STUDIES ON ERYTHROPOIETIN RESPONSES IN CATTLE EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA CONGOLENSE*

By

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DECLARATION

This is my original work and has not been submitted for a degree at any other university or institution.

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This work has been submitted to the University of Nairobi for examination with our approval as University Supervisors.

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This work is dedicated to my father, Josephat Buza, my mother, Georgina Karumuna, my wife, Teddy, my son, Joe and my daughter Janet.

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ABSTRACT

Since the anaemia of *T. congolense* infection, which has been shown to be mainly due to erythrocyte destruction by macrophages of the mononuclear phagocytic system, has been shown to be associated with poor reticulocyte response, a study was designed to verify if the unresponsiveness is attributable to low erythropoietin levels. In the absence of commercially available bovine erythropoietin, a radioimmunoassay based on human urinary erythropoietin was assessed in measuring bovine erythropoietin levels, but was found to be unsuitable, presumably because the polyclonal antibody to human erythropoietin could not recognise bovine erythropoietin.

An *in vitro* bioassay which indirectly measures erythropoietin levels by utilising erythropoietin's stimulatory effect on ^{125}I-deoxyuridine (IUDR) incorporation in DNA of spleen cells from phenylhydrazine treated mice was able to measure erythropoietin levels in bovine plasma. The assay could detect minimum of 62.5 mU/ml of erythropoietin in plasma. Using the *in vitro* bioassay, increased erythropoietin levels were detected in calves as early as 6 hours post-bleeding (hpb) of 50% of their estimated total blood volume. The peak levels of 1225 mU/ml was reached at 33hpb and dropped below the detection limit of the assay by 72hpb. Reticulocytes appeared in the blood of the calves by 72hpb and this was followed by a macrocytic hypochromic anaemia during the recovery period.

From 18 day post-infection (dpi) onwards, undiluted plasma collected from *T. congolense* infected animals suppressed IUDR incorporation into spleen cells. Diluting the plasma five fold decreased its suppressive effect and allowed the detection of erythropoietin.

Using diluted plasma in the assay, distinct erythropoietin peaks were observed both in the acute stage of the disease when the packed cell volume (PCV) was dropping rapidly and in the chronic stage when the PCV had stabilised between 16-17%. Increased erythropoietic response occurred throughout the acute stage of the disease indicated by progressive increase in mean corpuscular volume. During the chronic stage of the disease (39-76 dpi) the anaemia was macrocytic hypochromic and there was very few reticulocytes (0.2-0.4%). From 76dpi, despite low PCV and peaks of elevated erythropoietin, the erythropoietic response waned as indicated by a normocytic normochromic anaemia and an absence of reticulocytes. This suggests the bone marrow was unresponsive to elevated plasma erythropoietin.

Cattle treated in the chronic stage of *T. congolense* infections recovered their normal PCV although their erythropoietin levels were below the detection limit of the assay. The animal treated on 57dpi recovered its pre-infection PCV four weeks after treatment while an animal treated on 85dpi had only recovered 84% of its pre-infection PCV 11 weeks after treatment.

CHAPTER ONE

1. INTRODUCTION

Animal trypanosomiasis is a disease caused by several species of protozoan parasites of the genus *Trypanosoma*. The disease is endemic in sub-Saharan Africa, and in large areas of Asia and South America. In Africa, trypanosomes are mainly transmitted cyclically to animals by tsetse flies of the genus *Glossina* while in South America and Asia, trypanosomes are mainly transmitted mechanically to animals by biting flies of of genus *Stomoxys* and *Tabanus*. The disease in cattle (bovine trypanosomiasis) is caused by *Trypanosoma congolense*, *T. vivax* and *T. brucei brucei*. In Africa, *T. congolense* and *T. vivax* cause the greatest economic loss (Losos and Ikede, 1972).

Bovine trypanosomiasis is one of the major hindrances to livestock development in Africa. Approximately, 37% of the African continent is tsetse fly infested and consequently most of this land can not be effectively used for livestock production (ILRAD, 1990). As a result, it has been estimated that Africa produces 70-times less animal protein per hectare than Europe (Allsopp *et al.*, 1985). The human population in sub-Saharan Africa is estimated to be 500 million and its growth rate of 3.1 % is the fastest of any region of the world (Winrock report, in preparation). Therefore in order to provide the rapidly growing sub-Saharan Africa population with much needed animal protein, the control of trypanosomiasis is essential. Current control methods for trypanosomiasis are primarily based on the reduction of tsetse fly population density using insecticides and the use of trypanocidal drugs for prevention and treatment of the disease. The success of these approaches is limited by a number of factors. Trypanocidal drugs are expensive, not always readily available and their use has resulted in the development of drug resistant trypanosomes (Mbwambo *et al.*, 1988). The

application of insecticides to tsetse fly habitats, apart from being expensive and laborious, may result in environmental pollution (Nantulya and Moloo, 1988). Therefore there is a need to develop alternative control methods.

