DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES (MCABS) TO *TRYPANOSOMA (TRYPANOZOON) BRUCEI EVANSI* AND THEIR APPLICATION AS IMMUNODIAGNOSTIC REAGENTS

BY

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DECLARATION

(a) This Thesis is my original work and has not been presented for a degree in any other University

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(b) This Thesis has been submitted for examination with my approval as a University Supervisor

Professor W.K. Munyua, Ph.D.

(c) This Thesis has been submitted for examination with my approval as a supervisor

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DEDICATION:

This Thesis is dedicated to my late

Father Philip Mukani.

He was more than a father to me

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SUMMARY

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES (MCABS) TO *TRYPANOSOMA (TRYPANOZOON) BRUCEI EVANSI* AND THEIR APPLICATIONS AS IMMUNODIAGNOSTIC REAGENTS.

Parasitological techniques employed in the diagnosis of patent trypanosome infection often fail to detect animals with low or cryptic infection. Yet, serological tests lack precision and accuracy in detecting patent infection due to the presence of antibodies in circulation for a long time. So, there arises a need for a better test for the diagnosis of patent trypanosome infection in the field.

In an effort to improve on the diagnosis of infection with *T.(T).b. evansi*, murine McAbs were developed against antigens of the bloodstream forms of *T.(T).b.evansi*, KETRI 1342 and used as antigen detecting probes for diagnosis of patent *T.(T).b.evansi* infection in experimental goats and field camels. The murine McAbs were produced by immunizing Balb/c mice with plasma antigen I and membrane antigen II preparation from bloodstream forms of *T.(T).b. evansi* KETRI 1342. Later, spleen cells from the immunized Balb/c mice were harvested and fused with NS-I myeloma cells. A total of 69 hybridomas secreting McAbs to the above antigens were raised. The hybridomas were cloned and where applicable, recloned by limiting dilution. The classes and subclasses of McAbs secreted by hybridoma reclones were determined by the double immunodiffusion and immunoelectrophoresis techniques and their stability to freezing and thawing, and salt precipitation investigated.

Twenty two hybrid reclones were selected and inoculated intraperitoneally into pristane-primed Balb/c mice so that

antibody rich ascites could be produced from each hybrid reclone. Mouse IgM class McAbs were purified from the antibody rich ascites by gel filtration on Sepharose 6 column. Mouse IgG-rich fractions were obtained by ammonium sulphate precipitation.

In an indirect ELISA and/or IFAT, the McAbs showed a wide range of cross-reaction with antigens of other mammalian African trypanosome spp, but no cross reaction was observed with *Theileria parva, Babesia bigemina, Anaplasma marginale, Plasmodium falciparum* or *Leishmania donovani* lysate antigens. Two McAbs TE M5/17.4.6. and TE M3/12.3.6, showed high specificity for KETRI 1342 stock but no reaction with other trypanosome species. However, their reaction with thirteen *T.(T).b.evansi* Stocks of defined serodemes did not show that they were *T(T).b.evansi* specific. McAb TEAI/23.4.6. was selected for application in the sandwich antigen - ELISA (Ag-ELISA) studies for the detection of circulating trypanosome antigens in serum or plasma and cerebrospinal fluid of experimental goats infected with KETRI 1342. This McAb is a mouse IgM antibody which cross-reacts with all the species of mammalian African trypanosomes. In this experiment, group one goats in which each goat was inoculated intravenously by needle and syringe challenge with $2x10^6$ trypanosomes of KETRI 1342 stock, the sandwich Ag-ELISA technique was able to detect circulating trypanosome antigens in plasma or serum 24 hours after the inoculation of the trypanosomes. In the group two goats, in which the trypanosomes were inoculated by intramuscular route, antigens could not be detected until day 6 after inoculation. In both groups, there was no parasitological or serological evidence of trypanosomes in cerebrospinal fluid. In these experiments, a high positive correlation was observed between plasma Ag-ELISA and serum Ag-ELISA values. A low positive

correlation was observed between serum Ag-ELISA values and parasitaemia or rectal temperature. A negative correlation was observed between serum Ag-ELISA values and PCV or total haemolytic complement. A positive correlation was observed between serum Ag-ELISA and antibody-ELISA (Ab-ELISA) values. After treatment with the trypanocidal drug, Berenil, antigen levels dropped more sharply in group one than in group two goats. By day 12 and day 41, antigen levels had fallen to pre-infection levels in group one and two, respectively. However, by day 56 and 44, IgG antibody levels were still very high in group one and group two respectively. On the other hand, IgM antibodies had fallen almost to pre-infection levels by this period. Field studies were carried out in four camel herds to evaluate the usefulness of McAb TEA1/23.4.6.as an antigen detecting probe in the diagnosis of patent trypanosome infection in camels. In an Ag-ELISA sandwich assay designed as for the goat experiment, a very high significant difference ($P<.0001$) was observed between plasma or serum Ag-ELISA values of infected and non-infected camels. In the third camel herd, it was observed in 25% of camels, that by day 14 after treatment, there was no evidence of detectable trypanosome antigenaemia, while in only few camels antigenaemia persisted beyond day 28. On the other hand, by day 48, anti-trypanosome antibody levels were still very high in about 95% of the previously infected camels.

Thus McAb TEA1/23.4.6. was a very successful probe in detecting circulating *T.(T).b evansi* antigens in plasma or serum of both experimental goats and field camels. Since antigens were detected much earlier than the antibodies, it was concluded that Ag-ELISA technique employing McAb TEA1/23.4.6. was a superior technique to Ab-ELISA in detecting patent infection. There was a very high agreement between TEA1/23.4.6. Ag-ELISA and the

parasitological test (HCT and MI) used. Since it is possible to modify the assay conditions so that Ag-ELISA technique can be used as a field test, McAb TEA1/23.4.6. could be employed in such a test as a diagnostic reagent in trypanosomiasis control programmes.

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

Evans (1880) described the first causative agent of mammalian trypanosomiasis, *Trypanosoma (Trypanozoon) brucei evansi*. Later, Bruce (1895) established that trypanosomes caused disease in livestock and that the disease could be transmitted by tsetse-flies. There are a number of pathogenic trypanosomes causing the disease in man and animals. The disease in man is known as sleeping sickness and in animals as nagana. Wild animals suffer inapparent infection but act as reservoirs for the disease in man and his domestic animals.

The tsetse-transmitted pathogenic trypanosomes of mammals (*T.(T). brucei rhodesiense, T.(T).b. gambiense, T.(T).b. brucei; T. (Nannomonas) congolense, T.(N). simiae; T. (Duttonella) vivax* and *T. (Pycnomonas) suis* are largely confined to the African continent, although *T.(D). vivax* subspecies have been found outside Africa (Gardiner and Wilson, 1987). The distribution of these species coincides with their vectors, the flies of the genus *Glossina*. From the medical and veterinary point of view, these trypanosome species are also among the most important because they include the aetiological agents of sleeping sickness (*T.(T).b. rhodesiense* and *T.(T).b. gambiense)* in man and nagana (*T.(T).b. brucei, T.(P). suis, T.(N). congolense, T.(N). simiae* and *T. vivax* in animals. Chagas' disease of man and animals is caused by *T. (Schizotrypanum) cruzi* which is widely distributed throughout Southern and Central America and extends into Mexico and Southern United States. *T.(S). cruzi* is transmitted by bugs of the family Reduviidae and is the only cyclically transmitted trypanosome of man outside the African Continent.

Among the pathogenic trypanosomes of mammals, there are also two species *T.(b).b. evansi* and *T.(T).b. equiperdum* which are of Veterinary importance. These two are not transmitted cyclically by tsetse-flies, although they are closely related to *T.(T).b. brucei* and are considered probably to have descended

from the latter through the replacement of *Glossina* by mechanical transmitters such as Tabanidae flies in case of *T.(T).b. evansi* or through the complete loss of a vector, in case of *T.(T).b. equiperdum*. Trypanosomiasis caused by *T.(T).b. evansi*, commonly known as Surra, is essentially a disease of camels but it may also have a serious effects on variety of domestic animals including horses, donkeys, cattle, goats, sheep, dogs, cats, elephants and Asiatic buffaloes. Natural infections with *T.(T).b. equiperdum* occur only in equines, in which it causes a disease known as dourine. Transmission occurs from stallion to mare during the act of coitus.

Mammals may also harbour non-pathogenic trypanosomes many of which have a cosmopolitan distribution. These include *T. (Megatrypanum) theileria, T.(M). ingens* commonly encountered in domestic and wild ruminants. These latter species, though non pathogenic, may be encountered during surveys and be confused with pathogenic species mentioned earlier (Hoare, 1970).

Trypanosomiasis manifests itself as an acute, subacute, or chronic disease (Losos and Ikede, 1972). For clinical and epidemiological purposes, early detection and identification of the species of the infecting trypanosome is advantageous.

During the acute stage of the disease, parasitaemia is usually high in peripheral circulation and diagnosis can be based on the detection and morphological identification of the infecting trypanosome using wet-blood films and Giemsa-stained thin-blood smears, respectively. In the chronic disease, peripheral parasitaemia is scanty or absent and the demonstration of parasites in the blood films or stained thin smears is difficult (Godfrey and Killick-Kendrick, 1961; Killick-Kendrick, 1968). Alternative techniques such as those based on the buffy-coat examination or sub-inoculation of blood into rodents are less convenient, unsuitable for *T.(D). vivax, T.(N). simiae, T.(P). suis*, certain strains of *T.(N). congolense* and may fail to detect infected animals(Rae and Luckins, 1984). Moreover, serological assays based on the detection of circulating anti-trypanosome antibodies lack parasitological precision in

that they cannot show with certainty the presence of active infection nor identify the species of infecting trypanosome. This is due to the fact that common anti-trypanosome antibody levels persist for a long time even after cure has been effected (Luckins *et al*, 1978, 1979; Boid *et al* 1981).

Ultimately, the most reliable approach to the diagnosis of trypanosomiasis, is to develop techniques which can detect and identify the parasite at the lowest level of infection, so that the right chemotherapeutic regimen can be choosen.

1.2. OBJECTIVES

1.2.1. To employ hybridoma technology as a means of producing monoclonal antibodies (McAbs) to *T.(T).b. evansi*.

1.2.2. To characterize the above produced McAbs using biochemical and immunological methods.

1.2.3. To employ McAbs against *T.(T).b. evansi* as parasite typing reagents for the *in vitro* differentiation of *T.(T).b. evansi* species from other trypanosome species, especially those of the sub-genous Trypanozoon.

1.2.4. To employ McAbs generated above as antigen detecting probes in the sera of goats and camels infected with *T.(T).b. evansi*. Furthermore, to evaluate the level of such detectable antigenaemia in relation to:-

1.2.4.1. the preparasitaemic, parasitaemic and post-parasitaemic phases of infection.

1.2.4.2. the level of parasitaemia, specific antibody response (with respect to IgM and IgG), complement level and clinical disease.

1.2.5. To evaluate the relevance and potential of such McAbs as:-

1.2.5.1. trypanosome typing reagents

1.2.5.2. immunodiagnostic reagents for the detection of patent *T.(T).b. evansi* infection in camels.

CHAPTER TWO

LITERATURE REVIEW - GENERAL

2.1 CLASSIFICATION

Trypanosomes are flagellate haemoprotozoan parasites belonging to the genus *Trypanosoma*. The genus *Trypanosoma* was first described by Gruby in 1842 (Gruby,1843). The systematic position of the genus *Trypanosoma* among other protozoa and the revised classification of mammalian trypanosomes has been given by Hoare (1970). According to this classification, the genus *Trypanosoma* belongs to the family *Trypanosomatidae*, of the suborder *Trypanosomatina*, of the order *Kinetoplastida*, of the class *Zoomastigophora*, of superclass *Mastigophora*, of the subphylum *Sarcomastigophora* and phylum *Protozoa*. The names :"Stercoraria" were introduced by Hoare (1972) as the names of two groups described earlier as "Sections" at the level between genus and subgenus and referring only to the trypanosomes of mammals. Though this nomenclature is not permissible according to the 1964 International Code of Zoological Nomenclature, Article 42(d) (WHO, 1978), the terms "Stercoraria" and "Salivaria" have proved so useful in a descriptive sense that they should be provisionally retained (though not as formal taxa). Hoare (1964), recognized four subgenera namely: *Megatrypanum*, *Herpetosoma*, *Schizotrypanum* and *Endotrypernum*, as belonging to Section "Stercoraria".

According to Hoare (1964) the Section "Salivaria" is composed of four subgenera namely: Duttonella (Comprising, *T.(D). vivax vivax*, *T.(D). vivax viennei* and *T.(D). vivax uniforme*), Nannomonas (Comprising, *T.(N). congolense* and *T.(N) simiae*), *Pycnomonas* (Comprising, *T.(P). suis*) and *Trypanozoon* (Comprising, *T.(T).b. rhodesiense*, *T.(T).b.b. gambiense*, *T.(T).b. brucei*, *T.(T).b. evansi* and *T.(T).b. equiperdum*). These trypanosomes complete their cyclical development (with the exception of *T.(T).b. evansi* and *T.(T).b. equiperdum*) in the "anterior station" and transmission of the metacyclic forms is inoculative through saliva. These four subgenera contain species of Veterinary and Medical importance. The confirmation of the

four subgenera by Hoare (1972) materially clarified evolutionary relationships within the salivarian trypanosomes and substantially simplified their description and discussion. It was pointed out by Hoare that the citation of the subgeneric names is not necessary in publications dealing only with species of a single subgenus, but only when comparisions are being made with members of other subgenera; the specific binominal or subspecific trinominal nomenclature being adequate identification. According to the Linnaean taxa, a species is defined as an assemblage of organisms that can be distinguished from other species by one or more stable discontinuous morphological characters, and subspecies as assemblages of organisms within a species that cannot be separated from each other by morphological characters but only by other stable characters (WHO, 1978). In considering the validity of the taxa of salivarian trypanosomes at species and subspecies level and taking into account the definition of species proposed above, it is evident that more convincing evidence is required for the differentiation of many of the commonly accepted taxa. Purely mensural characterization of species is often inadequate unless a thorough statistical treatment of data from a wide range of materials is available and, in the absence of other morphological criteria, supporting evidence of other kinds (e.g. macromolecular and immunological characteristics) should be sought. Also, many of the differential morphological characters currently used are clinical rather than discontinuous in geographical (e.g. length in the subgenus *Nannomonas* and the incidence of dyskinetoplasty in *Trypanozoon*) distribution. Because of this, it was suggested that for the present, each salivarian subgenus should be regarded as unispecific unless and until further definite evidence to the contrary is forthcoming (WHO, 1978).

2.2 CHARACTERIZATION OF SALIVARIAN TRYPANOSOMES

Methods for the characterization of trypanosomes are developing rapidly at present and are more advanced with these organisms than with any other protozoan parasite. In the characterization of trypanosomes, both intrinsic and extrinsic characters have been used.

2.2.1 INTRINSIC CHARACTERS

Morphological characteristics have been used to distinguish subgenera and species of trypanosomes. These include: differences in length (e.g. *T.(D). vivax vivax* and *T.(D). vivax uniforme*), size and position of the kinetoplast (all genera) and the sustained presence or absence of the flagellum (subgenera *Duttonella* and *Nannomonas*) (Hoare, 1972). Ultrastructural differences also occur at subgeneric level. The surface coat is compact in *Nannomonas* and *Trypanozoon* and diffused in *Duttonella*. The mitochondrion lies on abflagellar side of the nucleus in *Nannomonas* and *Trypanozoon* and on the adflagellar side in *Duttonella*. Tubular mitochondrial cristae are present in all bloodstream stages and plate-like cristae in all vector stages of *Duttonella* and *Nannomonas*. They are absent in dividing bloodstream stages, tubular in non- dividing bloodstream stages and vector salivary gland stages and plate-like cristae are present only in the vector midgut stages in *Trypanozoon* (Vickerman, 1974; Vickerman and Preston, 1976). Structural and ultrastructural characters have not so far been helpful in distinguishing the subspecies of *Trypanozoon*. Dyskinetoplastic stocks of *T.(T).b. evansi* and *T.(T).b. brucei* which are indistinguishable by conventional light microscopy are found at the level of electron microscope to differ in the distribution of the kinetoplast remnant material, which may be unitary, central or fragmented and dispersed within the mitochondrion (Vickerman and Preston, 1976). New cytochemical staining employing DNA-Specific fluorescent stains may enable these differences to be discerned at light microscope level (Hadjuk, 1976), especially when specific and standardized reagents like monoclonal antibodies are used.

The oxidative metabolism of salivarian trypanosomes reveal differences at subgeneric level (Lumsden and Ketteridge, 1979). These differences correlate with mitochondrial characters and cyclical mitochondrial changes referred to above.

There is a clear evidence for the differences in drug-sensitivity between different trypanosome subgenera, species and even between different subspecies within the same species of

salivarian trypanosomes (Lumsden and Ketteridge, 1979). Drug-resistance is now a common phenomenon among the salivarian trypanosomes and may preclude the use of this technique to differentiate trypanosomes.

With the analytical techniques now available for characterization of macromolecules, many of the problems basic to the identification and inter- relations of salivarian trypanosomes are being studied. Deoxyribonucleic acid (DNA) characterization, by buoyant density (Newton and Burnette, 1972), high- resolution melting analysis (Steinert and Van Assel, 1974; Steinert *et_al,* 1976) and restriction endonuclease target sequence analysis (Fairlamb *et al.*, 1978, Borst *et al.*, 1980) has been used to characterize trypanosomes with some degree of success. DNA hybridization is one of the most promising innovations in molecular biology, for the characterization of parasites. Parasite identification using, DNA probes is now possible and soon will be introduced at field level for field epidemiologists and control organizations (Borst *et al* , 1987; Greig and Ashall, 1987; Ole-Moi Yoi, 1987).

Isoenzyme analysis has been widely employed for the characterization of trypanosomes (Kilgour and Godfrey, 1973; Gibson *et al.*, 1978, 1983; Otieno and Darji, 1985; Allsopp and Newton 1985 and Gashumba *et al.*, 1986). Isoenzyme characterization methods have the advantage that they are sufficiently simple to allow their use in relatively poorly equipped laboratories.

Trypanosomes being protozoan of complex molecular structure may have other macromolecular antigens which could be used for their identification at species and subspecies level. Parish *et al.* (1985) using monoclonal antibodies showed that the *T.(N). congolense* specific antigen was associated with cell membrane. Recently, Nantulya *et al.*, (1987) have developed and used monoclonal antibodies to characterize *T.(N). congolense, T.(D). vivax vivax* and *T.(T). brucei* subspecies. Such reagents may be used for the immunological characterization of trypanosome species and subspecies.

2.2.2 EXTRINSIC CHARACTERS

The behaviour of trypanosomes in hosts or *in vitro* culture systems have been used as criteria for the differentiation of salivarian trypanosomes with identical or closely similar morphological features within one species whose distinction may be desirable for practical purpose. Such criteria include host range (Onyango *et al.*, 1966; Geigy *et al.*, 1975; Gibson *et al.*, 1978; Lumsden and Ketteridge, 1979), clinical characteristics and pathogenicity (Ormerod, 1970), resistance to human plasma (Rickman and Robson, 1970), the site of development in the invertebrate host (Lumsden and Ketteridge, 1979) and behaviour in culture (Trager, 1975 and Hirumi *et al.*,1977). Though some of these criteria were valid in the past, today they have become less valid. This is because the trypanosome has been studied in more detail and additional information has revealed what was not known in the past.

2.3 TRYPANOSOME ANTIGENS

2.3.1 CHEMICALLY EXTRACTED ANTIGENIC FRACTIONS OF TRYPANOSOMES

Trypanosomes are complex organisms and possess many antigens. This is clearly seen from the fact that they can induce the production of a wide variety of different antibodies e.g. agglutinins, precipitins, complement fixing antibodies, etc. Until recently, little was known about the fundamental nature of trypanosome antigens because of the lack of suitable means for analysing them in the past. Earlier observations on trypanosome antigenic properties were made on crude or whole organisms. This problem was further complicated by the great capacity of trypanosomes for antigenic variation and the occurrence of a multiplicity of strains of common trypanosome species.

The earliest attempts to characterize trypanosome antigens were made by Kligler and Olitzki (1936) and Kligler *et al.*, (1940). They prepared lipid, protein and carbohydrate extracts from *T.(T).b. evansi* and *T.(H). lewisi* by chemical methods which were common at that time for antigenic analysis of bacterial cells. Kligler and Olitzki (1936) and Ikejiani (1947) extended their work onto *T.(T).b. equiperdum.* The work of Kligler and

Olitzki (1936), however, did not produce conclusive results as they did not test the fractions for ability to stimulate antibody production or react with antibody. Brown and Williamson (1962) however, were able to determine the precipitinogenic constituents of all the variants of the same strains they examined. As a result of their work, the soluble components of homogenates of trypanosomes, belonging to the subgenus *Trypanozoon* were categorized into feebly antigenic nucleoproteins, a major group of variable precipitinogenic antigens - the "4S" antigens, and the poorly defined weak common antigens - common to different strains and subspecies of *T.(T). brucei* species. They found at least three nucleoproteins in trypanosomal homogenates which exhibited little precipitinogenic activity, but featured prominently in physical and chemical analyses. Williamson (1963) found that these nucleoproteins were mainly ribonucleoproteins. A similar group of nucleoproteins had been observed in *T.(D). vivax* by Williamson and Desowitz (1961). Williamson and Brown (1964) showed that the so called "common antigens" contain two to three antigens. There also exist uncharacterized antigens in trypanosome homogenates. Weitz (1960) called them "bound precipitinogens" as well as soluble "exo-antigens". Gray (1961) detected two and sometimes three antigens in the homogenate of a rat adapted strain of *T.(D). vivax*. Seed and Weinman (1963) described a group of three precipitinogens in homogenates of a number of variants of a strain of *T.(T).b. rhodesiense*. They found that the same antigens occurred in culture forms of the same strain. They called them "common antigens" to distinguish them from variant specific antigens found in the homogenates of trypanosomes and serum from infected animals. Common antigens were also found in strains of *T.(T).b. brucei* and *T.(T).b. equiperdum* and in reduced amounts in *T.(T).b. gambiense* .

2.3.2 TRYPANOSOME ANTIGENS IN SERUM OF ANIMALS INFECTED WITH TRYPANOSOMES

Thillet and Chandler (1957) reported the immunizing properties of what they called metabolic products of *T.(H).*

lewisi. Later, Weitz (1960) described the properties of an exo-antigen in the serum of experimental animals infected with other species of trypanosomes. Gray (1961) also observed that trypanosome antigens occur in serum of animals infected with tsetse-transmitted trypanosomes. Chemically, the serum antigens seem to be proteins or glycoproteins (Cross, 1978; Vickerman and Tetley, 1978). A carbohydrate-rich antigen was also detected in the serum of mice infected with *T.(M). cruzi* (Gottlieb, 1977). The specificity of serum antigens was considered by several workers. Gray (1962) found that two strains of *T.(D). vivax* with different histories, and biological characteristics produced several serum antigens in common. He also found that strains of *T.(T).b. gambiense, T.(T).b. brucei* and *T.(D). vivax* had common serum antigens. Although the variant specificity of serum antigens is now known, there is no reason why different strains of one or more species and subspecies should not produce common soluble antigens.If this is true, then the possibility of soluble serum species specific antigens also exists. These may not be necessarily immunogenic, but could be useful in immunodiagnostic assays. Theoretically, quantitative assays for released trypanosome molecules may give clues to the level of infection and also be more responsive to the parasite elimination after chemotherapy. Any parasite molecule which has at least some epitopes to which high affinity antibodies are induced in the infected host will be unsatisfactory if circulating antigen-antibody complexes are removed or if the bulk of the antigen is masked. The ideal parasite molecule for detection in serum would be one which is poorly if at all immunogenic in the natural host, is species specific as well as produced by established parasites and is present in sufficient quantities for detection. However, molecules which are poorly immunogenic in large animals may be immunogenic in rodents (especially mice) with, or without antigen modification and exploitation of linked antigen recognition, making possible the production of murine hybridomas for use in sensitive immunoassays (Mitchell, 1981).

Gottlieb (1977) and Araujo (1982) were able to detect circulating *T.(M). cruzi* antigens in plasma of mice acutely

infected with the parasite using immunochemical and enzyme immunoassay techniques,respectively. Araujo (1982) was able to detect as low as 17ng of protein lysate of *T.(M). cruzi* and a high parasitaemia did not seem necessary for the detection of antigenaemia. Recently, Rae and Luckins (1984) were able to detect circulating antigens of *T.(T). brucei, T.(T). b. evansi, T.(N). congolense* and *T.(D). vivax* in goats and rabbits infected with these parasites. They used polyclonal antibodies in a double antibody enzyme linked immunosorbent assay (ELISA). for the detection of circulating antigens reported above. As low as 1.5ug/ml of protein could be detected. *T.(T).b. evansi* antigens were first detected in serum four to eight days after infection and increased in levels thereafter. However, seven days after treatment with the trypanocidal drug Suramin, antigens were no longer detectable.

The enzyme assays for the detection of circulating antigens are attractive for several reasons: they are simple to perform, can be specific and sensitive, are applicable to field conditions and do not require expensive equipment like the radioactive counting equipment used in radio-immunoassay (RIA). They may be more useful because they do not require access to freshly collected blood. They may also offer a means by which it would be possible to detect relapse-infections in animals undergoing trypanocidal drug therapy during a period at which it is not possible to detect parasite in the peripheral circulation. The usefulness of conventional polyclonal antibody preparations as antigen detecting probes may be of limited value because of the variability of the immune response to various trypanosomal antigens and the heterogeneity of the antibody affinity and avidity (Haque *et al.*,1981). However, by means of Hybridoma Technology, it is possible to produce antibodies from individual clones of normal antibody-producing cells immortalised by fusion with myeloma cells. Virtually unlimited quantities of homogenous, exquisitely specific antibodies can be produced even if the immunizing antigen is not pure. With such reagents one can avoid the frequent problems of cross-reactivity between trypanosome species (Rae and Luckins, 1984), as well as the usual

contamination of anti-parasite antisera with antibodies to host antigens.

An important feature of the use of such monoclonal antibodies (McAbs) is the possibility of obtaining perfectly standardized reagents which may be maintained stable and produced indefinitely as diagnostic and parasite typing reagents. Parish *et al.*,(1985) identified an antigen specific for *T.(N). congolense* by the use of McAbs. Nantulya *et al.*,(1987) and Nantulya *et al.*, (1989) were able to generate McAbs which were *T.(N). congolense, T.(D). vivax* and *T.(T). brucei* specific and which could detect the species specific circulating serum antigens in sera of animals and human patients.

T.(T).b. evansi infection in camels takes a generally chronic course, characterized by an apparent absence of parasitaemia. This may seem to indicate that parasites and/or parasite antigens are released intermittently into circulation and therefore suggests that antigens would be difficult to detect under such circumstances. Apparently, a high parasitaemia is not necessary for the detection of antigenaemia (Araujo, 1982). Wilson *et al.*,(1975) and Houba *et al.*,(1976) were able to demonstrate parasite antigens in serum which also contained antibodies. However, the relationship between such circulating antigens, humoral response, complement level and the clinical disease needs further elucidation.

2.4 DIAGNOSIS OF TRYPANOSOMIASIS

Rapid and accurate diagnosis is of supreme importance in any clinical condition, so that a suitable therapeutic regimen can be instituted, or a proper chemotherapeutic regimen can be instituted, or a proper chemotherapeutic control strategy can be set up. Diagnosis of trypanosomiasis is at present not very satisfactory in that no single test is sufficiently specific, sensitive and reproducible to warrant its widespread application in the field (Vickerman and Barry, 1982). Besides the need for such a test, it would be useful to be able to differentiate between infections with the different species and subspecies of trypanosomes since the chemotherapy and pathogenesis of different

species and subspecies differ.

Several papers have reviewed the diagnostic techniques available for animal trypanosomiasis over the last three decades (Leach and Roberts, 1965; Killick-Kendrick, 1968; Wilson and Dar, 1972; Soltys and Woo, 1972; Luckins *et al.*,1979; Paris *et al.*, 1980). The review by Killick-Kendrick discussed at length parasitological methods of diagnosis and assessed the value of each in relation to each of the trypanosome species infecting animals and also dealt with the information and reliability of the serological techniques available at the time. From these reviews diagnostic techniques in human and animal trypanosomiasis were thus conveniently divided into two categories namely: Parasitological (by the direct demonstration of the parasites) and serological or immunological (by the indirect demonstration of the parasites).

2.4.1 PARASITOLOGICAL TECHNIQUES

Parasitological techniques available for the diagnosis of animal trypanosomiasis were described and their value assessed by Killick-Kendrick (1968). Since then, considerable progress has been made on this aspect of diagnosis. Wilson (1969) referred to the parasitological techniques commonly used at that time as standard Trypanosome Detection Methods (STDM). These techniques can be listed as:

(a) Thick, thin and wet blood film examination
(b) Sub-inoculation of susceptible laboratory animals, and
(c) Concentration methods.

Murray *et al.*,(1977) described the Blood-buffy coat darkground-phase technique, which was a considered improvement on the concentration methods. The methods for making and staining thin and thick smears, and for wet blood films were described by Baker (1970) and Paris *et al.*,.(1980). Examination of wet and thick blood films is more sensitive than thin blood smear examination. However, the latter technique allows a better preservation of the morphology of the trypanosomes present in the blood, than the former two techniques. Robson and Askar (1972) found that examination of lymph gland smears was of great value

in the diagnosis of *T.(D). vivax* in cattle. However, Zwart *et al.*,.(1973) found that lymph smears from sheep and goats had no advantage over blood film examination as a means of diagnosing this parasite, confirming the earlier observations of Adams (1936).

Paris *et al.*,.(1980) evaluated seven parasitological diagnostic techniques namely: blood buffy coat dark ground-phase contrast technique (DG) (Murray *et al.*,1977), haematocrit concentration technique (HCT) (Woo, 1969), wet blood film examination, thick blood smear and thin blood smear examination, haematocrit centrifugation technique and sub-inoculation into mice. They found that the blood buffy coat darkground-phase technique was more sensitive that the other techniques in detecting *T.(N). congolense* and *T.(D). vivax* trypanosomes, while the HCT was superior to the other techniques, apart from sub-inoculation of mice for the diagnosis of *T.(T). brucei* subspecies. Similar observations were made by Olaho and Wilson (1981) and Olaho *et al.*,(1987) for the field diagnosis of cameline trypanosomiasis.

The techniques of tissue culture have been successfully used for the diagnosis of stercorarian trypanosomes (Martinez-Silva *et al.*,1969). To date, salivarian blood forms of mammalian trypanosomes can be grown in vitro (Hirumi *et al.*,1977; Zweygarth and Rottcher, 1986). However, techniques of tissue culture have not been successfully used for the diagnosis of African trypanosomiasis on routine basis (Molyneux, 1975). The ability of *T.(M). theileria* to grow in such cultures as a contaminant (Malmquist, 1968) and other practical problems of contamination, preclude the use of such cultures for routine field diagnosis of pathogenic trypanosomes.

2.4.2 SERO-IMMUNOLOGICAL TECHNIQUES

Animals infected with trypanosomes produce most of the classical types of antibodies and many different immunological techniques have been used to study and diagnose trypanosomiasis. Many of the serological tests have a long history and undergone modifications over the years.

One of the earliest immonological methods used in trypanosomiasis was devised by Ehrlich and Shinga (Gray, 1965). They found that mice which had been infected with trypanosomes and cured by treatment with a therapeutic drug, produced protective antibodies and were immune to re-infection with homologous strains.

