TRYPANOSOME NON-SPECIFIC ANTIBODY RESPONSES DURING TRYPANOSOMA CONGOLENSE INFECTION OF CATTLE

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ABSTRACT

EXPERIMENT ONE

Trypanosome infections of cattle are characterized by concomitant increase in serum IgM, development of antibodies reacting with non-trypanosome antigens and an increase in the proportion of $CD5^+$ B cells in peripheral blood and spleen. It is not known whether the three events are related. In mice and humans, $CD5^+$ B cells have been shown to predominantly produce IgM antibodies that are polyreactive in nature. This experiment was initiated first to confirm whether trypanosome non-specific antibodies develop during the course of *Trypanosoma congolense* infections of susceptible Boran or resistant N'Dama cattle. In addition, to investigate whether a different trypanosome species, *Trypanosoma vivax,* can also induce these antibodies. Secondly, to investigate whether the $CD5^+$ B cells, which increase during trypanosome infections of cattle, are the source of the trypanosome non-specific antibodies observed.

Experimental infections were initiated *Trypanosoma congolense* by tsetsefly bite in 13 susceptible Boran and 6 resistant N'Dama cattle. A separate group of 4 Boran cattle was also infected with a different trypanosome species *T. vivax*. Serum samples were collected from infected cattle at different time points and tested in ELISA for trypanosome-specific and trypanosome non-specific antibodies. Seven Boran cattle from the *T. congolense*-infected group were killed between 31-51 after

infection and mononuclear cells prepared from spleen tissue. The cells were double-stained for CD5 using monoclonal antibody IL-A67 and surface immunoglobulins using monoclonal antibody IL-A58. Separate populations of $CD5^+$ and $CD5^-$ B cells were obtained by sorting using a flow-cytometer. Equal numbers of $CD5^+$ and $CD5^-$ B cells were tested in the Silver Immunogold (SIG) blot assay for enumeration of number of cells secreting IgM, IgG and antibodies reacting with non-trypanosome antigens ß-galactosidase, ovalbumin and lysozyme.

ELISA tests on sera from both Boran and N'Dama cattle infected with *Trypanosoma congolense* revealed an increase in antibodies which react with a number of non-trypanosome antigens such as ß-galactosidase, ferritin and cytochrome, but less or no reactivity was found for antigens such as ssDNA and TNP. A similar development of trypanosome non-specific antibodies reacting with ß-galactosidase was found in *T. vivax* infections of Boran cattle. The trypanosome non-specific antibodies were exclusively IgM, while the trypanosome-specific antibodies were both IgM and IgG. Results from the SIG blot assay revealed that numbers of IgM- and IgG-secreting cells were not different between $CD5^+$ and $CD5^-$ populations ($P > 0.05$). However, significantly more cells in the $CD5^+$ population secreted antibodies reacting with non-trypanosome antigens than in the $CD5^-$ population ($p < 0.05$).

It is concluded from these studies that trypanosome non-specific antibodies develop during trypanosome infections of both in Boran and N'Dama cattle, they can be induced by different trypanosome species, are exclusively IgM and mainly secreted by the $CD5^+$ B cells.

EXPERIMENT TWO

In the first experiment, IgM antibodies reacting with a number of unrelated non-trypanosome antigens were detected in serum of trypanosome infected cattle. These antibodies were mainly secreted by the $CD5^+$ B cells. However, the specificity of these antibodies is not known. Two alternative hypotheses have been put forward to explain the reactivity to unrelated antigens observed in serum of trypanosome infected cattle. The first one ascribes reactivity to unrelated antigens due to presence of different antibody clones, each one possessing different specifity as observed in cases of polyclonal activation. The second one attribute reactivity to unrelated antigens due to presence of antibody molecules, each one capable of binding more than one unrelated antigens, such as the polyreactive antibodies secreted by murine and human $CD5^+$ B cells. This experiment was initiated to investigate whether the trypanosome non-specific antibodies are polyclonal or polyreactive.

A pool of serum was made from samples of 6 Boran cattle on 30 days after infection, when trypanosome non-specific antibody levels were highest. The serum

pool was passed through immunoaffinity colums conjugated with either trypanosome antigens or non-trypanosome antigen ß-galactosidase. Antibody fractions that bound to the column and those which did not bind were collected and tested in ELISA for their reactivity to trypanosome and non-trypanosome antigens.

The IgM fraction purified on ß-galactosidase reacted with ß-galactosidase, cytochrome, ferritin and the trypanosome lysate. Similar results were obtained for IgM fraction purified on a trypanosome lysate column. The IgM fraction that exhibited reactivity to different antigens was present in both pre-and post-infection sera. In contrast, the IgG fraction purified on trypanosome lysate column reacted only with trypanosome lysate but not with the non-trypanosome antigens. The trypanosome-specific IgG fraction was only found in post-infection sera.

These results conclude that trypanosome non-specific IgM antibodies are polyreactive. Their presence in pre-infection sera indicates that the infection does not specifically induce them, but helps to amplify their production. In contrast, trypanosome-specific IgG antibodies are monoreactive and specifically induced by trypanosome infection.

EXPERIMENT THREE

Trypanosome infections in cattle induce production of both trypanosome-specific and the trypanosome non-specific antibodies. It is known that specific antibodies

that are directed at the exposed determinants of the variable surface glycoprotein coat play a role of destruction of trypanosomes and eventual elimination of infection. However, the significance of specific antibodies, which recognize various antigens released after the destruction of trypanosomes, and the trypanosome non-specific antibodies are not known. Some workers suggested that antibodies recognizing products of lysed trypanosomes contribute to immunopathological processes such as development of anaemia. However, others suggested that some of these antibodies, such as those binding to trypanosome enzyme cystein protease may play a protective role to the host by neutralizing the enzymatic function of the enzyme. The polyreactive trypanosome non-specific antibodies may potentially bind to host or trypanosome antigens; and both pathogenic and protective consequences are possible. It is therefore, important to study regulation of antibody responses which takes place during trypanosome infections in cattle. Information obtained may help in designing means by which protective antibody responses can be selectively upregulated at the expense of pathogenetic responses. T lymphocytes play an important regulatory role on antibody responses. $CD4^+$ T cells provide helper function to antibody production by B cells during T-cell dependent antibody responses. $CD8^+$ T cells sometimes suppress antibody production. The role played by these two T cell subsets on antibody responses during trypanosome infections of cattle is not known. The aim of this experiment was to investigate the role played by $CD4^+$ or $CD8^+$ on antibody response during *T. congolense* infection of Boran cattle.

Cattle were depleted of $CD4^+$ or $CD8^+$-T cells subpopulations by intravenous injection of specific monoclonal antibodies IL-A11 or IL-A105 respectively, before infection. The levels of the two cell subsets in peripheral blood were monitored by flow-cytometric analyses. Serum samples collected at various time points were tested in ELISA for determination of levels of trypanosome-specific and trypanosome non-specific antibodies.

Flow-cytometric analyses of peripheral blood mononuclear cells revealed a complete depletion of these T cells subpopulations over a period of two weeks. Serum samples collected at various time points were tested in ELISA assay for specific antibodies reacting with whole lysate of trypanosomes, non-specific antibodies reacting with a non-trypanosome antigen ß-galactosidase, and total IgM. Trypanosome-specific antibodies were detected in both IgM and IgG isotypes. In contrast, non-specific antibodies reacting with ß-galactosidase were exclusively IgM. Depletion of $CD4^+$-T cells significantly reduced levels of specific, non-specific and total IgM ($p < 0.05$) while depletion of $CD8^+$-T cells no effect on these antibody types ($p > 0.05$).

These results show that $CD4^+$-T cells play a crucial role in production of trypanosome-specific as well as the trypanosome non-specific antibody responses to

T. congolense infection in susceptible Boran cattle. CD8 T cells have no effect on antibody responses to trypanosome infections in cattle.

DECLARATION

I, Joram Josephat Buza, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work and has not been submitted for a degree award in any other University.

Signature:.....

Date: 22/09/1998

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DEDICATION

This work is dedicated to my parents Mr. Josephat Buza and Mrs. Georgina Buza, my wife Teresia and our kids Joe, Janet and Steve.

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CHAPTER ONE

1.0 INTRODUCTION

Trypanosomiases are a complex of diseases of man and domestic animals caused by several different species of a haemoprotozoan parasite of the genus *Trypanosoma*. Bovine trypanosomiasis is mainly caused by three trypanosome species namely *Trypanosoma congolense, T. vivax* and *T. brucei*. The disease is cyclically transmitted by tsetse flies of the genus *Glossina* (Mulligan, 1970).

Trypanosomiasis is considered to be the most important disease constraint to livestock development in infested areas (Morrison et al., 1985). Enormous International measures to control the disease such as the use of trypanocidal drugs and insecticide spraying of tsetse fly habitats has been employed but little success has been achieved. The failure is due to the widespread distribution of the tsetse flies which infest more than 30% of the African continent (Jordan, 1974). In addition, trypanosomes infect a large number of wild animals which, although they do not develop severe clinical disease, nevertheless become carriers and are a source of infection to domestic animals (Ford, 1970).

The ability of the trypanosome to survive in the host and perpetuate the often fatal infection is mainly due to their ability to change their surface coat, thus varying surface antigens, and therefore evade the immune response of the host (Gray, 1965; Vickerman, 1978). Antigenic variation has made difficult the attempts to develop an immunoprophylaxis. Because in addition to the presence of unknown numbers of variable antigen types (VATs) which are expressed in an infection, there is also a large number of different strains or serodemes, each one capable of expressing a different repertoire of VATs (Vickerman, 1978).

Despite the apparent advantage the trypanosome possesses over the mammalian hosts, some wild bovidae have managed to suppress the infection to low levels of parasitaemia and subsequently suffer little effect (Murray et al., 1982). The ability of these animals to elicit responses which are effective at limiting the numbers of circulating parasites is believed to have been acquired due to selection pressure owing to being exposed to infections over many generations. Trypanosusceptible cattle breeds, which have not been kept under the selection pressure of trypanosome infection, develop a severe disease which often terminates fatally. In contrast, some cattle, especially those belonging to the trypanotolerant breeds, survive and are productive under trypanosome challenge without the aid of trypanocidal drugs

(Vickerman, 1978). Trypanotolerant breeds of cattle include N'Dama, Muturu and some other West African taurine breeds of cattle (Murray et al., 1982).

It has been suggested that immunological mechanisms contribute greatly in the expression of trypanotolerance (Murray et al., 1982; Akol et al., 1986). The quality and quantity of the immune response of trypanotolerant animals may efficiently reduce the number of parasites with subsequent reduction in pathological damage. However, it is not yet clear which immunological mechanisms, if any are responsible for the expression of trypanotolerance. Identification of these mechanisms may assist in devising ways by which the immune may be manipulated to enhance resistance of susceptible and trypanotolerant breeds.

To date, a number of differences in the immune response to trypanosome infection between susceptible and tolerant cattle breeds have been revealed. N'Dama cattle eliminated trypansomes much more rapidly than Zebu cattle following a secondary infection, suggesting a superior secondary immune response (Desowitz, 1959). Some investigators have associated the superior immune response of trypanotolerant animals with the ability to recognise and produce antibodies to certain trypanosome antigens. During *T. brucei* infection, the ability of N'Dama cattle to resist infection as compared to Zebu cattle, was associated with the ability to recognize at least one of the three

common trypanosome antigens of molecular weights 110, 150 and 300 Kd (Shapiro and Murray, 1982). In another study, the ability of trypanotolerant animals to resist infection was related to their ability to recognize a *T. congolense* cysteine protease enzyme (congopain) (Authie *et al.*, 1993). However, it is clear that other differences remain to be studied.

One such difference between the immune response of N'Dama and Boran cattle is the finding that the specific antibody response of susceptible Boran cattle, but not trypanotolerant N'Dama cattle, is compounded by development of high titre of antibodies which are not specific for trypanosomes (trypanosome non-specific antibodies) (Williams *et al.*, 1996). Although the role played by the trypanosome non-specific antibodies during the course of the disease is not known, the fact that in humans and laboratory animals some of these antibodies react with self tissues (Houba and Allison, 1966; Klein *et al.*, 1970; Lindsey et al., 1974; Mackenzie and Boreham, 1974; Dressler and Popham, 1976; Hudson et al., 1976; Kobayakawa et al., 1979) suggest that they may play a role in the pathogenesis of the disease.

While it is agreed that trypanosome non-specific antibodies invariably develop during the immune response of humans and laboratory animals, there has been differing opinions as to whether these antibodies develop in cattle. Trypanosome non-specific

antibodies could not be detected in serum of cattle infected with *T. brucei* (Musoke et al., 1981) and *T. congolense* (Masake et al., 1983), but they were detected in serum from *T. vivax*- (Assoku and Gardiner, 1992) and *T. congolense*-infected cattle (Williams et al., 1996). These contrasting results may have been caused by differences in methodology or sensitivity of assays used. Therefore, further studies in this subject are still required.

A number of hypotheses have been put forward to account for the development of trypanosome non-specific antibodies. These include polyclonal activation of B cells by trypanosome antigens (Hudson et al., 1976; Kobayakawa et al., 1979), cross-reactive trypanosome antibodies (Musoke et al., 1981; Masake et al., 1983) and bystander responses of memory B cells due to action of antigen-specific T-helper cells (Morrison et al., 1985). However, due to lack of experimental evidence, these hypotheses are still debatable.

Polyclonal activation of B cells by trypanosome antigens does not seem to directly account for the development of trypanosome non-specific antibodies. Several investigations using laboratory animals either failed to demonstrate the trypanosome mitogen or detected very weak mitogenic activity in trypanosome antigens (Esuruoso, 1976; Mansfield et al., 1976; Corsini et al., 1977; Assoku and Tizard, 1978). In

addition, it was shown in trypanosome infected cattle that polyclonal activation does not occur (Rurangirwa et al., 1982; Nantulya et al., 1985).

Trypanosome-specific antibodies which develop during an infection are both IgM and IgG. But the trypanosome non-specific antibodies are exclusively IgM. Therefore, if the trypanosome non-specific antibodies were cross-reactive trypanosome antibodies, they would be present in both IgM and IgG isotypes. For the same reason, bystander responses of B cells does not seem to adequately account for the development of trypanosome non-specific antibodies.

Recently, following the observation that $CD5^+$ B cells increase in blood and spleen concomitantly with an increase in serum IgM during *T. congolense*-infection of cattle, it was suggested (Naessens and Williams, 1992) that trypanosome non-specific antibodies might come from polyreactive antibodies secreted by the $CD5^+$ B cell subpopulation, similar to those secreted by the B-1 subpopulation in humans and mice (Hayakawa et al., 1983).

The objectives of this study were to confirm whether trypanosome non-specific antibodies develop in cattle during infection with trypanosomes. In addition, to investigate whether the $CD5^+$ B cells were responsible for the production of these

antibodies and whether $CD4^+$ or $CD8^+$ T cells played a role in the development of trypanosome non-specific antibodies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African trypanosomiasis

2.1.1 General

African trypanosomiases are a group of diseases of man and animals caused by protozoan parasites called trypanosomes. Trypanosomiasis is regarded as a major disease constraint to livestock development in sub-Saharan Africa (Jahnke et al., 1987). The disease is characterized by intermittent parasitaemia and fever, anaemia, loss of weight, reduced productivity, and death in untreated cases.

African trypanosomiasis is transmitted by tsetse flies of the genus *Glossina* (Mulligan, 1970). The tsetse vector of trypanosomes is widely distributed, infesting 37% of Africa south of the Sahara and leaving about 50 million people at risk of contracting sleeping sickness. In addition, one-third of the cattle population of Africa is at risk of the disease. In cattle, trypanosomiasis causes slow growth, weight loss, poor milk yield, loss of tractive power, abortion, infertility and death

(Paling et al., 1991). It was estimated that the disease cause an annual loss of $ 5 billion in meat production alone (ILRAD, 1989).

2.1.2 Etiology

Trypanosomes are spindle-shaped organisms which are 8.0-39.0μ long. They possess a flagellum which originates at the basal body located at the posterior end of the trypanosome. The flagellum extends to the anterior end of the body and is attached along its length to a pellicle to form an undulating membrane. In a stained specimen, a single centrally placed nucleus is evident. Adjacent to the flagellar pocket is the kinetoplast, a small structure which contains the DNA of the single mitochondrion. Under electron microscopy, a 12-15 μm thick surface coat overlying the plasma membrane is seen on trypanosome stages found in the mammalian host and infective stages found in the vector.

The genus trypanosoma comprises all known trypanosomes (Hoare, 1970). Trypanosomes of importance to human and livestock are subdivided into two sections; Stercoraria and Salivaria depending on the mode of development in the insect vector or mode of transmission into the vertebrate host (Hoare, 1970). Trypanosomes in the Stercoraria section are transmitted through the posterior station (hind gut) and include *T. cruzi* which causes Chagas' disease in South America. Salivarian trypanosomes are trasmitted through saliva when the vector

feeds on the mammalian host, and include many trypanosome species of importance to man and livestock in Africa.

In man, the disease, also called sleeping sickness, is caused by two trypanosome species. *T. brucei rhodesiense* causes predominatly acute infection in East Africa while *T. brucei gambiense* infections leads to a more chronic human trypanosomiasis in West Africa. The economically important species of trypanosomes causing disease in livestock are *T. brucei brucei, T. congolense, T. vivax, T. evansi and T. simiae*. These species are not infective to man. While each of the three species pathogenic to livestock is capable of inducing disease on its own, mixed infections involving two or three species are common under field conditions (Sileghem et al., 1994).

2.1.3 Geographical distribution

Tsetse flies are found in Africa between latitudes 14 ^{0} North and 29 ^{0} South. Outside Africa, tsetse flies are also found in Saudi Arabia. The distribution of vectors is generally related to the distribution of the disease. However, some trypanosome species such as *T. evansi* can be transmitted in the absence of tsetse flies and are therefore found outside the tsetse belt such as in Asia, South America and in tsetse-free areas of North Africa. *T. evansi* only exists as a blood stream form and can be transmitted mechanically by biting flies. In Central and South

America, which are tsetse-free areas, incidences of *T. vivax* infections occur due to mechanical transmission.

2.1.4 Transmission

Wild animals are natural hosts of many salivarian trypanosomes. They serve as a reservoir from which the parasites are transmitted to livestock by tsetse flies. These wild animals include warthog (*Phacochoerus*), bush pig (*Potamochoerus*), duiker (*Sylvicapra* spp), eland (*Taurotragus*), bush buck (*Tragelaphus*), buffalo (*Syncercus*) and others (Ford, 1970). Within wild animals, trypanosomes are maintained by transmission cycles involving tsetse flies. Animals are infected by trypanosomes through a bite by an infected tsetse fly. Tsetse flies in turn become infected when they feed on an infected animal. In the tsetse fly, the ingested trypanosomes undergo a cycle of development lasting 8 to 35 days before the infective metacyclic trypanosomes are produced (Vickerman, 1985). The infected vector may remain capable of transmitting trypanosomes for the rest of its life.

Apart from cyclical transmission, both tsetse flies and biting flies can mechanically transmit trypanosomes. Typical mechanical transmission of trypanosomes involves *T. evansi* in Asia, South America and North Africa and *T. vivax* in Central and South America.

Carnivores such as lions, hyenas and cats can be infected by *T. brucei* through eating infected carcasses. Transplacental infection is theoretically possible but there are no reports of its occurrence.

2.1.5 Life cycle of trypanosomes

The life cycle of trypanosomes alternates between two hosts: the vertebrate host and the tsetse fly vector (Vickerman, 1985). The vertebrate host is infected when a tsetse fly feeding on it injects into its skin saliva containing metacyclic trypanosomes. A local inflammatory "chancre" reaction develops at the site of a fly bite. After a period of development in the chancre, the metacyclic trypomastigotes transform into blood stream trypomastigotes and eventually enter the draining lymphatics and later the blood vessels. At this stage, *T. congolense* and *T. vivax* remain largely intravascular while *T. brucei* escapes into the soft connective tissues and multiplies in the tissue fluid. *T. brucei* exists in blood in two forms: the dividing long slender form which constitutes the majority of parasites during the ascending parasitaemia, and the non-dividing short stumpy form when parasitaemia is in decline.

Following ingestion of a blood meal from the infected vertebrate host, trypanosomes enter a procyclic phase of development in the fly midgut where they undergo a proliferative phase. The multiplicative forms in the midgut are known

as epimastigotes. For *T. brucei,* the epimastigotes migrate to the salivary gland where they attach to the epithelium by the flagellum. Here they differentiate into the non-multiplicative metacyclic forms which are released into the fly's saliva ready to be injected into the host during feeding. Differentiation of *T. congolense* epimastigotes into metacyclic forms takes place in the proboscis. Eventually the metacyclics invade the hypopharynx for discharge. The procyclic phase of *T. vivax* is similar to that of *T. congolense*, except that the development in the fly's mid gut does not occur.

2.1.6 Antigenic variation

Trypanosomiasis is characterized by waves of parasitaemia in the blood. Each wave is due to the appearance of a trypanosome population with a surface coat made up of a different variable antigenic type (VAT) (Gray and Luckins, 1976). The host immune response eliminates trypanosomes of a particular VAT, but others which have changed their surface coat survive and initiate another wave of parasitaemia.

The surface coat of trypanosomes is 12-15 μm thick overlying the plasma membrane. It is made up of one major glycoprotein, the variable surface glycoprotein (VSG). A single trypanosome has over 10^7 VSG molecules composing its surface coat (Cross, 1990; Turner et al., 1985). Antigenic variation

starts in the tsetse fly vector when the trypanosomes differentiate into vertebrate-infective metacyclic forms in the salivary glands (Tetley et al., 1987). The metacyclic trypanosomes extruded in the salivary glands contain a mixture of metacyclic VATs (M-VATs), which is about 27 for *T.b. rhodesiense* (Turner et al., 1988) and 12 for *T. congolense* (Crowe et al., 1983). The exact number of blood stream VATs which are expressed by a single clone is not known, but may be as high as 100 for *T. equiperdum* (Capbern et al., 1977).

Antigenic variation of trypanosomes is genetically determined, each VSG being a product of one gene. About 1000 VSG genes were found for *T.b. brucei* using hybridization studies (Van der Ploeg et al., 1982). It is not known what stimuli signal the switch from one VAT to the other. However, it was shown that the host's antibody response is not a stimulus for antigenic variation as new VATs occur *in vitro* in the absence of antibodies (Doyle et al., 1980).

2.1.7 Parasite control

The first wave of parasitaemia reaches a peak and then subsides as a result of an antibody-mediated trypanosome destruction. However this is followed by a fluctuating parasitaemia which develops into chronic infection as the immune system eliminates parasites of particular VATs only to be confronted by new VATs. Antibodies responsible for parasite clearance are of the IgM and IgG

isotypes, and are directed against VSG surface-exposed epitopes (Nantulya et al., 1979; Musoke et al., 1981; Morrison et al., 1982). Studies of neutralization of infection indicate that IgM is much more effective in mediating trypanosome destruction than IgG (Morrison et al., 1982). It is believed that elimination of parasites is mainly effected through phagocytosis of antibody-coated trypanosomes by liver macrophages rather than complement-mediated lysis (MacAskill et al., 1980; Dempsey and Mansfield 1983; Ngaira *et al.*, 1983; Urquhart and Holmes, 1987). In mice, the protective antibody response appears to be T-cell independent since it occurs in nude mice which are deficient in T cells (Clayton et al., 1979).

2.1.8 Control of trypanosomiasis

Trypanosomiasis remains the major constraint to livestock development in tsetse infested areas, despite enormous efforts employed to control it. The major disease control approaches currently in use fall in two categories; those directed at the trypanosomes and those directed at the vectors.

The use of chemotherapy or chemoprophylaxis of animals exposed to the risk of trypanosomiasis has been successful but it is expensive (Dwinger, 1985). In addition, few trypanocidal drugs are available and their continued use has inevitably led to development of drug resistant trypanosomes (Holmes and Scott, 1982). The other problem associated with the use of trypanocidal drugs is the

inability of these drugs to penetrate to some tissue sites (privileged areas) where trypanosomes lodge (Poltera, 1983; Rudin et al., 1984). These sites, which include the central nervous system act as foci from which relapse infections later arise.

The major trypanosomiasis control method currently in use is aimed at attacking the tsetse vectors by spraying their habitat with insecticides. This method has successfully eradicated tsetse flies in some localized areas in Nigeria (Spielberger et al., 1977), South Africa and Zimbabwe (Chapman, 1976) and Botswana (Davies and Bowles, 1979). Other related methods involve the use of traps and targets impregnated with insecticides (Hargrove and Vale, 1979; Vale, 1980). These measures are however made difficult by the vast size of tsetse infested areas, the presence of more than 30 tsetse species adapted to different climatic conditions and vegetations, and ecological and environmental constraints (Jordan, 1974).

Bush clearing of tsetse habitats has also been used to control trypanosomiasis. But this method is very expensive and is only effective in geographically restricted areas (Akol, 1985). Furthermore, vigorous efforts are required to prevent re-invasion of cleared areas by trypanosomes.

Biological control of tsetse flies by release of sterile males has also been used (Simpson, 1958). Male tsetse flies which are sterilized by irradiation can not breed

successfully. The sterile males compete with wild males for females. The overall effect is reduction in the tsetse population in the target area. However, this method is expensive and can only be used successfully in areas where tsetse populations have been reduced by other methods, such as insecticide spraying (Roelants and Williams, 1982). In addition, sterile males are also capable of cyclical transmission of the disease (Moloo, 1982). Therefore, release of large numbers of these sterile flies may increase the incidence of the disease.

The development of vaccines for immunoprophylaxis against trypanosomiasis has been hampered by the ability of the trypanosomes to vary their surface antigens. Protective anti-trypanosome antibodies are directed at the VSG antigens (Morrison et al., 1982) which are highly immunogenic (Cross, 1978). However, the immunity induced by the VSG is only specific for trypanosomes whose VATs are identical in VSGs (Doyle, 1977; Cross, 1978). A cyclically transmitted infection followed by treatment with a trypanocidal drug Berenil was only effective against all M-VATs comprising the serodeme of the infecting trypanosome species (Nantulya et al., 1980). The number of M-VATs in each trypanosome species is conserved (Crowe et al., 1983). But within each trypanosome species, there exist a large number of serodemes in the field, making it difficult to produce an M-VAT-based vaccine which will be effective for all existing serodemes.

The use of inherently trypanotolerant animals is one of the measures employed to enhance cattle productivity in trypanosome-infested areas. Trypanotolerant breeds are capable of surviving and producing better in tsetse-infested areas than susceptible breeds (Murray et al., 1981). Resistant breeds include N'Dama, Muturu and some other West African taurine breeds of cattle (Murray et al., 1982). Trypanotolerance is not limited to cattle since some indigenous breeds of sheep and goats in East and West Africa also have a degree of natural resistance to trypanosomiasis (Murray et al., 1982). However, the numbers of trypanotolerant cattle available is very small and largely limited to West Africa. Introducing these animals to other tsetse-infested areas will require vast financial commitment.