Attempts to develop vaccines in the control of trypanosomiasis have not produced successful results because trypanosomes possess the ability to vary their glycoprotein surface coat, thus evading the immune response of the host (Doyle, 1977). However it has long been recognised that some cattle breeds as well as some species of wild animals are able to survive and remain productive in tsetse fly infested areas without the aid of trypanocidal drugs (Murray *et al.*, 1986). This trait is known as trypanotolerance and is exhibited by West African Long Horn (e.g. N'Dama) and Short Horn (e.g. Baoule) cattle breeds. Trypanotolerant animals have the ability to limit the numbers of trypanosomes in their blood and control the development of anaemia; both of these features are heritable, although not genetically linked to each other (Paling *et al*, 1987; Trail *et al.*, 1991a). Trail *et al.* (1991a) observed that the ability of trypanosome-infected animals to control the development of anaemia, measured by the packed cell volume of erythrocytes (PCV), is correlated with economically important traits such as growth rate and reproductive performance. Studies have been initiated in the field to select trypanotolerant animals based on their ability to maintain a high PCV during the course of infection (Trail, 1991b). The mechanisms through which trypanotolerant animals control the development of anaemia are not well understood. A better understanding of the host-parasite interactions which enable trypanotolerant animals to limit the development of anaemia, might facilitate the production of animals which are resistant to trypanosomiasis using a number of ways. Means may be designed by which the development of anaemia in trypanosusceptible animals can be blocked by using therapeutic or immunological methods. In addition, the genes responsible for limiting the development of anaemia in trypanotolerant cattle may be identified and they may be incorporated into the genome of susceptible animals. Studies such as the one reported in

this thesis are an attempt to understand what effects trypanosome infections have on erythropoiesis.

Anaemia which occurs in bovine *T. congolense* infections is mainly due to destruction of erythrocytes by the expanded and active mononuclear phagocytic system (MPS) and by ineffective erythropoiesis (Dargie *et al.*, 1979; Murray and Dexter, 1988). Histopathological examination of lesions from trypanosome infections revealed massive deposits of haemosiderin (a form of storage iron which is not readily available for iron metabolism) in the bone marrow, spleen, liver and haemolymph nodes (Murray and Dexter, 1988), hypoferraemia (Dargie *et al.*, 1979) and low rate of plasma iron turn over (Wellde *et al.*, 1989). This means that the iron is retained in the MPS and hence the bone marrow is deprived of iron which is essential for haemoglobin synthesis. Decreased availability of iron interferes with the capacity of the bone marrow to respond to increased demand for erythrocytes. Consequently, the erythroid population in the marrow is either decreased or an inadequate erythroid response for the degree of anaemia occurs (Valli and Mills, 1980). Erythrocytes which are smaller than normal (microcytes) may be produced (Fiennes, 1970; Wellde *et al.*, 1989), and in more chronic cases the femoral marrow become yellow and gelatinous (Dargie *et al.*, 1979), a sign of almost total unresponsiveness. In such situations the erythroid response following trypanocidal drug treatment becomes poor (Murray and Dexter, 1988).

Ineffective erythropoiesis due to retention of iron by the MPS is also a feature of anaemia of chronic disorders in humans (Cartwright and Lee, 1971; Feldman and Kaneko, 1981; Murray and Dexter, 1988; Suliman and Feldman, 1989). In addition, it has been shown that patients with anaemia of chronic disorders have low erythropoietin titres relative to their degree of anaemia and this may contribute to the pathogenesis of their anaemia (Baer *et al.*, 1987). This was confirmed when the administration of erythropoietin in these patients resolved their anaemias (Oster *et al.*, 1990; Pincus *et al.*, 1990). Since the anaemia of chronic disorders is "morphologically, biochemically and kinetically similar to anaemia of chronic bovine trypanosomiasis"

(Dargie *et al.*, 1979), it is therefore possible that anaemia of bovine trypanosomiasis is associated with low plasma erythropoietin. Igbokwe and Anosa (1989) observed that plasma from anaemic *T. vivax* infected sheep elicited a poor reticulocyte response when injected into mice, compared to plasma from normal sheep. This suggested low levels of erythropoietin in anaemic *T. vivax* infected sheep. A similar situation may prevail in cattle infected with *T. congolense*. These infections takes more chronic course than T.vivax infections of sheep with progressive erythroid unresponsiveness due to the iron blockade by the MPS (Dargie *et al.*, 1979; Murray and Dexter, 1988). The relationship between iron blockade and erythropoietin is not known but if erythropoietin can resolve the anaemias of chronic disorders, then in addition to regulating erythropoiesis, erythropoietin may directly or indirectly be able to release iron from MPS. This suggests that erythropoietin may have therapeutic potential for anaemia of trypanosomiasis especially in chronic or prolonged cases which respond poorly to trypanocidal therapy.

The purpose of the study reported in this thesis was to establish an assay which is capable of detecting bovine erythropoietin in serum or plasma and to use this assay in measuring the levels of this hormone in serum or plasma from cattle infected with *T. congolense*. A more clear understanding of erythropoietin responses in trypanosome-infected cattle would allow us to better understand why cattle suffering from anaemia associated with trypanosome infections exhibit a poor bone marrow erythropoietic response.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. *T. congolense* infections of cattle.

2.1.1. General Introduction

Trypanosomes of medical and veterinary importance are protozoa belonging to the family Trypanosomatidae. The family Trypanosomatidae is divided into two sections, namely the salivaria and the stercoraria, depending on the location of their development in the insect vector and their mode of transmission to their mammalian hosts. Trypanosome species in section salivaria include *T. congolense, T. simiae, T. vivax, T. uniforme, T. brucei, T. rhodesiense, T. gambiense, T. evansi, T. equiperdum* and *T. suis*. Section stercoralia comprises of *T. cruzi* and *T. theileri*.

The hosts of *T. congolense* include domestic animals such as bovines, equines, sheep, goats, camels, pigs and dogs (Hoare, 1970). It is the smallest of the African trypanosomes, measuring 9-18 μm in length. It has a blunt posterior extremity, lacks a free flagellum, and the kinetoplast is marginal. In wet mounts, *T. congolense* has sluggish intermitent twisting movements (Soulsby, 1982).