The trypanolysis test, which is based on the lysis of trypanosomes by antisera in the presence of complement has been used since the discovery of trypanolytic antibodies by Schilling (Schilling, 1904). This test was of great value in early studies on antigenic variation and later, in the study of immune response of animals to infections and in attempts to type strains (Chandler, 1958). In another development, Soltys (1957) found that incubated trypanosomes - antisera mixtures frequently contained live trypanosomes which were not infective for mice, their infectivity having been neutralized. This neutralization test is described by Lumsden *et al.*,(1973) and is still applicable in studies of antigenic variation.

Following the work of Ouchterlony (1964), precipitation tests were revived for use in the research of trypanosomiasis. These tests were more suitable for use in the trypanosomes of *Trypanozoon* and *Duttonella* subgenera than those of *Nannomonas* subgenus (Gray, 1965). The capillary agglutination test originally described by Ross (1971) was later recommended by WHO (1976) as a reliable test for *T.(D). vivax, T.(T). brucei brucei, T.(T).b. gambiense, T.(T).b. rhodesiense, T.(T).b. evansi, T.(N). congolense* and *T.(N). simiae*. An immune precipitin test with trypanosome antigens was described by WHO (1976). The test employs an Ouchterlony-type agar gel double diffusion technique. The precipitin antibodies detected in this test are invariably IgG globulins. Quantitative estimation of serum and cerebrospinal fluid IgM by the radial immunoprecipitation technique (Mancini *et al.*,1965) has been used with other specific techniques to diagnose trypanosomiasis in man and at times, in animals (Cunningham, *et al.*,1967; Luckins, 1972; Luckins, *et al.*,1979). The test is particularly important in establishing the central nervous system (CNS) stage of

trypanosome infection in man and animals (Itazi, 1981).

Agglutination tests are among the most simple serological tests used for the diagnosis of trypanosomiasis. Two types of agglutination tests have been used in trypanosomiasis namely: the Direct agglutination test (DAT) and the Indirect haemagglutination test (IHA) or the Passive haemagglutination test. The DAT which consists of the direct agglutination of whole trypanosomes by antibodies was described in detail by Cunningham and Vickerman (1962) and Lumsden *et al.*,1973). Apart from its application in studies on the *T.(T).b. brucei* infections and related trypanosomes of the subgenus *Trypanozoon*, it has been of little practical application in infections with other trypanosome species (Toure, 1980). However, the card agglutination test for trypanosomiasis (CATT) is now widely used as a field test for the diagnosis of Gambian sleeping sickness in West Africa (Croft, 1985). The Procyclic Agglutination Trypanosomiasis test (PATT) (Pearson *et al.*,1986) which employs the use of procyclic forms of *T.(T).b. rhodesiense*, has been used successfully to detect anti-trypanosome antibodies in the sera of rhodesian sleeping sickness patients.

The IHA, despite its recognized sensitivity, is not a routine test for the diagnosis of animal trypanosomiasis (Magnus *et.al.*, 1978). Gill (1964) used IHA for the diagnosis of *T.(T).b. evansi* infection in rabbits. He found the test to be specific, sensitive, easy to perform and gave reproducible results. Clarkson *et al.*,(1971) found IHA to be a sensitive test in the diagnosis of *T.(D). vivax* infection in sheep. Verma and Gautam (1977) compared serological tests for the diagnosis of *T.(T).b. evansi* infection in bovines namely: IHA, gel diffusion and the Indirect Fluorescent Antibody test (IFAT). They found that IHA detected haemagglutinating antibodies against *T.(T).b. evansi* between the 4th and 8th day of infection much earlier than the other tests. The test gave no false positive and was more sensitive and more reliable. Olaho and Wilson (1981) used the IHA test in an epidemiological study of camel trypanosomiasis in Kenya. The test was sensitive, specific,

reproducible and detected more than 90% of the positive camels.

A modification of IHA, the capillary passive agglutination test, was proposed by Bone and Challier (1975) for the epidemiological study of sleeping sickness. This test was found to give reliable results in *T.(T).b. gambiense* infection, but not in *T.(T).b. rhodesiense* (WHO, 1976).

The haemolysis test or the Complement Fixation Test (CFT) was one of the first serological tests to be used for the diagnosis of animals and human trypanosomiasis. It was successfully used for the diagnosis of Surra by Sabanshiev, (1973).

Coons and Kaplan (1950) and Coons (1956) introduced the fluorescent antibody test. The direct fluorescent antibody test (DFAT) was employed by Weitz (1963) to demonstrate the species specific antigens of *T.(T).b. brucei* and *T.(D). vivax*. This technique is useful for the demonstration of localized trypanosomal antigens in tissues. The indirect (or sandwich) fluorescent antibody test (IFAT) is one of the most widely used tests for the diagnosis of animal trypanosomiasis. The antigen for IFAT may be prepared differently depending on the laboratory (Van Meirvenne *et al.*,1975). Recently, Katende *et al.*,(1987) have described an improved method for preparing trypanosome antigen for IFAT. Sadun, *et al.*,(1963) used IFAT to demonstrate antibodies in sera of human patients with a history of sleeping sickness. Similarly, antibodies were found in the CSF of man and chimpanzees at different stages of infection with *T.(T).b. gambiense* and *T.(T).b. rhodesiense* (Lucasse, 1970). Similar results were obtained from samples of blood dried on filter paper and found to be a suitable field technique by Bailey, *et al.*, (1967). Wilson *et al.*,(1967) and Schindler and Sachs (1970) reported that the subgenera of *Trypanozoon, Duttonella* and *Nannomonas* could be differentiated by IFAT on the basis of differences in titre of antisera to homologous and heterologous antigen. Wery *et al.*,(1970) also claimed that they could differentiate *T.(T).b. brucei, T.(T).b. gambiense* and *T.(T). rhodesiense* on these grounds. However, Zwart, *et al.*,(1973)

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refuted this claim and reported that the test failed to detect early infections. Luckins *et.al.*, (1979) compared IFAT and Micro-ELISA with tests for the detection of raised euglobulin levels,including the measurement of IgM levels, in the development of improved diagnostic techniques for the detection of *T.(T).b. evansi* in camels, in the Sudan. They found that there was a high correlation between ELISA and IFAT. The two tests gave results agreeing more closely with the presence of active infection and there was evidence, using ELISA and IFAT that antibody levels showed a fall after a successful treatment contrary to what Wilson *et al.* (1967)had earlier found in cattle. Despite its usefulness, in practice, IFAT is not easy to quantify since it is dependent on subjective visual assessment of fluorescence.

Before the introduction of enzyme techniques by Engvall and Perlmann (1971, 1972) and Van Weemen and Schurs (1971) radioimmunoassay (RIA) and immunofluorescence were the only widely accepted assays that employed labelled antibodies and antigens. Some of the disadvantages of immunofluorescent techniques have been mentioned. Then there is the disadvantage of expensive microscopy associated with immunofluorescence. Although RIA is highly sensitive and permits precise quantification, isotope labels may decay rapidly so that the conjugates have a short shelf-life. Furthermore, complex equipment is necessary for their assessment; and, because of the medical hazards, they must be handled by highly trained personnel in specialized laboratories. Enzymes immunoassay (EIA) techniques offered an attractive alternative to labelled antibody/antigen methods mentioned previously and since their introduction, they have become some of the most widely used techniques in diagnostic medicine (Bidwell and Voller, 1981). Enzyme immunoassay techniques are easy to perform, require no expensive equipment, and most of the latest reagents are not biohazardous compared to RIA (Goka and Farthing, 1987). Voller *et al.*, (1975) showed that EIA could be successfully applied in the diagnosis of both American and African trypanosomiasis. Later, Luckins (1977) used a micro- ELISA to detect antibodies in

trypanosome infected cattle. Enzyme linked immunosorbent assay technique is now a routine diagnostic test for both animal and human trypanosomiasis. Luckins *et al.* (1979) showed that ELISA was as sensitive as IFAT in the diagnosis of Surra in the Sudan. In Kenya, Olaho *et al.* (1984) developed and used ELISA for the diagnosis of Surra in Kenya camels. It has been reported that ELISA is more ideal for detection of circulating trypanosome antigens in the sera of infected people and animals (Araujo, 1982; Rae and Luckins 1984; Nantulya, *et al.* 1987).

CHAPTER THREE

LITERATURE REVIEW: HYBRIDOMA TECHNOLOGY

Polyclonal antibodies are a product of hundreds or thousands of different antibody secreting clones. Many of these clonal products may be directed against undesired antigens, such as impurities in the immunizing antigen or molecules which are closely related to it. Despite the fact that serology predates many branches of medicine, the above considerations limited what could have been achieved by conventional serology.

The derivation of cell lines capable of permanent production of specific antibody directed against a predefined immunogen was first reported by Kohler and Milstein (1975). The method was based on fusion between myeloma cells and spleen cells from suitably immunized animals. In this way, individual clones of normal antibody-secreting cells (in this case spleen cells) could be immortalised. The resulting "hybridoma" approach has moved serology into a new area of precision. With hybridoma technology, virtually unlimited quantities of homogenous, exquisitely specific antibodies can be produced, even if the immunizing antigen is not pure.

Today, the use of hybridoma antibodies has moved into almost every area of biology and related sciences (Melchers *et al.*, 1978; Kennett *et al.*, 1980; Secher and Burke, 1980; Staines and Lew, 1980; French *et al.*, 1986; Aquilla *et al.*, 1986). It has even penetrated areas where a serological approach had not previously been considered feasible. Monoclonal antibodies have been used as probes for the fine structure of proteins, radioimmunoassay of hormones and drugs, histocompatibility testing, tumour localisation and classification, immunotherapy, purification of molecules by affinity chromatography, microbial and parasitic diseases, neurochemistry and embryology. They have also proven invaluable as a means of "Fishing" for previously unidentified molecules, (Goding, 1980). As effector molecules of pathogen immunity, they have been reported to inhibit, and remove or destroy *in vivo* or *in vitro Plasmodium berghei* and *Plasmodium*

yoelii parasites (Yoshida *et al.*, 1980; Freeman *et al.*, 1980; Epstein *et al.*, 1981). As probes for the detection of antigens or antigenic determinants (epitopes) they have proved very promising in immunoparasitology. Such monoclonal antibodies have been successfully used for immunodiagnosis (Araujo, *et al.*, 1980; Nantulya *et al..*, 1987) and as parasite typing reagents (Pinder and Hewett, 1980; Pratt, *et al.*, 1982; Parish *et al.*, 1985; Nantulya *et al.*, 1987).

3.1 PROPERTIES OF MONOCLONAL ANTIBODIES

Cotton and Milstein (1973) and Kohler and Milstein (1975) showed that antibodies secreted by myeloma hybrids contained light and heavy chains from both parental cells and that these could associate in a random fashion. For this reason not all the antibodies were functional (i,e. bind antigen). Therefore, parental myeloma cells were derived which synthesize no immunoglobulin (Pearson, 1981).

It is now possible to derive hybridomas which secrete monoclonal antibodies which are 100% active (Pearson, 1981). Cotton and Milstein (1973) also showed that mixing of the variable and constant regions of the heavy and light chains does not occur in hybridomas. This means that antibodies derived from fusions between non-synthesizing myeloma parental cells and immune lymphocytes have the properties of antibodies from the immune lymphocyte only. That is, the dual role of recognition (antigen-binding to mast cells, etc.) are found in one polypeptide chain which is synthesized under the control of independent genetic loci of the parental spleen cell and this is true for both heavy and light chains. The type of antibody (as defined by its specificity and effector function) obtained in cell fusion experiments depends on the immune status of the animal used as lymphocyte donor. It is also important to note that hybridomas do not represent random samplings from all lymphoid cells of the immune donor, a random representation from the antibody producing cells or precursors (Pearson, 1981).

Essentially monoclonal antibodies are chemically homogenous reagents with the same amino acid sequence which bind to single

antigenic detaminants on a molecule and can be produced in unlimited quantities without using pure antigens for immunization. The result is that each monoclonal antibody is a unique protein with its own biochemical and immunochemical characteristics, unlike conventional antisera which are composed of antibodies of different classes and subclasses, affinities and specificities. This biochemical uniformity of monoclonal reagents results in their exquisite specificity and high titres obtained after growth of cloned hybridomas. Because they are insignificantly contaminated by other irrelevant antibodies, monoclonal antibodies can be easily purified so that radionuclides enzymes or fluorochrome labelling and conjugation are easily accomplished. A low background in immunoassays or stained sections is characteristic of such reagents. In contrast with conventional antisera, it is easy to incorporate radionuclides of hydrogen,carbon or sulphur (^{3}H, ^{14}C or ^{35}S) into monoclonal antibody molecules biosynthetically by growing the hybridoma cells in a medium containing labelled amino acids. Because monoclonal antibodies can be produced and distributed indefinitely and their properties will always remain the same, radioimmunoassay and enzyme immunoassay and related techniques can be permanently standardized on an international basis. Quality control of such reagents may also be easier because the presence of monoclonal antibodies can be checked e.g. by isoelectric focusing pattern and their activity measured by a convenient titration. They may be expensive to produce initially, but subsequently, they are cheaper than producing antigens or conventional antisera (Edwards, 1981). It must be noted carefully that monoclonal antibodies have some shortcomings over conventional antisera. They do not work in most basic immunological tests e.g. the Ouchterlony double diffusion assay. A monoclonal antibody may be unable to distinguish a group of different molecules that all bear the antigenic determinant it recognizes or even between determinants that have sufficient structural similarity to bind the antibody, e.g. a hormone's peptide portion. Their failure to precipitate target antigens is because they will only cross-link antigens in dimers rather than

form lattice. That is why classical antisera fix complement more readily than do monoclonal antibodies, because complement requires at least two bound antibody molecules on neighbouring determinants. A solution to this is to use suitable blends of monoclonal antibodies for both precipitation and cytoxicity (Howard *et al.*, 1979). These workers showed that a mixture of two monoclonal antibodies is sufficient to effect precipitation or cytotoxicity. Conventional antiserum contains a variety of antibody molecules of different immunoglobulin classes and subclasses with different physical properties, different affinities for antigens, etc. Many practical procedures rely on this for example, some of the antibodies will be IgG and a proportion will have a high affinity. A monoclonal antibody has a unique structure and so it may not satisfy some of these conditions, e.g. it may be unusually susceptible to denaturation by freezing, iodination or salt precipitation. It may elute from DEAE-cellulose column at an atypical point. In assays with monoclonal antibodies, it is important to select appropriate concentrations of antibody and antigen and the time and temperature requirements of the reactions must be carefully chosen (Edwards, 1981). In selecting a monoclonal antibody for use in immunoassay and related studies Ghosh and Campbell (1986) suggest these guidelines:-

(1) Did the antibody have a high affinity on the original selecting antigen? If not, it may have potential for non specific cross- reactions.

(2) If the monoclonal antibody recognizes several antigens, does either of the antigens involved have a repeated structure (DNA, LPS, Dextran, etc) either naturally or in presentation to the animal (i.e. haptens at high substitution ratio)? Alternatively, is it present in assays at high concentrations. Any of these cases may lead to non specific cross-reactions.

(3) Is the antibody of IgM class? If so, then there is an increased likelihood of non specific cross-reactions.

(4) If the antibody is of the IgG class and one of the antigens is a cell surface protein, has the possibility of Fc receptor binding been tested?

(5) Has the antibody been tested on the antigens and

cross-reactions been demonstrated under identical experimental conditions employing identical amount of antigens and pure antibody?

(6) Has a Kinetic analysis of intrinsic interactions between antibody isotype and antigenic determinant been performed?. Evidently a careful consideration of experimental results is required in the final characterization of a monoclonal antibody.

3.2 PRODUCTION OF MONOCLONAL ANTIBODIES

Since Kohler and Milstein (1975) described the hybridoma technology, the technique has greatly expanded the usefulness and application of immunoassays. From the very onset, there was no question that monoclonal antibodies would significantly improve the quality and reliability of immunoassays.

3.2.1 MECHANISMS OF CELL FUSION

In nature spontaneous fusion of cells is usually rare. However, there are notable exceptions which include that of sperm and egg,oesteoclasts and myotubes. Almost any two cell types can be made to fuse by the addition of Sendai virus, lysolecithin or polyethylene glycol (PEG). Polyethylene glycol is now the most used agent in most cell fusion protocols. The mechanisms involved in cell fusion are poorly understood (Poste and Nicolson, 1978; Knutton and Pasternak (1979). However, during fusion, first the cell membranes fuse into the formation of heterokaryons which possess two or more nuclei. At the next division, the nuclei fuse and hybrid cells result (Goding, 1980).

Even when PEG or Sendai virus are used, fusion is still a relatively rare event. If tumour cells are fused with normal cells, the culture will be rapidly overgrown by unfused tumour cells. What is needed is a way to ensure that only hybrids will grow. By far the most celebrated strategy is that devised by Littlefield (1964). Its rationale is outlined below. The main biosynthetic pathway for purines and pyrimidines can be blocked by the folic acid antagonist, aminopterin. However cells can still synthesize DNA via the so called salvage pathways by depending on the enzymes thymidine kinase (TK) and hypoxanthine

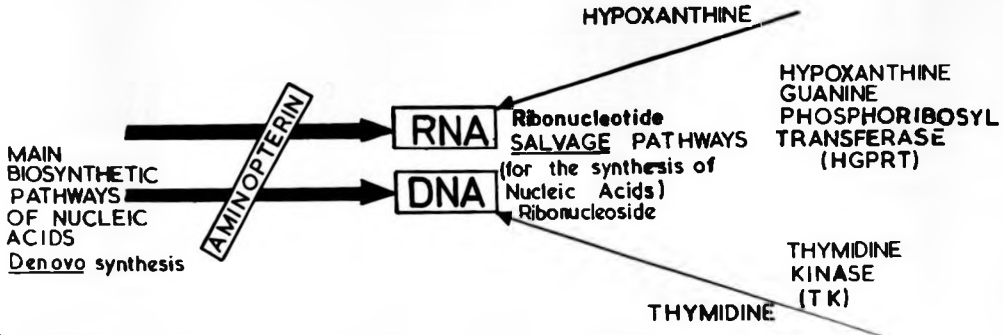
guanine phosphoribosyl transferase (HGPRT). Thus, if a cell is provided with thymidine and hypoxanthine, DNA and RNA synthesis can still take place provided the enzymes TK and HGPRT are present. If one or other enzyme are absent, DNA synthesis ceases. The cell can, however, be rescued by fusion with another cell which supplies the missing enzyme - thus, if spleen cells (which possess TK and HGPRT, but die in culture after 10 days) are fused with myeloma cells lacking TK or HGPRT, only the hybrid cells will grow in a medium containing hypoxanthine, aminopterin and thymidine (HAT medium). This is the principle of HAT selection of hybridomas and is well illustrated in Fig. 1.

Mutant myeloma cells lacking TK or HGPRT are produced by the use of toxic drugs. For example, thioguanine is incorporated into DNA via HGPRT, resulting in cell death. Only HGPRT-lacking cells will survive in thioguanine selection. Production of HGPRT-lacking cells is relatively easy since the enzyme is coded for by a gene on the X-chromosome. Mammalian cells possess only one active X chromosome (Lyonisation) and thus only a single mutation is needed to result in total loss of the enzyme. TK-lacking cells are much difficult to select, because two simultaneous rare events are required. It must be remembered that not all thioguanine resistant cells are HAT sensitive. In order to express a differentiation cell function,two parental cells should be at a similar stage of differentiation . Fusion of unlike cells frequently results in the extinction of differentiated cell function (Ringertz and Savage, 1976). Thus, antibody secretion is only maintained if normal antibody - secreting cells are fused with myeloma cells (Goding, 1980).

3.2.2 IMMUNIZATION STRATEGIES

Before producing a set of monoclonal antibodies it is important to define the animal model to be used and the potential use of such monoclonal antibodies. For example, if they are to be used on glutaraldehyde fixed antigen, then the animal should be immunized and the fusion screened with glutaraldehyde fixed antigen. It is also important to recognize that a large number of hybrids will have to be screened over a short period of time

PRINCIPLE OF HAT SELECTION SYSTEM



* Aminopterin blocks the main Pathway for the synthesis of nucleic acids
* The myeloma cell line NS−1 lacks TK or HGPRT, so dies because cannot synthesise nucleic acids by the Salvage Pathways.
* Hybrids can continue to synthesise Nucleic acids by the salvage pathways because they have HGPRT or TK (inherited from the Plasma cells at hybridization).
* Plasma cells die off naturally 10 − 14 days.

Fig 1 Principle of HAT Selection

therefore sufficient antigen and appropriate screening assay must be available. Other considerations being equal, the simplest and most commonly used animal model is that of Balb/c mice or Lou rats (Galfre and Milstein, 1981). Other suitable mouse myeloma cell lines are widely available and growth of hybridomas in mice is usually no problem (Goding, 1980). If the animal chosen is of a strain different from that of the myeloma parent, the growth of tumour cells will require the use of F_1 hybrid animals, for example if C57BLX spleen cells are fused with a Balb/c F_1 mice. A considerable variation in the immune responsiveness between different inbred strains of mice does exist. In particular, SJL mice are usually vigorous responders. If difficulty is encountered in producing active hybrids, a trial of different mice strains may be rewarding (Goding, 1980). The use of rats rather than mice as spleen cell donors may be more promising, in that rats are larger and thus can generate much greater volumes of ascites or serum when hybridomas are grown *in vivo*. One practical disadvantage of rats is that few rat IgG molecules bind to staphylococcal protein A, which means that alternative and more cumbersome methods of antibody purification must be used (Ledbetter and Herzenberg, 1979). Other species have received relatively little attention. Experience has usually indicated that the greater the phylogenetic distance between normal donor and myeloma cell, the more unsuitable the resulting hybrids. While the rat-mouse hybrids have been very successful, rabbit-mouse hybrids have proven very disappointing.

Immunization of the normal spleen donor serves two main purposes. The first is to expand the desired clones, and thus to increase the chance of obtaining a relevant hybrid. However, it is possible to obtain hybrids secreting antibody to some antigens without intentional immunization (Goldsby, *et al.*, 1978). The second function of immunization is to cause B lymphocytes to divide and differentiate into cells which will fuse and form useful hybrids. Although the exact nature of the normal cell which fuses to form active hybrids is not known, there are indications that recently activated B cells fuse preferentially. Thus, velocity sedimentation experiments indicate that larger

(dividing?) cells are much more efficient in fusion (Andersson and Melchers, 1978). It has been found that the best time for fusion is 3 or 4 days after the last boosting (Oi *et al.*, 1978) supports this concept. This is the time when antigen-induced proliferation is strong and blast transformation is at peak. Fusion at the peak of antibody secretion (say 7-8 days after boosting) seems to result into fewer active hybrids (Goding, 1980). There is also evidence that hyperimmunization by multiple injections may be counter-productive (Oi *et al.*, 1978). If multiple injection are considered necessary, it is important to 'rest' the animal for a few months and then boost 3 days prior to fusion (Goding, 1980). In many cases however, short immunizations have been successful (Trucco *et al.*, 1978). However, Stahli *et al.*, (1980) presented evidence to suggest that the frequency of specific hybrids against soluble antigens was greatly increased by immunization protocols which cause marked blast transformation. They obtained excellent results by using several conventional immunization courses followed by four very high doses of antigen in saline on each of the four last days before fusion. There is some evidence that the final boost is more effective if given intravenously (Goding, 1980). Kubagawa *et al.*, (1982) suggested that lymphnode fusions be carried out the same day after the last boost. Most investigators have not found it necessary to use adjuvant for cell surface antigens (Oi *et al.*, 1978). On the other hand, soluble proteins require adjuvants (Oi *et al.*, 1978; Kearney *et al.*, 1979). If Freund's adjuvant is used, it is important to ensure that small amounts of the antigen-adjuvant mixture are injected (Farr and Dixon, 1960). Also, the injected material must be true water-in-oil emulsion (Weir, 1973). It is advisable to immunize as many as 10 mice and test for activity. The animals showing the highest titre are the ones selected for fusion. Many different immunization schemes have been proposed (Damjanov and Knowles, 1983) and different protocols may work better for different immunogens. In general, it is advisable to use the simplest immunization protocol available (French *et al.*, 1986).

3.2.3 FUSION PROTOCOLS

A number of points must be considered during fusion. The first point to consider is the species. Unless there are specific reasons against it, the myeloma should be of the same species as the immunized animal, so that easy development of tumours occurs (Galfre and Milstein, 1981). The next point to consider is the chain composition of the myeloma. Hybrid myeloma codominantly express the immunoglobulin chains of both parental cells. As a general rule, the non producer myelomas are the best choice for fusion. Not all tissue culture lines of mouse myeloma are suitable for fusion and not all sublines of suitable cells are good partners for cell fusion. If difficulty is experienced, it is better to obtain a new batch of cells from a different source (Goding, 1980).

Cells may be grown in RPMI-1640, MEM or Dulbecco's modified Eagle's medium. The medium requires supplementation with serum. Until recently it was common to include 10-20% foetal bovine serum (FBS) in all parts of hybridoma production (Goding, 1980). The shortage of serum has provided the necessary incentive to isolate the factors which allow the growth of cells and therefore opened the way for the synthesis of artificial supplements. Today, a number of artificial supplements are on the market (e.g. hybri- max,Sigma,U.S.A; Hybri-clone, ICN Biochemicals, U.S.A.;GIT Wako Chemicals,Germany;SFRI-4, France) . It is advisable to choose the batch of serum carefully since all batches may not be suitable for cell growth. Also, if cells are grown in a particular medium, then growth should be continued in that medium until the end.

Though there are a number of other fusion agents, virtually all hybridoma work uses polyethylene glycol (PEG) as a fusing agent. The exact molecular weight of PEG seems not to be important (Goding, 1980). However, most investigators have used PEG of molecular weight ranging from 500-6000. Individual batches of PEG vary considerably in their toxicity. Typical concentrations of PEG for fusion range from 30-50%. Below 30% the fusion is low and above 50%, toxicity becomes overwhelming. According to Goding(1980), PEG solution (P) is made according to

the formular:

$$P = \frac{100M}{M + V}$$

Where M is the weight of PEG (in g) and V the volume of medium (in ml)added. The myeloma cells must be in exponential growth phase. If the cells are overgrown or if the viability is less than 98-99% the chance of successful fusion is low (Goding, 1980). Assessment of viability by trypan blue, oesin or nigrosin exclusion is not very accurate and the use of one or more modern methods such as staining with acridine orange and ethidium bromide is preferable. Myeloma cells may be grown in stationary rectangular flasks which usually allow a cell density of around 10^5 per ml. while somewhat higher densities are possible using roller bottles. The precise details of fusion protocols vary greatly between laboratories (Galfre, *et al.*, 1977; Gefter *et al.*, 1977; Oi *et al.*, 1978; Kohler, 1979; French *et al.*, 1986). It is fairly well agreed that the precise ratio of spleen cells to myeloma cells is not very important and good results have been achieved with ratios between 1:1 to 10:1 (Spleen cells: myeloma cells). It does seem that the initially fused cells are rather fragile and should not be subjected to rough or unnecessary pipetting (Goding, 1980).

A number of protocols for the enrichment of antibody forming cells have been suggested (French *et al.*, 1986). The protocols described by Pollock *et. al* (1984) are quite promising and easy to apply.

3.2.4 INITIAL GROWTH AND HAT SELECTION

After fusion there are many ways in which the cells can be cultured. Some investigators prefer bulk cultures (about 10^7 cells per 2.0 ML well). Others prefer microcultures (10^6 cells per 0.2 ML well). Some prefer to use HAT medium from the time of fusion, while others prefer to wait a day or two. The HAT medium may be added all at once (Stahli *et al.*, 1980) or gradually over a few days (Oi and Herzenberg, 1980). Yet another approach is to culture the fused cells in one flask for a day or two, and then

harvest by centrifugation and resuspend in HAT followed by plating out into microcultures (Goding, 1980). Amongst the hybrid cells there is a great variation in growth rates and susceptibility to chemical and physical changes. It would therefore seem logical to culture the cells in a large number of small wells such that, on average, only one hybridoma grows in each. This approach was adopted by Oi and Herzenberg (1980) and others (Goding 1980).

Within a day or two of the commencement of HAT selection there is massive cell death. After an additional 5-15 days, macroscopically visible colonies of hybrid cells should appear and the medium turn yellow at this stage. As soon as visible colonies are seen, screening of supernates begins. The cultures need no feeding until vigorous growth is evident. Feeding consists of removal of 20-50% of the spent medium and replacement by fresh medium. Once it is certain that all parental tumour cells are dead (5-10 days) HAT selection can be discontinued. It is a good idea to remove the aminopterin before hypoxanthine and thymidine. This is accomplished by the change from HAT medium to Hypoxanthine Thymidine Medium (HT) for a few days, before reversion to the regular culture medium (Goding, 1980).

3.2.5 SCREENING

The screening procedure is the key to success in hybridoma production (Goding, 1980). It occupies the greatest amount of time. whichever assay is chosen, it should be worked out before hybridisation is commenced. The requirements for the screening assay are that it can be reliable and quick. It should be able to be performed in large numbers and exact quantitation may not be necessary. However, the sterile removal of supernates is very important.

Perhaps the most widely used screening assays are the solid phase radioimmunoassay (RIA) and enzyme immunoassay (EIA) (Catt and Tregear 1967; Klinman 1972; Engvall and Perlmann, 1971; Tsu and Herzenberg, 1980). Because of the biohazards of RIA, coupled with its associated technical complications, EIA has become the most widely used assay, in the form of Enzyme linked

immunosorbent assay (ELISA). It must be emphasized that alkaline phosphatase is present in many cell types particularly B lymphocytes. If lymphocytes are used as the target antigen, it is preferable to use another enzyme (e.g. B-galactosidase). Secondly, it has been observed that only high affinity antibodies will bind to ELISA plates containing a low density of antigen (Herzenberg *et al.*, 1980). Furthermore, ELISA plates containing a high density of antigen may amplify the level of cross-reactions. Solid phase binding whether in RIA or EIA systems may induce antigen conformational alterations (Ghosh and Campbell, 1986). It is therefore wise to use where possible, several tests for screening hybridoma supernates or ascites.

Haemagglutination assays (Coombs, 1980) have also been described. These assays are based on the ability of an antibody to agglutinate red cells carrying the specific antigen. They have all the advantages in terms of extreme simplicity, speed and direct visual reading of the results. The disadvantages they have are the inhibitory effects due to excess antibody (Prozone effects) and quantitative inaccuracy. In practice, they have been reported to fail in detecting a number of antibody secreting clones (Galfre and Milstein, 1981). Lytic Assays based on the lysis of cells by antibody and complement have been described by Pearson *et al.*, (1977) and Galfre and Milstein (1981). It should be noted that assays based on complement may be good for IgM monoclonals but not IgG monoclonals. Assays based on the Biological activity of the antigen may too be applicable in screening monoclonal antibodies (Secher and Burke, 1980). Antibody-secreting cells under appropriate conditions, bind a certain amount of antigen. It has been possible to detect such cells secreting specific antibody by attaching the antigen to fluorescent microspheres (Parks *et al.*, 1979) or by the complete-dependent, localized lysis of antigen-coated red blood cells (Galfre and Milstein, 1981).

