et al.*, 1987a) suggests that it is not necessary to fragment the allogeneic mhc molecule in order to expose the necessary residues for alloreactive T cell recognition.

When the precise mechanism for this allorecognition is finally clarified it may provide an important contribution to our understanding of the intended function of the mhc molecule as well as being perhaps the most intensely studied artefact in immunology.

1.2.5.3 *Immune responsiveness*

The ability of an individual to respond to a particular antigen is known to be controlled by the immune response genes located within the mhc gene complex

(Gonwa, Peterlin and Stobo, 1983). Direct implication of mhc class II genes in determining immune responsiveness comes from the work of Le Meur *et al.* (1985), where the introduction of cloned specific mhc class II genes into transgenic mice effectively turned a non responder strain into responders. Non responder class I alleles have also been defined for recognition of influenza nucleoprotein by CTL in inbred strains of mice (Pala and Askonas, 1986).

Understanding the mechanisms involved in the genetic control of immune responsiveness has obvious applications in the design of effective vaccines and in understanding the immunological control of resistance or susceptibility to disease.

Considered within the framework of what we already understand about the mechanisms of the induction and effector function of the immune response there are three points whereby immune response genes may exert their effect:

i) Unresponsiveness may reflect the absence of an appropriate antigen receptor on a T cell. This hole in the repertoire may be a consequence of cross reaction between self mhc and self mhc plus the specific antigen (Schwartz, 1978). Clones of T cells potentially reactive against the specific antigen would also be potentially autoreactive and would be eliminated during T cell maturation in the thymus (Marrack *et al.*, 1988).

ii) Unresponsiveness may reflect the inability of particular mhc proteins to bind specific antigens. Recent experiments demonstrating the physical association of a peptide antigen and class II molecules (Babbit *et al.*, 1985) provide direct evidence for the idea that a high responder phenotype results from the high affinity of a particular class II allele and peptide, whereas a low responder phenotype reflects the failure of a particular class II allele to associate with the appropriate antigen. Murine cytotoxic T cell responsiveness to influenza nucleoprotein epitopes was found to be dictated by the capability of a given mhc classI protein to bind a specific antigenic determinant (Taylor *et al.*, 1987).

iii) A third possibility to explain unresponsiveness is the effect of immunosuppressive influences. It is known that exposure to antigen results in the induction of both helper and suppressor T cells. Suppressor epitopes within the antigenic structure may prevent the establishment of all T helper function thereby resulting in effective non responsiveness (Jensen, Pierce and Kapp, 1984). This has obvious implications in the selection of peptides as potential vaccines. Fortunately suppressor and helper T cell epitopes are generally non overlapping (Sercarz *et al.*, 1978) and if the suppressor epitope is "removed" from the antigen, responsiveness is restored. It has also proved possible to restore "responsiveness" by the addition of a T helper cell epitope from a foreign protein (Francis *et al.*, 1987).

Non mhc genes have also been shown to play an important role in the response to infectious organisms; resistance to *Salmonella typhimurium* has been mapped to the *Ity* gene on the murine chromosome I (Plant and Glynn, 1979). Genes on this chromosome have also been implicated in murine resistance to *Leishmania donovani*, where it appears that the gene involved (*Lsh*) influences macrophage function (Bradley *et al.*, 1979).

Linking immune responsiveness to particular genes is much more difficult in an outbred species such as man or cattle due to the extensive polymorphism of the mhc haplotypes within a population, the influence of other genes, and various contributory environmental factors. In man genetic predisposition to certain diseases, like Type 1 or insulin-dependent diabetes and ankylosing spondylitis, has been associated with certain HLA phenotypes (Batchelor and McMichael, 1987). Most of the disease associations have been found with the class II genes, the DR region in particular (Svejgaard, Platz and Ryder, 1983).

In veterinary studies a classic example of successful intervention by immunogeneticists is in Marek's disease, a lymphomatous condition of poultry caused by a herpes virus (MDV). Research has linked certain mhc haplotypes to disease resistance and improved vaccine response, leading to improved disease resistance by selectively breeding for particular mhc types (Powell, 1986).

Immune response genes have also been recently identified in cattle (Glass and Spooner, 1988). In this study an mhc class II dependent T cell proliferation assay was used to demonstrate within a group of cattle that animals could be divided into responders and non responders to the simple antigen ovalbumin, which is known to be under immune response gene control in mice. Further studies have also indicated a link between diferences in antibody response in cattle to human serum albumin and possession of particular mhc class I alleles (Lie *et al.*, 1986). Disease association linked to particular BoLA class I antigen specificities has been reported for bovine leukaemia virus (Lewin and Bernoco, 1986), and mastitis. In a recent study in Norway (Solbu, Spooner and Lie, 1982) a putative link between BoLA A w16 and susceptibility, and between BoLA A w2 and resistance, was suggested. A follow up report from Iceland also suggested a link between various BoLA A specificities and mastitis related traits (Oddgeirsson *et al.*, 1987).

The identification of genes with the potential to improve disease resistance has great attraction in the control of livestock disease. Once these genes are established within cattle populations they are not dependent on sophisticated management or other disease control methods for their continued effectiveness.

CHAPTER 2

General Materials and Methods

2.1 BOLA TYPING

2.1.1 Materials

2.1.1.1 *The BoLA typing antisera panel*

The panel of alloantisera used to define the BoLA specificities of the animals and *Theileria* infected cell lines used in this study were produced at the department of Immunogenetics, AFRC Institute of Animal Physiology and Genetics Research in Edinburgh, from parous or skin grafted cattle (Spooner, Millar and Oliver, 1979).

These operationally monospecific sera have been tested in the three international comparison tests carried out to date (Spooner *et al.*, 1979, Anon., 1982, Bull *et al.*, 1989) and have been assigned workshop (w) specificities. The Edinburgh alloantisera typing panel listed in Table 2.1 includes sera defining all the presently agreed workshop specificites (Bull *et al.*, 1989). The workshop specificities are believed to be coded for by alleles at a single mhc class I BoLA A locus (Spooner *et al.*, 1979).

The BoLA typing sera were stored neat in small aliquots at -20°C or -70°C. Control serum, which was selected for non-reactivity with any of the test cells, was included in the lymphocytoxicity test to estimate baseline test cell viability.

TABLE 2.1 **BoLA workshop specificities (w) and defining sera**

w specificity	Previous designation	Defining Sera (Edinburgh)
1		1,8,83
2		2,62,64,65
3		39
4		82,117
5		7,90
6		9,10,66
7		11,17,67,68
8		13,63,80
9		14
10		69,71,96
11		73,76
12		47
13		5,16,101
14	8.1	80
15	8.2	105
16		38,43,131
17	6.1	93
18	6.2	86
19	6.4	100
20		78
21		107,123
25		111
30		74,98
32		40

2.1.1.2 *Hanks balanced salt solution*

The HBSS was supplied in powder form (Gibco Biocult) containing phenol red, and reconstituted with deionised distilled water. Sodium bicarbonate was added at the rate of 0.35g per litre and the pH of the HBSS solution was adjusted to 6.8 (as measured with a Philips PW9409 pH meter) using 1N HCL and 1N NaOH. The HBSS solution was sterilised by filtration (0.22 micrometre, Millipore Corporation) and stored at 4°C until required.

2.1.1.3 *Ficoll/Hypaque*

To prepare a solution of Ficoll/Hypaque with a specific gravity of 1.069, 67.5g of Ficoll 400 (Pharmacia Fine Chemicals) was dissolved in 400ml of boiling deionized distilled water, then made up to 750ml and left to cool at room temperature; 79.8g of sodium Hypaque (Winthrop) was dissolved in 120ml of warm deionised distilled water, made up to 200ml and left to cool at room temperature. The Hypaque solution was then added, slowly with constant stirring, to the Ficoll solution until a specific gravity of 1.069 (determined using a hydrometer) was obtained. The resultant Ficoll/Hypaque solution was then sterilised by filtration (0.22 micrometre, Millipore Corporation) or autoclaved and stored at 4°C until required.

2.1.1.4 *Complement*

A source of complement was obtained from pooled rabbit sera obtained from a commercial slaughterer.

Batches of complement were screened for lack of inherent cytotoxicity and for potency in a standard lymphocytotoxicity test using test cells and antisera of known reactivity. Suitable batches of complement were then stored at -70°C until required in the lymphocytotoxicity test.

2.1.1.5 *Eosin dye*

A 5% weight/volume stock solution of Eosin powder (Koch Light Laboratories Ltd) in deionised distilled water was stored at -20°C. A working solution comprising 1 volume of Eosin stock solution 1 volume of deionised distilled water and 2 volumes of double strength HBSS was prepared for use in the lymphocytotoxicity test and could be stored for up to one week at 4°C.

2.1.1.6 *Fixing solution*

Fixing solution was prepared by adding 5ml of 0.15M disodium hydrogen phosphate in deionised distilled water (phosphate buffer) to 95ml of formalin (40%

formaldehyde in 0.9% saline). The resultant fixing solution was stored at 4°C until required.

2.1.1.7 *Terasaki typing plates*

Terasaki 60-well plastic typing plates (Sterilin) were prepared for use by the addition of appropriate dilutions (in HBSS) of both the control and typing sera (1 microlitre per well) dispersed in a known order under liquid paraffin using a multidispenser Hamilton syringe. Batches of typing plates were stored at -20°C until required.

2.1.2 Methods

2.1.2.1 *Preparation of test cells*

The preparation of lymphocytes for use in the lymphocytotoxicity test was performed as described by Spooner *et al.*, (1979) using the following procedure.

Lymphocytes were separated from venous blood, collected in sterile vacutainers containing lithium heparin (Becton Dickinson), by layering 3ml of whole blood onto 2.5ml of 1.069 Ficoll/Hypaque solution followed by centrifugation for 15 minutes at 1500g.

The resultant lymphocyte layer at the plasma/Ficoll-Hypaque interface was removed and washed in HBSS by centrifugation at 400g for 5 minutes. The resultant cell pellet was resuspended in a small volume of HBSS and lysis of any contaminating erythrocytes was achieved by the addition of 2ml of deionised distilled water to the cell sample with constant mixing, followed rapidly by 2ml of double strength HBSS. The cell suspension was then washed twice by centrifugation at 100g for 5 minutes (to remove platelets) and resuspended in HBSS. Cells were counted in a haemocytometer (Neubauer chamber) using a phase contrast microscope (Nikon) and adjusted in HBSS to give a final concentration of 2.5×10^6 cells per ml. *Theileria* infected cells were adjusted to 10^6 cells per ml in HBSS for testing.

2.1.2.2 *Lymphocytotoxicity test*

The lymphocytotoxicity test was conducted as described by Spooner *et al.*, (1979). The Terasaki typing plates were thawed prior to use and kept at room temperature. The test cell suspension (1 microlitre) and complement (1 microlitre, diluted 1:2 in HBSS) were added to the plate containing the typing sera and incubated for 1 hour and 20 minutes at 37°C. Following incubation 1 microlitre of eosin solution (working stock) was added to each well and incubated for 5 minutes at room temperature before

adding 2 microlitres of fixing solution. The plates were then read immediately using an inverted phase contrast microscope (x 100 magnification) or stored at 4°C.

Test plates were scored as follows using the system described in the second international BoLA workshop (Anon, 1982).

Score	% killed cells
8	80-100
6	60-79
4	30-59
2	10-29
1	0-9
0	reaction unreadable

2.2 *THEILERIA* PARASITES

2.2.1 General

The species and stocks of *Theileria* used in this study are detailed in the relevant chapters. The majority of the work involved a Moroccan isolate of *T. annulata* described by Ouhelli (1985). The parasite material used included lymphoblastoid cell lines (LCLs), cryopreserved tick derived stabilates and ground up tick supernatant (GUTS) prepared by staff at the Centre for Tropical Veterinary Medicine, Edinburgh.

2.2.2 Lymphoblastoid cell lines

Theileria infected LCLs were established *in vitro* using the methods described by Brown (1983).

2.2.2.1 *Peripheral blood mononuclear cells (PBM)*

Peripheral blood mononuclear cells (PBM) were separated from venous blood by centrifugation over Ficoll paque (Pharmacia Fine Chemicals) at 800g for 35 minutes at 15°C. The mononuclear cells were removed from the interface of the plasma and the Ficoll paque, and washed twice in PBS by centrifugation at 300g for 10 minutes. The cells were resuspended in 10ml of cold complete culture medium, counted, and adjusted to the required cell concentration.

2.2.2.2 *Complete culture medium*

The LCLs were initiated and maintained in complete culture medium (sterile) comprising RPMI 1640 with 25mM Hepes and 2mM L-glutamine, supplemented with 10-20% foetal calf serum (FCS), penicillin (100iu per ml) and streptomycin (100mg per ml) (Gibco Biocult). Aliquots of complete culture medium were tested for sterility prior to use in cultures.

2.2.2.3 *Ground up tick supernatant (GUTS)*

The procedure for obtaining a sterile preparation of ground up tick supernatant as a source of *Theileria* sporozoite material (Brown, 1983) is described briefly as follows.

Adult *Hyalomma anatolicum anatolicum* ticks infected with *T. annulata* (Walker *et al.*, 1985) were fed for 3 days on rabbits' ears to stimulate maturation of the sporozoites. Gorged ticks were then removed and washed, once in 1% benzalkonium chloride (Roccal, Winthrop) and a further three times in 70% ethanol. They were then transferred to a sterile container and washed three times in warm Eagles minimal essential medium (MEM) with Hanks salts, double strength antibiotics (penicillin, 200 iu per ml and streptomycin 0.2mg per ml) (Gibco Biocult), and nystatin (0.1mg per ml) (E.R. Squibb and Sons, Inc., New York). They were left for ten minutes in the fourth wash. The medium was then discarded, and 2-5ml of cold MEM with 3.5% Bovine Plasma Albumin (BPA) (Fraction V, Sigma Chemical Co.) containing antibiotics as described above was added to the ticks, which were then transferred to a sterile mortar. They were then enthusiastically ground with a sterile pestle, with aliquots of supernatant being removed, and measured quantities of MEM and 3.5% BPA solution (as described above) added. This was repeated until the required concentration of supernatant material (measured as tick equivalents per ml) was reached.

The supernatant was spun in the centrifuge for 5 mins at 100g; the supernatant (containing the sporozoites) was removed and filtered through a sterile 25mm or 47mm Millipore Swinnex filter with an AP prefilter and 8 micrometre MF filter (Millipore Corporation). The resultant filtrate is a preparation of sterile GUTS.

2.2.2.4 *In vitro infection of bovine PBM with Theileria sporozoites*

This technique is described in detail by Brown (1983). It involves mixing equal volumes of PBM cell suspension at $2x10^6$ per ml with the sporozoite suspension (GUTS filtrate) at 1.0 tick equivalent per ml in a multiwell culture plate (Costar). According to the amount of material available and the purpose for which the cultures are intended this can be done with varying numbers of PBM and volumes of GUTS.

The cultures are incubated at 37°C in a 5% CO_2 humidified incubator (Vindon Scientific Ltd). After 24 hours, complete culture medium is added to give double the original volume per well and the culture medium is subsequently changed according to the metabolic demands of the culture. Aliquots are removed at regular intervals for cytocentrifuge preparations to monitor infection and transformation rates. Once 30% or more of the cells are infected the cultures should be transferred to a larger culture area, the most convenient container for maintaining infected LCLs being a $25cm^2$ culture flask (Nunc, Gibco Biocult). The cells are usually maintained at a concentration between 10^5 and $2x10^6$ per ml.

2.2.3 Cryopreservation of parasite material

Both parasite infected LCLs and GUTS can be successfully cryopreserved using the methods described by Brown (1983).

The LCLs are cryopreserved using a solution of 10% dimethyl sulphoxide (DMSO) (BDH biochemicals) in complete culture medium at a cell concentration of 10^7 per vial, and stored in liquid nitrogen (BOC Ltd).

The GUTS are cryopreserved as a tick stabilate using 7.5% glycerol as the cryoprotectant and stored in liquid nitrogen.

2.2.4 Clinical observations on infected cattle

2.2.4.1 *The indirect fluorescent antibody test (IFAT)*

This test was carried out to determine levels of anti-macroschizont or anti-piroplasm antibodies in sera taken from cattle prior to inoculation of the *T. annulata* infected LCLs in the cell line immunisation trial in Morocco described in Chapter 3. It was carried out by staff at the department of Parasitology, Institute Agronomique et Veterinaire (IAV), Hassan II, Rabat, Morocco using the method described by Burridge and Kimber (1972). Macroschizont antigens were prepared from cell cultures of *T. annulata* (Burridge and Kimber, 1972) and piroplasm antigens were prepared according to the method of Burridge (1971).

2.2.4.2 *Haematology*

The haematological parameters routinely measured during the experimental infection of cattle described in chapters 3 and 4 were packed red cell volumes (PCV) and leucocyte (WBC) count. PCV was estimated using a haematocrit tube (in Morocco) or a Hawksley microcentrifuge technique. WBC counts were estimated using a haemocytometer (Neubauer chamber) or a coulter counter (Coulter ZBI electronic

particle counter, Coulter Electronics Ltd). These haematological parameters were measured on the day of sampling the cattle by staff at the department of Parasitology IAV, Hassan II, Rabat, Morocco (Chapter 3) and the staff at the department of Protozoology, Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh.

2.2.4.3 *Parasitology*

Both blood and lymph node smears were examined for evidence of *Theileria* parasites in the experimentally infected cattle.

Blood smears were prepared by spreading a small drop of freshly obtained venous blood onto a clear glass slide, using the edge of a second slide, followed by rapid air drying.

Lymph node smears were prepared from needle biopsies of infected nodes. This involved the insertion of a 19G sterile disposable needle into the lymph node where it was rotated several times and withdrawn following closure of the syringe attachment part of the needle with a finger or thumb. The material within the needle was then expressed onto a clean glass slide and was spread and dried as described above for the blood smear. The smears were fixed in methanol for 2 minutes and then stained in a working solution of 5% stock Giemsa stain in Giemsa buffer (Gurr buffer tablets pH 7.2, BDH Biochemicals) for 40 minutes. The slides were then rinsed in Giemsa buffer and dried prior to microscopic examination.

2.2.5 Giemsa stock solution

10g of Giemsa powder (Merck) was added to 540ml of Glycerol (BDH Biochemicals) and heated to 60°C and maintained at this temperature with constant stirring for 1 hour. After cooling to room temperature 840ml of methanol (BDH Biochemicals) was added and the mixture left on a stirrer overnight. Azur II (Merck) was then added at a concentration of 0.2g per 100ml and stirred for a further 48 hours. The Giemsa stock was then filtered (Whatmans No. 4) and stored in a dark bottle.

2.2.6 Cytocentrifuge preparation of cells

The morphology of cells within a culture was examined using Giemsa stained smears of cytocentrifuge preparations. These were prepared by spinning 50 microlitre aliquots of culture suspension onto clean glass microscope slides at 400 rpm for 10 minutes in a cytocentrifuge (Cytospin, Shandon, Southern Instruments). The slides were first "primed" by a preliminary centrifugation of complete culture medium at 800rpm for 5 minutes. The cytocentrifuge smears were air dried, fixed in methanol (2 minutes), and stained for 40 minutes with 5% Giemsa stock (diluted in Giemsa buffer).

The stained slides were rinsed in Giemsa buffer and dried prior to examination using an Ortholux II microscope (Leitz).

2.3 BOVINE LEUCOCYTE SPECIFIC MONOCLONAL ANTIBODIES

Monoclonal antibodies (MAbs) which react with distinct bovine leucocyte subpopulations were used in Chapter 5. The putative specificities of these antibodies, characterised by their cellular distribution and function within the bovine immune system, was reviewed by Baldwin, Morrison and Naessens (1988). A summary of the MAbs and the cell surface markers they detect is given below.

MAbs IL-A11 and IL-A17 identify two distinct subpopulations of T lymphocytes in cattle, bearing the BoT4 (Baldwin *et al.*, 1986; Teale *et al.*, 1986) and BoT8 (Ellis *et al.*, 1986; Teale *et al.*, 1986) molecules respectively. BoT4 is expressed on approximately 30% of bovine PBM with a range of 20-40%, and BoT8 is found on about 18% (range 10-32%) of PBM. These molecules are believed to be analogous to human CD4 and CD8 respectively (Baldwin, Morrison and Naessens, 1987). CD4 and CD8 molecules have been postulated to interact with nonpolymorphic portions of mhc class II and class I respectively (Reinherz, Meuer and Schlossman, 1983). Current evidence suggests that the analogous bovine molecules may have a similar function (Baldwin, Morrison and Naessens 1987). MAb B5/4 is specific for bovine IgM and hence for bovine B cells (Pinder *et al.*, 1980). MAb IL-A24 recognises a molecule present on bovine monocytes/macrophages (J. Ellis, personal communication). This MAb recognises functional bovine antigen presenting cells (E.J. Glass, personal communication). MAb J11 recognises a monomorphic determinant on bovine mhc class II molecules (Baldwin, Morrison and Naessens, 1988). This MAb will block proliferation of bovine PBM in mixed lymphocyte culture (E.J. Glass, personal communication).

2.3.1 Indirect immunofluorescence test

An indirect immunofluorescence test was used to stain the cells prior to their analysis using a fluorescence activated cell sorter (FACS IV, Becton Dickinson).

In brief, 10^6 cells in 50 microlitres of FACS medium (RPMI 1640 with 25 mM Hepes and 5% gammaglobulin-free horse serum, Gibco Biocult) were mixed with 0.1ml of the MAb at a predetermined optimal dilution and incubated for 30 minutes at 4°C in a 96-well round-bottomed culture plate (Nunc, Gibco Biocult). The cells were washed three times by repeated suspension and centrifugation for three minutes at 100g and 4°C. The supernatant was removed, 0.25ml of a fluorescein isothiocyanate (FITC) conjugated antimouse immunoglobulin (RAMIg) (Nordic) at a dilution of 1:40 in

FACS medium was added to each well, and the test plate was incubated for 30 minutes at 4°C.

2.3.2 Fluorescence activated cell sorter (FACS)

Flow cytometric analysis was performed with a FACS IV (Becton Dickinson, FACS Systems, Sunnyvale, CA).

The 488nm line of an Argon ion laser run at an output power of 400mW was used for excitation of scatter and fluorescence signals. The FACS IV was calibrated using a mixture of green fluorescent (0.86 micrometre diameter) and non-fluorescent (1.10 micrometre diameter) latex microspheres (Polysciences, Northampton, England).

For analysis, cell samples were run at approximately 2000 cells per second and normally 10,000 cells were analysed. Forward angle scatter signals (using a gain setting of 1.0-3.0) were used to exclude debris and dead cells. The remaining viable cells were analysed by simultaneous 90° scatter and green fluorescence using a 520nm long pass filter and 488nm band pass filter for the 90° scatter.

CHAPTER 3

Immunisation of cattle using lymphoblastoid cell lines infected with a Moroccan stock of *Theileria annulata*

The effect of cell dose, cell line and mhc compatibility between parasite infected cell line and recipient

Aims

To examine the safety and efficiency of using low passage lymphoblastoid cell lines infected and transformed with a Moroccan stock of *T. annulata* (Ouhelli, 1985) to immunise susceptible cattle in Morocco against a subsequent lethal homologous challenge.

To estimate the relative importance of histoincompatibility, cell dose and cell line when using this method of vaccination.

3.1 INTRODUCTION

Tropical theileriosis, a tick borne disease caused by the protozoan parasite *Theileria annulata*, has always presented a formidable barrier to the survival of exotic cattle imported into parts of Asia and Africa (Gilbert, 1935; Pipano, 1977; Purnell, 1978). This has become an increasing problem in recent years, due to the interest in cross breeding and the demand for exotic cattle in developing countries in an attempt to improve dairy and beef production (Gill *et al.* 1980). It is estimated that about 250 million cattle are at risk throughout a wide geographical area covering parts of Spain and Morocco in the West, through the Mediterranean coast of Europe and North Africa, and east to Southern Russia, India and China (Purnell, 1978). In view of the economic importance of this disease the search for appropriate control methods is paramount.

It was noted in the early part of this century that animals which recovered from theileriosis developed a durable immunity and that the disease could be transmitted to susceptible cattle when infective blood or organ suspensions were used as an inoculum (Sergent *et al.*, 1924). Workers in North Africa followed this up by extensive immunisation trials of cattle using a blood vaccine containing the "Kouba" or other parasite stocks of low virulence (Donatien and Lestoquard, 1938). Problems with these early vaccines were the variable reactions and occasional mortality induced in the recipient animals. There was also the danger of transmitting other diseases via the vaccinal blood (Sergent *et al.*, 1945).

An important breakthrough in vaccine development came with the *in vitro* culture of *Theileria* infected lymphoblasts, first reported by Tchernomoretz (1945). Subsequent development of tissue culture systems made it possible to isolate and maintain indefinitely *in vitro*, *Theileria* infected cell lines from the tissues of infected animals (Tsur and Adler, 1962). This technology exploited the fact that the presence of the parasite within the host cell induces synchronous division of both parasite and host cell (Hulliger *et al.*, 1964). This property of the parasitised cell lines allows them to be maintained *in vitro* as continuously growing cell lines. Lymphoblastoid cell line vaccines have been used to infect and immunise animals in the field with notable success (Pipano and Tsur, 1966; Hashemi- Fesharki and Shad-Del, 1973; Gill *et al.*, 1976; Pipano, 1977; 1981). One of the main problems in using such vaccines has been the difficulty of inducing predictable levels of infection that result in the induction of protective immunity without killing the animal (Pipano, 1974). There are many reports of the variation in virulence between various stocks of *T. annulata* (Sergent *et al.*, 1945; Rafyi, Maghami and Hooshmand-Rad, 1965; Pipano, 1974; Hooshmand Rad and Hashemi-Fesharki, 1968). The problem of virulence of parasite material can be overcome by prolonged *in*

vitro cultivation of infected cell lines, leading to their "attenuation" (Pipano and Tsur, 1966). The attenuation procedure did not appear to reduce the immunogenic capacity of the infected cell lines (Pipano and Tsur, 1966). However the process of attenuation may take two years or more and the mechanisms involved in this process are not well understood.

In contrast to the reports on virulence of various parasite stocks, there has been very little work done on the role of the donor cell in macroschizont cell line immunisation and what effect this may have on the safety and efficiency of vaccination. In cell line vaccination the parasite is effectively introduced to the recipient animal in the context of a foreign graft. Histoincompatibility between cell line and recipient may influence the successful establishment of the parasite within the host, which is believed to be a prerequisite for the development of protective immunity (Wilde, Hulliger and Brown, 1966; Pipano *et al.*, 1977; Brown *et al.*, 1978a).

A recent study involving BoLA (Bovine mhc) defined lymphoblastoid cell lines and recipients suggested that the mhc may be a barrier to the infection of cattle using *T. parva* cell lines (Teale, 1983, Dolan *et al.* 1984b). This study confirmed earlier observations of a "self preference" in the infection of cattle using *T. parva* infected cell lines (Brown *et al.*, 1978a and Morrison *et al.*, 1981). However one of the major differences between *T. annulata* and the closely related parasite *Theileria parva*, which causes East Coast fever, is that it is much easier to infect and immunise animals with *T. annulata* infected cell lines than with *T. parva* infected cell lines. This implies that infection of animals using *T. annulata* cell lines is not inhibited by assumed histoincompatibility between cell line and recipient, although this has never been properly examined.

The major histocompatibility complex of cattle (BoLA) has been partially characterised and the serologically defined products have been assigned workshop specificities at the three international comparison tests to date, (Spooner *et al.*, 1979; Anon, 1982; Bull *et al.*, 1989). The BoLA specificites are believed to be polymorphic mhc Class I determinants encoded by alleles at a single locus. They have been shown to be the targets for bovine alloreactive cytotoxic cells (CTL) (Teale *et al.*, 1986; Spooner *et al.*, 1987) and to act as restricting elements in *Theileria* specific CTL clones (Goddeeris *et al.*, 1986). It was shown that BoLA class I specificities present on normal bovine lymphocytes were still expressed after infection with *Theileria* parasites (Spooner and Brown, 1980).

In this study we examined the ability of low passage *in vitro* established cell lines infected and transformed with a Moroccan stock of *T. annulata*, to infect and subsequently protect susceptible animals in the field against a lethal sporozoite

challenge. Four different cell doses and four different cell lines were used to examine whether there were upper or lower limits as regards a safe vaccinal dose and whether there were any differences between the reactions caused by the four different cell lines. The effect of histocompatibility between cell line and recipient was also considered.

In designing this experiment we were constrained by the numbers of animals we could use and by the fact that we had to find recipient animals in Morocco BoLA class I matched to the animals in Edinburgh used to provide the parasite infected cell lines. We therefore had the option of limiting the number of parameters examined, thereby increasing the number of animals in each group and the chance of obtaining statistically significant results, or of including all the parameters in the experimental design and possibly sacrificing statistical significance. As this was a preliminary experiment and the relative importance of each factor (mhc incompatibility, cell dose and cell line) was not known, we decided to examine all the parameters as a pointer for future research.

3.2 MATERIALS AND METHODS

3.2.1 Cattle

Thirty six Friesian Holstein crossbred calves aged between 8-14 months were selected from 120 on the basis of their BoLA specificity (Appendix 1.1). All the animals were sero-negative, prior to immunisation when tested against *T. annulata* piroplasm antigen in an indirect fluorescent antibody test using the technique of Burridge (1971). The cattle were bred and maintained on a farm near Sidi Slimane approximately 200 km north of Rabat (Morocco). There had been no reported cases of theileriosis on the farm in the previous three years. The experiment was conducted during the months of May and June 1985 (Figures 7a and 7b).

3.2.2 Histocompatibility testing (BoLA typing)

All the animals and cell lines were assigned BoLA types according to their reactivity with a panel of operationally monospecific bovine alloantisera developed at IAPGR, Edinburgh (Table 3.1). The BoLA reagents included in the panel detect all of the internationally agreed specificities to date (Spooner *et al.*, 1979; Anon, 1982) which behave as if they were alleles at a single locus (Oliver *et al.*, 1981). The animals and infected cell lines were tested using the microlymphocytotoxicity test described by Spooner *et al.* (1979) (section 2.1).

FIGURE 7a. Field work in Morocco

Top Figure

Freisian cow with tropical theileriosis during terminal stages of the disease.

Bottom Figure

Young bull diagnosed as having tropical theileriosis and being treated with chemotherapy.

FIGURE 7a. Field work in Morocco

FIGURE 7b. Field work in Morocco

TABLE 3.1 **BoLA specificities of experimental animals**

Animal Number	Sex	BoLA Specificity
1	F	11, 20
2	F	6.4, 11
3	F	11, 12
4	F	8, 16
5	F	10, -
6	F	8, -
7	F	6.4, 11
8	M	6.4, 8
9	F	6.4, 11
10	F	11, -
11	F	6.4, 13
12	F	16, -
13	F	6.4, 10
14	F	8, 11
15	F	13, 20
16	F	20, -
17	F	8, -
18	F	13, -
19	F	11, 20
20	F	11, 20
21	F	13, 20
22	F	8, 11
23	F	10, 11
24	F	20, -
25	F	6.4, -
26	F	10, 13
27	F	13, 20
28	F	6.4, 11
29	M	6.4, 11
30	M	8, 11
31	M	6.4, 20
32	M	12, -
33	F	6.4, 8
34	M	11, 20
35	F	8, 11
36	M	13, 20
37	F	6.4, 11
38	F	8, 11
39	F	11, 20
40	M	13, 20

3.2.3. Parasite material

Theileria annulata Gharb (Ouhelli, 1985) was isolated in the region of Kenitra, Morocco in 1980. This stock has subsequently been maintained within lymphoblastoid cell lines, passaged through cattle and cryopreserved in liquid nitrogen. In Morocco a calf was infected with this parasite and uninfected nymphal ticks of the species *Hyalomma anatolicum anatolicum* from the closed Edinburgh colony (Walker *et al.*, 1985) were fed on this animal. In Edinburgh adult infected ticks derived from those nymphs (batch 1) were used to infect bovine lymphoid cells *in vitro* using the method of Brown (1983). Further animals were inoculated with one of these cell lines and more *H. a. anatolicum* (batch 2) were fed on these animals. The resulting infected ticks were then used to prepare the infected cell lines and stabilate material to be used in the experiment.

3.2.4 Macroschizont infected cell lines

Peripheral blood mononuclear cells (PBM) from four animals on the farm at IAPGR Edinburgh were infected *in vitro* with sporozoites of the Gharb stock of *T. annulata* and maintained in tissue culture as infected lymphoblastoid cell lines (Brown 1983) (section 2.2.2.4). Donor animals were selected by matching their BoLA mhc class I specificities to the appropriate recipient animals in Morocco. Cell lines A, B and C were established using tick batch 2 and were maintained in culture for 3 weeks prior to the experiment. Cell line D had been prepared previously with sporozoites from tick batch 1 and had been stored in liquid nitrogen and resuscitated prior to the experiment.