T. congolense is cyclically transmitted by several species of tsetse flies of the genus *Glossina*. These include *G. morsitans, G. tachinoides, G. palpalis* and *G. austeni* (Soulsby, 1982). It produces the most economically important form of trypanosomiasis in East Africa (Losos and Ikede, 1972).

T. congolense infection in cattle produces a disease which may be acute or chronic, or may be an asymptomatic carrier state, depending on a number of factors. These include parasite factors such as the stock and virulence of the *T. congolense* involved and the host factors such as the breed, age, sex, pregnancy, state of nutrition, overwork, previous exposure to trypanosomes and the presence of concomitant infections (Losos and Ikede, 1972; Logan-Henfrey *et al.*, 1992). After a variable

incubation period following infection, the acute disease usually develops and lasts for 4-6 weeks. The commonly observed clinical signs are lethargy, weakness, anaemia, weight loss, intermitent fever, and a "stary" (dry and bristling) hair coat (Losos and Ikede, 1972). The acute disease may end in death, a chronic disease or in some rare cases, recovery of the infected animal (Nantulya *et al.*, 1984). The chronic disease may last for months or years and is characterised by weakness , anaemia and emaciation. The chronic disease usually terminates in either death, spontaneous recovery or a persistent carrier state (Logan-Henfrey *et al.*, 1992).

Haematological changes in *T. congolense* infected cattle begins during the first wave of parasitaemia. These changes include anaemia (Murray and Dexter, 1988), leucopaenia (Naylor, 1971; Wellde *et al.*, 1974; Maxie *et al.*, 1979; Valli and Mills, 1980) and thrombocytopaenia (Wellde *et al.*, 1978; Preston *et al.*, 1982). Leucopaenia is largely a result of decreased numbers of lymphocytes and neutrophils (Naylor, 1971; Valli and Mills, 1980; Ellis *et al.*, 1987; Williams *et al.*, 1991). Later in infection, a lymphocytosis develops while neutrophil numbers usually remain low (Naylor, 1971; Ellis *et al.*, 1987; Williams *et al.*, 1991). The numbers of monocytes and eosinophils are largely unaffected (Murray and Dexter, 1988).

The following mechanisms have been suggested as possible causes of the haematological changes observed. Lymphocytopaenia may be a consequence of the movement of lymphocytes from circulation to trypanosome-stimulated inflammation sites in a variety of tissues including the lymph nodes and spleen (Anosa, 1988). Neutropaenia may result from a number of mechanisms such as marrow granulocyte hypoplasia, trapping of neutrophils in the enlarged spleen and phagocytosis of neutrophil progenitors by bone marrow macrophages (Anosa, 1988; Anosa *et al.*, 1992). Thrombocytopaenia may be associated with ineffective platelet production in bone marrow and coagulation abnormalities which may cause excessive consumption of platelets (Anosa, 1988). Changes affecting the erythrocytes will be discussed under anaemia.

There are no pathognomonic gross or histological lesions at post-mortem examination of an animal that has died of trypanosomiasis. Thus, a definitive diagnosis depends on finding trypanosomes in blood smears taken before or after the death of the animal. In the acute form of the *T. congolense* infection, there are usually few gross lesions. If the animal is anaemic, the carcass appears pale with variable atrophy of adipose and muscular tissue. There is generalised oedema which is very prominent in mesentery, abomasal mucosa and perirenal fat (Losos, 1986). There may also be ascites, hydrothorax and hydropericardium. Enlargement of lymph nodes, haemolymph nodes, spleen and liver is usually prominent. Red marrow is found in ribs and sternum, and the distal ends of the long bones from where in some cases, it partially displaces fatty marrow in the long bones (Logan-Henfrey et al., 1992).

In chronic *T. congolense* infections, the carcass is emaciated and may have generalised oedema (Murray et al., 1979; Losos, 1986). There is frequent ascites, hydrothorax, hydropericardium and serous atrophy of body fat deposits. The heart is flabby and has rounded ventricles. The lungs may show evidence of bronchopneumonia and generalised oedema. The liver is enlarged and lobulation is prominent due to chronic passive congestion. The spleen and lymph nodes may be normal, enlarged or relatively reduced in size. The thymus is usually markedly atrophied. Femoral marrow may lack evidence of erythropoiesis and much of the marrow may have undergone serous atrophy (Losos and Ikede, 1972; Murray *et al.*, 1979; Morrison *et al.*, 1981; Losos, 1986; Logan-Henfrey *et al.*, 1992).

By light microscopy, trypanosomes may be seen lodged in small blood vessels of various organs and tissues, especially the heart and brain. This is associated with swelling of endothelial cells and dilation of small blood vessels (Valli and Forsberg, 1979). The acute stage of the disease is associated with hyperplasia of the lymphoid organs. During the acute disease, the lymph nodes may be enlarged with hyperplasia of large lymphoblasts and macrophages in the paracortical area (Logan-Henfrey *et al.*, 1992). This is followed by development of secondary follicles in the cortex. There are increased numbers of lymphocytes and plasma cells in the medullary cords. Increased