Trypanotolerance is thought to have arisen through natural selection, resulting from constant exposure of the animals to infection over many generations (Murray et al., 1982). Trypanotolerant animals are genetically capable of suppressing the numbers of parasites in the blood and of limiting the development of anaemia (Murray and Dexter, 1988).

The information available indicates that the immune system plays a role in the expression of trypanotolerance. After a prior exposure to trypanosomes, the resistant N'Dama cattle eliminated trypanosomes much more rapidly during re-challenge infection as compared to the susceptible Zebu cattle (Desowitz, 1959). This suggests that resistant N'Damas elicited an effective secondary immune

response. In another study, the ability of N'Dama as compared to Boran cattle to resist an infection with *T. brucei* was associated with their ability to recognize at least one of the three trypanosome antigens of molecular weights 110, 150 and 300 Kd (Shapiro and Murray, 1982). Furthermore, it was shown that N'Dama cattle respond better than Borans, in the production of neutralizing antibodies to congopain, a *T. congolense* cysteine protease enzyme which may play a role in pathogenesis (Authie et al., 1993). Recently, it has been shown that during infection with *T. congolense* the specific antibody response of Boran but not N'Dama cattle is accompanied by the development of high titre of antibodies which are not specific for the infecting trypanosomes (Williams et al.,1996).

2.1.9 Immunopathology

Following the tsetse fly inoculation of trypanosomes into the skin, an inflammatory reaction, the chancre, develops at the site of bite. The chancre develops 5-10 days after fly bite, reaches peak size at 10-13 days and disappears after one month (Luckins and Gray, 1978; Emery and Moloo, 1980). Initial reactions in the chancre represent an inflammatory reaction associated with infiltration of neutrophils and small lymphocytes (Akol and Murray, 1982). Later, an immunological response ensues, characterised by infiltration of lymphoblasts, plasma cells and macrophages. Also present in the chancre are numerous dividing trypanosomes (Emery et al., 1980; Akol and Murray, 1982). After a

multiplicative phase in the chancre, trypanosomes enter the blood through the draining lymph nodes. The entrance of trypanosomes into the blood and eventual distribution to various organs, is followed by a lymphoproliferative reaction which is characterized by marked proliferation of B lymphocytes in the lymph nodes, spleen, and bone marrow (Mayor-Withey et al., 1978; Morrison et al., 1981; Morrison et al., 1982). Although the lymphoid organs of cattle are markedly enlarged (Masake and Morrison, 1981), this reaction is more marked in murine infections that are characterized by rearrangement of the normal architectural organisation of lymphoid organs, particularly the spleen. The profound proliferation of B cells is associated with excessive increase in serum immunoglobulin concentration (hypergammaglobulinaemia), especially of the IgM isotype (Seed et al., 1969; Hudson et al., 1976; Corsini et al., 1977; Baltz, 1981; Rose et al., 1982). Hypergammaglobulinaemia is due to increase in amount of both trypanosome-specific as well as non-trypanosome-specific antibodies (Nantulya et al., 1985). As parasitaemia enters a chronic phase, the lymphoproliferative phase ceases and instead, cellular depletion of lymphoid organs ensues (Fiennes, 1970; Morrison et al., 1979). The major immunological feature occuring in this stage is immunosuppression. The immune dysfunctions associated with immunosuppression include depression of antibody responses to unrelated antigens, depression of cell-mediated immune responses, increased susceptibility to other diseases and depression of responses to the infecting trypanosomes (Morrison et al., 1985). Immunosuppression was suggested to be

the cause of increased incidence of opportunistic infections in sleeping sickness patients (Greenwood et al., 1973).

The mechanisms underlying polyclonal B cell activation and immunosuppression have been subjects of many research endeavors. The association between the two phenomenon led many investigators to suggest that polyclonal activation of B cells led to clonal exhaustion of potential antigen responsive cells, and hence the observed unresponsiveness to antigen challenge (Greenwood et al., 1973; Hudson et al., 1976). However, subsequent studies show that the two phenomenon may not have causal relationship. First, evidence from studies conducted experimental murine models and host bovine infections show that generalised immune suppression observed is mediated by suppressor cells of macrophage phenotype (Sileghem and Flynn, 1992; Steinbergh and McGuigan, 1992; Schleifer et al., 1993; Schleifer ad Mansfield, 1993). Secondly, Diffley (1983) could induce polyclonal activation by injection of mice with purified VSG. However, this protocol could not induce suppressor cells. Therefore, polyclonal activation and generation of suppressor cells may be two co-existing phenomenon caused by different mechanisms or different trypanosome antigens. Although Diffley's protocol failed to induce suppressor cells in mice injected with VSG, spleen cells from these mice failed to produce specific antibody to sheep red blood cells, but remained responsive to stimulation by mitogens. It would seem therefore that polyclonal activation of B cells might be responsible for the observed

unresponsiveness to antigenic challenge such as vaccinations, but is not responsible to unresponsiveness to mitogenic stimuli. Suppressor cells on the other hand are responsible for generalised unresponsiveness to both antigens and mitogens (Diffley, 1983; Sileghem and Flynn, 1992; Steinbergh and McGuigan, 1992; Schleifer et al., 1993; Schleifer ad Mansfield, 1993).

The nature of immunosuppression has been studied extensively in murine models of the disease. Data obtained show that trypanosome infections activate T cells, which although do not proliferate, nevertheless secrete cytokines IL-2 and IFN-γ (Schleifer et al., 1993). The IFN-γ secreted in turn activates macrophages that secrete nitrous oxide and prostaglandins. It is these two products of activated macrophages that suppress T cell clonal expansion (Steinberg and McGuigan, 1992; Schleifer ad Mansfield, 1993; Steinberg et al., 1994).

In cattle, immunosuppression seems to be mediated by activated macrophages as in mice, but through a different mechanism. It was shown that macrophages from lymph nodes of infected cattle suppressed T cell responsiveness through their inhibitory effects on IL-2 receptor expression as well as IL-2 secretion (Sileghem and Flynn, 1992). In contrast to the situation in mouse models, nitrous oxide secretion by macrophages from infected cattle was suppressed, suggesting that it is not involved in T cell unresponsiveness (Taylor et al., 1996).

2.1.10 Hypergammaglobulinemia

A common feature of trypanosome infections in natural hosts and in experimental animals, is the enormous increase in serum immunoglobulin concentra

tions (hypergammaglobulinemia), especially of the IgM isotype. The elevation in serum IgM has been consistently observed in sleeping sickness patients since 1961 when the disease was first reported by Mattern. Increased IgM in cerebrospinal fluid, secreted directly by plasma cells, is diagnostic of sleeping sickness (Morrison et al., 1985). In trypanosome infections of mice, increased immunoglobulin secretion by spleen cells as well as elevated serum IgM, were observed (Seed et al., 1969; Hudson et al., 1976; Corsini et al., 1977; Askonas et al., 1979; Baltz, 1981; Rose et al., 1982). Elevation of serum immunoglobulin concentration was also observed in *T. gambiense* infections of monkeys (Houba *et al.*, 1969) and a trypanosome-infected bushbuck (*Tragelaphus scriptus*) (Luckins, 1976). A 2-to 10-fold increase in serum IgM concentrations was observed during experimental infections of cattle with *T. brucei*, *T. congolense* and *T. vivax* (Clarkson et al., 1975; Kobayashi and Tizard, 1976; Luckins, 1976; Luckins and Mehlitz, 1976; Nielsen et al., 1978; Masake and Morrison, 1981). The concentration of IgM decreased following treatment with a trypanocidal drug Berenil, indicating that these antibodies are induced by the presence of trypanosomes (Luckins et al., 1976). Changes in IgG were about two fold during *T. congolense* and *T. vivax* infections of cattle (Luckins, 1976). Other isotypes, IgA and IgE, remained unchanged (Nielsen et al., 1978).

It was suggested by Houba et al.(1969) that much of the serum IgM developing in infected animals is not parasite-specific. Studies to measure the specificity of antibodies induced by trypanosome infections have been conducted mainly in laboratory animals. Homologous trypanosomes absorbed less than 10% of the immunoglobulins secreted *in vitro* by spleen cells from *T. brucei*-infected mice (Corsini et al., 1977). Spleen cells from *T. brucei*-infected mice secreted antibodies which reacted to a variety of unrelated antigens such as sheep erythrocytes (sRBC), pneumococcal polysaccharide (SIII) and hapten-coated erythrocytes (Hudson et al., 1976; Kobayakawa et al., 1979). Increases in antibodies expressing a T-15 idiotype which is specific for phosphoryl choline and a corresponding increase in anti-T15 idiotype-bearing antibodies was also reported (Rose et al., 1982).

In addition, antibodies with reactivity to self-antigens (autoreactivity) were found in trypanosome infections of different animal species including rabbits (Muschel et al., 1961), mice (Popham and Dresser, 1980), rats (Rickman and Cox (1980), monkeys and man (Lindsey et al., 1974). Antibodies with rheumatoid-like activity (anti-IgG) were detected in monkeys (Houba et al., 1969) and mice (Popham and Dresser, 1980; Klein et al., 1970). Some antibodies appeared to react with antigens on tissues or organs such as the liver, kidney and heart, thymocytes,

erythrocytes, complement (C3) and fibrin/fibrinogen product (Mackenzie and Boreham, 1974; Kobayakawa et al., 1979).

In infected cattle however, some investigators failed to demonstrate the presence of trypanosome non-specific antibodies leading to the conclusion that these antibodies do not develop during trypanosome infections in this species. Heterophile antibodies to chicken or sheep erythrocytes could not be detected in serum from trypanosome-infected cattle (Tabel, 1981). Furthermore, antibodies reactive to TNP-BSA could not be detected in serum from *T. congolense*-infected cattle containing high levels of IgG and IgM specific for trypanosome VSG (Masake et al., 1983). However, in other experiments, antibodies to autologous erythrocytes and platelets were found in serum from *T. vivax*-infected cattle (Assoku and Gardiner, 1992). In addition, antibodies reactive to a number of non-trypanosome antigens, including a bacterial enzyme ß-galactosidase from *Escherichia coli*, were detected in serum from *T. congolense* infected cattle (Williams et al., 1996).

The origin and mechanisms leading to the development of trypanosome non-specific antibodies and their subsequent role are still unknown. Trypanosome non-specific antibodies developed in infected athymic (*nu/nu*) and thymectomised mice (Clayton et al., 1979; Kobayakawa et al., 1979) indicating that the production of these antibodies is T-cell independent.

Some investigators attributed these antibodies to polyclonal activation of B cells, in response to a stimulus by a mitogen-like molecule from the parasite (Hudson et al., 1976; Kobayakawa et al., 1979). Suggestions of polyclonal activation were based on the enormous increases in the size and number of cells in affected organs of infected animals. Splenomegaly and rearrangement of the spleen structure, is a common feature of murine trypanosomiasis (Goodwin, 1970; Morrison et al., 1981). Marked proliferation of plasma cells but not T cells, occurred in lymph nodes and spleen of mice infected with *T. b. brucei* (Murray et al., 1974). An increase in spleen size up to 20-fold over normal was observed in mice infected with *T. b. rhodesiense* (Mansfield and Bagasra, 1978). This splenomegaly was attributed mainly to an increase B cells and macrophages rather than T cells. In *T. congolense*- and *T. b. brucei*- infected mice, the increased splenic cellularity was due to increase in B cells and non-T non-B cells and H^3 uptake assay showed that most of the cells were dividing (Mayor-Withey et al., 1978; Morrison et al., 1978). Several investigators have attempted to demonstrate a trypanosome mitogen-like molecule but the results have not been encouraging (Esuruoso, 1976; Mansfield et al., 1976; Corsini et al., 1977; Assoku and Tizard, 1978). However, Diffley (1983) managed to induce polyclonal activation in mice by repeated injection with purified VSG at concentrations that mimicked VSG released by T.b. rhodesiense infection *in vivo*. Therefore, information available suggests that in murine models, the development of trypanosome non-specific antibodies is a results of polyclonal activation of B cells.

In trypanosome infections of cattle, many investigators have argued against the development of polyclonal activation. Rurangirwa et al. (1979; 1982) used the argument that polyclonal activation of B cells implies that antibodies comprising the entire B cell repertoire in the animal will be secreted. The authors concluded that polyclonal activation does not occur during bovine trypanosomiasis when they failed to detect antibodies to *Leptospira biflexa* and *Brucella abortus* in serum from trypanosome-infected cattle. Yet when these animals were challenged with *L. biflexa* and *B. abortus* after treatment with Berenil, they elicited a good antibody response to the two bacteria indicating that specific B cells for these bacteria were present but were not activated by the trypanosome infection.

However, failure to detect antibodies to *Leptospira biflexa* and *Brucella abortus* in serum of trypanosome-infected should not be used to rule out polyclonal activation. Polyclonal activation does not mean the entire B cell repertoire will be involved. Some polyclonal activators, such as superantigens, select only B or T cells whose variable region receptors express particular products of conserved genes (Rosen, 1997).

As in murine infections, trypanosome-infected cattle makes a poor response when challenged with other antigens, vaccines or infections (Greenwood et al., 1997; Rurangirwa et al., 1979). This unresponsiveness was earlier attributed to

polyclonal activation based on as in murine model. Nantulya et al. (1992), however found that the unresponsiveness observed in trypanosome infected cattle fitted antigenic competition rather than polyclonal activation (Nantulya et al., 1992). This competition occurs at the levels of macrophages or /antigen presenting cells which finds themselves engaged in processing of one type of antigen and therefore fails to effectively process other antigens given. There is therefore no sufficient evidence for or against trypanosome non-specific antibodies being a result of polyclonal B cell activation in cattle.

Other investigators attributed the non-trypanosome specificities in cattle to cross-reactive antibodies that were generated against various VSGs or other parasite molecules present during the infection. Evidence for this was obtained from absorption studies (Seed et al., 1969; Musoke et al., 1981; Nantulya, 1985). Musoke et al. (1981) removed more than 80 % of antibodies from serum by absorption on trypanosomes collected at 3-day intervals during the course of *T. brucei* infection of cattle. However, the procedures used in these experiments might have resulted in non-specific absorption of large quantities of non-specific antibodies (Morrison et al., 1985).

It has been observed that in many animal species, the specific antibody response following antigen administration is always accompanied by development of "small quantities" of non-specific antibodies (Loor, 1971; Miller, et al., 1975; Poskit et

al., 1977). Therefore, it was suggested that the trypanosome non-specific antibodies appear through the same mechanism (Morrison et al., 1985). This response particularly involves antigens to which the animal has recently been exposed. Based on this hypothesis, the non-trypanosome antibody response would preferentially be directed to trypanosome antigens encountered in previous waves of parasitaemia. However, this suggestion does not account for trypanosome non-specific antibodies being exclusively IgM.

Recently, following the observation that $CD5^+$ B cells increase in blood and spleen concomitantly with an increase in serum IgM during *T. congolense*-infection of cattle, it was suggested that non-trypanosome and autospecificities are products of the $CD5^+$ B cells (Naessens and Williams, 1992). An argument in favour of this hypothesis comes from the observations that the $CD5^+$ (B-1) B cells in humans and mice are characterised by production of IgM antibodies which are polyspecific and autoreactive (Hayakawa et al., 1984).

2.1.11 The role of T cells on antibody responses to trypanosome infections

The role played by T lymphocytes in the antibody responses induced by trypanosome infections were conducted since late nineteen seventies using mouse strains lacking T cells. Results indicated that IgM hypergammaglobulinaemia and development of trypanosome-non-specific antibodies occur in trypanosome

infections initiated in athymic and thymectomised mice (Kobayakawa et al., 1979; Mansfield et al., 1981; Pinder et al., 1986). This indicated that the latter responses take place independent of T cells. In addition, athymic mice are capable of mounting an effective anti-trypanosome antibody response which, however, is exclusively IgM (Campbell et al., 1978; Pinder et al., 1986; Mansfield et al., 1981). While these results indicated the role of T cells in switching of IgM response to IgG, they suggested a lesser role of T cells in protection against trypanosome infections. However, it was observed later that normal thymic sufficient mice responded with a significantly higher antibody titre to the exposed trypanosome antigens than their athymic counterparts (Reinitz and Mansfield, 1990) indicating that T cells may still be important in protection. T cells specific for trypanosome VSG in mice are have recently been characterised. They are $CD4^+$, localised in the peritoneal cavity, do not proliferated in response to stimulation by VSG, instead large amounts of Th1 (T-helper one) cytokines IL-2 and IFN-γ with no Th-2 cytokines IL-4 or IL-5 even at transcription level (Schleifer et al., 1993). This cytokine pattern suggested the predominance of a switch of IgM response to IgG2a. However, in addition to IgG2a, a switch response to IgG1 also predominated (Schopf et al., 1998). The authors suggested the presence of non T cell, non-parasite-specific cell which secrete sufficient quantities of IL-4 to induce a switch to IgG1.

The advent of gene knockout technology helped to shed more light on the role of different T cell subpopulations to the immune response induced by trypanosome infections. Mice lacking CD4 expression were capable of producing trypanosome-specific IgM but not IgG indicating the role of this T cell subpopulation in isotype switching (Rottenberg et al., 1993). Based on evidence accumulated from different experiments, Mansfield (1994) suggested a model for the interaction between trypanosome VSG, antibody-producing B cells and T-helper (Th) cells. Trypanosome VSG exposed on the surface of the trypanosomes presents a repetitive conformational structure that resembles the T helper-independent antigens. This interaction leads to the predominantly IgM T-cell independent B cell responses which are observed in athymic or CD4-gene knockout mice. In contrast, molecules derived from dissociated VSG will only stimulate B cells after being processed and presented to T-helper cells. Therefore, normal mice will mount a Th-independent B cell response and in addition, a Th-dependent B cell response characterised by a switch from IgM to IgG.

The CD8 T cell subpopulation do not seem to have any influence on antibody responses during trypanosome infections as evidenced from experiments initiated in CD8 gene knockout mice (Rottenberg et al., 1993). However, these mice responded with lower parasite load as compared to their normal counterparts, suggesting that CD8 T cells promote the growth of trypanosomes.

In cattle, studies to address the role T cells play in vivo during trypanosomiasis were hampered by unavailability of a suitable model. The breakthrough came when Howard et al. (1989) described a method of depleting T cell subpopulations in cattle by injection of with mouse monoclonal antibodies which are specific for bovine T-cells. Using this method, a study was conducted in which CD8+ T cells were completely depleted and the impact of these cells on resistance to bovine trypanosomiasis examined (Sileghem and Naessens, 1995). Results from this study showed that CD8 T cells play no role in development of anaemia and, contrary to the mouse model, these cells do not have any impact on parasitaemia. Immediately thereafter, another study was conducted to examine the significance of CD4 T cells on trypanosomiasis in trypanotolerant N'Dama and susceptible Boran cattle (Naessens and Sileghem, in preparation). CD4 depletion caused a reduction in titres of antibodies directed to the exposed parasite antigens in both N'Dama and Boran cattle and in addition, a slight increase in parasitaemia was observed in depleted Boran cattle. However, in these two studies, the impact of CD8 and CD4 T cells on levels of specific antibodies reacting with lysate of trypanosomes, trypanosome non-specific antibodies and total serum IgM were not examined.

2.2 $CD5^+$ B Cells

2.2.1 General

A CD5 B cell is a B lymphocyte which in addition to possessing all characteristics of B cells, expresses the CD5 surface antigen which is a marker for all T lymphocytes (Hayakawa et al., 1983; Herzenbergh et al., 1986). The CD5 antigen is a 67 Kd glycoprotein molecule determined by reactivity of cells to CD5-specific monoclonal antibodies. It was initially defined as a marker for the helper T-cells (Cantor and Boyse, 1975). Subsequent studies using CD5-specific monoclonal antibodies and sensitive flow-cytometric analyses, demonstrated the presence of this antigen on all T cell subpopulations (Ledbetter et al., 1981).

That some B cells from chronic lymphocytic leukemia (CLL) patients also express T cell marker was first demonstrated by Boumsell et al., (1978). Flow-cytometric analyses of malignant B cells (CLL cells) showed that they also express CD5 (Boumsell et al, 1980). In mouse, CD5 was found to be expressed on several B cell lines (Lanier et al., 1978, 1981). Finally in both humans and mice, CD5 B cells were found to be a minor subpopulation in normal individuals (Hardy et al., 1982; Hardy and Hayakawa, 1986).

A marked interest in the $CD5^+$ B cells was stimulated by the finding that in mice the $CD5^+$ subpopulation is responsible for secreting autoreactive antibodies

(Hayakawa et al., 1984), thereby opening the possibility of a role of these cells in the pathogenesis of autoimmune diseases.

The major function of the CD5 molecule is not yet established. The B-cell specific CD72 molecule is believed to be the natural ligand for CD5 (Lydyard et al., 1993). Since CD5 is present on both $CD5^+$ B cells and all T cells, it has been suggested that this molecule may be involved in communication among CD5 B cells or between T cells and B cells (Hardy and Hayakawa, 1994). In humans, the CD5 molecule on B cells is associated with the B-cell receptor complex (BRC), which is made up of membrane immunoglobulin and the non-covalently associated invariant Igα/Igβ heterodimer. It was shown that CD5 serves as a substrate for BCR-induced tyrosine kinase activity and therefore may be involved in the modulation of BCR signals (Lankester et al., 1994).

2.2.2 Nomenclature of B cells

Initially the $CD5^+$ B cell was referred to as the Ly-1 B cell in mouse and Leu-1 B cell in human. The nomenclature currently in use assigns any defined cell differentiation antigen, a CD (cluster of differentiation) number. Thus, a Ly-1 or Leu-1 B cell is known as a $CD5^+$ B cell.

2.2.3 Murine B cell lineages

Two definitions for cell lineage are commonly used. The first defines a lineage as the descent in line from a common progenitor. The second one defines a lineage as a set of cells deriving from distinct, relatively undifferentiated (unrearranged) progenitors that have at least a limited capacity for self renewal and give rise to progeny which are committed to differentiate into cells distinguishable by particular functional or phenotypic characteristics (Herzenberg and Kantor, 1993). Based on the latter definition, three B cell lineages can be identified; named the conventional B cells (B-2 cells) and the two very similar B-1 lineages, B-1a (formerly called Ly-1 B) and B-1b (formerly "sister cells").

2.2.4 Comparison of mouse B-1a and B-1b B cell subpopulations

The two B-1 B cell lineages have an almost identical phenotype but differ in two major aspects. Firstly, B-1a cells express the CD5 antigen whereas B-1b cells do not (Herzenberg et al., 1986). Secondly, progenitors for B-1a cells are mainly located in the foetal liver and omentum and very rarely in the adult bone marrow. Transfer of bone marrow cells to irradiated recipients reconstitutes very few B-1a cells (Kantor et al., 1992). In contrast, progenitors for B-1b cells are found mainly in the adult bone marrow, and when transferred to irradiated recipients they reconstitute as much as 50% of B-1b B cells found in normal animals (Kantor et al., 1992).

2.2.5 Characteristics of mouse $CD5^+$ and $CD5^-$ B cell subpopulations

Extensive work has been on the characterisation of the $CD5^+$ and $CD5^-$ B cell subpopulations in mice. Some of the important features of the two subpopulations are listed in Table 1.

Table 1. Features of the murine $CD5^+$ and $CD5^-$ B cells.

	$CD5^+$	$CD5^-$
PHENOTYPE		
IgM	+++	+
IgD	+	+++
B220	+	-
CD23	-	+
Size	large	small
MAIN LOCATION	peritoneal cavity	lymphoid organs
ONTOGENY	arise first in foetal liver	arise later in bone marrow
LIFESPAN	self renewing constitutive production of IL-10	replaced by IgM^- precursors in BM
GROWTH	propensity to expansion	die easily
IMPAIRED DEVELOPMENT		
	Xid (CBA/N)[1]	M^v (motheaten)[2]
Ig GENES	remain germ-line little N-nucleotide insertion	Mutate, common N-nuleotide insertion
ANTIBODY PRODUCTION		
Serum IgM, IgG3	+++	+
IGG1	+	+++
IgG2a, IgG2b	+ to ++	++ to +++
IgM autoantibody	+++	?
IgM anti-Id	+++	?
IgM anti-bacterial antibodies	+++	+ to +++
Anti hapten/protein	?	+++
T-dependence	-	++
Affinity maturation	-	++

1. CBA/N mice have an X-linked immunodeficiency gene (Xid) associated with defective CD5 B-cell matuaration and poor response to type II T-independent antigens.
2. Motheaten mice have wide spread autoimmunity and most of their B cells are $CD5^+$

Source: Roit (1994)

2.2.5.1 Phenotype

$CD5^+$ B cells expresses the CD5 surface antigen which is a marker for all T lymphocytes (Hayakawa et al., 1983; Herzenbergh et al., 1986). They resemble other B cells, so called $CD5^-$ B cells, in the expression of major features of the B cell lineage such as surface IgM, CD19, CD20 and CD21, Fcγ RII and class II major histocompatibility complex (MHC) (Ling et al., 1987; Kipps, 1989). They differ from T lymphocytes because they do not express markers associated with other T cells such as CD4, CD8 and CD3 (Kipps, 1989). B cells express the CD5 antigen at about 20% of the level expressed by T lymphocytes. The CD5 B cells may also express low levels of CD11b, which is a feature of cells of myelomonocytic lineage (Reviewed by Kipps, 1989; Lydyard et al., 1993). Some human CD5 B^+ cells also express the receptor for murine erythrocytes (ME), which is associated with the CD22 molecule (Lydyard et al., 1993).

A comaparison of phenotype for murine B-1 and conventional B-2 cells was reviewed by Kantor et al., (1991). Murine B-1 cells are bright for surface IgM and dull to moderate for IgD and B220. In contrast conventional B cells are dull to moderate for surface IgM and bright for IgD and B220. B-1 cells found in the peritoneal cavity are $Mac1^+$ (CD11b) and FcεR $(CD23)^-$ while conventional B cells are $Mac1^-$and $Fc\varepsilon R^+$. However, in the spleen, B-1 cells do not express Mac1 and both B-1 and marginal zone B cells lack FcεR.

2.2.5.2 Ontogeny and anatomical localisation

$CD5^+$ B cells appear early in ontogeny. They are evident with the first appearance of surface IgD expressing cells (Kipps, 1989). The proportion of $CD5^+$ B cells in the spleen of Balb/c mice soon after birth is 20% of all B cells (Dexter and Corley, 1987). This percentage decreases progressively reaching 9% at 3 weeks and 5% at 3 months of age. A similar decrease is seen in the peritoneal cavity of mice where from 100% $CD5^+$ B cells at birth, the proportion decreases to 46% at 3 weeks and 16% after three months of age in mice (Kantor, 1991). However, this proportional decrease is not associated with decreased production of these cells, but is due to dilution by the $CD5^-$ lymphocyte subpopulations (Hayakawa et al., 1986). Indeed, the absolute numbers of $CD5^+$ B cells increases both in spleen and peritoneal cavity up to one year (Hayakawa et al., 1986). $CD5^+$ B cells comprise about 1% of total B cells in adult mice. These cells are very rare in lymph nodes, blood and adult bone marrow. They are found in small numbers in the spleen where they are localised in the marginal zone. Very few B cells are found in thymus, but the majority of them are $CD5^+$ (Miyaba-Inaba et al., 1988). The major area of localisation in mice is the peritoneal cavity where in terms of percentage they are the major population (Kantor, 1991). Another area of localisation is the intestinal lamina propria from where they are said to contribute to mucosal IgA response (Kroese et al., 1989; Kroese et al., 1993). Intestinal $CD5^+$ B cells are seeded from the subpopulation in the peritoneal cavity (Kroese et al., 1993). The site of proliferation for the intestinally associated $CD5^+$ B cells is

the mesenteric lymph nodes (Bos et al., 1996). Furthermore, these authors found that antibodies secreted by these cells react with faecal bacterial populations and suggested a role of these cells in the maintenance of homoestasis between host and gut flora.