The cell lines were inoculated subcutaneously above the right parotid lymph node using a 2 ml volume of cell suspension. All four cell lines were at passage 3 at the time of inoculation.

3.2.5 Transportation of cell lines

Each of the cell lines was harvested in Edinburgh, at a logarithmic phase of growth, and resuspended at a concentration of $5x10^7$ cells per ml in 2x2ml volumes of RPMI 1640 supplemented with 25mM Hepes and 2mM L-glutamine, 20% foetal calf serum, penicillin 100 iu per ml and streptomycin, 0.1 mg per ml (all Gibco Biocult). A separate vial from each line containing 10^7 cells was taken to make the lower cell dose dilutions. The vials were kept at 4°C in polystyrene containers with ice bags. After arrival in Rabat, Morocco the infected cells were maintained at 4°C overnight. The following morning the cell dilutions for the lower doses were prepared and cell viability was checked using a trypan blue exclusion test. The infected cell lines were inoculated

subcutaneously above the right parotid lymph node using a 2ml volume of cell suspension. This was achieved within 24 hours of the cell lines leaving Edinburgh.

3.2.6 Stabilate challenge

The sporozoites used for the challenge were prepared as a stabilate from *H. a. anatolicum* ticks (batch 2) infected with the Gharb stock of *T. annulata* (STAB, 35) using the method of Cunningham *et al.* (1973a) (section 2.2.3). The stabilate material was removed from gas phase liquid N_2, stored and transported to Morocco in solid CO_2, and thawed rapidly 30 mins prior to use. One ml of the stabilate at a dose representing 2.5 tick equivalents was inoculated subcutaneously over the left parotid lymph node of all the experimentally immunised animals and four naive controls.

3.2.7 Clinical observations

The clinical condition of the animals was assessed by rectal temperature (a febrile response being 39.5°C), haematocrit values (packed cell volume), the occurrence of macroschizonts in cells from the lymph node draining the site of inoculation, and the percentage of erythrocytes infected with piroplasms as assessed by Giemsa stained smears (section 2.2.4.3). Base line recordings were taken prior to inoculation of the cell lines. Clinical data was collected three times weekly except for the temperature responses which were recorded daily. The parasitaemia recordings were assessed at the Institut Agronomique et Veterinaire, Hassan II, Rabat and the complete data were not made available. The (incomplete) parasitaemia recordings shown in the text were obtained from Dr H. Ouhelli (personal communication).

3.2.8 Experimental design

The recipient animals were allocated into four groups on the basis of the immunising cell line. On day 0 each cell line was inoculated at four different dose concentrations (10^8, 10^6, 10^4 and 10^2 cells) into two recipient animals which were either BoLA matched or mismatched to the cell line (Table 3.2). On day 29 all the animals were challenged with a homologous sporozoite stabilate, along with four susceptible control animals.

TABLE 3.2 **Experimental design - animal numbers**

Cell Dose	BoLA Relationship	Cell lines: Line A (37) w(6.4,11)	Line B (38) w(8,11)	Line C (39) w(11,20)	Line D (40) w(13,20)
10^8	M	29	14	20	36
	MM	10	26	13	33
10^6	M	2	22	19	15
	MM	4	11	32	3
10^4	M	28	35	34	21
	MM	12	16	17	25
10^2	M	9	30	1	27
	MM	18	24	5	7

Control animals: 23, 8, 31, 6
M : BoLA class I matched
w : BoLA workshop specificity
MM : BoLA class I mismatched

3.3 RESULTS

3.3.1 Macroschizont cell line infections

3.3.1.1 *General*

All four *Theileria* infected cell lines (A, B, C and D) established infections in the animals at each dose level regardless of whether the recipients were BoLA matched (M) or mismatched (MM) to the cell line. The temperature and haematocrit values for each animal throughout the experiment are shown in Appendix 1.2. One of the BoLA MM recipients (No. 7) died of theileriosis within 25 days of having been inoculated with 10^2 cells of line D. This animal showed a 50% drop in haematocrit value from base line recordings, and 24% of lymphoid cells taken from the right parotid lymph node were infected with *theileria* macroschizonts. Animal No. 27 had to be treated with the theilericidal drug, parvaquone at 20 mg per kg (Coopers Animal Health), after reacting very severely to 10^2 BoLA M cells of line D.

3.3.1.2 *Effect of cell dose*

There was no obvious linear correlation between cell dose and severity of disease symptoms (Figure 8a and b). Although the 10^8 dose generally appeared to be the most pathogenic, severe disease responses were recorded in some of the recipient animals in the two lowest dose groups, 10^4 (No. 12 and 21) and 10^2. Indeed, as mentioned above, one recipient (No.7) in the 10^2 dose group died of theileriosis and another animal in the same group had to be drug treated to prevent death. Of the four different cell doses 10^6 consistently provoked the least severe clinical reaction. There did appear to be a correlation between cell dose and onset of an increase in temperature associated with the patent disease response. The incubation period for the disease, as assessed by measuring the start of the temperature peak occurring between days 12 and 25, was shortest in the 10^8 dose group and increased with decreasing dose (Figure 8a). The initial temperature peak in the 10^8 cell dose group, which occurred in all except the cell line D recipients (Figure 9), was ignored when estimating the disease incubation period as it did not coincide with other clinical symptoms.

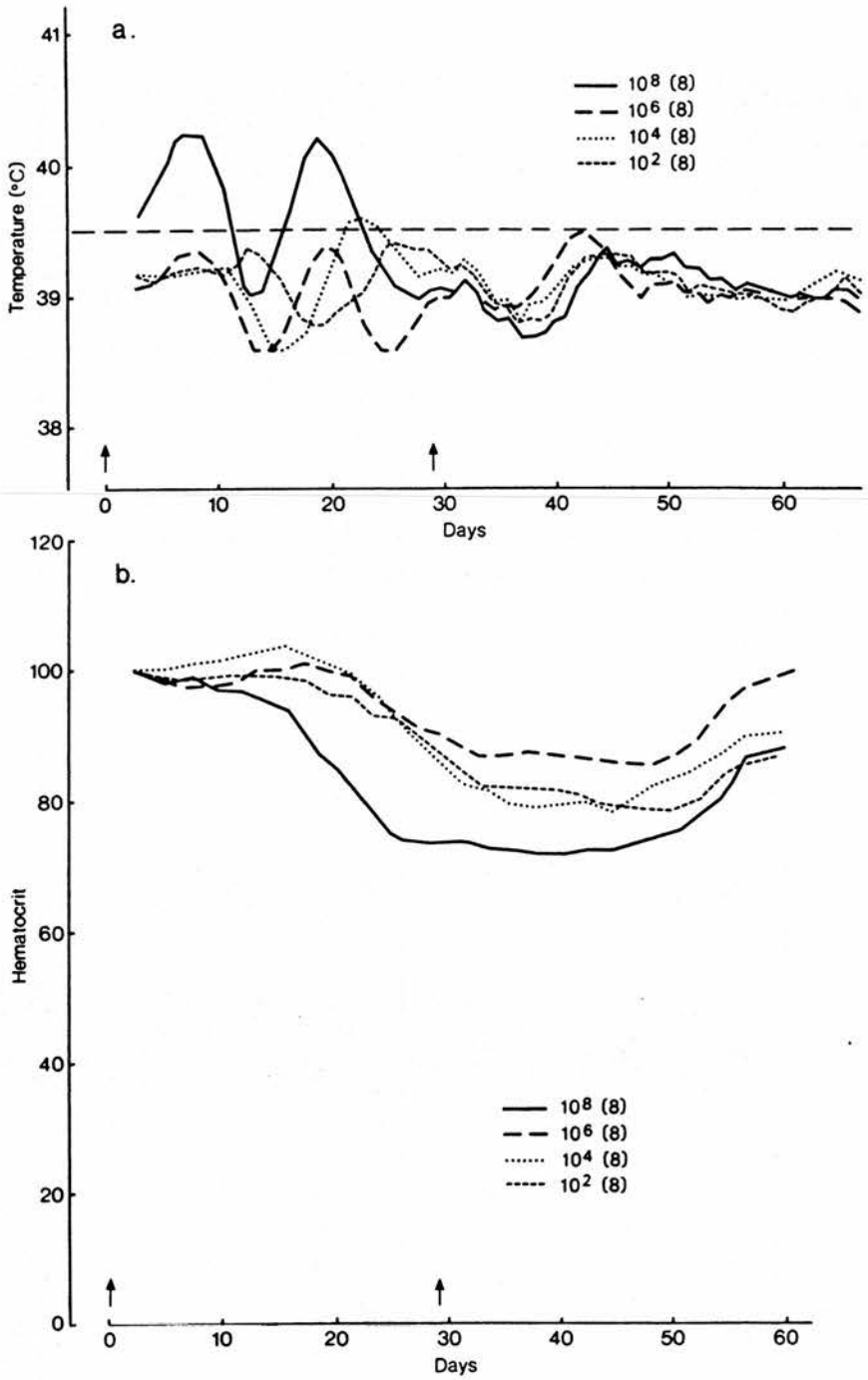
3.3.1.3 *Differences between cell lines*

All four *Theileria* infected cell lines established infections in all the recipient animals. Line D was, in general, more pathogenic than the other three lines, between which there was little variation (Figure 10a and b). The two animals that showed the most severe reactions had both been inoculated with 10^2 cells of line D.

Fig. 8

a. The average daily temperature responses of the collated recipients at each cell dose. The broken line represents a temperature of 39.5°C, temperatures above this line were considered febrile. The arrows indicate when the animals were inoculated with the T.annulata infected cell lines (Day 0) and challenged with a lethal dose of sporozoites (Day 29).
The number in brackets indicate the number of animals in each group.

b. The average haematocrit values of the collated recipients at each cell dose. Base line values recorded prior to inoculation were taken as 100% and the values recorded susequently were expressed as a % relative to the base line value.


a.
41
40
39
38
Temperature (°C)
10^8 (8)
10^6 (8)
10^4 (8)
10^2 (8)
0
10
20
30
40
50
60
Days
b.
120
100
80
60
40
20
0
Hematocrit
10^8 (8)
10^6 (8)
10^4 (8)
10^2 (8)
0
10
20
30
40
50
60
Days

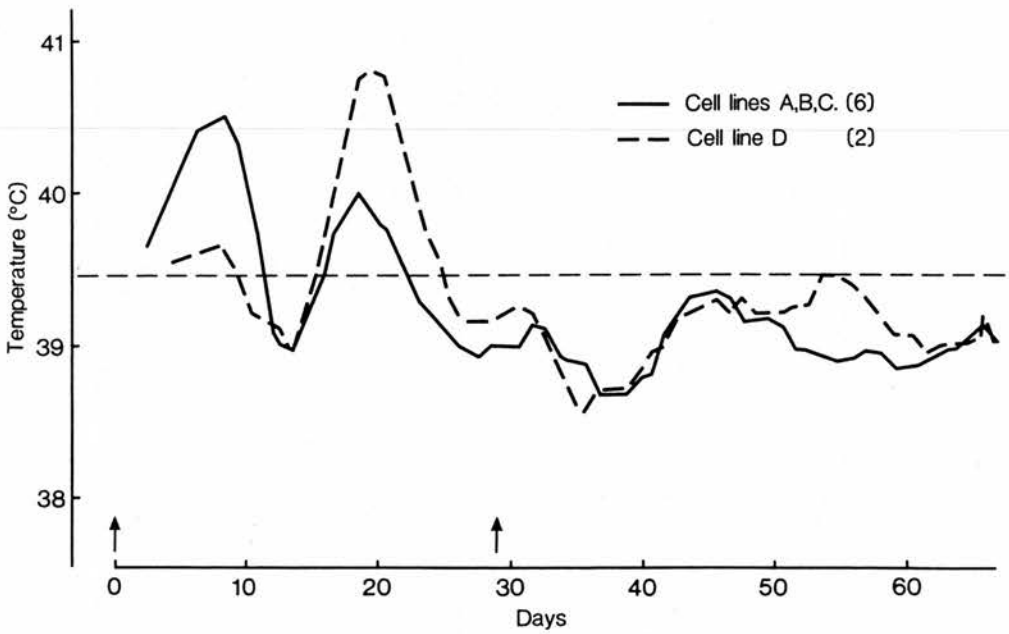


Fig. 9

The average daily temperature responses from the collated recipients of T.annulata infected cell lines A, B and C at a cell dose of 10^8 and the cell line D recipients (10^8 cells).
The temperature responses of the recipients of lines A, B and C showed a similar pattern and were therefore pooled for comparison with the responses of the recipients of line D at this cell dose.

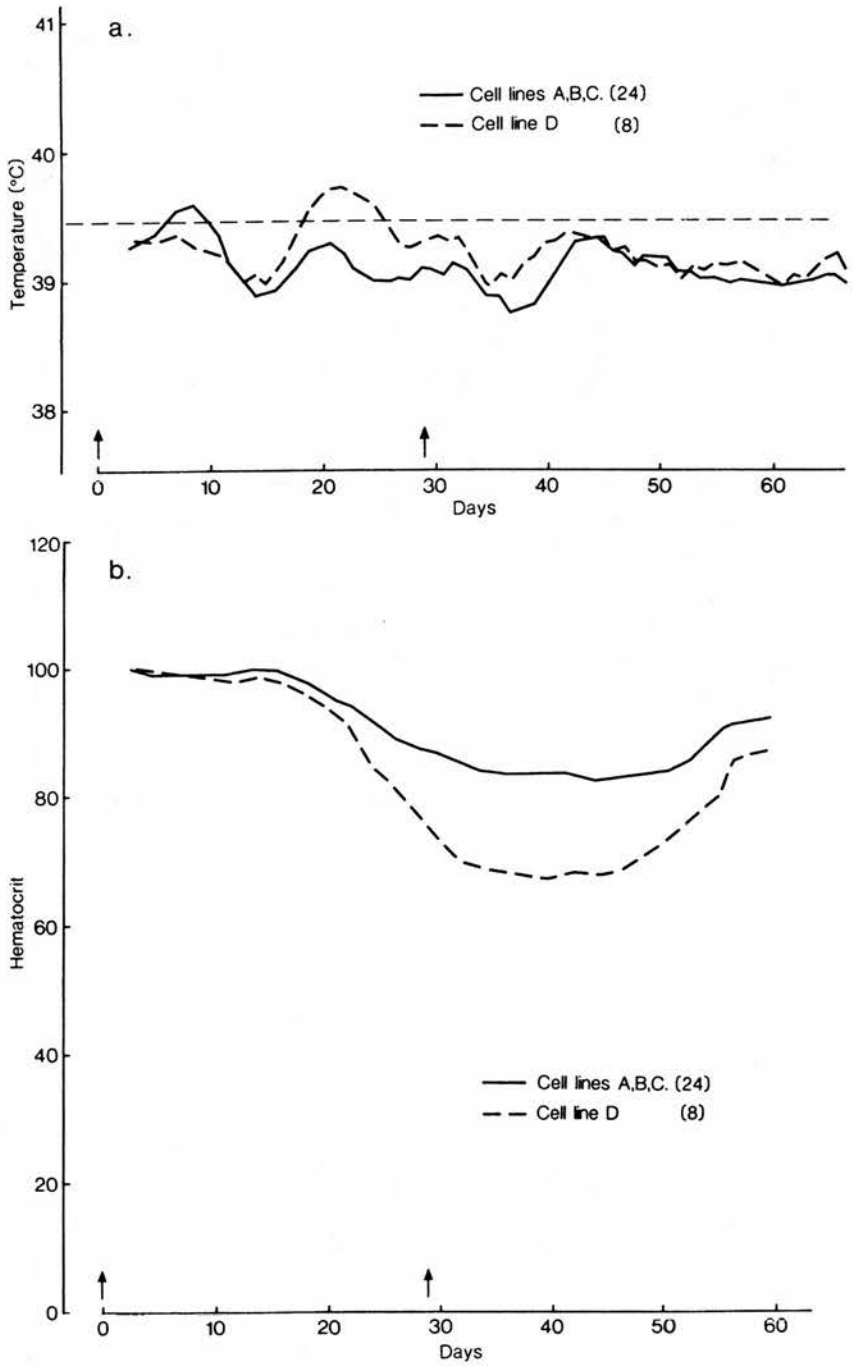


Fig. 10
The average temperature and haematocrit values of the pooled recipients of T.annulata cell lines A, B and C compared with those of cell line D. (All cell doses are included)

3.3.1.4 *mhc incompatibility*

With three of the cell lines (A,B and C) the BoLA MM recipients showed more severe clinical responses than the BoLA M, but with cell line D the response of the BoLA M and MM recipients were very similar (Figure 11a and b). Accordingly, in examining the effect of mhc matching at each cell dose, line D was excluded. The most striking effect of BoLA matching cell line and recipient occurred in the animals receiving 10^8 infected cells, where the BoLA MM recipients reacted more severely than the BoLA M (Figure 12a). This effect was observed to a lesser extent in the other dose groups (10^6 and 10^4) but in the lowest dose group (10^2) there was no apparent difference between the BoLA M and MM groups (Figure 12b, c and d). Temperature and haematocrit values during the patent disease period were examined and there is an obvious difference in the temperature peaks (Figure 12a) and a drop in the haematocrit value (not shown) in the 10^8 dose group. A t-test was used to assess differences between the BoLA M and MM recipients at each dose level and the results were found not to be significant, probably due to the large variation between animals and the small sample size.

3.3.2 Sporozoite challenge

The homologous stabilate challenge was administered to the 31 animals surviving the cell line inoculations and to 4 naive control animals. The four control animals all died within 20 days of receiving the challenge material, exhibiting symptoms of tropical theileriosis. They showed a severe and prolonged temperature response, a drop in haematocrit of 50%, 30% of cells from the lymph node draining the site of inoculation contained macroschizonts and 20% of erythrocytes were infected with the piroplasm form of the parasite.

In contrast the animals that had been previously immunised with the infected cell lines were all immune to the challenge. There were no obvious differences in the response between the BoLA M and MM recipients to the challenge.

3.3.3 Effects on productivity

The animals from this experiment have been followed for two years and their growth rate is similar to that of untreated animals kept in similar conditions. Three heifers and three bulls from this experiment have been mated together. Calves were produced and subsequent normal milk production was observed in the dams, who showed no ill effects throughout the pregnancy.

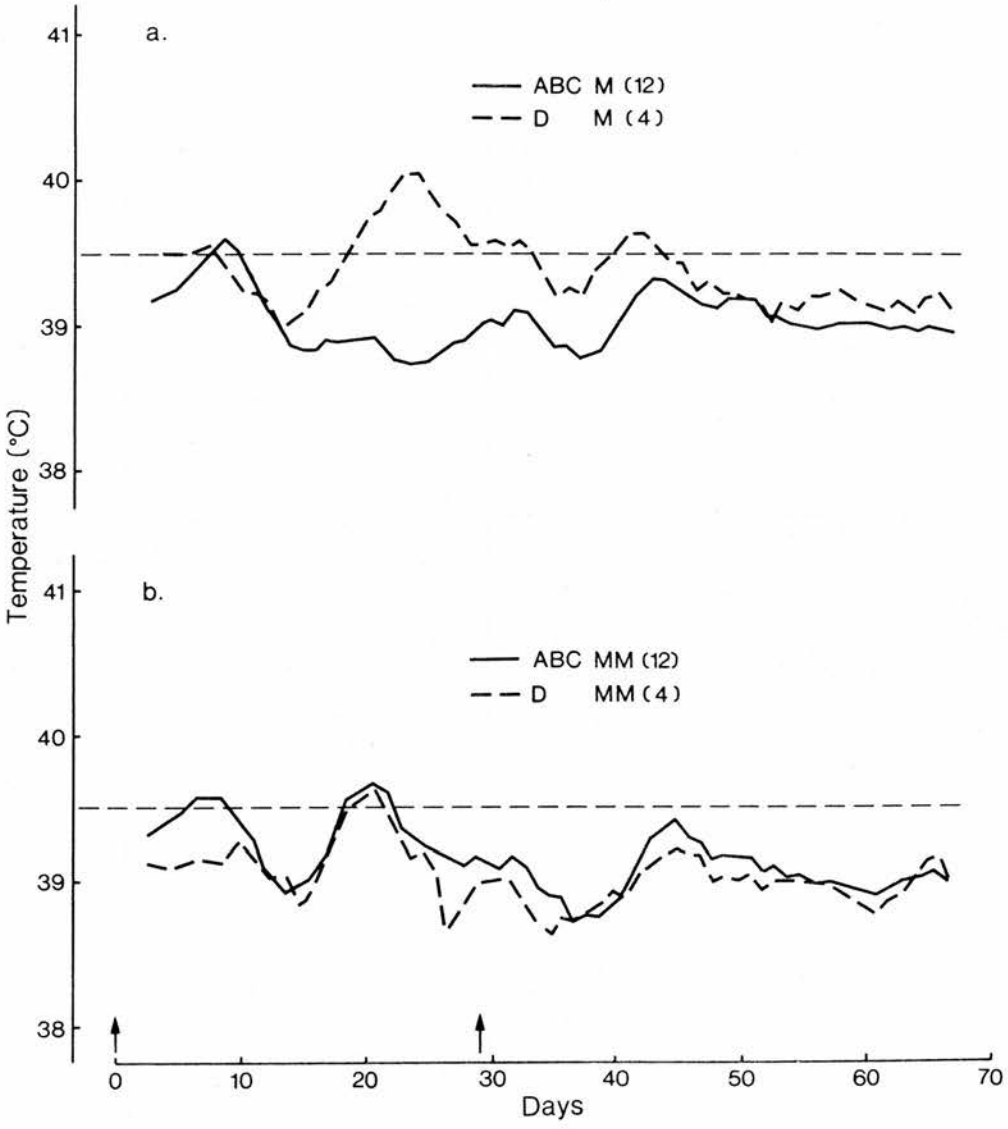
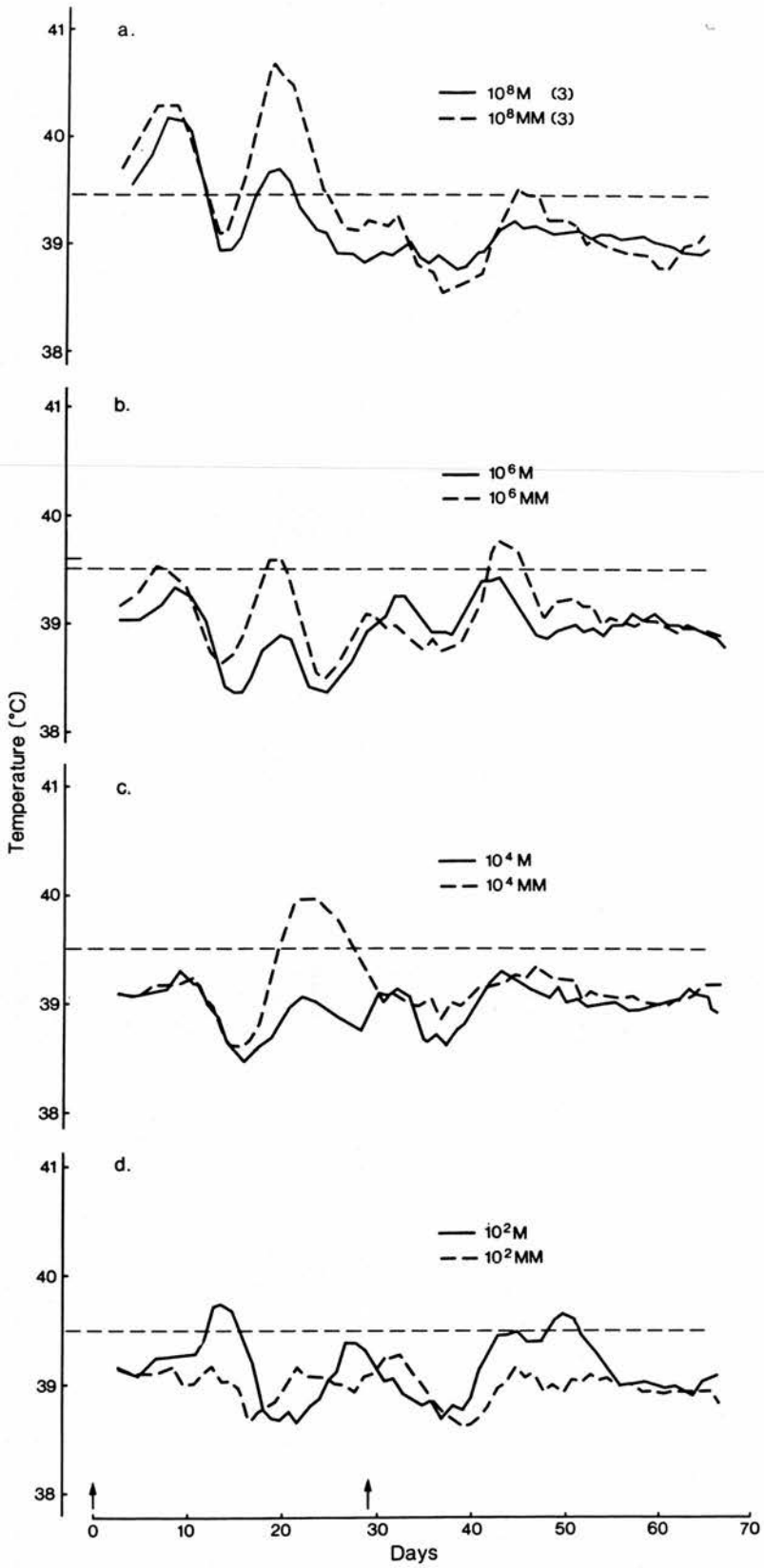


Fig. 11
a. and b. illustrate the average daily temperature responses of the collated recipients of cell lines A, B and C compared to cell line D. As there was little variation between lines A, B and C the results were collated.
a. Represents the BoLA matched recipients and b. represents the BoLA mismatched recipients.The broken line indicates a temperature of 39.5°C temperatures above this line were considered febrile. Febrile responses corresponded with a drop in haematocrit levels and detection of parasitaemia (not shown). The arrows indicate when the animals were inoculated with the cell lines (day 0) and challenged (day 29). The numbers in brackets indicate the numbers of animals in each group.

Fig. 12

a. b. c. and d. represent the average daily temperature responses of the 10^8,10^6,10^4 and 10^2 dose groups respectively. The black line represents the BoLA matched recipients.The broken line represents the BoLA mismatched recipients. This figure represents results taken from cell lines A, B and C only. Cell line D was excluded as it behaved differently from the other three lines.


a.
10^8M (3)
10^8MM (3)
b.
10^6M
10^6MM
c.
10^4M
10^4MM
d.
10^2M
10^2MM
Temperature (°C)
41
40
39
38
0
10
20
30
40
50
60
70
Days

3.4 DISCUSSION

The results of this experiment raise a number of questions about immunisation with *Theileria* infected lymphoblastoid cell lines. The cell lines used in this study were not attenuated, were established *in vitro*, and had been maintained in culture for only three weeks prior to their use. All four lines, at cell doses of 10^8, 10^6, 10^4 and 10^2, infected animals and conferred protection against a lethal, homologous sporozoite challenge. In countries where vaccination is well established the recommended dose, using cell lines initiated *in vivo* and attenuated by long term culture *in vitro*, is 1-5 x 10^6 cells (Hashemi-Fesharki and Shad-Del, 1973; Pipano, Klopfer and Cohen,1973; Pipano, 1977). Interestingly, in our experiment a cell dose of 10^6 was found to produce the least severe clinical reaction. The response of calves to varying numbers ($5x10^3$ to $5x10^6$) of attenuated schizont infected cells was investigated by Pipano (1974), who found that infection and immunisation was only achieved at cell doses of 10^5 or more. The superior infectibility of the cell lines used in our study may reflect the length of time they were maintained in culture. With the closely related parasite *T. parva* one of the observed changes in infected cell lines after long term maintenance *in vitro* was their loss of infectivity and immunogenicity for cattle *in vivo* (Brown, 1979).

An important consideration when looking at the effect of cell dose is that, once inoculated, the parasite infected cells multiply and establish infection within the cells of the host (Brown *et al.*, 1978a). In this experiment the onset of patent disease was delayed in the lower dose groups presumably because the infected cells had to multiply substantially to reach a level where the recipient animal showed disease symptoms. However, there was no obvious decline of severity of disease symptoms from the recipients of the highest dose of cells (10^8) to the lowest (10^2). Indeed none of the animals in the 10^8 dose group died of the infection and most surprisingly the only recorded death was in the 10^2 dose group.

It would therefore appear that infection and immunisation of animals can be achieved over a very wide range of cell doses. Interestingly, a different response occurs when animals are inoculated with titrated doses of *T. parva* sporozoites, where it is possible to induce predictable levels of infection over a narrow dose range (Cunningham *et al.*,1974; Radley *et al.*, 1974; Morrison *et al.*, 1981). This difference between the responses induced by inoculating parasitised cell lines and sporozoites may reflect the fact that sporozoites rapidly infect host cells (Fawcett *et al.*, 1982) without being impeded to any great extent by the host's immune system. The quantity of host cells that become infected can thus be directly correlated to the amount of sporozoite material inoculated. The unpredictability of the responses observed in the recipients of

parasitised cell lines may reflect the fact that the quantity of parasite the animal actually becomes infected with is not known. It will depend on the rate of parasite transfer from cell line, which may not necessarily correlate with the original cell dose inoculated. In the group of animals that received 10^8 infected cells, except the cell line D recipients, an initial temperature peak occurred between days 2-5 after inoculation of the cell lines. This was thought to be a reaction caused by the inoculation of large numbers of cells as opposed to an anti-parasite response, as the temperature response occurred in isolation of other disease symptoms. This same type of response was observed by Sharpe and Langley (1983) when they inoculated $1.5x10^9$ *Theileria annulata* infected cells into recipient animals. Anti-lymphocyte responses have been detected serologically as early as day 5 after inoculation of 10^8 *T. parva* infected cell lines (Wagner and Duffus, 1974).

Of the four cell lines used in the experiment line D appeared to be more pathogenic than lines A, B and C, showing marked differences both *in vitro* and *in vivo*. *In vitro* the cells grew in a suspension as opposed to the more adherent form observed in lines A, B and C. Line D also grew less rapidly in culture. A study described by Hooshmand-Rad and Hashemi- Fesharki (1968) noted that the more virulent strains of *T. annulata* were harder to establish and maintain as lymphoblastoid cell lines in culture. When line D was inoculated into the calves it was found to be the most pathogenic of the four cell lines. All four cell lines were established from the same stock of parasite material although line D was established using the first batch of infected ticks (batch 1) and stored in liquid nitrogen prior to use. Lines A, B and C were established from batch 2 ticks, which had been infected by feeding on an animal previously inoculated with a cell line established from batch 1. It could be argued that the parasite material became attenuated during passage through the animal - tick - cell line cycle, except that the sporozoites used to infect cell lines A, B and C were from batch 2, which provided the lethal challenge. A factor which may contribute to the virulence of a particular cell line is its composite lymphocyte subpopulation or subpopulations. Cell lines of differing phenotypes may exhibit different *in vitro* and *in vivo* characteristics. As cell line D was stored in liquid nitrogen and resuscitated prior to use, perhaps the process of cryopreservation selected for a particular lymphocyte subpopulation. At the time this experiment was done we did not have reagents which would allow identification of lymphocyte subpopulations within a cell line. Variation in the responses of cattle when inoculated with phenotypically distinct autologous *T. parva* infected cell lines has been reported (Lalor, Morrison and Black, 1986).

In this study all the animals became infected as a result of inoculation of various doses of *T. annulata* infected cells, regardless of whether they were BoLA M

or BoLA MM to the donor cell line. *Theileria annulata* thus differs from *T. parva* where it has been shown that very high doses of allogeneic infected cell lines (10^8) are required to consistently infect animals (Brown, 1981). When lower cell doses are used *T. parva* is only transmissible if the cell line is autologous or BoLA M to the recipient (Teale, 1983; Dolan *et al.*, 1984b). In the study reported here we were able to infect and immunise animals with as few as 10^2 allogeneic *T. annulata* infected cells.

Although all the surviving animals were consistent in their response to the challenge material their reactions to cell line immunisation were variable. There were differences observed between the BoLA M and BoLA MM recipients which were most apparent in the 10^8 dose group. Within this group the BoLA MM animals showed a more severe clinical response than the BoLA M group. A possible explanation for this difference may be in the specific priming of the immune system. It is generally accepted that in order to induce protective immunity the parasite must establish infection within the cells of the recipient (Pipano *et al.*, 1977; Brown *et al.*, 1978a; Emery *et al.*, 1981a).