3.2.6 CLONING.

As soon as positive cells are identified, it is advisable to clone the cells. This reduces their chance of being overgrown by irrelevant cells. Multiple clones in single culture compete for growth and this together with chromosome segregation, conspires against stability of expression. However, hybrid lines are easier to clone after some time after active growth (Galfre and Milstein, 1981). Several methods for cloning are in current use. Cloning by limiting dilution has been described by Galfre and Milstein (1981). The presence of "feeder" cells will greatly enhance the cloning efficiency (Coffino *et al.*, 1972; Lernhardt *et al.*, 1978). The function of "feeder" cells is to add unknown growth factor to the medium rather than to remove waste product and dead cells, as has been suggested (Zola and Brooks, 1981). An alternative method of cloning hybridomas involves the use of semisolid agar (Coffino *et al.*, 1972; Galfre and Milstein, 1981). While cloning in agar is very popular, it requires the additional step of reculturing in liquid medium before biological activity can be assessed. In contrast, cloning by limiting dilution allows direct testing. In some cases, however, actively secreting colonies can be identified by haemolytic overlay technique (Kohler and Milstein, 1975; Kohler, 1979), or formation of immune precipitates surrounding the colonies (Cook and Scharffe, 1977). Parks *et al.*, (1979) described a modification of fluorescent activated cell sorter which allows individual cells to be cloned. The method allowed the authors to actively clone living healthy cells. More importantly, it was also possible to label hybrids with antigen-coated fluorescent latex microspheres and to clone them on the basis of their antigen binding properties.

3.2.7 LARGE SCALE PRODUCTION OF MONOCLONAL ANTIBODIES

Once positive hybrids have been successfully cloned, they may be grown in bulk. Cultures should be expanded gradually (diluting cells 1:3 to 1:5) especially in the early phases. Some hybrids are particularly intolerant to dilution. In this case, the addition of feeder cells may help (Goding, 1980). Typical antibody concentrations that can be achieved in culture

supernates are 0.1-1.0mg/ml. Cells used to propagate the line should always be kept in exponential growth phase, to minimize stress and adverse selective pressure which might favour the growth of non-productive variants. In contrast, if the only purpose of a culture is antibody production, the cells may be allowed to overgrow to the point of death. They are then discarded and the supernatant retained. It is not advisable to use cultures as a way of storing cells. As soon as feasible, aliquots of cells should be frozen in liquid nitrogen. This will ensure against loss of the cells by overgrowth of non-producers or by infection (Goding, 1980).

Cells may also be grown in mice or rats. The need for histocompatibility between the hybrid and the recipients of hybridomas has already been mentioned. Growth of hybridomas in mice or rats will allow the production of much larger amounts of antibody. Typical IgG antibody levels in serum or ascites are 3-15mg/ml (Goding,1980;Edwards,1981)which is many times higher than the culture supernates. A typical dose of cells is 10^5 - 10^6 , administered intraperitoneally and tumour growth should be evident 2-4 weeks. In some cases the growth of tumour cells will result in ascites up to 10ml per mouse. The production of ascites may be favoured by injecting recipients with 0.5ml of pristane (2, 6, 10, 14 - tetramethyl pentadecane) intraperitoneally, one week prior to the injection of cells (Goding, 1980). Ascites may be induced using Freund's adjuvant (Tung *et al.*, 1976). Occassionally, tumours will fail to appear after injection of cells. Sublethal irradiation (about 350R) of recipients may help growth (Goding, 1980).

3.2.8 PURIFICATION OF MONOCLONAL ANTIBODIES

Often, purification of monoclonal antibodies will not be required. The ease of purification will depend on the class of antibody. It is therefore helpful to test the antibody class. This may be done by Ouchterlony analysis of culture fluid which has been concentrated ten times by precipitation with 50% saturated ammonium sulphate (SAS), followed by dialysis in phosphate buffered saline. Several methods have been developed

for the purification of monoclonal antibodies.

Procedures for the purification of mouse monoclonal antibodies were reviewed extensively by Parham *et al.*, (1982). By a combination of clarification, ammonium sulphate precipitation, Sephadex G-200 and DEAE-52 cellulose chromatography, a purity in excess of 90% may be achieved for mouse IgM, IgG1, IgG2a and IgG2b. DEAE-cellulose has the advantage of removing non-specific IgG, and having a less denaturation effect on the monoclonal antibodies. Affinity chromatography on protein A Sepharose (Pharmacia) or anti-immunoglobulin coupled to cyanogen bromide activated Sepharose - 4B is a convenient and effective method (Parikh and Cautrecasas, 1975), but could subject monoclonal antibodies to denaturation conditions.

3.2.9 NUMBERING OF HYBRIDOMA SYSTEMS

A monoclonal antibody laboratory is a nightmare of inventory control (Letchworth and Appleton, 1984).
It is therefore essential to follow a comprehensive identification scheme beginning with the first fusion and never changing. A number of schemes have been suggested by different workers. For example at ILRAD Lab. 7 (Nantulya, Personal communication) the following numbering is used:

1. Designate the fusion type with a symbol
2. Designate the fusion number, then stroke (/)
3. Designate "Pot" number showing activity, then " ." (dot)
4. Designate Clone number, then " ." (dot)
5. Designate recloning number,e.g.A1.
6. Designate immunoglobulin chains secreted ab. e.g.

anti-H-fusion	= H
fusion NO. 6	= 6/
Pot number	= 31
Clone number	= A4.
Reclone number	= A1
Ig Chains	= HLK

H6/31.A4.A1 HLK

B. Letchworth and Appleton (1984) have suggested the following numbering scheme:

1. A series of numbers and letters are used to indicate the antigen used in the fusion, that is, BTV 17, for bluetongue virus type 17. This is followed by an "X" and the identity of the myeloma used, for example, BTV 17X653 or BTV 17XP3.

2. The 96-well tissue culture plates in which the colonies are growing are numbered by the investigator; rows and columns have letter and number assignment printed in the plastic. Each colony can then be identified by the plate number, a letter indicating the row of the well, and a number for the column of the well, for example, 2B4 (plate 2, row B column 4). The colony designation can be added to the fusion name following a dash, for example, BTV 17x653 - 2B4.

3. Clones and reclones are numbered consecutively and numbers are added to the previous derived designation following a dot, for example, BTV 17X653-2B4.12. Clones that are reclones receive numbers following another dot, for example, BTV 17X653-2B4-12.3

4. If possible, the antigens and cell lines used should be defined in the publication's materials and methods sections, then abbreviated laboratory numbers (for example, 2B4.12 and 4C1.3) are used in the text. If this is too confusing, a chart may be made, listing on one side the full laboratory designation for all hybridomas to be published and on the other side the simple consecutive numbers, for example:

BTV 17x653 - 2b4.12 - 1
BTV 17x653 - 4CL.3 - 2
BTV 4XP3 - 1A 4.8 - 3.

These simple numbers (1,2,3, etc.) may then be used in the text. In the text, the nomenclature of Nantulya was adopted.

3.2.10 STORAGE OF MONOCLONAL ANTIBODIES AND HYBRIDOMA CELLS

While antibodies are quite tough molecules, they can lose activity if stored incorrectly. Repeated freezing and thawing will lead to denaturation, especially if the concentration of the immunoglobulin is low. Lyophilization of culture fluid, serum or purified hybridoma proteins often results in loss of activity.

In general, culture supernatants, are extremely stable at 4^{o} C with addition of 0.1% sodium azide. Culture fluid may usually be frozen and thawed once. Serum and ascites from hybridoma-bearing mice are generally quite stable and may be stored at - 70^{o} C although storage at -20^{o} C may be satisfactory for short periods. Storage of serum or ascites at 4^{o} C in the form of ammonium sulphate precipitates is an economical and safe way of storing antibodies over long periods (Goding, 1980). Purified hybridoma proteins may be kept for many months at 4^{0}C in phosphate-buffered saline with 0.1% sodium azide. The optimum concentration for storage under these conditions should be 1-10mg/ml (Goding, 1980). There is a critical difference between monoclonal and conventional antibodies as regards stability. Conventional antisera contain many different monoclonal antibodies each with different stability. It is therefore advisable to test the stability of a given monoclonal antibody to any given treatment before committing a large batch (Galfre and Milstein, 1981). Many methods for freezing myeloma and hybridoma cells have been described (Galfre and Milstein, 1981, Hurrel, 1982; Letchworth and Appleton, 1984). However, it is important to use a method which is simple, and ideally suited to the specific needs of derivation of hybrid myelomas.

3.2.11 DIFFICULTIES IN HYBRIDOMA PRODUCTION

Careful planning before the fusion is started will reduce many of the difficulties experienced in monoclonal antibody production. It is extremely important that the assay procedure for screening the primary cells and the detection of antibodies during subsequent cloning, be worked out well in advance (Goding, 1980; Galfre and Milstein, 1981). With good tissue technique, bacterial infection should not occur. However, infection with moulds can be very troublesome and can occur with the best of the technique (Goding, 1980).

Cell hybrids have a tendency to lose chromosomes (Ringertz and Savage, 1976), although it seems likely that some hybrids are inherently more stable than others (Goding, 1980). It is realistic to expect 50-70% of all the culture that are initially

positive will be lost. However, recloning may save a failing clone. Perhaps the most important element in hybridoma production is the constant monitoring of progress.

Failure of growth in a previously growing culture can have many causes. In early phases cells should be diluted gradually, 1:3 or 1:5 as cultures are expanded. Feeder cells may help. Occassionally, batches of tissue culture flasks have been found to be toxic for cells. Similarly, certain batches of agar, agarose and polyethylene glycol may be toxic (Goding, 1980).

If cells are growing well yet all supernates are negative for antibody, one explanation can be the failure of HAT selection. This might be due to the back mutation of the myeloma cell, although the reversion frequencies usually very low. A more likely cause is the deterioration of aminopterin. The effectiveness of HAT selection may be tested by growing the myeloma cells in HAT medium for a few days. If 10^7 cells are grown there should be survivors (Goding, 1980). Some workers regularly passage their myelomas through medium containing 6-thioguanine (40mg/ml) or 8-azoguanine (20ug/ml) to kill the aminopterin-resistant cells (Zola and Brooks, 1981).

The quality and quantity of monoclonal antibodies can be improved by increasing the immunogenecity of the antigen, selecting the best animals, enriching for B cells, making the desired antibody, increasing the efficiency of fusions and screening judiciously. Additional improvements in each of the steps outlined above would be useful and may ultimately make it possible to recover the best and most useful antibodies (French *et al.*, 1986).

CHAPTER FOUR

4.1 GENERAL MATERIALS AND METHODS

4.1.1 EXPERIMENTAL ANIMALS

4.1.1.1 Mice

Two strains of mice were used. Swiss-White albino mice were obtained from KETRI mouse colony or the Veterinary Research Department (of the former East African Veterinary Research Organization) at Muguga. The KETRI Swiss-white strain originated from London through Walter-Reed Army Medical Research Project based at the Veterinary Research Laboratories (VET. LABS.) at Kabete, Nairobi. This Swiss-white strain originated from Carworth Farm in New York (U.S.A.). This strain was then taken to Rio de Janeiro laboratory for the Rockefeller Foundation, from where it was taken to Lagos, Nigeria. From Lagos, it was brought to Entebbe, Uganda, in 1936. In 1956, it was brought to the East African Veterinary Research Organization (EAVRO)-Muguga., - which is the present National Veterinary Research Centre (NVRC), Muguga.

The second strain of mice used was Balb/c. It was obtained from the International Laboratory For Research in Animal Diseases (ILRAD) at Kabete, Nairobi. Some of the Balb/c mice were also obtained from the International Centre for Insect Physiology and Ecology (ICIPE), Nairobi. The ICIPE Balb/c mice were originally obtained from ILRAD. Balb/c mice were also obtained from the National Public Health Laboratories, Kenyatta Hospital Nairobi, The origin of the Kenyatta Balb/c mice was VET. LABS.

4.1.1.2 Rats

Rats were obtained from the KETRI small animal Breeding Unit or the NVRC small animal unit at Muguga. This strain of rats was brought from the National Public Health Laboratories, Kenyatta, to Muguga in 1960. It is probably a wistar strain (Opiyo, 1987).

4.1.1.3 Rabbits

Rabbits were obtained from the KETRI or the NVRC rabbit

colonies.

4.1.1.4 Goats

The goats used were crosses between the East African Maasai and Galla breeds. They were purchased from a tsetse-free area near Nairobi. They were screened for anti-trypanosome antibodies, and parasitologically examined for the presence of trypanosomes, related haemoparasites and helminths before they were used.

4.1.2 TRYPANOSOME SPECIES AND RELATED HAEMOPARASITES

The species and stock or stabilate numbers as well as the origin of the trypanosomes and related haemoparasites used are presented in Tables 1(a) and 1(b).

Table 1(a): *T.(T). brucei* subgroup trypanosome stocks used in the study and their origin.

Sub-species	Trypanosome stock		Origin
T.(T).brucei evansi	KETRI	1342	London School of Tropical Medicine
		2472	Khartoum, Sudan.
		2540	Carimagua, Columbia S.America
		2531	Galana, Kenya
		2485	" "
		2458	Rumuruti "
		2455	Galana "
		2481	Mt. Kulal "
		2477	" " "
		2476	Ngurunit "
		2462	" "
		2467	Galana "
		2450	" "
		2471	Mt. Kulal "
		2470	"
		2469	"
		2468	"
		2466	"
		2447	"
		2443	"
T.(T). brucei		2535	Berlin.W. Germany der. of TGZ8/18
		2637	Galana Kenya
		2505	Matuga Kenya
		2623	Serengeti, Tanzania
	EATRO	617	Ankole, Uganda
		1718	Lambwe Valley, Kenya
		2486	Illulaba, Ethiopia
T.(T). rhodesiense	KETRI	2590	West Nile, Uganda
		2600	Busoga, Uganda
		2602	Kitanga, Tanzania
		2399	Buinja, Busoga, Uganda
T.(T).b. gambiense	KETRI	2566	Yambio, S. Sudan
		2347	Sakwa, Central Nyanza, Kenya
		2596	Yambio, S. Sudan

EATRO = East African Trypanosomiasis Research Organization.

Table 1(b). *T.(N). congolense, T.(N). simiae and T.(D). vivax* trypanosome stocks used in the study and their origin.

Sub-species	Trypanosome stock	Origin
T.(N). congolense	ILRAD 1180	Serengeti, Tanzania
	KETRI 1785	Acholi, Uganda
	KETRI 1896	Lugala, Uganda
	" 209	Busoga, Uganda
	G 870	Galana, Kenya
T.(N). simiae	KETRI 2431	Ukunda, Kenya
T.(D). vivax	KETRI 2633	Zaria, Nigeria
	" 2619	Zaria, Nigeria
	" 2627	Zaria, Nigeria
	" 2626	Zaria, Nigeria
	ILRAD 1392	Zaria, Nigeria
	KETRI 2500	Teso, Uganda
	" 2430	Galana, Kenya
	" 2501	Aitong, Kenya

Table 2. Other Protozoal Haemoparasites used in the study.

Protozoal haemoparasite	Developmental stage used	Source
Anaplasma marginale	intra-erythrocytic stage	ILRAD
Babesia bigemina	intra-erythrocytic stage	ILRAD
Leishmania donovani	Promastigotes	KEMRI
Plasmodium falciparum	intra-erythrocytic stage	KEMRI
Theileria parva	intra-lymphocytic (Schizonts)	ILRAD

KEMRI: Kenya Medical Research Institute.

ILRAD: International Laboratory for Research in Animal Diseases.

4.1.3 STABILATE PREPARATION

Mice inoculated with the KETRI Bank trypanosome stabilates or isolates were bled daily from tails and wet blood films for the microscopic determination of parasitaemia. As soon as parasitaemia was evident, passages were made into several more mice. When sufficiently high parasitaemia was observed (usually 10^7 - 10^8 trypanosomes/ml, stabilates were prepared. Parasitaemic blood containing Ethylenediamine tetra acetic acid (EDTA) was quickly mixed with Phosphate Saline Glucose (PSG) buffer pH 8.0 containing 20% glycerol (as the cryoprotectant) in equal proportions. Glass capillaries were filled with the preparation and sealed with cristoseal. The capillaries were then put into ampoules and suspended in liquid nitrogen vapour overnight and then transferred into liquid nitrogen (-196^{o} C) for permanent storage.

4.1.4 CLONE PREPARATION

Clones were obtained by the microscopic isolation of a single trypanosome which was allowed to multiply during three-day successive passages in mice as described by Otieno and Darji (1985). Blood from a heavily parasitaemic mouse was diluted in PSG pH 8.0 and serially diluted to give approximately 10^3 trypanosomes per ml. Several microdroplets of this dilution were then placed on a coverslip and quickly put into a moistened siliconized grooved microscope slide (droplets being on the underside of the coverslip). Droplets were viewed under the microscope at 160x magnification. In order to ensure a single organism had been isolated, only microdroplets that had a diameter smaller than the microscope field were examined. A microdroplet containing a single organism (confirmed by a second and third person) was flooded with PSG buffer and sucked into a one ml syringe and injected (i/p) into a mouse using a 25G needle. The mouse was bled to death three days later and the whole blood collected was injected (i/p) into another mouse. The three-day passages were repeated until a sufficiently high level of parasitaemia was obtained. At this juncture, stabilates were made as described above in 4.1.3. All mice used for the three-day passages were lethally irradiated before use.

4.1.5 PREPARATION OF SOLUBLE TRYPANOSOME ANTIGEN (STA) FOR ELISA

Trypanosomes were harvested from heavily parasitaemic mice or rats as described by Lanham and Godfrey (1978). Non-rodent adapted *T.(D). vivax* species were separated from infected cow blood and *T.(N). simiae* from pig blood. The harvested trypanosomes were then washed three times with cold PSG buffer pH 8.0 and then once in cold 0.01M PBS pH 7.2. Subsequently, they were suspended in cold PBS and subjected to twenty- seconds ultra-sonication at maximum amplitude on an MSE ultrasonic Disintegrator (Safam Electrical Instruments Co. England). The resulting trypanosome suspension was centrifuged at 10,000g for 30 minutes at 4^{0}C. The protein concentration of the supernatant was determined by the method of Lowry *et al.*, (1951) as modified by Peterson (1979). This supernatant was stored in liquid nitrogen at -196^{o} C and later used as antigen for ELISA.

4.1.6 IMMUNIZATION PROCEDURES

4.1.6.1 Raising Antibodies against Soluble Proteins:

Rabbits were used to raise antibodies against mouse IgG, Goat IgG (Heavy chain) and Goat IgM (Heavy chain) and camel IgG. Two adult male rabbits were used per protein. In the primary immunization each rabbit was injected with 50ug of the protein in Freund's Complete Adjuvant (FCA) intradermally into six sites on the back (0.2ml/site). The rabbits were boosted after one month with 100ug of protein/rabbit in Freund's Incomplete Adjuvant (FIA), intramuscularly (i/m) at six sites (0.2ml/site). The rabbits were reboosted after another month with 100ug of protein/rabbit in 0.01M PBS pH 7.2, i/m (0.2ml/site). The antibody titre was checked by the Double immunodiffusion method of Ouchterlony (1964). Rabbits showing a titre of 1/16 and above were finally bled to death and serum separated and stored as in 4.1.7 below.

4.1.6.2 Raising Antibodies against Sheep Red Blood Cell Stroma (SRBCS).

Four male rabbits were immunized with SRBCS (see Appendix 12) by eleven intravenous (i/v) injections over a period of about two weeks. On day one each rabbit was injected i/v with 0.1 ml of the SRBCS suspension in normal saline. On each of the following five days each rabbit received 1.0ml (i/v) of the same stroma. Thereafter, each rabbit received 2ml of the stroma for five successive days. Rabbits were bled on the fourth and fifth day after the last injection and serum separated as indicated in 4.1.7 below. The serum was then heat inactivated at 56° C for 30 minutes and stored at -20° C until used. The method used here is that of Kabat and Mayer (1961).

4.1.7. BLEEDING AND SERUM PREPARATION

Rabbits were bled from the marginal ear vein. Rats and mice were bled by cardiac puncture, and goats and camels from the jugular vein. Blood in bijou or universal bottles was allowed to stand at room temperature for 2-3 hours until the clot had formed. For each blood sample, the clot was separated from the sides of the container using a glassrod or a wooden applicator stick. The blood samples were then left overnight at 4° C. Subsequently, the serum was then decanted and centrifuged for 30 minutes at 3000g at 4° C. Pasteur pipettes were used to remove serum into clean tubes for storage at -20° C until required.

4.1.8 ISOLATION AND PURIFICATION OF IMMUNOGLOBULINS

4.1.8.1 Partial purification of Immunoglobulins by Salt Franctionation

Saturated ammonium sulphate, $(NH_4)_2SO_4$ (SAS) was prepared by heating 100g of ammonium sulphate in 100ml of distilled water at 50° C, until most of the salt was dissolved. The solution was then allowed to stand overnight at room temperature (RT) and the pH adjusted to 7.0 using either ammonium solution or sulphuric acid to avoid introduction of foreign ions. This SAS was then used to precipitate the gamma fraction from serum, hybridoma

supernatant fluid or mouse ascites fluid. To a given volume of the above fluids SAS was added dropwise with constant stirring on ice to give a final 45% SAS (v/v). After adding the last drop of SAS, stirring was continued for 30 minutes and the salt precipitate left at 4° C for 4 hours. The precipitate was suspended in 45% SAS, the centrifugation process repeated and the supernatant discarded. Finally, the precipitate was dissolved in a minimum volume of 0.01M PBS pH 7.2 and dialysed at 4° C with many changes against the same buffer. The protein was then stored at -20° C until required.

4.1.8.2 Preparation of Goat and Camel IgG (Modified after Johnstone and Thorpe, 1982)

Fifty mls of goat or camel serum was warmed to 25° C and then 9.0g of sodium sulphate was added to make an 18% (w/v) solution and stirred. The mixture was incubated for 30 minutes at 25° C and centrifuged at 4000g for 30 minutes at RT (for the goat serum 4.4g of sodium sulphate was added to make a 14% (w/v) solution. The solution was stirred, incubated and centrifuged as above. The precipitate was retained, redissolved in minimum 0.02M phosphate buffer pH 7.5 and dialysed against 0.02M phosphate buffer pH 7.5 (for camel serum) or against 0.02M phosphate buffer pH 7.8 (for goat serum). The above salt fractions were then applied onto a DEAE-52 cellulose column already equilibrated with the same buffer and the IgG fraction eluted with the same buffer at RT. The absorbance of 5.0ml fractions was monitored at 280nm using a Bechmann spectrophotometer (Beckmann, USA). IgG was eluted in a single asymmetric peak, the rest of the protein contaminants having been held on the cellulose. The first half of the IgG peaks was pooled, concentrated and protein concentration determined by the method of Lowry, *et al.*, (1951) as modified by Peterson (1979) and kept at -20° C.

4.1.8.3 Preparation of Goat IgM (H & L) (Modified after Johnstone and Thorpe, 1982)

Three mls of the 45% Ig (gamma fraction from) (4.1.8.1)

containing 15mg of protein was dialysed overnight against Tris-buffered saline (TBS) pH 7.3 at 4° C and centrifuged at 3000g for 10 minutes to remove debri. The protein solution was then applied to the column of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with TBS at RT. Three-ml fractions were collected and absorbance monitored at 280nm. Each tube of the first half of the peak was kept separately. Five such runs were carried out. Corresponding tubes of the first half of the peak were pooled and concentrated by Ultrafiltration in an Amicon Unit (Amicon, USA) and stored at -20° C until required.

4.1.8.4. Preparation of Goat IgG(H) and IgM (H) (Modified after Johnstone and Thorpe, 1982).

The disulphide bonds between heavy (H) and light (L) chains are easily split by reducing agents (under conditions which leave interchain bonds intact) and they can be alkylated to prevent their re-oxidation. However, the two chains are still held together by non-convalent forces and a dissociation agent is required during chromatographical separation (Fleschman *et al.*, 1963).

1. 0.275ml of dithiothreitol (BDH, England) solution (0.1M dithiothreitol in 0.5M Tris-Hcl buffer pH 8.0 containing 2mM EDTA - freshly mixed), were added to the IgG or IgM (50mg in 2.5ml of the 0.5M Tris-Hcl buffer pH 8.0), flushed with nitrogen and incubated in a sealed tube at RT for 1 hour with stirring.

2. The mixture was cooled on ice and covered with foil and then 0.275ml of iodoacetic acid solution (0.21M iodoacetic acid in Tris-Hcl buffer above freshly mixed) and the mixture incubated in an ice-bath for 30 minutes with stirring.

3. 20ul of dithiothreitol solution were added and the mixture incubated at RT for 15 minutes. The mixtures was then applied onto a column of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with 1.0M propionic acid. One ml fractions were collected and absorbance monitored at 280nm.

4. The first protein peak above was concentrated in an Amicon

ultra-filtration unit and applied directly onto a column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution was again carried out with 1.0M propionic acid. Fractions of 2.5ml were collected and the absorbance monitored at 280nm. Five runs were carried out for IgG and IgM.

5. According to this protocol, the first peak is supposed to consist of aggregated protein and any intact IgG or IgM, the second peak is heavy chains and the third peak is light chains. Corresponding tubes for the second peak were pooled, concentrated and the protein concentrations determined. These were used to immunize rabbits. Sera raised against them was used to test for cross-reactions against all the IgG(H) and IgM(H) fractions to check the purity of the fractions.

4.1.9 GEL PRECIPITATION (DOUBLE-IMMUNODIFFUSION - DID)

The Ouchterlony (1964) DID technique was employed to determine the antibody response in sera of animals and the antibody class and subclass in the hybridoma culture supernatant fluids. Agarose (Merck,West Germany) was used at 1.2 % (w/v) in 0.01M PBS pH 7.2 containing 0.1% sodium azide was used.

4.1.10 IMMUNOELECTROPHORESIS (I.E.P.)

An immunoelectrophoresis test (IEP) modified after the method of Schiedegger (1955) was used to test the purity of the separated immunoglobulins and monoclonal antibodies. The following procedure was followed:

(1) 1.5% agarose (Merck, West Germany) in barbital buffer pH 8.6 (see Appendix 13) containing 0.1% sodium azide was used. Three mls of melted agarose were pipetted per pre-coated microscope slide (see Appendix 1) and allowed to set on a levelling table and the slides transferred to humid chamber.

(2) The pattern for immunoelectrophoresis was then punched out according to Graber and Burtin (1964) and the agar removed from the wells but not the troughs.

(3) Samples (antigens) were then applied in the sample wells using plain glass capillary tubes.

(4) The slides were then placed into an electrophoresis chamber containing barbital buffer pH 8.6, and moistened wicks attached to the slides.

(5) Electrophoresis was allowed to continue for one hour at approximately 8mA per slide under constant voltage.

(6) Thereafter, the slides were removed from the chamber and the agar removed from the troughs. The troughs were then filled with the antisera corresponding with the antigens in the wells.

(7) The slides were then placed in a humid chamber and the reaction allowed to proceed overnight.

8) After reading the wet preparation, the slides were washed, dried and stained, read again and the final results recorded (see Appendix 2).

4.1.11 PROTEIN DETERMINATION

Protein concentrations were measured by the method of Lowry *et al.*, (1951) as modified by Peterson (1979). The method below was used.

(1) 1.0mg/ml solution of the standard protein Bovine serum albumin (BSA), (Sigma, USA) was made and the exact concentration from its absorbance at 280 calculated:

$$\text{Concentration of sample} = \frac{\text{absorbance at 280nm}}{\text{Extinction Coeficiency at 280nm}} \times 10\text{mg/ml}$$

$$E_{280}^{1\%} \quad \text{for BSA} \quad = \quad 6.7$$

(2) An aliquot of unknown solution containing 5-50ng of protein, the same volume of buffer blank, and 0,2,5,10,20,35 and 50, ul of the standard protein solution (the zero is the water blank) was put into separate tubes.

(3) Distilled water added to bring the contents of each tube to the same volume (ideally less than 200ul but the assay will cope with up to 1ml).

(4) 1.0ml of the Copper Sulphate solution (2% (w/v) Copper Sulphate $5H_2O$) and 1 ml of the tartrate solution (4% (w/v)

sodium potassium tartrate), with 48 ml of the carbonate solution (3% ($w/_v$) sodium carbonate in 0.2M sodium hydroxide) were mixed (freshly each time) and 1 ml of this added to each tube, mixed and incubated for 10 minutes at RT.

(5) Then 50 ul of the Folin and Ciocalteu's reagent (BDH, England) were added to each tube, mixed again and 5 minutes later, the absorbance of each tube read at 640nm, using water blank to zero the spectrophotometer.

(6) The absorbance of the protein standard was plotted against the protein concentration and from this the amount of the protein in the unknown read off. Any absorbance of the buffer blank was subtracted and the protein concentration in the original solution calculated.

4.1.12 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

4.1.12.1 Peroxidase Conjugation

Conjugation of McAb, the IgG-fraction of immune sera or 45% salt precipitated immune sera to horseradish peroxidase (HRPO) was carried out by a modified method of Nakane and Kawaoi (1974) as described by Henning and Nielsen (1987). Ten milligrams HRPO (type VI-Sigma Chemical Co., Lois, USA) was dissolved in 2.5ml of double distilled deionized water and mixed with 0.5ml of 0.1M sodium meta-periodate for 20 minutes at room temperature. The aldehyde was dialysed against 0.001m sodium acetate buffer, pH 4.4 at 4^{o} C for 18 hours. Fifty microlitres of 0.2M sodium carbonate buffer pH 9.5 was added to 10mg of HRPO followed immediately by the addition of 2mg of purified McAb, IgG or 45% salt precipitated antibody. The mixture was stirred at room temperature for 2 hours and the reaction stopped by the addition of 0.25ml of ascorbic acid (4mg/ml).

4.1.13 IFAT METHODOLOGY

Commercial goat antimouse IgG (G1, G2a, G2b,G3, Fc and Fab), and Ig (IgG1,G2b,G3, IgM, Fc and Fab) flourescein isothiocyanate isomer1 (FITC) conjugates were purchased from Nordic Immunological Laboratories, Tilburg, the Netherlands.

Frozen fixed blood-stream trypanosomes ($2x10^6$ tryps/ml) prepared as described by Katende *et. al.* (1987), were applied onto Teflon-coated multitest slides (Cooper Wellcome, Frembodgen, Belgium) using a 10-100ul Eppendorf pipette (Eppendorf, Hamburg, West Germany). By dispensing 50 ul and sucking up quickly the trypanosome suspension, a thin layer of trypanosomes remained in each spot. The slides were allowed to dry at RT(21-25^{o} C). Optimal dilutions of monoclonal antibodies were added and the slides incubated at RT for 25 minutes. The slides were then washed three times in 0.01M PBS pH 7.2 (15 minutes/wash). The flourescein conjugate in PBS was mixed with an equal volume of 0.45% Nigrosin (W.S) (S) stain (SDS,Lab.Chem. Industry, Bombay) to give a final dilution of 1/100. This was applied onto the spots and slides incubated at RT for 25 minutes, washed as before, mounted in 50% glycerol 0.01M PBS buffer pH 7.2 and examined for fluorescence, using a Leitz Laborlux K Microscope equiped with epiploem illumination (50W Mercury Lamp), UV filter block, 6.3x eye-pieces and Phaco F1 $40/_{1.3}$ oil objective.

4.1.14 METHODS OF STATISTICAL ANALYSIS

Statistical analysis of sequential time data from different groups of experimental goats and camels was carried out using a two factor repeated measures analysis of variance design (Winer, 1971).

Differences between groups were analysed by the student "t" test for unequal variances (Snedecor and Cochran, 1982) as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}}$$

Using a 5% significance level, the "ta" value was calculated using the following formula:

$$ta = \frac{t(V_1)\frac{SD_1^2}{n_1} + t(V_2)\frac{SD_2^2}{n_2}}{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}$$

The difference between the two samples will be significant at 5% level if "t" value falls below -ta or beyond +ta and non-significant if it falls within the -ta and +ta values. Graphs were drawn from means (x) calculated separately from each group.