In humans, $CD5^+$ B cells constitute 100% of B cells in the developing lymph node (Bofill et al., 1985). In foetal spleen at 19-22 weeks of age, 40-60% of B cells are $CD5^+$ (Kantor, 1991). The numbers and percentages of $CD5^+$ B cells decrease with age up to adulthood (Lydyard et al., 1993). In adult humans, $CD5^+$ B cells represent 5-30% of circulating B cells. Not more than 10% of splenic B cells and less than 30% of lymph node and tonsil cells are $CD5^+$ and these organs they are mainly found scattered in the marginal zone (Lydyard et al., 1993). It seems human $CD5^+$ B cells may be enriched in peritoneal cavity as in mice. Study of peritoneal lymphocytes in patients undergoing peritoneal dialysis and women undergoing bilateral tubal ligation showed that $CD5^+$ B cells represented up to 63% of peritoneal B cells (Donze et al., 1997).

In sheep, the foetal ileal Peyer's patches, a place where B cell diversification takes place, have an age-dependent increase in $CD5^+$ B cells (Lydyard et al., 1993).

2.2.5.3 Lifespan

Murine adult B-1 cells sustain their numbers by self-replenishment. This is in

contrast to conventional B cells that are replenished by precursors from bone marrow (Kantor, 1991). Initially, B-1 cells are seeded by progenitors in the foetal liver. However, After 6-8 month of life, a feedback mechanism start operating which impedes entry of B-1a and B-1b into the peripheral pool. Furthermore, when stimulated with lipopolysaccharide, B-1 cells produce IL-10 and this is in contrast to conventional B cells. It seems IL-10 may be essential for the development of B-1 cells. Treatment of animals from birth with monoclonal antibody against IL-10 caused deficiency of B-1 cells (Kantor, 1991).

2.2.5.4 Immunoglobulin genes and antibody repertoire

Recombination events involving V, D and J immunoglobulin gene segments precedes the formation of the immunoglobulin gene and determines the variability in its antigen-combining site. However, in addition to the variability encoded by the three segments, there are other factors which contributes to the specificity of the immunoglobulin. These derive from variability in the sites of joining, addition of non-templated nucleotides at their junctions (N-regions) and from templated nucleotides, known as P sequences (Kantor et al., 1997).

B-1 cells use a restricted set of immunoglobulin variable genes specific for autoantigens and some bacterial antigens. Studies in mice show that non-templated N-region sequences are rarely inserted in B-1 cells arising early in ontogeny (Gu et al., 1991). Because of self-replenishment, some of these B-1 cells lacking N-

region insertions survive and express themselves in the adult repertoire. However, B-1 cells arising later (more than one month of age) show N-region insertions comparable to conventional B cells. It has been suggested that lack of N-region insertions in B-1 cells arising earlier in life is due to absence of the enzyme terminal deoxynucleotide transferase (TdT) which is responsible for insertion of N nucleotides (Kantor et al., 1997).

Antigen-driven selection has also been suggested to contribute to the restricted repertoire in the B-1 cells (Kantor, 1991). The development of B-1 cells early in life might expose them to positive selection by endogenous antigens present in foetal but not adult life. This might explain the predominant secretion of anti-idiotype and autoantibodies to thymocytes and phosphatidylcholine (on bromelain trated erythrocytes) by B-1 cells.

However, it has recently been shown that the extent of restriction in the immunoglobulin V gene usage by the B-1 cells might have been exaggerated. Previous studies on V gene repertoire were based on immunoglobulin RNA prepared from bulks of cells, hybridomas or LPS stimulated B-1 cells. These methods might have preferentially selected some clones, and not others, resulting in overrepresentation of some genes. Kantor et al., (1997) studied immunoglobulin gene representations on single FACS sorted B-1a, B-1b and B-2 cells from the peritoneal cavity. Results showed that a bigger proportion of B-1 cells (62%), than

previously thought, express N-region insertions. However, this proportion was still less than B-2 cells that express N-insertions in about 93% of cells.

2.2.6 Separate lineage or activatiom marker?

It is still debated whether $CD5^+$ B cells represent a separate B cell lineage or a state of B cell activation.

Evidence supporting $CD5^+$ B cells being a separate lineage came from experiments involving cell transfer into irradiated or severe combined immunodeficient (SCID) recipient mice. It was found that transfer of bone marrow stem cells and peritoneal cells into irradiated recipients reconstituted the $CD5^+$ B cell population (Herzenberg et al., 1986). In other experiments, reconstitution of $CD5^+$ B cells in SCID mice was only achieved by transfer of foetal liver cells (Hardy and Hayakawa, 1986) or foetal omental cells (Salvason et al., 1992).

Although results from transfer experiments indicate clearly that $CD5^+$ B cells are a separate lineage with separate progenitors, critics of this model claim that the SCID mouse recipients in the transfer experiments might have provided an environment which favours development of $CD5^+$ and not $CD5^-$ from the common progenitor.

Data indicating that CD5 antigen is an activation marker came from experiments demonstrating that human $CD5^-$ B cells acquire CD5 when activated by PMA (Youinou et al., 1987; Miller and Gragow, 1984). Similarly, treatment of mouse splenic $CD5^-$ B cells with anti-IgM and IL-6 increased expression of CD5 (Ying-Zi et al., 1991).

However, it is not known whether cells acquiring the CD5 antigen *in vitro* possess other characteristics of B-1 cells such as self-replenishment when transferred in irradiated recipients. Until these controversies are solved by experimental evidence, it seems at present that the CD5 antigen is developmentally expressed on some B cells and can also be induced on others.

2.2.7 Possible lineage relationship between $CD5^+$ B cells and macrophages

Although B cells and macrophages are generally regarded as originating from different lineages and exhibiting different functions, there is accumulating body of evidence to show that some subpopulations of the two cell types may have common origin as recently reviewed by Borello and Phipps (1996).

A number of studies have shown that malignant $CD5^+$ pre-B cell lines switched to macrophage cells (B/Mc) when cultured *in vitro* alone or cultured in the presence of inducing substances such a demethylating drug 5-azacytadine, phorbol ester or growth factors such as IL-3 and colony stimulating factor. The B/Mc cells co-

express features of the original pre-B cell such as CD5, CD19, BB20 and macrophages surface markers Mac-1 (CD11b), CD13, CD15, F4/80. In addition, B/Mac exhibit macrophages functional activities such as phagocytosis of opsonized sheep red blood cells and non-specific esterase like activity. The original pre-B and the biphenotypic B/Mac had identical immunoglobulin gene rearrangement demonstrating that the B/Mac originated from the pre-B cell line. The Pre-B cell switch to macrophages is not limited to malignant cells alone. Co-culture of purified normal adult splenic cells with normal splenic fibroblasts or culture in fibroblast conditioned media yielded B/Mac cells. Sorted $CD5^+$ but not $CD5^-$ B cells yielded B/Mac when cultured in fibroblast conditioned media indicating the $CD5^+$ B cell origin of the B/Mac.

The function of the B/Mac is not yet known. But unlike the macrophages that originate from the bone marrow that thrives in IFN-γ rich environment, the B/Mac activity is inhibited in the presence of this cytokine. Instead, the presence of IL-4 that dampens Th1 activity did not have negative effect on B/Mac activity, suggesting that the latter cells would function better in Th2 environment. Possession of antigen-specific immunoglobulins and phagocytic activity by B/Mac cells may allow it to function as antigen presenting cells for certain bacterial and self antigens as for the $CD5^+$ B cells. In addition, its expression of CD5 may allow it to stimulate other B cells via the CD72 receptor. The anti-self and anti-bacterial repertoire and phagocytic activity may make this cell important in gut

associated immune defence. In addition, the cell may be a component of the foetal and neonatal immune system dominated by primitive cells such as $CD5^+$ and the γδ-T cells.

The authors suggest the following lineage relationship between B cells and macrophages. In adult bone marrow, myeloid precursors generate monocytes that mature into conventional macrophages. In contrast, during foetal life, precursors of B/Mac generates a primitive pool of B-1 and B-2 cells and macrophages that do not have rearranged immunoglobulin gene. It is the B-1 cells which then give rise to B/Mac cells.

2.2.8 Functions of $CD5^+$ B cells in health and disease

2.2.8.1 Source of natural and autoantibodies

$CD5^+$ B cells are the source of natural IgM antibodies which bind to isologous bromelain-treated erythrocytes in mice (Hayakawa et al., 1983). These antibodies are specific for trimethylammonium determinants or phosphatidyl choline (PtC), which is exposed on senescent or bromelain treated erythrocytes (Kawaguchi, 1987).

CD5 B cells have been implicated in the pathogenesis of autoimmune disease. Autoimmune strains of mice, such as the New Zealand Black (NZB) which

naturally develop autoimmune disease, have markedly increased numbers of CD5 B cells compared to other strains of mice (Hayakawa et al., 1983; 1984). Serum from such autoimmune strains contains high amounts of autoreactive antibodies reactive with autologous erythrocytes, thymocytes, and single stranded DNA (ssDNA) (DeHeer et al., 1978; Izui et al., 1978; Andrews et al., 1978; Smith and Steinberg, 1983; Theophilopoulos and Dixon, 1985). It was concluded that $CD5^+$ B cells are the source of the pathogenic autoantibodies when pure $CD5^+$ B and not $CD5^-$ B cells were found to secrete antibodies to autologous T lymphocytes or ssDNA *in vitro* (Hayakawa et al., 1983; 1984).

Further evidence for involvement of $CD5^+$ B cells in autoimmunity comes from motheaten mouse strains. These strains are genetically predisposed to develop autoimmune disease and have a remarkably shortened life span. Serum from these animals is hypergammaglobulinemic due to elevated IgM and IgG3 antibodies (Sidman et al., 1986) and increased amounts of IgM autoantibodies (Sidman et al, 1984). It was found that most of the B cells from this strain are $CD5^+$ B cells (Sidman et al., 1986).

In contrast, CBA/N mice which genetically lack CD5 B cells are deficient in mature lymphocytes and are hypogammaglobulinemic owing to extremely low IgM levels (Kipps, 1989). Furthermore, they are not responsive to soluble polysaccharides (T-cell independent type 2 antigens) or phosphatidyl choline on

any carrier. This immunodeficiency in CBA/N mice is due to *Xid* genes located on the X chromosome (Berning et al., 1980).

In humans, $CD5^+$ B cells have a higher capacity than CD5- for production of IgM autoantibodies, particularly IgM anti-IgG autoantibodies or rheumatoid factor (RF). Sorted $CD5^+$ B cells from normal and rheumatoid individuals were significantly enriched for production of RF after stimulation with *Staphyloccocus aureus* Cowan I (SAC) (Hardy and Hayakawa, 1987).

B cells in chronic lymphocytic leukemia, known to express CD5, also have been shown to produce RF and other autoantibodies. Patients with CLL had B cells bearing IgM antibodies which bound IgG, ssDNA, double stranded DNA (dsDNA) (Preud'homme and Seligmann, 1972; Sthoeger et al, 1989).

It is not clear what are the major functions of the natural antibodies. However, since they are reactive with lipids, carbohydrates and DNA, their function might be to clear cell debris. In addition, they might be involved in setting up the idiotype network or first line of defence against a wide array of micro-organisms (Lydyard et al., 1993).

Production of natural polyreactive antibodies is dramatically increased in response to some antigen such as tetanus toxoid ot bacterial LPS or in course of some viral,

bacterial and parasitic infections (Casali and Notkins, 1989). Natural antibodies possessing reactivity to the Fc portion of IgG were shown to potentiate the protective effect of *T. lewisi*-specific IgG in suckling rats and their pups (Clarkson and Mellow, 1981). Natural antibodies were also demonstrated to potentiate antibody-dependent viral neutralisation (Casali and Notkins, 1989).

2.2.8.2 Role in idiotype network, tolerance and maintenance of immunological memory

It has been observed that many of the antibodies produced by B cells during early stages of foetal development are autoreactive (Holmberg et al., 1986). Hybridomas generated from splenocytes of newborn Balb/c produce antibodies which are reactive with idiotypes of immunoglobulins encoded by non-diversified immunoglobulin V genes or germ line genes (Vakil and Kearney, 1986). The recognition of these mainly IgM anti-idiotypic autoantibodies is directed at more than one germ line encoded antibody idiotypes.

These antibodies are of physiological significance to normal development of the adult B and T cell repertoire. Injection of the anti-idiotypic antibodies at a particular time during neonatal development either enhanced or suppressed the utilization of the corresponding idiotype bearing antibodies in the adult immune responses (Vakil and Kearney, 1986).

The interacting $CD5^+$ B cells are suggested to be involved in the idiotype network that was a subject of Jerne's hypothesis (Jerne, 1955). Jerne hypothesised that all antibody diversities are pre-formed in the individual before encountering an external antigen. He suggested that production of these antibodies in the absence of the external antigens was driven some internal images of antigen, in the form of cells bearing corresponding anti-idiotype antibodies. Based on Jernes's hypothesis, (Cohen and Young (1991; Coutinho, 1995) further explored the physiological function of the network to include other relevant phenomenons such as tolerance without deletion or inactivation of autoreactive clones as earlier suggested by Burnett (1957). These authors suggest that the network consist of enteracting $CD5^+$ B cell but other cells such as T-cells and macrophages may be involved. Some of these interactions are stimulatory while others are suppressive. The network involves $CD5^+$ B cells bearing autoantibodies that predominantly recognize immunodominant proteins such as thyroglobulin, tubulin, actin, myoglobulin, albumin, collagen and heat shock proteins in man. Each of these dominant antigens is immunologically represented in a cellular network comprising of interacting set of T and B cells. The overall organization of immune network around dominant antigens represents the immune system picture of self, named the *immunologocal homunculus* or little man.

Organization of the idiotype network around immunodominant self antigens represents a pre-formed mechanism for tolerance, defence against possible self reactivity or autoimmunity. Some invading microbes posseses both specific determinants and determinants shared with host tissues. The antigen presenting cells (macrophages or autoimmune B cell) will process and present the shared epitope and the microbe-specific epitope. The response to the shared epitope will be suppressed by the lymphocyte network controlling autoimmunity while cells recognising the foreign epitope will react vigourously to eliminate the invading microbe. In this context, the authors suggest that the immune system exist in two fuctional units, one made up of a controlled network of outoreactive lymphocytes and the other made up of non-autoreactive lympocytes prepaired to respond quickly and eliminate foreign invadors. However, sometimes the regulatory or suppressive functions of the network fails and instead, an aggressive reaction to self or autoimmunity occurs.

It has been suggested that the cellular network can contribute to the maintenance of the immunological memory in the absence of antigen (Uytde Haag et al., 1991; Van der Heijden et al., 1991). Memory B cells specific for microbial antigens are not part of the idiotype network. However, on encountering specific antigen, these cells undergo clonal proliferation and selection of B cell mutants that possess high affinity for the antigen. Mutation may alter the variable region (V-region) of the microbial-specific memory B cells and thereby allowing them to interact and

stimulate network $CD5^+$ B cells whose immunoglobulin V-region mimick the antigen. These $CD5^+$ B cels also undergo clonal expansion and high affinity mutants are selected. The interaction of the antigen-specific memory B cells and V-regions of the anti-idiotype $CD5^+$ B cells stimulate clonal expansion of mature antige-specific B cells and keep them alive. Therefore, even In the absence of antigen, immunological memory is maintained by self-renewing $CD5^+$ B cells whose somatically mutated V-regions mimick the antigen.

2.2.8.3 Antigen presentation

It has been established that B cells are effective antigen presenting cells. Antigen is captured through the immunoglobulin surface receptor, processed and peptides displayed in association with MHC class II molecules to antigen-specific T cells (Lanzavecchia, 1985). However $CD5^+$ B cells may be more involved in antigen presentation than $CD5^-$ considering the various ways by which they gain access to antigen. First, $CD5^+$ B cells are polyreactive and therefore are capable of presenting a variety of antigens provided they bind these antigens with sufficient avidity. Secondly, $CD5^+$ B cells are enriched for reactivity to the Fc portion of IgG (Rheumatoid factor) (Fong et al., 1983), therefore will be able to process any form of antigen if it is complexed with IgG (Roosnek and Lanzavecchia, 1991).

It has been suggested that murine thymic $CD5^+$ B, acting as antigen presenting cells, may play a role in the induction of tolerance. Presentation of certain autoantigens, derived from disintegrated cells, to $CD4^+$ T cells in association with class II MHC antigens may result into deletion or anergy of the corresponding autoreactive $CD4^+$ T cells, and hence tolerance to these antigens (Inaba et al. 1991; Lydyard et al., 1993).

2.2.8.4 Immunosuppression

A number of studies have indicated that $CD5^+$ B cells possess immune-suppressive properties. $CD5^+$ B cells from B cell CLL patients activated with PHA-P and Con A, or from long term cultures, inhibited mixed lymphocyte reaction (Farkas et al., 1987). The mechanism through which the $CD5^+$ B cell exerts its suppression is still unknown. However, it is known that CD5 B cells produce IL-10. This cytokine, which is also produced by Th2 cells, inhibits the production of IFN-γ by Th1 cells through antigen presenting cells (Fiorentino et al., 1991). This mechanism may be responsible for the observed suppression of the mixed lymphocyte reaction. It has been suggested that $CD5^+$ B cells plays an important regulatory role in the pathogenesis of the autoimmunity associated with *T. cruzi* infections in mice (Minoprio, 1991). In this disease, protective Th1 responses involves production of IFN-γ which kills the parasites, while pathology is mediated by Th2 responses marked by secretion of autoreactive IgG2a and IgG2b. It is

suggested that $CD5^+$ B cells secrete IL-10 which suppresses protective Th1 responses while at the same time promoting Th2 responses leading to increased secretion of autoreactive antibodies by conventional B cells.

In addition, human $CD5^+$ B cells from myeloma patients inhibited immunoglobulin secretion from normal B cells (MacKenzie et al., 1987). The search for $CD5^+$ B cell-secreted factors that may be responsible for suppression of antibody production is still going on but so far several factors have been eliminated. These include IL-1, IL-4, IL-10, IL-11, IFN-α, IFN-γ, IFN-β, TNF-α and TNF-β and TGF-β (MacKenzie and Paglieroni, 1995).

2.2.9 Bovine CD5 B cells

Presence of $CD5^+$ B cells in cattle was first observed by Depelchin et al.(1989) when developing monoclonal antibodies to various bovine blood cells. They found that some blood B cells from cattle infected with bovine leukemia virus (BLV) expressed a pan T cell marker which was identified to be CD5.

Monoclonal antibodies for bovine CD5 detect two allelic forms of this antigen. One form which is expressed in *Bos taurus* is detected by monoclonal antibodies CC17 (IgG1) (Howard et al., 1988) or 8C11 (IgG2a) (Depelchin et al., 1989) and its allele expressed in *Bos indicus* is detected by monoclonal antibody IL A-67

(IgG1) (Howard et al., 1989). The phenotype and anatomical localization of bovine $CD5^+$ B cells were reported by Naessens and Williams (1992). They are found in blood and spleen but not in tonsils and Peyer's patches. Compared to the $CD5^-$ B cells, bovine $CD5^+$ B cells are larger in size, express high amounts of IgM and express CD11b. The two subpopulations were similar in the expression of other B cell specific antigens.

It is still not established whether or not the bovine $CD5^+$ B cells represent a separate B cell lineage similar to the B-1 B cells in humans and mice or whether they represent an activation state of B cells. More studies on ontogeny, V-H gene rearrangements and antibody repertoire are still required.

In diseases of cattle which are associated with changes in $CD5^+$ B cells, alterations in serum IgM concentrations have been observed but no direct link between the two and the establishment of pathology has been made. During infections with *T. congolense*, profound increase in proportion and numbers of $CD5^+$ B cells was correlated with the increase in the concentration of serum IgM (Naessens et al., 1992). However, it has not been established whether the $CD5^+$ B cells or the IgM produced play any role in the pathology of the disease.

Increased numbers of circulating $CD5^+$ B cells are a common feature of bovine leukemia virus infection (Depelchin et al., 1989). This disease is characterized by

unresponsiveness to heterologous antigens (Ungar-Waron and Trainin, 1987). However, there appears to be controversy regarding the changes in serum IgM concentrations during the disease. Pierce et al. (1977) observed an increase whereas Ishihara et al., (1980) did not detect any change and Gatei et al. (1990) observed a significant decrease. In another study, it was found that there is a significant increase in surface expression of IgM but not IgG on B cells from BLV infected cattle (Levkut et al., 1995).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental animals

3.1.1 Cattle

Thirty five Boran and 6 N'Dama cattle aged between 6 months and 3 years were used. The animals were born and reared at the International Livestock Research Institute's (ILRI) Kapiti Plains Ranch, Athi River, Kenya, an area known to be free from trypanosomiasis. Six weeks before experimental infection with trypanosomes, they were moved to the ILRI farm compound in Nairobi and kept indoors at the ILRI Large Animal Unit. The animals tested free of antibodies to *Babesia bigemina*, *B. bovis*, *Theileria mutans* and *Anaplasma marginale* using ELISA (Katende et al., 1990) and tested free of antibodies to *T. parva* using an indirect fluorescent antibody test (Goddeeris et al., 1982). Throughout the experimental period, the animals were fed hay and concentrates (Unga Feeds Limited, Nairobi, Kenya). Drinking water was supplied *ad libitum*. The animals were routinely sprayed with an acaricide, dioxathion (Delnav DFF, Welcome, Kenya) once weekly, except one week before they were experimentaly infected with trypanosomes by tsetse fly bite.

3.1.2 Rats

Sprague Dawley rats between 2 and 3 months of age were used. These rats were bred and maintained at the Small Animal Unit at ILRI. They were kept in rat cages (North Kent Plastic Cages Ltd., Dartford, England) and fed mouse cubes (Unga Feeds Limited). Water was supplied *ad libitum.*

3.2 Trypanosomes

The following trypanosomes were used;

3.2.1 ***T. congolense*** **clone IL 1180.** This is the ILRI name for clone *T. congolense* IL Nat 3.1. It is a double cloned derivative of STIB 212 (Geigy and Kauffmann, 1973), which was isolated from a lion in Serengeti, Tanzania in 1971 (Moloo et al., 1992).

3.2.2 ***T. vivax*** **stock IL 2337.** This stock was derived from GALANA/78/TRYPS 392, which was isolated from a Zebu cow between Galana and Malindi, Kenya in 1978 (Moloo et al., 1993).

3.3 Infection of cattle with trypanosomes

Cattle were infected by allowing five infected *Glossina morsitans centralis* flies to feed on their left flanks as described by Dwinger et al. (1987).

3.4 Preparation of serum

Blood was obtained by jugular venipuncture and collected in silicone-coated vacuum tubes (Vacutainer, Beckton Dickinson, New Jersy, USA). The blood was incubated in a water bath at 37 °C for 30 minutes to hasten clotting and then kept at 4°C for 2 hours to allow the separation of serum. The tubes were then centrifuged at 200 (xg) for 15 minutes and serum collected in microtubes (Micronic tubes, BDSL, Lelystad, Netherlands) in 1ml aliquots. Serum tubes were stacked in Micronic tube holders (BDSL) and stored at -70 °C until use.

3.5 Monoclonal antibodies

The following monoclonal antibodies were used in these studies. They were all produced at ILRI.

3.5.1 IL-A2: a mouse IgG_1, recognises bovine IgG (IgG_1 and IgG_2 (Naessens et al., 1988).

3.5.2 IL-A11: a mouse IgG_2a, recognises bovine CD4 (Baldwin et al., 1986).

3.5.3 IL-A30: a mouse IgG_1, recognises bovine IgM (Naessens et al., 1988).

3.5.4 IL-A50: a mouse IgG_2a, recognises a bovine IgM allotype present in most cattle (Naessens et al., 1988).

3.5.5 IL-A58: a mouse IgG_2a, recognises bovine immunoglobulin light chain (Williams et al., 1990)

3.5.6 IL-A59: a mouse IgG_1, recognises bovine immunoglobulin light chain (Williams et al., 1990).

3.5.7 IL-A67: a mouse IgG_1, recognises a polymorphic CD5 determinant present in *Bos Indicus* cattle (Howard et al., 1989).

3.5.8 IL-A105: a mouse IgG2a, recognises bovine CD8 (MacHugh et al., 1993).

3.6 Purification of immunoglobulins from ascitic fluid

Immunoglobulins were purified from murine ascitic fluid by passage through a diethylaminoethyl (DEAE) cellulose ion exchange column as described previously (Jurd, 1981). Ascitic fluid was centrifuged at 12,000 (xg) for 5 minutes on a

Beckman centrifuge model J2-21M (California, USA) to remove precipitates, and dialysed against 0.02M phosphate buffer (pH 8.0) overnight at 4°C. Swollen DEAE powder (Whatman DE 52, Maidstone, England) was equilibrated with the phosphate buffer and the slurry formed was packed in a 10 ml chromatography column (Pharmacia, Uppsala, Sweden). Dialysed ascitic fluid was then introduced to the DE52 column (at a ratio of 1 ml ascites for 3 ml packed DE52) and the absorbance$_{280nm}$ of eluted fractions monitored on a U.V. spectrophotometer (Pharmacia, Uppsala, Sweden). The unbound fraction was discarded and the bound mouse IgG fraction was subsequently eluted by running phosphate buffer containing 0.05M NaCL through the column. The collected pure mouse immunoglobulins were dialysed in Dulbecco's phosphate buffered saline (DBPS), pH 7.4 and their absorbance determined on the Ultrospec II Spectrophotometer (LKB Biochrom, Cambridge, England) at 280 nm. The concentration of each immunoglobulin fraction was calculated based on the absorbance of immunoglobilin samples of known concentration.

3.7 Conjugation of monoclonal antibodies to biotin

Preparation of biotinylated monoclonal antibody IL-A59 for use in the silver immunogold (SIG) blot assay was done according to the method described by Naessens and Nthale (1993). Three ml of 1 mg/ml solution of monoclonal antibody IL-A59 was dialysed against 0.1 M sodium carbonate buffer (pH 8.3)

overnight at 4°C. Three mg of NHS-LC-biotin powder (Pierce, Illinois, USA) was dissolved in 60 μl of dimethylsulphoxide and incubated with the antibody for 2 hours at room temperature. The biotinylated IL-A59 was then dialysed against DPBS and stored at 4°C.