We may consider that the induction of immunity occurs in two stages. Initially the immune response is primed by the antigens of the donor cell line which include both mhc antigens (Spooner and Brown, 1980) and some form of infection specific antigens (Shiels *et al.*, 1986a). After the parasite transfers and establishes infection in the host cells there is a second priming of the immune system by infection specific antigens seen in the context of self mhc. There is evidence that an important component of protective immunity in *Theileria* infections involves BoLA Class I restricted cytotoxic cells directed against the macroschizont infected cell (Preston, Brown and Spooner, 1983; Morrison *et al.*, 1986b; Goddeeris, Morrison and Teale, 1986). The difference between the BoLA M and MM groups may involve the specificity of the initial priming of the immune system to the antigens of the donor cell line. One may speculate that with the BoLA M group the primary stimulation of the immune system is by infection specific antigens "seen" in the correct mhc class I background on the donor cell line. After parasite transfer and infection of the recipient's cells the immune system will already have been primed to this particular parasite antigen/mhc class I combination (unlike the BoLA MM group) and will therefore be better able to control the resultant infection. In the lower dose groups the differences between the BoLA M and MM groups were not so distinctive. Perhaps with the lower cell doses parasite transfer and infection of host cells takes place before the antigens of the donor cell line can prime the immune system.

As already described the cell line D recipients in general had more severe disease symptoms than the other cell line groups. Interestingly, there was no difference in reaction between the BoLA M and MM recipients of line D. The BoLA matching in this case was based on the reactivity of PBM with sera recognising the BoLA workshop

specificities w13 and w20. However the PBM from the cell line donor WB81, was found to only react with one of the three sera that recognise w20. Therefore it is possible that the recipient animals matched to this cell line were only matched at the w13 allele. The relatively poor matching of this cell line to the recipient animals may explain the similarity in response between the BoLA M and MM recipients within this cell line group. More complete mhc matching would have included the Class II antigens, however at the time this experiment took place it was not possible to identify these antigens to the same degree of specificity as the Class I antigens. The logistics of the experiment also made it impossible to include autologous infected cell lines.

3.5 CONCLUSION

In conclusion this study has shown that susceptible animals can be protected against a lethal challenge using lymphoblastoid cell lines infected and transformed *in vitro* using a Moroccan stock of *T. annulata* (albeit of limited antigenic variety) at doses ranging from 10^8 to 10^2 cells. The differing responses observed in the recipient animals indicate that cell line/recipient interaction is an area of extreme complexity, the outcome of which involves many contributory factors, some of which have been discussed above. This study has also conclusively demonstrated that there is no histocompatibility barrier in the infection and immunisation of animals using *T. annulata* infected lymphoblastoid cell lines, which can be achieved over a very wide range of cell doses. Following the success of this preliminary experiment Dr H. Ouhelli and colleagues at Institut Agronomique et Veterinaire in Rabat, have obtained permission to test this cell line vaccine on a larger scale on selected government farms.

The ability of allogeneic *T. annulata* infected cell lines to immunise cattle at low cell doses illustrates one of the major differences between the two closely related parasites *T. annulata* and *T. parva*. The superior infectibility of *T. annulata* may be because this parasite can more readily transfer from one cell to another, or because the two parasites preferentially infect different cells of the immune system. Comparative studies between the two parasites may lead to a better understanding of cell line/recipient interactions and suggest ways of circumventing the histocompatibility barrier which arises in the case of *T. parva*.

Some of the work reported in this chapter has been accepted for publication:

1. The effect of MHC compatibility between parasite infected cell line and recipient in immunisation against tropical theileriosis. E.A. Innes, H. Ouhelli, R.A. Oliver, S.P. Simpson, C.G.D. Brown and R.L. Spooner, *Parasite Immunology* (1988) 10

2. The effect of dose and line on immunisation of cattle with lymphoblastoid cells infected with *Theileria annulata*. H. Ouhelli, E.A. Innes, C.G.D. Brown, A.R. Walker, S.P. Simpson and R.L. Spooner, *Veterinary Parasitology* (In press).

CHAPTER 4

The development and specificity of cytotoxic effector cells in animals infected with *T. annulata* sporozoites, autologous infected cell lines or allogeneic infected cell lines

AIMS

To measure the development and specificity of cytotoxic effector cells, against the intracellular stage of the parasite, in animals immunised with sporozoites, autologous *T. annulata* infected cells or BoLA mismatched *T. annulata* infected cells and subsequently challenged with a lethal dose of heterologous sporozoites.

To establish whether the detection of cytotoxic effector cells correlates with recovery of the animal.

To determine the specificity of the cytotoxic cells.

4.1 INTRODUCTION

The purpose of a vaccine is to induce an immune response that will be protective against challenge with the virulent pathogen. In the case of *Theileria annulata* it is possible to infect and immunise animals against the disease using bovine lymphocytes which have been infected and transformed with *T. annulata* sporozoites (Pipano, 1977). Although such parasitised cell lines have been successfully used to protect animals in both field and experimental conditions the mechanisms of protective immunity induced are poorly understood. Studies on animals recovered from natural infection or by controlled infection with sporozoites have emphasised the importance of cell mediated immune mechanisms (Singh, Jagdish and Gautam, 1977; Ahmed *et al.*, 1981b; Samed *et al.*, 1983), in particular a role for putative BoLA restricted cytotoxic cells (Preston, Brown and Spooner, 1983) similar to the HLA restricted CTL in infectious mononucleosis, a lymphoproliferative disorder in man (Moss *et al.*, 1981). The BoLA mhc class I antigens are known to act as targets for alloreactive CTL (Teale *et al.*, 1985, Spooner *et al.*, 1987) and restricting elements for *Theileria parva* specific cytotoxic T lymphocyte CTL clones (Goddeeris *et al.*, 1986).

In contrast to the limited information on immune responses in *T. annulata* infection, extensive work has been done with the closely related parasite *T. parva*, the causative agent of East Coast fever, where it has been shown that an important component of protective immunity involves mhc restricted CTL directed against the macroschizont infected cell (Morrison *et al.*, 1986b). Interest in *T. parva* has probably arisen because of the relative difficulty (compared to *T. annulata*) of infecting and immunising animals using infected cell lines (Brown, 1981). Recent work has suggested a role for the mhc in this context which has different implications for both parasites. In the case of *T. parva*, cell line immunisation was consistently successful if autologous or BoLA matched cell lines were used (Morrison *et al.*, 1981; Teale, 1983; Dolan *et al.*, 1984). Immunisation with allogeneic infected cell lines was only possible using very high cell doses, 10^7-10^9 (Pirie, Jarrett and Crighton 1970; Brown *et al.*,1978b; Brown 1981). The results presented in chapter 3 of this thesis show that with *T. annulata* as few as 10^2 allogeneic infected cells are sufficient to infect and protect animals.

Clearly there are differences between the two parasites so direct extrapolation of the data from *T. parva* infection to *T. annulata* may be misleading.

As there are no reports in the literature of the measurement of cytotoxic effector cells in animals immunised with *T. annulata* infected cell lines it was decided to examine this area in an attempt to understand the mechanisms which confer on these cell lines the ability to induce protective immunity. We chose to examine cytotoxic cells

as these are known to be effective in other intracellular infections (Zinkernagel and Doherty 1979) including *T. parva* infection (Morrison *et al.* 1986b).

In this study we wished to ask the following questions:

Does the detection of cytotoxic cells correlate with recovery of the animal?

What is the specificity of the cytotoxic cells?

Are there any differences in the magnitude and/or specificity of cytotoxic cells between groups of animals immunised with sporozoites (natural infection) or autologous or BoLA mismatched *T. annulata* infected cell lines?

4.2 MATERIALS AND METHODS

4.2.1 Preparation of effector cells

4.2.1.1 *Isolation of peripheral blood mononuclear cells (PBM)*

An isolation technique was used to prepare effector cells for the cytotoxicity assay, responder cells for *in vitro* culture with parasitised stimulator cells, and uninfected blast cells for use as targets in the cytotoxicity assay. The technique is as described by Spooner *et al.*(1987) with a few modifications.

Venous blood was collected aseptically in 20% v/v acid citrate dextrose (ACD). Mononuclear cells were separated from the blood by layering 25ml of blood in ACD onto 15ml of sodium metrizoate/ficoll solution, SG.1.077 (Lymphoprep, Nycomed), followed by centrifugation for 30 minutes at 1030g. The leucocyte layer was harvested at the plasma/ficoll interface and washed three times in Alsevers solution by repeated centrifugation (10 mins at 170g) and resuspension and washed once in RPMI 1640 (Gibco Biocult). The Alsevers solution was prepared from deionised distilled water by the addition of citric acid (550mg per litre), glucose (20500mg per litre), sodium chloride (4200mg per litre) and sodium citrate (8000mg per litre).

4.2.1.2 *Effector cells*

Effector cells for the cytotoxicity assay were prepared as described above (section 4.2.1.1.) from blood taken from infected animals throughout the experimental period (direct assay). The cells were maintained in RPMI 1640 supplemented with 10% foetal calf serum (FCS), (Assay medium). The FCS was from a batch that had been previously screened to give low levels of spontaneous ^{51}Cr release from the target cells.

4.2.1.3 *In vitro stimulation of effector cells*

As the duration and the magnitude of the cytotoxic response in the experimental animals was unknown at the start of the experiment a second assay system was developed in an attempt to enrich for cytotoxic effector cells. In this assay (indirect assay) a proportion of the PBM effector cells (section 4.2.1.2.) were cultured *in vitro* at a cell concentration of $2.5x10^6$ cells per ml with gamma- irradiated (5000rads from a ^{137}Cs source) *T. annulata* infected stimulator cells. The cells were cultured in a mixed lymphocyte culture (MLC) medium comprised of RPMI 1640 supplemented with 2mM L-glutamine and 10% FCS, $5x10^{-5}g$ per ml gentamycin (Gibco Biocult) and $5x10^{-5}M$ 2-mercaptoethanol (BDH Biochemicals).

4.2.1.4 *Creation of cytotoxic T cell line*

A cytotoxic T cell line was generated from animal N53 by stimulating PBM collected on day 72 of the experiment (9 days after the final sporozoite challenge), with irradiated N53 *T. annulata* (Morocco) infected cells as described in section 4.2.1.3. After 6 days the cells were harvested and viable cells were separated using density gradient centrifugation over sodium metrizoate/ficoll solution, as described for preparation of PBM (section 4.2.1.1), and washed once in RPMI 1640. The cells were then stimulated for a second time with irradiated N53 parasitised cells as in the primary culture. After a further 6 days viable cells were separated as before and stimulated for a third time with irradiated N53 parasitised cells supplemented with 20% T cell growth factor (TCGF) obtained as a supernatant from a transformed gibbon lymphoid cell line, MLA-144, which releases a factor biologically and chemically similar to human TCGF. (Rabin *et al.* 1981). Viable cells recovered following this third stimulation were tested for cytotoxic function using the cytotoxicity assay described in section 4.2.4. The cytotoxic T cell line was maintained by restimulation at weekly intervals with irradiated N53 parasitised cells and supplements of TCGF.

4.2.2 *Theileria* infected cell lines

Parasite infected lymphoblastoid cell lines were established *in vitro* by infection of normal bovine peripheral blood mononuclear cells (PBM) with *T. annulata* sporozoites using the method of Brown (1983) described in section 2.2.2.4.

The parasite material used was from the Gharb stock of *T. annulata* which was isolated in Morocco (TaM) (Ouhelli, 1985). The resultant persistently infected cell lines were used both to infect the recipient animals and to act as target cells in the cytotoxicity assay.

4.2.3 Uninfected lymphoblasts

Uninfected lymphoblasts were established by stimulating normal bovine PBM once with 2mg per litre of concanavalin A (Sigma Type IV-S) in MLC culture medium as described previously (section 4.2.1.3.). The cells were incubated for 48-72 hours in a 5% CO_2 humidified incubator at 37°C, then washed twice in phosphate buffered saline (PBS) by repeated suspension and centrifugation (10 minutes at 170g). The blast cells were separated by centrifugation over sodium metrizoate/Ficoll solution S.G. 1:077 (Lymphoprep, Nycomed) for 30 minutes at 1030g then washed in culture medium (10 minutes at 170g). The resultant blast cells were cultured at a concentration of 2.5×10^6 per ml in MLC culture medium containing a supplement of 10-20% of TCGF (section 4.2.1.4.). Uninfected lymphoblasts were used as target cells in the cytotoxicity assay.

4.2.4 Cytotoxicity assay

Effector cells were prepared as described in sections 4.2.1.2., 4.2.1.3. and 4.2.1.4.

Target cells comprised *T. annulata* infected lymphoblastoid cell lines in log phase growth (24 hours after a 2:10 subculture) and uninfected lymphoblasts (24-48 hours after stimulation by TCGF). The cells were washed in assay medium by centrifugation for 10 mins at 300g and resuspended at a concentration in the assay medium of $2x10^7$ per ml. An equal volume of isotope in medium (^{51}Cr as sodium chromate, Amersham international Ltd., added to give a 1 mCi per ml solution) was added to the cell suspension which was then incubated with agitation for 1 hour at 37°C. Following incubation the labelled cells were washed three times in assay medium (300g for 10 mins), after which the cells were resuspended to give a final cell concentration (based on prelabelling counts) of $2.5x10^5$ per ml.

The cytotoxicity assay was performed by adding 0.05 ml of target cell suspension to 0.2 ml of effector cell suspension in duplicate in 96-well round-bottomed tissue culture plates (Sterilin). The concentration of effector cells used was varied to give different effector:target ratios (10^7 per ml for a 40:1 ratio, $5x10^6$ per ml for 20:1, and $2.5x10^6$ per ml for 10:1). As a control, to measure spontaneous release of ^{51}Cr, the labelled target cells were incubated in triplicate in assay medium alone. Maximum ^{51}Cr release was determined by the addition of 0.2 ml of aqueous 1% sodium dodecyl sulphate (SDS, Sigma) to 0.05 ml of labelled target cells in triplicate. The test plate was incubated at 37°C for 4 hours in a 5% CO_2 incubator after which time the plates were centrifuged (300g for 10 mins) and 0.125 ml of supernatant from each well was removed

to be counted over a period of one minute in a Packard auto-gamma 5650 (Canberra Packard).

The % ^{51}Cr release from the target cells caused by cytotoxic effector cell function was calculated as follows:

$$\text{specific cell lysis} = \frac{\text{(test release - spontaneous release)}}{\text{(maximum release - spontaneous release)}} \times 100\%$$

4.2.5 Monoclonal antibodies and immunofluorescence test

The cytotoxic T cell line created as described in section 4.2.1.4. was analysed using a panel of monoclonal antibodies (MAbs) which react with distinct bovine lymphocyte subpopulations. Details of the MAbs used are given in section 2.3. An indirect immunoflourescence test based on that described by Lalor *et al.* (1986) was used to stain the cells prior to their analysis using a fluorescence activated cell sorter (FACS IV, Becton Dickinson) as described in sections 2.3.1 and 2.3.2.

4.2.6 Inhibition of cytotoxicity with antisera

Monospecific alloantisera defining the BoLA w10 specificity and a monoclonal antibody (MAb) J11 which recognises a monomorphic bovine mhc class II determinant were tested for their capacity to block the effector function of the cytotoxic T cell line, created from N53, by interaction with the target cells. For blocking, 0.1 ml of an appropriate dilution of antibody in assay medium (BoLA w10 alloantisera 1:4; J11 MAb 1:100) was added to 0.05 ml of ^{51}Cr labelled target cells in the wells of a 96-well round-bottomed culture plate and incubated for 1 hour at 37°C. This procedure resulted in no loss of viability of effector cells as assessed by trypan blue exclusion. The cells were then incubated and harvested as for a normal ^{51}Cr release assay (section 4.2.4).

4.2.7 Histocompatibility (BoLA) typing

All the recipient animals and infected cell lines to be used in the study were assigned BoLA specificities (Table 4.1) according to their reaction to a panel of alloantisera (developed at IAPGR Edinburgh) including all the internationally agreed workshop specificities to date (Spooner *et al.*, 1979; Anon, 1982). The method is described in section 2.1.

4.2.8. Animals and experimental design

The animals used in the experiment were Ayrshire or Friesian x Ayrshire calves aged 4-5 months. Prior to inoculation PBM were taken from the animals and

TABLE 4.1 **BoLA specificities of animals and parasitised cell lines**

Peripheral Blood Mononuclear Cells	BoLA Specificity
Animal	
N53	10, 20
N54	13, -
N57	7, 20
N58	2, 6.1
N60	2, -
N61	10, 20

T. annulata Infected Cell Lines	BoLA Specificity
Cell line	
N53TaM	10, 20
N54TaM	13, -
N57TaM	7, 20
N58TaM	2, 6.1
N60TaM	2, -
N61TaM	10, 20
110TaM	3, 4

TaM : *Theileria annulata* (Morocco)

used to establish the cell lines and evaluate base line cytotoxicity measurements. The animals were divided into three pairs and on day 0 one pair received 0.1 ml of a sporozoite stabilate of *T. annulata* (Morocco) at 0.2 tick equivalents per ml, another 10^6 autologous *T. annulata* Morocco (TaM) cells (1 ml), the third 10^6 BoLA mismatched (MM) TaM infected cells (1 ml) (Table 4.2). A fourth pair of animals were used as controls for the sporozoite stabilate challenge. The inoculum was injected subcutaneously over the right shoulder of each animal. On day 30 the same inoculum was injected contralaterally and on day 62 all the animals, along with the two susceptible controls, were challenged with a pool of stabilate material comprising *T. annulata* sporozoites of the Ankara stock from Turkey (TaA) (Schein, 1975) and the Hissar stock from India (TaH) (Gill *et al.*, 1980) . Throughout the experimental period the animals were monitored for their clinical reactions, including daily rectal temperature (a febrile response being 39.5°C) and haematocrit values, white blood cell counts and parasitaemia recordings three to seven times weekly, as described in section 2.2.4. The animals were also monitored for the development of cytotoxic responses, the specificity of which was determined using both autologous and allogeneic parasite infected and uninfected blast cells of known BoLA specificites.

4.3 RESULTS

4.3.1 Primary inoculation

4.3.1.1 *Sporozoite recipients*

4.3.1.1.1 Cytotoxic response - direct assay (Figure 13)

In the direct assay the effector function of the PBM was assessed immediately after the blood sample was taken from the animal.

No cytotoxic activity was apparent against any of the target cells prior to inoculation of the sporozoites. The first response appeared on day 9 after inoculation, reaching a peak betweeen days 16-20. The target cells killed most efficiently by the cytotoxic effector cells were both the autologous and BoLA class I matched TaM. infected cells.

4.3.1.1.2 Cytotoxic response - indirect assay (Figure 14)

In the indirect assay the effector function of PBM extracted from the animals was examined after culture for 6 days *in vitro* with irradiated TaM infected stimulator cells. Prior to inoculation of the sporozoites it was possible to generate cytotoxic cells

TABLE 4.2 **Experimental design**

Inoculum	Source of effectors	Target cell	Relation between target and effector cells
Sporozoites	N53	N53 TaM	Autologous TaM
	N53 - *N53 TaM*	N61 TaM	BoLA M TaM
		N58 TaM	BoLA MM TaM
		N53	Autologous blast
	N61	N61 TaM	Autologous TaM
	N61 - *N61 TaM*	N53 TaM	BoLA M TaM
		N58 TaM	BoLA MM TaM
		N61	Autologous blast
Autologous *T. annulata* infected cells	N57	N57 TaM	Autologous TaM
	N57 - *N57 TaM*	N58 TaM	Autologous TaM
		N57	Autologous blast
	N58	N58 TaM	Autologous TaM
	N58 - *N58 TaM*	N57 TaM	BoLA MM TaM
		N58	Autologous blast
BoLA mismatched *T. annulata* infected cells	N54	N54 TaM	Autologous TaM
	N54 - *N54 TaM*	N57 TaM	Cell line TaM (N57 TaM)
	N54 - *N57 TaM*	110 TaM	BoLA MM TaM
		N54	Autologous blast
		N57	Cell line blast (N57)
	N60	N60 TaM	Autologous TaM
	N60 - *N60 TaM*	N57 TaM	Cell line TaM (N57 TaM)
	N60 - *N57 TaM*	110 TaM	BoLA MM TaM
		N60	Autologous blast
		N57	Cell line blast (N57)

TaM : *Theileria annulata* (Morocco)

N53 - *N53 TaM* : N53 effector cells cultured for 6 days *in vitro* using N53 TaM cells as stimulators

BoLA M : BoLA class I matched BoLA MM : BoLA class I mismatched

blast : uninfected blast cell Cell line blast : blast cells derived from N57

Cell line TaM : the BoLA MM cell line (N57 TaM) used to immunise

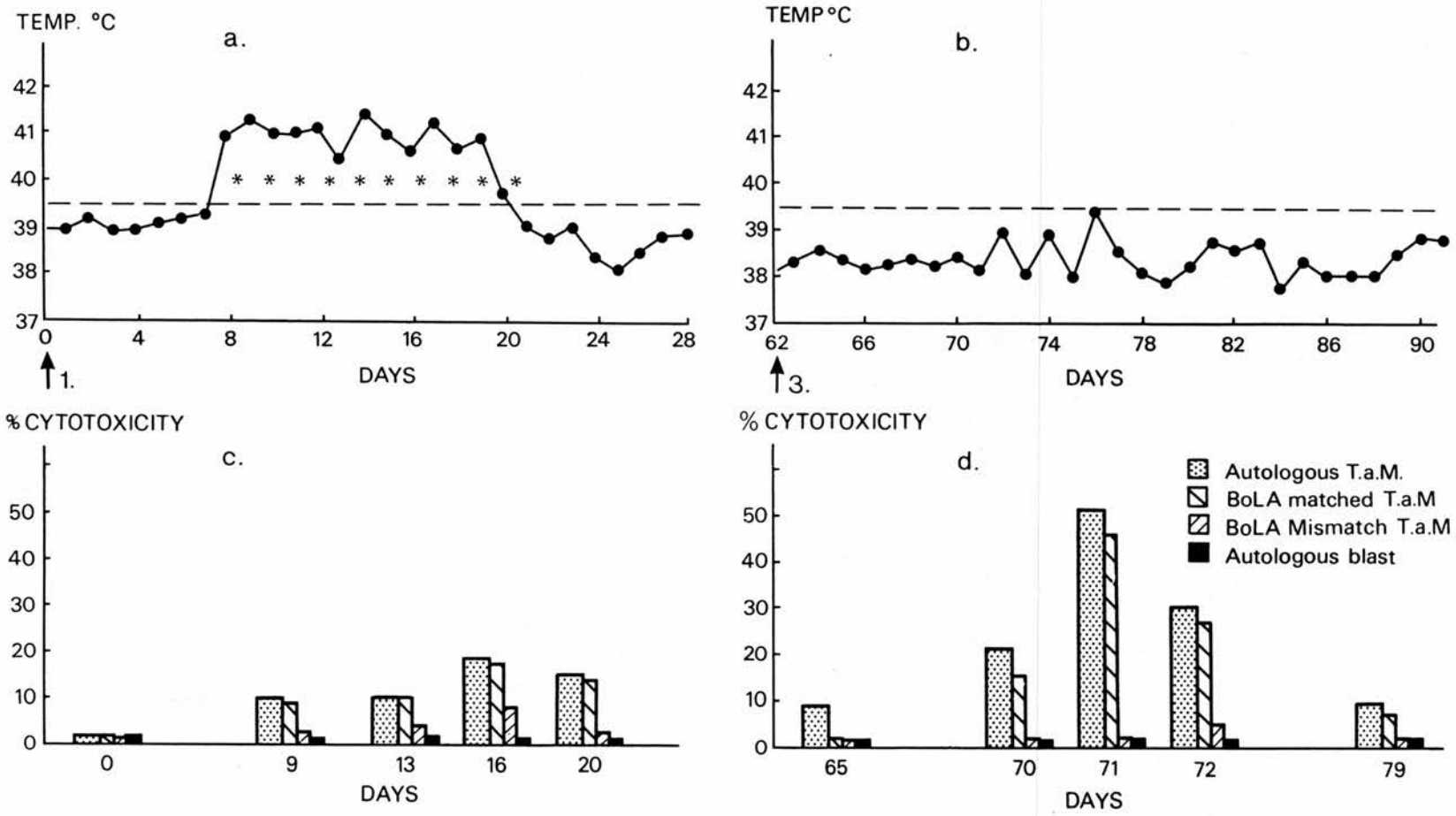
The counts in replicate wells in the Cr^{51} release assay were all within $\pm$ 5% of the mean value.

In figures 13, 15 and 16 the cytotoxicity values for each animal within a group were all within $\pm$ 5% of the mean value.

Fig. 13

a. and b. The average daily temperature response of the sporozoite recipient group after inoculation 1. (0.1ml TaM stabilate at 0.2 tick equivalents/ml) on day 0 and inoculation 3. (2ml TaA stabilate + 2ml TaH stabilate) on day 62. The arrows represent the timing of the inoculation. Temperatures aboove 39.5°C were considered febrile and detection of parasitaemia is indicated by *.

c. and d. The direct cytotoxic function of effector cells taken from the sporozoite recipient group on various days throughout the experimental period. Each animal within the group was assayed separately and the results were averaged. The effector:target ratio was 40:1 and the key indicates the various target cells (illustrated in the histograms) used in the assay.


TEMP. °C
a.
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DAYS
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b.
42
41
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38
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62
66
70
74
78
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86
90
DAYS
3.
% CYTOTOXICITY
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50
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10
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9
13
16
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DAYS
% CYTOTOXICITY
d.
Autologous T.a.M.
BoLA matched T.a.M
BoLA Mismatch T.a.M
Autologous blast
50
40
30
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10
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65
70
71
72
79
DAYS

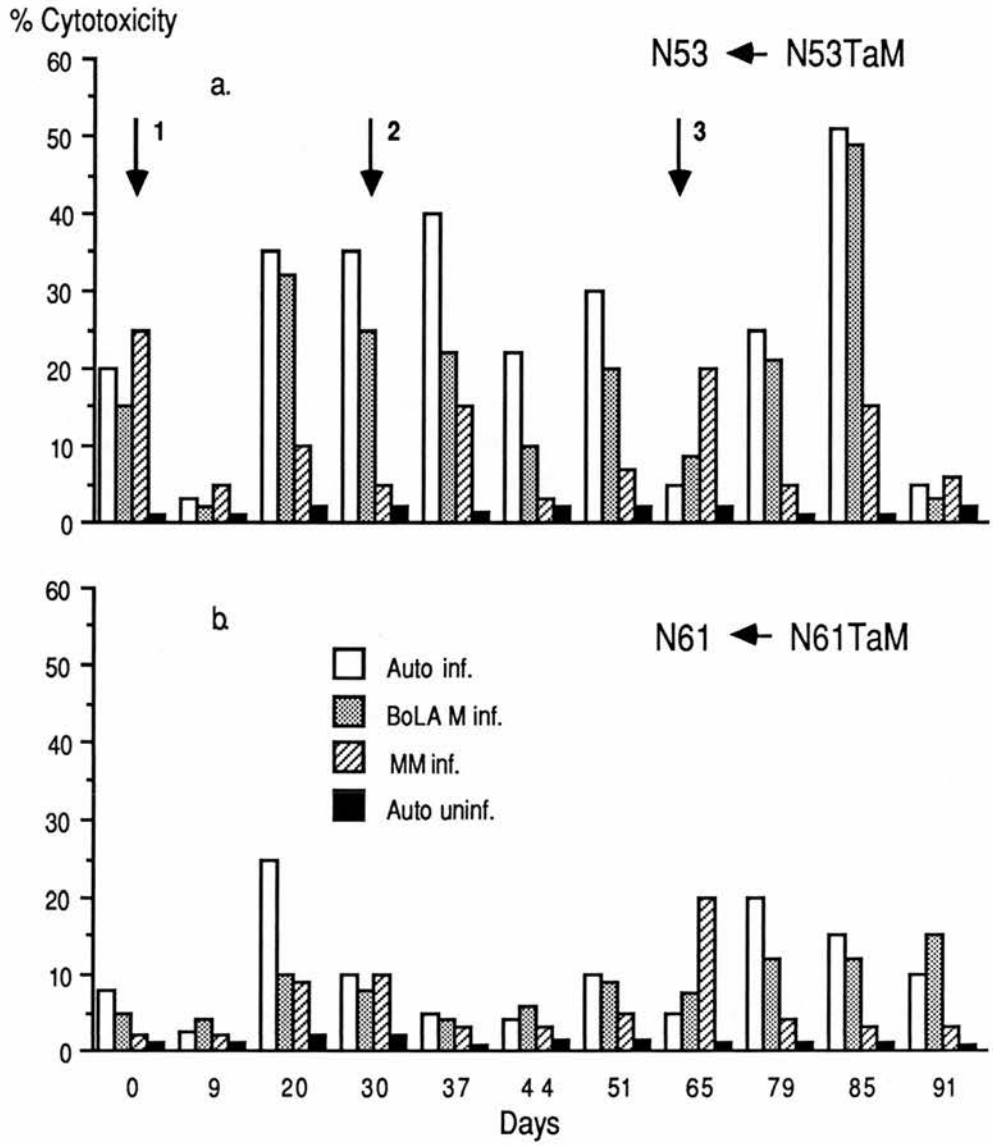


Fig. 14

The cytotoxic responses of effector cells taken from the recipient group (animals N53 and N61 respectively) after culture for 6 days in vitro using autologous infected cells (irradiated) as stimulators.The resultant functional activity is illustrated on the day the effector cells were isolated from the animal. The effector: target ratio was 20:1 and the key indicates the various target cells used in the assay.
The arrows indicate inoculation 1. (0.1ml TaM.stabilate at 0.2 tick equivalents 1ml), 2. (As inoculation 1) and 3. (2 ml TaH. stabilate + 2ml TaA. stabilate).

by co-culture of PBM with irradiated TaM infected cells but they were promiscuous in their functional capacity, being able to kill all of the parasite infected targets regardless of their mhc specificity. This was most apparent in the response of animal N53 (Figure 14a). With animal N61 (Figure 14b) very little cytotoxic effector function was generated prior to inoculation of the sporozoites. After infection with the sporozoites both animals in the group showed an increased cytotoxic response when PBM extracted on day 20 were cultured *in-vitro* for 6 days. In both cases the predominant response was against the autologous TaM infected target cells (stimulator cells) and interestingly with animal N53 the cytotoxic response was directed equally against the BoLA matched TaM infected target cell.

4.3.1.1.3 Clinical response (Figure 13 and Appendix 2)

In both animals a febrile response was detected from day 7 after inoculation to day 20, during which time severe parasitaemia values were recorded, reaching a peak on day 15 with 10-15% of red blood cells infected. Macroschizonts were first detected in both animals on day 6, reached peak numbers between days 14 and 15, and were undetectable after day 22. Haematocrit values dropped to almost 50% of pre-inoculation recordings by day 17 and recovered to pre-inoculation levels by day 26. There was a transient drop in white blood cell count (WBC), between days 6-10 in both animals, to 50% of pre-inoculation recordings. Both animals had to be treated once on day 15 with 5mg per kg of the theilericidal drug buparvaquone (720c Coopers Animal Health, Berkhamsted). By day 27 post-inoculation both animals had recovered.

4.3.1.2 *Autologous TaM cell line recipients*

4.3.1.2.1 Cytotoxic response (direct assay) Figure 15

No cytotoxic activity was detected in the animals prior to inoculation of the autologous TaM infected cell lines. The first cytotoxic response was detected on day 9 after inoculation and was directed against the autologous TaM infected target cell line. Cytotoxicity reached a peak on day 16, the response being equally effective against the autologous and the BoLA mismatched TaM infected target cell lines. On day 20 the only detectable response was against the autologous TaM infected cell line.