4.2 EXPERIMENT ONE

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *TRYPANOSOMA (T) B. EVANSI*

4.2.1 INTRODUCTION:

The derivation of cell lines capable of permanent production of specific antibody directed against a predefined immunogen was reported more than a decade ago (Kohler and Milstein, 1975). This novel method is based on the fusion between specifically selected myeloma cells and plasma cells from suitably immune animals (rats, mice or humans). Plasma cells die in a short time (10-14 days) under ordinary tissue culture conditions. Myeloma cells have been adapted to grow permanently in culture and mutants were isolated that lacked the enzymes hypoxanthine guanine ribosyltransferase (8-azaguanine resistant) or thymidine kinase (bromodeioxyuridine-resistant). Such mutants cannot grow in medium containing aminopterin supplemented with hypoxanthine and thymidine (HAT), because they are unable to utilize the salvage pathway (see Fig.1). Therefore, hybrids between such cells and plasma cells can be easily selected from the parental components as the only cells that actively multiply in HAT selective medium. From the growing hybrids, individual clones can be selected that secret the desired antibodies. Antibodies generated this way are called Monoclonal Antibodies. The selected clones can be

maintained indefinitely. This basic methodology has been used to prepare antibodies against a large variety of antigens as reported in the literature. Fig. 2 summarises the stages of hybridization from immunization to characterization of the monoclonal antibody.

4.2.2 MATERIAL AND METHODS

4.2.2.1 EXPERIMENTAL ANIMALS

Female Balb/c mice from ILRAD were used. They ranged from 8-9 weeks in age.

4.2.2.2 MYELOMA CELL LINE

The parental cell line used was NS-1 (P31/1.Ag4.1). It was derived from P3(MOPC-21) Balb/c mouse myeloma cell line (Kohler and Milstein, 1975). This cell line was obtained from Dr. V.M. Nantulya, ILRAD. Cells of NS-1 are resistant to 8-azoguanine and therefore susceptible to HAT selection. They do not synthesize the MOPC-21 heavy chain. Although the NS-1 cells synthesize MOPC- 21 *K* chain, it is internally degraded (Oi and Herzenberg, 1980).

4.2.2.3 TRYPANOSOME ANTIGENS

Trypanosome (T) b. evansi (KETRI 1342) was used to prepare antigens for immunization. Antigen ONE (1) was prepared by harvesting plasma from Balb/c mice infected with KETRI 1342 and showing parasitaemia of more than $1x10^8$ trypanosomes/ml. Blood was harvested from such mice into bijou bottles containing 10% disodium-EDTA as an anti-coagulant. This blood was then centrifuged at 6,000g for 30 minutes at 4^o C. This plasma rich in circulating trypanosome antigens was stored in liquid nitrogen until used.

Antigen TWO (II) was prepared by harvesting trypanosomes from heavily parasitaemic Balb/c mice infected with KETRI 1342 as reported by Lanham and Godfrey (1970). The harvested trypanosomes were washed three times in cold PSG buffer, pH 8.0 and once in cold 0.01M PBS pH 7.2. The trypanosomes were then suspended in the latter buffer and lysed by repeated freezing in liquid nitrogen and thawing at 37^o C. Thereafter, the

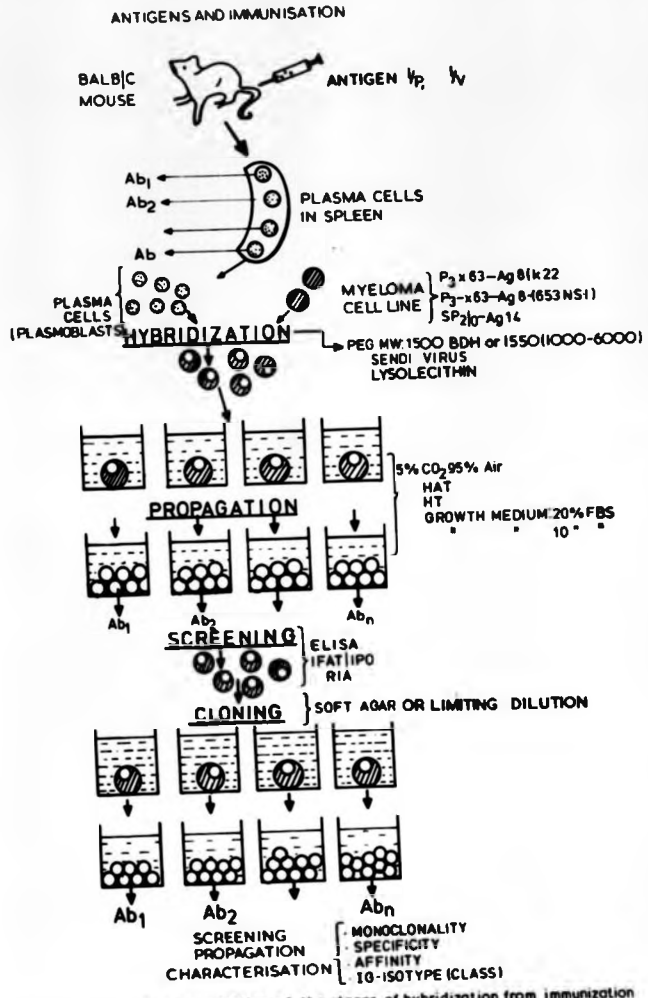


FIG. 2 Digrammatic representation of the stages of hybridization from immunization to characterization of monoclonal antibody.

trypanosome lysate was centrifuged at 6,000g for 30 minutes at 4° C. The supernatant was discarded and the pellet washed three times with cold PBS by suspension and centrifugation as indicated above. The pellet was then suspended in cold PBS and subjected to twenty-seconds ultrasonication of maximum amplitude on an MSE ultrasonic Disintegrator (Safam Electrical Instrument Co. England). The resulting sonicate was centrifuged at 10,000g for 30 minutes at 4° C, the protein concentration determined as reported in 4.1.11 and kept in liquid nitrogen.

4.2.2.4 IMMUNIZATION

Ten adult female Balb/c mice (8-9 weeks old) from ILRAD were each immunized (i/p) with 0.2ml of antigen I in Freund's complete Adjuvant (FCA). Four weeks later, each mouse was boosted (i/p) with 0.2ml of the antigen in Freund's incomplete Adjuvant (FIA) and repeated after another four to sixteen weeks. The 3rd, 4th and 5th boosts were done on day one, two and three before fusion, respectively and fusion carried out on day four. For the 3rd, 4th and 5th boosts, each mouse received 0.2ml of the antigen i/p and 0.2m i/v without adjuvant being added.

Immunization with antigen II followed the same protocol described above except that the number of mice was doubled and the initial antigen dose was 50ug in CFA per mouse, followed by 50ug in IFA per mouse, then 400ug per mouse in PBS for each of the last three days before fusion. For each of the last three days, each mouse received 200ug i/p and 200ug i/v. This aggressive immunization protocol was modified after Stahli *et al.* (1980). The antibody titre for each mouse was determined by ELISA. Only mice with the highest ELISA readings were selected for fusion.

4.2.2.5 FUSION

The fusion protocol employed was modified after Pearson *et al.* (1980). Four days after the last boost, spleens were asceptically removed from the immunized Balb/c mice, teased with curved forceps and spleen cells suspended in warm (37° C),

serum-free RPMI-1640 (Flow Laboratories, Irvine KA 12 8NB, Scotland). The spleen cells were washed once with the same medium. Meantime, NS-1 cells which had been grown earlier (in RPMI-1640 containing 10% Fetal Bovine Serum (FBS) (Flow Labs. Irvine, Scotland) and maintained in a logarithmetic phase, were similarly washed. Spleen cells and NS-1 cells were counted in a haemocytometer to determine their concentration in cells/ml. Spleen cells and NS-1 cells were then mixed to the ratio of $2X10^8$:$2X10^7$ and suspended in serum free RPMI-1640 as above. The cell mixture was centrifuged at 600g for 5 minutes and the medium drained as much as possible. The cells were suspended and fused by adding drop-wise 0.5ml of polyethylene glycol (PEG) solution I (41.6% PEG, 15% DMSO), over a period of one minute to allow mixing and agglutination of cells. Immediately, an additional 0.5ml of PEG solution II (25% PEG in serum-free RPMI-1640) was added dropwise over a period of 2-3 minutes and the cell mixture centrifuged at 600g for one minute. The cells were then suspended gently without decanting the supernatant and 4mls of Fusion medium (containing 20% FBS) added slowly over a period of 2-3 minutes. Finally, 20ml of Fusion medium were added, the cells mixed gently, 0.5ml amounts of the cell suspension dispensed into 48 wells of multiwell culture plates (Linbro, Flow Laboratories, U.K.) and 1.5ml amounts of the fusion medium added. A drop of spleen cells (in Fusion medium) containing $1x10^5$ cells was added to each well to act as feeder cells. The cells were then grown at 37^o C in a Heraeus Model B 5060/k/Co_2, incubator (Heraeus, West Germany) in a 5% Co_2 95% air atmosphere.

4.2.2.6 THE ELISA TECHNIQUE FOR SCREENING HYBRIDOMA SUPERNATANTS

The antigen for this ELISA technique was prepared as described in 4.1.5. The wells of flat-bottomed 96-well microtitre polystyrene plates (Linbro, U.K.) were each coated with 200ul containing 10ug of the soluble antigen in carbonate buffer pH 9.6 for 3 hours at 37^o C and overnight at 4^o C. The plates were then washed three times at 5 minutes intervals using 0.01M PBS pH 7.4 containing 0.05% Tween-20. Thereafter, 0.5% Bovine Serum Albumin (BSA) in the PBS buffer was added and

incubated for one hour at 37° C. The washing process was repeated as before. Rabbit antimouse Ig gamma/horse radish peroxidase conjugate (diluted 1/500 in the PBS buffer) was added at a rate of 200 ul per well and the plates incubated at 37° C for 1 hour. The washing process was repeated as stated above, and the plates shaken dry. The enzyme substrate (0.1% O-phenylenediamine, Sigma, USA) was added at a rate of 200ul per well and incubated at room temperature in the dark for 30 minutes. The reaction was stopped by adding 100ul per well of 1.0M Sulphuric acid before reading the optical density (OD) in a micro-ELISA auto-reader (Model MR-580, Dynatech, USA) at a wavelength of 492nm. All samples were tested in duplicates. This protocol was also used for testing ascites fluids, and monoclonal antibodies.

4.2.2.7 GROWTH OF ANTIBODY-PRODUCTION CELL HYBRIDS

Two days post-fusion, 1.5ml amounts of the Fusion medium were removed from each well and replaced with 1.5ml of HAT medium (see Appendix 9). As soon as wells showed clones of growing cells, supernatants were tested for antibody activity using a solid phase ELISA, and the medium switched to HT (See Appendix 9). Clones of hybrids showing positive antibody activity were immediately cloned and recloned by limiting dilution in cloning medium (see Appendix 10) and retested using ELISA. Positive hybrid reclones were expanded in $25cm^2$ (50ml) tissue flasks (Costar, USA) and grown until $2x10^7$ cells could be frozen in liquid nitrogen (see Appendix 11). Once selected hybrids were growing well, the HT medium was replaced by normal growth medium (NGM) (see Appendix 3).

4.2.2.8 CLONING BY LIMITING DILUTION

Peritoneal macrophages were harvested from Balb/c mice by flushing the peritoneal cavity of Balb/c mice with 4.5ml of cold 0.34M sucrose using an 18- gauge needle. The cells were then centrifuged at 600g for 10 minutes, resuspended with the cloning medium (Appendix 10) at 37° C and counted in a haemocytometer. The macrophages were seeded into 48-well tissue culture plates

used above (at a rate of 10 cells/well) and cloning medium added to a final 1.1mls per well. The cells were then cultured overnight in a CO_2 incubator as stated earlier. The next day, 0.1ml of hybrids in the same cloning medium as above were added at a rate of 1 cell per well, 5 cells per well and 10 cells per well and allowed to grow for 3-5 days. Then 1.0ml of the cloning medium was added without removing the spent medium. The cells were checked twice a week for the growth of clones.

Once clones were evident, they were retested, using ELISA for antibody activity, expanded and frozen as above.

4.2.2.9 THE CLASSES AND SUBCLASSES OF THE MONOCLONAL ANTIBODIES

The classes and subclasses of the monoclonal antibodies was determined by the gel double immunodiffusion technique of Ouchterlony (1964) as described in 4.1.9.

4.3. EXPERIMENT TWO
APPLICATION AND EVALUATION OF ANTI-*T.(T).B. EVANSI* MCABS AS TRYPANOSOME TYPING REAGENTS

4.3.1. INTRODUCTION:

In an effort to improve on the diagnosis of trypanosomiasis McAbs were developed against antigens of *T.(T).b. evansi* bloodstream forms. The pattern of reactivity of these McAbs with African Trypanosome species as well as other parasites of man and domestic animals was investigated.

4.3.2. SEROTYPING

The indirect enzyme linked immunosorbent assay (ELISA) and the Indirect immunofluorescent antibody test (IFAT) were used to study the pattern of reactivity of all the McAbs produced with African trypanosome species as well as other parasites of man and domestic animals as indicated in Tables 11 and 12.

4.3.2.1 ELISA

The assay was performed on STA antigen prepared from

lysates of bloodstream forms of the species of African trypanosomes shown in Table 12. The Elisa protocol was as stated in 4.2.2.6. The STA from each trypanosome species was prepared as described in 4.1.5.

4.3.2.2 IFAT

The antigen for each trypanosome species used in the study was prepared by the method of Katende *et al*; (1987) and the test carried out as stated in 4.1.13. Results were scored according to the intensity of fluorescence and the number of trypanosomes stained as follows:-

(+++) = all trypanosomes stained (very strong fluorescence).
(++) = all trypanosomes stained (strong fluorescence).
+ = only slight staining (weak fluorescence).
- = No observable staining (no fluorescence).

4.4. EXPERIMENT THREE

APPLICATION OF ANTI-*T.(T).B. EVANSI* MONOCLONAL ANTIBODIES AS ANTIGEN DETECTING PROBES:

4.4.1. INTRODUCTION

As the prime objective of this study was to apply the produced McAbs as probes for the detection of circulating trypanosomal antigens in sera of infected animals, this experiment was designed in an attempt to detect by sandwich ELISA the relevant antigens in lysates of bloodstream forms of *T.(T).b. evansi* KETRI 1342 using monoclonal antibody-coated micro-ELISA plates. This approach would then serve as a useful prelude for designing an assay for the study of circulating trypanosomal antigens in relationship to parasitaemia, IgG and IgM antibodies, total haemolytic complement and clinical disease (see Experiment 4.5). The findings of this experiment and Experiment 4.5 would then be used to design an assay for the detection of circulating trypanosome antigens in infected field camels.

4.4.2. PREPARATION OF SOLUBLE TRYPANOSOME ANTIGEN

Soluble trypanosome antigen (STA) was prepared as described in 4.1.5. Protein concentration of the final STA preparation was determined as described in 4.1.11. This final STA preparation was then divided into 200ul aliquots and then stored in liquid nitrogen until required for use.

4.4.3. PURIFICATION OF MONOCLONAL ANTIBODIES (MCABS)

McAb-rich ascites fluid was produced in Balb/c (6-8 weeks old). Of the three groups of Balb/c mice mentioned in 4.1.1.1., the ILRAD mice were the best producer of McAb-rich ascites fluid. This strain was the one used for subsequent production of McAb-rich ascites fluid. Balb/c mice were first primed with pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Company, Milwaukee, Wisconsin, USA). After 2 weeks, $1x10^7$ viable hybrid cells suspended in medium (RPMI - 1640) were inoculated per mouse. For each McAb-hybrid, 25-50 pristane primed Balb/c mice were inoculated. The mice were then kept under observation until ascites fluid was noticed to be accumulating in the abdominal cavity (about 2-4 weeks after inoculation). The ascites fluid was then removed by "tapping" the mouse' abdominal cavity using a sterile 16-gauge needle. The fluid that dripped off was collected in a tube and centrifuged at 600g at room temperature for 10 minutes. The supernatant was further spun at 5000g, but the cells were either stored in liquid nitrogen as described in Appendix 11, or used to inoculate more pristane-primed Balb/c mice. IgM fraction was obtained from ascites fluid as described by Rurangirwa *et al.* (1986). Salt precipitation with 45% ammonium sulphate was used to obtain IgG-rich fractions from ascites fluids of mice inoculated with hybrid clones secreting IgG mouse immunoglobulin subclasses.

Final McAb fractions were concentrated by ultrafiltration in an Amicon Unit (Amicon, USA) and protein concentration determined as in 4.1.11.

4.4.4. PREPARATION OF PEROXIDASE-MONOCLONAL ANTIBODY CONJUGATES

Horse-radish Peroxidase Type VI (Sigma Chemical Company, USA) was used to label the following selected monoclonal antibodies (McAbs): TE A1/23:4.6, TE A5/48.28.5, TEM2/3.3.16, TEM8/2.4.10, TEM5/14.4.16. The class and subclass of these McAbs are given in Tables 9 and 10. The method employed in the conjugation is as described in 4.1.12.1.

4.4.5. TITRATION OF ENZYME CONJUGATES BY DIRECT ELISA

Soluble trypanosome antigen (STA) described in 4.1.5. was used in a block titration to determine the optimal conjugate dilution. The method was modified after Nantulya *et al.* (1987). Briefly, the wells of flat- bottomed 96-well microtitre plates (Dynatech, USA) were coated with 100 ul containing 10 ul of the STA in carbonate buffer pH 9.6 for one hour at 37° C. Thereafter,the plates were left at 4° C overnight. The plates were washed three times at 5 minute interval using 0.01M PBS pH 7.4 containing 0.05% Tween 20. Thereafter, the plates were then shaken dry and serial dilutions of the conjugate in 0.01M PBS containing 0.05% Tween 20 and 1% normal Bovine serum added to the wells (100ul per well). The plates were then incubated at 37° C for 30 minutes with intermittent shaking and thereafter, washed as before. The enzyme substrate OPD (0-phenylenediamine, Sigma, USA) was then added and incubated at room temperature in the dark for 30 minutes, before reading the optical density in a micro-ELISA autoreader (Model MR 580, Dynatech Instruments, Inc., Torrance, California, USA) at 492nm.

4.4.6. DETERMINATION OF THE OPTIMAL AMOUNTS OF McABs FOR COATING OF MICRO-ELISA PLATES FOR USE IN SANDWICH ELISA:

A block titration of the McAbs listed in 4.4.4 above was carried out in order to determine the optimal amount of antibody required to coat the micro ELISA plates for use in subsequent sandwich antigen - ELISA. Plates were coated with the unlabelled McAbs in coating buffer as in 4.4.5 above. After washing, STA was added to the plates in serial dilutions to allow the coating antibody to bind the relevant antigen present in the STA solution. Subsequently, the corresponding horse

radish peroxidase-labelled McAbs were added at the optimal dilutions determined in 4.4.5 above and the reaction of the conjugate with bound antigen revealed by the addition of the substrate. The ELISA protocol was similar to the one used in 4.4.5. above.

4.4.7. DETERMINATION OF THE LOWEST AMOUNT OF SOLUBLE TRYPANOSOME ANTIGEN DETECTABLE BY OPTIMAL CONCENTRATIONS OF McABs GIVEN IN 4.4.4.:

Micro-ELISA plates were coated with optimal concentrations of McAbs (see Table 14) and incubated as stated in 4.4.5 above. After washing as stated in 4.4.5, doubling dilutions of *T.(T).b. evansi* KETRI 1342 lysate were made in the diluting buffer (0.01M PBS pH 7.4 containing 0.05% Tween 20) and 100 ul applied into each well. Subsequent incubation and washing was as stated in 4.4.5. above and enzyme optimal dilutions of McAb conjugates applied, plates incubated, washed and the enzyme substrate added and the plates read as in 4.4.5 above.

4.5. EXPERIMENT FOUR

THE RELATIONSHIP BETWEEN CIRCULATING TRYPANOSOME ANTIGENS, PARASITAEMIA, IgG AND IgM ANTIBODIES, TOTAL HAEMOLYTIC COMPLEMENT AND CLINICAL DISEASE:

4.5.1. INTRODUCTION

Any trypanosome molecule which has at least some epitopes to which high affinity antibodies can be induced in an infected host may form immune- complexes if released into the circulation of an infected host. It is readily conceivable that at certain points during the course of infection, the antigen-antibody equilibrium in tissue fluids might shift such that the free antigen may not be detected (Nantulya *et al.*, 1987). Such an antigen can only be detected if some of its epitopes are not masked during immune-complex formation. However, Wilson *et al.*

(1975) and Houba *et al.* (1976) were able to demonstrate parasite antigens in sera which also contained antibodies. It is possible that some of the parasite molecules released into circulation may not be immunogenic or highly immunogenic. Such molecules can easily be detected in sera of infected hosts using high affinity polyclonal or monoclonal antibodies as detecting probes. Nevertheless, to design immunoassays for detecting such antigens in sera of infected hosts, it is necessary to study the course of such antigenaemia in relationship to antibody response, complement and related clinical parameters before their application in field diagnosis can be recommended. This experiment was designed with this in mind.

4.5.2. MATERIALS AND METHODS

4.5.2.1 Experimental Goats:

Three groups of goats were used (see Table 3). The goats ranged in weight from 20-25kg. Initially all the goats were eartagged and treated with diminazene aceturate (Berenil, Hoechst, Germany) at a dose rate of 7mg/kg body weight, Imidocarb dipropionate (Imizol, Wellcome, Kenya Ltd.) at a dose rate of 0.5mg/kg body weight, Oxytetracyline (Terramycin, Pfizer, Canada) at a dose rate of 10mg/kg body weight and drenched with 10% Fenbendazole (Panacur,Hoechst, Germany) at a dose rate of 7.5ml/100kg body weight. The goats were then confined in a fly-proof barn for two weeks before the start of the experiment. Before goats were infected, they were checked for a possible trypanosome and helminth infection using standard parasitological techniques. Throughout the experiment, the goats were fed on hay, ranch cubes and given water *ad libitum*.

4.5.2.2. Experimental Procedures

4.5.2.2.1. Clinical Examination

Before the goats were infected, they were clinically examined for temperature, respiratory and heart rate changes. All the goats were then bled from the jugular vein and blood used for plasma and serum separation, packed cell volume (PCV) determination and parasitological examination. Subsequently,

daily rectal temperatures, PCV and parasitological examination of jugular blood were done except where indicated (see Tables 16-18). Blood for serum plasma was taken on the days indicated in Tables 18-22. Blood for plasma was collected and plasma harvested as described below (4.5.2.2.2.). Serum for antigen and antibody - ELISA, and plasma for antigen-ELISA were kept at -20^{o} C and serum for total haemolytic complement in liquid nitrogen.

4.5.2.2.2. PCV Determination and Parasitological Examination:

Blood for plasma was collected in bijou bottles containing 5mg of disodium ethylene diamine tetracetic acid (EDTA). This was enough for 5mls of blood. After thorough gentle mixing, non-heparinized capillaries were filled with EDTA-blood and sealed with cristoseal. The capillary tubes were then placed symmetrically in a microhaematocrit centrifuge (Hawksley, England) with sealed ends outmost and centrifuged for 5 minutes. The tubes were then placed in a haematocrit reader (Hawksley, England) and PCV readings expressed as a percentage. After PCV determination, the same capillaries were cut just below the buffy-coat red blood cell junction and the buffy-coat applied onto glass microscope slides, and covered with a 22 x 22 glass cover slips. The preparations were then examined using a Zeiss Lab 16 microscope with a 11/Z condenser, Ph 2 40/0.65 objective and CPL W 10x18 eyepieces. In each case the whole field of the buffy-coat preparation was examined, the number of trypanosomes per preparation recorded and the concentration of trypanosomes/ml scored as reported of Murray *et al.* (1977).

Table 3. Experimental groups of the goats used to study the levels of circulating trypanosome antigens parasitaemia, IgG and IgM antibodies, total haemolytic complement and clinical disease.

Group	Goat No.	Experimental details
ONE	1 3 5 14 15	All goats were infected with *T.(T).b.evansi* KETRI 1342($2x10^6$ trypanosomes per goat) by syringe and needle i/v inoculation.
TWO	2 4 6 8 10 12	All goats were infected with *T.(T).b. evansi* KETRI 1342 ($2x10^6$ trypanosomes per goat) by syringe and needle i/m inoculation.
THREE	7 11 13	Non-infected control group

4.5.2.2.3. Serum, Plasma and Cerebrospinal fluid (CSF) Preparation:

Serum and Plasma were prepared as reported in 4.1.7. Cerebrospinal fluid was collected by lumbar puncture of goats in the lumbo-sacral space, using 11/2 F 18 sterile needles. Goats were anaesthetized with Rompun 2% solution (2- (2.6.-Oxylidino_--5,6,-dihydro-4H-1, 3-thiazine-hydrochloride). Each goat was injected i/m with 0.2ml of Rompun. Before CSF was centrifuged (at 4000g for 10 minutes at 4^{o} C), the colour, turbidity or evidence of coagulation were checked. Any CSF sample which was contaminated with blood was discarded. After centrifugation, the clear supernatant was kept at -20^{o} C for antigen and antibody assay. The precipitate was checked for evidence of trypanosomes.

4.5.2.2.4. Quantitation of Total Haemolytic complement in Goat Sera:

Total Haemolytic complement in goat sera was determined by the method of Lachmann and Horbart (1978) and the results expressed as 50% haemolytic units (CH_{50}) per ml of goat serum. The procedures are as described below.

4.5.2.2.4.1. Determination of Optimal concentration of Haemolysin:

Sheep red blood cells (SRBC) were added to serial dilutions of haemolysin in tubes as shown in Table 4. After incubating the tubes for ten minutes at 37^{o} C, 5.5. ml of Veronal buffered saline (VBS) pH 7.4 and 1.0ml of normal goat serum (NGS) were added to all tubes while +ve and -ve controls had 6mls of distilled water and 6mls of VBS, respectively. The tubes were then incubated again at 37^{o} C for one hour, centrifuged at 400g for 10 minutes and the optical density (OD) of the supernatant read at 415 nm using a Bechmann Spectrophotometer (Bechmann, USA). The minimum dilution of haemolysin (1:64) that gave the OD similar to that of the positive control (100% lysis of the one minimal haemolytic dose, 1MHD) was selected and used to sensitize SRBC for subsequent complement assays.

Table 4. Determination of the optimal concentration of haemolysin (Rabbit anti-SRBC).

Tube No. in duplicate	1	2	3	4	5	6	7	8
0.5ml of SRBC 10^{9}/ml	+	+	+	+	+	+	+	+
0.5ml of haemolysin in dilution of	1/2	1/4	1/8	1/16	1/32	1/64	-	-
Incubate the tubes at 37^{0}C and then add 5.5ml of VBS	+	+	+	+	+	+	+	+
6.0ml Distilled water	-	-	-	-	-	-	-	+
1.0ml 1:10 Normal goat serum	+	+	+	+	+	+	+	+

4.5.2.2.3.2. Sensitization of SRBCs:

Twenty to thirty ml of packed SRBC were washed with Veronal-buffered saline (VBS) pH 7.4 (containing $5x10^{-4}$M $Mgcl_2$, $1.5x10^{-4}$M $CaCl_2$ and 0.1% gelatin) three or more times by centrifugation at 400g for 5 minutes, until the supernatant was clear. The cell concentration was then adjusted to $1x10^9$ per ml (by haemocytometer counting) after which equal volumes of this cell suspension and a diluted one-minimal haemolytic dose (1MHD) rabbit anti-SRBC (haemolysin) were mixed gently before EDTA was added to a final concentration of 10^{-3} Molar. The mixture was incubated at 37^0C for 15 minutes and then on ice for 15 more minutes. After this, the cells were washed three times with VBS and the concentration adjusted to $2.5x10^8$ cells/ml as above. These cells were used in the quantification of total haemolytic complement.

4.5.2.2.3.3. Quantification of Total Haemolytic Complement in Test Samples:

Samples of each serum dilution ranging from 25-125ul were transferred into glass-test tubes and each sample volume was made upto 200ul by adding VBS. Two hundred microlitres of sensitized SRBC were then added to each tube and the mixture incubated at 37^o C for 30 minutes with occasional shaking of tubes. Before centrifugation, 2ml of cold VBS were added to the test samples and the negative control, while 2ml of distilled water were added to the positive control. The OD of the supernatants was then read in a Bechmann Spectrophotometer at 415nm to determine the amount of haemoglobulin released and the % haemolysis (Y) for each supernatant calculated as below:

$$\% \text{ haemolysis } (Y) = \frac{= \text{OD of sample (at 415nm)}}{\text{OD of +ve control (at 415nm) (100\% lysis)}} \times 100$$

All samples (25,50,75,100 and 125ul) obtained from 1:40 dilution gave values much below 54% lysis. In order to calculate the total haemolytic complement in each sample of goat serum only "Y" values of samples from 1:20 dilution were used. Total haemolytic complement was then calculated as follows:

The values of Log_{10} (Y/100-Y) and $Log10^X$ where X stands for

25,50,75,100 or 125 ul were determined and a graph of $Log10^{X}$ versus Log_{10} (Y/100⁻Y) plotted. From the graph, the values of the intercept of the $Log10^{X}$ axis was read and its anti-log determined. Dividing 1000 by the anti-log of the intercept gave the values of CH_{50} units of the complement per ml of whole goat serum, one CH_{50} unit being defined as the quantity of complement in the serum which was required to lyse 50% of the sensitized SRBC (Lachmann and Hobart, 1978). In the subsequent assays of total haemolytic complement in all goat sera the initial serum dilution was 1:20 and the assay was perfomed as above (see Table 5).

Table 5. Quantification of total goat Haemolytic complement in experimental and negative control goats. (Scheme of testing).

Tube No. in Duplicate	1	2	3	4	5	-ve 6	+ve 7
VBS (ml)	2	2	2	2	2	2	-
Normal saline (ul)	175	150	125	100	75	200	-
Distilled water (ml)	-	-	-	-	-	-	2
Diluted serum (ul)	25	50	75	100	125	-	-
SRBC $2.5x10^{8}$ cells/ml	+	+	+	+	+	+	+

4.5.2.2.4. Serological testing:

4.5.2.2.4.1. ELISA for the quantification of goat anti-trypanosome IgM and IgG - antibodies-(IgM Ab-ELISA and IgG Ab-ELISA)

STA antigen from *T.(T).b. evansi* KETRI 1342 was used to coat Dynatech (Dynatech, USA) micro-ELISA plates at an optimal concentration of 10 ug/well of STA antigen in 200 ul/well of carbonate buffer pH9.6, by incubating plates at 37° C for 3 hours and at 4° C overnight. The plates were then emptied, rinsed

three times with PBS - Tween 20 buffer pH 7.4, washed three times (by soaking for 5 minutes per wash) and dried on absorbent tissue paper. To each micro-well, 200ul of 0.5% bovine serum albumin (in PBS Tween 20 buffer) was added and plates incubated at 37^{O} C for 30 minutes. The rinsing, washing and drying process was repeated as above, before 200ul/well of test and control goat sera diluted 1/200 in PBS - Tween 20 buffer were added and plates incubated for an hour at 37^{O} C. The rinsing, washing and drying process was repeated and 200ul/well of rabbit anti-goat IgM-PO or Rabbit anti-goat IgG-PO conjugates were added at the optimal dilution of 1:500 and 1:1000, respectively. The plates were again incubated at 37^{O} C for an hour as above before the enzyme substrate, ortho-phenylenediamine (OPD, Sigma, USA) was added (200 ul/well) and plates incubated at room temperature in the dark for 30 minutes. The enzyme- substrate reaction was stopped by adding 100 ul/well of 1.0M Sulphuric acid and optical density (OD) readings of each well read on a Dynatech Micro-ELISA autoreader (Model MR 580) at a wave length 492nm. All goat sera were tested in duplicates while negative and positive control sera were tested in quadriplicates per microplate.