3.8 Conjugation of horseradish peroxidase to monoclonal antibodies

Enzyme conjugates of IL-A2 and IL-A30 for use in ELISA studies were prepared according to the method of Wilson and Nakane (1978). Four mg of horseradish peroxidase (HRPO) enzyme (Sigma, St. Louis, USA) was dissolved in 1 ml distilled water and the enzyme oxidised by addition of 0.2 μl of 0.1 M sodium meta-periodate (Sigma) and the solution stirred for 20 minutes at room temperature. The formed HRPO-aldehyde solution was dialysed against 1 mM sodium acetate buffer (pH 4.4) overnight at 4°C. The pH of the HRPO-aldehyde solution was raised to 9.0-9.5 by addition of 20 μl of 0.2 M sodium carbonate buffer (pH 9.5), after which, 8 mg of mouse IgG (IL-A2 or IL-A30) in 1 ml 0.1 M sodium bicarbonate buffer (pH 9.5) was added and the reaction mixture stirred for 2 hours at room temperature. The HRPO-monoclonal antibody conjugate solution was stabilised by addition of 0.1 ml of 4 mg/ml sodium borohydrate solution and incubated for 2 hours at 4 °C. The conjugates were stored at -20°C in 50% glycerol.

3.9 Antigens

3.9.1 Non trypanosome antigens

The non-trypanosome antigens used in ELISA and the silver immunogold blot assay were as follows; ß-galactosidase from *Escherichia coli*, bovine serum albumin (BSA), ssDNA from calf thymus, haemocyanin from keyhole limpets (KLH) (*Megathura crenulata*), ovalbumin from hen egg (OVA), trinitrophenol (TNP) and trypsin from soybean (all from Sigma, Montana, USA), blue dextran, aldolase from rabbit muscle, catalase from bovine liver, ferritin from horse spleen and thyroglobulin from bovine thyroid (all from Pharmacia, Uppsala, Sweden), cytochrome C from horse heart and myoglobin from horse skeletal muscle (from Schwarz/Mann, New York, USA) and hen egg lysozyme (from Polysciences Inc., Pa, USA).

3.9.2 Trypanosome antigens

A mixture of trypanosome antigens prepared by lysis of live trypanosomes as described by Authie et al.(1993) was used to test FOR specific antibodies.

Rats were sublethally irradiated by exposure to 900 rads in a caesium source and infected by intraperitoneal injection of a stabilate of *T. congolense* IL 1180 suspended in phosphate saline glucose (PSG) "rat buffer" (1% glucose, pH 8.0, ionic strength 0.217). Parasitaemia in blood was monitored daily by counting the

number of live trypanosomes using a haemocytometer. About 5 μl of blood was diluted 1/100 in PSG rat buffer containing 10 i.u. of heparin, before it was introduced to the haemocytometer. At peak parasitaemia (approximately 10^9 trypanosomes/ml), the rats were killed and blood collected in heparinized PSG buffer. Trypanosomes were separated from blood on DEAE-cellulose according to the method of Lanham and Godfrey (1970). The trypanosomes were then washed several times in PSG buffer before lysis.

The trypanosome lysate was prepared by subjecting the trypanosome pellet containing about 10^9 trypanosomes to three freeze-thaw cycles in liquid nitrogen. The lysate was prepared in the presence of the following protease inhibitors, 10 μg/ml leupeptin, 1 mM phenyl-methyl-sulphonylfluoride, 0.2 mM N-tosyl-L-phenylalanine chloromethyl ketone and 0.05 mM N-alpha-p-tosyl-lysine chloromethyl ketone (all from Sigma) as previously described by Authie *et al.* (1993).

3.10 The silver immunogold (SIG) blot assay for detecting antibody secreting cells

3.10.1 Preparation of spleen mononuclear cells

Seven cattle infected with *T. congolense* IL 1180 were slaughtered between 31-51 days post infection (dpi) and a 20 g piece of spleen removed immediately. The

piece of spleen was brought to the laboratory in a Sterilin Petri dish (Middlesex, England) containing 30 ml of RPMI 1640 + HEPES, where it was gently teased using forceps and a plunger of a 5 ml syringe to obtain a single cell suspension. After sedimentation of tissue fragments, 30 ml of the cell suspension was layered onto 20 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden) in a sterile 50 ml Falcon tube (Beckton Dickinson, New Jersy, USA) and centrifuged at 1000 (xg) for 25 minutes in a Beckman J6B centrifuge (California, USA). The mononuclear cells were collected from the interphase between RPMI 1640 + HEPES and Ficoll-Paque and washed in Alsevers solution by centrifugation at 200 (xg) for 10 minutes. If the mononuclear cells were contaminated with erythrocytes, the contaminating cells were lysed by incubation in tris-ammonium chloride lysis buffer (pH 7.2) for 5 minutes at 37°C. The lysis buffer was removed by washing cells three times in Alsever's solution at 200 (xg) for 10 min. After the last wash, the cells were resuspended in supplemented RPMI 1640, containing 10% normal rabbit serum, 5 x 10^{-3} M 2-mercaptoethanol (Merck, Munich, Germany), 1% penicillin-streptomycin and 1% L-glutamine.

3.10.2 Fluorescent staining and flow-cytometry

Staining of B cells for CD5 was done using equal volumes of monoclonal antibodies IL-A50 (IgG2a, anti bovine IgM) and IL-A58 (IgG2a, anti-bovine immunoglobulin) and IL-A67 (IgG1, anti CD5) as described previously (Naessens

and Williams, 1992). Equal volumes of IL-A50/IL-A58 mixture and IL-A67, each diluted 1/500 in supplemented RPMI 1640 were added to cells at the ratio of 25 μl per 10^6 cells and incubated for 30 minutes on ice in a 10 ml Sterilin tube (Bibby Sterilin Ltd, Starfordshire, England). Excess antibodies were washed away by resuspension of cells in supplemented RPMI 1640 and centrifugation at 200 (xg) for 10 minutes at 8^0C. Thereafter, 1/30 diluted fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG1 (Sigma, Poole, U.K.) and 1/200 diluted phycoerythrin (PE)-conjugated anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL, USA) were added to the cells at the ratio of 25 μl of conjugate per 10^6 cells and staining left to continue on ice for 30 minutes. Excess fluorescent conjugates were washed away by twice resuspending cells in supplemented RPMI 1640 and centrifuging the tubes at 200 (xg) for 10 minutes. The stained cells were then transferred into 6 ml Falcon tubes (Beckton Dickinson, New Jersy, USA). Populations of $CD5^+$ and $CD5^-$ B cells were obtained by cell sorting using a flow-cytometer (FACStar, Becton Dickinson, California, USA). The concentrations of $CD5^+$ and $CD5^-$ B cells were adjusted to 10^6/ml in supplemented RPMI 1640 for determination of antibody secreting cells.

3.10.3 Determination of antibody secreting cells

To determine cells secreting antibody of a given specificity, purified B cell fractions were tested in a SIG blot assay as described previously (Taylor et al.,

1994). Wells of a 96-well Dynatech (Dynatech, Plochingen, Germany) ELISA plate were coated with either mouse anti-bovine immunoglobulin light chain (IL-A58) or with non-trypanosome antigen (ß-galactosidase or ovalbumin). One hundred μl of the protein at 2.5 μg/ml in coating buffer (0.05M bicarbonate buffer, pH 9.6) were added to each well and plates incubated overnight at 4°C. The plates were washed three times with washing buffer (0.015 M Tris-HCl, pH 9.6). Uncoated sites were blocked by addition of 100 μl of 5% casein hydrolysate (Sigma) in washing buffer and plates incubated for 1 hour at 37 °C. $CD5^-$ and $CD5^+$ B cells were added in duplicate wells ranging from 100,000/well down to 3,125/well in two-fold dilutions. The plates were incubated for 2 hours in an incubator (W.C. Heraeus GmbH, Hanau, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Thereafter, the plates were washed and the secreted antibodies localised by addition of biotinylated IL-A2 (anti-bovine IgG) or IL-A30 (anti-bovine IgM). The blots were visualised by addition of silver initiator and enhancer reagents (Amersham International, Buckinghamshire, England) and counted using an inverted microscope at × 40 magnification. Differences in the number of antibody-secreting cells between $CD5^+$ and $CD5^-$ B cells were tested for significance using Student's t-test.

3.11 *In vivo* $CD4^+$-and $CD8^+$-T cell depletion

For $CD4^+$ T cell depletion, a mouse IgG2a monoclonal antibody IL A11, specific for bovine CD4 antigen was used. Ascitic fluid was raised in 50 F1 (Balb/C × Swiss) mice and sterilised by first passing it through a glass fibre filter (Whatman, Maidstone, Great Britain) followed by filtration through a 0.22 μm filter (Sartorius, Gottingen, Germany). To estimate the antibody concentration in the pooled ascites fluid, immunoglobulin from a sample (1 ml) of fluid was purified by anion chromatography on a DE52 (Whatman) column as described previously (section 3.6) and the concentration estimated by spectrophotometry at 280 nm. The immunoglobulin concentration in IL-A11 ascites was 5.2 mg/ml. Complete depletion was achieved by intravenous (i.v.) injection, into jugular vein, of ascitic fluid containing a total of 137.67 mg of immunoglobulins in doses divided over a period of three. On the first day of depletion, a total of 0.52 mg of immunoglobulins were administered i.v. in four injections of 25 μl of ascites each at 1-2 hours interval. On day two, a total of 4.03 mg of immunoglobulins were given in four injections of 25, 50, 200 and 500 μl of ascites respectively at 1-2 hours interval and on day three, a total of 133.12 mg of immunoglobulins were injected in volumes of 100, 500 μl, 5 ml and 20 ml of ascites at intervals of 1-2 hours.

Cattle were depleted for $CD8^+$ T cells using monoclonal antibody IL-A105 specific for bovine CD8 α-chain as described by Sileghem and Naessens (1995).

3.11.1 Monitoring of $CD4^+$ T cells

Levels of $CD4^+$ T cells were monitored in jugular blood samples twice weekly. Blood collected in Alsevers solution was layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden) and mononuclear cells obtained by centrifugation at 1000 (xg). The mononuclear cells were then washed four times in Alsever's solution by centrifugation at 200 (xg) to remove platelets and finally resuspended in L-15 medium (Flow Laboratories, Irvine, Great Britain) at a concentration of 10^7/ml. Staining of $CD4^+$ T cells was done by mixing 25 μl of 1/1000 diluted IL A11 ascitic fluid with 25 x 10^4 cells in a well of a U-bottomed 96-well plate (Costar, Cambridge, MA, USA) and incubating on ice at 4°C for 30 minutes. The cells were washed twice at 200 (xg) and a 1/30 diluted FITC-conjugated polyvalent anti-mouse immunoglobulin added on ice for 30 minutes. The cells were then washed twice, resuspended in 2% formalin in normal saline and the fluorescence analysed on a flow cytometer (FACStar PLUS or FACSCAN, Becton Dickinson, California, USA).

3.12 Purification of immunoglobulin fractions from serum

3.12.1 Coupling of proteins (antigens) to CNBr-activated Sepharose gel

Coupling of the ligands to CNBr-activated sepharose beads was done according to manufacturer's instructions. One gram of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) powder was resuspended and allowed to swell in 1

mM HCl. The gel was washed with 400 ml of 1 mM HCl over a sintered glass filter for 15 minutes and transferred into a 20 ml chromatography column (Eco-column, Biorad, California, USA). Three ml of a 5 mg/ml solution of ligand (monoclonal antibody IL-A30 or IL-A2, trypanosome lysate, ß-galactosidase, cytochrome or ferritin) in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.5) were added, and the mixture rotated end-over-end for two hours at room temperature. The unbound sites on the gel were blocked by incubation with 1% OVA (Sigma) in DPBS for 2 hours at room temperature. The product was then washed IN three cycles of alternating pH, each cycle starting with a wash with 0.1 M acetate buffer (pH 4.0) followed by a wash with DPBS (pH 7.4). The product was stored in DPBS containing 0.2% Sodium azide at 4°C until use.

3.12.2 Affinity purification of bovine antibodies from serum

A pool of serum collected from 6 Boran cattle, 30 dpi with *T. congolense* was dialysed in DPBS overnight at 4°C. About 20 ml of the serum was passed through a CNBr-activated Sepharose 4B affinity column coupled to monoclonal antibody IL-A30 (specific for bovine IgM). The bound IgM was eluted with 0.1 M glycine-HCL (pH 4). The eluted antibodies were immediately placed into dialysis membranes (Spectra/por, Spectrum, Los Angeles, USA) and dialysed against DPBS overnight at 4°C.

For purification of IgM reacting with trypanosome and non-trypanosome antigens, the IgM fraction was affinity purified on CNBr-activated Sepharose 4B coupled to either trypanosome lysate or ß-galactosidase respectively. The affinity purified IgM fractions were immediately dialysed at 4°C overnight and subsequently stored at -70 °C until use. Unbound IgM fractions were re-run through the respective columns until no more binding could be detected on ELISA.

Bovine IgG wa affinity purified from a serum pool using a CNBr conjugated IL-A2 (mouse anti-bovine IgG) column and IgG. The affinity purified IgG was then passed over antigen-conjugated columns and the bound and unbound fractions collected as described above. The collected bound and unbound IgM and IgG fractions were tested in ELISA for their capacity to bind trypanosome lysate, ß-galactosidase, cytochrome and ferritin.

3.13 ELISA for bovine antibodies

ELISA assay was used for measuring amount of trypanosome non-specific antibodies and trypanosome-specific antibodies in serum of cattle infected with *T. congolense* IL 1180 and *T. vivax*. Each well of a 96-well Dynatech ELISA plate was coated with 100 µl of either 40 µg/ml of trypanosome lysate or 1 µg/ml solution of a non-trypanosome antigen in coating buffer (0.05M bicarbonate buffer, pH 9.6) and the plates incubated overnight at 4 °C. The plates were then washed

three times with washing buffer (DPBS, containing 0.1 % Tween 20). Thereafter, 100 μl of bovine serum diluted 1/100 in washing buffer was added to duplicate wells and incubated for 2 hours at 37 °C. The plates were then washed and 100 μl of 1/6,400 diluted HRPO-conjugated IL A2, specific for bovine IgG, or 1/1,600 diluted HRPO-conjugated IL-A30, specific for bovine IgM, was added for 1 hour at room temperature. After washing, 100 μl of substrate K-blue (ELISA Technologies, Lexington, USA) was added to each well and the optical density determined at 650 nm on a Titertek Multiskan MCC/340 ELISA plate reader (Flow, Oy, Finland) after a 30 minute incubation.

When measuring the binding of affinity-purified IgM and IgG fractions in ELISA, their immunoglobulin concentrations were brought to 10 μg/ml so that they could be directly compared.

3.14 Statistical analyses of ELISA results

Significant changes in mean levels of antibodies reacting with various non-trypanosome as well as trypanosome antigens over the infection period (0-35 dpi) were determined by subjecting optical density values obtained for each serum to one-way analysis of variance (ANOVA) using Minitab Release 9 for Windows. Probability of $p < 0.05$ was considered significant. Where a significant change was

detected, the mean optical densities were compared using the Least Significant Difference method at a rejection level of $p = 0.05$.

Optical density values obtained for sera from $CD4^+$ or $CD8^+$ T-cell depleted and non-depleted control cattle were compared using analysis of variance. Each analysis consisted of the following terms; group (depleted and control), animal within group, time, and group × time interaction. Differences between averages of treatment groups were compared with the variation among animals in the group. Individual one-way analysis of variance was also undertaken at each time point.

CHAPTER FOUR

4.0 RESULTS

4.1 The presence of trypanosome non-specific antibodies in serum of trypanosome-infected cattle.

In order to confirm whether antibodies reacting with non-trypanosome antigens develop in cattle during trypanosome infections, trypanosome infection was initiated in cattle and trypanosome non-specific antibodies measured in serum. In addition, trypanosome-specific antibodies were measured to assess whether there exists any relationship in kinetics and antibody isotypes between trypanosome non-specific and specific antibodies.

4.1.1 Trypanosome non-specific antibodies in *T. congolense* IL 1180 infection of cattle

Twelve cattle (6 Boran and 6 N'Dama) were infected with *T. congolense* IL 1180. Serum was collected at weekly intervals and tested for antibody binding activity to non-trypanosome antigens as well as trypanosome antigens in ELISA. The results are presented as absorbance (O.D. at 650 nm) of each sample minus the absorbance obtained with the pre-infection serum. Differences between optical

density values obtained for various non-trypanosome antigens in six animals over 35 days of infection were analysed for significance by one-way analysis of variance.

In Boran cattle, significant differences in optical density values over 35 days of infection were detected for IgM reacting with cytochrome ($p<0.01$), ferritin ($p<0.01$), β-galactosidase ($p<0.01$) and thyroglobulin ($p=0.07$) (Fig. 1). IgM reacting with cytochrome and β-galactosidase was significantly increased between 21-35 dpi ($p<0.05$). However, IgM reacting with thyroglobulin was only increased on 31 dpi ($p<0.05$) and declined by 35 dpi. There was no significant change in IgM reacting with other non-trypanosome antigens including aldolase, KLH, myoglobin, ssDNA and TNP over the infection period ($p>0.5$) (Fig. 1).

In N'Dama cattle, a significant change in IgM reacting with cytochrome ($p<0.01$), ferritin ($p=0.02$), ß-galactosidase ($p<0.01$), ovalbumin ($p=0.018$) and lysozyme ($p<0.01$) was detected over 35 days of infection (Fig. 2). IgM reacting with cytochrome was significantly increased between 21-31 dpi ($p<0.05$), and by 35 dpi, had declined. For ß-galactosidase and lysozyme, IgM-associated antibody was significantly increased between 21-35 dpi ($p<0.05$). However, antibodies reacting with ferritin and ovalbumin were only increased on 21 dpi ($p<0.05$), and decreased by 31-35 dpi. There was no significant change in IgM to thyroglobulin,

TNP, ssDNA, myoglobin, KLH and aldolase over the infection period ($p < 0.05$) (Fig. 2).

However, in both Boran (Fig. 3) and N'Dama (Fig. 4), there was no change in IgG antibodies which reacted with the non-trypanosome antigens cytochrome, ferritin, ß-galactosidase and BSA ($p > 0.05$).

Analysis of variance for trypanosome-specific IgM in Boran (Fig. 5) and N'Dama (Fig. 6) cattle revealed significant changes during the 35 days of infection ($p < 0.01$). In Boran, trypanosome-specific IgM was increased between 14-21 dpi ($p < 0.05$) and subsequently decreased, while in N'Dama, IgM antibodies were significantly increased between 14-31 dpi ($p < 0.05$) and declined by 35 dpi.

In contrast to the trypansome non-specific antibodies, a significant change trypanosome-specific IgG ($p < 0.01$) was detected over the infection period in both Boran (Fig. 7) and N'Dama (Fig. 8). In Boran cattle, trypanosome-specific IgG was significantly increased between 14-35 dpi ($p < 0.05$). In N'Dama cattle, trypanosome-specific IgG was significantly increased by 14 dpi ($p < 0.05$) and thereafter, increased further was and was highest between 21-35 dpi ($p < 0.05$).

4.1.2 Trypanosome non-specific antibodies in *T. vivax* infection of cattle

To test whether other trypanosome species induce trypanosome non-specific antibodies, four Boran cattle were infected with *T. vivax* IL 2337, and the antibodies binding to ß-galactosidase measured. IgM antibodies reactive to ß-galactosidase were induced in *T. vivax* infections (Fig. 9). Again, no IgG antibodies binding to ß-galactosidase could be detected.

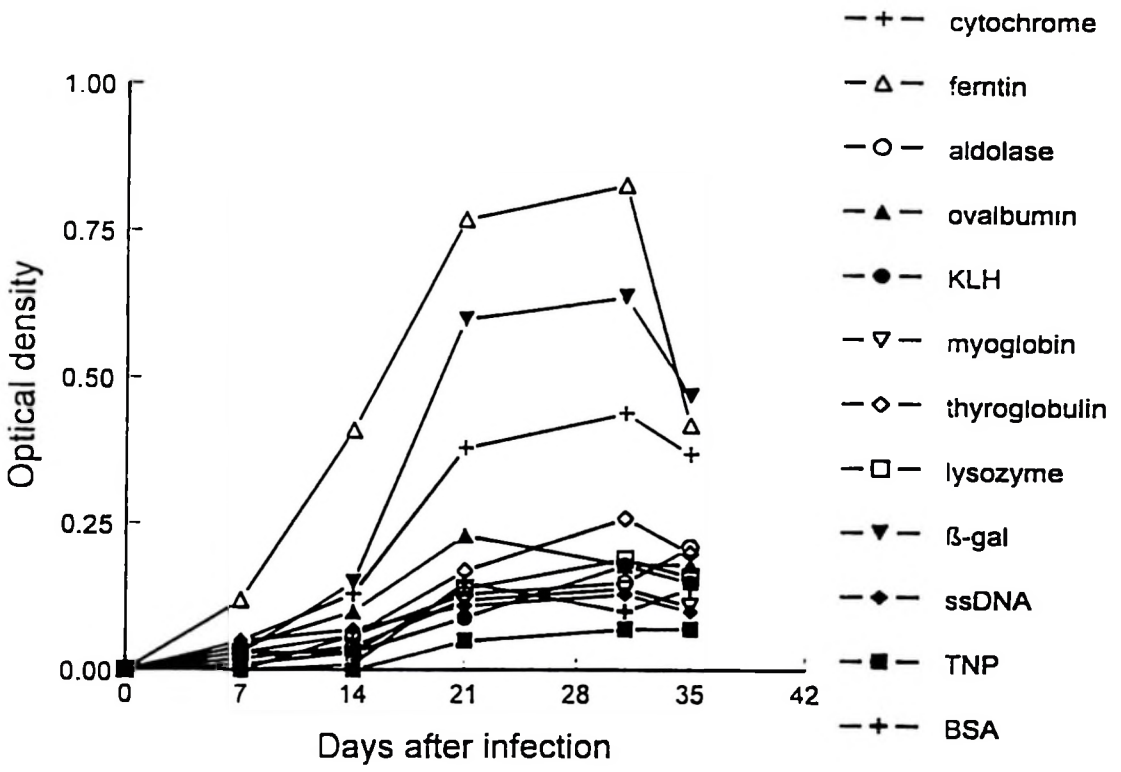

Figure 1. Kinetics of IgM antibodies in sera from *Trypanosoma congolense* IL 1180-infected Boran cattle which reacts with the twelve non-trypanosome antigens shown.

Each point represents the mean optical density of sera from six animals measured in ELISA. (KLH=key limpet haemocyanin, ß-gal=ß-galactosidase, ssDNA=single strand DNA, TNP=trinitrophenol, BSA=bovine serum albumin).

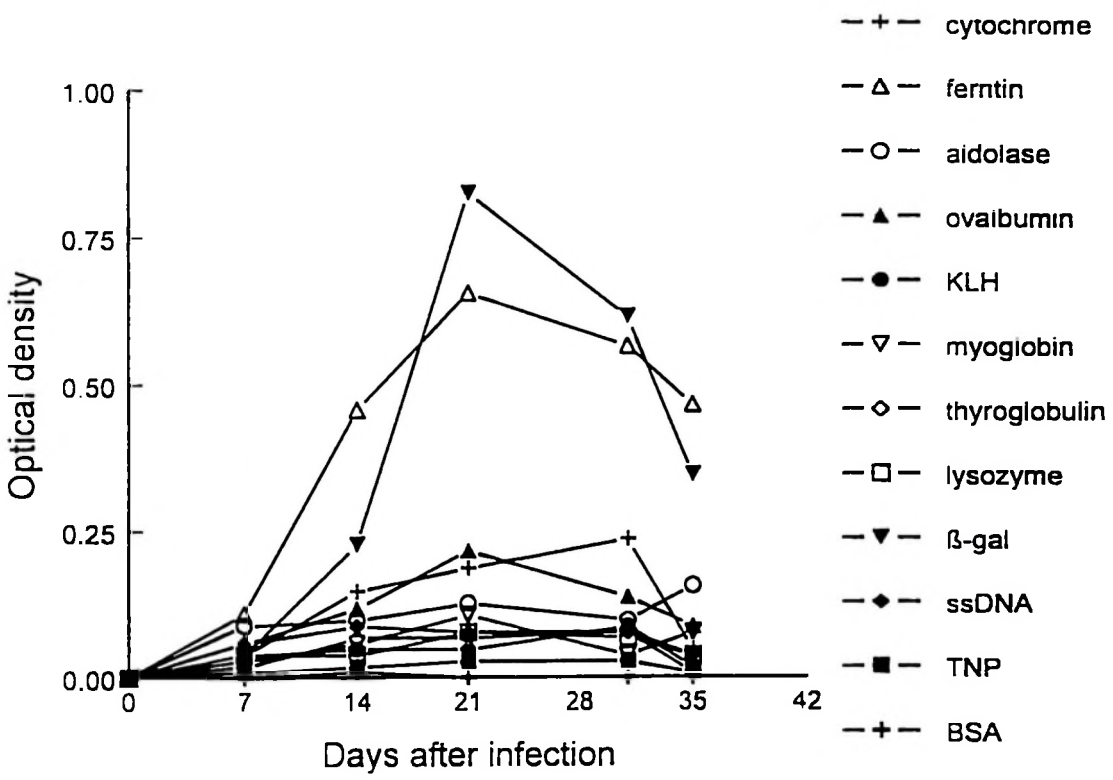

Figure 2. Kinetics of IgM antibodies in sera from *Trypanosoma congolense* IL 1180-infected N'Dama cattle which reacts with the twelve non-trypanosome antigens shown. Each point represents the mean optical density of sera from six animals measured in ELISA. (KLH=key limpet haemocyanin, ß-gal=ß-galactosidase, ssDNA=single strand DNA, TNP=trinitrophenol, BSA=bovine serum albumin).

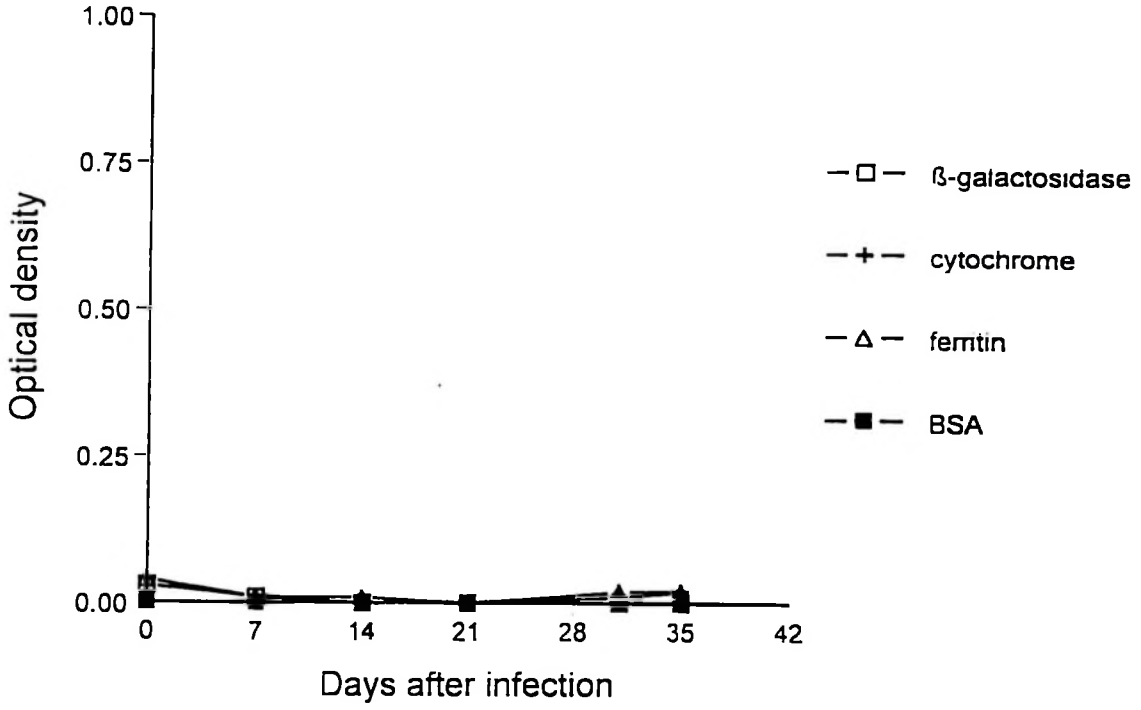

Figure 3. Kinetics of IgG antibodies in sera from *Trypanosoma congolense* IL 1180-infected Boran cattle which reacts with the four non-trypanosome antigens shown. Each point represents the mean optical density of sera from six animals measured in ELISA. (BSA=bovine serum albumin).