numbers of macrophages and evidence of erythrophagocytosis and development of haemosiderosis may be seen in lymphoid sinuses. Spleen sections show hyperplasia of lymphoblasts in the periarteriolar sheath accompanied by the development of secondary lymphoid follicles. There is hyperplasia of the splenic cord with the proliferation of macrophages, lymphocytes and plasma cells as well as marked erythrophagocytosis and development of haemosiderosis. Early changes in the bone marrow include increased numbers of erythroid cells, lymphoid cells and macrophages while granulocytes are decreased (Anosa *et al.*, 1992). There is hyperplasia of Kupffer cells in the liver and depletion of cortical lymphoid cells in the thymus of young animals (Murray *et al.*, 1979; Logan-Henfrey *et al.*, 1992). There myocarditis associated with myocardial infiltration by mononuclear cells. There is also centrolobular ischaemic necrosis in the liver. Focal infiltration of mononuclear cells is evident in the pituitary. The chronic stage of the disease is associated with decreased cellularity of lymphoid organs. However, generalised hyperplasia of MPS and erythrophagocytosis continues in lymph nodes, haemolymph nodes, spleen, liver, lungs and bone marrow. In the lymph nodes, the lymphoid follicles become depleted and the organ may eventually undergo atrophy. The bone marrow becomes hypocellular, and ultimately serous atrophy of marrow tissue occurs (Losos and Ikede, 1972; Valli and Forsebergh, 1979; Losos, 1986; Logan-Henfrey *et al*, 1992).

2.2. Anaemia in *T.congolense* infections

Anaemia is a reduction in numbers of circulating red blood cells, packed cell volume (PCV) and haemoglobin concentration. It is the common consequence of trypanosome infection in cattle (Horny, 1921). The lesions associated with most trypanosome infections are thought to be largely due to anaemia (Losos and Ikede, 1972). Anaemia is consequently used to monitor the disease status of trypanosome infected cattle (Murray *et al.*, 1979). The severity of anaemia of trypanosomiasis depends on the species and strain of the parasite within the species and on host factors such as breed, age and state of nutrition (Murray and Dexter, 1988).

The pattern of development of anaemia of trypanosome infected cattle can be divided into two phases (Murray and Dexter, 1988). The first phase, also called the acute or parasitaemic phase commences following the appearance of detectable parasitaemia and is characterised by a progressive decrease in packed cell volume (Naylor, 1971; Wellde *et al.*, 1974).

In general, the onset of the anaemia is closely associated with the appearance of detectable parasitaemia while the extent to which PCV falls correlates well with the height and duration of parasitaemia (Dargie *et al.*, 1979). The progressive development of anaemia takes place over a period of 4 to 12 weeks (average 6 weeks) after infection and it may result in the death of the animal if PCV falls to 15% or less. Alternatively, the PCV of the infected animal may stabilise at around 20% (Murray and Dexter, 1988). The progress of anaemia in this stage is dependent on the presence of trypanosomes in the circulation. If animals are treated in the acute stage, the haematological values recover to reach pre-infection values. The earlier animals are treated the more rapid haematological response is. Wellde *et al.* (1989) observed that it took only 6 weeks for an animal treated in parasitaemic phase, to recover its normal PCV value while animals treated later in the chronic stage had not recovered their pre-infection PCV values even 52 weeks after treatment.

Animals surviving the acute stage progress into the chronic stage of the disease that may last for months or years. It may end in death, spontaneous recovery or survival with persisting low grade anaemia. Unlike the acute phase, the chronic stage is characterised by low, transient or undetectable parasitaemia and PCV values which stabilise between 20 to 25%. At this stage, some animals mainly of trypanotolerant breeds e.g. N'Dama (Murray and Dexter, 1988) and a few individuals of trypanosusceptible breeds (Wellde *et al.*, 1989), make progressive spontaneous recovery and may attain their pre-infection PCV values 2-4 months after infection (Murray and Dexter, 1988; Wellde *et al.*, 1989).

A number of factors, acting singly or in concert have been implicated in the pathogenesis of anaemia of trypanosomiasis. These are haemodilution (Naylor, 1971),

increased red cell destruction (Mamo and Holmes, 1975) and ineffective erythropoiesis (Fiennes, 1970; Murray and Dexter, 1988). The roles of these causative factors were investigated by injecting radioactive isotope tracers, ^{51}Cr-labelled erythrocytes, ^{125}I-labelled albumin and ^{59}Fe-labelled transferrin in trypanosome-infected animals in order to measure directly the plasma and red cell volumes and the rates at which red cells are added to and withdrawn from the circulation by synthesis and breakdown. These were supplemented with microscopic and gross pathological findings. It was observed that the erythrocyte survival was reduced in infected animals during the parasitaemic phase of the disease and was a result of massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (Murray *et al.*, 1979; Dargie *et al.*, 1979;). At postmortem examination, the spleen was found to be enlarged and to weigh up to four fold its normal weight as a result of red cell sequestration and large macrophage population phagocytosing red blood cells (Murray *et al.*, 1979; Murray and Dexter, 1988). Erythrophagocytosis has also been demonstrated in lungs, haemolymph nodes, bone marrow, in circulation and is particularly marked in the liver which is usually swollen and histologically shows a marked increase in active Kupffer cells (Dargie *et al.*, 1979; Murray *et al.*, 1979). Previous studies using ^{59}Fe-labelled transferrin to measure plasma volume concluded that trypanosome infections caused an increase in plasma volume whose dilution effect to the blood contributed to the development of anaemia (Naylor, 1971). However, Dargie *et al.* (1979) observed that while there was a significant increase in plasma volume (measured using ^{125}I-labelled albumin) and a decrease in total circulating erythrocyte volumes (measured using ^{51}Cr-labelled erythrocytes), the total blood volume was not changed by the infection. Thus, Dargie *et al.* (1979) concluded that haemodilution is not a contributory factor to anaemia of trypanosomiasis. It was suggested that the increase in plasma volume in anaemic trypanosome infected animals was a physiological compensatory mechanism for maintaining blood volume and pressure following a massive loss of erythrocytes from the circulation. It was further observed that the use of ^{59}Fe-labelled transferrin to measure plasma volume gives misleading results, since ^{59}Fe is rapidly transferred from

the circulation before it equilibrates with the entire plasma space, thus giving an overestimation of plasma volume (Dargie *et al.*, 1979).