4.5.2.2.4.2 Enzyme-Linked immunosorbent assay for the quantification of circulating trypanosome antigens in serum, CSF and Plasma of goats infected with *T.(T).b. evansi* KETRI 1342 (Ag-ELISA):

Monoclonal antibody TEAI/23.4.6. was used for the detection of circulating trypanosome antigens because of its high titre and stability to freezing and thawing and salt precipitation. Dynatech micro-ELISA plates were coated with 25ug/well of the McAb in 200ul/well of the coating buffer (carbonate buffer, pH 9.6) by incubating plates at 37^{O} C for 1 hour and then overnight at 4^{O} C. The plates were then emptied, rinsed three times with PBS- Tween 20 buffer pH 7.4, washed three times (5 minutes/wash) and dried on absorbent tissue paper. To each micro-well, 200 ul of undiluted serum CSF or plasma was applied and plates incubated at 37^{O} C for 1 hour. The rinsing, washing and drying process were repeated as above, before 200 ul/well of TEA1/23.4.6.- PO conjugate diluted 1:1000 was added and the

incubation process repeated for 1 hour at 37° C. Thereafter, the plates were rinsed, washed and shaken dry on absorbent tissue paper as above. The remaining stages of the test remained as in 4.5.2.2.4.1 above.

4.6. EXPERIMENT FIVE

APPLICATION OF ANTI-*T.(T).b. EVANSI* McABs FOR THE DIAGNOSIS OF PATENT TRYPANOSOME INFECTION IN CAMELS:

4.6.1 INTRODUCTION

In this study, McAb TEA/23.4.6. and McAb TEA1/23.4.6 - horseradish peroxidase conjugate were used in a double antibody sandwich immunoassay technique (Ag-ELISA) for the detection of trypanosomal antigens in sera and plasma of field camels.

4.6.2 MATERIALS AND METHODS

4.6.2.1 Camel Herds

Table 6 shows the camel herds sampled during the study.

Table 6. Camel Herds examined during the Application of anti *T.(T).b. evan* McAb TEA1/23.4.6 in an Antigen-ELISA sandwich immunoassay for t diagnosis of Camel trypanosomiasis.

Herd No.	Location	Frequency of sampling	No. of Camels in the herd (n)	Remarks
1	Ngare Ndare (Meru District Kenya)	2	14 (14)	Experimental Government camels Trypanosomiasis was endemic in the area.
2	Athi-River (Machakos District of Kenya	1	50 (31)	All camels privately owned. No record of camel trypanosomiasis in the herd.
3	Athi-River	4	70 (70)	Some of the camels belonged to the Kenya Govt., the rest were privately owned. Trypanosomiasis was endemic in the herd. Drug resistance to suramin suspected.
4	Athi-River	1	83 (83)	

NB. (n)=number sampled

4.6.2.2 Herd One

This camel herd was on an experimental drug trial (Dr. Mary Otsyula). The 14 camels were all positive on buffy coat examination (HCT). The infecting trypanosome species in all the 14 camels was determined by the morphological identification of Giemsa stained thin smears and found to belong to *T.(T). brucei* - subgroup. On day 0 of this experiment, the camels were all bled from the jugular vein and blood collected for PCV determination, serum and mouse sub-inoculation (MI), before they were treated by the experimental trypanocide, x RM110 (May and Baker) according to the groups shown in Table 27. Subsequently, the camels were bled and examined for the evidence of parasitaemia (by HCT and MI tests) and for serum. Unfortunately only serum for day 0 and day 90 could be available for testing, so only the results of day 0 and day 90 are included (see Table 27).

4.6.2.3 Herd Two

This herd was examined only once. Only 31 camels could be examined (by permission of the owner). All the 31 camels were bled from the jugular vein and blood collected for PCV determination, HCT and MI and serum for serology.

4.6.2.4 Herd Three

Camels in this herd had been moved from Ngare Ndare in Meru District to Athi-River. All the camels were males. On day 0, all the camels were bled from the jugular vein and blood collected for PCV determination, HCT and MI for parasitological examination and serum and plasma for serology. Subsequently, only 55 identifiable camels were selected for further examination on days 14, 28 and 48.

The 55 camels were selected for further observation because they had proper identification and could therefore be identified on subsequent examination. The camels in herd 4 were bled and examined only once. Because of the high prevalence of trypanosome infection in herd 3 and because both herd 3 and 4 were being kept on the same ranch, all camels were treated with the trypanocidal drug trypacide (May and Baker) (quinapyramine Sulphate) at 5 mg/kg body weight (by subcutaneous injection of 10% solution of the reconstituted drug in sterile distilled

water. This approach provided an ideal opportunity to evaluate the trypanocidal efficacy of the drug from the results of parasitological examination and Ab-ELISA and Ag-ELISA results. At the same time, it was possible to determine how long trypanosome antigens remained in circulation after successful chemotherapeutic treatment.

4.6.2.5 Herd Four

This herd had also been moved from Ngare Ndare to Athi-River. It was composed of female camels only. These camels were examined only once and treated on the same day (Day 0) when herd 3 camels were being bled.

4.6.2.6 Serology

4.6.2.6.1 Antibody-ELISA (Ab-ELISA)

In order to carry out Ab-ELISA on the test serum samples, a block titration of the rabbit anti-camel IgG-peroxidase conjugate, the negative and positive sera and STA antigen prepared from KETRI 1342 was performed to determine the optimal dilutions of sera, antigen and conjugate. Subsequently, Dynatech micro-ELISA plates were coated with 10 ug/well of STA antigen in 200 ul/well of the carbonate buffer pH 9.6 by incubating plates at 37° C for 3 hours and at 4° C overnight. The plates were then emptied, rinsed three times with PBS-Tween 20 buffer pH 7.4, washed three times (5 minutes/wash) and dried on absorbent tissue paper. To each micro-well, 200 ul of 0.5% BSA in PBS-Tween 20 was added and plates incubated at 37° C for 30 minutes. The rinsing, washing and drying process was repeated as above before 200 ul/well of test and control sera diluted to 1:100 in PBS-Tween 20 buffer above were added and plates incubated for 1 hour at 37° C. The rinsing, washing and drying process was repeated and the conjugate added (200 ul/well) at an optimal dilution of 1:500. The plates were again incubated for 1 hour as above before the enzyme substrate (OPD) added (200 ul/well) and plates incubated at room temperature in the dark for 30 minutes. The enzyme - substrate reaction was stopped by adding 100 ul/well of 1.0M Sulphuric acid and optical density (OD) readings of each

micro-well read on a Dynatech Micro-ELISA auto reader (Model MR 580) at a wavelength of 492nm. All test sera were tested in duplicate while negative and positive were tested in quadruplicates per plate.

4.6.2.6.2 Antigen - ELISA (Ag-ELISA).

Antigen-ELISA (Ag-ELISA) was perfomed as described in 4.5.2.2.4.2. The test was performed on both camel plasma and serum.

4.6.2.7. PCV and Parasitological examination:

Packed cell volumes and buffy-coat examination by the micro haematocrit centrifugation technique (HCT) of camel blood for trypanosome infection was carried out as described in 4.5.2.2.2. Mouse sub-inoculation (MI) was done in the field immediately after bleeding the camels. For each EDTA camel blood sample (see 4.5.2.2.2) two mice were injected 1/p with 0.5 mls of the blood. The mice were then placed in clean cages and given food and water *ad-libitum*. Parasitaemia was checked daily by the examination of wet smears made from mice tail blood. Infecting trypanosome species were identified by their morphological characteristics in Giemsa stained thin smears. The mice were kept for a period of 60 days.

CHAPTER FIVE

RESULTS

5.1. PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *T.(T).B. EVANSI* KETRI 1342:

5.1.1. GROWTH OF NS-1 CELLS IN RPMI-1640 MEDIUM SUPPLEMENTED WITH 10-20% FBS:

NS-1 cells grew very well in RPMI-1640 supplemented initially with 20%FBS. After cells showed evidence of monolayer growth, the medium was supplemented with 10% instead of 20% FBS(see photograph in Fig. 3.). It was necessary to subculture every second or third day to keep the cells in the logarithmic phase.

5.1.2. FUSION AND GROWTH OF ANTIBODY-PRODUCING CELL HYBRIDS:

Tables 7 and 8 show the results of sixteen cell fusions performed during the whole period of the study. A reasonably high fusion success was obtained with both antigens. The period at which growing hybrid clones showed evidence of active growth varied from 5-20 days (see Fig.4a and 4b). As shown by Tables 7 and 8, the % hybrid clones ranged from 24-100% and was higher with antigen I than antigen II. Only three fusions were contaminated with (*Staphylococcus* species and yeast organisms). A reasonably high percentage of positive hybrid clones was achieved. A total of 140 positive hybrid clones was obtained out of which 71 were lost due to a week-end sudden drying of the liquid nitrogen pot into which the hybrid cells had been preserved. This was the reason for the high loss of positive clones.

The growth of cell hybrid clones was generally good, but Balb/c mice peritoneal or splenic macrophages had to be added at a concentration of $1x10^5$ macrophages/ml of medium or 10 cells/well to facilitate an initial cell growth. Once growth was established, hybridomas attained the logarithmic growth phase between 2-4 days. Cloned hybridomas also grew quite well in RPMI- 1640 containing 20% FBS.Culture flasks were first seeded with Balb/c mice macrophages. One particular hybridoma TEM5/17

FIGURE :3

Growing NS-1 Myeloma cells in Growth Medium (RPMI-1640 containing 10% FBS)

FIGURE 4(a):

Hybrid colony, day 5, growing in HAT selection medium.

FIGURE 4 (b):

Multiple Hybrid colonies, (day 7), growing in HAT selection medium.

and its cloned derivatives showed very unstable growth characteristics, occasionally showing spontaneous death even when macrophage cells were added.

Table 7. Fusion Results: Antigen I

Fusion No.	Immunizing Antigen	Total No.of Wells	Total No. of Wells with growing Hybrid Clones	Total No. of Wells with +ve Hybrid Clones	No. of +ve Clones Lost	% Hybrid Clones	% +ve Hybri Clone
1	I	48	17	3	1	35	18
2	"	48	25	0	0	52	0
3	"	48	14	11	8	29	79
4	"	24	C o n t a m i n a t e d			-	-
5	"	48	28	14	12	58	50
6	"	24	C o n t a m i n a t e d			-	-
7	"	48	31	22	10	65	71
8	"	24	16	10	3	67	63

Table 7. Fusion Results: Antigen II.

Fusion No.	Immunizing Antigen	Total No. of Wells.	Total No. Wells with growing Hybrid Clones	Total No. of wells with +ve Hybrid Clones	No.of +ve Clones lost	% Hybrid Clones	% Hyb Clon
MI	II	24	24	4	1	100	1
M2	"	24	24	6	0	100	2
M3	"	24	20	10	1	83	5
M4	"	24	13	12	5	54	9
M5	"	24	24	14	4	100	5
M6	"	24	C o n t a m i n a t e d			-	-
M7	"	24	24	19	20	100	7
M8	"	24	15	15	6	63	10

5.1.3. ISOTYPING AND NOMENCLATURE OF ANTIBODY-PRODUCING CELL HYBRIDS:

Tables 9 and 10 show the results of isotyping of 69 McAbs. Table 10 shows McAbs which did not react with the antibodies against specified mouse classes and subclasses. These antibodies showed strong reaction against rabbit anti-mouse Ig(gamma). Since they were not tested against anti-mouse IgG_3 (reagent was not available) and chances that they are of mouse IgA or IgE classes are slim, they are probably of IgG_3 subclass. However, they are still unclassified until tested.

Antibody producing hybrids were named according to the nomenclature used by Dr. V.M. Nantulya ILRAD with minor changes. Examples of McAb TEA1/23.4.6. and TEM8/2.4.10 will be used to explain the meaning of letters and numbers used:

TE	=	*Trypanosoma. (T).b. evansi*
A	=	I antigen
M	=	II antigen
1/or8/	=	Fusion No.1 or Fusion No.8
/23 or /2	=	Hybrid was first obtained from well No. 23 or well No.2 of the culture plate.
.4 or .4	=	Hybrid was obtained from well No. 4 after the first cloning.
.6 or .10	=	Hybrids were obtained from wells No.6 or No.10 after the second cloning.

Fig. 5a and 5b show the reaction of McAb TEA1/23.4.6. with rabbit anti- mouse IgM(u) antiserum in a DID and IEP reaction.

FIGURE 5 (a):

DID reaction of McAb TEA1/23.4.6. with anti-mouse IgM(u)-chain specific anti-serum. The monoclonal antibody is in the central well: anticlockwise in the peripheral wells, from top well are: anti-mouse IgG1, IgM, IgG2a IgG2b and PBS in the last two wells.

FIGURE 5(b):

IEP reaction of McAb TEA1/23.4.6. with anti-mouse IgM(u)-chain specific anti-serum. In the central trough is the MCAbs and anti-mouse IgM (u) chain specific anti-serum in the opposite wells.

Table 9. Classes and subclasses of McAbs produced against T.(T).b.evansi KETRI 1342,antigen I and II frozen and/or salt precipitated and tested against STA in an ELISA test.

McAb Class/	Subclass	ELISA OD values (E_{492nm})	Effect of Salt pptn.	Effect of Freezing
TEAI/23.4.6	IgM	.488	Stable	Stable
TEM3/3.4.7	IgM	. 265	Unstable	Unstable
TEA5/48.28.5	IgM	.476	Stable	Stable
TEM2/3.3.16	IgM	.463	"	"
TEM8/2.4.10	IgM	.390	"	"
TEA7/11.2.8	IgM	.273	"	"
TEM5/9.8.7	IgM	.206	Unstable	Unstable
TEM3/13.12.8	IgM	.389	Stable	Stable
TEM5/8.9.12	IgM	.347	"	"
TEM8/24.4	IgG1	.234	"	"
TEM3/17.5	IgG1	.367	"	"
TEM8/4.6	IgG1	.288	"	"
TEM5/17.4.6	IgG2a	.731	"	"
TEM5/20.2.7	IgG2a	.265	"	"
TEM5/14.4.16	IgG2a	.381	"	"
TEM5/15.4.8	IgG2a	.261	"	"
TEM5/7.3.11	IgG2a	.299	"	"
TEA8/7.6.12	IgG2a	.480	"	"
TEM5/16.7.6	IgG2a	.350	"	"
TEA8/23.1.9	IgG2a	.266	"	"
TEM3/5.5.21	IgG2a	.275	"	"
TEA7/20.3.17	IgG2a	.189	"	"
TEA8/22.2.7	IgG2b	.314	"	"

Normal mouse serum showed OD readings of less than 0.090.

pptn. Precipitation

Table 10. Reaction of unclassified McAbs with *T.(T).b. evansi* KETRI 1342 STA McAb in ELISA test.

McAb	Class/ Subclass	Gamma-globulin (in DID)	(OD values E_{492} nm.)
TEA7/39.4	IgG3,IgA,IgE ?	+	.269
TEM3/12.3.6	"	+	.136
TEM3/18.7	"	+	.234
TEM3/14.16	"	+	.368
TEM8/13.6	"	+	.357
TEM5/2.12	"	+	.178
TEM5/7.13	"	+	.266
TEM5/11.4	"	+	.311
TEA8/24.6	"	+	.287
TEM5/15.4	"	+	.389
TEM5/3.7	"	+	.286
TEM4/8.6	"	+	.288
TEM4/4.15	"	+	.267
TEM4/7.13	"	+	.314
TEM4/6.6	"	+	.290
TEM4/9.19	"	+	.320
TEM4/18.7	"	+	.355
TEM7/16.2	"	+	.211
TEM7/29.6	"	+	.352
TEM2/4.3	"	+	.263
TEM1/16.5	"	+	.189
TEM8/5.3	"	+	.194
TEM8/15.2	"	+	.163
TEM8/7.11	"	+	.246
TEM8/11.6	"	+	.277
TEA8/2.6	"	+	.214
TEM7/33.8	"	+	.391
TEA8/7.10	"	+	.255
TEA7/12.6	"	+	.342
TEM3/15.5	"	+	.214
TEM2/18.4	"	+	.256
TEA7/7.6	"	+	.188
TEA3/23.7	"	+	.169
TEA8/1.11	"	+	.210
TEA8/22.4	"	+	.230
TEA3/20.17	"	+	.174
TEA7/3.16	"	+	.300
TEA3/40.5	"	+	.381
TEA7/4.16	"	+	.168
TEA7/1.10	"	+	.146
TEA7/14.6	"	+	.212
TEM2/14.4	"	+	.270
TEM8/16.4	"	+	.224
TEA1/16.3	"	+	.233
TEA7/2.7	"	+	.191

Optical readings of 0.09 nm and above were considered positive.

5.1.4. EFFECT OF FREEZING AND PRECIPITATION WITH 50% AMMONIUM SULPHATE SOLUTION:

Only McAbs TEM3/3.4.7 and TEM5/9.8.7 showed a marked drop in antibody activity after precipitation with 50% ammonium sulphate solution (see Table 9). The rest of the McAbs were stable even after the salt treatment. There was no marked drop in antibody activity on thawing after the first freezing. Subsequent freezing and thawing showed a deterioration in antibody activity as would be expected with any other antibody.

5.2 APPLICATION AND EVALUATION OF ANTI-*T.(T).B. EVANSI* McABs AS TRYPANOSOME TYPING REAGENTS:

All the 69 McAbs showing reactivity with KETRI 1342 (see Tables 9 and 10) showed a wide cross reaction with STA - lysate antigens and IFAT - antigens tested in ELISA and IFAT, respectively. In ELISA, using lysate antigens from *Leishmania donovani (L.d.), Babesia bigemina (B.b.) Anaplasma marginale (A.m.), Plasmodium fulciparum (P.f.)* and *Theileria parva parva (T.p.p.)* no cross reaction was observed with all the McAbs. Ten ug protein of the respective antigens were used to coat the ELISA microplates. Tables 11 and 12 show the reactivity of ten McAbs in ELISA and IFAT tests. Two McAbs TEM5/17.4.6. and TEM3/12.3.6 showed a high specificity for the *T.(T).b. evansi* KETRI 1342 STA antigen and IFAT - antigen in ELISA and IFAT tests, respectively. These two were further tested on proven *T.(T).b. evansi* stabilates in the KETRI Trypanosome bank. Table 13 shows the pattern of reactivity by ELISA and IFAT of TEM5/17.4.6. and TEM3/12.3.6 McAbs with lysates of blood stream forms of *T.(T).b. evansi* stocks of defined zymodemes. These two McAbs did not react with any of the *T.(T).b. evansi* stocks in the test except for fluorescence with stock 2469 and 2468 in IFAT test.

Table 11. The reactivity of selected McAbs with bloodstream forms of African trypanosomes as defined by the indirect immunofluorescent antibody test(IFAT)

Monoclonal antibody	Reactivity of the McAbs with fixed bloodstream forms of:						
	T.(T). b.b.	*T.(T). b.ev.*	*T.(T). b.rh.*	*T.(T). b.gamb.*	*T.(D). vivax*	*T.(N). simiae*	*T.(N). congo.*
TEA8/22.2.7	+++	+++	+++	+++	++	++	++
TEM5/17.4.6	-	+++	-	-	-	-	-
TEA7/11.2.8	++	+++	+++	+++	++	++	++
TEM3/12.3.6	-	++	-	-	-	-	-
TEM5/9.8.7	++	++	++	++	+	+	+
TEM2/3.3.16	+++	+++	+++	+++	+++	+++	+++
TEM3/13.12.8	+++	+++	+++	+++	++	++	++
TEM3/3.4.7	++	+++	+++	++	+++	+++	+++
TEM5/18.9.12	+++	+++	+++	+++	++	++	++
TEA1/23.4.6	+++	+++	+++	+++	+++	+++	+++
TEM8/24.4.3	++	++	++	++	+	+	+
TEA5/48.28.5	+++	+++	+++	++	++	++	++
TEM3/17.5.4	+++	+++	+++	++	++	+	+
TEM8/2.4.10	+++	++	++	++	++	++	++
TEM8/4.6.6	+++	+++	+++	++	+++	++	++
TEM5/20.2.7	++	+++	++	++	++	++	++
TEM5/15.4.8	++	++	++	++	+	+	+
TEM5/14.4.16	++	++	++	++	++	++	++
TEM5/7.6.12	++	++	++	++	++	++	++
TEA5/39.7.4	+++	+++	+++	+++	+++	+++	+++
TEM5/16.7.6	+++	+++	+++	+++	++	++	++
TEA8/23.1.9	++	++	++	++	++	++	++

(-) No observable staining (no fluorescence).

(+) Normal staining (normal fluorescence).

(++) Most trypanosomes stained (strong fluorescence).

(+++) All trypanosomes stained (very strong fluorescence).

Table 12. The reactivity of selected McAbs with STA-antigen from bloodstream forms of African trypanosomes as defined by the ELISA test.

Monoclonal antibody	Reactivity of the McAbs with STA-Ag from bloodstream forms of:						
	T.(T). b.b.	*T.(T). b.ev.*	*T.(T). b.rh.*	*T.(T). b.gamb.*	*T.(D). vivax*	*T.(N). simiae*	*T.(N). congo.*
TEA8/22.2.7	.298	.314	.320	.285	.150	.148	.136
TEM5/17.4.6	.027	.731	.029	.025	.017	.020	.020
TEM3/12.3.6	.043	.136	.019	.017	.021	.026	.020
TEM5/9.8.7	.211	.206	.241	.189	.095	.095	.101
TEM2/3.3.16	.395	.463	.448	.461	.399	.389	.352
TEM3/13.12.8	.361	.389	.401	.316	.241	.236	.249
TEM3/3.4.7	.211	.265	.278	.193	.261	.214	.233
TEM5/18.9.12	.362	.391	.347	.343	.265	.222	.195
TEA1/23.4.6	.491	.488	.451	.466	.421	.433	.399
TEM8/24.4.3	.195	.234	.198	.211	.175	.098	.111
TEA5/48.28.5	.461	.476	.488	.432	.311	.322	.304
TEM3/17.5.45	.362	.367	.338	.394	.286	.233	.207
TEM8/2.4.10	.402	.390	.302	.324	.216	.192	.176
TEM8/4.6.6	.293	.288	.303	.344	.265	.298	.301
TEM5/20.2.7	.211	.265	.198	.204	.126	.133	.106
TEM5/15.4.8	.202	.261	.188	.163	.085	.115	.143
TEM5/14.4.16	.362	.381	.333	.341	.366	.359	.341
TEM5/7.6.12	.287	.299	.211	.264	.243	.206	.217
TEA5/39.7.4	.422	.437	.460	.416	.395	.401	.429
TEM5/16.7.6	.366	.350	.349	.374	.320	.311	.298
TEA8/23.1.9	.241	.266	.214	.257	.221	.231	.211
TEA7/11.2.8	.289	.273	.301	.333	.216	.200	.198

OD readings of $\geq$ 0.09 were considered positive.

Table 13. Reactivity by ELISA of TEM5/17.4.6 and TEM3/12.3.6 McAbs with lysates (STA) of blood forms of defined T.(T).b.evansi stocks of defined Zymodemes.

KETRI stabilate No.of *T.(T).b. evansi* stocks.	Zymodeme	Reactivity by IFAT		Reactivity by ELISA (OD, $E_{492\ nm}$).	
		TEM5/ 17.4.6	TEM3/ 12.3.6	TEM5/ 17.4.6	TEM3/ 12.3.6
1342	58	+++	++	0.731	0.136
2472	58	-	-	0.022	0.029
2540	58	-	-	0.029	0.025
2531	58	-	-	0.020	0.027
2485	58	-	-	0.027	0.037
2458	58	-	-	0.027	0.022
2455	58	-	-	0.029	0.026
2481	64	-	-	0.031	0.023
2477	41	-	-	0.039	0.031
2476	64	-	-	0.040	0.039
2462	64	-	-	0.038	0.027
2457	58	-	-	0.031	0.028
2450	64	-	-	0.043	0.036
2371	64	-	-	0.033	0.030
2470	64	-	-	0.028	0.029
2469	64	+	+	0.049	0.039
2468	64	+	+	0.046	0.039
2467	64	-	-	0.042	0.045
2466	66	-	-	0.033	0.031
2447	64	-	-	0.045	0.038
2443	64	-	-	0.022	0.018

Optical density readings (OD) of 0.090 and above were considered positive.
Each value represents an average of two readings.

Zymodemes were defined by W.C.Gibson (KETRI trypanosome Bank Records).

5.3. APPLICATION OF ANTI-*T.(T.).B. EVANSI* McABs AS ANTIGEN DETECTING PROBES:

Because of the scarcity of reagents, only five McAbs shown in Table 14 and 15 were selected. Table 14 shows the optimal conjugate dilutions and McAb protein concentrations of the 5 McAbs. TEA1/23.4.6 had the highest activity (see Table 15) and was able to detect as low as 1.25 ug/ml of STA - antigen. Because of its superiority, this antibody was produced in larger quantities and used for other studies as reported below.

Table 14. Optimal conjugate dilution and McAb protein concentration for use in Sandwich ELISA for the detection of STA antigen.

McAb.	TEA1/ 23.4.6	TEA5/ 48.28.5	TEM2/ 3.3.16	TEM8/ 2.4.10	TEM5/ 14.4.16
Optimal conjugate dilution.	1:1000	1:500	1:500	1:250	1:250
Optimal protein Concentration ug/well for coating micro-wells	25	40	35	63	30

McAb TEA1/23.4.6. was selected for use in subsequent Ag-ELISA studies.

Table 15. ELISA values (OD, E_{492nm}) of STA of *T.(T).b evansi* 1342 in a Sandwich ELISA reaction with McAbs: TEA1/23.4.6. TEM5/48.28.5 TEM2/3.3.16 TEM8/2.4.16 and TEM5/14.4.16 at various serial dilutions of the antigen

Concentration of STA antigen (ug/ml)	TEA1/ 23.4.6	TEM5/ 48.28.5	TEM2/ 3.3.16	TEM8/ 2.4.16	TEM5/ 14.4.16
0.000	0.006	0.006	0.006	0.002	0.000
0.625	0.060	0.006	0.014	0.001	0.003
1.250	0.090	0.024	0.024	0.018	0.003
1.500	0.204	0.058	0.063	0.036	0.006
3.000	0.513	0.106	0.139	0.081	0.014
6.000	0.816	0.198	0.250	0.156	0.031
12.000	1.126	0.397	0.483	0.315	0.058
24.000	1.336	0.711	0.822	0.622	0.106
48.000	1.386	1.201	1.293	1.117	0.224
96.000	1.441	1.307	1.348	1.236	0.563
192.000	1.490	1.411	1.442	1.461	1.236

OD readings of $\geq$ 0.09 were considered positive in subsequent Ag-ELISA tests.

5.4. THE RELATIONSHIP BETWEEN SERUM ANTIGEN LEVELS AND PCV, TEMPERATURE, PARASITAEMIA, HAEMOLYTIC COMPLEMENT, IgG AND IgM - ANTIBODY LEVELS:

5.4.1 SERUM ANTIGEN-ELISA AND PCV CHANGES:

Tables 16 and 22 show the mean (+SE) values of PCV and serum antigen-ELISA values, respectively in the groups of goats during the study period.

In the two experimental groups (group 1 and 2) PCV values dropped gradually, but not below 20%. PCV drop was more marked in group 1 (range: 45-23) than group 2 (range: 40-25). Tables 22 and 23 show serum and plasma antigen ELISA values (OD, E492nm) during the course of infection and after treatment. For group 1 which received i/v inoculation of the parasites, antigens were detectable by day one, though mean ELISA values were very low (.089+.006).In group 2 antigens were detectable by day six, except for goat No. 6 in which antigens could not be detected until day 13. The mean serum antigen ELISA levels showed a steady increase, although minor fluctuations could be observed in all the experimental goats. After treatment (day 58 for group 1 and day 70 for group 2), antigen levels dropped to pre-infection levels by day 77 in group 1 and day 111 in group 2 (see Fig. 6). Analysis of variance and multiple range testing (see Table 25) showed a high significant difference in mean PCV and antigen ELISA values between the infected groups and the control growth ($P<0.001$). Multiple regression analysis based on antigen ELISA as the dependent variable (see Table 26) showed a negative correlation ($r = -0.65$ for group 1 and -0.53 for group 2). One of the control goats (No. 7) became infected during the course of the experiment, perhaps, mechanically by *Musca domestica* flies. This goat was therefore eliminated from the group.

5.4.2. SERUM ANTIGEN - LEVELS AND TEMPERATURE CHANGES.

Table 17 shows the mean (+SE) daily temperature changes in the three groups of goats during the study period. Fig. 7 shows changes in temperature and serum antigen levels during the period. In the experimental groups changes were more marked in group 1 (range: 38-41^{o} C) than group 2 (range: 37.9-40.8^{o} C). Analysis of variance and multiple range testing (see Table 25) showed a significant difference in mean temperature values of infected (group 1 & 2) and non-infected group (group 3). Regression analysis showed a weak positive correlation ($r = 0.42$ for group 1,and $r = 0.28$ for group 2) between temperature and serum antigen levels (See Table 26).

FIGURE 6:

PCV (%)(A) and serum Ag-ELISA values (B) (OD $E_{492\ nm}$) in the goats of group 1(I/V challenge),2(I/M challenge) and 3 (non-infected control) during the experimental period.

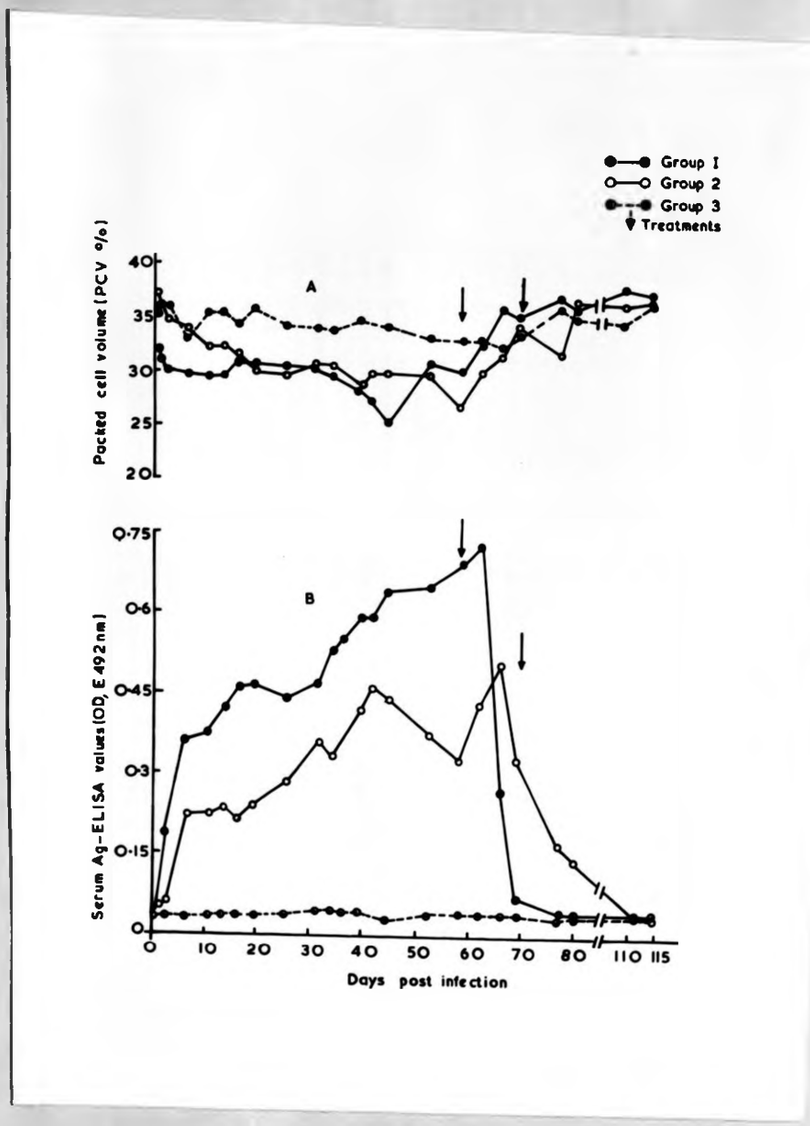


FIGURE 7:

Temperature (^{o}C) changes (A) and serum Ag-ELISA values (B) (OD, 492 nm) of the goats of group 1(I/V challenge),2(I/M challenge) and 3(non-infected control during the experimental period.