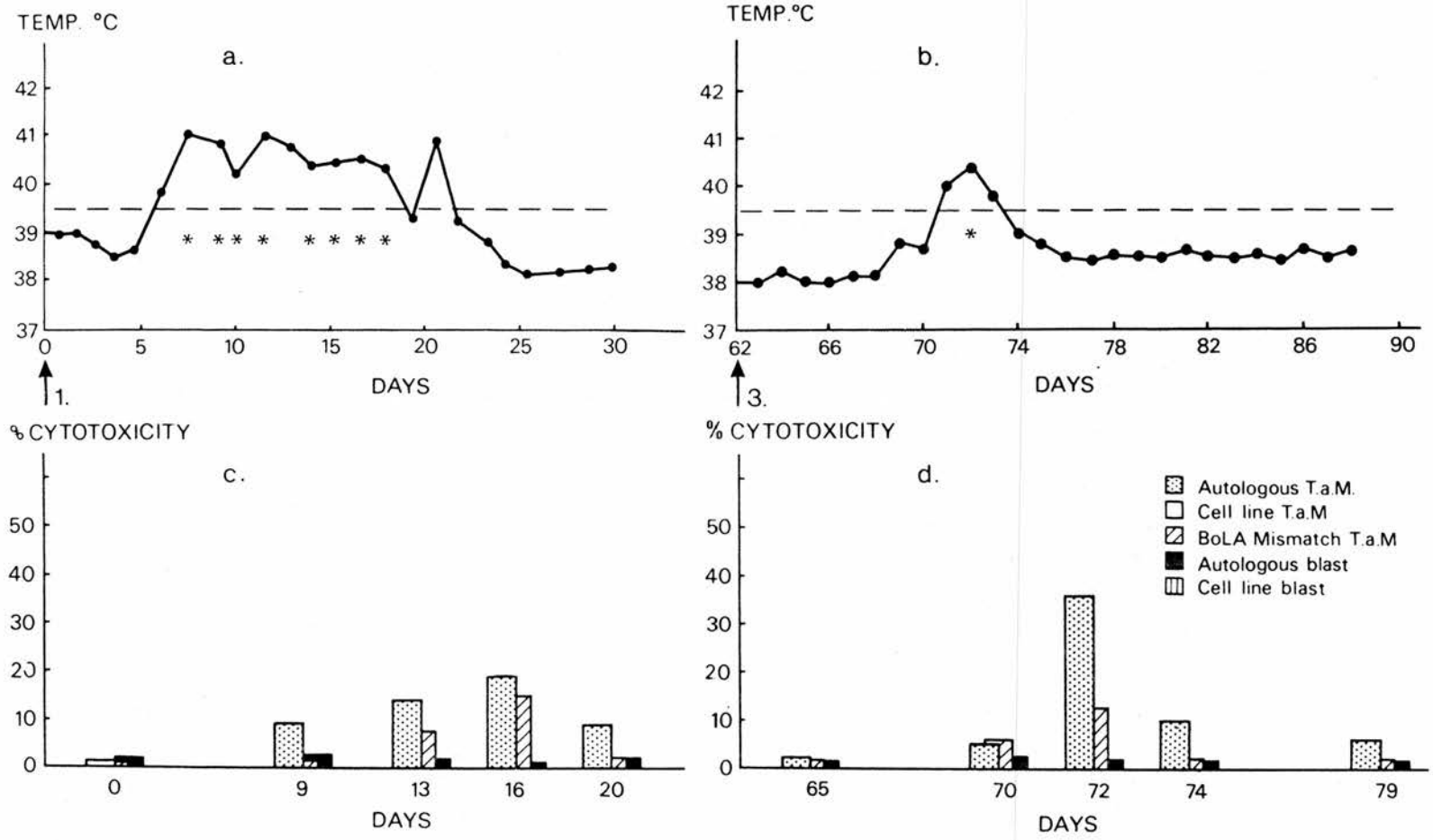
4.3.1.2.2 Cytotoxic response (indirect assay)

Cytotoxic effector cells generated after *in vitro* stimulation from both animals in this group did not differ markedly either in efficiency or specificity from those generated before inoculation (results not shown).

Fig. 15

a. and b. The average daily temperature response of the autologous T. annulata infected cell line group after inoculation 1. (10^6 autologous infected cells) on day 0 and inoculation 3. (2ml TaA stabilate + 2ml TaH stabilate) on day 62.

c. and d. The direct cytotoxic function of effector cells taken from the autologous T.annulata infected cell line group on various days throughout the experimental period. Each animal within the group was assayed separately and the results were averaged. The effector:target ratio was 40:1 and the key indicates the various target cells (illustrated in the histograms) used in the assay.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9
13
16
20
DAYS
% CYTOTOXICITY
d.
50
40
30
20
10
0
65
70
72
74
79
DAYS
Autologous T.a.M.
Cell line T.a.M
BoLA Mismatch T.a.M
Autologous blast
Cell line blast

4.3.1.2.3 Clinical response (Figure 15 and Appendix 2)

In both animals a febrile response was detected from day 6; this lasted for approximately 12 days. During this time severe parasitaemia recordings were detected (days 13-15), with up to 25% of red blood cells infected with the parasite, and the haematocrit values dropped to almost 50% of base line values. One animal (N57) was treated on day 14 with buparvaquone, administered as before. By day 27 the animals had recovered, with temperature and haematocrit levels returned to normal.

4.3.1.3 *BoLA mismatched TaM cell line recipients (Figure 16 and Appendix 2)*

4.3.1.3.1 Cytotoxic Response (direct assay) Figure 16

On day 9 there was a substantial peak of cytotoxicity which was specifically directed against the cell line (N57 TaM) used to immunise the animals. Interestingly the same degree of lysis was also observed against the uninfected lymphoblasts derived from N57. On day 13 the main response was still directed against the parasite infected and uninfected target cells derived from N57, but a moderate cytotoxic response was now developing against the autologous TaM infected target. By day 23 the predominant response was against the autologous TaM infected target cell.

4.3.1.3.2 Cytotoxic response (indirect assay) Figure 17

In the BoLA MM group effector cells were stimulated in the *in vitro* cultures both by autologous and by N57 TaM infected cells (the cell line used to immunise the animals). Pre-inoculation PBM cultured with either stimulator tended to produce effector cells that would kill all of the parasite infected targets in an apparently non-specific manner, although the response of the PBM cultured with N57 TaM was predominantly directed against the N57 TaM target. By day 23 the cytotoxic responses generated in both cultures were more specific. The PBM cultured with autologous TaM infected stimulator cells produced effector cells which would specifically kill the autologous TaM target cell (Figure 17a and c) and the PBM cultured with N57 TaM infected cells would specifically kill both the N57 TaM target and the N57 uninfected blast target (Figure 17b and d). The pattern of target cell killing in the effector cells generated by co-culture with irradiated N57 TaM cells, suggests that the response is directed against target antigens on the N57 cell and is not a parasite specific response.

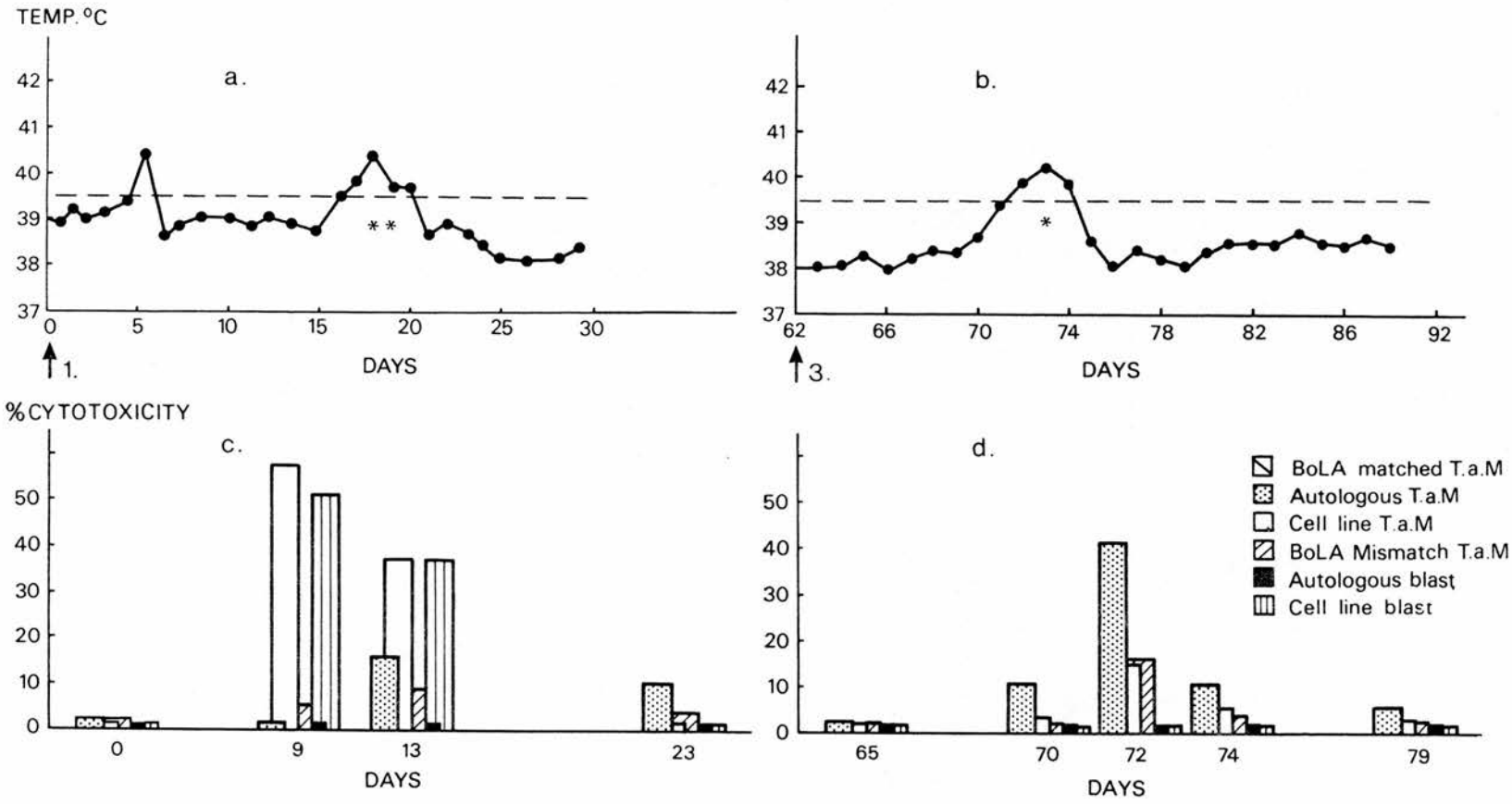
4.3.1.3.3 Clinical response (Figure 16 and Appendix 2)

In contrast to both the sporozoite and autologous TaM infected cell line recipients the BoLA MM TaM infected cell line recipients showed two transient febrile temperature peaks, one at day 6 post inoculation the other between days 17-20. At day

Fig. 16

a. and b. The average daily temperature response of the BoLA mismatched (MM) T.annulata infected cell line group after inoculation 1. (10^6 BoLA MM infected cells (N57 TaM) on day 0) and inoculation 3. (2ml TaA stabilate + 2ml TaH stabilate on day 62).

c. and d. The direct cytotoxic function of effector cells taken from the BoLA MM T.annulata infected cell line group on various days throughout the experimental period. Each animal within the group was assayed separately and the results were averaged. The effector:target ratio was 40:1 and the key indicates the various target cells (illustrated in the histograms) used in the assay.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74
78
82
86
92
*
3.
DAYS
%CYTOTOXICITY
c.
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40
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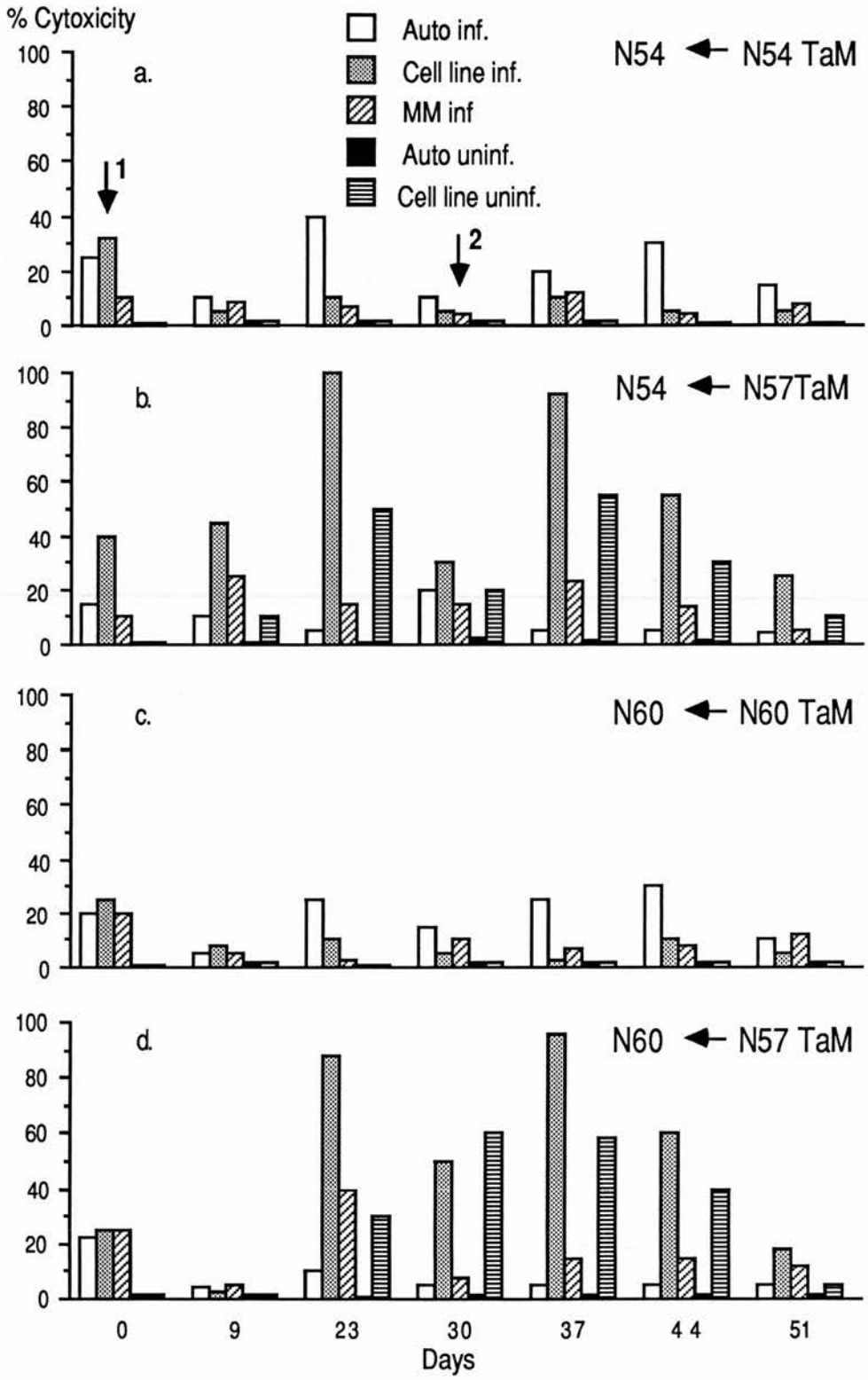
20
10
0
0
9
13
23
DAYS
d.
65
70
72
74
79
DAYS
BoLA matched T.a.M
Autologous T.a.M
Cell line T.a.M
BoLA Mismatch T.a.M
Autologous blast
Cell line blast

Fig. 17

The cytotoxic responses of effector cells taken from the BoLA MM infected cell line group (animals N54 and N60 respectively) after culture for 6 days in vitro using as stimulators both autologous (a. and c.) and BoLA MM infected cell line (N57 TaM) used to inoculate the animals (b. and d.) The resultant functional activity is illustrated on the day the effector cells were isolated from the animal.
The effector:target ratio was 20:1 and the key indicates the various target cells used in the assay. The arrows indicate inoculation 1 (10^6 BoLA MM infected (N57 TaM) and 2 (as 1).


% Cytoxicity
Auto inf.
Cell line inf.
MM inf
Auto uninf.
Cell line uninf.
a.
N54 ← N54 TaM
1
2
b.
N54 ← N57TaM
c.
N60 ← N60 TaM
d.
N60 ← N57 TaM
100
80
60
40
20
0
0
9
23
30
37
44
51
Days

20 low levels of parasitaemia were recorded (1% of erythrocytes infected) with an average haematocrit drop of 20% from base line recordings. The only clinical symptom that accompanied the first temperature peak was a 50% drop in the white blood cell count; this recovered to base line levels at the same time as the temperature returned to normal, only to fall again at around day 20 when other clinical symptoms of the disease were apparent. By day 28 both the animals had recovered.

4.3.1.3.4 Parasite transfer

Evidence for parasite transfer from the donor cell line to recipient cells was obtained from both the animals in the BoLA MM group. On day 20 the parasite was reisolated *in vitro* by establishing a parasite infected lymphoblastoid cell line from PBM taken from the recipient animals in this group using the methods described by Brown (1983). The resultant cell lines were BoLA class I typed and it was found that the parasite was now in the cells of the recipient animals.

4.3.2 Secondary inoculation

On day 30 all the groups of animals received a second inoculation which was a repeat of the primary immunisation. The animals were monitored for a further 33 days during which time no clinical symptoms of the disease or cytotoxic responses were detectable using the direct cytotoxicity assay (results not shown).

4.3.2.1 *Cytotoxic response (indirect assay)*

4.3.2.1.1 Sporozoite recipients

The responses of the effector cells generated in the sporozoite group after *in vitro* culture are shown in Figure 14a and b. In animal N53 the response is predominantly directed against the autologous and BoLA matched TaM target cells, peaking at day 37 (day 7 after the second inoculation) and returning to baseline levels by day 44. With animal N61 (Figure 14b) insignificant levels of cytotoxicity were detected.

4.3.2.1.2 BoLA mismatched TaM infected cell line recipients

When the effector PBM were cultured with irradiated autologous TaM infected cells a specific response against the autologous TaM target cell was detected on day 44 (14 days after the second inoculation), but this response was not much higher than pre-inoculation levels (Figure 17a and c). However when the same effector PBM were cultured with irradiated N57 TaM infected cells a very strong and specific response was generated against both the N57 TaM target cell and the uninfected N57 target cell

This response peaked on day 37 (day 7 after inoculation of the cell lines), returning to baseline levels by day 51 (Figure 17b and d).

4.3.3 Heterologous sporozoite challenge

The responses of each group after a heterologous challenge comprising sporozoites of *T. annulata* Ankara and *T. annulata* Hissar are shown in Figures 13, 15 and 16 and Appendix 2. In all groups the clinical and cytotoxic responses (direct assay) were very similar. On days 9-10 post challenge there was a transient peak of cytotoxicity predominantly directed in all cases against the autologous TaM infected cell line target. The strongest response was in the sporozoite recipient group, with equal killing against both the autologous and BoLA matched TaM infected cell targets. Very mild clinical responses were detected in all three groups, with the sporozoite group showing the least clinical response. The two susceptible control calves died of acute theileriosis within 20 days as a result of the heterologous sporozoite challenge. The *in vitro* culture assay (indirect) was discontinued after inoculation 3 as heterologous parasites had now been introduced to the animals. It was decided to concentrate on the direct cytotoxicity assay and the generation of a CTL line.

4.3.4 Cytotoxic T cell line

A cytotoxic T cell line was generated from animal N53 as described in the materials and methods section (section 4.2.1.4.). The effector function of this cell line is shown in Figure 18. The cytotoxic response was directed towards the autologous TaM infected target cell and two different BoLA mhc class I matched TaM infected targets. To confirm the involvement of bovine mhc class I molecules in restricting the cytotoxicity, experiments were conducted to test whether or not lysis could be blocked by pre-incubation of the target cells with antibodies recognising the restricting mhc class I molecule. Pre-incubation of the autologous TaM infected target cell with alloantiserum recognising BoLA w10 specificity reduced cytotoxicity by approximately 95% (Figure 18). A MAb J11, which recognises a monomorphic determinant on bovine mhc class II molecules, did not block the cytolysis (Figure 18).

A phenotypic analysis of the CTL line is given as follows:

MAb	putative specificity	% of cells positive
J11	monomorphic class II	52
IL-A11	BoT4	62
IL-A17	BoT8	37
IL-A24	monocyte/macrophage	1
B5/4	B cell	1

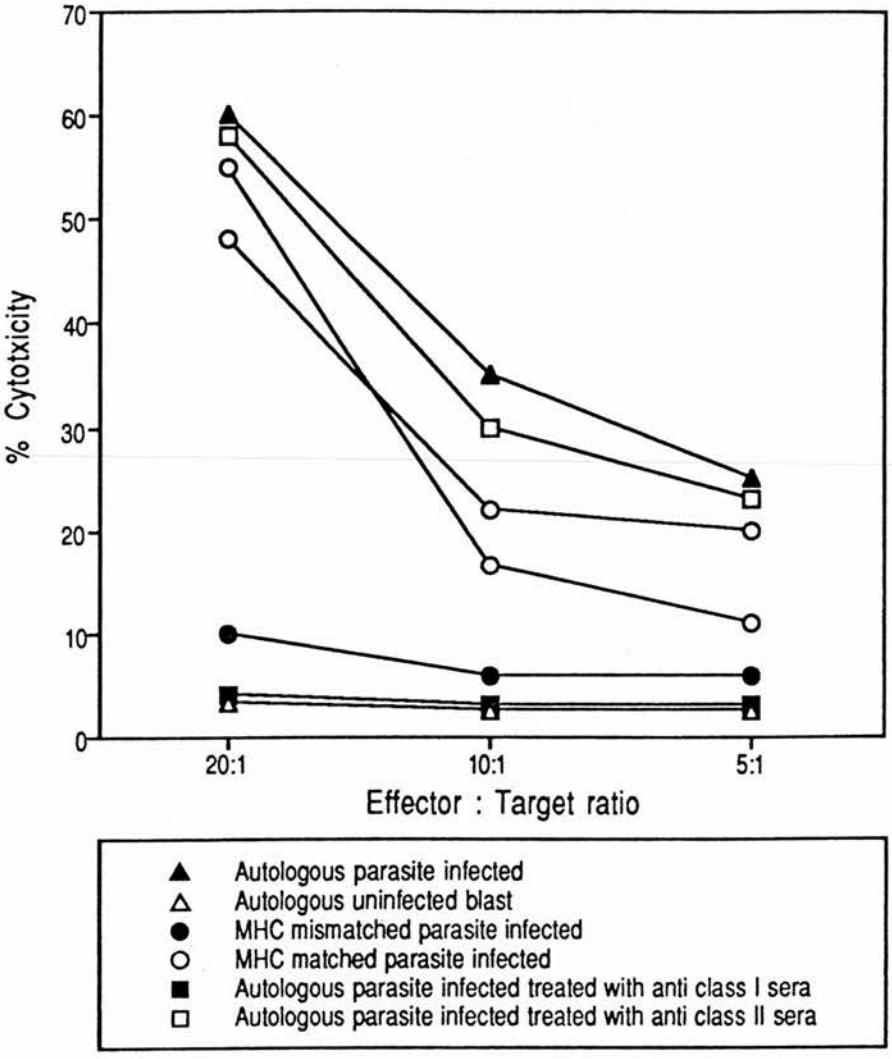


Fig. 18
The effector function of a CTL line, (created by stimulating PBL from N53 (sporozoite group) with irradiated infected cells and IL2), at various effector:target ratios.
The target cells are indicated in the key.

Evidence that the BoT8 subpopulation may be mainly responsible for the cytotoxic activity was obtained by removing the BoT4 population of T cells by treatment with IL-A11 and rabbit complement as follows: $2x10^6$ cells (0.5 ml) were incubated for 30 minutes at room temperature with MAb IL-A11 (0.5 ml) diluted 1:100 in MLC culture medium. Following the incubation 0.5 ml of rabbit complement was added and incubated at room temperature for a further 30 minutes. The cells were washed and resuspended in MLC culture medium. The resultant BoT4- CTL line showed a magnitude and specificity of response similar to the untreated CTL line (results not shown).

4.4 DISCUSSION

These results show that it is possible to detect cytotoxic effector cells in animals infected with *T. annulata* and that the occurrence of these cells coincides with remission of infection. The three groups of animals which had been immunised by different inoculums of parasite material all showed the same cytotoxic response after receiving the final sporozoite challenge (lethal to two naive control animals). This response was parasite specific, apparently mhc class I restricted, and coincided with resistance to the challenge. By creating a CTL line it was possible to confirm that the cytotoxic effector cells were of (BoT4-) T cell origin and were restricted by bovine mhc class I antigens. However each of the groups of animals was effectively immunised using different inoculations of parasite material. The sporozoite group, representative of naturally infected animals, was compared with the two groups of animals immunised with infected cell lines. The autologous infected cell line recipients were included as a control in examining the effect of allogeneity when immunising animals with BoLA MM infected cells.

This chapter raises a number of points for discussion concerning the complex interaction between *T. annulata* infected cell lines and recipients which results in the induction of protective immunity. Although this study deals primarily with *T. annulata* some reference to *T. parva* is made as a comparison.

The most dramatic difference between the three groups occurred after the primary inoculation where the response of the BoLA MM group differed markedly from the other two groups. By analysis of the target cells lysed *in vitro* it appeared that the initial cytotoxic response in the BoLA MM group was some form of graft rejection directed against the lymphocyte antigens of the donor cell line (Cerottini and Brunner, 1974). It is of interest to note that a serological anti-lymphocyte response was reported in animals immunised with *T. parva* infected cell lines derived from unrelated animals (Wagner and Duffus, 1974; Duffus, Wagner and Preston, 1978). Later in the course of

infection, in the experiment reported here, the specificity of the cytotoxic response in the BoLA MM group changed, becoming directed against the recipient's own cells infected with the parasite (autologous TaM). Presumably the changing specificity of the cytotoxic response reflects a change in the antigens being "presented" to the immune system, the initial response being directed against the antigens of the donor cell line (N57 TaM). After parasite transfer and infection of the recipient's cells, which is believed to be a prerequisite for the development of protective immunity (Pipano *et al.*, 1977; Brown, 1981), the immune response received a second priming, this time by infection specific antigens in the context of self mhc antigens. In contrast one might expect that (provided no novel antigens are acquired in culture) autologous infected cell lines may be accepted as autografts and that parasite infection of the recipient's cells need not occur for the infection specific antigens to be presented to the immune system in the correct mhc background. It was not possible using our BoLA typing methods to ascertain whether any schizont infected cell lines, reisolated from animals within the autologous group, originated from the donor cell line or as a result of infection of the recipient's own cells. However it may be possible to determine whether schizont transfer takes place in immunisation with autologous infected cell lines by immunising animals with cloned cells of a particular phenotype, then subsequently phenotyping schizont infected cells reisolated from the animal.

In comparing the clinical responses of the three groups of animals after the first inoculation of the infected cell lines both the sporozoite recipients and the autologous cell line recipients became very ill with theileriosis (3 animals had to be treated) whereas the BoLA MM group showed comparatively mild clinical symptoms. The major difference in the cytotoxic response of the three groups was the specific graft rejection response detected in the BoLA MM group. Although this response is not parasite specific it may play an important role in the development of the infection within the animal and consequently in the induction of protective immunity. This may be achieved as a result of direct cytotoxic action on the donor parasite infected cells by the alloreactive CTL, thereby effectively reducing the amount of parasite material for transfer and infection of host cells, making it easier for the immune system to cope with the resultant infection. Alternatively this alloreactive response may be beneficial indirectly by recruiting cells of the immune system to the site of infection by the induction and action of non-specific mediators such as T cell growth factors (Parmiami, Sensi and Fossati, 1985). This "non specific" stimulation or mobilisation of the immune system (which includes T cells, macrophages, B cells, NK cells, and so forth) may be advantageous in the subsequent priming of a parasite specific immune response, presumably after the parasite establishes infection within the cells of the recipient.

Previous workers have attempted to protect animals against *Theileria* infection using non-specific immunopotentiating agents such as *Bacillus* Calmette Guerin (BCG) or *Propionebacterium acnes* (*C. parvum*) which are known to stimulate macrophage or Natural Killer cell function (Roitt, Brostoff and Male, 1986). It was interesting to note that while moderate success was achieved when *C. parvum* was used to protect against *T. annulata* sporozoite challenge (Manickam, Dhar and Singh, 1983) prior immunisation with BCG did not afford protection against a *T. parva* sporozoite challenge (Dolan, Brown and Cunningham, 1980).

A second challenge with the same dose of infected cells as in the first inoculation produced no detectable clinical or cytotoxic reaction in any of the recipients. This may be because the immune response generated by the first inoculation effectively prevented infection by this second homologous challenge (Pipano *et al.*, 1977; Hooshmand- Rad, 1978). No cytotoxicity was detected, which may reflect the prompt action on the infected cells of other components of the immune response, such as humoral immunity which was not measured in this study.

Complement fixing antibodies specific to the mhc class I antigens on the immunising cell line N57 were detected only in the BoLA MM group (M. Kachani, personal communication). Serological anti-cell responses have also been reported in animals immunised with *T. parva* infected cell lines (Wagner and Duffus, 1974; Duffus, Wagner and Preston, 1978). It is possible, however, that a transient cytotoxic response may have been missed by sampling on the wrong day. Results from the *in vitro* culture of PBM (indirect assay) suggest that in the BoLA MM group precursor or primed cytotoxic cells specific for N57 cells (not parasite specific) were being "boosted" as a result of the second inoculation. In the same group of animals precursor cytotoxic cells specific for the autologous TaM infected targets were not so prevalent, which provides further evidence that the animals may not have become infected as a result of the secondary inoculation. This result may have important implications for revaccination using the same allogeneic infected cell line.

The heterologous sporozoite challenge produced a very similar response in all three groups of cattle, with a peak of cytotoxicity commencing at day 9 after challenge and restricted to the autologous parasite infected target. This peak of cytotoxicity coincided with remission of a mild infection and supported a role for parasite specific CTL restricted to the mhc antigens of the recipient in protective immunity (Preston, Brown and Spooner, 1983). Interestingly immunisation with *T. annulata* Gharb (Morocco) afforded protection against both *T. annulata* Ankara (Turkey) and *T. annulata* Hissar (India). As a full battery of autologous target cells infected with the three different *T. annulata* stocks was not available for each animal it was difficult to

assess the cross reactivity of the cytotoxic cells between each parasite stock (Goddeeris *et al.*, 1986). However, a suitable autologous target cell infected with *T. annulata* Ankara was available for one of the recipients in the autologous group. In this case there were similar levels of cytotoxicity detected against both the autologous *T. annulata* Gharb infected cells and the autologous *T. annulata* Ankara infected cells (results not shown). This may suggest some antigen common to these two *T. annulata* stocks which is recognised by the cytotoxic cells. Sergent *et al.* (1945) noted that immunity to a challenge with a homologous stock of *T. annulata* was stronger than that of a heterologous stock although it was not clear whether this should be attributed to antigenic differences between the stocks or simply to differences in virulence. In a more recent study (Gill *et al.*, 1980), it was shown that variable but nonetheless effective cross protection was achieved between different *T. annulata* stocks of similar virulence. The ability of geographically distinct stocks of *T. annulata* to cross protect has important implications for vaccination; lack of cross protection between various *T. parva* strains is an important limiting factor in vaccination against East Coast Fever (Irvin *et al.*, 1983).

The functional specifity and phenotypic analysis of the CTL line created from one of the sporozoite immunised animals provides further evidence that the cytotoxic response found in these animals was mhc class I restricted. Firstly the CTL line only recognised and killed infected target cells which were autologous to or shared BoLA class I specificities with itself. Secondly the effector function of the cells appeared to reside in the BoT4- subpopulation. Recent studies on bovine alloreactive T cell clones has indicated that BoT4- CTL are mhc class I restricted and BoT4+ CTL are mhc class II restricted (Teale *et al.*, 1986). Thirdly the effector function of the CTL line was effectively blocked by the addition of anti-mhc class I sera.

4.5 CONCLUSION

In conclusion this study shows that it is possible to demonstrate cytotoxic effector cell function from PBM isolated from animals infected with *T. annulata*. Recovery from a heterologous sporozoite challenge lethal to two naive control animals was accompanied by the detection of parasite specific mhc class I restricted cytotoxic cells in animals immunised with sporozoites, autologous and BoLA mismatched infected cells.

The generation of a CTL line was achieved by repeated stimulation *in vitro* with autologous irradiated parasitised cells and TCGF. The specificity of this line suggested that it was analogous to the cytotoxic cells detected *in vivo* during recovery from *T. annulata* infection. The ability to propagate and enrich for cytotoxic cells *in vitro*

will allow more detailed analysis of this response and provide an important tool in identifying the target antigens on the surface of the infected cell.

Animals immunised with 10^6 BoLA MM infected cells exhibited very mild clinical symptoms compared with the severe responses in those animals immunised with sporozoites or 10^6 autologous *T. annulata* infected cells. The major difference in the immune responses between these groups was the specific alloreactive cytotoxic response, directed against the antigens of the immunising cell, which was detected only in the allogeneic infected cell line recipients. The precise role of this "graft rejection" response in the subsequent infection of host cells and development of protective immunity is unclear. The relative ease of immunisation using allogeneic *T. annulata* infected cell lines compared to allogeneic *T. parva* infected cell lines is unlikely to be a direct effect of mhc incompatability between cell line and recipient, which is common in both cases, but in the ability of each parasite to subsequently transfer and infect the host's cells (Morrison *et al.*, 1986a). The limiting factor in this reaction may be the availability of suitable cells in the immediate microenvironment permissive to infection by either parasite.

Preliminary *in vitro* studies have shown that *T. parva* preferentially infects T cells and can infect B cells but not macrophages (Lalor, Morrison and Black, 1986; Baldwin *et al.*, 1988), whereas the host cell preferences of *T. annulata* have never been properly examined. In the final chapter of this thesis the comparative ability of *T. annulata* and *T. parva* sporozoites to infect phenotypically and functionally defined cell subpopulations *in vitro* is examined.

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The development and specificity of cytotoxic cells in cattle immunised with autologous or allogeneic *Theileria annulata* infected lymphoblastoid cell lines. Innes, E.A., Millar, P., Brown, C.G.D. and Spooner, R.L., *Parasite Immunology* (1989) 11.