A number of possible factors which render erythrocytes prone to increased levels of phagocytosis by the MPS during the acute stage of the disease have been suggested (Dargie *et al.*, 1979; Murray and Dexter, 1988). These include damage of erythrocytes, opsonisation of erythrocytes by immunoglobulins and complement, and the activated and expanded MPS. It has been demonstrated that disintegrating *T. congolense* and *T. brucei* release active enzymes such as proteases and phospholipases. *In vitro* studies have also shown that *T. vivax* produces neuraminidase, an enzyme capable of cleaving sialic acid residues from red cell membrane (Esievo, 1983; reviewed by Murray and Dexter, 1988). The presence of such enzymes *in vivo* may lead to red cell damage. Infections caused by *T. vivax* and in some cases *T. congolense* in cattle lead to coagulation abnormalities which result in widespread fibrin deposits in the microvasculature (Wellde *et al.*, 1978; Wellde *et al.*, 1983). Such fibrin deposits may cause damage to red cells as they pass through the blood vessels (Murray and Dexter, 1988). *In vitro* exposure of erythrocytes to temperatures above normal for a few hours leads to erythrocyte damage through increased osmotic fragility, increased permeability and reduced plasticity (Murray and Dexter, 1988). A similar situation may occur *in vivo* in trypanosome infected animals which exhibit periodic fever which may lead to erythrocyte damage. Erythrocytes damaged through the above mechanisms may bind immunoglobulins and complement, facilitating their phagocytosis through immunoglobulin and complement receptors on macrophages.

Trypanosome specific antibodies, erythrocyte specific antibodies and complement have been demonstrated on the surface of erythrocytes from *T. congolense* and *T. vivax* infected cattle (Murray and Dexter, 1988). It has been suggested that soluble antigens from dying trypanosomes may adsorb on erythrocyte surface, resulting in opsonization of erythrocytes with trypanosome specific immunoglobulins, mainly of the IgM and IgG classes. On the other hand, erythrocyte specific antibodies were demonstrated on erythrocytes of cattle infected with *T. vivax* (Assoku and Gardiner, 1989; Murray and

Dexter, 1988). These autoantibodies might have been directed to hidden epitopes, exposed as a result of erythrocyte membrane damage, probably through the mechanisms already discussed. It has also been demonstrated that trypanosomes activate the complement in the absence of antibody, and it is suggested that this mechanism may play a role in the development of anaemia before the antibody response to trypanosomes occurs (Murray and Dexter, 1988). Furthermore, a complement component was demonstrated on erythrocytes from trypanosome infected cattle (Kobayashi *et al.*, 1976). Erythrocytes opsonised by immunoglobulins and complement could be easily phagocytosed by cells of MPS through the immunoglobulin and complement receptors on the macrophages. In addition, it has also been suggested that a passive deposition of antigen-antibody complexes on the surface of normal erythrocytes may result in their phagocytosis.

The acute stage of trypanosomiasis is associated with an activated and expanded MPS which is widespread throughout the body and is partly responsible the development of splenomegaly (Dargie *et al.*, 1979). Such an expansion and activation of the MPS may by itself induce these cells to phagocytose erythrocytes. Splenomegaly is associated with hyperplasia of cells lining the splenic cords, inluding macrophages, resulting in lengthening of splenic cords through which blood passes. A lengthened passage exposes erythrocytes to the numerous activated macrophages lining the splenic cords and this renders them prone to phagocytosis.

In the acute parasitaemic phase, there is no evidence of overt ineffective haemopoiesis. Rather, a number of investigators reported increased erythropoietic activity marked by elevation of erythrocyte mean corpuscular volume (MCV) (Naylor, 1971; Valli *et al.*, 1978; Wellde *et al.*, 1989). However, others did not observe an increase in MCV (Murray and Dexter, 1988). Increased erythropoietic activity was also marked by erythroid hyperplasia in the bone marrow, a drop in myeloid:erythroid ratio (Valli *et al.*, 1978; Anosa *et al.*, 1992) and increased plasma iron turnover and red cell iron utilization which was up to three fold by the 7th week following infection (Dargie *et al.*, 1979; Wellde *et al.*, 1989). Although there is evidence of increased erythropoietic

response in the acute phase of the disease, this response is considered inadequate for the degree of anaemia. In spite of there beeing macrocytosis, the reticulocyte response was little or absent and it is known that haemolytic anaemias in ruminants elicits a considerable reticulocyte response (reviewed by Igbokwe, 1989). Although the maximum haematopoietic response of cattle is not known, the erythropoietic response in trypanosome infected cattle has been considered inadequate for the degree of anaemia, since it is known that sheep, man and pigs can increase their haematopoietic response six to ten times in response to persistent demand for red cells (reviewed by Murray and Dexter, 1988; reviewed by Papayannopoulou and Abkowitz, 1991). Further more, microscopic examination revealed no evidence of extramedullary erythropoiesis or expanded haemopoiesis in long bones in contrast to the response in mice infected with *T. congolense* (Murray and Dexter, 1988).