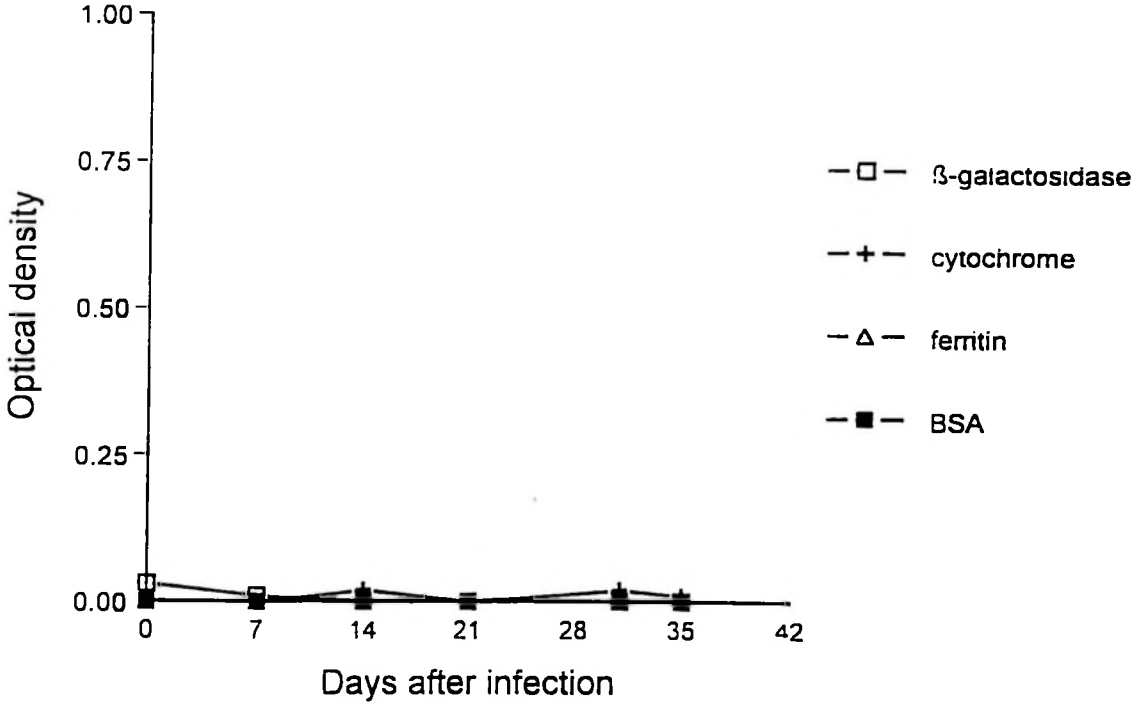

Figure 4. Kinetics of IgG antibodies in sera from *Trypanosoma congolense* IL 1180-infected N'Dama cattle which reacts with the four non-trypanosome antigens shown. Each point represents the mean optical density of sera from six animals measured in ELISA. (BSA=bovine serum albumin).

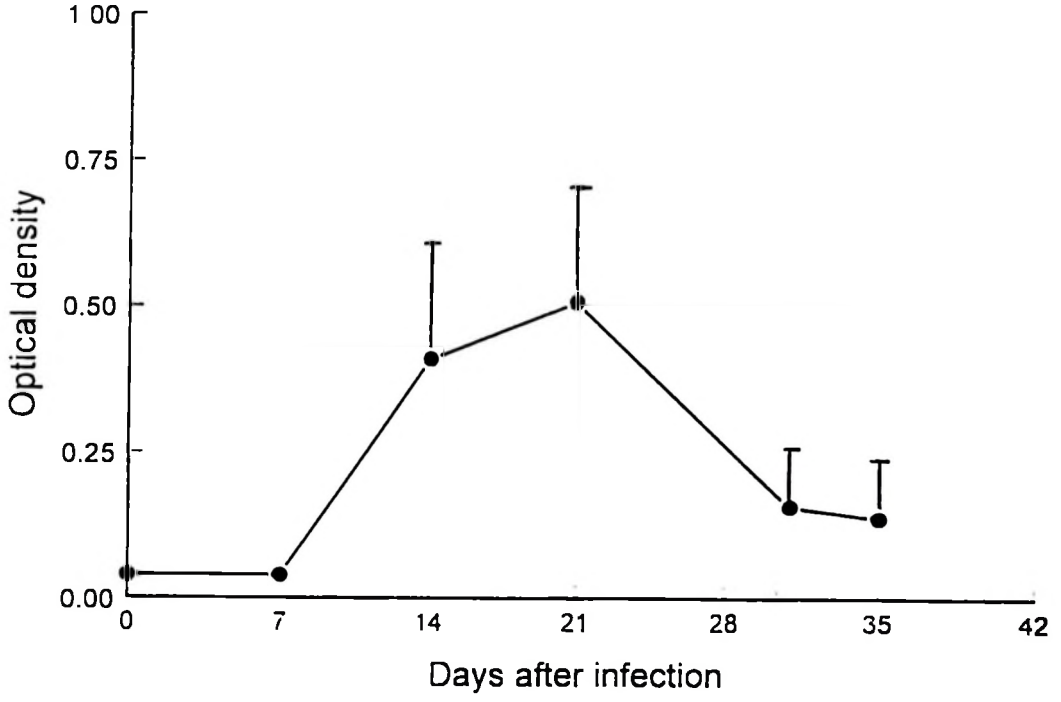

Figure 5. Kinetics of IgM antibodies in sera from *Trypanosoma congolense* IL 1180-infected Boran cattle which react with whole trypanosome lysate.

Each point represents the mean optical density (± standard deviation) of sera from six animals measured in ELISA.

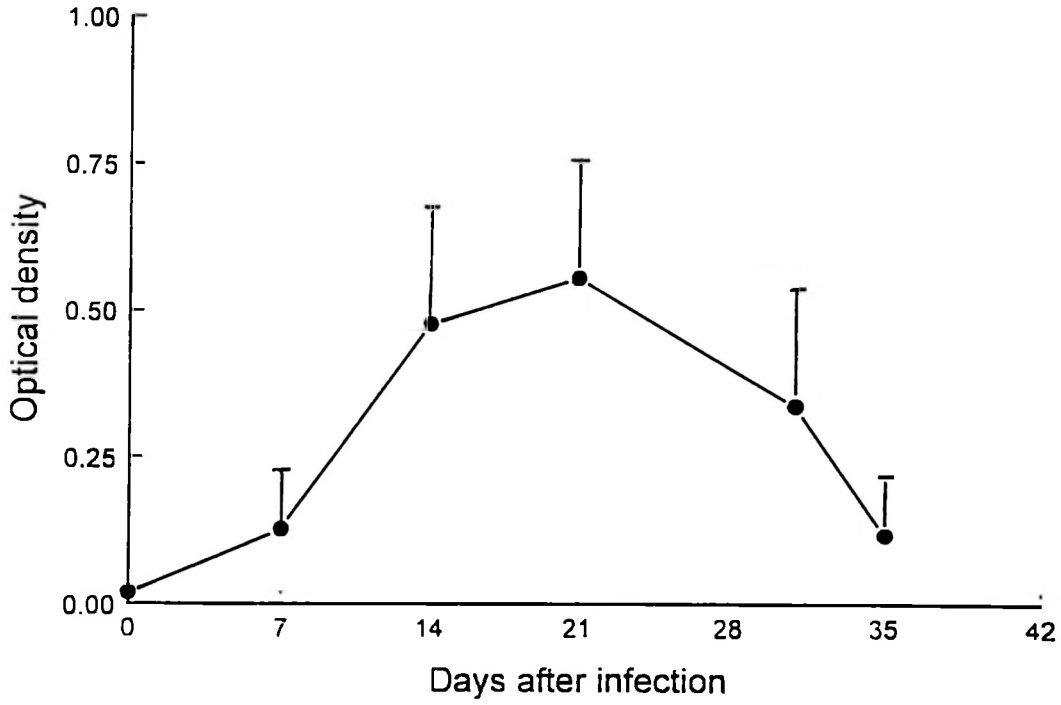

Figure 6. Kinetics of IgM antibodies in sera from *Trypanosoma congolense* IL 1180-infected N'Dama cattle which react with whole trypanosome lysate.

Each point represents the mean optical density (± standard deviation) of sera from six animals measured in ELISA.

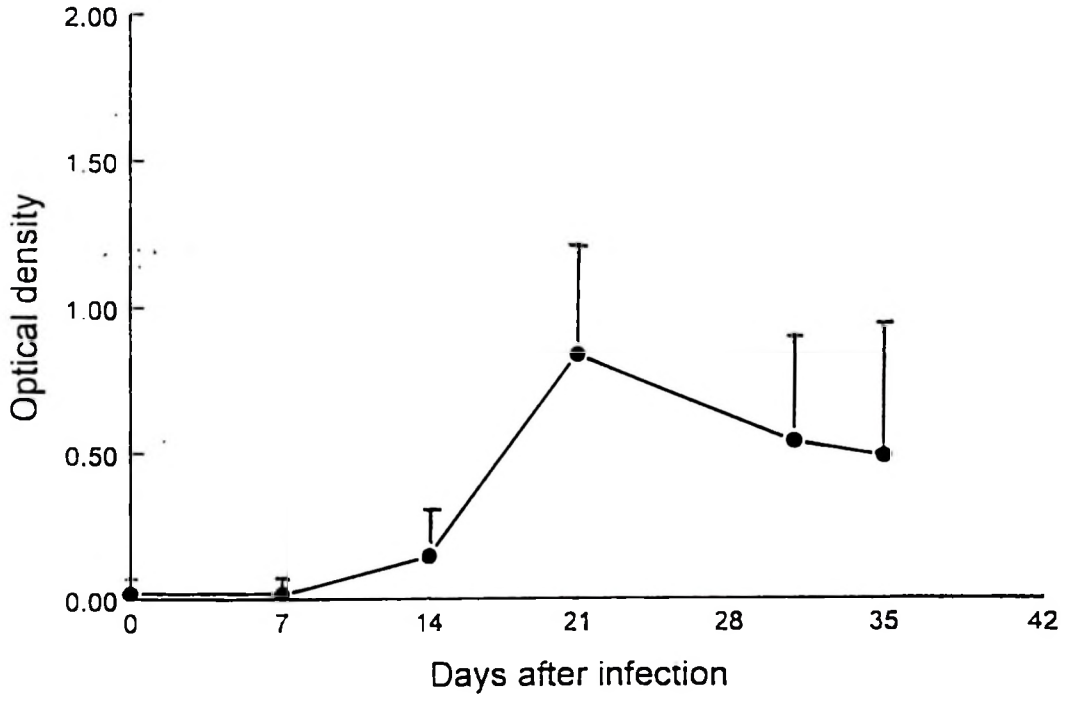

Figure 7. Kinetics of IgG antibodies in sera from *Trypanosoma congolense* IL 1180-infected Boran cattle which react with whole trypanosome lysate.

Each point represents the mean optical density (± standard deviation) of sera from six animals measured in ELISA.

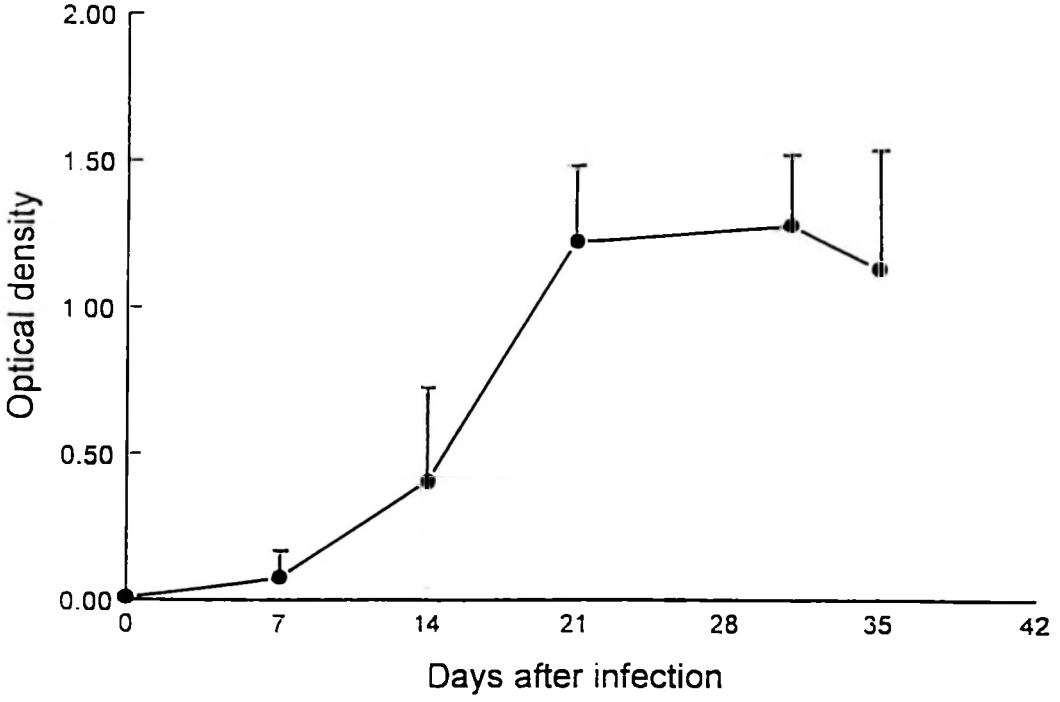

Figure 8. Kinetics of IgG antibodies in sera from *Trypanosoma congolense* IL 1180-infected N'Dama cattle which react with whole trypanosome lysate.

Each point represents the mean optical density (± standard deviation) of sera from six animals measured in ELISA.

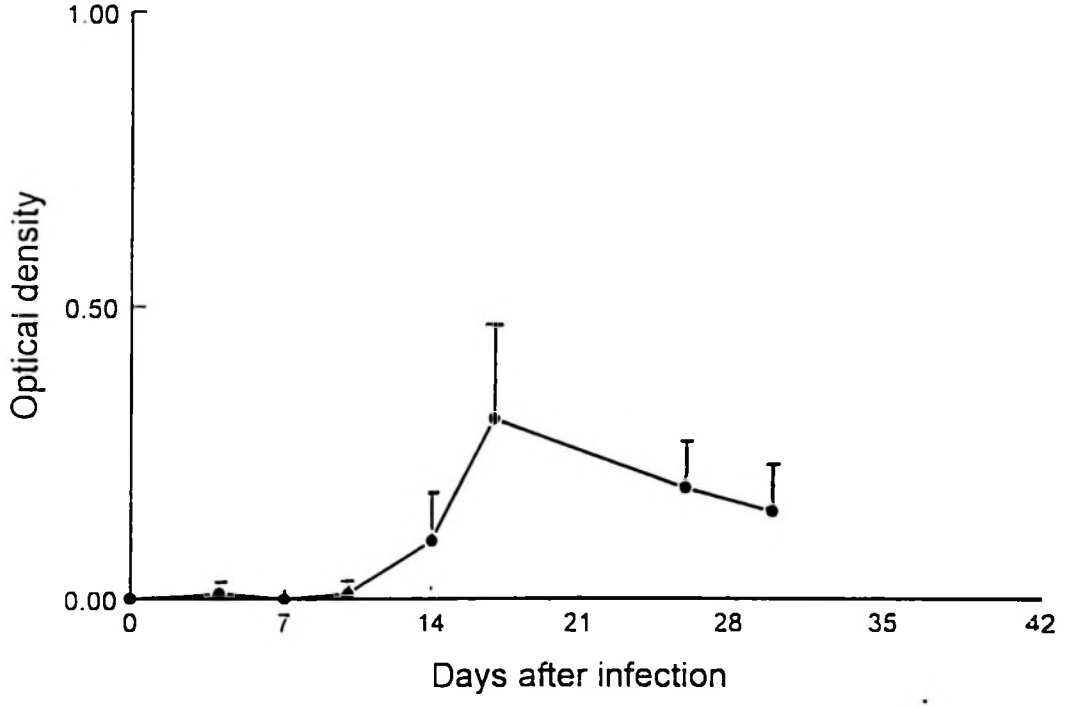

Figure 9. Kinetics of IgM antibodies in sera from *Trypanosoma vivax* IL 2337 - infected Boran cattle which react with a non-trypanosome antigen, ß-galactosidase. Each point represents the mean optical density (± standard deviation) of sera from four animals measured in ELISA.

4.2 Isotypes and specificity of antibodies secreted by $CD5^+$ and $CD5^-$ B cells

4.2.1 Immunoglobulin secreting cells in spleen

The objective of this experiment was to investigate whether the trypanosome non-specific antibodies found in serum of trypanosome-infected cattle were secreted by $CD5^+$, $CD5^-$ or both B cell populations. Spleen cells were derived from 7 Boran cattle between 31 and 51 dpi with *T. congolense* IL 1180 and populations of $CD5^+$ and $CD5^-$ B cells obtained by sorting on FACS. Sorted cells from each subpopulation were tested on the SIG blot assay to identify the isotype and specificity of antibodies secreted by each B cell subpopulation. The FACS profile of spleen cells stained for CD5 and IgM revealed three distinct subpulations, labelled number 1 to 3. (Fig. 10). These populations include (1) the $CD5^+$ B cells which express both surface IgM and CD5, (2) the $CD5^-$ B cells which express surface IgM but not CD5 and, (3) the T-cell subpopulation which express high levels of CD5 but no surface IgM. CD5 expression on splenic B cells was variable and ranged from very low to high, but always remained lower compared to expression on T cells. Cells sorted in the $CD5^+$ and $CD5^-$ categories were those which were most positive and most negative for fluorescence with the CD5 reagents, respectively (Fig. 10). To confirm that only the targeted cells were sorted, the sorted $CD5^+$ and $CD5^-$ were reanalysed. Cells from the positive fraction were almost all CD5 positive, but some cells in the negative fraction displayed a low amount of CD5 (Fig. 11).

Equal numbers (100,000 cells) of sorted $CD5^+$ and $CD5^-$ B cells were tested in the SIG blot assay for comparison of the percentage of cells secreting IgM and IgG, and antibodies reactive with ß-galactosidase and ovalbumin (Table 2). The proportion of cells secreting IgM in both $CD5^+$ and $CD5^-$ B cells ranged from around 400 up to 9,000 per 10^5 in the seven animals, while the number of IgG secreting cells was about ten fold lower in both populations (between 14 and 600 per 10^5). There were no significant differences ($p > 0.05$) in the number of IgM or IgG secreting cells between $CD5^+$ and $CD5^-$ B cell subpopulations.

In the two B cell populations, only between 0.5 % and 10 % of the IgM-secreting cells, produced antibodies with reactivity for ß-galactosidase (Table 2). These percentages were even lower for anti-ovalbumin secreting cells. No cells secreting anti-ß-galactosidase or anti-ovalbumin IgG were found (Table 2). Significantly ($p < 0.05$) more cells secreting ß-galactosidase-reactive antibodies were found in sorted $CD5^+$ B cells than in an equal number of $CD5^-$ B cells (Table 2). Since this difference may be due to the presence of more IgM secreting cells in the $CD5^+$ than in equal numbers of $CD5^-$ B cells, the number of anti-ß-galactosidase and anti-ovalbumin secreting cells were calculated as a proportion of the total number of IgM secreting cells. Again, the proportions of these cells were significantly ($p < 0.05$) higher in the $CD5^+$ B cells than in the $CD5^-$ B cells (Fig. 12). Similar results were obtained for ovalbumin (Fig. 13).

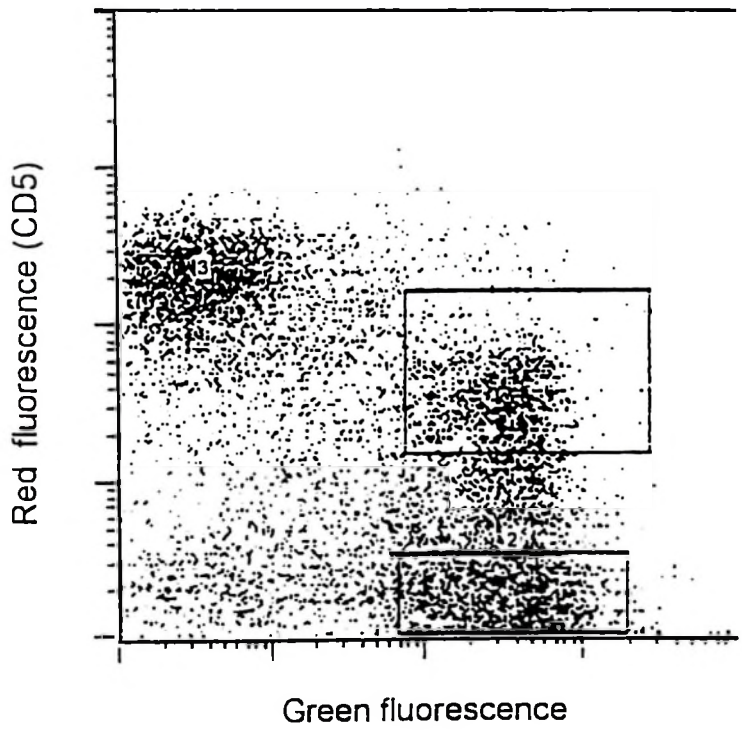

Figure 10. Two-colour fluorescence profiles of cells from whole bovine spleen stained for CD5 (y-axis) and IgM (x-axis), with the windows used to sort $CD5^+$ (1) and $CD5^-$ B cells (2).

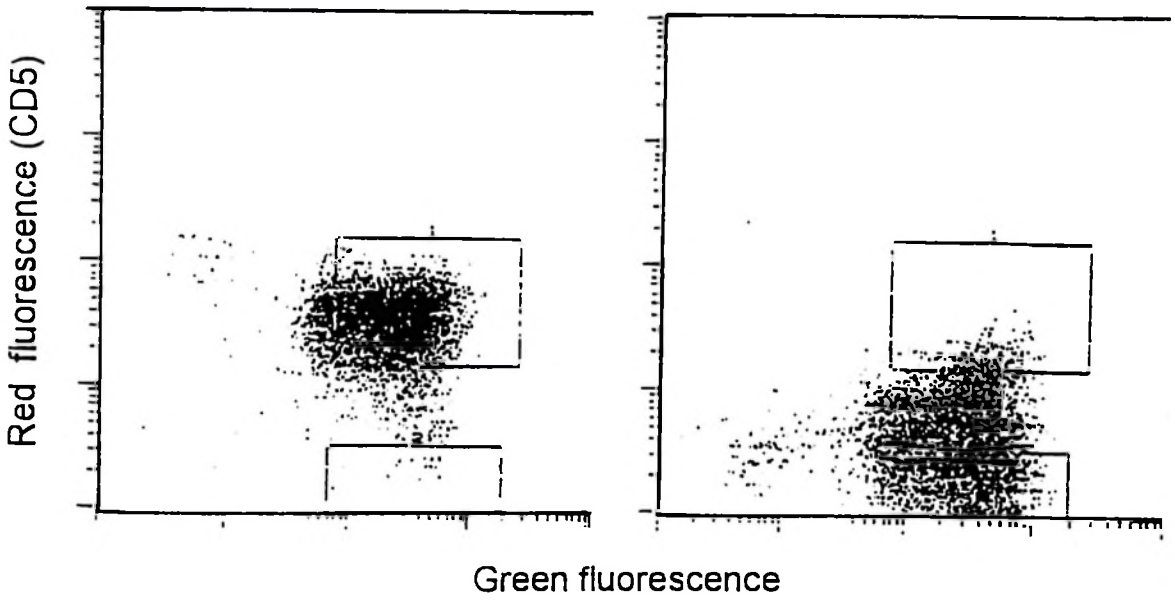

Figure 11. Profile of the the sorted $CD5^+$ and $CD5^-$ B cells during flow-cytometric re-analysis. Sorted $CD5^+$ and $CD5^-$ B cells were re-analysed by fluorescence activated cell sorter and their CD5 expression compared with the same windows which were used for sorting. The fraction of $CD5^-$ B cells contained cells which stained weakly for CD5 (right).

Table 2. Number of cells secreting IgM, IgG, anti-ß-galactosidase IgM and anti-ovalbumin IgM per 100,000 $CD5^+$ or $CD5^-$ B lymphocytes from Boran cattle.

	Total IgM		Total IgG		IgM anti-ß-gal.		IgM anti-OVA	
Animal	$CD5^+$	$CD5^-$	$CD5^+$	$CD5^-$	$CD5^+$	$CD5^-$	$CD5^+$	$CD5^-$
BL209	9455	4929	364	103	182	23	82	10
BL351	1617	1562	35	24	80	23	-	-
BM218	594	970	125	370	29	4	6	0
BM219	912	1050	387	634	26	19	5	3
BM221	1710	746	400	191	28	4	51	8
BM222	550	400	280	150	212	87	103	39
BM326	2070	2455	90	144	204	144	98	50

ß-gal. = ß-galactosidase

OVA. = Ovalbumin

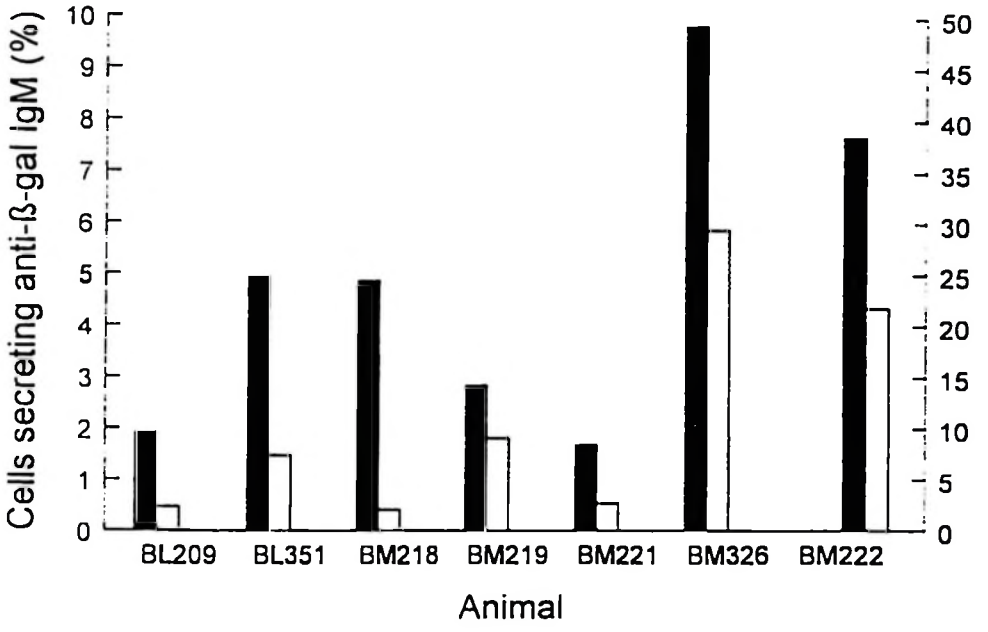

Figure 12. Percentages of IgM-secreting $CD5^+$ (dark bars) and $CD5^-$ B cells (open bars) with reactivity for ß-galactosidase (ß-gal) in seven *T. congolense*-IL 1180-infected Boran cattle. The right y-axis refers to the data from BM222. In each animal, the $CD5^+$ B cell population has a higher percentage of cells secreting anti-ß-gal. antibodies ($p < 0.05$).