CHAPTER 5

Characterisation of the target cell for infection *in vitro* **by** *Theileria annulata* **and** *Theileria parva*

Aims

To determine the phenotype of established *T. annulata* or *T. parva* infected cell lines using monoclonal antibodies specific for bovine leucocyte subpopulations.

To use the same monoclonal antibodies to purify bovine cell subpopulations to test their susceptibility to infection *in vitro* by *T. annulata* or *T. parva* sporozoites.

5.1 INTRODUCTION

The tick borne diseases tropical theileriosis and East Coast fever are caused by the protozoan parasites *Theileria annulata* and *Theileria parva* respectively. Tropical theileriosis is transmitted by ticks of the genus *Hyalomma* and occurs over a vast area stretching from Morocco to China. East Coast fever, transmitted by *Rhipicephalus appendiculatus*, is endemic in East and Central Africa and has a mortality rate of 95-100%, compared to 40-60% for tropical theileriosis (Brown, 1983).

A feature common to both parasites is their ability to infect and transform cells of the immune system, with the parasite dividing in synchrony with the host cell (Hulliger *et al.*, 1964). This property of the infected cells, which may be established by infection either *in vivo* or *in vitro*, allows them to be maintained *in vitro* as continuously growing cell lines without the addition of exogenous growth factors. It is, however, easier to establish and maintain *T. annulata* infected cell lines than *T. parva* infected lines in culture (Brown, 1987). Macroschizont infected cells are of central importance in the pathogenesis of both *T. annulata* and *T. parva* infection (Irvin and Morrison, 1987) and there is evidence that they are targets for immune attack by cytotoxic T lymphocytes (CTL), which are believed to be an important protective immune mechanism in both diseases (Preston, Brown and Spooner, 1983; Morrison *et al.*, 1986b). *T. annulata* infection has been successfully controlled in both experimental conditions and large scale field trials using macroschizont infected lymphoblastoid cell lines as vaccines (Pipano, 1977). However this method has proved difficult in the case of *T. parva*, where it has been shown that histoincompatibility between cell line and recipient may prevent immunisation (Teale, 1983; Dolan *et al.*, 1984b). Histoincompatibility does not appear to pose the same problem in the case of *T. annulata*, where there is no evidence that an allogeneic infected cell line is any less infective to the host than an autologous or a BoLA matched infected cell line (Chapter 3, this thesis). With either parasite the induction of protective immunity is believed to depend on the establishment of infection in the cells of the recipient. This must involve some form of parasite transfer from the donor cell line to the host cell (Pipano *et al.*, 1977; Brown *et al.*, 1978b; Emery *et al.*, 1982). It is not known whether the observed differences between *T. annulata* and *T. parva* lie in the intrinsic capacity of the parasites to transfer to host cells (Allison, 1981; Morrison *et al*, 1986a) or whether they reflect differences in the host cell types that permit infection by either parasite.

The characteristics of the target cells each parasite infect are poorly understood. Recent studies have examined the surface phenotype markers on established *T. parva* cell lines, which were negative for surface immunoglobulin (Ig)

(Duffus, Wagner and Preston, 1978) but positive for various T cell markers (Pinder, Withey and Roelants, 1981; Naessens *et al.*, 1985; Lalor, Morrison and Black, 1986) and both mhc class I and class II antigens (Spooner and Brown, 1980, Morrison *et al.*, 1986a). To our knowledge, no data has been published on the phenotype(s) of *T. annulata* infected cells.

Recently, monoclonal antibodies (MAbs) which react with distinct cell subpopulations within the bovine immune system have been produced and characterised (Lalor *et al.*,1986; Baldwin, Morrison and Naessens, 1988). These have been used to examine the heterogeneity of target cells for *in vitro* parasitosis by *T. parva*. The results suggest that *T. parva* sporozoites infect and transform *in vitro* phenotypically defined T cells and (in certain circumstances) B cells (Lalor, Morrison and Black, 1986; Baldwin *et al.*, 1988). There are conflicting reports about the ability of *T. parva* sporozoites to infect and transform monocytes/macrophages. Two separate studies suggest that it was not possible to infect and transform peripheral blood monocytes (as defined by MAb P8) with *T. parva* (Lalor, Morrison and Black, 1986; Baldwin *et al.* 1988). Another report (Moulton *et al.*, 1984) indicates that *T. parva* transforms adherent cells derived from peripheral blood mononuclear cells, but in this study the cells had been cultured for a few days *in vitro* prior to infection.

Following *T. parva* infection and transformation T cells retain their appropriate differentiation antigens as defined by the MAbs, but transformed B cells lose their surface immunoglobulin (Ig) (Lalor, Morrison and Black, 1986). Less work has been done on characterization of the target cells for infection by *T. annulata* sporozoites. Preliminary work has shown that it is possible to infect and transform both T cells and B cells with *T. annulata* sporozoites (Ahmed, Rehbein and Schein, 1984). However, target cells in this study were separated using nylon wool (Julius *et al.*, 1973) or rosetting techniques (Ahmed *et al.*, 1981a) and the percentage purity of each population was not defined. *T. annulata* has also been reported to infect adherent cells (Musiime, 1983).

As part of a study to investigate whether the observed differences in pathogenicity and efficiency of immunisation between *T. annulata* and *T. parva* were caused by the two parasites infecting different cells of the immune system, a preliminary study was conducted *in vitro* to characterise the target cells infected by either parasite. In the initial part of the study, established cell lines infected *in vitro* by *T. annulata* or *T. parva* were analysed using a panel of MAbs specific for bovine leucocyte subpopulations. However, as the parasitised cells may cease to express surface phenotypes characteristic of the cell lineage from which they were derived, identification of the pre-infection cell phenotype by analysing the post-infection phenotype may not always be accurate. Therefore in the second part of the study the ability of *T. annulata*

and *T. parva* sporozoites to infect and transform defined target populations of cells *in vitro* was compared.

5.2 Materials and Methods

5.2.1 Target cell populations

5.2.1.1 *Separation of peripheral blood mononuclear cells (PBM)*

Peripheral blood mononuclear cells (PBM) were separated from bovine venous blood as described in section 4.2.1.1. These cells were used for immunofluorescence staining and cell sorting using the fluorescence activated cell sorter (FACS), and for the preparation of alloreactive T lymphocyte cell lines and adherent cells.

5.2.1.2 *Adherent Cells (Adh)*

Adherent cells were separated from the above PBM by an adaption of the method of Ackerman and Douglas (1978).Briefly, baby hamster kidney (BHK) cells (a gift from ICRF Lincoln's Inn Field, London) were grown to confluence on 9mm plastic petri dishes (Nunc, Gibco Biocult). The cells were detached with 10mM EDTA (Fisons) in phosphate buffered saline (PBS) leaving behind extracellular microexudate (fibronectin). $5x10^7$ PBM were layered onto a fibronectin coated petri dish in 10 ml of RPMI 1640 with 10% foetal calf serum (FCS) (Gibco Biocult) and incubated for 1 hour at 37°C to allow attachment of the adherent cells. After washing off the nonadherent cells, adherent cells were detached by incubating the cells with 3mM EDTA in RPMI 1640 with 10% FCS for 5 minutes at 37°C. The detached adherent cells were decanted, washed and resuspended in RPMI 1640 with 10% FCS.

5.2.1.3 *Generation of alloreactive cytotoxic cells*

All the alloreactive cytotoxic cell lines (CTL) were generated as described by Spooner *et al.* (1987). In brief, mixed lymphocyte cultures were established by the addition of equal volumes of peripheral blood mononuclear cells (PBM) at $5x10^6$ cells per ml and irradiated allogeneic PBM (5000 rads from a ^{137}Cs source) at $2.5x10^6$ cells per ml in 6-well tissue culture plates (Nunc, Gibco Biocult) in MLC medium as described in section 4.2.1.3. In order to increase the number of CTL precursor cells, skin from potential stimulator cell donors was implanted into potential responder animals using the technique of Prignitz *et al.* (1982). Specific details of the CTL lines used in the

experiment are as follows; (BoLA designations with the prefix w signify workshop bovine mhc class I assigned specificities, Spooner *et al.*, 1979; Anon, 1982).

CTL Line A

The responder animal was 3916 (BoLA w8, w12) and the stimulator cells came from animal 7168 (BoLA w6.1, w10). The resultant alloreactive CTL line was restimulated three times at weekly intervals, the last restimulation occurring 3 days before the day of infection.

CTL lines B(i) and B(ii)

The second alloreactive CTL generation was made using the same responder and stimulator combination as CTL line A. Alloreactive CTL line B was restimulated twice at weekly intervals, the last restimulation occurring 9 days before infection. Part of this cell line was treated with anti BoT4 MAb (Baldwin *et al.*, 1986) and complement to remove the BoT4 population prior to infection as described in section 4.3.4. The untreated CTL line is referred to as CTL B(i) and the treated line as CTL B(ii).

CTL lines C(i), (ii) and (iii)

In the third alloreactive CTL generation, which had animal 3420 (BoLA w6.2, w10) as the responder and 7168 (BoLA w6.1, w10) as the stimulator, the line was restimulated once after it was established and split into three parts which were subsequently restimulated at varying intervals prior to infection. Line C(i) was restimulated 7 days, C(ii) 4 days, and C(iii) 2 days prior to infection.

5.2.1.3.1 Cytotoxicity Effector Function

The effector function of the alloreactive CTL lines was assessed where possible, using a ^{51}Cr release assay on labelled target cells of known BoLA specificity infected and transformed with *Theileria* parasites as described by Spooner *et al.* (1987) and in section 4.2.4.

5.2.2 Monoclonal antibodies (MAbs)

Monoclonal antibodies which react with distinct bovine lymphocyte subpopulations were used in the phenotypic analysis of the cell lines both before and after sporozoite infection. The putative specificities of these antibodies, characterised by their cellular distribution and function within the bovine immune system, are described in section 2.3 and shown in Table 5.1.

TABLE 5.1 **Bovine leucocyte subpopulation specific monoclonal antibodies**

Monoclonal antibody	Dilution	Putative specificity
IL-A11	1:1000	BoT4 (Baldwin *et al.*, 1986; Teale *et al.*, 1986)
IL-A17	1:2000	BoT8 (Ellis *et al.*, 1986)
J 11	1:2000	Monomorphic mhc class II (Baldwin, Morrison and Naessens, 1988)
IL-A24	1:800	Monocyte/macrophage (Baldwin, Morrison and Naessens, 1988)
B 5/4	1:800	Bovine immunoglobulin (Pinder *et al.*, 1980)

5.2.3 Indirect immunofluoresence staining

An indirect immunofluorescence test (section 2.3.1) was used to stain the cells prior to their analysis using a fluorescence activated cell sorter (FACS IV, Becton Dickinson) as described in section 2.3.2.

5.2.4 Cell sorting

Prior to sorting, PBM were indirectly stained with the appropriate MAb at twice that concentration of MAb which gave the highest number of detectable positive cells. The second-step reagent was fluorescein isothiocyanate (FITC) conjugated anti mouse immunoglobulin (RAMIg) (Nordic) at 1:40 dilution. Cells were sorted on a fluorescence activated cell sorter (FACS IV, Becton Dickinson). The equipment was calibrated as described in section 2.3.2. Cells were sorted at 3500 events per second in sterile phosphate buffered saline (PBS) at pH 7.2. Samples of negative and positive populations were analysed at the end of the sort to estimate the efficiency of the separation. Once sorted, the cells were washed and resuspended in complete medium (section 2.2.2.2) with twice the concentration of FCS and antibiotics. The cells were sorted using the appropriate MAbs into the following positive and negative cell populations: mhc class II, monocyte/macrophage, T cell and B cell.

MAb	Pre-sort (PBM) % positive cells	Post-sort % positive cells	
		+ve population	-ve population
IL-A24 (monocyte/macrophage)	6	99	4
J11 (mhc class II)	20	96	3
IgM (B cell)	23	91	10
IL-A11/17 (T cell; BoT4, BoT8)	64	N.D	N.D

N.D = not determined

5.2.5 Parasite material

The stocks of *Theileria* sporozoites used in these experiments were *T. annulata* Ankara (TaA) (Schein, 1975), (Experiment 1) and *T. annulata* Hissar (TaH) (Gill *et al.*, 1980) (Experiments 2 and 3). Sporozoite suspensions at 1 tick equivalent per ml were harvested from filtered ground up tick supernatant (GUTS) from *Hyalomma anatolicum anatolicum* ticks previously fed for 3 days on rabbits (Walker *et al.*, 1985; Brown, 1987). Sporozoites of *T. parva* Muguga (Brocklesby, Barnett and Scott, 1961)

were prepared as suspensions of 10 tick equivalents per ml from salivary glands dissected from previously infected *Rhipicephalus appendiculatus* ticks which had been fed for four days on rabbits (Brown, 1987).

5.2.6 *Theileria* infected cell lines

Parasite infected lymphoblastoid cell lines were established *in vitro* by infection of normal bovine peripheral blood mononuclear cells (PBM) with *T. annulata* sporozoites using the method of Brown (1983) described in section 2.2.2.4, using the stocks of parasite material described above.

5.2.7 Cell infections (experimental design)

The target cells for infection (sorted PBM, alloreactive CTL or adherent cells) were mixed at 10^6 per ml with equal volumes of the sporozoite preparation (*T. annulata* or *T. parva*) and incubated at 38°C for 1 hour. Successive 3:1 dilutions of this mixture were made in complete culture medium (section 2.2.2.2) and 0.1ml of each dilution was distributed (with 20 replicate wells at each dilution) into 96-well round bottomed culture plates (Nunc, Gibco Biocult). In the case of *T. parva*, filler cells (autologous PBM exposed to 5000 rads of gamma irradiation from a ^{137}Cs source) were added at a fixed concentration of $5x10^4$ cells per well; the dilutions of target cells and sporozoites were adjusted to take account of these added cells, so the total volume in each culture well remained constant at 0.1ml. The plates were maintained at 38°C in a 5% CO_2:air mixture.

Infection and transformation of the cells by *Theileria* sporozoites was assessed periodically between days 4 and 28 post-infection. The results obtained on day 7 were typical, and are discussed in section 5.3. Transformation was initially quantified by visual assessment of blast cells using an inverted phase contrast microscope (Diavert, Leitz), the transformed cells being 5-10 times larger than untransformed cells and clearly visible at 100x magnification. Infection was later confirmed by the presence of intralymphocytic schizonts in Giemsa stained smears of the cells prepared using a cytocentrifuge (Cytospin, Shandon) as described in section 2.2.6, and the establishment of *Theileria* transformed cell lines.

An estimate of the proportion of target cells infected and transformed by either parasite was calculated as described by Brett, Kingston and Colston (1987) based on the method of Lefkovits and Waldman (1979) for analysis of limiting dilution systems, assuming single hit phenomena and random distribution of target cells for parasite infection among the culture wells. Because it is difficult to quantify sporozoites it was not possible to ensure that the number present would always be sufficient to saturate

all of the potential target cells. The frequency of establishment of infected cells may thus vary from one experiment to another, so results obtained with different leucocyte populations are comparable only within each experiment. The limiting dilution analysis was used to compare relative and not absolute frequencies of *Theileria* transformation in different cell subpopulations.

On no occasion was it possible to infect and transform any of the irradiated PBM, used as filler cells, with the *T. parva* sporozoites.

5.3 RESULTS

5.3.1 Phenotypic analysis of established infected cell lines

The results are illustrated in Table 5.2. All the *T. annulata* and *T. parva* cell lines expressed mhc class II antigens, although there was variation in the proportion and intensity of positive cells within a line. *Theileria annulata* infected cell lines that were sorted using the FACS into mhc class II -ve populations re-expressed mhc class II molecules after 3-4 days in culture (results not shown), suggesting that the expression of the J11 determinant was linked to specific stages of the cell cycle. Therefore mhc class II molecules may be more indicative of cell activation than cell lineage. All of the *T. annulata* cell lines tested were effectively negative for both T cell MAbs (recognising BoT4 and BoT8). In contrast all the *T. parva* cell lines tested were positive for various combinations of the T cell MAbs used. Some lines were exclusively positive for the MAb IL-A11 (BoT4) or MAb IL-A17 (BoT8) and others positive for both T cell markers.

Expression of both the monocyte/macrophage and B cell markers (IL-A24 and B5/4 respectively) was effectively negative for all *T. annulata* or *T. parva* cell lines tested. If it is assumed that the cell phenotype post-infection is identical to the phenotype pre-infection then the conclusion from these initial results would be that *T. parva* infects T cells and *T. annulata* does not but infects a cell population that cannot be distinguished using the available MAbs.

5.3.2 Infection of sorted cell populations

The proportion of cells transformed with either *T. annulata* or *T. parva* in the various sorted populations is given in Table 5.3. In all cases infection of the observed transformed cells was confirmed by analysis of Giemsa stained cytospin preparations which indicated the presence of intracellular parasites.

The fraction of class II positive cells transformed by *T. annulata* was approximately 24 times that of the reciprocal class II negative cells. *T. parva* sporozoites

TABLE 5.2 **Phenotypic analysis of established *Theileria* infected cell lines**

MAb	Putative specificity	*T. parva* Number of lines tested	*T. parva* Mean % positive cells	*T. annulata* Number of lines tested	*T. annulata* Mean % positive cells	PBM Number of lines tested	PBM Mean % positive cells
J11	mhc class II	5	96 (90-99)	13	78 (40-98)	7	36 (20-45)
IL-A11	BoT4	2	95*				
		1	40	9	6	7	24
		2	1.3		(2-8)		(20-35)
IL-A17	BoT8	3	90*				
		1	60	6	2	6	23
		1	4				(17-25)
IL-A24	monocytes	2	3	12	0.5	4	6
B5/4	IgM	NT		2	0	8	13

Ranges are shown in brackets NT : not tested

*These figures illustrate the major differences between *T. parva* lines observed for the markers BoT4 and BoT8

TABLE 5.3 **Frequencies of precursor cells infected and transformed by** *T. annulata* **and** *T. parva* **sporozoites**

Experiment	Cell population	Frequency of transformed cells	
		T. parva	*T. annulata*
1	PBM	ND	1:690
	CTL A	1:741	1:960
	BoT4/T8 +ve	ND	1:2490
	BoT4/T8 -ve	ND	1:590
	mhc class II +ve	ND	1:590
	mhc class II -ve	ND	1:14000
2	PBM	1:190	1:590
	CTL B(i)	1:92	1:31000
	CTL B(ii)	1:150	1:19500
	monocyte/macrophage +ve	no infection	1:9
	monocyte/macrophage -ve	1:860	1:1300
	adherent	1:480	1:520
3	PBM	ND	1:1500
	CTL C(i)	1:140	no infection
	CTL C(ii)	1:150	no infection
	CTL C(iii)	1:60	1:84000

ND : not determined

were not tested in this experiment. Both parasites were tested with the monocyte/macrophage sort (distinguished by MAb IL-A24) and a dramatic difference in the infectibility of each population was observed. The IL-A24 positive population, which was very efficiently infected with *T. annulata*, was uninfectible by *T. parva* sporozoites. The reciprocal IL-A24 negative population was more easily infected by *T. parva* than *T. annulata*. With the IgM sort *T. annulata* preferentially infected the IgM positive population and *T. parva* preferentially infected the IgM negative population. The BoT4/BoT8 negative cells were preferentially infected by *T. annulata* compared with the BoT4/BoT8 positive cells. It was impossible to assess the infectibility of this cell sort with the *T. parva* sporozoites as the cultures unfortunately became contaminated.

Phenotypic analyses of the sorted cells and PBM both before and after infection are found in Table 5.4. The cells analysed post-infection were derived from single wells in the limiting dilution analysis, at the lowest dilution from which it was possible to establish cell lines. Thus cell lines derived from the readily infectible populations came from wells originally seeded with only three cells, whereas those populations of cells that were less readily infectible derived from wells originally containing 10^4-10^5 cells. As it was not possible to propagate any of the *T. parva* cell lines from the sorted cell populations only the *T. annulata* cells lines were phenotypically analysed.

5.3.3 Infection of adherent cells

The adherent cell fraction, which contained approximately half as many monocytes as the IL-A24 positive population (Table 5.4), was surprisingly infected equally efficiently by both parasites.

5.3.4 Infection of various alloreactive cytotoxic cell lines

5.3.4.1 *Alloreactive cytotoxic cell line A*

The phenotypic and functional analyses of this CTL line both before and after infection are shown in Table 5.5 and Figures 19a and b respectively.

Pre-infection, the CTL line was comprised mainly of T cells (61% BoT4, 24% BoT8) and had cytotoxicity values of 55% against the stimulating cell line at an effector:target ratio of 8:1. No cytotoxicity was detected against any BoLA mismatched targets. The comparative infection rates of *T. parva* and *T. annulata* parasites for this CTL line are shown in Table 5.3. The infection rates of the CTL in limiting dilution cultures were compared to that of PBM which acted as a positive control. In this case, which

TABLE 5.4 **Phenotypic analysis of sorted and adherent cells 19 days after infection with *T. annulata***

Sorted cell population	Fraction of infected cells positive for MAb (%)				
	J11 (class II)	IL-A11 (BoT4)	IL-A17 (BoT8)	IL-A24 (monocyte)	B5/4 (B cell)
Uninfected* PBM	20	31	40	5	10
J11 +ve	67 (48-73)	2	0.8	7 (5-10)	2
J11 -ve	26	8	0.1	9	0.5
Uninfected* adherent	63	19	11	53	2
Adherent	58 (35-80)	2 (2-3)	1	5	2
IL-A24 +ve	57 (38-86)	4	3	6 (0-14)	0.5
IL-A24 -ve	100	0.3	0.8	2	4
IgM +ve	82	5	6	3	6
IgM -ve	86	4	3	5	4

*Phenotypic analysis before infection included for comparison

Mean and range in brackets are shown if more than one cell line was tested

TABLE 5.5 **FACS analysis of CTL A (% cells positive)**

MAb specificity	Pre-infection	28 days post-infection		
		TaA (10^5)	TaA (10^2)	TpM (10^5)
Class II	33	44	61	70
BoT4	61	3	6	1
BoT8	24	2	7	0.4
monocyte/macrophage	1	4	ND	5
IgM	1	6	4	0.2

TaA : *T. annulata* Ankara TpM : *T. parva* Muguga ND : not determined

Post infection cell lines were established from single wells; the original cell dilution is indicated in brackets

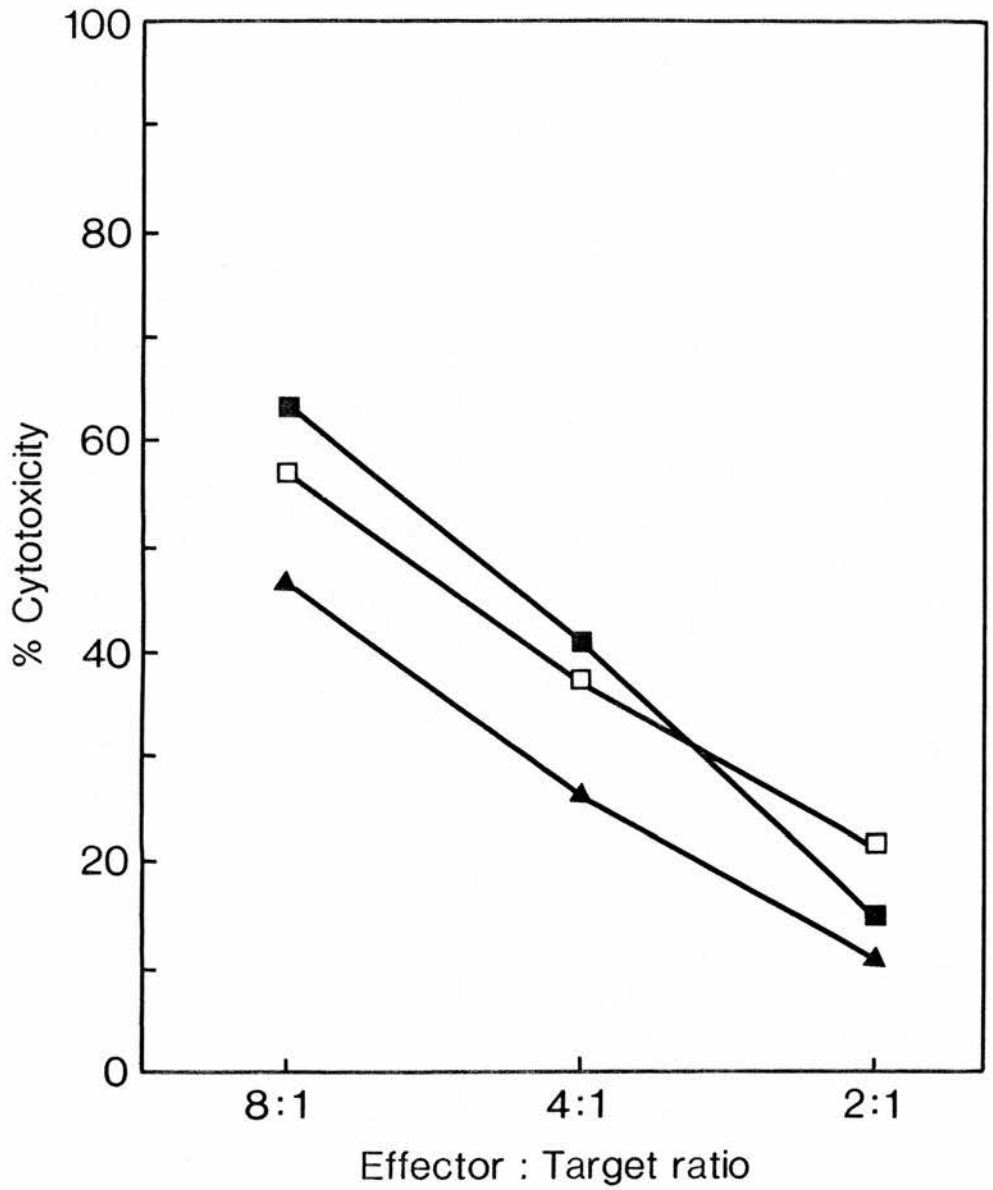

Fig. 19a
Effector function of alloreactive CTL lines A □, B(i)▲ and B(ii)■, prior to infection. The target is the cell line used to stimulate the CTL.

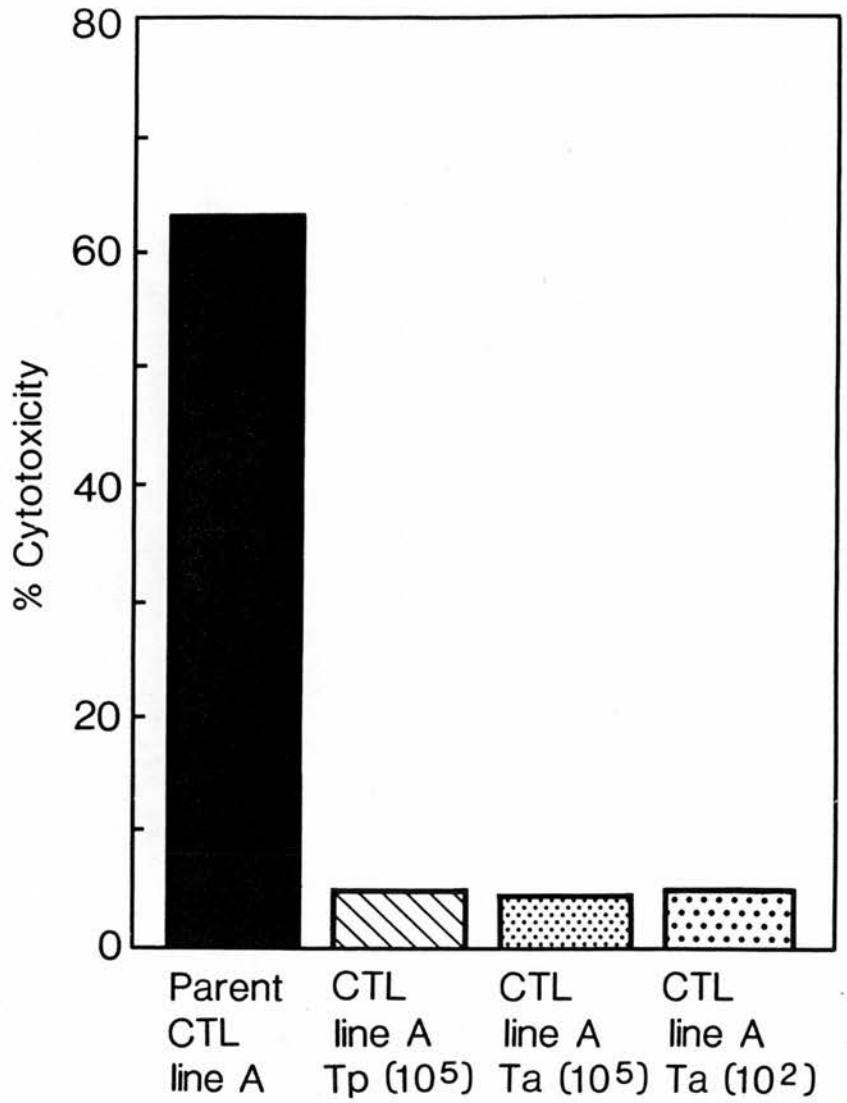

Fig. 19b

Effector function of alloreactive CTL line A after infection with either T.annulata (Ta) or T.parva (Tp). The numbers in brackets indicate the cell dilution from which the infected CTL line was derived. The parent CTL line A is uninfected and used as a control.

was later proved to be the exception, both parasites infected the CTL line with equal efficiency (Table 5.3). However, *T. parva* sporozoites infected the CTL line with greater efficiency than the control PBM. It appeared that the *T. parva* sporozoite preparation was cytotoxic for the PBM so no cell lines were established from this preparation.

Post-infection, cell lines were established from single wells and analysed for phenotype markers (Table 5.5) and function (Figures 19a and b). All the infected lines, regardless of whether they were infected with *T. annulata* or *T. parva*, lost their cytotoxic function when compared to the original uninfected CTL line (Figure 19 b), and the infected lines showed similar phenotypic characteristics. Class II expression increased from pre-infection levels, only a small percentage of the cells within each line expressed any T cell markers, no surface Ig was detected, and only one of the *T. annulata* lines expressed the monocyte marker.

5.3.4.2 *Alloreactive cytotoxic cell lines B(i) and B(ii)*

In the second alloreactive cytotoxic cell line generation, which was made from the same combination of responder and stimulator cell populations as CTL A, a proportion of the line was treated with the anti BoT4 MAb and complement to remove the BoT4 population and provide CTL B(ii), as a comparison with the untreated CTL line B(i). The phenotypic analyses of these lines both before and after infection are illustrated in Tables 5.6 and 5.7.

The degree of cytotoxicity of the lines before infection is shown in Figure 19a. The levels of infection of the two parasites in both these cell lines and control PBM is illustrated in Table 5.3. On this occasion there was a marked difference between the level of infection of the *T. parva* sporozoites and that of *T. annulata*. In both the untreated CTL B(i) and the treated CTL B(ii) a higher proportion of cells were infected and transformed by *T. parva* (Table 5.3).

The phenotypes of the resultant infected cell lines were examined at 7 and 19 days after infection (Tables 5.6 and 5.7). One of the *T. annulata* cell lines derived from the untreated CTL B(i) infection expressed T cell markers at 7 days, but when the same line was tested at 19 days the markers had been lost. The infected cell lines showed varying levels of mhc class II expression, which had increased from pre-infection levels. None of the lines expressed surface Ig or monocyte markers. All the *T. parva* lines expressed one or both of the T cell markers.

5.3.4.3 *Alloreactive cytotoxic cell lines C(i), C(ii) and C(iii)*

The phenotype and function of the alloreactive cell lines prior to infection are shown in Table 5.8 and Figure 19c.