In the chronic stage of the disease, although splenomegaly is no longer a feature, erythrophagocytosis continues in spleen,liver, lungs and bone marrow accompanied by haemosiderosis (Dargie *et al.*, 1979). The moderate erythropoietic response observed in the acute infection begins to wane despite persistent anaemia. The MCV was reported to reach its highest value by week four (Murray and Dexter, 1988) or seven after infection (Naylor, 1971), and then started decreasing progressively. By week 35 after infection, microcytosis was observed (Welde *et al.*, 1989). There was normal or low erythroid population in the bone marrow (Valli *et al.*, 1978) and grossly, in prolonged cases, the bone marrow had undergone serous atrophy resulting in yellow gelatinous appearance (Murray and Dexter, 1988). Ferrokinetic studies showed development of hypoferraemia (Dargie *et al.*, 1979) and low plasma iron turnover rate which was one third of the rate observed during the acute stage (Wellde *et al.*, 1989). These findings suggest that anaemia of chronic trypanosomiasis is ultimately complicated by some degree of marrow dysfunction, the basis of which could be a reticuloendothelial iron blockade. This condition in cattle has been compared to the reticuloendothelial iron blockade associated with chronic diseases in man (Dargie *et al.*, 1979; Murray and Dexter, 1988; Suliman and Feldman, 1989). Anaemia of chronic diseases is characterised by decreases in

serum iron, total iron binding capacity and percent transferrin iron saturation. In addition, there occurs an increase in amounts of stored iron in form of haemosiderin due to sequestration of iron in MPS. Ultimately a poor erythropoietic response ensues (Smith, 1989).

The bone marrow response to anaemia is regulated by the hormone erythropoietin. Although the relationship between iron blockade and erythropoietin is not clear, the observation that erythropoietin administration to patients with anaemia of chronic disorders such as malignances and rheumatoid arthritis resolved these anaemias, suggests that the hormone levels are low in these diseases (Oster *et al.*, 1990; Pincus *et al.*, 1990). Using a radioimmunoassay for erythropoietin, it was shown that patients with anaemia of some chronic disorders have low plasma erythropoietin compared to their degree of anaemia and that this may contribute to the pathogenesis of their anaemia (Baer *et al.*, 1987). It is therefore possible that the poor erythropoietic response to anaemia of *T. congolense* infection of cattle may be partly due to to low levels of erythropoietin. Igbokwe and Anosa (1989) observed that plasma from anaemic *T. vivax* infected sheep elicited a poor reticulocyte response when injected into mice when compared to plasma from sheep made anaemic by heat treatment of homologous erythrocytes. This suggests low levels of erythropoietin in anaemic *T. vivax* infected sheep. Although there is a degree of erythropoietic unresponsiveness in anaemic *T. vivax* infected sheep indicated by absence of reticulocyte and a progressive decrease in MCV (Anosa and Isoun, 1976, 1980; Igbokwe and Anosa, 1989), the erythropoietic unresponsiveness is even more evident in *T. congolense* infection of cattle which takes a more chronic course.

It is therefore important to evaluate the erythropoietin response in cattle infected with *T. congolense*. Studies in sheep (Igbokwe and Anosa, 1989) measured the erythropoietic potential of plasma on the basis of the reticulocyte response it elicited when injected into mice. This method is a crude estimate of the level of plasma erythropoietin. Currently immunoassays and bioassays which are specific and highly sensitive are being used to measure human erythropoietin. The adoption of these

techniques to measure bovine plasma erythropoietin will help clarify whether low erythropoietin level occur in the anaemia of *T. congolense* infections of cattle. Likewise, erythropoietin may be of therapeutic value in animals with chronic or prolonged anaemia caused by trypanosomiasis. Administration of erythropoietin might decrease the recovery time of the PCV and thus allow animals to become productive more rapidly following treatment.

2.3. Erythropoietin

Erythropoietin is a glycoprotein hormone produced primarily by the kidney, and is a principal growth factor regulating red blood cell production (Krantz, 1991).

The history of erythropoietin was reviewed by Erslev (1987). A French physician, Dennis Jourdanet, working in a highland area of Mexico in 1850's observed that the blood of his surgical patients was thick and viscous and contained an increased number of red blood cells. But he did not realise that low oxygen pressure in highlands initiated an increase in red blood cell production. At the end of the 19th century, mountain climbers found that a few weeks exposure to low atmospheric pressure stimulated red blood cell production. This stimulated a search for a humoral erythropoietic factor which continued unsuccessfully for about 50 years. In 1953, Erslev demonstrated the existence of the humoral erythropoietic factor by injecting large volumes of plasma from anaemic rabbits into normal rabbits and observing the increase in normoblasts, reticulocytes, erythrocytes and haematocrit in the normal rabbits. This humoral erythropoietic factor was referred to as erythropoietin (Erslev, 1987).

Early studies on erythropoietin were hampered by the failure to obtain the hormone in pure form and in sufficient quantities. Miyake *et al.* (1977) isolated and purified erythropoietin from the urine of anaemic patients. Later researchers cloned and expressed the gene for erythropoietin in mammalian cells (Jacobs *et al.*, 1985; Lin *et al.*, 1985). The availability of recombinant erythropoietin resulted in improved studies on the chemistry, site of production, mechanisms of action and therapeutic uses of the hormone.

The development of an easy, sensitive, specific and accurate radioimmunoassay using recombinant erythropoietin to replace the labourious bioassays has provided an important tool for studies on erythropoietin in various haematological disorders (Egrie *et al.*, 1987).