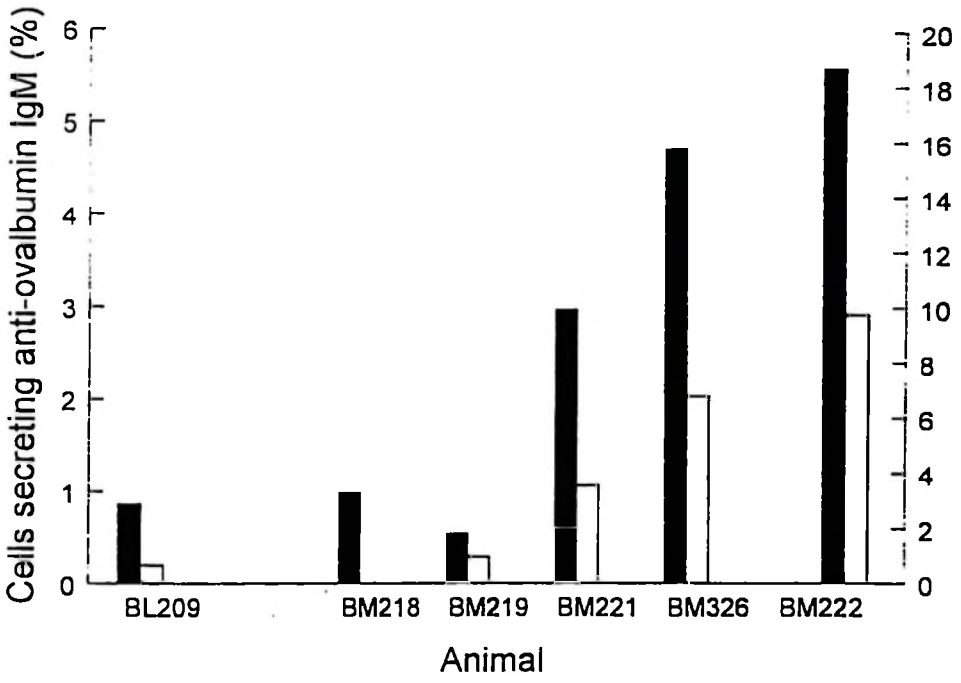

Figure 13. Percentages of IgM secreting splenic $CD5^+$ (dark bars) and $CD5^-$ (open bars) with reactivity for ovalbumin in six *Trypanosoma congolense* IL 1180-infected Boran cattle. The right y-axis refers to the data from BM222. In each animal, the $CD5^+$ B cell population has a higher percentage of cells secreting anti-ovalbumin antibodies.

4.3 The role of $CD4^+$ or $CD8^+$ T cells on the production of trypanosome non-specific antibodies

4.3.1 Efficiency of Depletion

Following the injection of monoclonal antibodies to deplete $CD4^+$ T cell, these cells were monitored in peripheral blood mononuclear cells by fluorescence staining and flow-cytometric analysis during the period of study. The $CD4^+$ T cells were completely eliminated in blood up to 12 dpi. Thereafter, they reappeared slowly and when the last samples were analysed on 28 dpi, the $CD4^+$ T cells were about half of the pre-treatment percentage.

4.3.2 Effect of $CD8^+$ T cells on antibody responses

This study investigated the role played by the $CD8^+$ T cells on antibody responses during trypanosome infection of cattle. Serum from four Boran cattle which were depleted of $CD8^+$ T cells before infection with *T. congolense* IL 1180 (Sileghem and Naessens, 1995) was tested in ELISA to determine changes in antibodies reacting with non-trypanosome antigens or with trypanosome antigens. Sera from 4 undepleted Borans were included as controls. Data obtained for sera collected between 12-28 dpi from the depleted and control groups were compared using analysis of variance (ANOVA). Results are presented as mean antibody ± standard deviations (Fig. 14-16). Both CD8-depleted and -undepleted cattle developed antibodies reacting with ß-galactosidase and these antibodies were exclusively IgM

(Fig.14). In contrast, trypanosome-specific antibody responses were found in both IgM (Fig.15) and IgG (Fig.16). Depletion of $CD8^+$ T cells had no significant effect on antibody responses ($p>0.05$).

4.3.3 Effect of $CD4^+$ T cells on antibody responses during *T. congolense* IL 1180 infection of cattle

The objective of this study was to understand the role of $CD4^+$ T cells in the production of trypanosome non-specific antibodies. A group of 5 Boran cattle was depleted of $CD4^+$ T cells before infection with *T. congolense* IL1180. Five cattle were included as infected but undepleted control animals. The trypanosome non-specific and specific antibodies in serum collected over the course of the infection were measured in ELISA. Results are presented as mean ± standard deviations of optical densities. Data obtained for sera collected between 12-28 dpi from the depleted and control groups were compared using analysis of variance (ANOVA).

Antibodies reactive with a non-trypanosome antigen, ß-galactosidase were of the IgM isotype only (Fig. 17). Analysis of variance revealed significant differences between groups over the period 12-28 dpi ($p<0.01$). IgM reactive to ß-galactosidase was first detected on 15 dpi in the two groups but was significantly lower in the CD4-depleted group ($p<0.01$). Antibodies in the control group were highest between 15-22 dpi and declined progressively thereafter.

The trypanosome-specific antibody response comprised both IgM and IgG classes (Fig. 18-19) Analysis of variance for trypanosome-specific IgM detected significant differences between groups over the period 12-28 days ($p<0.01$). Trypanosome-specific IgM was raised by 12 dpi in both groups, and was significantly higher in controls than in the depleted group ($p<0.05$) (Fig. 18). Antibody in the control group continued to increase more rapidly than in the depleted group up to 15 dpi ($p<0.001$). From 19 dpi, antibody in the control group gradually decreased to near pre-infection levels by 28 dpi. From 22-28 dpi there were no significant differences between control and depleted group.

Trypanosome-specific IgG was detected in both groups (Fig. 19). Analysis of variance revealed differences between groups over the period 12-28 dpi ($p<0.01$). Increased antibody was first detected on 15 dpi in both groups being significanltly more in the control group than in the depleted group ($p<0.01$). Thereafter, in the control group antibody increased more rapidly than in the depleted group and reached a plateau on 22 dpi. Antibody in the depleted group increased gradually from 15 to 24 dpi but remained significantly lower than the control group throughout the study period ($p<0.01$).

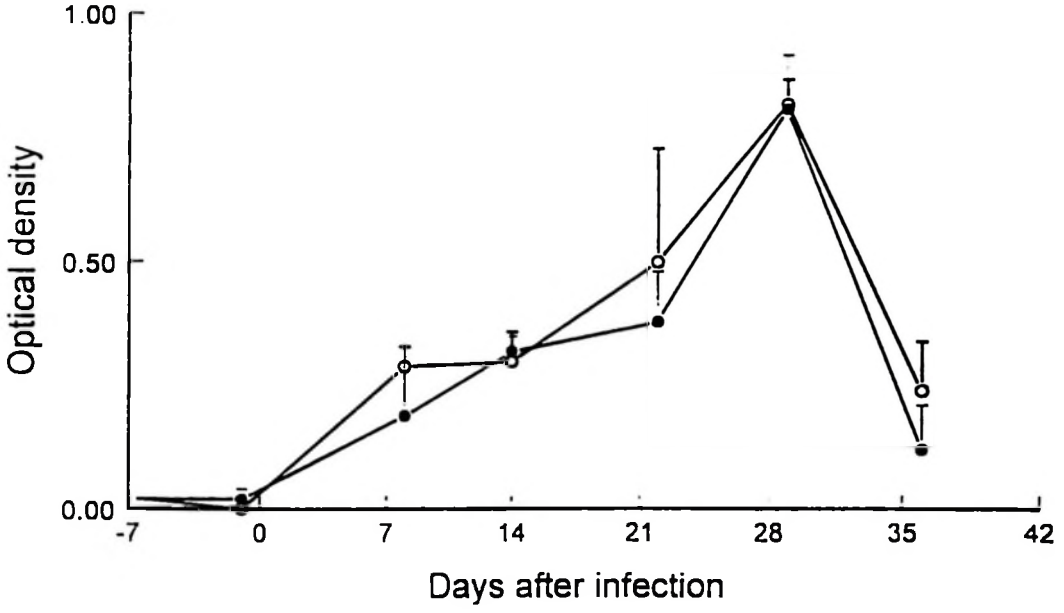

Figure 14. Responses of IgM antibody reacting with a non-trypanosome antigen, ß-galactosidase in $CD8^+$ T-cell depleted Boran cattle during infection with *Trypanosoma congolense* IL 1180. Each point represents the mean optical density (± standard deviation) of sera from five depleted (-o-) and five undepleted (-•-) animals measured in ELISA.

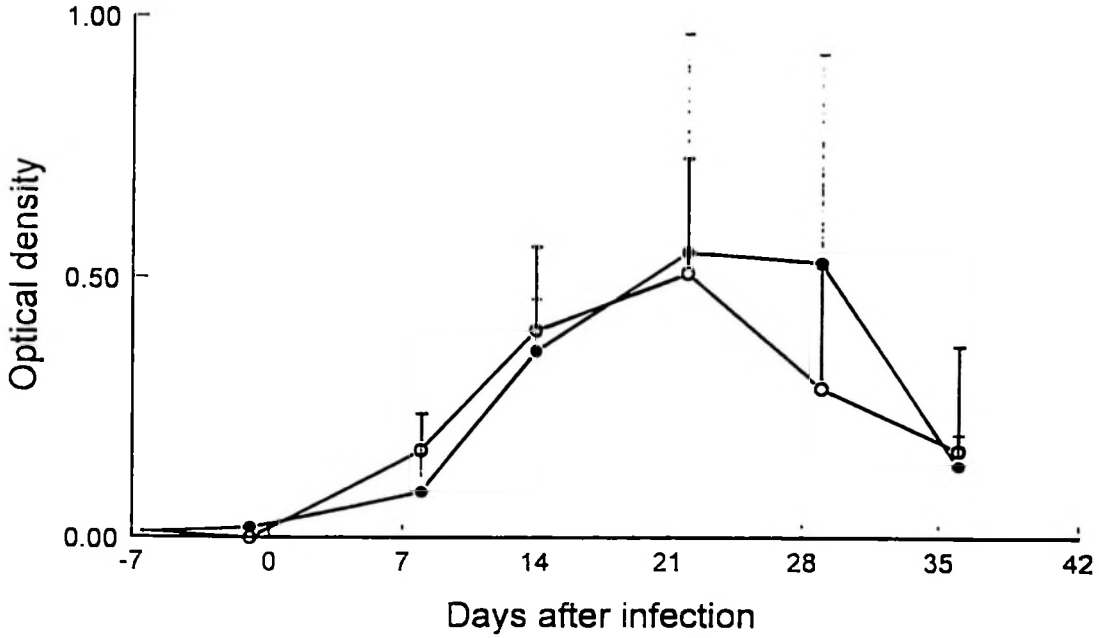

Figure 15. Responses of IgM antibody reacting with trypanosome antigens in $CD8^+$ T cell-depleted Boran cattle during infection with *Trypanosoma congolense* IL 1180. Each point represents the mean optical density (± standard deviation) of sera from five depleted (-o-) and five undepleted (-•-) animals measured in ELISA.

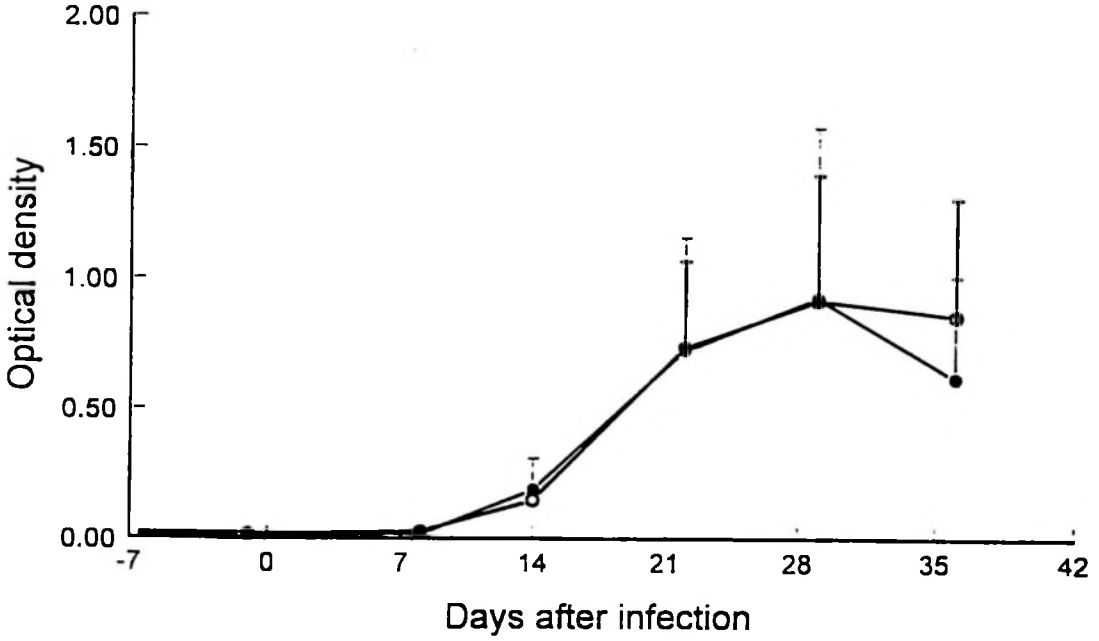

Figure 16. Responses of IgG antibodies reacting with trypanosome antigens in $CD8^+$ T cell-depleted Boran cattle during infection with *Trypanosoma congolense* IL 1180. Each point represents the mean optical density (± standard deviation) of sera from five depleted (-o-) and five undepleted (-•-) animals measured in ELISA.

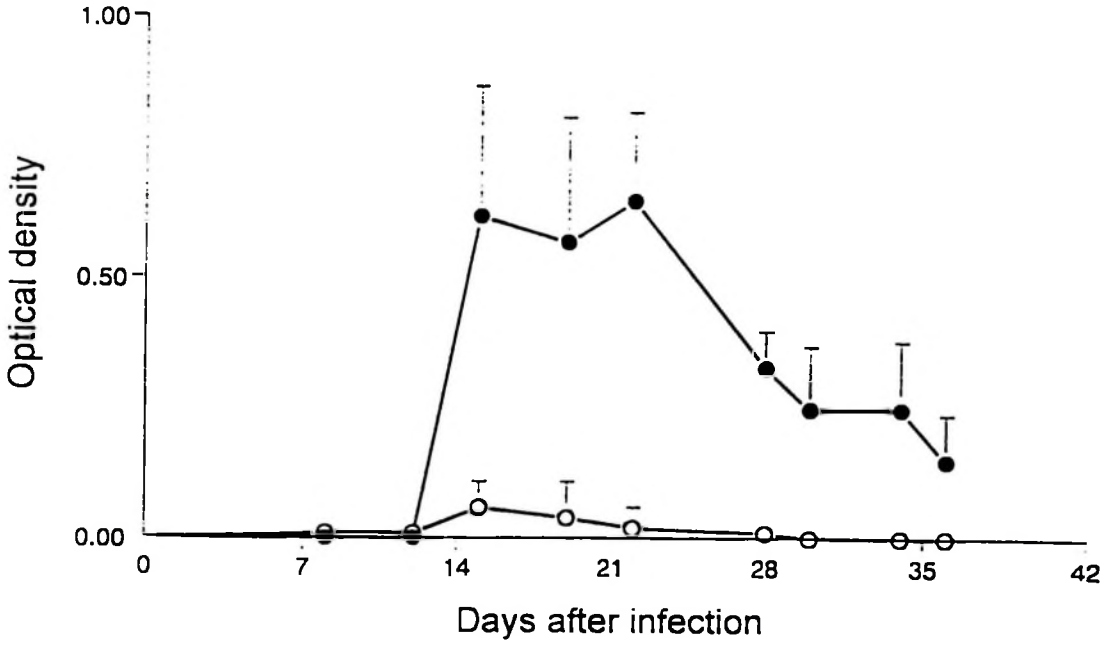

Figure 17. Effect of $CD4^+$ T cell depletion on IgM antibodies reacting with a non-trypanosome antigen, ß-galactosidase during *Trypanosoma congolense* IL 1180 infection of Boran cattle. Cattle were depleted of $CD4^+$ T cells before infection and antibody measured by ELISA. Results represents mean optical density (± standard deviation) of sera from five depleted (-o-) and five undepleted control (-•-) cattle.

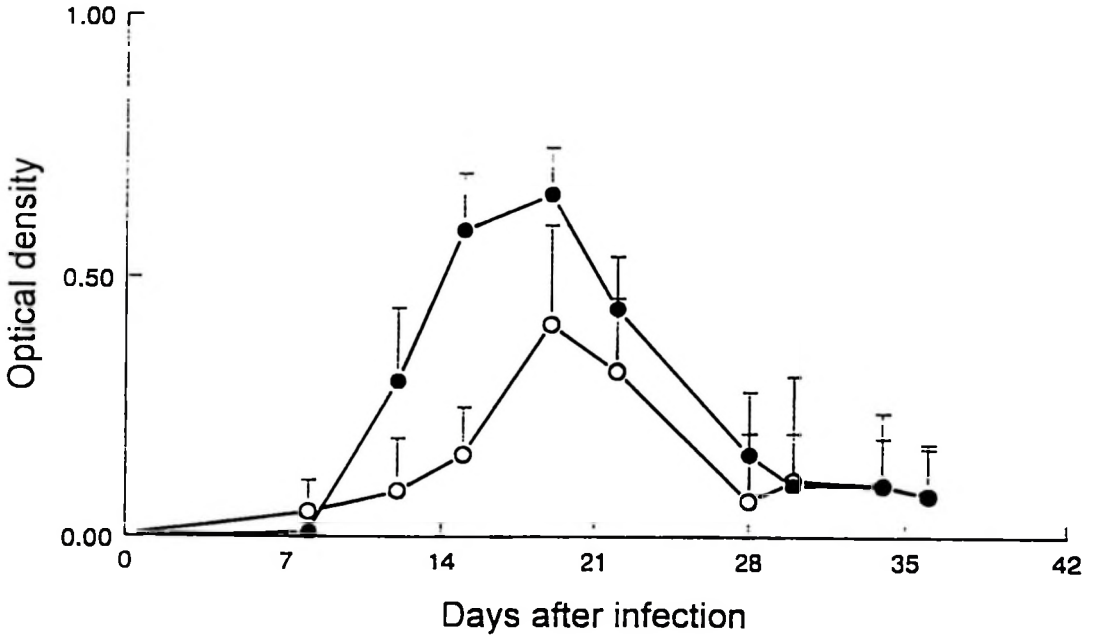

Figure 18. Effect of $CD4^+$ T cell depletion on IgM antibodies reacting with trypanosome antigens during *Trypanosoma congolense* IL 1180 infection of Boran cattle. Cattle were depleted of $CD4^+$ T cells before infection and antibody was measured in ELISA. Results represent mean optical density (± standard deviation) of sera from five depleted (-o-) and five undepleted control (-•-) cattle.

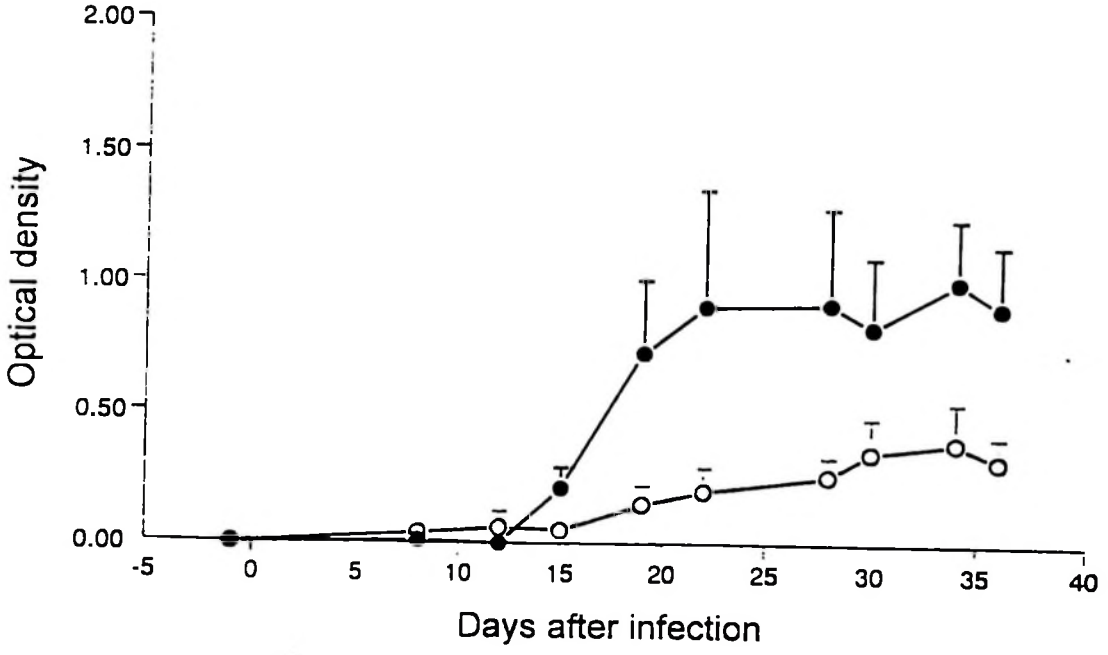

Figure 19. Effect of $CD4^+$ T cell depletion on IgG antibodies reacting with trypanosome antigens during *Trypanosoma congolense* IL 1180 infection of Boran cattle. Cattle were depleted of $CD4^+$ T cells before infection and antibody measured in ELISA. Results represent mean optical density (± standard deviation) of sera from five depleted (-o-) and five undepleted control (-•-) cattle.

4.4 Specificity of the non trypanosome-specific antibodies

This experiment was conducted to examine whether the trypanosome non-specific antibody activity detected in serum of trypanosome-infected cattle is due to polyclonal antibodies (antibody molecules with with different specificities) or polyreactive antibodies (antibodies with activity for more than one antigen). Total serum IgM was affinity purified from serum collected pre-infection and 30 dpi in Boran cattle. The 30 dpi IgM was then passed through affinity columns conjugated with a non-trypanosome antigen ß-galactosidase or whole trypanosome lysate respectively. The bound and unbound fractions were then tested in ELISA for reactivity to ß-galactosidase, cytochrome, ferritin and trypanosome lysate. The ß-galactosidase- or trypanosome lysate -unbound fractions were obtained after repeatedly passing the 30 dpi IgM pool from 6 cattle, through ß-galactosidase or trypanosome lysate affinity columns until the remaining binding activity could not be further reduced.

The reactivity of ß-galactosidase-bound and unbound IgM fractions in ELISA is shown in Fig. 20. Activity for trypanosome and non-trypanosome antigens was present in pre-infection IgM fraction but it increased after infection. The IgM fraction which bound ß-galactosidase also bound to trypanosome lysate as well as to the other non-trypanosome antigens, cytochrome and ferritin. The IgM fraction which did not bind to ß-galactosidase had reduced binding activity to ß-

galactosidase in ELISA. In addition, its activity to trypanosome lysate and to the non-trypanosome antigens, cytochrome and ferritin was also reduced.

The reactivity of ß-galactosidase-bound IgM for ß-galactosidase was expected to be higher than that of 30 dpi unfractionated IgM. However, the activity of the two fractions were similar.

The reactivity of trypanosome-bound and -unbound IgM fractions is shown in Fig. 21. Anti-trypanosome lysate activity increased in 30 dpi unfractionated IgM as compared to pre-infection IgM. Similar to ß-galactosidase-bound IgM, affinity purified anti-trypanosome antigen IgM was polyreactive, since it also reacted with both trypanosome antigen and all the non-trypanosome antigens tested. The unbound IgM fraction had reduced activity for trypanosome antigens but also for the non-trypanosome antigens. Therefore, removal of trypanosome-specific activity in the IgM fraction resulted in loss of activity to the non-trypanosome antigens.

Affinity purified anti-trypanosome lysate IgG was also tested on ELISA for reactivity to trypanosome and non-trypanosome antigens. No anti-trypanosome lysate activity was present in pre-infection IgG (Fig. 22), but was present in 30 dpi unfractionated IgG. The IgG bound to a trypanosome lysate column was about 8 times enriched in activity for trypanosome antigens in ELISA compared to unfractionated IgG from 30 dpi (Fig. 22). Unlike the affinity purified anti-

trypanosome-IgM, the anti-trypanosome IgG was very specific, since it did not recognise any of the non-trypanosome antigens tested (Fig. 22).