TABLE 5.6

FACS analysis of CTL B(i) (% cells positive)

Specificity of mAb	Pre-infection	7 days post-infection		19 days post-infection		
		TaH (10^5)	TpM (10^3)	TaH (10^5)	TaH ($3x10^4$)	TpM (10^3)
Class II	19	34	92	25	63	69
BoT4	72	54	85	1	1	66
BoT8	24	21	7	2	7	7
Monocyte/Macrophage	4	3	0.5	2	1	1.7
IgM	5	8	1	3	2	3

TABLE 5.7

FACS analysis of CTL B(ii) (% cells positive)

Specificity of mAb	Pre-infection	19 days post-infection			33 days post-infection	
		TaH (10^4)	TaH ($3x10^3$)	TpM (10^3)	TaH (10^4)	TaH ($3x10^3$)
Class II	53	8	28	68	74	65
BoT4	1	1	1	3	0.5	1
BoT8	66	13	2	66	0.2	1
Monocyte/Macrophage	1	1	2	2	1	4
IgM	2	1	7	3	0.1	0.4

TABLE 5.8

FACS analysis of CTL C(i), (ii) and (iii), pre and 19 days post infection (% cells positive)

Specificity of mAb	CTL c(i)			CTL c(ii)			CTL c(iii)		
	Pre-infection	Post infection		Pre-Infection	Post infection		Pre Infection	Post infection	
		TpM (10^5)	TpM (10^2)		TpM (10^5)	TpM (10^2)		TaH (10^5)	TpM (10^4)	TpM (10^2)
Class II	18	75	68	50	65	71	69	72	71	62
BOT4	60	56	66	62	60	52	58	5	59	60
BoT8	29	20	7	16	5	15	21	3	5	8
monocyte/ macrophage	2	3	3	1	3	1	2	3	3	1
IgM	1	2	3	1	2	1	2	2	1	1

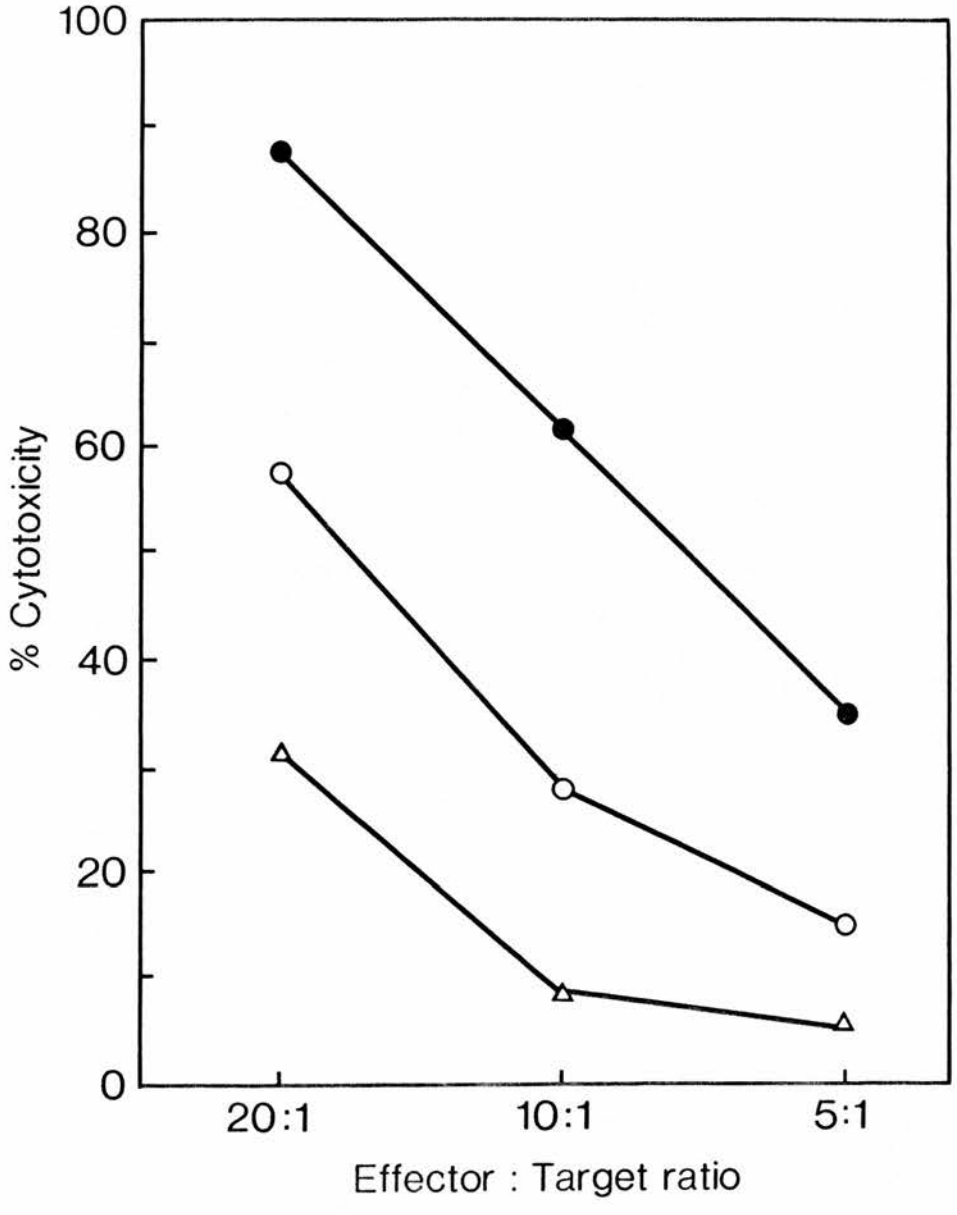

Fig. 19c
Effector function of alloreactive CTL lines c(i)△, c(ii)● and c(iii)○, prior to infection. The target is the cell line used to stimulate the CTL.

The main difference in phenotypic analysis between the three lines prior to infection was the level of class II expression, which was highest in the cell line C(iii) that had been restimulated most recently.

The frequencies of infection of the CTL lines by sporozoites of *T. parva* or *T. annulata* are illustrated in Table 5.3, which shows a dramatic difference in the infection levels. *T. parva* consistently infected all three CTL lines very efficiently whereas the *T. annulata* sporozoites failed to infect CTL lines C(i) and C(ii) and only infected C(iii) at low frequency. This cell line C(iii) had the highest pre-infection mhc class II expression. No cytotoxic activity was found in any of the cell lines after infection (Figure 19d).

5.4 DISCUSSION

These results indicate that *T. annulata* and *T. parva* preferentially infect distinct cell subpopulations *in vitro*. The most striking differences betweeen the two parasites were the marked preference of *T. annulata* for IL-A24 positive cells (monocyte/macrophage) and of *T. parva* for the alloreactive CTL lines. These studies confirm previous reports that *T. parva* preferentially infects T cells and, less efficiently, B cells, and will not infect monocytes (Lalor, Morrison and Black, 1986; Baldwin *et al.*, 1988). In contrast it appears that *T. annulata* preferentially infects mhc class II positive cells, monocytes (defined by IL-A24) and B cells, but will not readily infect any of the T cell populations.

An unexpected finding was the apparently equal ability of *T. annulata* and *T. parva* to transform the adherent cell population, which was previously thought to be more permissive to infection by *T. annulata* (Musiime, 1983). Phenotypic analysis of the adherent cell population prior to infection indicated the presence of a relatively high proportion of contaminating T cells and it is possible that these cells, as opposed to the monocyte fraction, were the targets for infection by *T. parva* sporozoites. Unfortunately this hypothesis could not be confirmed by phenotypic analysis as it proved impossible to propagate any of the *T. parva* infected cells derived from the adherent cell population.

This study has also shown that *T. parva* sporozoites consistently and efficiently infect and transform various alloreactive CTL preparations. By contrast the same alloreactive CTL were not infected and transformed by *T. annulata* except at very high cell and sporozoite concentrations. An exception occurred in the first experiment, in which CTL A was infected and transformed with equal efficiency by sporozoites of either parasite. Phenotypic analysis of the *T. parva* infected cells unexpectedly suggested that non T cells had become infected. The reasons for this are unclear and we have not been able to repeat the result. Comparing CTL A and the cell line B(i),

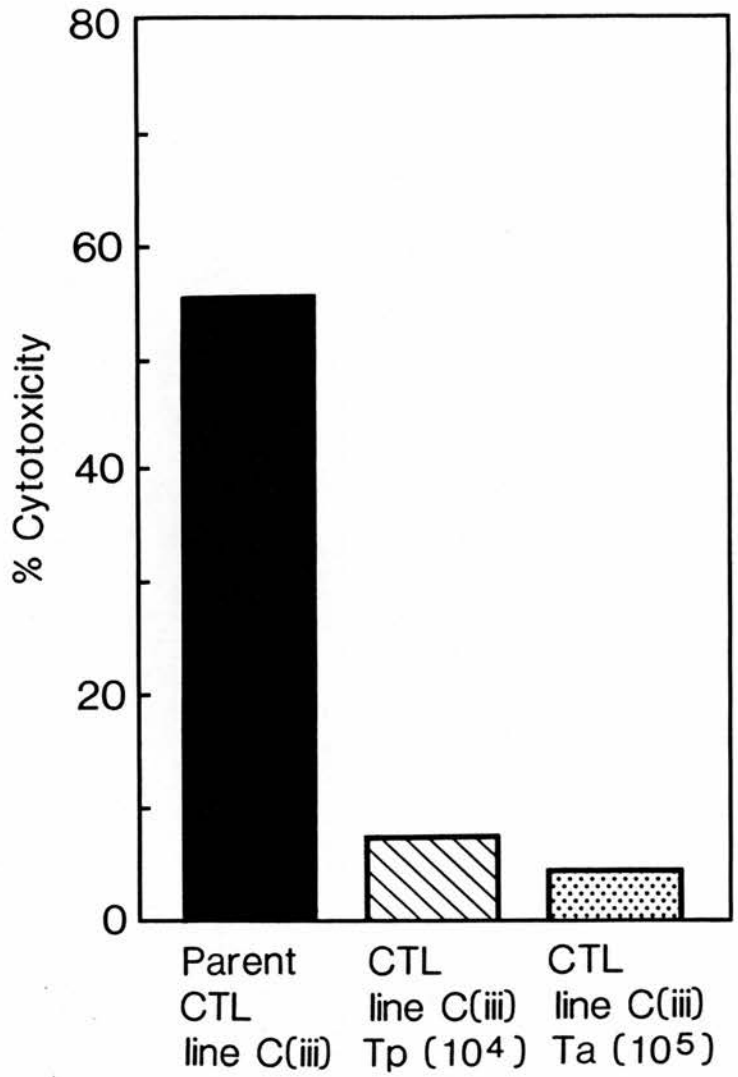

Fig. 19d
Effector function of CTL line c(iii) after infection with either T.annulata (Ta) or T.parva (Tp). The numbers in brackets indicate the cell dilution from which the infected CTL line was derived. The parent uninfected CTL line c(iii) is used as a control.

which in the subsequent experiment was not infected and transformed by *T. annulata*, the following similarities and differences were observed. Both CTL A and CTL B(i) were prepared from the same responder and stimulator combination, and showed very similar functional activity against a panel of target cells. However there were differences in the phenotypic analysis of CTL A and CTL B(i) at the time of infection. CTL A, which had been restimulated 3 days prior to infection, was composed of 83% T cells and had an overall mhc class II expression of 33%. CTL B(i), which had been restimulated 9 days prior to infection was composed of 95% T cells and had an overall mhc class II expression of 19.3%. These differences between the two CTL preparations may affect their susceptibility to infection by *T. annulata*.

With CTL C the relevance of the state of activation of the cell line to infection by sporozoites of either parasite was investigated. While *T. parva* infected each of the CTL preparations C(i), C(ii) and C(iii) with equal efficiency, *T. annulata* sporozoites most efficiently infected the CTL line C(iii) that had most recently been restimulated and showed the highest mhc class II expression. The *T. annulata* sporozoites also infected CTL line B(ii), which had been treated with anti BoT4 and complement, more readily than the untreated CTL line B(i). Again the common feature was the higher class II expression on the treated CTL preparation B(ii) prior to infection. These results may point to a positive correlation between the ability of *T. annulata* sporozoites to infect and transform the CTL line and its state of activation and degree of class II expression. The mhc class II expression or state of activation of the CTL line showed no correlation with the ability of the *T. parva* cells to infect and transform any of the lines.

It is not possible from these data to decipher with precision within the bulk CTL line the target cell for infection by either *T. annulata* or *T. parva*. Phenotypic analysis of the cells post infection suggests that *T. parva* and *T. annulata* were preferentially infecting different cell populations. Established *T. annulata cell* lines did not express any T cell markers while *T. parva* cell lines always expressed BoT4 or BoT8 or both phenotypic markers. Expression of T cell markers was seen on one occasion with a *T. annulata* line derived from CTL A at 7 days after infection, but when this same line was tested 19 days post infection it was negative for both T cell markers. It may be possible that at 7 days post infection there were some contaminating uninfected CTL cells that caused a false positive result, with *T. annulata* infected cells overgrowing the culture by day 19.

The functional activity of all tested CTL lines was lost after infection with either parasite, although in other studies with *T. parva*, alloreactive CTL clones (BoT8+) maintained their function for a limited length of time following infection (Baldwin and Teale, 1987). This apparent loss of functional activity may simply reflect the fact that

none of the CTL lines assayed for cytotoxic activity after infection in this study expressed the BoT8 phenotypic marker. Moreover we were infecting a bulk CTL line composed of a mixture of cell populations, as opposed to the cloned BoT8+ material used in the other study.

Another point to note was that *T. parva* sporozoites were able to infect and transform the CTL lines which could then be easily maintained *in vitro*. The same *T. parva* sporozoites appeared to infect and transform PBM, J11-ve, IL-A24-ve, IgM+ve and IgM-ve cell populations but it was much more difficult to propagate these as continuous cell lines. The majority were thus lost at an early stage. No problems of this kind were encountered with the *T. annulata* sporozoites. This illustrates one of the limitations of using this *in vitro* infection model, *viz* the lack *in vitro* of necessary growth factors which may be active *in vivo*. It may be that the presence of growth factors in the CTL line aided the establishment of *T. parva* lines in our system. *T. parva* also required a feeder cell layer to help provide suitable conditions for the propagation of infected cells whereas *T. annulata* did not.

It is not known whether the ease of cultivation *in vitro* of *T. annulata* cell lines compared to *T. parva* cell lines is a property of the two different parasites or of the *in vitro* culture requirements of the host cell type that becomes infected.

Considerable changes were observed in the expression of various cell phenotype markers following infection of the defined cell subpopulations with both parasites. If the phenotypes of *T. parva* and *T. annulata* infected cell lines are compared it can be shown that, unlike *T. parva* lines, established *T. annulata* lines did not express BoT4 or BoT8 antigens. Analysis of the *T. annulata* infected cell lines propagated from the limiting dilution experiment indicated that their phenotypes did not necessarily correlate with the original cell population phenotype. All of the lines tested post-infection including those derived from the IL-A24+ population expressed low or no detectable levels of the determinant recognised by the MAb IL-A24. It would therefore seem that this marker had been down regulated.

It was also interesting to note that established *T. parva* infected cell lines could co-express BoT4 and BoT8 phenotypic markers, which may suggest that the original precursor cell was not a mature T cell (Reinherz and Schlossman, 1980) or that the T cell differentiation antigens are acquired due to gene derepression following infection (Black *et al.*, 1981). The gain or loss of the expression of markers by which the resting cell populations within PBM were originally defined highlights the difficulties in characterising an infected cell type by correlation of phenotypes before and after infection. It should be emphasised that the results of this study are only relevant in the context of the *in vitro* system used, which may inadvertantly select against the

transformation of particular subpopulations of cells simply because of the lack of necessary growth factors as discussed above. An important follow up to these studies would be to define the cell types that become infected *in vivo*. Comparatively little work has been done in this area, probably because of the technical difficulties of simultaneously monitoring parasitosis and cell phenotype. A further limiting factor is the unavailability of suitable MAbs to define a specific cell population both pre and post infection. T cell markers are not lost as a result of infection and preliminary studies examining the *in vivo* infection of cells with *T. parva* sporozoites have shown that the majority do express T cell markers (Emery, Machugh and Morrison, 1988; Baldwin *et al.*, 1988). It should also be noted that this study did not determine whether permissiveness to infection by either parasite is determined at the level of entry to the cell or at some later stage in the transformation process.

However, the fact that *T. annulata* and *T. parva* have been shown to have distinct and different cell preferences for *in vitro* infection may help to explain some of the observed differences in the pathogenesis of *T. annulata* and *T. parva* infection and the ability of allogeneic *T. annulata* infected cell lines but not allogeneic *T. parva* infected cell lines to infect and immunise animals.

It is possible that the infection of a particular phenotype of host cell may elicit different pathogenic processes and may thus determine the outcome of infection by *T. annulata* or *T. parva*. It has been reported that supernatants from certain *T. parva* infected T cell lines can suppress mixed lymphocyte responses (Baldwin, *et al.*, 1988), or produce stimulatory factors with interleukin 2 like activity (Brown and Logan, 1986). This may have important implications for the use of infected cell lines as stimulators to create *Theileria* specific T cell lines *in vitro* and might help to explain the variation observed between responses of individual animals when cultured *in vitro* with their autologous infected cell lines (Goddeeris, Lalor and Morrison 1986; Innes, unpublished observations).

Cytotoxic T cells have been shown to lose their functional specificity after infection by *T. parva* and become promiscuous killers (Baldwin and Teale, 1987). Cytotoxic lymphocytes are believed to be an important component of protective immunity in both *T. parva* and *T. annulata* infection (Preston,Brown and Spooner, 1983;Morrison *et al.*, 1986b). Therefore infection of CTL *in vivo* by *T. parva* sporozoites may compromise the immune system or subvert the normal function of the CTL.

A report of a preliminary study to determine the pathogenic effects on naive hosts of inoculation of autologous defined cell types infected *in vitro* has suggested that *T. parva* infected B cells induce milder infections than *T. parva* infected T cells (Lalor,

Morrison and Black, 1986). This suggests that the severity of the disease may be related to the nature of the cells which initially become infected by the parasite.

In the case of *T. annulata* no such studies have been done, but we know from the work reported in this chapter that in contrast to *T. parva* this parasite will preferentially infect mhc class II positive macrophages. Antigen presenting cell function in cattle is known to involve IL-A24 and mhc class II positive adherent cells (E.J. Glass, personal communication). Therefore it is possible that *T. annulata* sporozoites, by infecting potential antigen presenting cells, present themselves to the immune system in an immediately recognisable "package". In contrast the evidence with *T. parva* suggests that it preferentially infects class II negative cells and susequently induces class II expression (Black *et al.* 1981; Baldwin, Goddeeris and Morrison, 1987). The parasite is thus not immediately recognisable to the immune system, which may have important implications in the prompt induction of an effector immune response.

Lastly, the enigma of allogeneic infected cell line immunisation: why is it possible to readily infect and immunise animals with allogeneic *T. annulata* infected cells but not with *T. parva*?

Current evidence suggests that mhc incompatibility between cell line and recipient is not the only limiting factor, but rather that the ability (or, in the case of *T. parva*, inability) of the schizont stage of the parasite subsequently to infect the host cells is also important. It is not known whether this involves active infection of host cells by the schizonts or some form of passive transfer, for example by phagocytosis. Evidence from the literature favours the latter mechanism, as no attempts to infect animals using purified schizont preparations have been successful (Wilde, Hulliger and Brown, 1966; Pipano *et al.*, 1977).

If phagocytosis is presumed to be the mechanism then the simplest explanation for the superior ability of *T. annulata* schizonts to transfer and infect host cells is that they can successfully survive in and transform the phagocytic cells (macrophages) whereas *T. parva* cannot. An additional factor might also be the availability of suitable host cells in the immediate microenvironment permissive to infection by either parasite. The work presented in chapter 4 of this thesis demonstrated a rapid and specific immune response directed against the donor allogeneic *T. annulata* infected cell line (a form of "graft rejection" response) detected after primary immunisation and immediately prior to parasite transfer to the host cells. A similar rapid immune response against the donor cell line has been observed in the attempted infection of animals using allogeneic *T. parva* infected cells (Emery, *et al.*, 1982). The infected donor cells presumably interact with activated cells of the host's immune system which are likely to express mhc class II antigens characteristic of activated cells

(Reinherz and Schlossman, 1980). Evidence from this chapter suggests that *T. annulata* preferentially infects mhc class II positive cells, whereas this does not appear to be the case for *T. parva*. Interestingly the results from this chapter suggest that alloreactive cytotoxic T cell lines, normally refractory to *T. annulata* infection, became susceptible immediately following restimulation with alloantigen when mhc class II expression was, comparatively speaking, at its highest.

Some of the work reported in this chapter has been accepted for publication:

1. *In vitro* infection of bovine alloreactive cytotoxic T cell lines with sporozoites of *T. annulata* and *T. parva*. E.A. Innes, P. Millar, E.J. Glass, C.G.D. Brown and R.L. Spooner, *Research in Veterinary Science* (In press).

2. Bovine mononuclear cell lines transformed by *T. parva* or *T. annulata* express different subpopulation markers(1989). R.L. Spooner, E.A. Innes, E.J. Glass, P. Millar and C.G.D. Brown *Parasite Immunology* 11 (In press).

3. *T. annulata* and *T. parva* infect and transform different bovine mononuclear cells. R.L. Spooner, E.A. Innes, E.J. Glass and C.G.D. Brown *Immunology* (In press).

CHAPTER 6

Summary and Conclusions

SUMMARY AND CONCLUSIONS

A general review of the literature concerning *Theileria annulata* and the disease it causes in cattle is given, emphasis being placed on immunology and immunoprophylaxis. Most of the literature on theileriosis concerns the extensively studied parasite *Theileria parva*, included here for comparison with what is known about *T. annulata* and to illustrate the differences between the two parasites. The immunology of *T. annulata* infection has been relatively little studied in recent years. A review of the general features of the major histocompatibility complex (mhc) is included, with particular emphasis on what is currently known about the genetic organisation, structure and function of the bovine mhc. This provides a background for the first two parts of this study, in which the role of the major histocompatibility antigens was examined in two different contexts: in the first section the effect of histoincompatibility between *Theileria* infected cell line and recipient; in the second the requirement for mhc antigens to provide the correct "context" for recognition of parasite infected cells by the host immune system.

Current methods used to immunise animals against theileriosis rely on inducing controlled levels of infection in cattle sufficient to induce immunity without killing the animal. This can be achieved by inoculating live sporozoites or macroschizont infected cells grown in culture. Live sporozoite material is not a very satisfactory immunogen as, apart from the difficulty of obtaining material derived from infected ticks, it is very pathogenic unless administered with concurrent chemotherapy by trained personnel. Macroshizont infected cell lines have the advantage of effectively infinite availability (maintained as continuously growing cell lines *in vitro*), and can infect and immunise animals after only one inoculation. Although infected cell lines tend not to be as pathogenic as the inoculation of live sporozoites, an unacceptably high death rate directly attributable to cell line induced infection is observed, especially when inoculating *T. parva* cell lines, where very large numbers of parasitised cells have to be used to achieve consistent results. Very much lower doses can be used to infect animals with *T. annulata* cells. Recent research has confirmed earlier hypotheses that histoincompatibility between the *T. parva* cell line and the recipient animal acts as a barrier to infection and hence to immunisation of animals. It is interesting that histoincompatibility does not seem to be so critical when infecting animals with *T. annulata* cell lines. This has never been properly examined.

The work presented in this thesis concerns various aspects of the relationship between the intracellular stage of the *T. annulata* parasite and its bovine host. The study is divided into three main areas:

1) Lymphoblastoid cell lines infected and transformed *in vitro* with a Moroccan stock of *T. annulata* were used to infect and immunise animals against a subsequent challenge lethal to naive control animals. Different cell doses and cell lines were examined as parameters that might affect the safety and efficiency of cell culture vaccination. The effect of histoincompatibility between cell line and recipient was also explored. The main results can be summarised as follows. Susceptible taurine cattle were successfully infected and immunised at cell doses of 10^8, 10^6, 10^4 and 10^2, regardless of whether the recipients were BoLA matched or mismatched to the donor cell line. In contrast to the situation with the closely related parasite *T. parva*, there was no histocompatibility barrier to immunisation using *T. annulata* infected cell lines. This could be achieved using as few as 10^2 allogeneic infected cells.

The mhc relationship between the cell line and recipient, while not acting as a barrier to infection, did appear to influence its severity: at the highest cell dose (10^8), the BoLA mismatched recipients reacted more severely than the BoLA matched. This may reflect differences in the specific priming of the immune response by the antigens of the donor cell line. It is possible that the combination of mhc class I molecule and parasite antigen on the cell surface will appear similar to the immune system whether the cells presenting the combination are BoLA class I matched inoculated cells or the host's own cells following parasite transfer. The host's immune system will therefore be more rapidly primed to the correct mhc-parasite combination, resulting in a quicker and more effective immune response. This will not occur in the BoLA mismatched group, where effective priming of the immune response can only take place after parasite transfer. Differences between BoLA matched and mismatched recipients were not so apparent in animals receiving lower cell doses, presumably because the initial response was relatively insignificant, fewer antigenic molecules having been injected.

Three years after the completion of this experiment the immunised animals have continued to show normal productivity traits with no relapse of infection, although the field challenge situation that the animals were exposed to during this time is not known. The ability of these animals to withstand challenge from different field isolates occuring in Morocco has not been tested, but a larger field trial using a cell culture vaccine in an area of high challenge, is being conducted in Morocco as a follow up to this experiment. At present the only treatment available against theileriosis in Morocco is chemotherapy. An effective cell line vaccine would have considerable application, as

theileriosis is a serious constraint on the use of exotic or crossbred animals introduced to increase animal food production to meet with increased demand.

2) As the induction of immunity by *T. annulata* cell culture vaccines is not well understood, the second part of this study compared the development and specificity of cytotoxic cells in animals infected with sporozoites and autologous or allogeneic infected cell lines. We chose to examine cytotoxic T lymphocytes as these are known to be important in other intracellular parasitic infections, including those caused by the closely related parasite *T. parva*. Three groups of animals were immunised with *T. annulata* sporozoites, 10^6 autologous or 10^6 allogeneic *T. annulata* infected lymphoblastoid cells cultured *in vitro*. The development and specificity of cytotoxic cells generated *in vivo* were measured throughout immunisation and challenge using a panel of target cells that were either *T. annulata* infected or uninfected blast cells of known BoLA specificities. After primary inoculation of sporozoites, autologous and allogeneic infected cell lines the three groups showed distinct differences in both their clinical responses and the target specificity of the cytotoxic cells detected. A summary of the main findings is given below.

The allogeneic (BoLA mismatched) *T. annulata* cell line recipients showed a very mild clinical response, and on day 9 after inoculation a strong cytotoxic response was detected. This response appeared to be directed against the allogeneic mhc antigens of the inoculated cell line in some form of graft rejection. By day 23 (when the parasite was shown to have transferred to and infected the cells of the recipient animal) the predominant cytotoxic response was directed against the recipient animal's own cells, now infected with the parasite. It appears that despite a strong graft rejection response, which presumably killed off a proportion of the intracellular macroschizonts, sufficient *T. annulata* parasites were able to transfer from the allogeneic inoculated cell line to infect the cells of the recipient animal.

Very severe clinical reactions and low levels of cytotoxicity were detected in both the sporozoite recipients and the autologous *T. annulata* cell line recipients. Cytotoxicity was directed against parasite infected targets but did not appear to be mhc restricted until day 20. The immune system of the sporozoite recipients was apparently overwhelmed by the infection: without treatment they would have died. This presumably reflects the fact that sporozoites are more efficient than macroschizont cell lines at infecting the host. Autologous infected cells may not be immediately recognised as foreign, and may therefore multiply significantly before the immune system responds. The more severe clinical reaction observed in the autologous than in the allogeneic cell line group may be a consequence of this.

These differences notwithstanding, all three groups were subsequently found to be immune to a heterologous sporozoite challenge lethal to two susceptible control animals. On day 10 after challenge a peak of cytotoxicity was detected, directed in all cases against autologous infected target cells. This suggests that the cytotoxic response was both parasite specific and mhc restricted. Peak cytotoxic activity coincided with the recovery of the animal from the lethal challenge infection.

A cytotoxic T lymphocyte (CTL) line was created from one of the sporozoite recipients, and used to analyse further the cytotoxic response. The CTL line would only kill autologous infected cells or parasitised cells which were matched with the effector cells for BoLA class I mhc antigens. Moreover, it was found that the cytotoxicity could be inhibited by monoclonal antibodies to class I mhc molecules but not mhc class II molecules, confirming that most of the cytotoxic cells were restricted in their function by mhc class I molecules. Phenotypic analysis of the cytotoxic cell line suggested that a subpopulation of T cells (BoT4-) was responsible for the effector function. These cells appear to be important in the recognition and destruction of macroshizont infected cells, but it remains to be seen whether or not they play the decisive role in determining the outcome of an infection.

The identification of the antigens that the CTL recognise would be a major advance in the search for candidate molecules for a non-infective vaccine. T cells are the only tool available to identify these antigens, which appear not to be recognised by immune serum. In order to achieve this it is essential to be able to produce and maintain *in vitro* parasite specific CTL lines and clones.

3) Finally, an experiment was conducted to identify the preferred target cells for *in vitro* infection by the two related parasites, in the hope that this might help to explain some of the observed differences in pathology and ease of immunisation between *T. annulata* and *T. parva*.

Established cell lines already infected with either *T. annulata* or *T. parva* were analysed using bovine leucocyte specific monoclonal antibodies (MAbs) in an attempt to identify cell phenotypes. MAbs recognising normal B cells and monocytes/macrophages did not react with any of the lines tested, whereas all lines expressed mhc class II antigens. *T. parva* infected cell lines expressed the BoT4 and/or BoT8 markers indicative of normal T cells; *T. annulata* lines, by contrast, do not. This suggests that the two parasites infect different cells, although the possibility remains that phenotypic markers may be lost following infection.

To elucidate further which cells become infected by *T. annulata* and *T. parva*, defined populations of cells including various alloreactive T cell preparations, adherent cells, and cells separated using a fluorescence activated cell sorter into

subpopulations defined by the leucocyte specific MAbs were incubated *in vitro* with sporozoites of the two parasites. *T. parva* sporozoites readily infected all the alloreactive T cell preparations but did not infect the sorted monocyte/macrophage population. *T. annulata* sporozoites preferentially infected the monocyte/macrophage cells, but on only one occasion was it possible to infect cells within the alloreactive T cell population. In this exceptional case no *T. annulata* infected cells exhibited the T cell markers, whereas *T. parva* infected T cells consistently did. This suggests that the *T. annulata* parasites may in fact have infected contaminating non T cells within the bulk alloreactive population. The B cell, adherent cell, and mhc class II positive cell populations were infected more readily by *T. annulata* sporozoites than by *T. parva*; non B cells and mhc class II negative populations were more readily infected by *T. parva*.

The implication is clear: *T. parva* sporozoites preferentially infect T cells whilst *T. annulata* sporozoites prefer monocytes and macrophages, at least *in vitro*.

If the preference observed for sporozoites *in vitro* is also exhibited by schizonts *in vivo*, this may explain the superior ability of schizonts from *T. annulata* infected cell lines to infect host cells (and consequently to immunise animals). Macrophages phagocytose whatever debris is left after the destruction of foreign cells by the immune system: if this debris contains *T. annulata* schizonts then the transfer from inoculum to host is complete. The animal is infected by passive transfer. If the schizonts are those of *T. parva*, on the other hand, they will not be able to transform the macrophage. An alternative mechanism, by which the *T. parva* schizont is transferred to a host T cell, would require active participation by the schizont, as T cells are not normally phagocytic. Until the mechanisms of parasite transfer from cell to cell are fully understood this remains a speculation.

ACKNOWLEDGEMENTS

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CC128/4	070385	I	MOROCCO	F	6				4888				8					4	8	
CC156/4 (22)	070385	B	MOROCCO	F									8888			8888				
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CT202/5 (11)	070385	I	MOROCCO	F					828				8					8	8	
CT223/4	070385	I	MOROCCO	F	8									88	8			66	8	
CC224/B2	070385	I	MOROCCO	F			4	4									84	84		
CC228/2	070385	I	MOROCCO	F	6		4		8 888				8				84	8		
CC233/1 (24)	070385	I	MOROCCO	F	8												86	8		
CC235/1	070385	I	MOROCCO	F	8		4						8			8888		8	4	
CC237/1 (13)	070385	D	MOROCCO	F							4		4		6	8		8	4	6
CC238/1	070385	I	MOROCCO	F									4					84		88 8
CC241/1	070385	I	MOROCCO	F	8				88888				8							88
CC244/1 (21)	070385	D	MOROCCO	F	8	6						4				8888		888	8	
CC246/1 (17)	070385	I	MOROCCO	F									8888					64		
CC050/4	070385	I	MOROCCO	F											888	8888				
CC080/4 (20)	070385	C	MOROCCO	F											8888			8888	868	
CC100/4	070385	I	MOROCCO	F											8888					
CC104/4 (25)	070385	I	MOROCCO	F	4		4		84888			4				4		64		

APPENDIX I.I (continued).