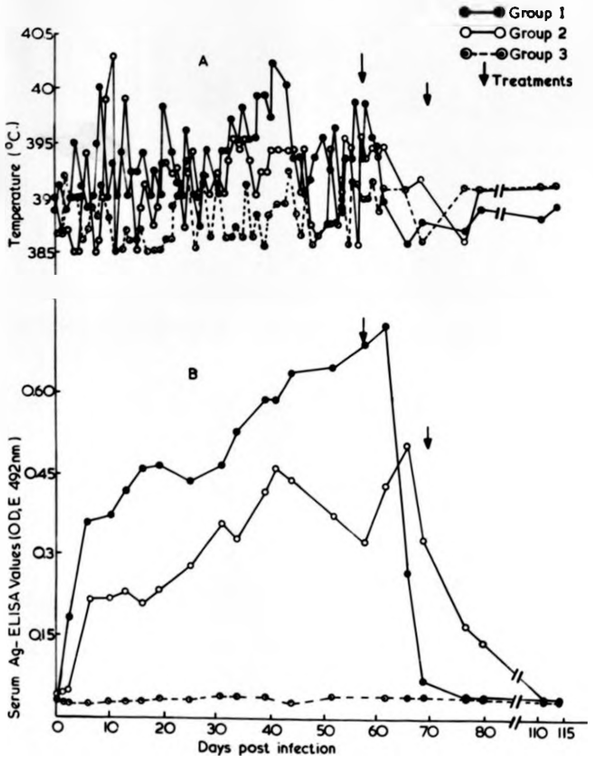


Table 16. Packed Cell Volume(X+SE) in the goats of group 1, 2 and 3 of experiment four ..

Goat No. Day	-3	-2	-1	0	1	2	6	10	13	16	19	25	31	34	3
1	34	34	34	32	29	29	28	28	30	29	29	31	29	30	2
3	36	35	36	29	30	32	32	32	31	31	32	32	32	32	3
5	45	40	39	34	35	32	33	33	32	32	31	29	30	29	3
9	35	31	32	27	30	30	28	28	27	28	29	30	30	31	2
14	38	37	36	32	28	28	27	27	26	37	28	30	35	27	2
15	36	37	37	39	35	34	31	27	30	34	35	32	27	30	3
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	37.3	35.7	35.7	32.2	31.2	30.8	29.8	29.2	29.3	31.8	30.7	30.7	30.5	29.8	28.
se	1.626	1.256	0.989	1.701	1.249	0.91	1.01	1.08	0.95	1.35	1.05	0.49	1.12	0.70	0.8
2	36	31	35	38	37	35	33	34	34	33	31	31	30	31	2
4	39	38	39	37	38	38	35	35	35	31	35	36	34	35	3
6	38	39	39	40	38	35	34	30	31	29	27	27	30	28	2
8	33	34	34	34	33	31	30	30	30	33	28	27	30	29	2
10	35	40	39	39	36	36	37	33	33	32	32	33	34	33	3
12	36	38	38	37	36	34	34	32	32	30	30	29	28	29	2
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	36.2	36.7	37.3	37.5	36.3	34.8	33.8	32.3	32.5	31.3	30.5	30.5	31.0	30.8	28.
se	0.872	1.406	0.919	0.847	0.760	0.95	0.95	0.84	0.76	0.67	1.18	1.45	1.00	1.11	0.9
11	35	36	37	34	35	34	32	34	36	34	36	33	34	34	3
13	34	37	37	37	36	38	34	37	35	35	36	36	34	34	3
n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
x	34.5	36.5	37.0	35.5	35.5	36.0	33.0	35.5	35.5	34.5	36.0	34.5	34.0	34.0	35.0
se	0.500	0.500	0.000	1.500	0.500	2.00	1.00	1.50	0.50	0.50	0.00	1.50	0.00	0.00	1.00

Table 17. Temperature changes (^{o} C) in the goats of group 1, 2 and 3 of experiment four.

Obs. day	GROUP 1 Mean	SE	GROUP 2 Mean	SE	GROUP 3 Mean	SE
-3	38.80	0.07	39.00	0.06	38.90	0.14
-2	38.90	0.06	39.20	0.09	38.80	0.07
-1	38.80	0.06	38.80	0.05	38.90	0.00
0	38.90	0.07	39.10	0.10	38.70	0.14
1	39.10	0.07	38.70	0.04	38.70	0.00
2	38.90	0.07	38.70	0.07	39.20	0.04
3	39.00	0.10	38.40	0.04	39.00	0.00
4	39.50	0.13	38.50	0.08	39.00	0.25
5	39.10	0.06	39.00	0.05	38.60	0.07
6	38.90	0.06	39.40	0.08	38.70	0.00
7	39.00	0.06	38.30	0.05	38.90	0.39
8	39.50	0.05	38.60	0.07	38.80	0.21
9	40.00	0.06	39.00	0.06	39.10	0.28
10	39.00	0.10	39.90	0.14	38.80	0.14
11	39.30	0.06	38.50	0.04	38.50	0.00
12	39.00	0.03	40.30	0.08	38.50	0.00
13	39.40	0.08	39.80	0.04	38.70	0.04
14	39.00	0.03	38.50	0.05	38.60	0.07
15	39.20	0.03	38.40	0.05	38.60	0.18
16	39.20	0.03	38.90	0.09	38.70	0.07
17	39.40	0.05	39.10	0.09	38.30	0.04
18	39.00	0.06	38.70	0.11	38.10	0.49
19	39.20	0.04	38.90	0.05	38.50	0.00
20	39.00	0.06	39.30	0.12	38.40	0.21
21	39.80	0.12	39.30	0.10	38.60	0.04
22	39.40	0.07	39.20	0.05	38.60	0.49
23	39.10	0.03	39.00	0.03	38.90	0.36
24	39.00	0.02	38.70	0.05	39.00	0.00
25	39.60	0.07	39.20	0.04	39.30	0.25
26	39.00	0.08	39.40	0.03	38.40	0.13
27	38.70	0.07	39.00	0.08	39.00	0.39
28	39.10	0.03	39.20	0.04	39.10	0.28
29	39.40	0.07	39.00	0.09	38.60	0.03
31	39.00	0.01	39.20	0.03	39.10	0.49
32	39.40	0.06	39.00	0.08	38.60	0.07
33	39.40	0.08	39.30	0.04	38.60	0.04
34	39.70	0.15	39.50	0.06	38.70	0.28
35	39.50	0.12	39.40	0.05	38.60	0.07
36	39.80	0.07	39.50	0.06	39.10	0.39
37	39.50	0.07	39.30	0.06	38.60	0.14
38	39.50	0.15	39.00	0.05	38.80	0.28
39	39.90	0.11	39.20	0.06	38.50	0.11
40	39.90	0.03	39.20	0.05	38.80	0.28
41	39.70	0.09	39.40	0.06	38.90	0.11
42	40.30	0.09	39.40	0.06	38.90	0.21
44	40.00	0.15	39.40	0.09	39.20	0.00
45	39.30	0.11	39.40	0.17	38.80	0.53
46	39.30	0.01	39.00	0.05	38.60	0.18
47	39.00	0.11	39.40	0.08	39.00	0.32
48	39.10	0.03	38.70	0.04	38.50	0.07
49	39.30	0.07	38.60	0.10	38.60	0.14
51	39.50	0.09	38.70	0.07	38.70	0.07
52	39.20	0.12	39.40	0.06	38.70	0.21
53	39.60	0.08	38.70	0.08	38.70	0.17
54	38.80	0.09	39.30	0.07	39.10	0.45
55	39.30	0.10	39.50	0.03	38.30	0.11

Table 17. (Continued)

Obs. day	GROUP 1 Mean	SE	GROUP 2 Mean	SE	GROUP 3 Mean	SE
56	39.30	0.13	39.40	0.07	38.10	0.35
57	39.80	0.12	38.50	0.05	38.10	0.11
58	39.30	0.12	39.50	0.04	38.90	0.43
59	39.80	0.11	39.30	0.06	38.90	0.28
60	39.50	0.08	39.40	0.06	39.10	0.42
61	39.40	0.14	39.30	0.02	38.80	0.46
62	38.90	0.03	39.40	0.06	39.00	0.21
66	38.50	0.08	39.00	0.08	39.00	0.07
69	38.70	0.07	39.10	0.07	38.40	0.14
77	38.60	0.05	38.40	0.02	39.00	0.00
80	38.80	0.06	39.00	0.08	39.00	0.14
111	38.60	0.08	39.00	0.02	39.00	0.28
114	38.80	0.07	39.00	0.09	39.00	0.39

5.4.3 SERUM ANTIGEN LEVELS AND PARASITAEMIA:

Parasitaemia was generally low in all the experimental groups and was lower in group 2 than group 1. In group 1, parasitaemia was detectable by day one, but later disappeared, until day 11, as shown in Table 18. In group 2, parasitaemia was evident by day 7 in one goat; day 16 in another and by day 45, all goats were evidently parasitaemic in this group. Fig. 8 and 9 show the levels of parasitaemia and serum antigen. During the course of infection, parasitaemia fluctuated in both groups. Rises in parasitaemia were not directly related to rises in detectable circulating serum or plasma antigens. After treatment, parasitaemia dropped to zero level in both groups by day one. The serum antigen levels started to fall sharply in group 2, but in group 1 there was an initial rise, followed by a very sharp fall. Multiple regression analysis of parasitaemia based on serum antigen-ELISA as a dependent variable (see Table 26) showed a weak positive correlation between group 1 (r= 0.050) and group 2 (r=0.10).

FIGURE 8:

Parasitaemia (X 10^3 trypanosomes /ml) (A) and serum Ag-ELISA values (OD,492nm) (B) in the goats of group 1(I/V challenge) during the experimental period.

FIGURE 9 :

Parasitaemia ($x10^3$ trypanosomes/ml) (A)
and serum Ag-ELISA values (OD,492nm) (B)
in the goats of group 2,(I/M challenge) during
the experimental period.

Table 18. Parasitaemia (Tryps/ml of blood) in infected goats of group 1, 2 and 3 of experiment four .

Observation day.	G R O U P 1 Mean	SE	G R O U P 2 Mean	SE
-3	0.00	0.00	0.00	0.00
-2	0.00	0.00	0.00	0.00
-1	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00
1	3666.00	467.00	0.00	0.00
2	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00
4	0.00	0.00	167.00	68.00
5	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00
9	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00
11	167.00	68.00	0.00	0.00
12	333.00	86.00	0.00	0.00
13	167.00	68.00	0.00	0.00
14	500.00	204.00	0.00	0.00
15	1830.00	670.00	0.00	0.00
16	2000.00	738.00	500.00	91.00
17	667.00	202.00	333.00	86.00
18	1333.00	360.00	333.00	86.00
19	1000.00	183.00	333.00	86.00
20	1500.00	391.00	500.00	91.00
21	1167.00	245.00	833.00	222.00
22	500.00	139.00	500.00	91.00
23	1167.00	400.00	167.00	68.00
24	333.00	86.00	0.00	0.00
25	1500.00	391.00	167.00	68.00
26	1330.00	292.00	0.00	0.00
27	2160.00	510.00	0.00	0.00
28	500.00	204.00	167.00	68.00
29	4500.00	1679.00	0.00	0.00
30	500.00	91.00	0.00	0.00
31	133.00	172.00	0.00	0.00
32	167.00	68.00	167.00	68.00
33	1400.00	149.00	167.00	68.00
34	833.00	267.00	0.00	0.00
35	500.00	204.00	167.00	68.00
36	0.00	0.00	500.00	91.00
37	500.00	204.00	333.00	86.00
38	833.00	340.00	333.00	86.00
39	1225.00	356.00	333.00	86.00
40	500.00	204.00	0.00	0.00
41	0.00	0.00	0.00	0.00
42	333.00	136.00	0.00	0.00
43	0.00	0.00	0.00	0.00
44	167.00	68.00	0.00	0.00
45	0.00	0.00	833.00	22.00
46	0.00	0.00	0.00	0.00
47	333.00	86.00	0.00	0.00
48	0.00	0.00	0.00	0.00
49	167.00	68.00	667.00	172.00
50	0.00	0.00	0.00	0.00
51	0.00	0.00	167.00	68.00
52	0.00	0.00	0.00	0.00

Table 18. (Continued)

Observation day.	G R O U P 1 Mean	SE	G R O U P 2 Mean	SE
53	0.00	0.00	0.00	0.00
54	0.00	0.00	0.00	0.00
55	333.00	86.00	0.00	0.00
56	167.00	68.00	0.00	0.00
57	0.00	0.00	0.00	0.00
58	667.00	172.00	0.00	0.00
59	0.00	0.00	500.00	204.00
60	0.00	0.00	0.00	0.00
61	0.00	0.00	667.00	172.00
62	0.00	0.00	0.00	0.00
63	0.00	0.00	500.00	204.00
64	0.00	0.00	0.00	0.00
65	0.00	0.00	0.00	0.00
66	0.00	0.00	0.00	0.00
67	0.00	0.00	167.00	68.00
68	0.00	0.00	333.00	86.00
69	0.00	0.00	500.00	204.00
70	0.00	0.00	333.00	86.00
71	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00

Group 1 goats were treated on day 58.

Group 2 goats were treated on day 70.

No parasitaemia was detected after treatment.

5.4.4 SERUM ANTIGEN LEVELS AND THE HAEMOLYTIC COMPLEMENT:

The levels of total haemolytic complement dropped sharply and then remained stagnant until after treatment when it rose back to pre-infection level (see Table 19 below and Fig. 10). The fall and rise in complement level was closely associated with evidence of post-infection parasitaemia and post- treatment parasitaemia. Analysis of variance and multiple range testing of mean haemolytic complement (CH_{50} Units/ml) showed a high significant difference between infected and non-infected group ($P < 0.0001$). There was a negative correlation ($r = -0.68$ for group 1 and $r = -0.68$ for group 2) between circulating serum antigens and total haemolytic complement.

5.4.5 SERUM ANTIGEN LEVELS AND IgG ANTIBODIES

IgG antibodies were detectable by day 6 in group 1 and by day 10 in group 2. The antibody levels rose steadily in all goats and remained high throughout the study period even after treatment (see Table 20 and Fig. 11). Analysis of variance and multiple range testing showed a high significant difference in IgG antibody levels between infected and control goats ($P<0.0001$). Regression analysis showed a positive correction between serum antigen levels and IgG antibodies ($r = 0.44$ for group 1 and $r = 0.60$ for group 2 goats).

FIGURE 10:

Total Haemolytic complement (CH_{50} units/ml) (A) and serum Ag-ELISA values (OD,492nm) (B) in the goats of group 1 (I/V challenge), 2(I/M challenge and 3(non-infected control), during the experimental period.

FIGURE 11:

IgG Ab-ELISA (A) and serum Ag-ELISA (B) values (OD.492nm), of the goats of group 1(I/V challenge),2(I/M challenge) and 3 (non-infected control) during the experimental period.

Table 19. Haemolytic Compliment values (CH50 Units/ml of serum) in the goats of group 1, 2 and 3 of experiment four ..

Goat No.	Day -3	-2	-1	0	1	2	6	10	13	16	19	25	31	34	39	41	44	52	58	64	66	69	77	80	111	114
1	143	144	142	140	139	142	130	125	121	123	119	125	126	125	120	118	104	114	105	110	115	118	126	134	133	136
3	132	140	140	142	135	143	138	122	120	120	116	119	123	125	121	120	108	112	100	95	95	114	119	130	136	138
5	127	137	130	136	132	141	121	124	118	95	85	100	115	116	100	116	102	106	90	105	100	116	125	130	133	136
9	138	132	136	130	136	135	127	123	121	125	125	125	126	125	128	120	105	110	105	109	106	119	128	132	134	139
14	155	153	150	152	154	154	142	124	118	119	121	126	128	125	126	120	118	116	106	108	109	100	104	129	132	137
15	147	148	152	150	145	144	130	126	122	128	119	125	126	129	125	114	99	102	94	103	105	119	126	135	130	136
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	140	142	142	142	140	143	131	124	120	118	114	120	124	124	120	118	106	110	100	105	105	114	121	132	133	137
se	4.161	3.106	3.403	3.403	3.301	2.522	3.095	0.577	0.683	4.856	5.958	4.131	1.915	1.759	4.187	1.033	2.696	2.129	2.720	2.266	2.840	2.974	3.685	0.989	0.816	0.516
2	148	143	146	144	141	142	130	130	124	126	122	116	117	115	119	112	115	113	105	117	116	109	116	122	128	130
4	156	151	157	153	148	146	132	130	129	124	126	119	115	119	121	120	125	117	114	119	118	111	114	120	126	129
6	139	143	141	142	143	148	126	125	125	130	127	126	124	122	120	125	125	115	116	120	120					
8	143	139	138	146	140	147	128	128	126	127	121	119	124	116	115	114	115	110	85	104	103	102	115	118	125	131
10	160	156	161	158	149	141	134	132	127	129	125	123	123	120	128	126	126	125	110	117	115	118	117	120	130	134
12	151	149	152	150	146	140	130	135	125	128	123	117	117	116	117	115	114	110	100	113	113	110	113	119	129	126
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	5	5	5	5	5
x	150	147	149	149	145	144	130	130	126	127	124	120	120	118	120	119	120	115	105	115	114	110	115	120	128	130
se	3.212	2.561	3.701	2.455	1.522	1.390	1.155	1.390	0.730	0.882	0.966	1.549	1.673	1.125	1.826	2.418	2.394	2.295	4.662	2.408	2.442	2.550	0.707	0.663	0.927	1.304
11	145	146	148	136	141	136	143	148	152	144	152	150	139	146	139	155	148	147	147	141	143	135	152	141	144	146
13	143	138	132	134	149	145	145	142	140	136	138	133	146	142	146	143	140	138	140	146	140	144	149	150	146	143
n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
x	144	142	140	135	145	141	144	145	146	140	145	142	143	144	143	149	144	143	144	144	142	140	151	146	145	145
se	1.000	4.000	8.000	1.000	4.000	4.500	1.000	3.000	6.000	4.000	7.000	8.500	3.500	2.000	3.500	6.000	4.000	4.500	3.500	2.500	1.500	4.500	1.500	4.500	1.000	1.500

Table 20. IgG-Antibody-ELISA values (OD, E492nm) in the goats of group 1, 2 and 3 of experiment four..

Goat No.	Day -3	-2	-1	0	1	2	6	10	13	16	19	25	31	34	39	41	44	52	58	64	66	69	77	80	111	114
1	.043	.031	.037	.045	.041	.065	.141	.228	.752	.369	.417	.549	.542	.536	.565	.567	.533	.630	.603	.668	.690	.601	.580	.561	.480	.481
3	.038	.045	.031	.033	.041	.057	.155	.310	.489	.219	.249	.357	.376	.376	.408	.386	.386	.454	.455	.490	.630	.568	.531	.530	.520	.500
5	.034	.045	.046	.036	.049	.053	.144	.206	.466	.206	.276	.576	.387	.415	.448	.452	.475	.575	.565	.630	.641	.530	.490	.487	.450	.420
9	.049	.034	.031	.049	.049	.047	.099	.139	.187	.177	.278	.256	.308	.348	.387	.456	.432	.497	.481	.589	.611	.612	.520	.521	.513	.500
14	.055	.033	.049	.053	.053	.053	.040	.206	.268	.259	.338	.354	.354	.411	.438	.427	.501	.533	.585	.638	.713	.639	.570	.510	.488	.493
15	.044	.042	.036	.038	.037	.031	.230	.268	.287	.254	.386	.386	.473	.589	.595	.611	.630	.591	.610	.740	.727	.688	.590	.560	.523	.521
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	.044	.038	.038	.042	.045	.051	.135	.226	.408	.247	.324	.413	.407	.446	.473	.483	.493	.547	.550	.626	.669	.606	.547	.528	.496	.486
se	.003	.003	.003	.003	.003	.005	.026	.024	.084	.027	.028	.051	.035	.039	.035	.035	.035	.026	.027	.034	.020	.022	.016	.012	.012	.014
2	.023	.036	.038	.038	.048	.029	.041	.127	.187	.258	.390	.382	.450	.470	.478	.500	.492	.487	.385	.468	.501	.611	.570	.533	.490	.488
4	.031	.033	.038	.041	.048	.029	.041	.137	.204	.299	.315	.542	.466	.502	.534	.579	.533	.541	.474	.554	.571	.623	.611	.603	.546	.537
6	.036	.027	.031	.027	.027	.026	.041	.130	.240	.252	.312	.492	.545	.596	.584	.616	.533	.539	.560	.545	.627					
8	.044	.033	.036	.031	.036	.034	.030	.141	.171	.194	.272	.550	.412	.450	.499	.520	.523	.525	.546	.606	.554	.691	.672	.621	.590	.530
10	.039	.029	.038	.030	.037	.036	.029	.143	.203	.242	.429	.403	.517	.534	.523	.478	.433	.522	.525	.565	.563	.650	.641	.606	.521	.511
12	.041	.043	.039	.044	.045	.033	.045	.157	.264	.300	.340	.480	.529	.574	.514	.568	.599	.522	.544	.584	.581	.706	.629	.587	.502	.499
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	5	5	5	5	5
x	.036	.033	.037	.035	.040	.031	.038	.139	.212	.258	.343	.475	.487	.521	.522	.543	.519	.523	.506	.554	.566	.656	.625	.590	.530	.513
se	.003	.002	.001	.003	.003	.002	.003	.004	.014	.016	.023	.028	.021	.024	.015	.021	.022	.008	.027	.019	.017	.019	.017	.015	.018	.009
11	.049	.038	.043	.039	.039	.039	.049	.033	.033	.033	.030	.031	.036	.030	.037	.035	.039	.039	.044	.037	.032	.032	.046	.033	.036	.031
13	.033	.033	.043	.037	.037	.039	.049	.033	.033	.033	.030	.031	.036	.030	.037	.030	.034	.037	.030	.031	.048	.037	.048	.036	.032	.047
n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
x	.041	.036	.043	.038	.038	.039	.049	.033	.033	.033	.030	.031	.036	.030	.037	.033	.036	.038	.037	.034	.040	.034	.047	.034	.034	.039
se	.008	.003	.000	.001	.001	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.003	.003	.001	.007	.003	.008	.003	.001	.002	.002	.008

5.4.6 SERUM ANTIGEN LEVELS AND IgM ANTIBODIES

IgM antibodies were detectable by day 6 in both group (see Table 21 and Fig. 12). Serum IgM antibodies increased steadily after infection with minor fluctuations during the course of infection. Following treatment, the level of IgM antibodies increased considerably (see Fig 12)and then dropped. By day 111, IgM antibody levels were back to pre-infection values. Analysis of variance showed a high significance of IgM antibodies between infected and non-infected goats ($P < 0.0001$). Regression analysis showed a high positive correlation between IgM antibodies and serum antigens ($r = 0.91$ for group 1 and $r = 0.66$ for group 2).

5.4.7 SERUM AND PLASMA - Ag LEVELS

For practical purposes, serum and plasma antigen levels were compared. Table 23 show the Plasma Antigen ELISA values in all the goats after infection and treatment. Analysis of variance and multiple range testing of mean($\pm$ SE) plasma Ag-ELISA values gave a very high statistical difference between infected and control groups. Regression analysis gave a very high positive correlation between plasma and serum antigen-ELISA values ($r = 0.99$ for group 1 and $r = 0.92$ for group 2).

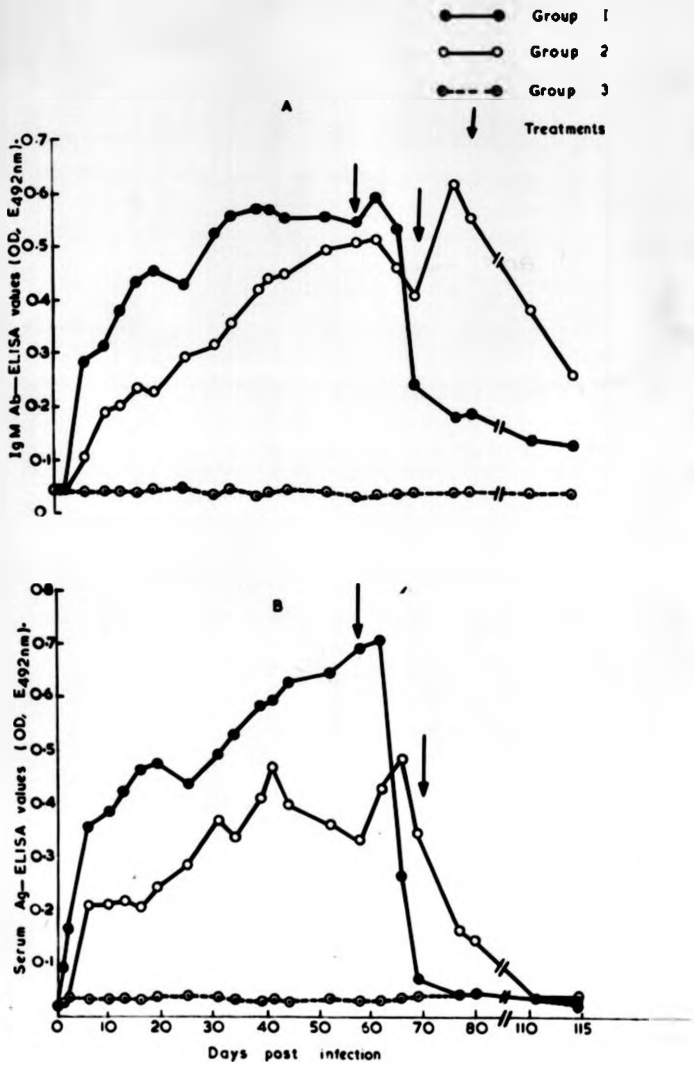

Group 1
Group 2
Group 3
Treatments
A
IgM Ab—ELISA values (OD, E492nm)
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
B
Serum Ag—ELISA values (OD, E492nm)
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0
10
20
30
40
50
60
70
80
110
115
Days post infection

Table 21. IgM Antibody-ELISA values (OD, E492nm) in the goats of group 1, 2 and 3 of expe

Goat No.	Day -3	-2	-1	0	1	2	6	10	13	16	19	25	31	34	39	41
1	.035	.035	.038	.041	.048	.039	.414	.303	.289	.308	.379	.378	.485	.493	.487	.476
3	.033	.033	.041	.037	.044	.036	.316	.370	.319	.378	.430	.491	.560	.583	.580	.511
5	.038	.038	.044	.043	.036	.043	.276	.414	.430	.470	.439	.478	.517	.591	.594	.567
9	.036	.038	.029	.033	.026	.043	.211	.316	.511	.433	.491	.399	.495	.536	.569	.611
14	.030	.022	.030	.029	.019	.048	.218	.320	.318	.517	.506	.403	.577	.598	.601	.670
15	.026	.036	.034	.027	.021	.056	.284	.396	.418	.504	.489	.431	.541	.567	.584	.612
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	.033	.034	.036	.035	.032	.044	.286	.353	.381	.435	.456	.430	.529	.561	.569	.574
se	.002	.002	.002	.003	.005	.003	.030	.019	.035	.033	.020	.019	.015	.016	.017	.029
2	.023	.024	.022	.022	.029	.021	.111	.228	.214	.211	.200	.292	.263	.300	.271	.239
4	.031	.036	.035	.034	.029	.021	.097	.189	.191	.193	.171	.211	.268	.311	.393	.413
6	.036	.040	.037	.034	.037	.032	.103	.166	.163	.223	.189	.241	.269	.293	.329	.422
8	.036	.027	.038	.033	.027	.028	.114	.187	.210	.311	.271	.333	.368	.414	.564	.526
10	.028	.027	.026	.027	.033	.037	.121	.193	.244	.220	.300	.391	.404	.438	.484	.501
12	.036	.038	.037	.037	.044	.091	.194	.190	.278	.231	.340	.317	.397	.491	.560	.548
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	.032	.032	.033	.031	.033	.038	.123	.192	.217	.232	.245	.297	.328	.375	.433	.441
se	.002	.003	.003	.002	.003	.011	.015	.008	.016	.017	.028	.027	.028	.034	.050	.046
11	.049	.039	.051	.038	.044	.041	.043	.043	.031	.047	.047	.048	.031	.047	.039	.027
13	.047	.038	.037	.043	.047	.038	.037	.041	.032	.033	.038	.049	.040	.051	.031	.053
n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
x	.048	.038	.044	.040	.046	.040	.040	.042	.032	.040	.042	.048	.036	.049	.035	.040
se	.001	.001	.007	.003	.002	.002	.003	.001	.001	.007	.005	.001	.005	.002	.004	.013

Table 22. Serum Antigen-ELISA values (OD, E492nm) in the goats of group 1, 2 and 3 of experiment four .

Goat No.	Day -3	-2	-1	0	1	2	6	10	13	16	19	25	31	34	39	41	44	52	58	62	66	69	77	80	111	114
1	.026	.018	.019	.026	.071	.129	.316	.300	.400	.437	.481	.430	.471	.541	.602	.593	.593	.548	.550	.561	.211	.091	.031	.035	.034	.028
3	.023	.027	.022	.029	.084	.138	.301	.291	.393	.403	.423	.351	.491	.480	.540	.567	.592	.607	.691	.699	.304	.086	.041	.041	.022	.021
5	.027	.019	.016	.029	.077	.143	.377	.381	.423	.471	.507	.467	.452	.499	.566	.613	.661	.693	.713	.760	.317	.071	.048	.048	.036	.037
9	.019	.021	.017	.020	.091	.181	.389	.394	.461	.511	.487	.511	.516	.530	.601	.649	.671	.693	.788	.804	.289	.077	.036	.035	.041	.033
14	.022	.027	.029	.036	.101	.193	.369	.390	.471	.588	.561	.498	.487	.521	.589	.513	.538	.596	.671	.741	.240	.061	.047	.048	.019	.026
15	.029	.030	.031	.034	.111	.179	.371	.399	.371	.416	.381	.350	.528	.600	.614	.622	.711	.720	.728	.750	.216	.031	.028	.031	.027	.029
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	.024	.024	.022	.029	.089	.161	.354	.359	.420	.471	.473	.434	.491	.529	.585	.593	.628	.643	.690	.719	.263	.069	.039	.040	.030	.029
se	.001	.002	.003	.002	.006	.011	.015	.020	.016	.028	.026	.029	.011	.017	.011	.020	.026	.028	.032	.035	.019	.009	.003	.003	.003	.002
2	.022	.041	.041	.042	.043	.046	.112	.127	.216	.313	.380	.420	.511	.540	.531	.316	.197	.211	.307	.416	.413	.453	.169	.147	.026	.029
4	.031	.025	.025	.022	.043	.036	.198	.234	.314	.268	.321	.366	.390	.421	.493	.511	.559	.268	.299	.427	.404	.519	.173	.156	.033	.039
6	.034	.044	.044	.043	.043	.041	.026	.015	.093	.120	.183	.110	.369	.189	.280	.592	.263	.510	.352	.375	.566					
8	.043	.031	.038	.022	.021	.029	.359	.274	.209	.120	.133	.096	.093	.093	.360	.367	.235	.352	.205	.413	.486	.239	.163	.143	.041	.038
10	.040	.033	.033	.041	.031	.031	.360	.288	.171	.189	.211	.310	.480	.366	.381	.491	.460	.389	.361	.511	.540	.241	.144	.133	.029	.026
12	.038	.022	.022	.031	.028	.038	.186	.311	.281	.204	.210	.389	.366	.391	.410	.520	.571	.411	.433	.616	.689	.261	.139	.138	.031	.031
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	5	5	5	5	5
x	.035	.033	.034	.033	.035	.037	.207	.208	.214	.202	.240	.282	.368	.333	.409	.466	.381	.357	.326	.460	.516	.343	.158	.143	.032	.033
se	.003	.004	.004	.004	.004	.003	.054	.047	.032	.032	.038	.058	.060	.067	.037	.042	.069	.043	.031	.036	.044	.060	.007	.004	.003	.003
11	.029	.022	.030	.019	.029	.027	.027	.048	.036	.029	.035	.033	.027	.033	.019	.027	.019	.027	.028	.030	.033	.039	.041	.026	.037	.027
13	.019	.023	.023	.016	.021	.029	.034	.044	.029	.033	.034	.040	.041	.031	.038	.036	.034	.035	.040	.036	.036	.038	.039	.039	.038	.039
n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
x	.024	.023	.027	.017	.025	.028	.031	.046	.033	.031	.034	.036	.034	.032	.029	.032	.027	.031	.034	.033	.034	.038	.040	.033	.038	.033
se	.005	.001	.004	.002	.004	.001	.004	.002	.004	.002	.001	.004	.007	.001	.010	.005	.008	.004	.006	.003	.002	.001	.001	.007	.001	.006

Table 23. Plasma Antigen ELISA values (OD, E492nm) in the goats of group 1, 2 and 3 of experiment four.