2.3.1. The physiology of erythropoietin and erythropoiesis

Erythropoietin is an obligate growth factor for erythroid progenitor cells, without which these cells die. It is therefore always present in plasma at levels between 10-30 mU/ml in humans (Erslev, 1991). The induction of hypoxia by hypobaria or anaemia activates two physiological responses aimed at restoring normal tissue oxygen tension. First, there is a rapidly responsive hyperventilation and a decrease in haemoglobin affinity for oxygen which occur within hours of the development of hypoxia. Secondly, an increase in plasma erythropoietin concentration occures and lead to an increase in erythrocytes, a few days later (Spivak, 1991). Hypoxia stimulates increases in alveolar ventilation, heart rate and tissue blood flow in order to maintain the diffusion gradient between the blood and tissues. In addition, hyperventilation causes a reduction in carbon dioxide tension which results in respiratory alkalosis. The change in pH stimulates synthesis of an organic phosphate termed 2,3 diphosphoglycerate (2, 3-DPG) in erythrocytes. This compound binds to haemoglobin and reduces its oxygen affinity, resulting in enhanced oxygen delivery in tissues. Simultaneously, with activation of hyperventilation and decreased haemoglobin affinity for oxygen, hypoxia activates the tissue oxygen sensor which is thought to be a heme containing protein located in or around erythropoietin producing cells in the kidney (Goldsberg *et al.*, 1988). It is this oxygen sensor which triggers the synthesis of erythropoietin and its release into the bloodstream. In the bone marrow, erythropoietin binds to erythropoietin-receptor bearing cells including the burst-forming-unit erythroid (BFU-E) and colony-forming-unit erythroid (CFU-E). This interaction causes increased proliferation and differentiation of erythroid progenitors into mature stages of erythrocytes, so that ultimately the number of circulating erythrocytes increases. The minimum time taken

from induction of hypoxia to detectable increase in plasma erythropoietin has been estimated to be 10 to 15 minutes in mice, and it is probably similar in other animals (Bauer., 1991). This "trigger phase" involves mainly the transduction process by which hypoxia lead to accumulation of erythropoietin messenger ribonucleic acids (mRNA). The increased plasma erythropoietin concentration produced in response to hypoxia reaches a transient peak after 20-24 hours (Bauer, 1991). If hyperventilation and decreased haemoglobin affinity for oxygen manage to correct the hypoxia, then the stimulus for erythropoietin production is removed and erythropoietin levels return to normal. However, if the hypoxia is extreme and cannot be compensated for, the elevated erythropoietin levels persist.

The degree of erythropoietin response depends on the residual arterial blood oxygen content which is a function of partial atmospheric oxygen pressure, number of erythrocytes, haemoglobin concentration and packed cell volume (Schuster *et al.*, 1987). For anaemic patients, with the exception of those suffering from anaemia of chronic disorders, there is an overall inverse and exponential relationship between plasma erythropoietin and packed cell volume (Erslev, 1991). Erythropoietin production depends on a feedback mechanism, by which hypoxia causes increased erythropoietin production and hence increased erythrocyte production whereas hyperoxia causes a reduction in erythropoietin synthesis resulting in reduced red cell production. This relationship between plasma erythropoietin concentration and PCV is very apparent in severely anaemic patients but becomes less clear when PCV are above 33% (Erslev, 1991).

The hypoxia-induced erythropoietin production can be affected by a number of factors. Studies using Hep3b, a human hepatoma cell line which produces erythropoietin when exposed to hypoxia, suggest that inflammatory cytokines have an effect on hypoxia-induced erythropoietin production (Faguin *et al.*, 1992). The production of erythropoietin by Hep3B cell cultures in hypoxic condition was inhibited in a dose-dependent manner by addition of interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1ß), tumour necrosis factor alpha (TNF-α) or transforming growth factor beta (TGF-

ß). Conversely, the addition of interleukin 6 (IL-6) under the same conditions caused a dose-dependent increase in erythropoietin production. However, IL-6 did not stimulate erthropoietin synthesis in non-hypoxic conditions. The mechanisms through which these cytokines affect erythropoietin production are not known but these observations suggest that these cytokines somehow have an effect on synthesis and secretion of this hormone. Furthermore, since increased amounts of these cytokines are produced during the course of various inflammatory disorders, their effect on erythopoietin production may play a role in the pathogenesis of anaemia of chronic disorders. Other studies have shown that the hypoxia-induced erythropoietin production can be inhibited by metabolic or respiratory acidosis, probably due to an acidosis-induced decreased oxygen consumption by renal tissues (Bauer, 1991). Also the expression of the erythropoietin gene in the kidney of hypophysectomised mice under hypoxic conditions is inhibited unless insulin growth factor (IGF-1), tri-iodotyrosil (T3) and testosterone are present (Bauer, 1991). These hormones may have an influence on the sensitivity of the kidney to hypoxia.