NAME	DATE	BREED	LOCTN	SE	W1	W2	W3	W4	W5	W6		W7	W8	W9	W10	W11	W12	W13	W16	W20	EU12
					1832	22455	99	2277	70	90675	223	163	77	0	17786	13300	144	916	3620	7731	5566
CC128/4	070385	X	MOROCCO	F																	
CC144/4 (7)	070385	A	MOROCCO	F																	
00150/4 (26)	070385	X	MOROCCO	F																	
CC153/4 (28)	070385	A	MOROCCO	F																	
CC162/3 (3)	070385	X	MOROCCO	F																	
CC170/4 (18)	070385	X	MOROCCO	F																	
CC192/4 (27)	070385	D	MOROCCO	F																	
CT201/5 (19)	070385	C	MOROCCO	F																	
CT204/6	070385	X	MOROCCO	F																	
CC206/4 (2)	070385	A	MOROCCO	F																	
CC223/2 (4)	070385	X	MOROCCO	F																	
CC225/2 (5)	070385	X	MOROCCO	F																	
CC239/1	070385	X	MOROCCO	F																	
CC242/1 (13)	070385	X	MOROCCO	F																	
CC243/1 (12)	070385	X	MOROCCO	F																	
CC 16/4A	080385	X	MOROCCO	M																	
CC 53/4	080385	X	MOROCCO	M																	
CC 67/3	080385	X	MOROCCO	M																	
CC 70/4	080385	X	MOROCCO	M																	
CC 72/4	080385	X	MOROCCO	M																	
CC 77/3 (36)	080385	C	MOROCCO	M																	
CC 87/4	080385	B	MOROCCO	M																	
CC100/4	080385	X	MOROCCO	M																	
CC105/3	080385	X	MOROCCO	M																	
CC107/4	080385	X	MOROCCO	M																	
CC113/3	080385	X	MOROCCO	M																	
CC168/4	080385	X	MOROCCO	M																	
CC169/4	080385	A	MOROCCO	M																	
CT178/6A (32)	080385	X	MOROCCO	M																	
CC179/3 (30)	080385	B	MOROCCO	M																	
CT213/4	080385	X	MOROCCO	M																	
CC217/2	080385	X	MOROCCO	M																	
CT218/4	080385	X	MOROCCO	M																	
CC221/2	080385	X	MOROCCO	M																	
CC 51/4	080385	X	MOROCCO	M																	
CE 13/2	120385	X	MOROCCO	F																	
CE 19/2 (1)	120385	CC	MOROCCO	F																	
CE 20/1	120385	X	MOROCCO	F																	
CE 21/1	120385	X	MOROCCO	F																	
CC 22/1	120385	X	MOROCCO	F																	
CE 23/1 (33)	120385	X	MOROCCO	F																	
CE 26/1	120385	X	MOROCCO	F																	
CE 27/1	120385	X	MOROCCO	F																	
PF292/3	120385	X	MOROCCO	F																	

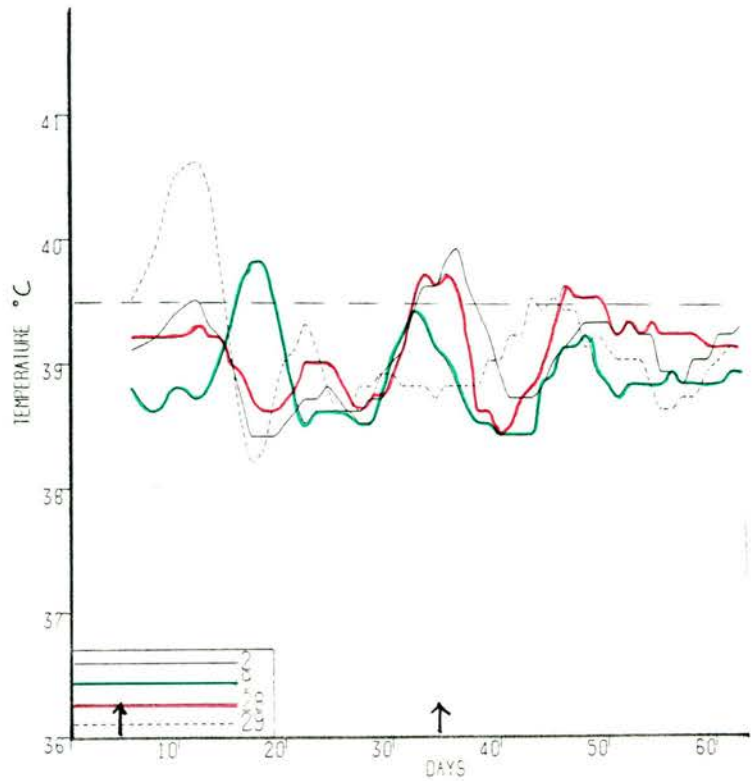
Print out of file typ1eb85.pas
Serum batch is bfeb85.for
Using serum sequence sfall.for

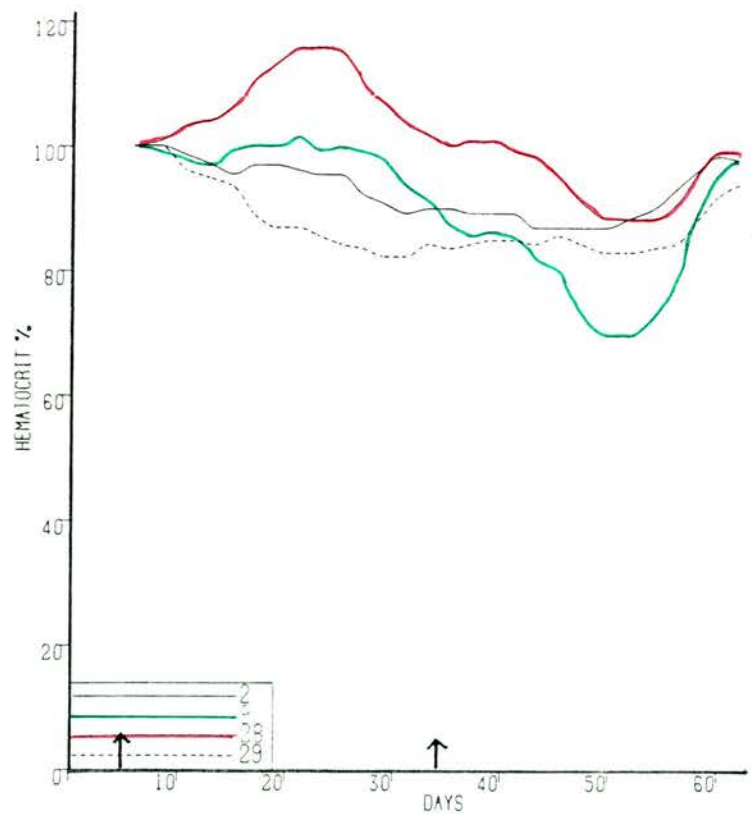
NAME	DATE	BREED	LOCTN	SE	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	W16	W20	EU12
					IEEEE	IEEEEE	IEE	IEEEE	IEE	IEEEEE	EEE EEE EE	E	IEEEEE	IEEEE	IEE	IEEE	IEEEE	IEEE	IEEEE	EEE
					IDDDD	IDDDDD	IFF	IFFDD	IDF	IDDDDD	DDD FDD DD	B	IDFDDD	IDDDD	IDD	IDDD	IDDDB	IFFN	IDDFF	DFFFFDD
					IAAAA	IAAAAA	IAB	IABAB	IAA	IAAAAA	AAA AAA AB	A	IAAAAA	IAAAB	IAB	IAAA	IAAAA	IABA	IABAB	AABABAA
					I0001	I00001	I00	I0011	I00	I00000	000 001 00	1	I00001	I0000	I00	I000	I0011	I001	I0000	0001111
					I0081	I06661	I33	I8811	I09	I01697	199 881 88	0	I11661	I1688	I11	I6791	I7701	I443	I0011	9882200
					I1832	I22455	I99	I2277	I70	I90675	223 163 77	0	I17786	I3300	I44	I916	I3620	I773	I5566	8888819
CE 16/2	I120385	AA	MOROCCO	M	I----I	-----I	--I	----I	--I	88888	--- --- --	8	I-----I	----I	--I	---I	88888	I---I	----	-------
CE 24/1	I120385	I	MOROCCO	M	I----I	---8-I	--I	----I	--I	88-88	--- --- --	8	I----4I	8888I	--I	---I	--4-I	---I	----	-------
CE 25/1	I120385	I	MOROCCO	M	I---4I	-----I	--I	----I	--I	88888	--- --- --	8	I-----I	----I	--I	---I	----I	---I	----	-------
CC 35/4	I120385	I	MOROCCO	M	I8---I	-----I	-4I	----I	--I	-----	--- --- --	-	I-----I	8888I	--I	---I	---4I	---I	--88	8----44
CK146/1	I120385	I	MOROCCO	M	I----I	--8--I	-4I	----I	--I	66888	6-- --- --	8	I68888I	-4--I	--I	--8I	--6-I	---I	----	-------
CT219/3	I120385	CC	MOROCCO	M	I----I	-----I	--I	----I	--I	-----	--- --- --	-	I----4I	----I	--I	---I	8888I	---I	----	-------
CC232/1	I120385	I	MOROCCO	M	I---8I	-----I	-8I	----I	--I	-----	--- --- --	-	I-----I	----I	--I	---I	8888I	---I	8882	888--8-I
CT226/3	I120385	I	MOROCCO	M	I--8-I	-8---I	-8I	----I	--I	-----	--- --- --	-	I-----I	----I	--I	---I	----4I	---I	--88	8----44
CJ362/1	I120385	I	MOROCCO	M	I----I	--4--I	--I	----I	--I	88888	--- --- --	8	I-----I	8888I	--I	---I	----I	---I	----	-------
CD366/3	I120385	I	MOROCCO	M	I8---I	-----I	--I	----I	--I	88-88	--- --- --	8	I-----I	----I	--I	---I	----I	---I	88--	--888--

APPENDIX I.2

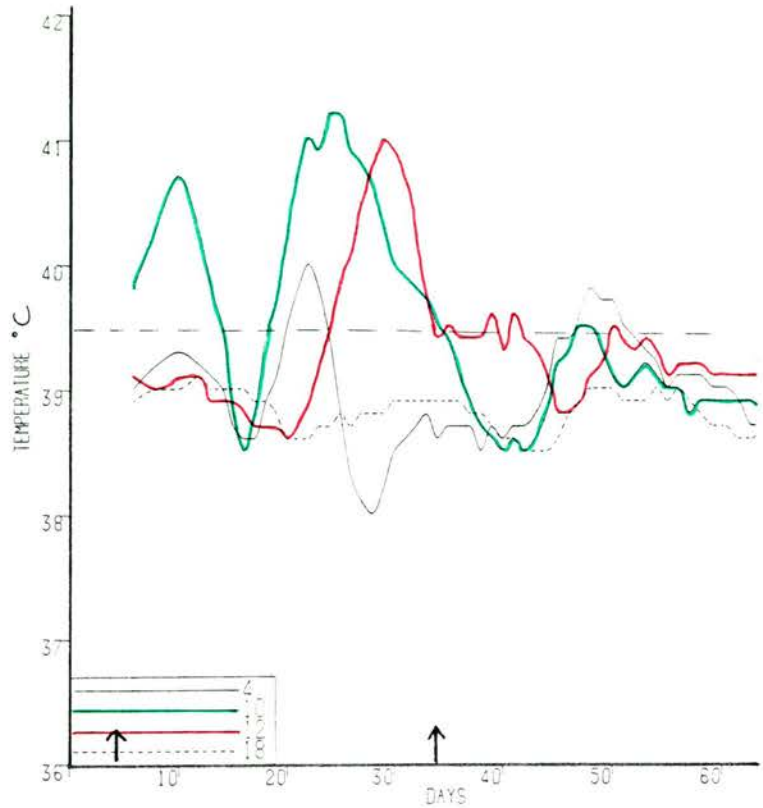
The temperature and haematocrit responses of individual animals immunised as described in Chapter 3. The arrows indicate immunisation and challenge.

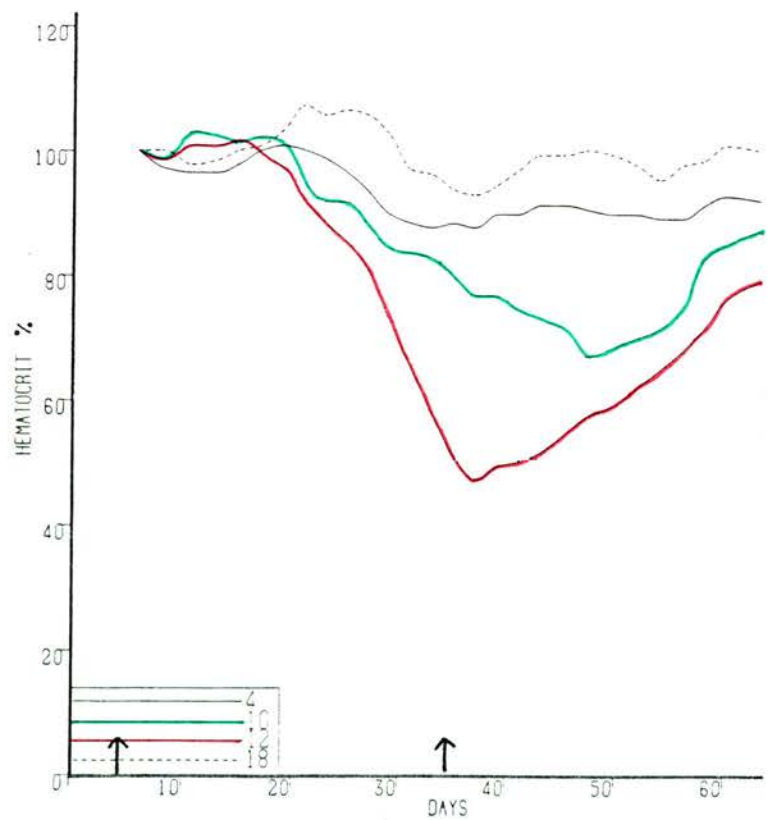
Cell line A recipients (BoLA matched).



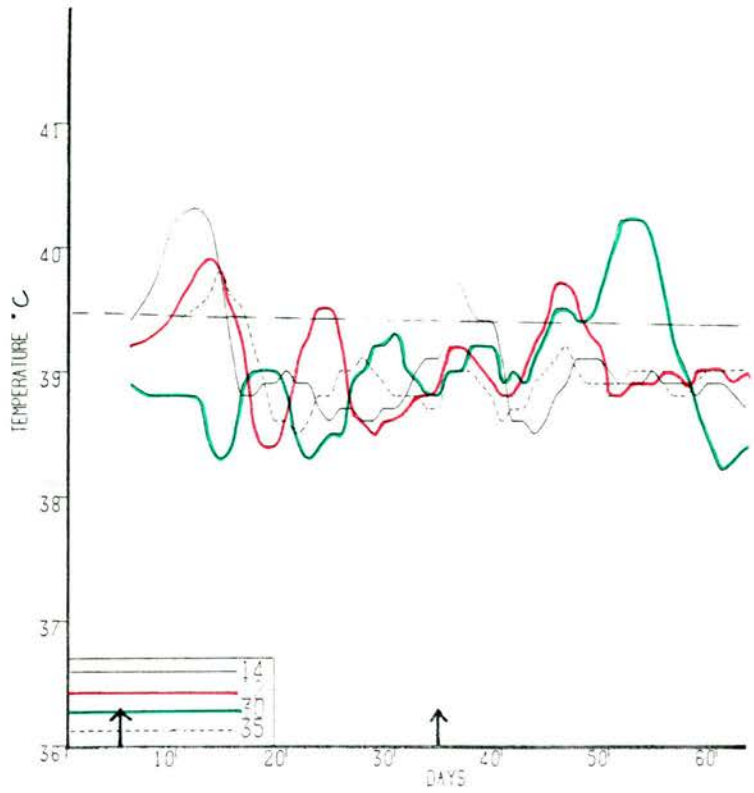


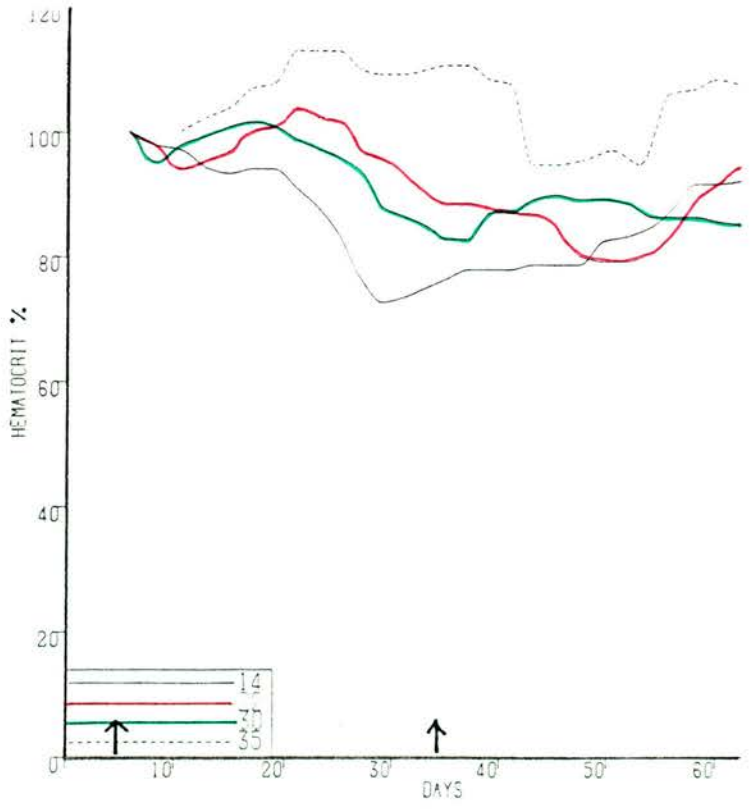
APPENDIX I.2 (continued). Cell line A (BoLA mismatched)



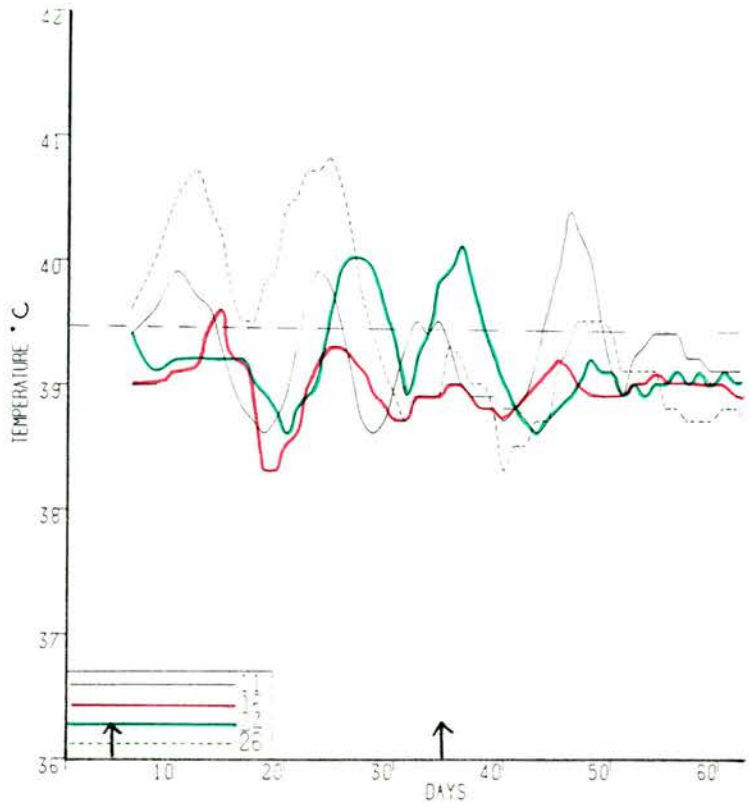


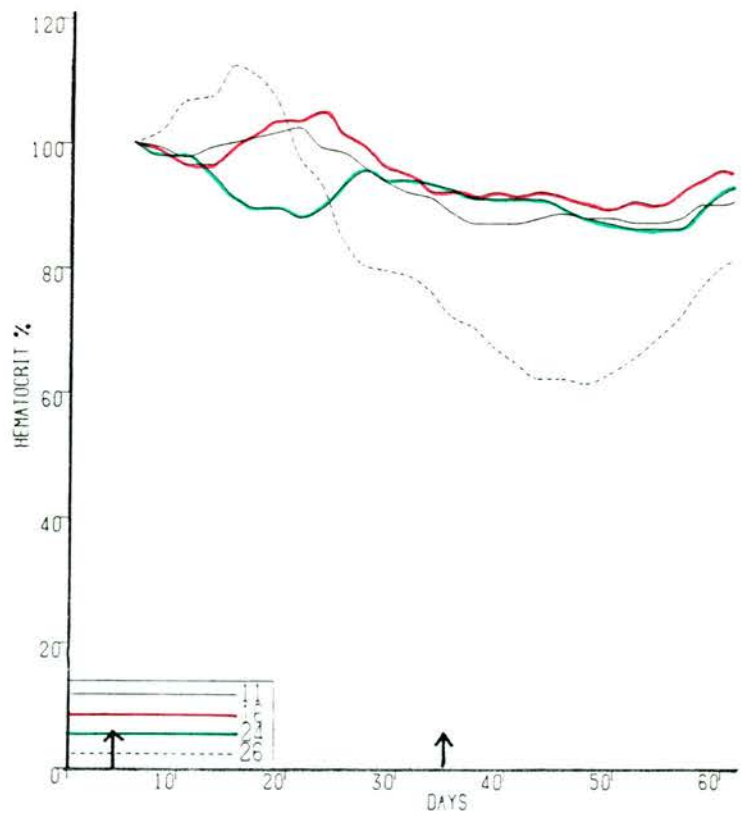
APPENDIX I.2 (continued). Cell line B (BoLA matched).



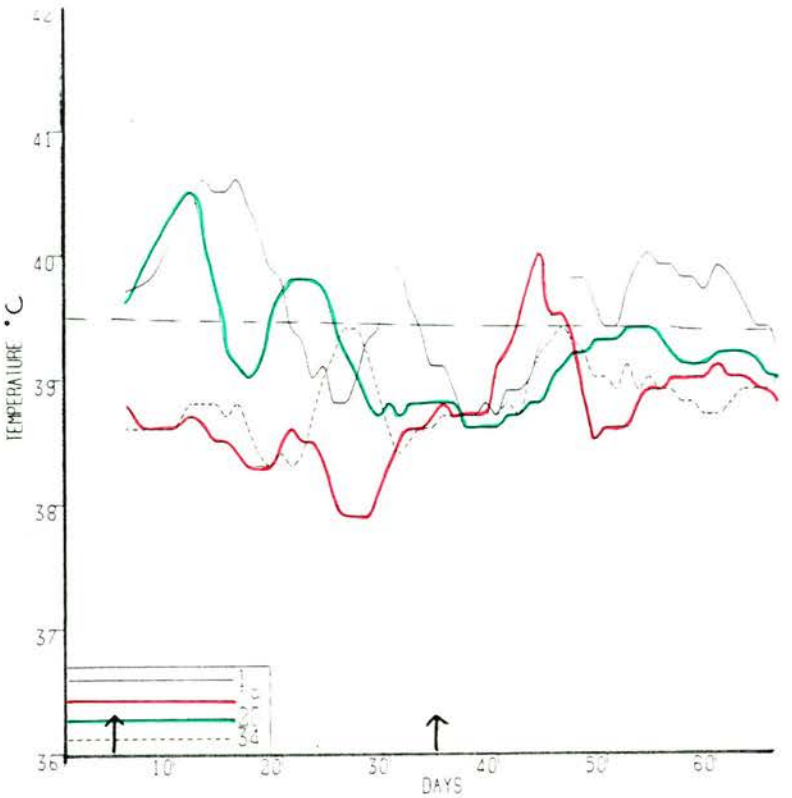


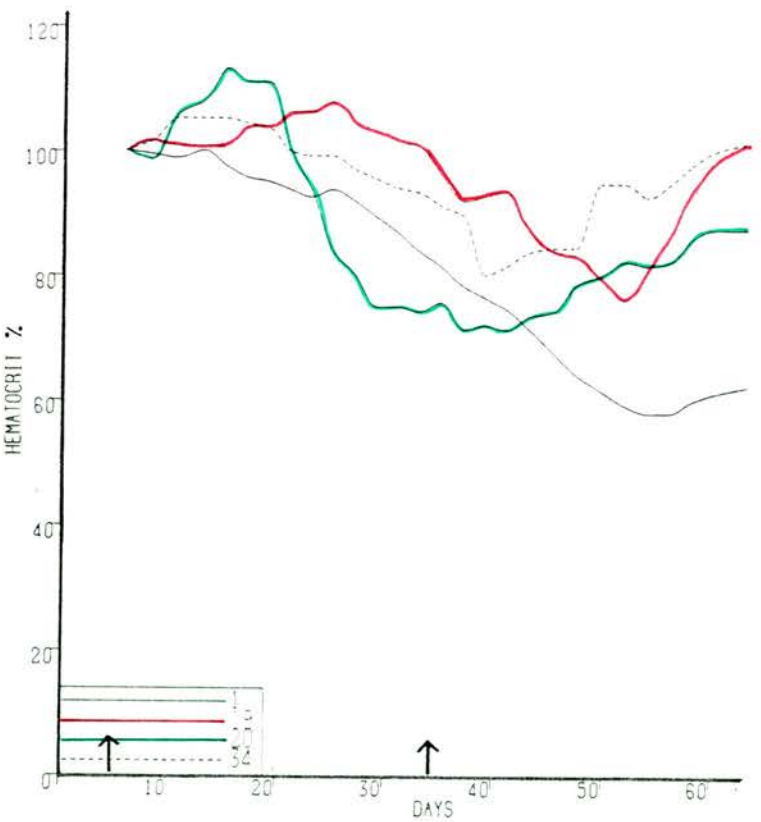
APPENDIX I.2 (continued). Cell line B (BoLA mismatched).



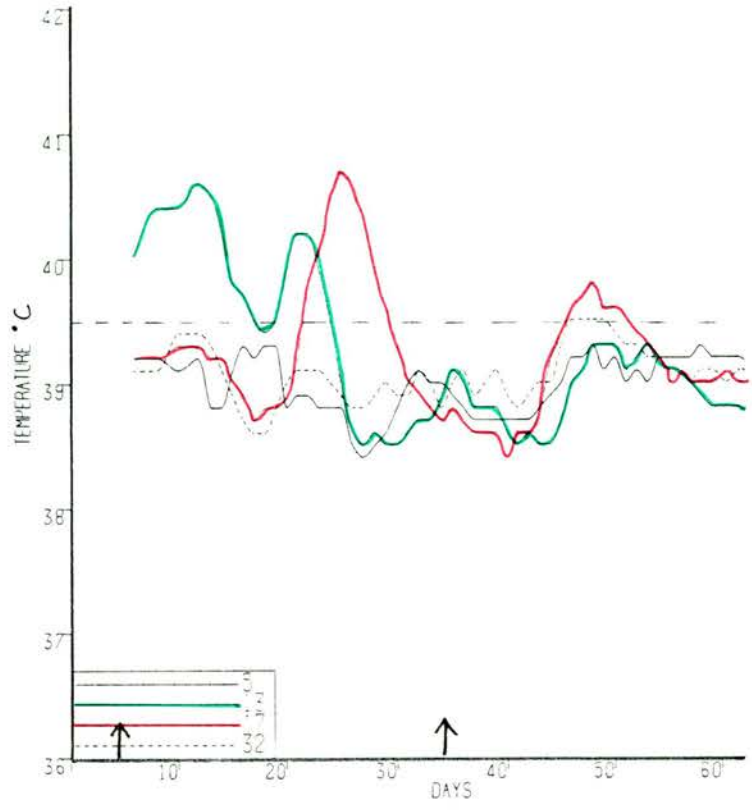


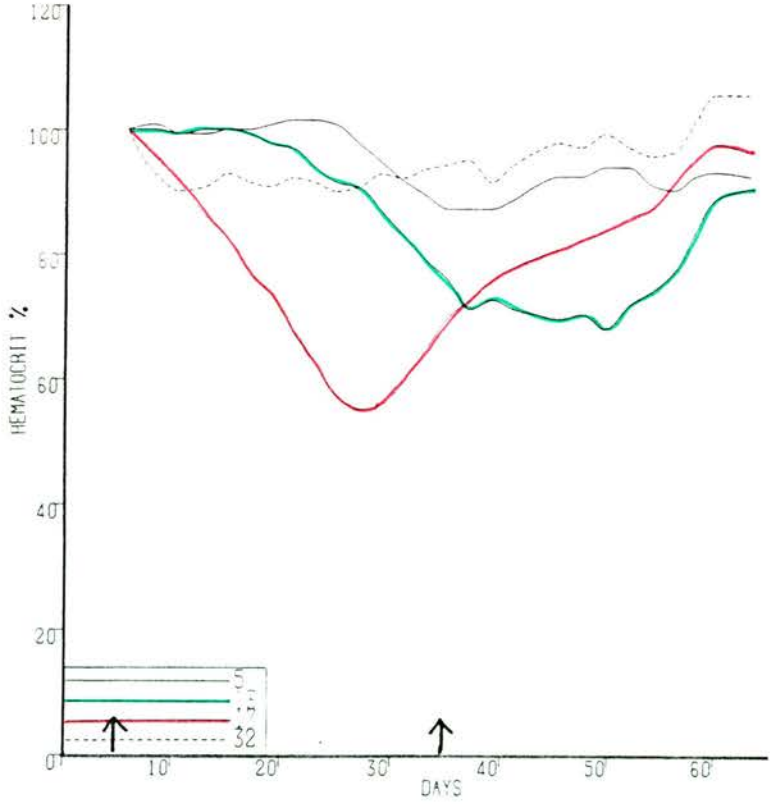
APPENDIX I.2 (continued). Cell line C (BoLA matched).



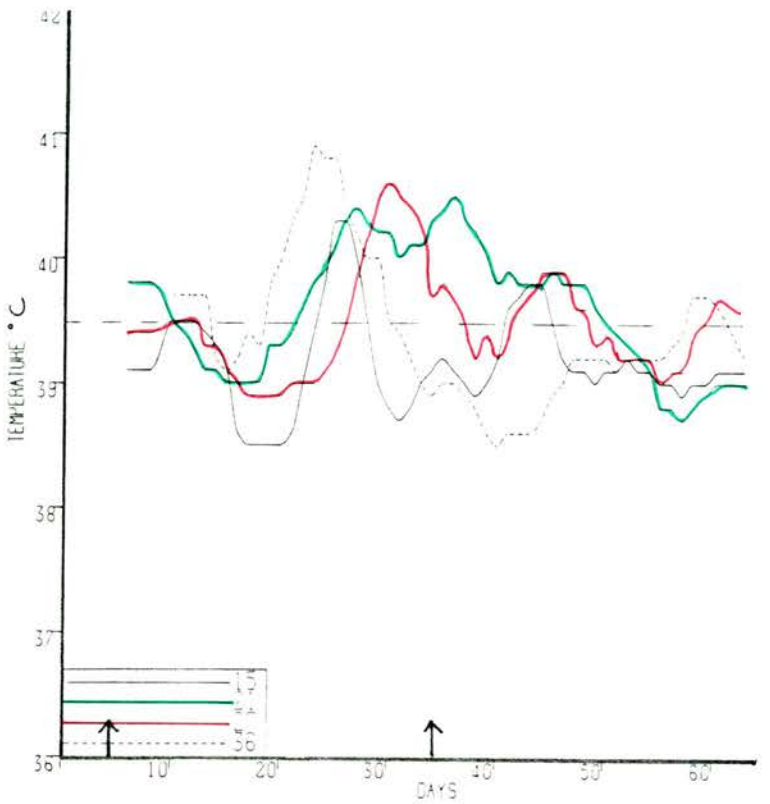


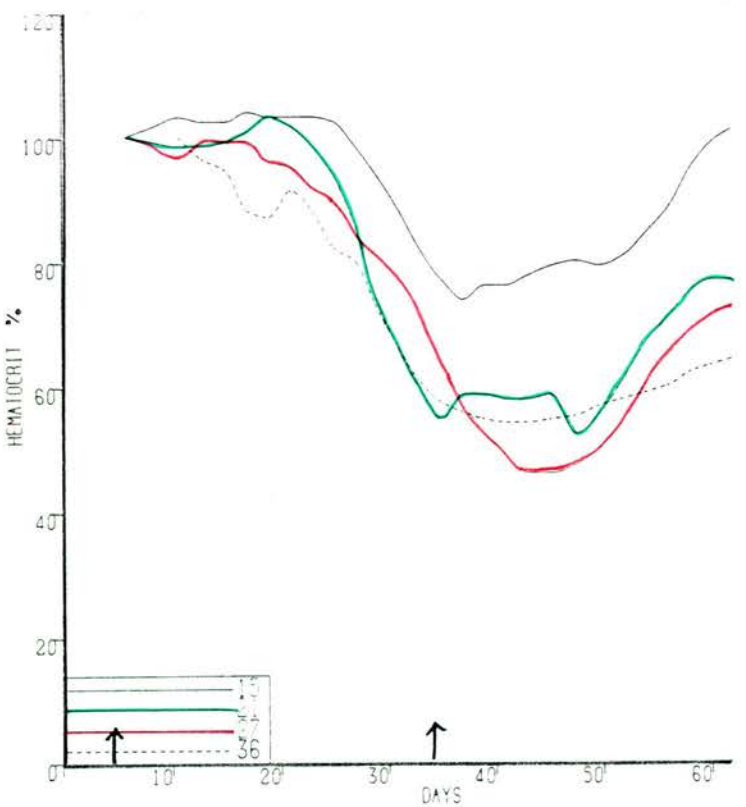
APPENDIX I.2 (continued). Cell line C (BoLA mismatched).