Goat No.	Day -3	-2	-1	0	1	2	6	10	13	16	19	25	31	34	39	41	44	52	58	64	66	69	77	80	111	114
1	.027	.022	.026	.026	.089	.131	.327	.398	.413	.424	.496	.521	.480	.530	.604	.587	.583	.550	.580	.581	.200	.086	.031	.033	.036	.033
3	.018	.026	.028	.029	.110	.149	.367	.394	.361	.413	.488	.389	.496	.483	.564	.561	.597	.621	.707	.700	.311	.073	.037	.038	.026	.026
5	.029	.026	.028	.028	.121	.200	.398	.404	.383	.460	.511	.470	.462	.501	.570	.600	.681	.701	.800	.806	.326	.088	.041	.031	.036	.039
9	.019	.031	.031	.029	.131	.218	.399	.413	.411	.427	.501	.521	.518	.539	.611	.649	.683	.701	.800	.806	.291	.068	.033	.022	.037	.035
14	.019	.020	.018	.023	.109	.198	.400	.416	.440	.371	.461	.372	.486	.526	.592	.527	.541	.603	.636	.628	.241	.073	.048	.040	.027	.031
15	.023	.029	.029	.030	.111	.183	.383	.289	.379	.420	.386	.356	.530	.607	.622	.630	.724	.736	.711	.750	.234	.027	.033	.029	.032	.028
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
x	.022	.026	.027	.028	.112	.180	.379	.386	.398	.419	.474	.438	.495	.531	.594	.592	.635	.652	.706	.712	.267	.069	.037	.032	.032	.032
se	.002	.002	.002	.001	.006	.014	.012	.020	.012	.012	.019	.031	.010	.017	.009	.018	.029	.029	.036	.038	.020	.009	.003	.003	.002	.002
2	.026	.042	.044	.045	.040	.042	.136	.108	.208	.321	.391	.420	.521	.551	.536	.360	.207	.233	.311	.459	.200	.146	.171	.144	.027	.026
4	.033	.026	.020	.037	.040	.041	.200	.220	.330	.281	.360	.368	.401	.431	.489	.516	.561	.279	.306	.441	.213	.259	.174	.153	.034	.038
6	.034	.041	.044	.040	.043	.041	.030	.031	.073	.160	.186	.121	.056	.191	.300	.598	.278	.531	.381	.571	.541					
8	.038	.030	.024	.028	.021	.036	.361	.491	.300	.167	.128	.086	.099	.098	.386	.374	.251	.364	.208	.430	.493	.536	.141	.139	.041	.038
10	.040	.035	.031	.030	.015	.045	.300	.287	.207	.197	.207	.311	.369	.388	.415	.486	.479	.400	.366	.527	.461	.544	.138	.136	.033	.028
12	.040	.026	.029	.029	.041	.046	.200	.268	.320	.236	.231	.295	.371	.390	.403	.509	.578	.421	.430	.640	.690	.557	.141	.140	.036	.036
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	5	5	5	5	5
x	.035	.033	.032	.035	.033	.042	.205	.234	.240	.227	.251	.267	.303	.342	.422	.474	.392	.371	.334	.511	.433	.408	.153	.142	.034	.033
se	.002	.003	.004	.003	.005	.001	.048	.065	.040	.026	.042	.055	.075	.068	.034	.037	.068	.043	.031	.034	.078	.086	.008	.003	.002	.003
11	.028	.030	.022	.035	.020	.030	.031	.034	.047	.047	.046	.034	.034	.028	.019	.027	.020	.028	.028	.030	.034	.041	.036	.027	.036	.027
13	.031	.024	.025	.027	.018	.023	.028	.035	.030	.033	.033	.042	.042	.031	.036	.036	.034	.350	.040	.036	.036	.038	.039	.039	.038	.039
n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
x	.030	.027	.024	.031	.019	.027	.030	.034	.039	.040	.040	.038	.038	.030	.028	.032	.027	.189	.034	.033	.035	.040	.038	.033	.037	.033
se	.002	.003	.002	.004	.001	.004	.002	.001	.009	.007	.007	.004	.004	.002	.009	.005	.007	.161	.006	.003	.001	.002	.002	.006	.001	.006

5.4.8. ANTIBODY (IgG AND IgM) ANTIGEN VALUES AND PARASITOLOGICAL EXAMINATION OF CSF FROM EXPERIMENTAL AND CONTROL GOATS.

Table 24 shows the results of the serological and parasitological examinations of CSF from experimental and control goats. There was no evidence of anti-trypanosome antibodies or trypanosomal antigens in the CSF of infected goats. Parasitological examination (HCT and MI) also showed no evidence of trypanosome in the CSF.

Table 24. Antibody (IgG and IgM) and Antigen-ELISA values (OD, E_{492nm}) and the parasitological examination results of the cerebrospinal fluid from goats of Group 1, 2 and 3.

Goat No.	ELISA OD Values (E_{492nm}) Ab-ELISA IgM	Ab-ELISA IgG	Antigen ELISA	Parasitological Exam. HCT	MI
1	0.022	0.013	0.027	-	-
3	0.034	0.023	0.018	-	-
5	0.011	0.022	0.019	-	-
9	0.013	0.016	0.029	-	-
14	0.012	0.021	0.019	-	-
15	0.023	0.023	0.026	-	-
2	0.022	0.020	0.026	-	-
4	0.026	0.023	0.033	-	-
6	0.027	0.035	0.034	-	-
8	0.031	0.023	0.038	-	-
10	0.033	0.026	0.022	-	-
12	0.035	0.033	0.019	-	-
11	0.028	0.035	0.028	-	-
13	0.031	0.019	0.031	-	-

OD readings of $\geq$ 0.05 were considered positive for IgM or IgG - Ab-ELISA
OD readings of $\geq$ 0.09 were considered positive for Ag-ELISA.

5.4.9 STATISTICAL ANALYSIS

Tables 25 and 26 shows statistical analysis of PCV, temperature, parasitaemia haemolytic complement (HC), IgG-Ab ELISA, IgM-Ab ELISA, and plasma and serum-Ag ELISA values.

Table 25. Analyses of Variance and Multiple range testing of Mean PCV, Temperature, Parasitaemia, Haemolytic complement, IgG Ab-ELISA, IgM Ab-ELISA, Serum Ag-ELISA and plasma Ag-ELISA of goats in groups 1, 2 and 3 throughout the experimental period.

Exp. Parameter	Group 1	Group 2	Group 3
PCV	32.53±33	32.97±.30	34.88±.22
	(F^2_{356} = 7.93. P < 0.001)		
Temperature	39.08±046	39.10±.042	38.79±.048
	(F^2_{356} = 7.37. P < 0.01)		
Parasitaemia *	455.12±113.72	72.85±32.69	0
	(F^2_{356} = 7.60. P < 0.001)		
Haemolytic complement	124.10±1.17	126.68±1.12	143.30±0.73
	(F^2_{356} = 42.02 P < 0.0001)		
IgG Ab-ELISA	.363±.018	.351±.019	.037±.001
	(F^2_{356} = 52.47. P < 0.0001)		
IgM Ab-ELISA	.325±.017	.288±.016	.041±.001
	(F^2_{356} = 44.71. P < 0.0001)		
Serum Ag-ELISA	.315±.020	.229±.015	.032±.001
	(F^2_{356} = 38.08. P < 0.0001)		
Plasma Ag-ELISA	.318±.020	.232±.015	.032±.001
	(F^2_{356} = 37.98. P < 0.0001)		

* The only parameter with statistical difference between the groups.

Table 26. Multiple Regression analysis of PCV,Temperature,Parasitaemia Haemolytic Complement (HC),IgG-Ab and IgM-Ab ELISA and Plasma Ag-ELISA based on Serum Ag-ELISA as the Dependent Vaviable.

	GROUP I STATISTICS				: GROUP II STATISTICS			
Independent Variable.	r	t	df	P	: r	t	df	P
PCV	.65	.6695	148	.540	-.53	.1006	143	.9200
Parasitaemia	.05	1.5300	148	.1290	.10	2.299	143	.0232
Temperature	.42	.0050	148	.9960	.28	1.063	143	.2899
HC -	.68	.4750	148	.6357	-.68	.920	143	.3593
IgG-Ab ELISA	.44	.3128	148	.7550	.60	.526	143	.5995
IgM-Ab ELISA	.91	4.608	148	.0000	.66	1.548	143	.1242
Plasma-Ag ELISA	.99	33.397	148	.0000	.92	15.502	143	.0000

5.5. APPLICATION OF McAB TEA1/23.4.6 IN THE FIELD DIAGNOSIS OF CAMEL TRYPANOSOMIASIS

5.5.1 CAMEL HERD ONE

Table 27 below summarizes PCV, HCT, MI, antibody and antigen-ELISA results after examination of camel Herd one. The 14 camels had all been proven positive on HCT. On mouse sub-inoculation (MI), only 13 were positive (day 0). All the 14 camels were positive on Ab and Ag-ELISA (day 0). By day 90, 2 out of 3 of the control camels (group E) were positive on MI and non was positive on HCT. All the 14 camels were positive on Ab-ELISA with even higher antibody ELISA values than day 0. Ag-ELISA values had dropped below zero in groups A and B which

had received the higher doses of the drug. However, one camel in group C (No 76) and all the camels in groups D and E were still positive on Ag-ELISA assay.

Table 27. PCV, HCT, MI, Ab-ELISA and Ag-ELISA values of Camels on an Experimental drug trial Herd 1.

Group	Camel No.	Observation Day 0 PCV	HCT	MI	Ab-ELISA	Ag-ELISA	Observation Day 90 PCV	HCT	MI	Ab-ELISA (OD,E492nm)	Ag-ELISA
A	12	23	+	-	.554	.126	30	-	-	.570	-0.020
	13	23	+	+	.499	.200	30	-	-	.436	-0.011
	52	21	+	+	.417	.303	25	-		.431	-0.025
B	D17	24	+	+	.434	.400	25	-	-	.308	-0.010
	D18	22	+	+	.496	.171	30	-	-	.688	-0.012
	D53	16	+	+	.317	.315	26	-	-	N/D	N/D
C	D20	27	+	+	.700	.125	30	-	-	.774	-0.014
	34	35	+	+	.226	.437	29	-	-	.340	0.000
	76	30	+	+	.552	.172	26	-	-	.364	0.111
	93	24	+	+	.470	.482	23	-	-	.657	0.097
D	86	34	+	+	.144	.118	28	-	-	.517	0.137
	45	22	+	+	.588	.298	27	-	+	.544	0.165
E	30	15	+	+	.337	.630	19	-	+	.523	0.167
	73	26	+	+	.713	.121	27	-	-	.286	0.062

Groups A - D = treated with the experimental trypanocide RM110
E = untreated controls
\+ = Parasitologically positive
\- = Parasitologically negative
N/D = Not done

Group A received 1.2 mg/kg; group B, 0.6 mg/kg, group C, 0.4 mg/kg and group D 0.2 mg/kg.

Analysis of variance multiple range testing showed no significant difference between the mean (+ SE) Ab-ELISA values of infected camels (On HCT or MI) ($\bar{X}$ = 0. 470 +.039) and parasitologically negative camels ($P< \bar{X}$ = 0.488 + 0.05). However, there was a very high significant difference ($P < 0.0001$) between Ag-ELISA values of parasitologically positive camels ($\bar{X}$=0.264 + 0.038) and parasitologically negative camels ($\bar{X}$=0.028 + 0.018).

5.5.2 CAMEL HERD TWO

Table 28 summarizes the PCV, HCT, MI, Ab-ELISA and Ag-ELISA results after the examination of herd two. All the camels were negative on HCT, MI, Ab-ELISA and Ag-ELISA except for two camels No. Z11 and Z12 whose Ab and Ag-ELISA values were higher than the rest. However, they were still negative at the cut-off point used in the assay systems ($\geq$ 0.09 for Ag-ELISA and $\geq$ 0.150 for Ab-ELISA). The PCV values of all the camels were within normal range ($\bar{X}$ = 31.3±0.62, range: 24-38).

Table 28. PCV, HCT, MI Ab-ELISA results obtained after the examination of Herd 2.

Camel No.	PCV%	HCT	MI	Ab-ELISA (OD, $E_{492\ nm}$ ± SE)	Ag-ELISA
A1	32	-	-	.032	0.000
A4	29	-	-	.032	-0.002
A8	34	-	-	.034	-0.009
B1	30	-	-	.015	-0.016
B4	33	-	-	.019	-0.007
B5	30	-	-	.015	-0.007
B8	36	-	-	.023	-0.008
D1	28	-	-	.026	-0.003
D5	38	-	-	.021	-0.003
D4	33	-	-	.009	-0.002
D8	35	-	-	.012	-0.000
P1	36	-	-	.015	-0.011
P4	30	-	-	.033	-0.004
P5	32	-	-	.023	-0.000
Z1	28	-	-	.049	-0.000
Z2	32	-	-	.030	-0.005
Z3	33	-	-	.088	-0.004
Z4	37	-	-	.046	-0.004
Z5	28	-	-	.020	-0.011
Z6	28	-	-	.031	-0.005
Z7	30	-	-	.050	-0.000
Z8	29	-	-	.028	-0.005
Z9	29	-	-	.023	-0.004
Z10	29	-	-	.025	0.009
Z11	24	-	-	.112	0.023
Z12	28	-	-	.110	0.006
Z13	Clotted	-	-	.014	-0.002
Z14	Clotted	-	-	.015	-0.008
Z15	29	-	-	.052	-0.006
Z16	33	-	-	.048	-0.002
Z17	35	-	-	.027	-0.007
$\bar{X}$ ± SE	31.3±.62			.036±.004	-0.009± -0.005

FIGURE 13:

Mean(+SE) PCV (%) (A) and Ab-ELISA, Plasma Ag-ELISA and serum Ag-ELISA values (OD,E_{492nm}) (B) in the camels of Herd 3. during the study period from day 0 of treatment with Trypacide, to the day 48 after treatment.

5.5.3 CAMEL HERD THREE

Tables 29(a) and 29(b) summarize all the results of PCV, HCT, parasitaemia, MI, serum Ag-ELISA, plasma Ag-ELISA and Anti-body ELISA after the first, second, third and fourth examination.

(a) PCV

Following treatment, there was a marked rise in mean PCV by day 14, see Tables 29(a), 29(b) and Fig. 13 (from 22.3+.64 to 29.9+.51). The PCV dropped slightly thereafter to 27.0+.33 on day 28 and 26.5+.30 by day 48.

(b) Trypanosome infection:

On day zero of examination, 23 out of 55 (42%) camels were positive on HCT; 47 out of 55(85%) positive on MI; 52 out of 55 (95%) positive on Ab- ELISA, 41 out of 55 (75%) positive on plasma Ag-ELISA and 47 out of 55 (85%) positive on serum Ag-ELISA (see Tables 29(a) and 29(b). All camels positive on HCT were also positive on MI. Of the MI positive camels (47) serum Ag-ELISA detected 44 camels (94%), plasma Ag-ELISA detected 41 (87%) and Ab-ELISA detected 47 (100%). This gives a false negative rate of 6%, 13% and 0%, respectively for the three tests.

By day 14, there was no parasitological evidence of trypanosome infection (on HCT and MI). However, Ab-ELISA still showed 100% (47 out of 47), serum Ag-ELISA showed 70% (33 out of 47) and plasma Ag-ELISA 74% (35 out of 47) trypanosome infection of the camels which were parasitologically positive on day 0. The percentages of herd trypanosome infection from day 48 are given in table 29(b). By day 28, 98% (46 out of 47) camels were positive on Ab-ELISA, 32% (15 out of 47) positive on serum Ag-ELISA and 42% (20 out of 47) positive on plasma Ag-ELISA. By day 48,95% (45 out of 47), 8.5% (4 out of 47) and 10.6% (5 out of 47) of the camels were positive on Ab-ELISA, serum Ag-ELISA and plasma Ag-ELISA, respectively. Fig. 13 compares the mean PCV, Ab - and Ag-ELISA values during the time of observation.

Table 29(a). PCV, HCT (Parasitaemia x 10^4/ml), MI, Serum Ag-ELISA, Plasma Ag- ELISA and Ab-ELISA results obtained after the examination of the camels in herd 3. (ELISA OD, E_{492}nm)

	PCV				HCT				MI				Serum Ag-ELISA				Plasma Ag-ELISA				Ab-ELISA			
Camel	Obs. Day				Obs. Day				Obs. Day				Obs. Day				Obs. Day				Obs. Day			
No.	0	14	28	48	0	14	28	48	0	14	28	48	0	14	28	48	0	14	28	48	0	14	28	48
1	27	27	31	32	500	-	-	-	+	-	-	-	.180	.049	.047	.027	.042	.045	.036	.032	.304	.332	.314	.357
2	15	25	21	24	500	-	-	-	+	-	-	-	.107	.050	.061	.032	.115	.060	.015	.032	.304	.332	.314	.357
3	20	30	26	26	-	-	-	-	+	-	-	-	.186	.142	.126	.024	.126	.054	.000	.032	.460	.422	.462	.238
4	26	32	29	25	-	-	-	-	+	-	-	-	.161	.100	.084	.033	.158	.015	.009	.024	.447	.311	.234	.359
5	23	34	26	25	-	-	-	-	+	-	-	-	.173	.115	.028	.028	.176	.132	.030	.032	.296	.313	.237	.235
6	21	31	25	24	-	-	-	-	+	-	-	-	.212	.194	.105	.025	.414	.426	.577	.065	.262	.226	.240	.156
7	23	29	27	27	-	-	-	-	-	-	-	-	.033	.073	.013	.024	.024	-.004	.006	.001	.315	.395	.352	.206
8	22	27	28	28	-	-	-	-	+	-	-	-	.331	.232	.107	.025	.419	.402	.180	.029	.272	.235	.282	.316
9	23	33	27	28	-	-	-	-	+	-	-	-	.140	.171	.111	.024	.135	.192	.063	.027	.232	.230	.237	.279
10	20	31	26	27	-	-	-	-	+	-	-	-	.085	.052	.031	.024	.095	.067	.051	.031	.301	.256	.283	.300
11	21	28	28	25	5	-	-	-	+	-	-	-	.247	.085	.055	.023	.383	.102	.102	.056	.281	.290	.241	.295
12	28	29	24	27	500	-	-	-	+	-	-	-	.480	.031	.159	.005	.570	.580	.615	.017	.253	.276	.252	.331
13	21	29	24	26	5	-	-	-	+	-	-	-	.630	.305	.084	.035	.716	.285	.204	.013	.197	.201	.187	.295
14	23	23	22	23	500	-	-	-	+	-	-	-	.125	.096	.044	.037	.079	.114	.045	.055	.197	.218	.206	.239
15	12	28	27	26	500	-	-	-	+	-	-	-	.228	.139	.099	.024	.202	.159	.093	.006	.301	.320	.294	.316
16	12	35	28	28	-	-	-	-	+	-	-	-	.058	.222	.109	.024	.067	.183	.083	.030	.164	.150	.135	.125
17	20	28	30	27	-	-	-	-	+	-	-	-	.144	.097	.047	.106	.180	.105	.036	.164	.355	.385	.311	.405
18	19	34	28	31	-	-	-	-	+	-	-	-	.223	.203	.115	.026	.318	.240	.072	.000	.284	.271	.237	.241
19	16	33	30	28	5	-	-	-	+	-	-	-	.109	.182	.106	.025	.058	.021	.030	.026	.325	.341	.306	.263
20	20	26	27	25	5	-	-	-	+	-	-	-	.124	.110	.130	.029	.267	.249	.187	.041	.439	.460	.421	.266
21	20	22	27	22	5	-	-	-	+	-	-	-	.279	.171	.078	.026	.233	.045	.009	.002	.308	.341	.295	.177
22	24	27	25	26	-	-	-	-	+	-	-	-	.163	.038	.014	.025	.427	.024	-.004	.030	.368	.411	.216	.217
23	22	26	29	27	500	-	-	-	+	-	-	-	.480	.245	.054	.019	.601	.546	.180	.003	.374	.412	.430	.207
24	20	32	29	29	500	-	-	-	+	-	-	-	.385	.225	.100	.027	.333	.345	.126	.029	.462	.480	.411	.227
25	22	25	27	28	500	-	-	-	+	-	-	-	.222	.197	.133	.024	.365	.345	.249	.022	.409	.430	.402	.152
26	18	29	27	27	500	-	-	-	+	-	-	-	.454	.215	.086	.002	.536	.338	.150	.050	.359	.381	.351	.274
27	15	23	25	26	500	-	-	-	+	-	-	-	.116	.060	.035	.029	.228	.210	.177	.006	.456	.490	.450	.310
28	21	28	25	24	-	-	-	-	+	-	-	-	.175	.081	.031	.027	.347	.105	.063	.049	.229	.300	.350	.158

Table 29(a). Continued.

Camel No.	PCV Obs. Day 0	14	28	48	HCT Obs. Day 0	14	28	48	MI Obs. Day 0	14	28	48	Serum Ag-ELISA Obs. Day 0	14	28	48	Plasma Ag-ELISA Obs. Day 0	14	28	48	Ab-ELISA Obs. Day 0	14	28	48
29	18	28	25	24	-	-	-	-	+	-	-	-	.310	.293	.246	.126	.755	.543	.360	.120	.184	.280	.200	.156
30	22	33	29	25	-	-	-	-	+	-	-	-	1.094	.901	.646	.895	.895	.700	.698	.825	.265	.296	.378	.269
31	18	35	29	26	-	-	-	-	+	-	-	-	.174	.009	.013	.029	.217	.144	.207	.030	.444	.480	.389	.206
32	22	33	29	25	500	-	-	-	+	-	-	-	.287	.150	.087	.020	.207	.216	.066	.036	.454	.461	.450	.259
33	20	30	29	26	500	-	-	-	+	-	-	-	.516	.279	.086	.017	.750	.392	.348	.090	.320	.398	.350	.235
34	16	25	25	24	5	-	-	-	+	-	-	-	.320	.142	.045	.020	.298	.144	.069	.066	.195	.250	.294	.243
35	27	32	30	30	5	-	-	-	+	-	-	-	.365	.625	.055	.296	.977	.905	.801	.495	.219	.264	.252	.320
36	25	35	25	25	5	-	-	-	+	-	-	-	.244	.033	.046	.023	.399	.201	.114	.039	.282	.320	.285	.353
37	16	24	19	24	-	-	-	-	+	-	-	-	.276	.111	.032	.044	.347	.153	.068	.037	.174	.198	.187	.225
38	30	37	30	31	-	-	-	-	-	-	-	-	.036	.056	.044	.019	.030	.033	.038	.031	.131	.106	.085	.095
39	26	34	27	24	-	-	-	-	-	-	-	-	.056	.088	.087	.028	.031	.034	.030	.021	.015	.010	.013	.004
40	33	37	25	27	-	-	-	-	-	-	-	-	.027	.030	.065	.016	.039	.057	.072	.039	.241	.235	.211	.226
41	28	28	30	29	5	-	-	-	+	-	-	-	.341	.266	.065	.032	.210	.036	.042	.057	.158	.260	.278	.227
42	25	30	36	28	5	-	-	-	+	-	-	-	.422	.374	.065	.020	.375	.243	.078	.030	.164	.290	.285	.259
43	23	34	26	25	-	-	-	-	+	-	-	-	.404	.404	.195	.061	.019	.150	.152	.014	.178	.290	.250	.115
44	23	28	25	25	500	-	-	-	+	-	-	-	.353	.397	.097	.116	.336	.447	.315	.161	.230	.295	.240	.156
45	18	28	28	26	-	-	-	-	+	-	-	-	.272	.384	.072	.029	.225	.138	.075	.045	.288	.305	.293	.319
46	32	30	32	30	-	-	-	-	+	-	-	-	.235	.229	.065	.023	.531	.450	.318	.018	.202	.218	.210	.298
47	28	35	28	29	-	-	-	-	-	-	-	-	.108	.055	.041	.020	.189	.105	.051	.021	.235	.300	.265	.158
48	25	34	29	23	-	-	-	-	+	-	-	-	.050	.074	.043	.020	.180	.111	.048	.019	.223	.285	.261	.238
49	21	30	28	26	-	-	-	-	+	-	-	-	.371	.129	.065	.025	.573	.258	.060	.031	.245	.295	.214	.185
50	26	35	26	30	-	-	-	-	-	-	-	-	.059	.041	.016	.029	.021	.045	.102	.000	.010	.020	.017	.009
51	25	32	27	24	-	-	-	-	+	-	-	-	.120	.048	.015	.031	.078	.048	.051	.049	.246	.265	.251	.186
52	26	32	28	27	-	-	-	-	+	-	-	-	.039	.086	.076	.024	.021	.063	.039	.036	.206	.281	.270	.210
53	28	30	28	27	-	-	-	-	+	-	-	-	.165	.062	.051	.025	.069	.069	.039	.035	.218	.295	.211	.229
54	26	28	26	26	-	-	-	-	-	-	-	-	.274	.106	.103	.018	.031	.071	.038	.040	.191	.243	.261	.136
55	30	32	29	29	-	-	-	-	-	-	-	-	.091	.065	.051	.028	.021	.084	.030	.043	.215	.211	.188	.236

obs.=observation day

Table 29(b). PCV, HCT, MI Ab-ELISA, Serum Ag-ELISA and Plasma Ag-ELISA results obtained after the examination of Herd 3.

Observation Day	0	14	28	48
No.of observations	55	55	55	55
No. +ve on HCT (%)	23(42%)	0(0%)	0(0%)	0(0%)
No. +ve on MI (%)	47(85%)	0(0%)	0(0%)	0(0%)
PCV ($\bar{X} \pm SE$)	22.3+.64	29.9+.51	27.0+.33	26.5+.30
No.+ve on Ab-ELISA(%)	52(95%)	52(95%)	51(93%)	49(89%)
$\bar{X} \pm SE$	(.271+.014)	(.298+.014)	(.270+.012)	(.232+.011)
No.+ve on Plasma Ag-ELISA(%)	41(75%)	35(64%)	21(38%)	5(9%)
$\bar{X} \pm SE$	(.285+.032)	(.204+.026)	(.137+.024)	(.059+.017)
No. +ve on Serum Ag-ELISA (%)	47(85%)	34(62%)	16(29%)	4(7%)
$\bar{X} \pm SE$	(.244+.024)	(.166+.021)	(.092+.018)	(.050+.017)

Out of the 8 parasitologically negative camels 5, 3 and 1 were positive on Ab-ELISA, serum Ag-ELISA and plasma Ag-ELISA, respectively. This gives a false negative of 62.5% for Ab-ELISA, 37.5% serum Ag-ELISA and 12.5% plasma Ag-ELISA.

(c) Statistical Analysis.

Analysis of variance and multiple range testing of mean Ag-ELISA and Ab-ELISA values of infected and non-infected camels of herd 3, showed a very high statistical difference between serum and plasma Ag-ELISA values of infected and non-infected camels ($P<.0.0001$). There was no statistical difference between Ab-ELISA values of infected and non-infected camels (see Table 30).

Table 30. Analysis of variance and multiple range testing of mean (+SE) Antigen-ELISA and Antibody-ELISA values of infected and non-infected camels in Herd 3.

	Source	df	Ms	F	P
A. Ab-ELISA	Between groups	1	.025	2.67	(P=0.105)
	within groups	218	.009		
	Group means				
	Infected		Non-infected		
	.288 +.013		.262 +.007.		
B. Serum Ag-ELISA	Between groups	1	1.036	45.03	(P<0.0001)
	within groups	218	.023		
	Group means				
	Infected		Non-infected		
	.268 + .026		.102 + .011		
C. Plasma Ag-ELISA	Between groups	1	1.385	39.25	(P<.0001)
	within groups	218	.035		
	Group means				
	Infected		Non-infected		
	.321+.033		.129+.0133		

Underlined group means (X) had no statistical significance.

5.5.4 CAMEL HERD FOUR

Table 31 summarizes the results of the examination carried out on Herd 4. All the 83 camels were negative on HCT and MI. However, 25(30%), 4(4.8%) and 4(4.8%) camels were positive on Ab-ELISA, serum and plasma Ag-ELISA, respectively. Considering that all camels were parasitologically negative, then the false positive rate by the three tests was 30%, 4.8%, and 4.8% for Ab-ELISA, Plasma and serum Ag-ELISA, respectively, which gives a very low rate of false positive for the antigen-ELISA tests. This agrees well with the previous observations.

Table 31. Mean(+SE) PCV,HCT, MI,Ab-ELISA, Serum Ag-ELISA and Plasma Ag-ELISA results obtained after the examination of Herd 4.

	HCT	MI	PCV	Ab-ELISA	Serum Ag-ELISA	Plasma Ag-ELISA
n	83	83	83	83	83	83
X	-	-	26.2	.108	.014	.005
+SE	-	-	+.320	+.001	+.001	+.001
No.+ve	0	0	-	25	4	4
%+ve	0	0	-	30%	4.8%	4.8%
No.-ve	83	83	-	58	79	79
%-ve	100	100	-	70%	95.2%	95.2%

CHAPTER SIX

DISCUSSION AND CONCLUSION

With a little experience in tissue culture techniques, and given the existence of more modern tissue culture facilities, hybridoma technology can be relatively simple. As additional standardization reagents become more commercially available, hybridoma technology is likely to become simpler, and its impact more widely felt in many aspects of clinical human and veterinary medicine.

One of the most important variables determining the success of a particular hybridization exercise, in many instances, is the immunization protocol (French *et al*, 1986). Empirically, the proportion of antibody producing hybrids depends on how well the animal is immunized (Hurrel, 1982). In addition, the outcome of such an exercise will also depend on the fusion protocol used, the health of cells involved throughout the procedure and the judicious screening for the hybrid clones producing the desired antibody (Pearson *et al.*, 1980; French *et al.*, 1986).