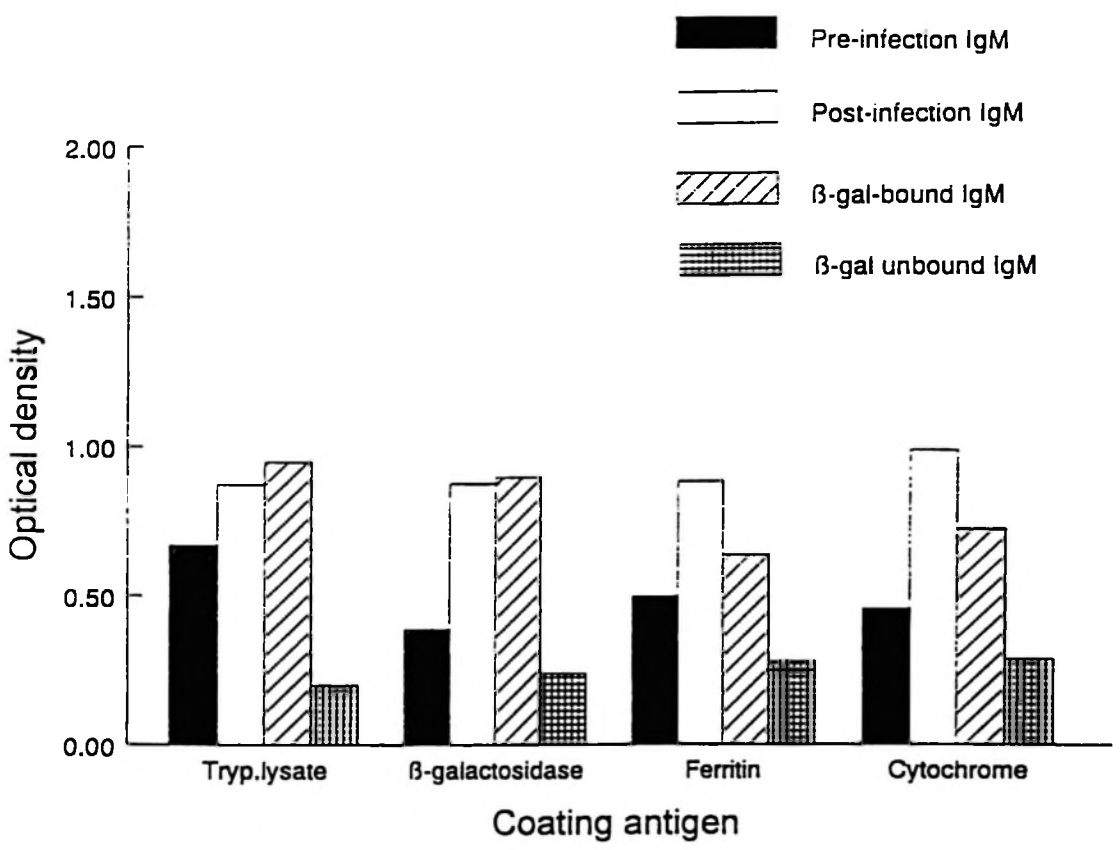

Figure 20. The reactivity of ß-galactosidase-bound and -unbound IgM fractions from *Trypanosoma congolense* IL 1180-infected Boran cattle. IgM was affinity purified from a pool of sera from 6 Boran cattle collected before (pre-infection IgM) and 30 days after infection (post-infection IgM). The ß-galactosidase-bound and -unbound IgM fractions were prepared by passage of the day 30 post infection IgM through an affinity column conjugated with a non-trypanosome antigen, ß-galactosidase. The reactivity of the various IgM fractions for trypanosome lysate, ß-galactosidase, ferritin, and cytochrome were determined in ELISA. Results represents the optical density of 10 μg/ml of each IgM fraction.

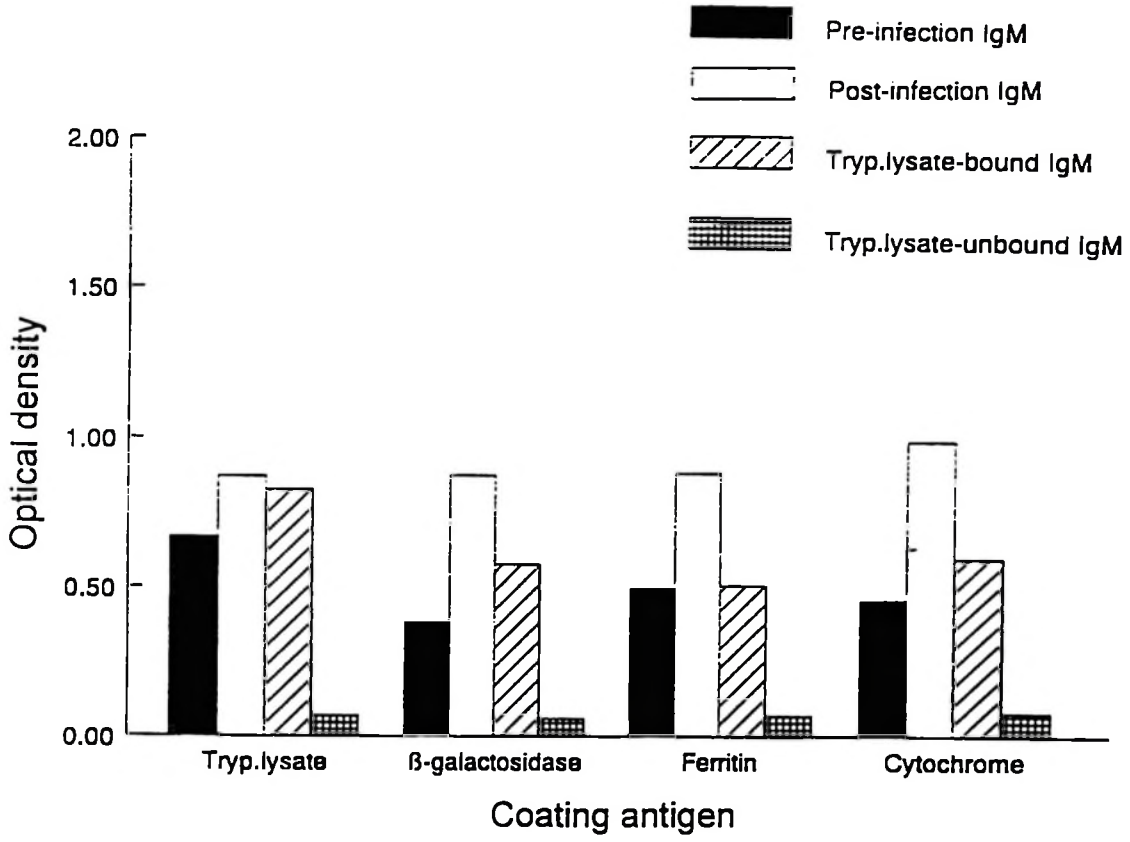

Figure 21. The reactivity of trypanosome lysate-bound and -unbound IgM fractions from *Trypanosoma congolense* IL 1180-infected Boran cattle. IgM was affinity purified from a pool of sera from 6 Boran cattle collected before (pre-infection IgM) and 30 days after infection (post-infection IgM). The trypanosome lysate-bound and -unbound IgM fractions were prepared by passage of the day 30 post infection IgM through an affinity column conjugated with trypanosome lysate. The reactivity of the various IgM fractions for trypanosome lysate, ß-galactosidase, ferritin, and cytochrome were determined in ELISA. Results represent the optical density of 10 μg/ml of each IgM fraction.

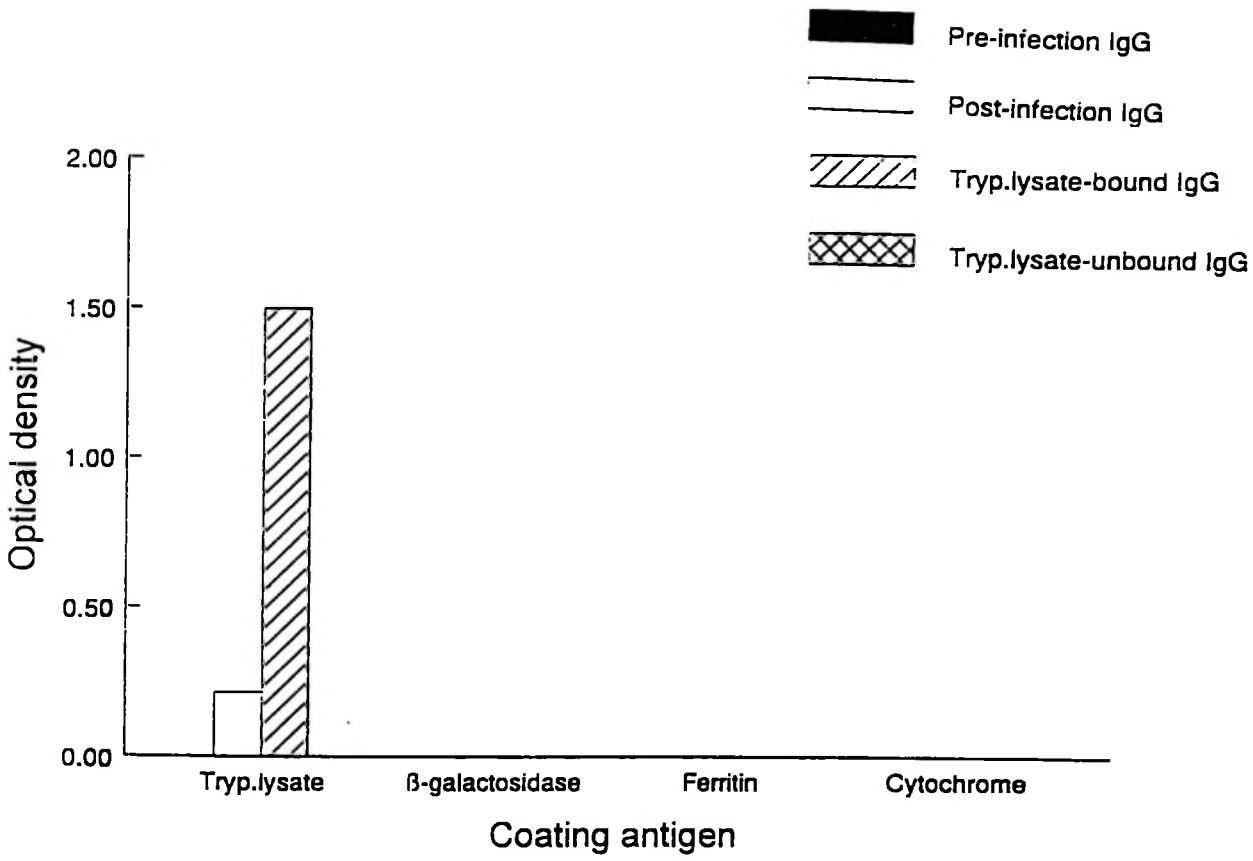

Figure 22. The reactivity of trypanosome lysate-bound and -unbound IgG fractions from *Trypanosoma congolense* IL 1180-infected Boran cattle. IgG was affinity purified from a pool of sera from 6 Boran cattle collected before (pre-infection IgG) and 30 days after infection (post-infection IgG). The trypanosome lysate-bound and -unbound IgG fractions were prepared by passage of the day 30 post infection IgG through an affinity column conjugated with trypanosome lysate. The reactivity of the various IgG fractions for trypanosome lysate, ß-galactosidase, ferritin, and cytochrome were determined in ELISA. Results represent the optical density of 10 μg/ml of each IgG fraction.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Presence of trypanosome-nonspecific antibodies and their source in *T. congolense*-infected cattle

Results from this experiment demonstrated that Boran and N'Dama cattle infected with *T. congolense* had elevated antibodies, exclusively of the IgM class, which recognized a number of non-parasite-derived antigens. The production of such trypanosome non-specific antibodies was not dependent on the trypanosome species, since antibodies reacting with a non-trypanosome antigen ß-galactosidase were also detected in cattle infected with *T. vivax*. These results extend the observation of a wide range of non-trypanosome specificities in sera from trypanosome-infected mice, monkeys and man to cattle infected with *T. congolense* and *T. vivax*. In a previous study, failure to detect antibodies binding to TNP-BSA in cattle led to the conclusion that non-parasite-specific antibodies did not develop in this species (Masake et al., 1983). Data from this experiment confirm that antibody reactivity to TNP is insignificant in sera from trypanosome-infected cattle. However, strong activity was present for other non-trypanosome antigens.

In the present study, antibodies reacting to non-trypanosome antigens were detected in both Boran and N'Dama cattle. These results contradict previous observations which indicated that trypanosome non-specific antibodies developed in Boran but not in N'Dama cattle (Williams et al., 1996). In the latter study however, only the kinetics of antibodies reacting with ß-galactosidase were studied in detail and moreover, the increased in titre in Boran cattle was not as marked as observed in this study. The discrepancy between the two results may be due to differences in sensitivity of assays used. Alternatively, the variations between the two studies may be due to differences in age of animals used; in this study, young N'Dama cattle aged six months were used in contrast to 1-3 year old N'Dama animals used by Williams et al., (1996).

There have been contrasting opinions regarding the nature of trypanosome non-specific antibodies in infected cattle. Some investigators concluded that all antibodies generated by infected cattle are specific for the infecting trypanosomes and that the apparent trypanosome non-specific reactivity observed is due to cross-reactive epitopes (Musoke et al., 1981). This view could not be corroborated in this study, since the trypanosome-specific and non-trypanosome-specific antibodies differed in two aspects. Firstly, antibodies against trypanosome antigens were of both IgM and IgG classes, while antibodies reacting with non-trypanosome antigens were exclusively of the IgM class. In this study, IgG activity to non-trypanosome antigens could not be demonstrated in serum or in spleen B cells. If

the antibody activity to the non-trypanosome antigens was due to cross-reactive epitopes shared with trypanosome antigens, reactivity against these antigens would also be expected to be present in the IgG subclass. Secondly, in Boran cattle, trypanosome-specific and trypanosome non-specific antibodies displayed different kinetics during the course of the infection. Trypanosome-specific IgM levels were increased between 14-21 dpi, and dropped thereafter while the trypanosome non-specific antibodies started increasing after 21 dpi. Antibody responses to variant (Williams et al., 1996) and to invariant trypanosome antigens (Authie et al., 1993) show similar kinetics to the trypanosome-specific antibodies in our study. These two differences, the restricted isotype and the different kinetics, strongly suggest that trypanosome-specific and non-specific antibodies are of different origins.

It is therefore likely that the two sets of antibodies are generated through different mechanism or by different B cell subpopulations. A previous study suggested that bovine $CD5^+$ B cells might be directly responsible for the high titres of IgM and for the production of auto- and trypanosome non-specific antibodies (Naessens and Williams, 1992). Immunoglobulin secreting B cells were monitored using the blot assay described by Taylor et al. (1994). Results showed that IgM-secreting cells occurred in both $CD5^+$ and CD5- B cell populations, without preference for either one. However, production of antibodies to non-trypanosome antigens was always greater in the $CD5^+$ B cell population. It is likely that these differences may even be greater, as the sorted $CD5^-$ fractions still contained some B cells expressing low

levels of membrane CD5. This bias for production of trypanosome non-specific antibodies by $CD5^+$ B cells, together with the fact that $CD5^+$ B cells constitute the largest B cell population in blood and spleen of infected cattle (Naessens and Williams, 1992), indicates that this B cell subset is the main source of IgM antibodies to non-trypanosome antigens in infected cattle.

The two characteristics of trypanosome non-specific antibodies, predominant secretion by the $CD5^+$ B cells and the exclusive presence in the IgM isotype indicates some resemblance to natural antibodies. Natural antibodies are antibodies which develop without any known antigenic stimulation, they are mainly IgM, polyreactive and are mainly secreted by the $CD5^+$ B cells in mouse and humans (Casali and Notkins, 1989; Casali and Schettino, 1996). Elevated numbers of $CD5^+$ B cells are found in patients with autoimmune diseases, cancer, AIDS, in transplant patients and in early life (Raveche, 1990; Talal et al., 1992). The same mechanism that upregulates $CD5^+$ B cells in immunodeficient states in human may be responsible for their upregulation in infected cattle (Naessens and Williams, 1992) and for the subsequent increase in trypanosome non-specific antibodies. Severe immunosuppression is characteristic of trypanosome infections (Sileghem et al., 1994).

To confirm whether the trypanosome non-specific antibodies belong to a group of polyreactive natural antibodies, further studies are required to characterise these

antibodies in terms of their specificity and the mechanisms which regulate their production in normal healthy state or during trypanosome infection in cattle.

5.2 Specificity of the trypanosome non-specific antibodies

In the first set of experiments, it was demonstrated that trypanosome non-specific antibodies increase in trypanosome-infected cattle, that they are exclusively IgM and are mainly secreted by the $CD5^+$ B cells. These characteristics indicated that they may be natural antibodies. The next set of experiments were initiated to test this hypothesis. The antigenic specificities of the trypanosome non-specific antibodies were analysed by affinity chromatography to determine whether they were composed of a mixture of antibodies with a variety of specificities, as would be expected from a polyclonal B cell stimulation, or whether they were a set of polyreactive antibodies, capable of binding with multiple antigens, as would be expected from natural antibodies.

The results from the affinity chromatography analysis supported the hypothesis that the trypanosome non-specific IgM antibodies were polyreactive, natural antibodies. First, like natural antibodies, they occurred in pre-infection sera, indicating that they were not induced by trypanosome antigen. Second, the IgM fraction eluted from immobilized ß-galactosidase also reacted with two other non-trypanosome antigens (cytochrome and ferritin) and a trypanosome lysate, suggesting that the same antibodies could bind multiple antigens. This agrees with results from a

previous study, which demonstrated that the antibody fraction which bound ß-galactosidase also reacted with trypanosome lysate (Williams et al. 1996). Similarly, IgM affinity-purified with the other non-trypanosome antigens or the trypanosome lysate could bind to ß-galactosidase and other antigens. Since the specificity of the bound fractions may have been changed by the acid-elution step, the reactivities of the non-bound fractions were also analysed. Preabsorption of the IgM fraction with ß-galactosidase, removed reactivity for cytochrome, ferritin and even trypanosome lysate. Similar results were obtained when other antigens were used for pre-absorption, suggesting that the same antibodies reacted with multiple antigens. These observations strongly indicate that the IgM antibodies which bind trypanosome and non-trypanosome antigens are basically the same, and must therefore be polyreactive. In contrast, the IgG fractions did not show evidence for polyreactivity. IgG antibodies binding the trypanosome lysate were not present in the pre-infection sera, but appeared after infection, suggesting that they were specifically induced by parasite antigens. They did not cross-react with the three non-trypanosome antigens tested (ß-galactosidase, cytochrome and ferritin), confirming that they were mono-specific.

Previous debates on the nature of IgM hypergammaglobulinaemia which develops during trypanosome infections, attributed the non-specific antibodies to polyclonal activation or to trypanosome-specific antibodies that bound cross-reactive epitopes on unrelated antigens. At that time the possibility of polyreactive antibodies was

not considered. In one study it was shown that absorption of sera from *T. brucei*-infected cattle with live trypanosomes resulted in removal of most of the IgM (Musoke et al., 1981). This led to the conclusion that most of the antibodies were specific for the infecting trypanosomes and that activity for unrelated antigens was due to anti-trypanosome antibodies which recognised cross-reactive epitopes. However, while this procedure could rule out the existence of polyclonal antibodies to non-trypanosome antigens, it could not be used to rule out the existence of polyreactive antibodies. Results from this study showed that the parasite non-specific IgM fraction was absorbed by a trypanosome lysate, and the same could have happened by absorption with a pellet of live trypanosomes.

Results from the absorption studies show that only a small percentage (5-7%) of total serum IgM was polyreactive. Similarly, the fraction of spleen cells secreting anti-ß-galactosidase IgM ranged from 0.5% to 5 % of the total of all IgM secreting cells. This suggests that the polyreactive IgM antibodies detected do not by themselves account for the IgM hypergammaglobulinaemia. As suggested by Musoke et al. (1981), most of the IgM is probably trypanosome-specific antibodies, directed against the exposed VSG-epitopes.

Affinity chromatography analysis of parasite non-specific antibodies from *T. cruzi*-infections in human, have also demonstrated the existence of polyreactive, natural antibodies (Ordonez et al. 1995). Decreased reactivity against actin, myosin,

thyroglobulin and parasite antigens was observed with all absorbed sera regardless of the absorbing antigen, similar to what was observed with sera from infected cattle.

The polyreactivity of trypanosome non-specific antibodies and the other two characteristics discussed in section 5.1 namely restriction to the IgM isotype and predominant secretion by $CD5^+$ B cells, strongly suggest that they are "natural" antibodies. Murine and human $CD5^+$ B cells have been shown to be the main source of natural antibodies. These natural antibodies are predominantly IgM and are polyreactive, binding to several antigens, including self-antigens, with low affinities (Casali and Notkins, 1989; Casali and Schettino, 1996).

It is therefore likely that the trypanosome non-specific antibodies detected in trypanosome-infected cattle are natural antibodies that are secreted at low levels by $CD5^+$ B cells in a normal healthy state. Since the repertoire of natural antibodies has been found to be species-specific (Nobrega et al., 1993), it is not surprising that the parasite non-specific antibodies in cattle and mice react with different antigens, as discussed above. Natural antibodies occur in the absence of any known antigen stimulation (Casali and Schettino, 1996), and this may explain why trypanosome non-specific antibodies are exclusively IgM. In contrast, production of trypanosome-specific antibodies is antigen-driven and can therefore be expected to undergo an isotype switch to IgG. An impairment in isotype switching has been

suggested to occur in infected Boran cattle to explain lower IgG1 antibodies to certain trypanosome antigens when compared to N'Dama (William et al., 1996). However, since trypanosome non-specific antibodies were restricted to the IgM isotype in both breeds, their occurrence can not be simply explained by the lesion in isotype switching proposed above.

Although parasite non-specific antibodies have been reported in both *T. cruzi* (Chagas' disease) and African trypanosome infections, these antibodies are associated with autoimmunity and disease only in chronic Chagas' disease (Tackle and Hudson, 1989). The biological significance of trypanosome non-specific antibodies in African trypanosomiasis remains unknown. Their functions can be speculated upon based on what is suggested to be the role of the polyreactive natural antibodies. It has been suggested that natural antibodies may act as first line of defence against viral, bacterial or parasitic infections by mediating antibody dependent phagocytosis, complement lysis or by amplifying the ongoing specific immune response (Casali and Schettino, 1996). In addition, natural antibodies possessing reactivity to the Fc portion of IgG were shown to potentiate the protective effect of *T. lewisi*-specific IgG in suckling rats and their pups (Clarkson and Mellow, 1981). Furthermore, natural antibodies were also demonstrated to potentiate antibody-dependent viral neutralisation (Casali and Notkins, 1989).

In addition, natural antibodies can also play an immunoregulatory role (Casali and Notkins, 1989; Raveche, 1990). According to one hypothesis, the anti-self and/or polyreactive antibodies produced by the $CD5^+$ population, which make up most of the "natural" antibodies found in the normal individual, are positively selected by a set of dominant auto-antigens known as the "immunological homunculus" (Cohen and Young, 1991). A stable network of low affinity autoantibodies and this set of autoantigens act as a controlling mechanism to prevent production of more dangerous high affinity anti-self antibodies following activation of $CD5^-$ B cells by cross-reactive epitopes on foreign antigens.

It is possible that trypanosome non-specific antibodies may be involved in some of the functions of natural antibodies mentioned above. The major mechanisms by which trypanosomes are removed from circulation is through antibody-mediated phagocytosis or complement lysis of opsonised trypanosomes (MacAskill et al., 1980; Dempsey and Mansfield 1983; Ngaira *et al.*, 1983; Urquhart and Holmes, 1987). Therefore, apart from amplifying the specific anti-trypanosome response, the polyreactive trypanosome non-specific antibodies may play a key role in the removal of trypanosomes early in infection before the specific response appears or before the specific response to a new VAT is induced following a change in trypanosome surface coat.

Following the destruction of trypanosomes by the immune response, a variety of antigens are released into circulation and some of them might share epitopes with self antigens. Trypanosome non-specific antibodies might play a crucial role in downregulating potential autoimmune responses induced by these antigens. Failure to do so could trigger pathogenic autoimmune responses, such as those seen in chronic *T. cruzi* infections (Cunha-Neto et al., 1995).

The IgM fractions which bound to ß-galactosidase or trypanosome antigens were found to have an unexpectedly weak activity to ß-galactosidase or trypanosome antigens respectively in ELISA. The activity of these two fractions were not greater than the unfractionated post-infection IgM. The reduced activity by these IgM fractions may have been due to denaturation following the acid elution used to remove them from the affinity columns. Loss of binding activity by the IgM fraction has been observed in other studies in which acid elution was used (Ordonez et al., 1995).

5.3 The role of $CD4^+$ and $CD8^+$ T cells

In the previous sections, arguments were presented that trypanosome non-specific antibodies belong to the natural antibodies. In these experiments the role played by $CD4^+$ and $CD8^+$ T cells in the production of trypanosome non-specific antibodies was analysed.

Two previous experiments, involving depletion of T cell subsets in trypanosome-infected cattle, had examined the role of $CD8^+$ (Sileghem and Naessens, 1995) or $CD4^+$ (Naessens and Sileghem, submitted) T cells in development of parasitaemia and anaemia. In the latter experiment, antibody response to trypanosome surface-exposed epitopes was also followed. Such antibodies are able to control parasitemia, as long as the trypanosomes have the corresponding variant antigen type. In this study, sera from cattle in $CD8^+$ and $CD4^+$ T cell depletion studies were analysed for presence of trypanosome non-specific antibodies, by monitoring binding to a bacterial enzyme, ß-galactosidase. In addition, trypanosome-specific IgM and IgG responses to a trypanosome lysate were measured. As the conformational, surface exposed epitopes, are destroyed by lysis of the trypanosome, antibodies measured in this assay will mainly recognise hidden VSG epitopes and internal antigens and are therefore not associated with parasite destruction and control of parasitaemia. However, they may play a role in protection against pathological parasite products (Murray and Dexter, 1988; Authie, 1994), or play a role in pathogenesis through immune complex formation (Murray and Dexter, 1988; Mansfield et al., 1990).

A previous study had shown that depletion of $CD8^+$ T cells did not have an effect on anaemia and parasitaemia in trypanosome-infected cattle (Sileghem and Naessens, 1995). Analysis of serum antibody from these animals shows that $CD8^+$

T cells had no effect on antibody responses, suggesting that they play no role in the production of the different types of antibodies. However, in murine *T. cruzi* infections, it has been suggested that $CD8^+$ T cells are suppressive for T-cell-independent antibody responses (Minoprio et al., 1987).

Results from the present studies have revealed some basic differences between cattle and mice with respect to the role of T-cells on antibody responses. In mice, the trypanosome non-specific antibodies are produced in athymic (nu/nu) mice indicating that this response is independent of T cells (Hudson et al., 1976; Kobayakawa et al., 1979). In contrast, the present study showed that the non-specific antibodies were completely eliminated in $CD4^+$ T cells-depleted cattle, indicating that $CD4^+$ T cells are required in this response. In addition, athymic mice produce an antibody response to the exposed parasite antigens, which mimic the T-independent antigens, while thymic sufficient mice respond to both the exposed parasite antigens and the epitopes present on disintegrated VSG and invariant antigens (Mansfield, 1994). Results reported here from cattle show that antibodies to disintegrated trypanosomes were suppressed in CD4 depleted cattle. Furthermore, in another $CD4^+$ T cell depletion experiment in cattle, antibodies to exposed parasite antigens were also reduced (Naessens and Sileghem, submitted). These results indicate that in cattle infected with trypanosomes, the role of the T-independent antibody response may be minor compared to the T-dependent one. The differences observed between the two species may be due to the marked

differences in parasitaemia and the way antigens are presented to the immune system. Due to much higher parasitaemias in mice than cattle (10^8 compared to 10^5 per ml of blood (Murray et al., 1982), the contribution of T cell-independent activation of B cells may be more important in mice than in cattle.