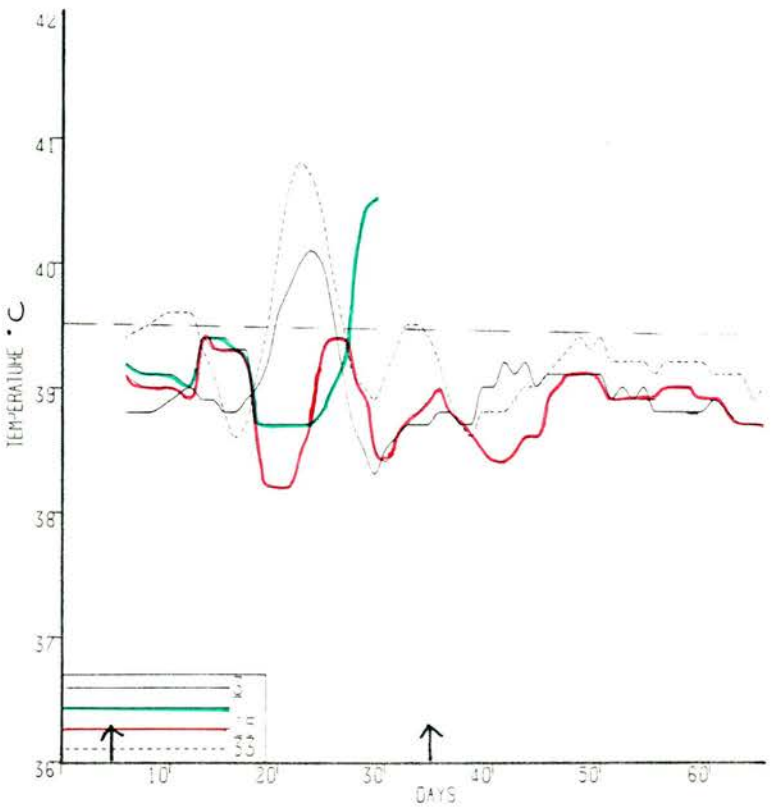


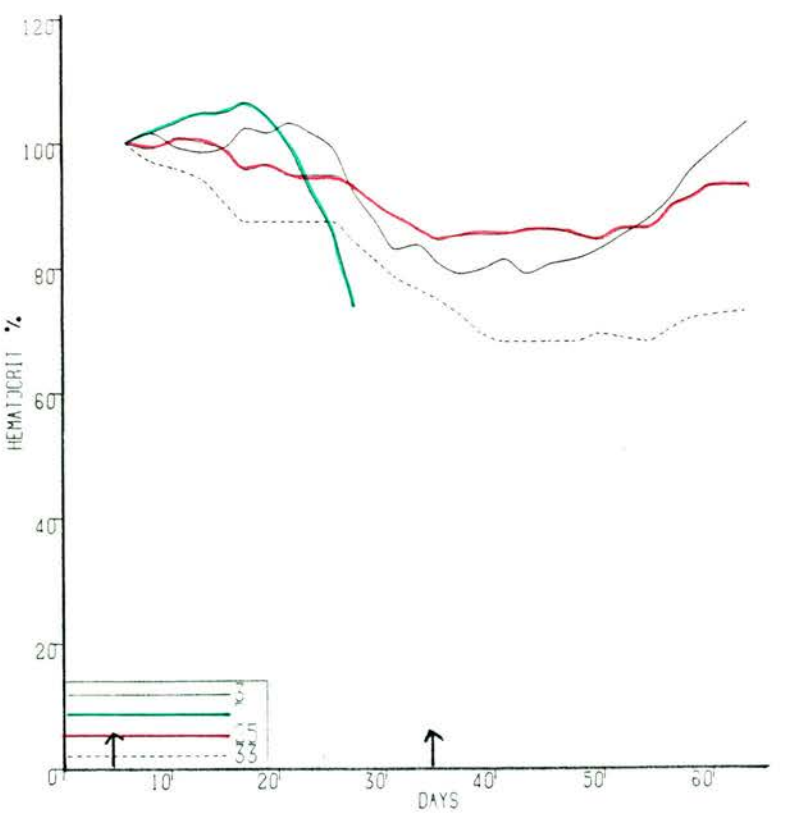
APPENDIX I.2 (continued). Cell line D (BoLA matched).

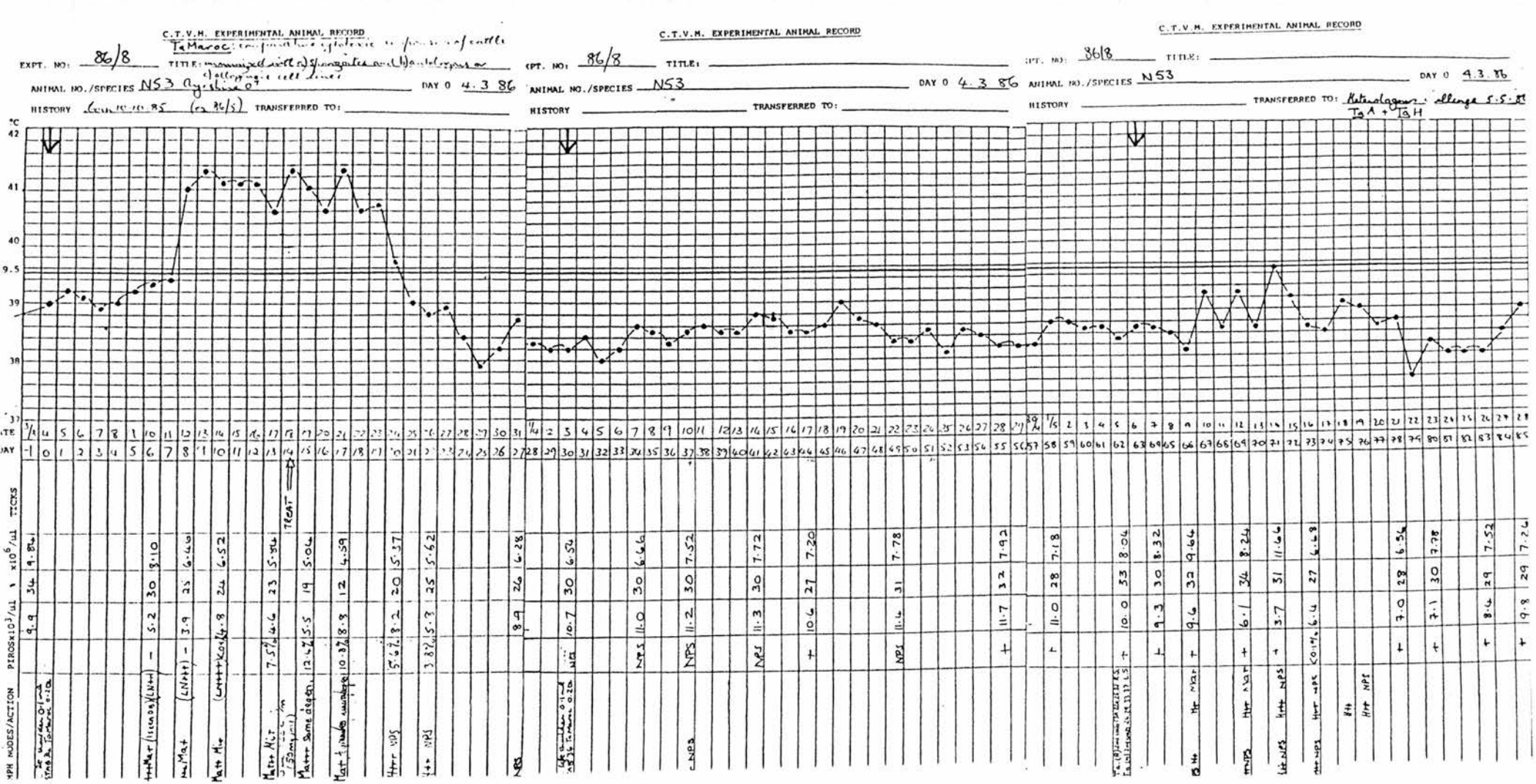




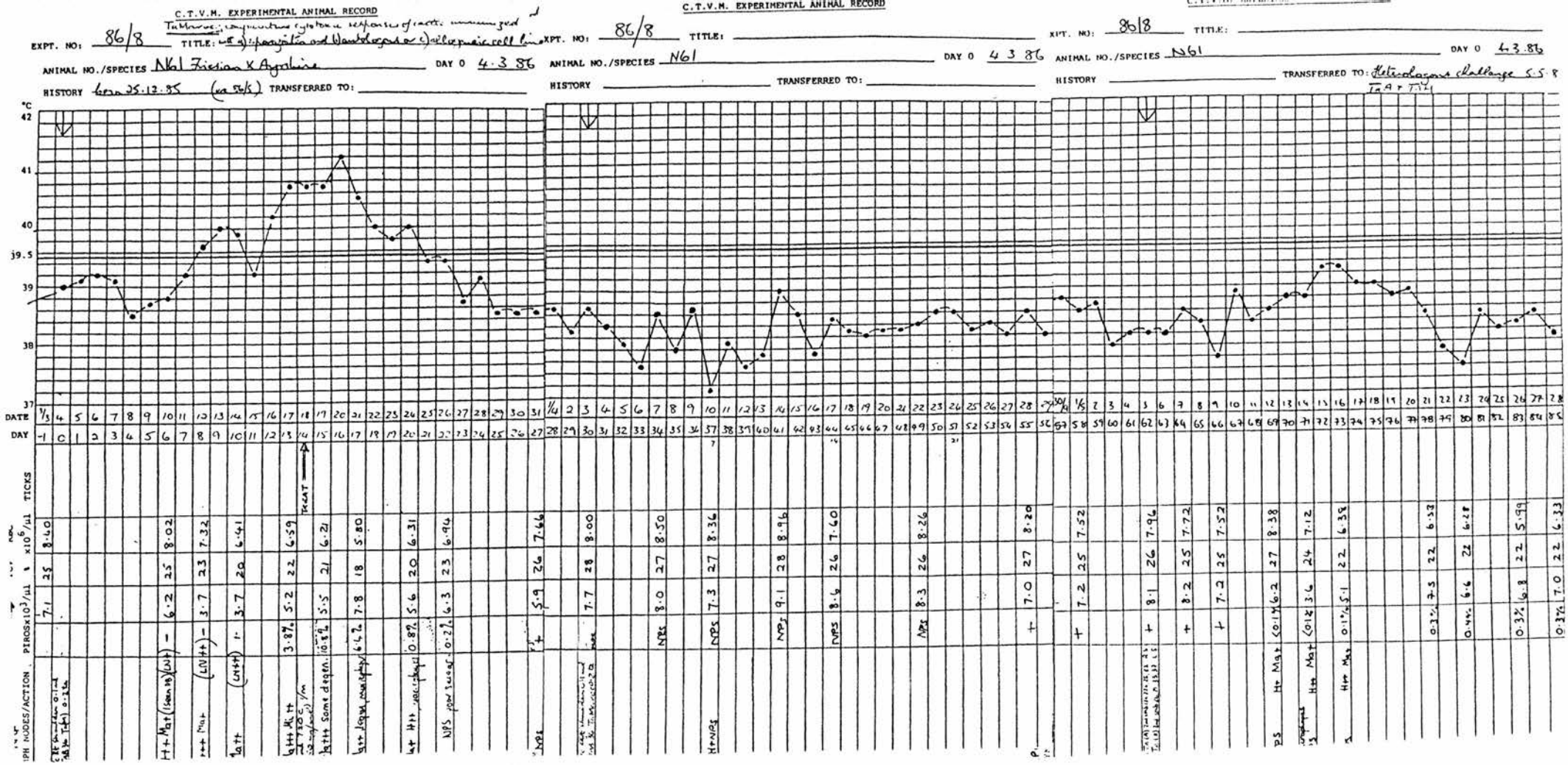
APPENDIX I.2 (continued). Cell line D (BoLA mismatched).



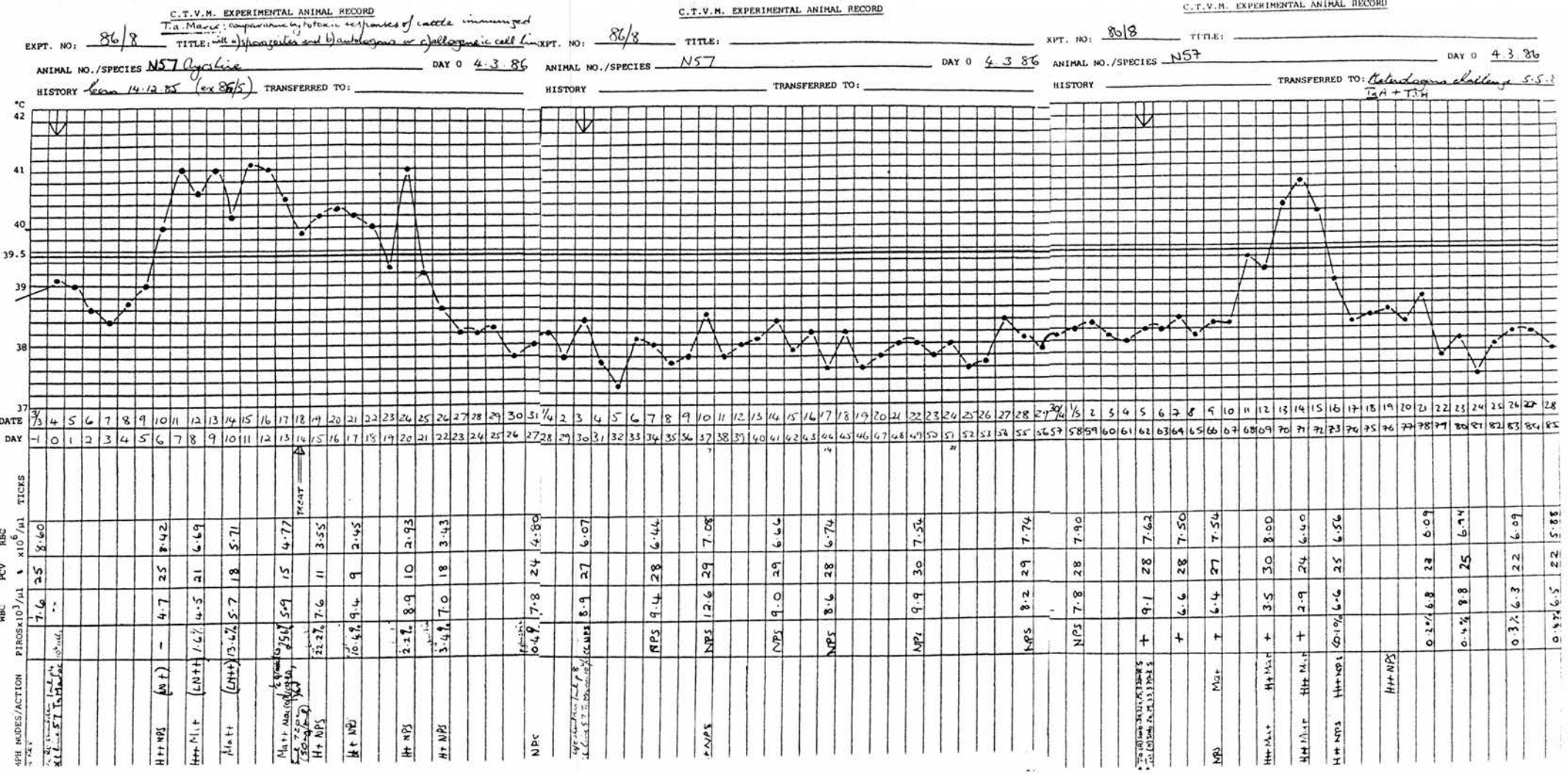




APPENDIX 2 The clinical response of N53 (Sporozoite group)

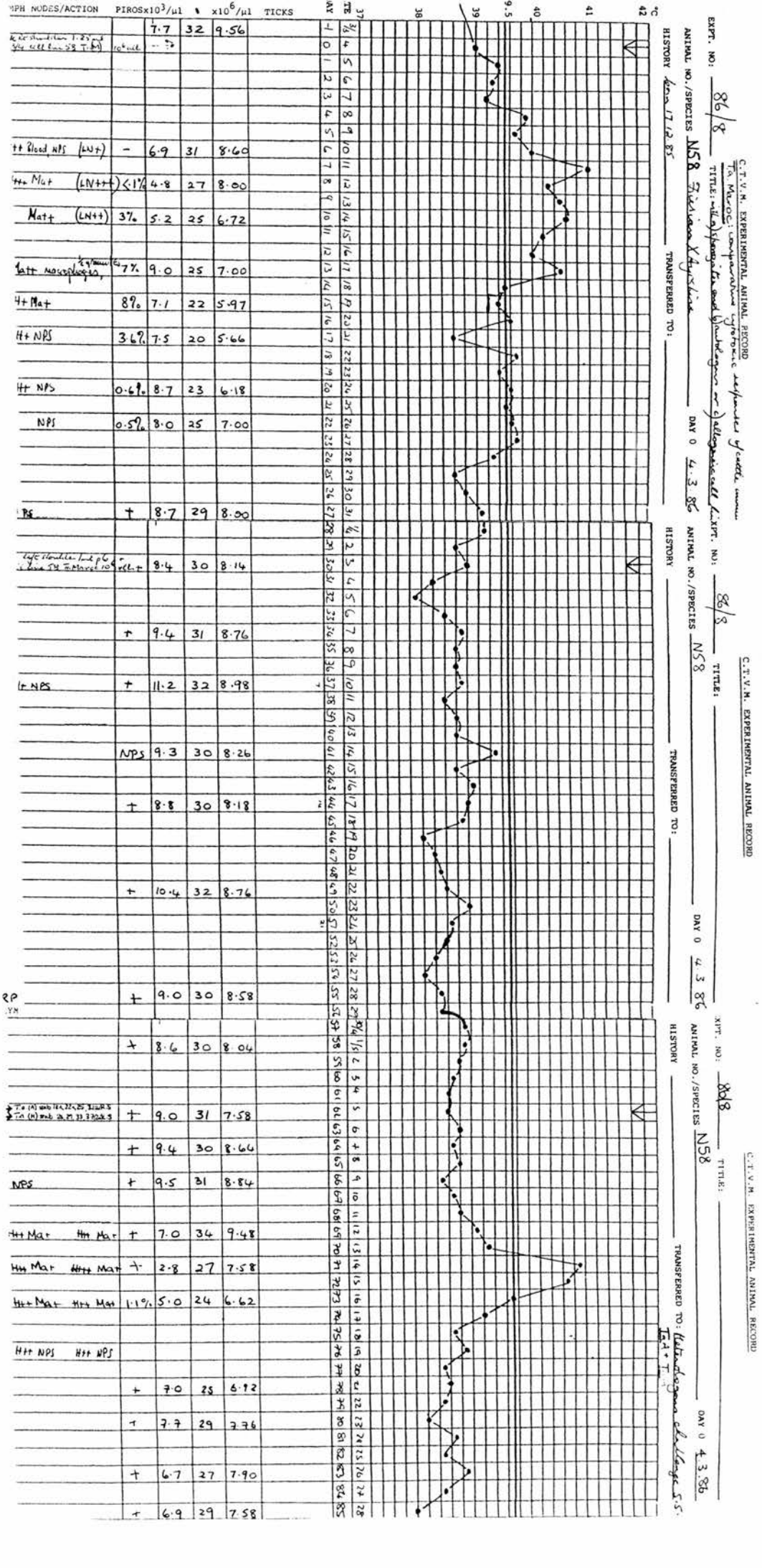


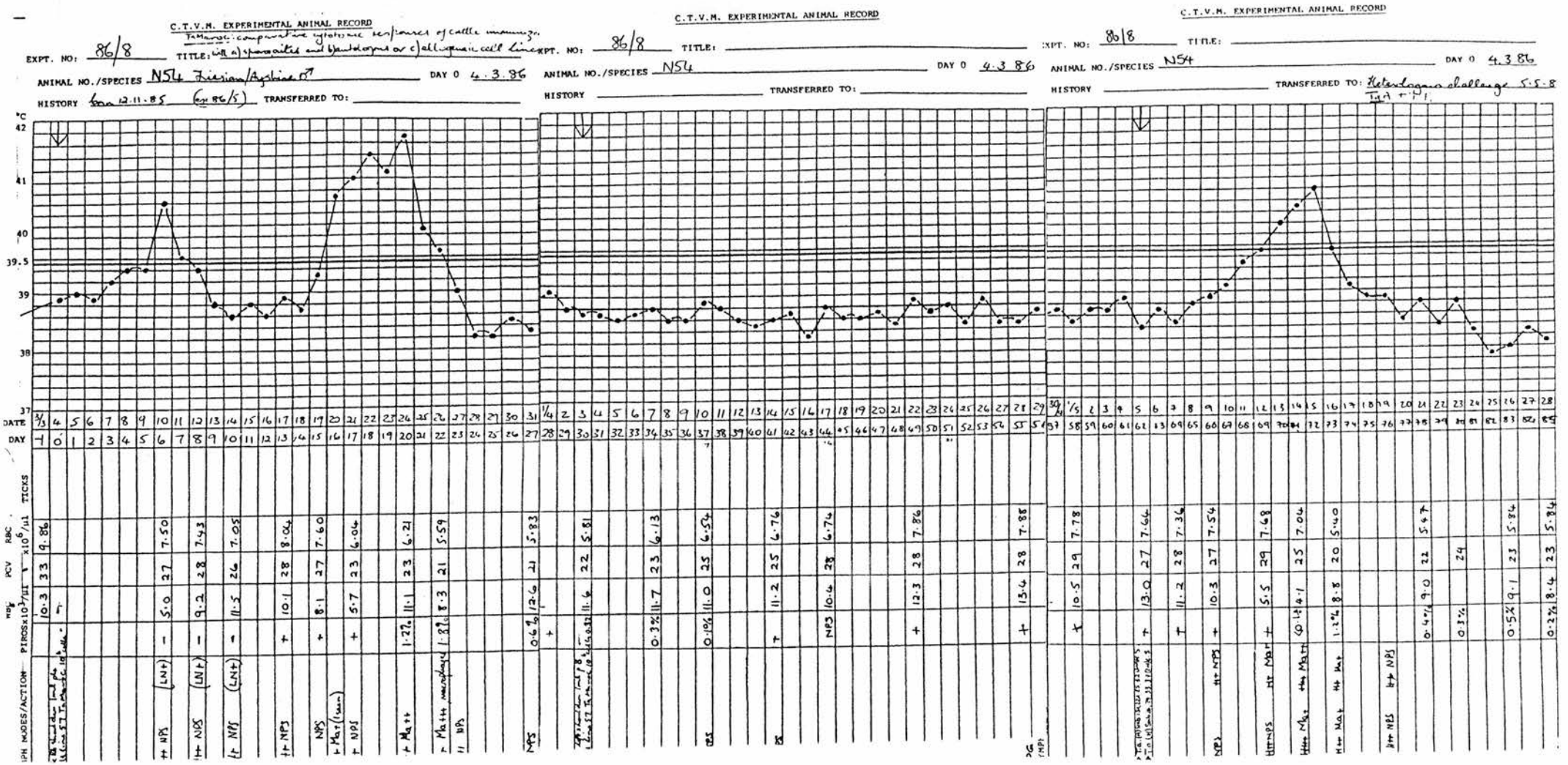
APPENDIX 2 (continued) The clinical response of N61 (sporozoite group)



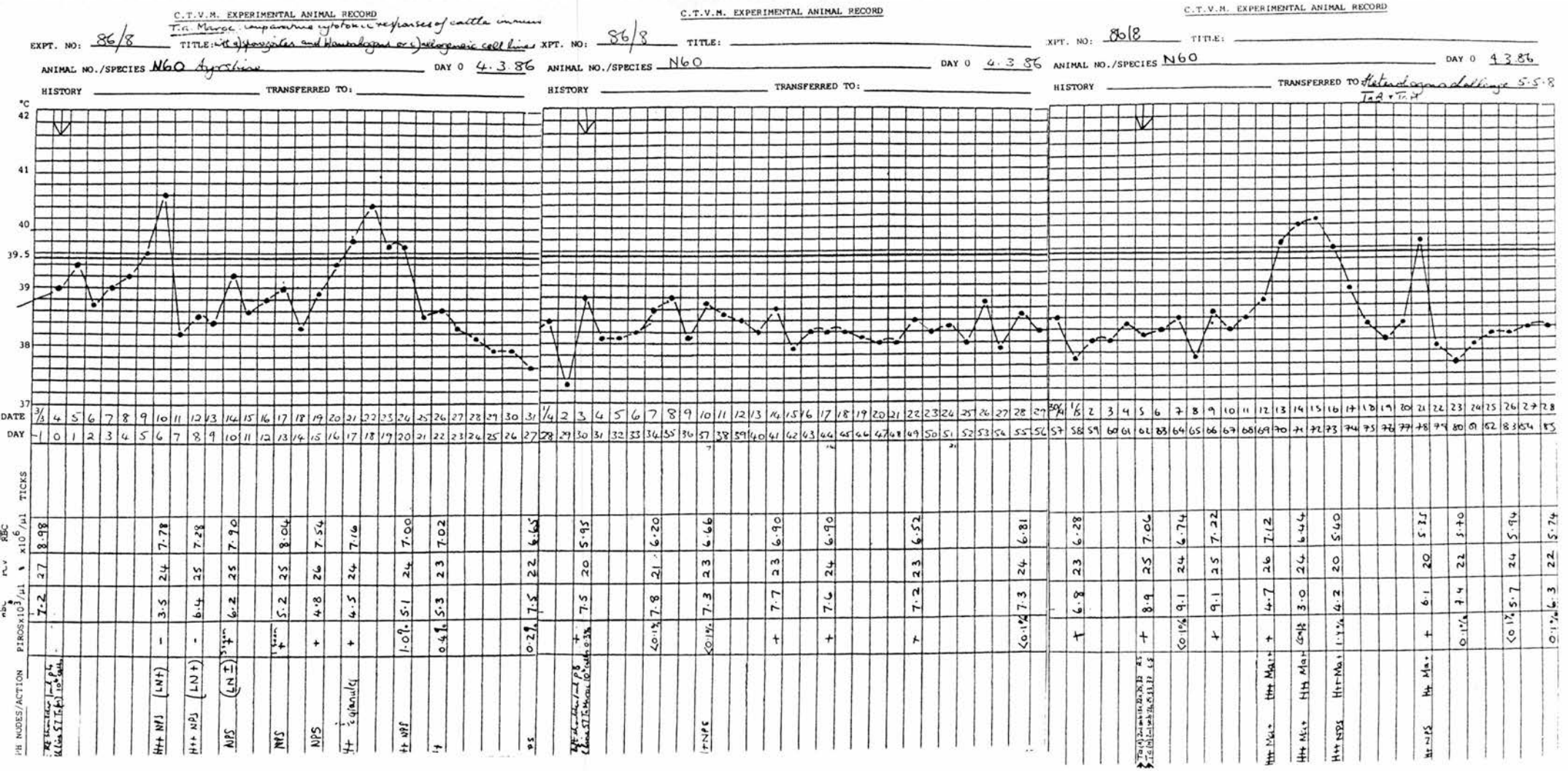
APPENDIX 2 (continued) The clinical response of N57 (autologous cell line group)

APPENDIX 2 (continued) The clinical response of N58 (autologous cell line group)

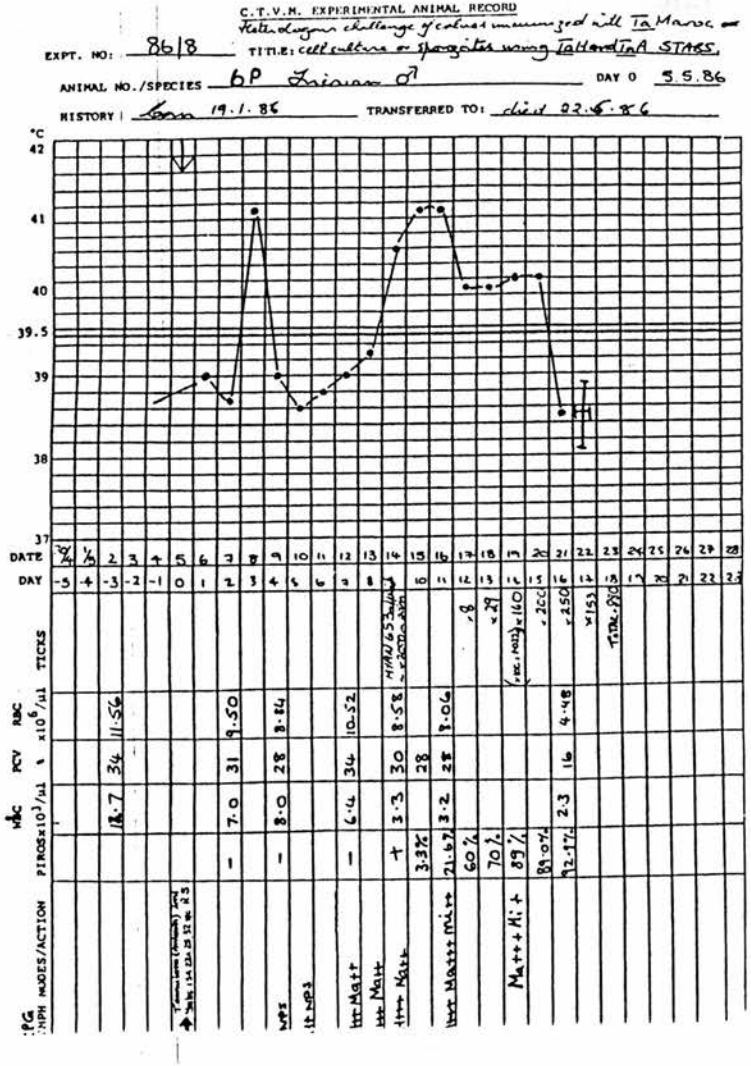


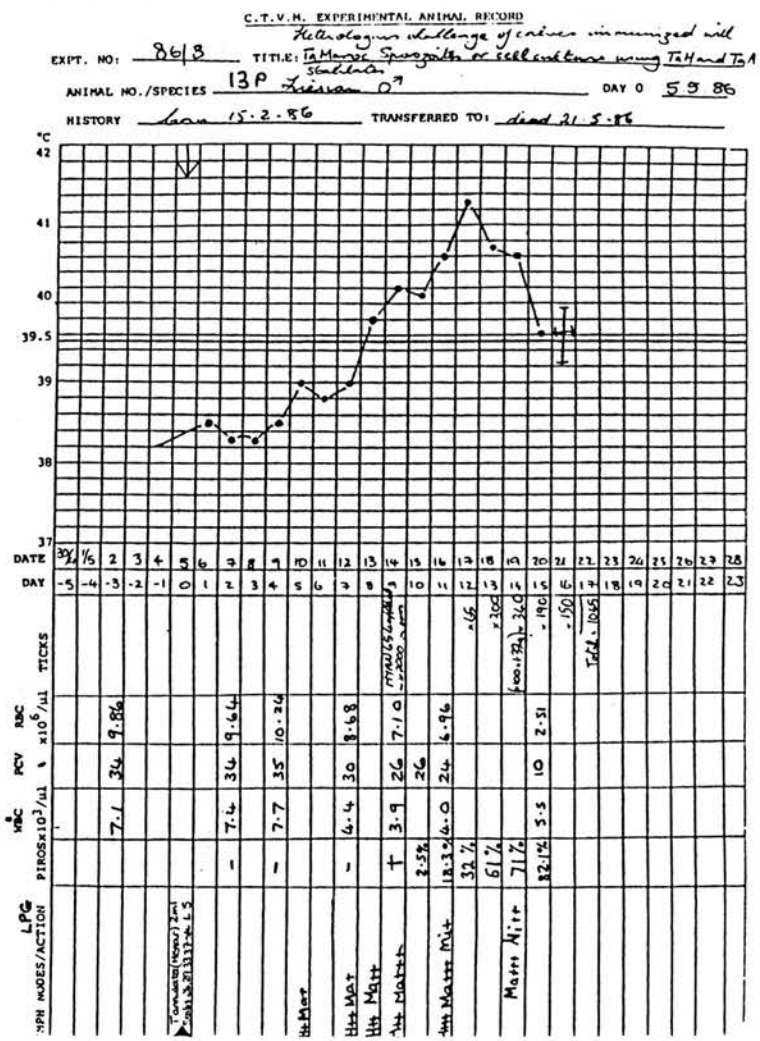


APPENDIX 2 (continued) The clinical response of N54 (alloeneic cell line group)



APPENDIX 2 (continued) The clinical response of N60 (allogeneic cell line group)





APPENDIX 2 (continued) The clinical response of the control animals (heterologous challenge)