The immunization and fusion protocols used in this study (4.2.2.4 and 4.2.2.5) gave a fusion success rate ranging from 29%-100% hybrid clones, and 0 - 100% positive hybrid clones secreting different classes of antibodies to trypanosomal antigens (see Tables 7 and 8). This was a high success rate by any standards. It is clear from this study that by paying attention to certain details of the protocols and techniques involved in hybridoma technology, it is possible to achieve a high increase in the number of myelomas secreting the desired antibodies. The immunization protocol which was used in this study aimed at increasing the rate of blast transformation and hence thereby increasing the fusion rate (Stahli *et al.*, 1980) without necessarily being counter-productive as was observed by Oi *et al.*, (1978). It will be noted that the first two fusions in the immunizations with both ARMP and MRA antigens the fusion success rates of positive hybrid clones were low (18% and 0%; 17% and 25%, respectively). Thereafter, the percentage of

positive hybrid clones generally increased. This observation agrees quite well with the view of Goding (1980), that if multiple injections are to be considered necessary during the immunization exercise, then the animals should be "rested" for a few months, before they are finally boosted for fusion. There was a time difference of one to two weeks between successive fusions. This appears to have improved on the "rest" period before the animals were given the final boost.

The screening test had to be carefully chosen because it plays a key position in the success of hybridoma production. The ELISA technique employed was easy to perform, quick, sensitive, specific and enabled a large number of samples to be tested. Since the McAbs derived would be used in a double sandwich ELISA as antigen detecting probes, by employing ELISA technique as an initial screening test, ensured that the McAbs with the right avidity, affinity and specificity were selected right from the start. This is important because discrepancies in the sensitivities of the screening assays may arise. A monoclonal antibody showing a strong reaction in an IFAT test may not necessarily show a high ELISA reading. The shortcomings of ELISA technique have already been cited (Herzenberg *et al.*, 1980; Ghosh and Campbell, 1986). Because of this, IFAT technique was employed to supplement ELISA in cross-reactivity and sensitivity studies (see Tables 11 and 12). There was a very high agreement between ELISA and IFAT tests. However, in some instances IFAT scores were lower than the ELISA values. This discrepancy may be attributed to the different methods employed in the preparation of antigens for the two tests. An antigen prepared by two different methods may undergo unknown conformational effects in its epitopes (Ghosh and Campbell, 1986) which may lead to differences in recognition by a given McAb or set of McAbs.

The classes and subclasses of the immunoglobulins secreted by the various hybridomas in this study were easily identified by the double immunodiffusion technique using commercial antisera against mouse IgG1, IgG2a, IgG2b and IgM. However, 46 McAbs reacting with anti-mouse whole gamma fraction, and with evidence of antibody activity against *T.(T).b. evansi* STA antigen, could

not be identified by the commercial antisera against the above classes and subclasses of mouse immunoglobulins. The fact that the 46 McAbs were not tested against anti-mouse IgG3 and IgA serum may suggest that the former belong to either of these two. However, sets of antisera available commercially for the McAbs may not be adequately specific, allowing the unequivocal identification of some, but not all subclasses (Hurrel, 1982).

Two McAbs TEM3/3.4.6. and TEM5/9.8.7 were unstable to precipitation with 50% ammonium sulphate. This shows why it is important to test the stability of a given McAb to any particular treatment before committing a large batch (Galfre and Milstein, 1981).

The antigens and the immunization strategy employed in this study aimed at deriving McAbs which would be used in the immunodiagnosis of *T.(T).b. evansi* infection. The majority of the McAbs derived against KETRI 1342 (which is a strain from London but believed to have originated from East Africa), also showed a .wide range of cross-reaction with other mammalian trypanosome species in an ELISA and IFAT tests. On the other hand, no cross-reaction was observed between these McAbs and the haemoparasites used in this study. It is to be noted that crude trypanosome antigen preparations were used in all the immunizations during the study. Trypanosomes being complex unicellular organisms, one would expect a wide variety of different antibodies to be elicited against different epitopes of the crude trypanosome antigen fractions. Some of these antibodies are directed against variant or strain specific antigens, while others are directed against species or genus specific antigens (Weitz, 1960; Gray, 1961; Brown and Williamson, 1962; Seed and Weinman, 1963; Williamson and Brown, 1964; Nantulya et al., 1987). Evidently therefore, the majority of the McAbs produced in this study are directed against antigen epitopes which are common to trypanosome species mentioned, but not present in the haemoparasites used in the study. Two McAbs TEM5/17.4.6 and TEM3/12.3.6 which only reacted with KETRI 1342 are directed against strain-specific antigens present in this

strain but not in any of the strains used except, probably, present in KETRI 2468 and 2469 at a very low level (see Table 13). No *T.(T).b. evansi* specific McAb was identified. However, ELISA and IFAT readings of many McAbs showed higher readings for the *T.(T).brucei* subgroup trypanosomes than the other species. (See Tables 11 and 12). This may mean that these antigens are more abundant in the former than the latter, rather than their absence in the latter. Other workers have been able to produce species-specific McAbs against *T.(T).congolense* and *T.(D).vivax* (Parish et al., 1985; Richardson et al., 1986 and Nantulya et al., 1987). Although *T.(T).brucei* subgroup specific McAbs have been reported (Richardson et al., 1986 and Nantulya et al., 1987), McAbs showing specificity for the different *T.(T).brucei* subspecies have not been reported. However, since nosological and biochemical differences exist between these subspecies (Hoare, 1972, Lumsden and Ketteridge, 1979), the presence of antigens peculiar to each subspecies cannot be ruled out.

By improving the specificity and immunogenecity of antigen fractions used for immunization, selecting the best responders, enriching for B lymphocytes producing the desired antibodies, increasing the efficiency of fusions and screening judiciously, it is possible to produce subspecies-specific McAbs (French et al., 1986). Such an exercise may require more fusions than the ones carried out in this study, together with very careful consideration of the above mentioned suggestions. According to Parish et al., (1985), Richardson et al., (1986) and Nantulya et al., (1987), some of the species specific antigens are associated with cell membrane of trypanosomes and are very well expressed on the cell membrane of the procyclic forms (coatless). This is why it has been easy to produce species-specific McAbs against tsetse- transmitted trypanosome species. In the case of *T.(T).b. evansi* and related subspecies which are mechanically transmitted but do not transform into the coatless insect forms it may be more difficult and may require a longer time to produce McAbs that are subspecies specific. However, in addition to membrane antigens, internal soluble species-specific or subspecies-specific antigens do also exist (Parish et al., 1985;

Nantulya et al., 1987). Enrichment for such antigens by biochemical or immunochemical procedures like two dimensional immunoelectrophoresis, sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE) or affinity chromatography, could be of added advantage.

Monoclonal antibodies are a powerful "short-in-the-arm" (Mitchell and Cruise, 1981). Many of the McAbs produced in this study could be used in many ways. They could be used as probes for antigenic determinants in the analysis of antigen location, organization and availability of the common trypanosome antigens. They could also be used to study antibody-mediated, parasite - inhibitory effects, *in vivo* and *in vitro*. Nantulya and Moloo (1988) were able to show evidence of suppression of cyclical development of *T.(T).b. brucei* in *Glossina morsitans centralis* by an anti-procyclics monoclonal antibody. The strain-specific McAbs TEM5.17.4.6 and TEM3/12.3.6 could be used in serodeme studies to identify strains belonging to the same serodeme from given *T.(T).b. evansi* stocks or isolates.

The application of polyclonal antibodies (PcAbs) or McAbs for the development of immunodiagnostic reagents is only a recent development. There are very few reports of the application of such reagents for the immunodiagnosis of trypanosomiasis. In the present study, the five McAbs selected and evaluated for use as trypanosome antigen detecting probes in lysates of bloodstream forms of *T.(T).b. evansi* were able to detect 12 ug/ml to 1.25 ug/ml of lysate antigen (see Table 15) and at an optimal conjugate dilution varying from 1:250 - 1:1000 and an optimal protein concentration of 25 ug to 63 ug for coating each micro-well of the ELISA microplate (see Table 14). This represents 250 ng to 126 ng of protein in each well of the ELISA plate containing 200 ul/well of lysate antigen and compares favourably with the findings of Araujo (1982), who, in a double antibody sandwich ELISA and employing PcAbs, was able to detect as low as 170 ng of protein antigen in *T.cruzi* lysate. Rae and Luckins (1984), also using PcAb and PcAb - Peroxidase conjugates in a double antibody sandwich ELISA were able to detect as low as 300 ng of trypanosome protein antigen in soluble reference

antigen preparations of *T.(T).b. evansi* and *T.(N). congolense*. On the other hand, Liu et al. (1988) also using a double antibody sandwich ELISA were able to detect trypanosome antigens from as few as 50-5000 trypanosomes/well or 1-5 ng of trypanosomal membrane proteins/well. This was much lower than what Nantulya et al. (1987) who, using McAbs in a double antibody sandwich ELISA were able to detect as low as 1 x 10^5 trypanosomes/ml or 10,000 trypanosomes per 100 ul.

In this study circulating trypanosomal antigens were demonstrated in the serum and plasma of goats experimentally infected with *T.(T).b. evansi* KETRI 1342, using McAb TEA1/23.4.6 in a double antibody sandwich ELISA. Circulating antigens were detected as early as one day after infection in the goats which were infected intravenously. In most of the goats which were infected intra- muscularly (group two) detectable antigenaemia was evident by day six after infection, except for goat number 6, in which the antigens could not be detected until day thirteen. This latter observation could be associated with very low antigen levels below the detectable values. In the infected goats, Ag-ELISA values showed a steady rise above the pre-infection level and, although they showed considerable fluctuation in both groups, they remained above the pre-infection level until the time of treatment. Rae and Luckins (1984) found that in goats experimentally infected with *T.(D). vivax*, *T.(T).b. brucei* and *T.(T).b. evansi*, circulating trypanosome antigens were detected within 10 - 40 days of infection, followed by a decline. This was more obvious in the goats infected with *T.(D). vivax* and *T.(T).b. brucei*. This finding was attributed to the rapid multiplication of large numbers of trypanosomes inoculated intravenously into susceptible hosts or the formation of immune complexes, in the later stages of infection, in which fewer antigenic determinants were exposed.

Following treatment, the parasitaemia disappeared and Ag-ELISA values also started to decline. In group two goats, antigen levels were already on the decline by the time of treatment. This trend continued until pre- infection level was attained. In group one goats, there was a high elevation of the

circulating antigens after treatment. This was followed by a very sharp fall to pre-infection levels. In both group one and two goats, antigen levels had fallen to pre-infection levels by day 19 and 31 after infection, respectively. However, the antibody level profile was quite different. The IgG antibodies were detected some days after the antigens had been detected in both groups. However, IgM antibodies were detectable at the same time as the antigens in group two goats but five days later in group one goats. The antibody levels showed less fluctuation and remained higher than pre-infection up to the end of the experiment. The difference in the Ag-ELISA values of detectable circulating antigens in the two groups could be attributed to the differences in the numbers of trypanosomes in the circulation or in the tissues in the individual goats of both groups. However, although antigenaemia was closely associated with parasitaemia, changes in parasitaemia levels were not correspondingly reflected in the levels of detectable antigenaemia, and in many cases antigens were detected in the absence of demonstrable parasitaemia (see Table 22 and 23). In all the infected goats, antigenaemia was demonstrable in the presence of antibodies.

Wilson et al. (1975) and Houba et al. (1976) were able to demonstrate parasite antigens in serum which also contained antibodies. Rae and Luckins (1984] and Liu et al. (1988) using a double antibody sandwich ELISA were also able to detect trypanosome antigens in sera of infected animals. Antigens of parasites may exist in circulation as free molecules or in the form of immune complexes. In the latter form they can only be detected in immunoassays like Ag-ELISA techniques, if some of their epitopes are free to be recognized by PcAbs or McAbs. Liu and Pearson et al. (1987) observed some false negatives when they used murine McAbs produced against procyclic membrane protein ("procyclin"). Indeed, parasite molecules which are likely to induce a strong immune response in the infected host may have all their epitopes completely masked by reacting with corresponding antibodies to form immunecomplexes, and thus making their detectability difficult in long-standing infections. Detectability of such antigens will therefore

depend on the balance between antibodies and antigens.

No trypanosomal antigens were detected in the CSF of infected goats. This was evidenced by the absence of trypanosomes in CSF on parasitological examination. Anti-trypanosomal antibodies could not be detected either. These findings show that the *T.(T).b. evansi* 1342 did not cross into the CNS. However,it is still a controversy as to whether *T.(T).b. evansi* crosses into the CNS or not, during infection (Olaho and Wilson 1981),although, in this study, there was no evidence of CNS infection.

The role of complement in antibody production has been reviewed by Peys (1976). In trypanosomiasis there is evidence of complement consumption (Nielsen *et al.*, 1978) which leads to hypocomplementaemia (Rurangirwa *et al.*, 1980). Hypocomplemetaemia in trypanosome-infected animals may lead to immunodepression (Rurangirwa *et al.*, 1978). In this study there was evidence of total haemolytic complement consumption which could have resulted into immune complex formation. However, detectability of antigenaemia was possible in all goats during the time the animals were infected, and for some time after treatment. This means that the antigen which was being detected by McAb TEA1/23.4.6 had a free epitope which was being recognized by the McAb. This finding served as the basis for applying the McAb TEA1/23.4.6 for the diagnosis of cameline trypanosomiasis.

The limitations of the parasitological and the serological techniques currently employed for diagnosis of trypanosomiasis have been reviewed. In the light of the promising results of Ag-ELISA (Araujo, 1982; Rae and Luckings, 1984; Nantulya et al., 1987 and Liu et al., 1988) McAb TEA1/23.4.6 was employed as an antigen detecting probe for the diagnosis of cameline trypanosomiasis in selected camel herds. The double antibody of sandwich ELISA employing the above named McAb compared very well with the parasitological tests (HCT and MI). In the first camel herd at Ngare-Ndare, the Ab-ELISA and Ag-ELISA tests were both 100% sensitive. It is important to note that all the camels in

this herd had been diagnosed positive on the HCT test. The MI test missed to detect one animal, proving that even the improved parasitological tests may fail to detect some positive animals (Luckins, *et.al* 1979). Following treatment, by day 90, the camels which had received the higher doses of RM110 (May and Baker) had no trace of trypanosome antigens as evidenced by negative ELISA values (see Table 27). However, three camels in the groups which had received lower doses of the drug, and two control camels, were still positive. One of the control camels (No. 73) had been treated earlier with "Trypacide" (May and Baker) to save it from dying. It will be noted that in all the groups, antibody levels remained high and, in some cases, even higher than pre-treatment levels. Although the HCT and MI tests could not detect the Ag-ELISA positive camels at day 90, it is probable that the Ag-ELISA positive camels were still parasitologically positive with parasites hidden in the tissues. Unfortunately, there was no follow-up to show whether they relapsed or not. But even if they did relapse after day 90, it would be difficulty to know whether this would be a relapse or re-infection. Nevertheless, these findings show that Ag-ELISA technique employing TEA1/23.4.6 McAb was more reliable in assessing the patent state of infection, than Ab-ELISA and could be successfully used to evaluate the success of field chemotherapeutic trials.

In another field study, at Athi - River in an area not known to have tsetse - flies (not surveyed yet), again Ag-ELISA results using the same McAb gave 100% negativity in camel herd (herd No.2) with no known record of trypanosome infection. This was well reflected in the camel PCV values, Ab- ELISA values and negative HCT and MI results (see Table 28). These results show clearly that the test can be very reliable and could be used to assess the health status of camel herds as far as trypanosome infection is concerned.

In herd No. 4, in which all the 83 camels were parasitologically negative on HCT and MI, Ab-ELISA diagnosed 25 camels as positive (30% positive, 70% negative). On the other

hand, Ag-ELISA diagnosed only 4 camels as positive (3.8% positive, 95.2% negative). Since HCT and MI tests are the only reliable tests used for the diagnosis of patent trypanosoma infection in camels, if it is held that the 83 camels were all parasitologically negative, then Ag-ELISA gave a much lower percentage of false positives than Ab-ELISA. It must be borne in mind that this camel herd had been moved from Ngare-Ndare to Athi-River and that at the time the herd, was examined, all the camels had been treated with Suramin (Bayer, West Germany) a couple of months before. Another important aspect to consider was the suspect of the existence of drug resistant strain of *T.(T).b. evansi* to Suramin, which could mean that the presence of antigens in the four camels could be associated with cryptic infection undetectable by HCT or MI.

In the third camel herd at Athi-River (which had also moved from Ngare- Ndare), a very high point prevalence of patent infection (as shown by HCT and MI) was observed on day 0. In this herd, over 95% ofthe camels were detected by Ag-ELISA or Ab-ELISA. However, of the camels diagnosed as positive by MI, 100% were positive on Ab- ELISA and 94% were positive on Ag-ELISA. Note carefully that camel No.16, though negative by Ag-ELISA on day 0, by day 14 it was positive. Furthermore, the Ag-ELISA value of camel No. 52 were elevated to near positive value by day 14 (0.039 to 0.086). By examining the Ag-ELISA values of all the camels in herd three (see Table 29(a)) it will be noticed that Ag-ELISA values of camels No.35, 44 and 45 were elevated above the day 0 value by day 14. The Ag-ELISA value of camel No.43 had not changed by day 14. In the goat experiment (group one goats), the Ag-ELISA values also rose after treatment, before declining sharply to pre-infection levels. It appears that after treatment, trypanosomes die releasing antigens into circulation. Many of these are strong immunogens and immediately form immune complexes with antibodies which had been formed earlier against them. However, the less immunogenic antigens or antigens whose epitopes are not completely masked by immune complex formation may remain detectable in the circulation for some time after treatment. It would therefore

be expected that the plasma or serum levels of such antigens will be elevated for some time after the treatment of trypanosome infected animals. This is what appears to be the case with the antigens being detected by McAb TEA1/23.4.6. Notwithstanding, a proper assay of the levels of such circulating trypanosome antigens in sera or plasma taken at shorter intervals after treatment could give a better post-treatment picture of the antigen profile.

From the results of the goat experiment and the camel field experiment, subsequent to treatment, circulating trypanosomes fall very rapidly. In some camels by day 14 antigen levels had fallen below the lower limit for positive Ag-ELISA value. In the experimental goats, antigen levels had fallen to pre- infection level by day 11 to 31 after infection. In the field camels (Herd three) by day 29 after treatment antigen levels had fallen to a mean negative value. In a few camels of herd 1 and 3, antigen levels persisted and remained high up to the end of the observation period. This could mean: persistence of tissue foci of the infecting trypanosomes, re-infection, or circulating immune complexes. Re-infection is ruled out in herd three on the grounds that the drug used had a longer prophylactic effect than the observation time. From the work of Rae and Luckins (1984), it was observed that after treatment the level of circulating trypanosome antigens in infected rabbits fell to pre-infection values within 7 days, while antibodies persisted for longer periods. Liu et al., (1988) also observed that in monkeys infected with *T.(T).b. rhodesiense* and then treated with the trypanocidal drug, Melarsoprol (Mel B), antigens could not be detected by as early as 27 days and none of the sera taken long after successful drug treatment (105 - 933 days post-treatment) showed any trace of detectable trypanosomal antigens in serum. Only one of the pre-infection sera gave a false positive. These workers also observed an oscillation in serum antigen levels with a much more stable level of antibodies.

In conclusion, a number of McAbs were generated which identify common trypanosome antigens in lysates of seven bloodstream-forms of African trypanosome species. Two McAbs were

strain-specific for *T.(T).b. evansi*, KETRI 1342. McAb TEA1/23.4.6 was successfully used as an antigen detecting probe in lysates of seven bloodstream-forms of African trypanosome species and later, in the cerebrospinal fluid, plasma and serum of goats experimentally infected with *T.(T).b. evansi*. In this experimental model, trypanosome antigens were detected earlier than the antibodies, and continued to be detected even in the presence of antibodies. Following treatment, trypanosome antigens disappeared much earlier than the antibodies. These experimental findings were well reflected in the field studies carried out on camel herds, where Ag-ELISA employing the above McAb showed sensitivity of 94-100%. The Ag-ELISA technique employing the above McAb also showed that even at field level, after treatment, circulating trypanosome antigens disappear from the circulation much earlier than the antibodies. The Ag-ELISA technique was much more sensitive in assessing the patent state of infection than Ab-ELISA.

Though Liu et al. (1988) expressed fears of employing McAbs in Ag-ELISA systems due to the fact that a given McAb is limited to the recognition of only one epitope, the results of this study show that this is not necessarily true for all McAbs. Thus TEA1/23.4.6 could be used as a reliable reagent together with other McAbs reported by Parish et al. (1985), Richardson et al., (1986) and Nantulya et al. (1987) in the study of the epidemiology of animal trypanosomiasis and trypanosomiasis control programmes. This McAb is particularly useful in that it will recognize other trypanosome species and therefore in areas where several trypanosome species are endemic, it saves the time and the expenses of having to run several tests. For species identification, the McAbs produced by workers quoted above may be used.

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APPENDICES

1. PRECOATING OF SLIDES WITH AGAR:

(a) Clean slides with chromic acid or detergent; then rinse several times in tap water and in distilled water. Allow to dry.

(b) Prepare a 0.3% solution of agarose.

(c) Dip slides in melted agar using a pair of forceps and allow to dry at room temperature.

(d) Coated slides can be marked on the underside with wax marking pencil or a glass marker.

(e) Precoated slides can be stored at room temperature wrapped in aluminium foil.

2. WASHING, DRYING AND STAINING AGAR SLIDES:

(a) Immerse slides in saline for 24 hours.

(b) Place the slides in distilled water for several hours with at least three changes.

(c) Place slides in an incubator at 37^{o} C until dry.

(d) Stain slides with Ponceu 'S' or Amido Black for 10 minutes.

(e) Wash slides with tap water.

(f) Destain in 2% acetic acid.

(g) Allow slides to dry at room temperature.

(h) Now they are ready for reading and recording.

3. NORMAL GROWTH MEDIUM (NGM)

RPMI-1640 Medium (Flow Laboratories, Irvin, Scotland)	444.5ml
Inactivated Foetal Bovine Serum (FBS Flow Lab. Irvin, Scotland)	50.0ml
Gentamicin 59mg/ml (Schering, Kenilworth, NJ, USA)	5.0ml
Glutamine, 200mM (Flow, Irvin, Scotland)	5.0ml
Total	500.0ml

(Filter again 0.045 um or 0.2 and store at 4^{o} C. Add fresh glutamine if kept more than 10 days).

4. POLYETHYLENE GLYCOL SOLUTION I. (PEG I)

Autoclave 50g polyethylene glycol (PEG) 1550(Serva,

Heidelberg, F.R.G. or 1500 BDH, England) until liquified. When it has cooled to about 60° C and add 70ml of serum-free RPMI-1640 containing dimethylSulphoxide (DMSO) (55ml RPMI-1640+15ml DMSO). This is 41.6% PEG/15% DMSO. store at 4° C.

5. POLYETHYLENE GLYCOL SOLUTION II (PEGII)

Autoclave 50g PEG 1550 or1500 PEG until liquified. When it has cooled to about 60° C add 150ml of serum-free RPMI-1640. This is 25% PEG. store at 4° C.

6. FUSION MEDIUM

RPMI-1640 medium	398.0ml
Inactivated (FBS)	100.0ml
Gentamicin (50mg/ml)	0.5ml
Glutamine (200mM)	5.0ml
N-2 hydroxyethyl-piprazine-N-2-ethane sulfonic acid (HEPES) IM	5.0ml
2-mercaptoethanol $5x10^{-2}$M	0.5ml
Total	500.0ml

(Store at 4° C. Keep no longer than 14 days)

PH must be 7.2 (approx. 1 i.e "Salmon" Coloured). Adjust pH to 7.2 with 1.5ml of Im Na OH, just before use.

7. HYPOXANTHINE/AMINOPTERIN/THYMIDINE (HAT) - 100 X SOLUTION

Hypoxanthine (Sigma, St. Louis, MO, U.S.A.)	130.0 mg
Aminopterin (" " " " ")	1.9 mg
Thymidine (" " " " ")	39.0 mg
Na OH (0.0IM)	100.0 ml

Dissolve the above chemicals in the NaOH, filter through a 0.45um filter and add 1.0ml HEPES buffer (1.0M). Store at 4° C. Stable for several months. Do not expose aminopterin to light.

8. HYPOXANTHINE/THYMIDINE (HT) - 100 X SOLUTION

Hypoxanthine (Sigma, St. Louis, MO, U.S.A.)	130.0 mg
Thymidine (" " " " ")	39.0 mg
Na OH (0.01M)	100.0 ml

Prepare as for HAT. Store at 4° C. Stable for several months.

9. HAT and HT Medium (IX).

Fusion medium (as in recipe 4)	99.0 ml
HAT or HT (100X)	1.0 ml

Store at 4° C. Keep no longer than 14 days.

10. CLONING MEDIUM

RPMI-1640 medium	78.9 ml
Inactivated FBS	20.0 ml
Gentamicin	0.1 ml
Glutamine (200mM)	1.0 ml
Total	100.0 ml

11. FREEZING CELLS FROM CULTURE

(a) Cells are put into sterile test-tubes and an aliquot taken for counting.

(b) Spin cells at 600g for 5 minutes.

(c) Suspend the cells in FBS containing 10% DMSO so that there are $5x10^5$-$5x10^6$ cells/ml.

(d) Aliquot 1-2ml per ampoule.

(e) Cool ampoules at 4° C for 30 minutes and then transfer to the vapour phase of liquid nitrogen for 24 hours.

(f) Transfer to liquid nitrogen for permanent storage.

12. PREPARATION OF BOILED RBC STROMA

(a) Collect 100ml of sheep red blood cells (RBC) in 25ml of 3.8% sodium citrate.

(b) Wash once with isotonic saline.

(c) Add 0.4ml of Glacial Acetic acid to 1 litre of ice-cold distilled water.

(d) Add the Packed red cells slowly, with constant and vigorous mixing and continue stirring for about 10 minutes to complete RBC lysis and allow the RBC stroma to settle in the cold room at 4° C overnight.

(e) Decant as much of the supernatant as possible.

(f) Transfer the stroma to at least six 25-ml centrifuge bottles and centrifuge at 500g at 4^{O} C for about 15 minutes until the stroma is packed and remove the supernatant fluid by suction.

(g) Wash the RBC stroma 4-6 x with 0.001M acetate buffer, ph 5.0 (1.48ml of 02.m acetic acid and 3.25ml of 0.2m sodium citrate trihydrate per litre). Use as much buffer as possible and centrifuge in the cold as above.

(h) Suspend the RBC stroma in equal volume of cold 0.15m NaCl and distribute among eight heavy wall 10ml centrifuge tubes and centrifuge at angle head of 0^{O} for 20 minutes at 2000g and make two similar washings with 0.15m NaCl. As the acetate is washed out the stroma may settle less readily, so you may increase the time of centrifugation.

(i) Finally suspend the washed stroma in 0.15m NaCl to a volume of 30-40ml.

(j) Transfer the suspension to a flask containing a magnetic stirring bar. Cup the flask and immerse in boiling water for one hour. Cool and then stir on a magnetic stirrer to obtain a smoothly dispersed suspension.

(k) Analyse a sample for Nitrogen by Micro-Kjeldahl analyses and dilute to 1mg Nitrogen/ml. Add merthiolate to a final concentration of 1:10,000(w/v)which is 1% of a 1% solution.

13. BUFFERS

(a) 0.01M PBS pH 7.2

A	0.2M $NaOH_2$ PO_4. $2H_2O$	14.0 ml
B	0.2M Na_2HPO_4	36.0 ml
C	1.5M NaCl	100.0 ml
D	Distilled water	850.0 ml

To make PBS pH 7.2, mix 14ml of solution A and 36ml of solution B and add 100ml of NaCl, adjust the pH to 7.2 and add 850ml of distilled water. Check the pH again.

(b) 0.01M PBS - Tween 20, pH 7.4

A	0.2M $NaOH_2PO_4$. $2H_2O$.	9.5 ml
B	0.2M. Na_2HPO_4.	40.5 ml
C	1.5M NaCl.	100.0 ml
D	Distilled water	850 ml

E Tween - 20 0.5 ml

Mix the solutions as in (i) above and then add Tween - 20. Adjust the pH to 7.4 and then add 850ml of distilled water. Check the pH again.

(c) PSG, Ph 8.0

A	$NaOH_2PO_4$.	0.414 g
B	Na_2HPO_4.	8.088 g
C	NaCl.	2.550 g
D	Glucose (Dextrose).	10.00 g.

Dissolve in 1000 ml of distilled water and adjust the pH to 8.0.

(d) 0.02 M Phosphate Buffer pH 7.5

A	0.2M $NaOH_2PO_4$	16.0 ml
B	0.2M Na_2HPO_4	84.0 ml

Mix A and B, adjust pH to 7.5 and add 900 ml of distilled water. Check the pH again.

(e) 0.02M Phosphate Buffer pH 7.8

A	0.2M $NaOH_2PO_4$	8.5 ml
B	0.2M Na_2HPO_4	91.5 ml

Mix A and B, adjust pH to 7.8 and add 900 ml of distilled water. Check pH again.

(f) 0.001M (1mM) Acetate Buffer pH 4.4.

A	0.2M Glacial acetic acid.	3.05 ml
B	0.2M Sodium acetate	1.95 ml

Add A to 950 ml of distilled water with stirring, then add B. Mix well and adjust the pH to 4.4.

(g) Barbital Buffer pH 8.6:

Dissolve 8.5 g of barbital sodium in 500 ml of distilled water and adjust the pH to 8.6 with 1M Hcl. Make the volume to 1 litre with distilled water. Check the pH again.

(h) Carbonate/Bicarbonate Buffer, pH 9.6 (Coupling Buffer)

A	$NaCo_3$	1.59 g.
B	$NaHCo_3$	2.93 g.

Dissolve A and B in 1000 ml of distilled water. The pH usually comes to 9.6 without adjusting.

(i) 0.01M Tris Buffered Saline (TBS), pH 7.3:

A	Tris (hydroxy methyl) aminoethane	1.21 g
B	1.0M HCl.	
C	1.5M NaCl.	100 mls.
D	Distilled water.	

Dissolve A in 500 ml of distilled water. Add C and adjust the pH to 7.3 with B. Top up to 1000 ml with distilled water and check the pH again.

(j) 0.5M TRIS - HCL Buffer containing 0.002M EDTA pH 8.0:

A	Tris (hydroxy methyl) aminoethane	60.5 g
B	EDTA - diSodium salt	.74 g

Dissolve A in 800 ml of distilled water, add B and stir. Adjust the pH to 8.0 with 1.0M Hcl and make up the volume to 1000 ml. check the pH again.

(k) Veronal Buffered Saline (VBS) pH 7.4:

(i) Prepare a 5 x concentrate stock (VBS) by dissolving in 1 litre:

A 5.095 g of Sodium barbital (Verona)

B 41.5 g of Sodium Chloride and then add,

C 17.75 ml of 1.0M Hcl, with stirring.

This stock solution is stable for 1 month at 4^{o} C.

(ii) 0.03M $Cacl_2$: dissolve 3.33 g of $Cacl_2$ (anhydrous) or 4.41 g of the dihydrate in 100 ml of water.

(iii) 0.1M $MgCl_2$: dissolve 20.33 g of $Mgcl_2 . 6H_2O$ in 100 ml of water.

(iv) Working buffer for use is to be prepared daily: blend 200 ml of (1) 5ml of (2) and 5 ml of (3) and dilute to 1000 ml, including 1 g of gelatin, Added inorganic divalent ions supply: 1.5×10^{-4} M Ca^{2+} and 5×10^{-4} M Mg^{2+}.

14. ADDITONAL REAGENTS FOR ELISA:

(a) 1% Orthophenylenediamine (1% OPD).

0.1 g of OPD is dissolved in distilled water.

Work quickly in a shaded position.

No heating should be done.

Dispense in 350 ul volumes into vials and store at -20□ C in the dark.

N.B OPD is light sensitive, and carcinogenic!!

(b) OPD - Diluent:

A. 0.2M Na_2HPO_4	126.3 ml
B. 0.1M Citric Acid	73.7 ml
C. H_2O_2 (30 Vol.)	334 ul

Mix A and B and then C and Filter.

Dispense in 10 ml volumes into universal bottles.