Because trypanosome non-specific antibodies develop by a T-cell independent mechanism in mice, it has been suggested that they are directly generated through polyclonal activation of B cells by a trypanosome mitogen-like molecule (Hudson et al., 1976; Kobayakawa et al., 1979). However, as the non-specific antibodies in cattle are T cell dependent, the direct role for a parasite mitogen seems unlikely. In the previous section (5.2), it was demonstrated that the trypanosome-non specific antibodies were present before trypanosome infection. Therefore, their increase during infection is due to $CD4^+$ T cell-mediated enhancement of their secretion. It has been demonstrated in other diseases that $CD4^+$ T cells may induce production of non-specific antibodies through mechanisms other than the conventional antigen-specific helper function. Anti-$CD4^+$ T cell monoclonal antibody treatment in *T. cruzi* (Minoprio et al., 1987), adenovirus and LDV (Coutelier et al., 1990) or cytomegalovirus (Price et al., 1993) infections of mice resulted in elimination of non-specific antibodies. It was suggested that $CD4^+$ T cells induced production of the non-specific antibodies through cytokine activity such as IFN-γ. In addition, it has been shown that HIV-infected human $CD4^+$ T cells expressing membrane TNF-α can induce polyclonal B cell activation through

a non-cognate, contact dependent mechanism (Macchia et al., 1993). Similar mechanisms may operate in the production of trypanosome non-specific antibodies in cattle. $CD4^+$ T cells activated by the trypanosome infection may enhance the secretion of the trypanosome non-specific antibodies by B cells present in their microenvironment through their cytokines or contact dependent mechanisms.

However, trypanosome non-specific antibodies demonstrated to date in cattle consistently bind other types of antigens such as ß-galactosidase and ferritin and not others such as TNP. It is therefore possible that there might exist a mechanism which selectively activate $CD5^+$ B cells secreting ß-galactosidase or ferritin. One possible mechanism might be the presence of a B cell superantigen originating from the infecting trypanosomes. B cell superantigens are a special category of B cells such as Staphylococcus aureus protein A and HIV gp 120 (Rosen, 1997). These antigens activate B cells by interacting with a receptor located on the immunoglobulin variable region, but outside the antigen binding groove. B cells secreting ß-galactosidase and ferritin may possess a common trypanosome superantigen binding site and with $CD4^+$ T cell help can be activated to secrete antibodies with corresponding specificities.

If antibodies were involved in pathogenesis of disease associated with trypanosome infections, their severe reduction in CD4-T cell depleted cattle would have had an influence on the severity of disease. However, despite the almost complete

elimination of trypanosome non-specific antibodies in $CD4^+$ T cell depleted cattle, anaemia was not exacerbated (Naessens and Sileghem, in preparation). This suggests that trypanosome non-specific antibodies may not have a direct role in pathogenesis, as suggested earlier (Kobayakawa et al., 1979; Murray and Dexter, 1988).

It has been suggested that the specific anti-trypanosome antibodies may be involved in the immunopathological processes which leads to the pathogenesis of anaemia of African trypanosomiasis (Murray and Dexter, 1988). One hypothesis suggested that erythrocytes coated with soluble VSG released from dying trypanosomes, may adsorb trypanosome-specific antibody and complement, leading to their elimination by the cells of the mononuclear phagocytic system through their Fc or complement receptors. Erythrocyte destruction may also be mediated by autoantibodies or deposition of immune complexes on their surface. However, despite a significant reduction in trypanosome specific IgG in the $CD4^+$ T cell-depleted cattle observed in this experiment, anaemia was not reduced (Naessens and Sileghem, in preparation). These observations suggest that in cattle, the development of anaemia is not directly associated with an antibody mediated destruction of erythrocytes, but may be directly caused by the parasites or their products. Another possible mechanism may be the erythrocyte destruction through complement activation in the absence of antibody (Murray and Dexter, 1988).

It has also been suggested that IgG responses directed to some invariant parasite antigens may play a protective role by neutralising their detrimental effects (Authie, 1994). Results from this study do not confirm this hypothesis since suppression of antibody responses observed in this experiment and a previous one (Buza et al., in preparation) did not result in exacerbation of anaemia or the course of the disease.

5.4. Conclusions

This study has shown that;

1. *Trypanosoma congolense* or *T. vivax* infections in cattle cause an increase in trypanosome non-specific antibodies which react with various proteins including ß-galactosidase, cytochrome and ferritin.

2. Trypanosome non-specific antibodies are present in pre-infection sera, indicating that they are not induced by trypanosome antigen.

3. The trypanosome non-specific antibodies are exclusively IgM, polyreactive, and mainly secreted by the $CD5^+$ B cells. These features strongly suggest that they are natural antibodies

4. The increase in trypanosome non-specific antibodies during trypanosome infection depends on the presence of $CD4^+$ but not $CD8^+$T cells.

5. Elimination of trypanosome non-specific antibodies during *T. congolense* infection did not result in exacerbation of anaemia or the course of disease suggesting that they are not directly involved in pathogenesis of disease.

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APPENDIX

Appendix 1. Reactivity of serum IgM from *T. congolense*-infected cattle to various non-trypanosome antigens. Values represents optical density obtained for each sera.

IgM anti-cytochrome in Boran cattle

DPI	BM241	BM322	BM326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.08	0	0	0	0	0	0.01	0.03
7	0.14	0.07	0	0.01	0.01	0.06	0.05	0.06
14	0.31	0.05	0.3	0	0.04	0.1	0.13	0.14
21	0.53	0.57	0.26	0.42	0.31	0.19	0.38	0.15
31	0.54	0	0.58	0.55	0.75	0.21	0.44	0.28
35	0.36	0	0.46	0.95	0.29	0.17	0.37	0.33

IgM anti-cytochrome in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.14	0.01	0	0	0.05	0.01	0.04	0.05
7	0.1	0.11	0.05	0	0	0	0.04	0.05
14	0.32	0.21	0.08	0.17	0.02	0.09	0.15	0.11
21	0.46	0.18	0	0.34	0.02	0.15	0.19	0.18
31	0.54	0.16	0	0.48	0.23	0.04	0.24	0.22
35	0	0.13	0	0.09	0	0.07	0.05	0.06

IgM anti-ferritin in Boran cattle

DPI	BK214	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.7	0	0	0.04	0	0	0.12	0.28
7	0.52	0	0.1	0	0.08	0	0.12	0.2
14	0.68	0.31	1.06	0	0.38	0.04	0.41	0.4
21	0.88	1.09	0.21	1.01	0.28	1.16	0.77	0.42
31	0.52	1.11	0.67	0.47	1.35	0.84	0.83	0.35
35	0.5	1.14	0	0.68	0.18	0.03	0.42	0.44

IgM anti-ferritin in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.44	0	0.25	0.28	0	0.05	0.17	0.18
7	0.21	0	0.17	0.15	0	0.16	0.11	0.09
14	0.24	0.91	0.3	0.51	0.63	0.18	0.46	0.28
21	0.61	0.72	0	1.14	1.07	0.45	0.66	0.42
31	0.16	1	0.55	1.57	0.01	0.11	0.57	0.61
35	0	0.73	0.98	1	0	0.09	0.47	0.49

IgM anti-aldolase in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0.17	0.03	0.07
7	0	0	0	0	0	0	0	0
14	0.07	0	0.06	0.22	0.03	0	0.06	0.08
21	0	0.23	0	0.47	0.04	0.06	0.13	0.19
31	0	0.09	0	0.26	0.57	0	0.15	0.23
35	0	0	0	1.25	0.01	0	0.21	0.51

IgM anti-aldolase in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.06	0	0.18	0	0.03	0	0.04	0.07
7	0.04	0.1	0.03	0	0.4	0	0.09	0.15
14	0.29	0.1	0.07	0.14	0	0	0.1	0.11
21	0.2	0.06	0.19	0.18	0	0.17	0.13	0.08
31	0.21	0.17	0.09	0.05	0.06	0	0.1	0.08
35	0.25	0.45	0.07	0.17	0	0	0.16	0.17

IgM anti-ovalbumin in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0.02	0	0.08	0.03	0.24	0.06	0.09
7	0	0	0	0.04	0.05	0.14	0.04	0.05
14	0	0	0	0.5	0	0.13	0.1	0.2
21	0	0.37	0.09	0.49	0	0.41	0.23	0.22
31	0	0.22	0	0.75	0	0.14	0.18	0.29

35	0	0.1	0	0.89	0.04	0.06	0.18	0.35

IgM anti-ovalbumin in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0.04	0.01	0.01
7	0.02	0	0.24	0.02	0.11	0	0.06	0.1
14	0	0	0.15	0.48	0.02	0.06	0.12	0.19
21	0.22	0.12	0.47	0.3	0.22	0	0.22	0.16
31	0.14	0.21	0	0.26	0.22	0.05	0.14	0.1
35	0.22	0.03	0.29	0	0	0	0.09	0.13

IgM anti-KLH in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0.02	0.03	0	0.01	0	0.01	0.01
7	0	0	0	0.03	0	0	0.01	0.01
14	0	0	0.13	0	0.05	0	0.03	0.05
21	0	0.28	0	0.1	0.14	0	0.09	0.11
31	0.09	0	0.29	0.19	0.49	0	0.18	0.19
35	0	0	0.17	0.6	0.12	0	0.15	0.23

IgM anti-KLH in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.12	0	0.01	0.01	0.06	0	0.03	0.05
7	0.12	0	0.02	0	0.09	0.01	0.04	0.05
14	0.2	0	0.09	0	0	0.01	0.05	0.08
21	0.24	0	0	0.04	0	0.02	0.05	0.09
31	0.43	0	0	0.08	0.03	0	0.09	0.17
35	0.09	0	0	0	0	0.01	0.02	0.03

IgM anti-myoglobin in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0.1	0.02	0.04
7	0	0.01	0	0.04	0.01	0.06	0.02	0.02
14	0	0.02	0	0.16	0.01	0.05	0.04	0.06
21	0	0.21	0	0.39	0.03	0.1	0.12	0.16

31	0	0.09	0	0.48	0.19	0.06	0.14	0.18
35	0	0.05	0	0.53	0.06	0	0.11	0.21

IgM anti-myoglobin in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0.02	0	0	0	0.01
0	0	0	0.07	0	0.02	0.03	0.02	0.03
7	0	0	0.02	0	0.17	0	0.03	0.07
14	0.07	0	0.11	0.08	0.09	0	0.06	0.05
21	0.08	0	0.17	0.24	0.1	0.06	0.11	0.08
31	0.11	0	0	0.05	0.06	0.01	0.04	0.04
35	0.28	0	0.11	0.06	0	0.01	0.08	0.11

IgM anti-thyroglobulin in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0.01	0	0	0	0	0	0
7	0.05	0.02	0.02	0	0	0.06	0.03	0.03
14	0.08	0.02	0.18	0	0	0.06	0.06	0.07
21	0.22	0.33	0.08	0.16	0.15	0.1	0.17	0.09
31	0.25	0	0.22	0.49	0.45	0.15	0.26	0.19
35	0.13	0	0.16	0.78	0.03	0.11	0.2	0.29

IgM anti-thyroglobulin in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.06	0.04	0	0.04	0.02	0.01	0.03	0.02
7	0.05	0.06	0	0.01	0	0	0.02	0.03
14	0.16	0.12	0.04	0.12	0	0.01	0.07	0.07
21	0.17	0.15	0	0.07	0.02	0.02	0.07	0.07
31	0.27	0.06	0	0.12	0.02	0	0.08	0.1
35	0	0.02	0.03	0	0	0.03	0.01	0.01

IgM anti-lysozyme in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0.01	0	0	0	0	0	0
7	0	0.01	0	0	0.06	0.13	0.03	0.05
14	0	0	0.09	0.04	0.04	0.03	0.03	0.03

21	0.03	0.14	0.08	0.36	0.18	0.05	0.14	0.12
31	0.18	0.03	0.02	0.56	0.28	0.07	0.19	0.21
35	0	0.02	0.02	0.75	0.13	0.06	0.16	0.29

IgM anti-lysozyme in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.02	0.06	0.04	0	0	0	0.02	0.03
7	0.01	0.06	0.04	0	0.11	0.02	0.04	0.04
14	0.03	0.09	0	0.05	0.06	0	0.04	0.03
21	0.13	0.11	0.05	0.04	0.13	0	0.08	0.05
31	0.11	0.15	0.05	0	0.11	0	0.07	0.06
35	0.07	0.16	0.03	0.01	0	0	0.04	0.06

IgM anti-ß-galactosidase in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.27	0.05	0	0	0.05	0	0.06	0.1
7	0.16	0	0	0	0	0	0.03	0.07
14	0.35	0.1	0.18	0.29	0	0	0.15	0.15
21	0.48	0.9	0	1.13	0.57	0.54	0.6	0.39
31	0.5	0	0	1.1	1.36	0.86	0.64	0.57
35	0.54	0.33	0.04	1.08	0.35	0.49	0.47	0.35

IgM anti-ß-galactosidase in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.16	0	0.08	0.11	0.02	0	0.06	0.07
7	0	0	0.02	0.22	0	0	0.04	0.09
14	0.22	0.54	0	0.5	0.1	0	0.23	0.24
21	1.02	0.79	0.87	0.69	1.09	0.53	0.83	0.21
31	0.47	0.46	0.77	0.85	0.71	0.46	0.62	0.18
35	0.59	0.32	0.5	0.18	0	0.49	0.35	0.22

IgM anti-ssDNA in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0.11	0	0	0	0	0.02	0.04
7	0	0.22	0.02	0.06	0	0	0.05	0.09

14	0.01	0.2	0.12	0.08	0	0	0.07	0.08
21	0.12	0.37	0	0.19	0	0	0.11	0.15
31	0.12	0.1	0.05	0.31	0.23	0	0.13	0.11
35	0	0	0	0.62	0	0	0.1	0.25

IgM anti-ssDNA in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.06	0	0.06	0	0.13	0	0.04	0.05
7	0.11	0	0.08	0	0.14	0	0.05	0.06
14	0.1	0	0	0.23	0.11	0	0.07	0.09
21	0.21	0.01	0	0.07	0	0	0.05	0.08
31	0.18	0	0	0	0	0	0.03	0.08
35	0.09	0	0	0.06	0	0	0.03	0.04

IgM anti-TNP in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
7	0	0	0	0.01	0	0	0	0
14	0	0	0	0.01	0	0	0	0
21	0	0.09	0	0.19	0.04	0	0.05	0.07
31	0	0	0	0.19	0.25	0	0.07	0.12
35	0	0	0	0.4	0	0	0.07	0.16

IgM anti-TNP in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
7	0.02	0	0	0	0.05	0	0.01	0.02
14	0.07	0	0	0.03	0.04	0	0.02	0.03
21	0.1	0	0.03	0.03	0	0	0.03	0.04
31	0.13	0	0	0.04	0	0	0.03	0.05
35	0.04	0	0	0	0	0	0.01	0.01

Appendix 2. Reactivity of serum IgG from *T. congolense*-infected cattle to various non-trypanosome antigens

IgG anti-BSA in Boran cattle

DPI	BK241	BL326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0
0	0.15	0	0	0	0.07	0.08	0.11
7	0	0	0	0	0	0	0
14	0	0	0.07	0	0	0.01	0.03
21	0	0	0.52	0.38	0	0.15	0.24
31	0	0	0.63	0	0	0.1	0.26
35	0	0	0.75	0	0.11	0.14	0.3

IgG anti-BSA in N'Dama cattle

DPI	ND60	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0
0	0	0	0	0	0.02	0	0.01
7	0	0	0.01	0	0	0	0
14	0.09	0	0.23	0	0	0.09	0.11
21	0	0	0	0	0	0	0
31	0	0.02	0	0	0	0	0.01
35	0	0	0	0	0	0	0

IgG anti-ß-galactosidase in Boran cattle

DPI	BK214	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0
0	0.07	0.02	0	0	0.11	0.03	0.05
7	0.04	0	0	0	0	0.01	0.02
14	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0

IgG anti-ß-galactosidase in N'Dama cattle

DPI	ND60	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0

0	0.14	0.01	0	0	0.04	0.03	0.05
7	0.1	0.07	0	0	0	0.03	0.04
14	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0

IgG anti-cytochrome in Boran cattle

	BK241	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0
0	0.02	0	0	0	0	0	0.01
7	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
31	0.04	0	0	0	0	0.01	0.02
35	0.27	0	0	0	0	0.06	0.11

IgM anti-cytochrome in N'Dama cattle

	ND60	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0
14	0	0	0.09	0.02	0.01	0.02	0.04
21	0	0	0	0	0	0	0
31	0	0	0.25	0.02	0	0.04	0.1
35	0	0.09	0.19	0	0	0.05	0.08

IgG anti-ferritin in Boran cattle

	BK241	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0
0	0.03	0	0	0	0.05	0.04	0.05
7	0	0	0	0	0	0.01	0.03
14	0	0	0	0	0	0.01	0.02
21	0	0	0	0	0	0	0
31	0	0.01	0	0	0	0.02	0.05
35	0	0.16	0.07	0	0	0.09	0.13

IgG anti-ferritin in N'Dama cattle

	ND60	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0

Appendix 3. Reactivity of sera from *T. congolense*-infected cattle to whole trypanosome lysate.

IgM anti-whole trypanosome lysate in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.21	0	0	0	0	0.03	0.04	0.09
7	0.17	0.04	0	0	0	0	0.04	0.07
	0.89	0.35	0.38	0.55	0.14	0.15	0.41	0.28
	0.48	0.56	0.06	0.71	0.83	0.43	0.51	0.27
31	0.2	0.08	0	0.49	0.07	0.16	0.16	0.17
	0.04	0.13	0.07	0.51	0.08	0.01	0.14	0.18

IgM anti-whole trypanosome lysate in N'Dama cattle

	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.12	0	0	0	0	0	0.02	0.05
7	0.15	0	0.07	0.08	0	0.52	0.13	0.19
14	0.54	0.6	0.54	0.69	0	0.49	0.48	0.24
21	0.82	0.5	0.64	0.73	0.36	0.3	0.56	0.21
31	0.36	0.29	0.64	0.62	0	0.15	0.34	0.25
35	0.3	0.08	0.33	0.01	0	0.01	0.12	0.15

IgG anti-whole trypanosome lysate in Boran cattle

DPI	BK241	BK322	BK326	BL37	BL209	BM1	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.12	0	0	0	0	0.01	0.02	0.05
7	0.12	0.01	0	0	0	0	0.02	0.05
14	0.33	0.09	0.03	0.38	0	0.05	0.15	0.16
21	1.21	0.52	1.08	0.48	0.5	1.21	0.84	0.37
28	1.05	0.33	0	0.77	0.6	0.5	0.54	0.36
35	1.02	0.29	0.05	0.05	0.45	1.06	0.49	0.45

IgG anti-whole trypanosome lysate in N'Dama cattle

DPI	ND60	ND66	ND72	ND73	ND74	ND75	Mean	STD
-3	0	0	0	0	0	0	0	0
0	0.07	0	0	0	0	0	0.01	0.03
7	0.07	0	0.01	0.2	0.02	0.2	0.08	0.09

14	0.18	0.56	0.67	0.83	0.03	0.19	0.41	0.32
21	1.32	1.39	0.73	1.45	1.3	1.22	1.23	0.26
28	1.36	1.38	1.49	1.46	1.17	0.85	1.29	0.24
35	1.14	1.43	1.5	1.48	0.64	0.64	1.14	0.41

Appendix 4. Effect of $CD8^+$ T cell depletion on antibody responses during *T. congolense* infection of cattle.

IgM anti ß-galactosidase in depleted Boran cattle

DPI	BM218	BM219	BM224	BM225	Mean	STD
-14	0	0.07	0.02	0.09	0.05	0.04
-1	0	0	0	0	0	0
8	0.34	0.24	0.31	0.28	0.29	0.04
14	0.3	0.21	0.34	0.34	0.3	0.06
22	0.4	0.37	0.38	0.84	0.5	0.23
29	0.85	0.8	0.87	0.76	0.82	0.05
36	0.25	0.26	0.1	0.33	0.24	0.1

IgM anti-ß-galactosidase in control non-depleted Boran cattle

DPI	BM220	BM221	BM222	BM223	Mean	STD
-14	0	0	0.06	0	0.02	0.03
-1	0.04	0	0	0.02	0.02	0.02
8	0.14	0.39	0.14	0.08	0.19	0.14
14	0.29	0.3	0.32	0.35	0.32	0.03
22	0.3	0.3	0.51	0.41	0.38	0.1
29	0.67	0.77	0.92	0.86	0.81	0.11
36	0.12	0.15	0.2	0	0.12	0.09

IgM anti-whole trypanosome lysate in depleted Boran cattle

DPI	BM218	BM219	BM224	BM225	Mean	STD
-14	0.03	0	0	0.08	0.03	0.04
-1	0	0	0.02	0	0	0.01
8	0.21	0.14	0.24	0.1	0.17	0.07
14	0.33	0.64	0.33	0.3	0.4	0.16
22	0.2	0.64	0.51	0.71	0.51	0.22
29	0	0.42	0.21	0.53	0.29	0.23
36	0	0.46	0.06	0.17	0.17	0.2

IgM anti-whole trypanosome lysate in control non-depleted Boran cattle

DPI	BM220	BM221	BM222	BM223	Mean	STD
-14	0	0	0.01	0	0	0
-1	0.02	0.32	0	0.05	0.1	0.15
8	0.07	0.22	0.06	0.02	0.09	0.09

14	0.41	0.48	0.29	0.27	0.36	0.1
22	0.06	0.99	0.38	0.78	0.55	0.42
29	0.01	0.97	0.53	0.62	0.53	0.4
36	0.1	0.18	0.08	0.21	0.14	0.06

IgG anti-whole trypanosome lysate in depleted Boran cattle

DPI	BM218	BM219	BM224	BM225	Mean	STD
-14	0	0.06	0	0.05	0.03	0.03
-1	0	0.01	0.02	0	0.01	0.01
8	0	0.04	0.03	0.05	0.03	0.02
14	0.07	0.17	0.18	0.17	0.15	0.05
22	0.55	1.24	0.56	0.62	0.74	0.33
29	0.69	1.63	0.77	0.59	0.92	0.48
36	0.69	0.92	1.45	0.38	0.86	0.45

IgG anti-whole trypanosome lysate in control non-depleted Boran cattle

DPI	BM220	BM221	BM222	BM223	Mean	STD
-14	0.03	0.02	0	0.02	0.02	0.01
-1	0	0	0	0.03	0.01	0.02
8	0	0.04	0.04	0	0.02	0.02
14	0.05	0.33	0.08	0.16	0.15	0.12
22	0.18	1.2	0.68	0.87	0.73	0.43
29	0.17	1.67	0.67	1.2	0.93	0.65
36	0.33	1.16	0.34	0.65	0.62	0.39

Appendix 5. Effect of CD4 T cell depletion on antibody responses during *T. congolense* infection of cattle.

IgM anti-ß-galactosidase in undepleted control cattle

DPI	BN14	BN16	BN18	BN19	BN20
-1	0	0	0	0	0
8	0	0	0	0	0
12	0	0	0	0	0
15	0.95	0.5	0.28	0.76	0.62
19	0.97	0.55	0.35	0.4	0.57
22	0.88	0.6	0.42	0.69	0.65
28	0.41	0.34	0.22	0.33	0.33
30	0.34	0.33	0.3	0.04	0.25
34	0.26	0.24	0.44	0.07	0.25
36	0.12	0.15	0.29	0.05	0.15

IgM anti-ß-galactosidase in depleted cattle

DPI	BN21	BN22	BN23	BN24	BN26
-1	0	0	0	0	0
8	0.01	0	0.05	0.01	0
12	0.01	0	0.05	0	0
15	0.02	0.03	0.13	0.08	0.09
19	0.02	0.02	0	0.19	0.02
22	0.01	0.01	0	0	0.1
28	0.03	0.01	0	0	0
30	0.01	0	0	0	0
34	0.01	0	0	0	0
36	0	0	0	0	0

IgM anti-whole trypanosome lysate control undepleted cattle

DPI	BN14	BN16	BN18	BN19	BN20
-1	0	0	0	0	0
8	0	0.02	0	0.02	0.02
12	0.4	0.22	0.13	0.28	0.49
15	0.63	0.49	0.49	0.58	0.74
19	0.62	0.54	0.71	0.69	0.75
22	0.41	0.32	0.45	0.59	0.46
28	0.07	0	0.24	0.21	0.29
30	0	0	0.2	0.11	0.21
34	0.03	0.02	0.19	0.05	0.2

36	0	0	0.19	0.06	0.16

IgM anti-whole trypanosome lysate in depleted cattle

DPI	BN21	BN22	BN23	BN25	BN26
-1	0	0	0	0	0
8	0.14	0	0.03	0.07	0
12	0.24	0	0.06	0.14	0
15	0.3	0.14	0.08	0.09	0.18
19	0.61	0.43	0.25	0.6	0.19
22	0.54	0.27	0.21	0.22	0.37
28	0.3	0.07	0	0	0
30	0.46	0.1	0	0	0
34	0.34	0.11	0	0	0.05
36	0.25	0.09	0	0	0.04

IgG anti-whole trypanosome lysate in control undepleted cattle

DPI	BN14	BN16	BN18	BN19	BN20
-1	0	0	0	0	0
8	0.02	0	0	0.02	0
12	0	0.01	0	0	0
15	0.2	0.31	0.14	0.27	0.11
19	1.09	0.84	0.47	0.85	0.42
22	1.53	0.99	0.34	1.04	0.67
28	1.52	0.76	0.61	1.02	0.67
30	1.24	0.78	0.64	0.93	0.56
34	1.35	1.08	0.8	1.07	0.76
36	1.26	0.96	0.78	0.93	0.63

IgM anti-whole trypanosome lysate in depleted cattle

DPI	BN21	BN22	BN23	BN25	BN26
-1	0	0	0	0	0
8	0.04	0	0.01	0.09	0.05
12	0.06	0	0.03	0.15	0.07
15	0.08	0	0.04	0.08	0.07
19	0.12	0.04	0.16	0.23	0.19
22	0.22	0.07	0.17	0.29	0.27
28	0.29	0.16	0.23	0.32	0.29
30	0.59	0.28	0.33	0.29	0.27
34	0.63	0.28	0.39	0.4	0.25
36	0.46	0.23	0.31	0.35	0.25

Appendix 6. Buffers

Alsevers solution

Citric Acid	0.55 Grams/L
Dextrose	20.50 Grams/L
Sodium Chloride	4.2 Grams/L
Sodium Citrate	8.0 Grams/L

Tris ammonium chloride

A. Tris hydroxymethyl aminomethane 20.60 Grams/L (0.17 Molar)
B. Ammonium Chloride 8.30 Grams/L (0.16 Molar)
Containing a mixture of solution A with solution B in the ratio 1:9. pH to 7.2.

Dulbecco's phosphate buffered saline

Na2 HPO4 Anhydrous	1.15 Grams/L
KH2 PO4	0.20 Grams/L
Na Cl	8.0 Grams/L
K Cl	0.20 Grams/L
Mg Cl2(H2O)6	0.10 Grams/L
Ca Cl2(H2O)2	0.13 Grams/L

pH between 7.2 and 7.4.

Phosphate saline glucose

RAT

Na2 HPO4	8.088 Grams/L
Na H2PO4	0.359 Grams/L
Na Cl	2.5 Grams/L
Glucose	10.0 Grams/L
Ionic Strength	0.217