The bone marrow produces 3×10^9 new erythrocytes per kilogram per day in order to maintain a stable haematocrit (Papayannopoulou and Abkowitz, 1991). The bone marrow has the capacity to respond quickly to both acute or chronic demands for oxygen. The normal time for maturation of BFU-E to CFU-E *in vivo* is 2-3 days (Papayannopoulou and Abkowitz, 1991). Acute demand for erythrocyte formation e.g. in sudden blood loss, is usually followed by rapid entrance of new erythrocytes into the circulation as early as 24 hours later (Papayannopoulou and Abkowitz, 1991). The rapidity of the erythrocyte response suggests that a transient peak of erythropoietin causes a shortening of the differentiation sequence from BFU-E to CFU-E to proerythroblasts. Such a hastened differentiation may be achieved either through shortening of intermitotic intervals, fewer mitotic divisions or by differentiation without division (Papayannopoulou and Abkowitz, 1991). This results in the premature release of reticulocytes in the circulation which appear macrocytic and basophilic on blood smear (reviewed by Finch, 1982). Therefore, increased size of erythrocytes (MCV) in the absence of vitamin B_{12} or folic acid deficiency and increased number of

reticulocytes in the peripheral blood are indicators of increased erythropoiesis. Once the CFU-E and precursor pools are amplified through these mechanisms, continued erythropoietic demands, such as those occurring in cases of chronic haemolytic anaemias, can be satisfied by this greatly expanded late erythroid pool i.e. CFU-E and their precursors. It is known at least from *in vitro* experiments that BFU-E require at least one thousand times as much erythropoietin as do CFU-E (Sawyer *et al.*, 1990; Papayannopoulou and Abkowitz, 1991). Thus the early unsustained increase in erythropoietin helps to recruit an additional late erythroid progenitor pool from the early progenitors while smaller quantities of the hormone can maintain this pool once it is expanded.

The erythropoietic capacity of sheep, pigs and humans can increase six to ten times above normal in response to persistent anaemia (Dargie *et al.*, 1979; Murray and Dexter, 1988; Papayannopoulou and Abkowitz, 1991). In addition to expanded erythropoietic pools, the sites of active erythropoiesis expand to include those which are inactive in normal circumstances. In adult animals, the bone marrow in axial bones i.e. vertebrae, pelvis, ribs and sternum are sufficient for normal erythropoiesis. Under an expanded erythropoietic activity, the femur, humerus, spleen, liver and rarely the thymus may also become involved in the production of erythrocytes (Papayannopoulou and Abkowitz, 1991).

It has recently been shown that factors other than erythropoietin may elicit an erythropoietic response. Infusion of insulin-like growth factor (IGF-1) stimulates erythropoiesis directly as well as indirectly through increased erythropoietin synthesis (Bauer, 1991). The mechanism through which IGF-1 exerts its influence on erythropoietin is not yet known. Mediators of the inflammatory response such as IL-1, TNF and τ-interferon inhibit erythropoiesis *in vitro* and *in vivo* and the inhibitory effect of IL-1 and τ-IFN can be reversed by increased concentration of erythropoietin (Means and Krantz, 1991). It is suggested that τ-IFN inhibits erythropoiesis by inducing the down regulation of erythropoietin receptor expression on erythroid progenitors and, likewise, erythropoietin may reverse the inhibitory effect of τ-IFN on erythropoiesis by

causing a down regulation of τ-IFN receptor expression on erythroid progenitors. The release of these cytokines by bone marrow macrophages in the course of inflammatory and chronic disorders, and their suppressive effect on erythropoiesis may play a role in the development of anaemia associated with chronic disorders.

2.3.2. Erythropoietin action

Erythropoietin responsive cells i.e. the BFU-E and the CFU-E are the descendants of the pluripotent stem cell (Erslev, 1991). BFU-E and CFU-E have been identified by the methyl cellulose culture technique that is capable of supporting discrete erythroid colonies (Basara *et al.*, 1988; Frischt and Nelson, 1990; Krantz, 1991). The CFU-E is highly responsive to low concentrations of erythropoietin and gives rise to erythroblast colonies (8 to 50 cells) in two days for mouse cells and seven days for human cells. The BFU-E is derived from a more immature cell and develops into grouped clusters of erythroblasts, or larger colonies of more than 500 erythroblasts in 8 days (mouse) or 15 days for human cells (Sawyer, 1990). Bovine CFU-E and BFU-E take 5 and 10-14 days respectively to develop (Fritch and Nelson, 1990).

The action of erythropoietin on responsive cells is mediated through erythropoietin-specific receptors (Chang *et al.*, 1974). Studies on mice and human erythroid progenitor cells show that these receptors develop at the BFU-E stage, attain maximum numbers at CFU-E/proerythroblast stage and eventually decline before the cells develop into late basophilic erythroblasts and their descendants (Sawyer, 1991). The number of erythropoietin receptors correlates well with the dependency of the cell on the hormone. Erythropoietins act as a mitogen, stimulating the proliferation of both BFU-E and CFU-E, and also acts as a differentiating factor for the transformation of CFU-E to proerythroblasts (Schuster *et al.*, 1987; Erslev and Caro, 1989).

In vitro studies on the biochemical effects of erythropoietin on progenitor cells show that erythropoietin increases DNA synthesis in BFU-E while increases total RNA synthesis, uptake of glucose, α and ß-globin gene transcription rate, numbers of transferrin receptors and synthesis of haemoglobin in CFU-E (Krantz, 1991). The

erythropoietin-receptor complex is important for survival of erythroid progenitor cells, because when it is present the cells develop into mature erythrocytes and in its absence they die. Erythropoietin prolongs the survival of progenitor cells by retarding DNA breakdown and thus prevents programmed cell death (Koury and Boudorant, 1990).

2.3.3. Biochemistry, molecular biology and biogenesis of erythropoietin

The human erythropoietin gene is located on chromosome 7 and its sequence is identical to that of mouse and monkey, but, there are minor differences in the final protein product which has 79% (mouse) and 94% (monkey) homologies with the human protein (Goldwasser, 1989).

Human urinary erythropoietin was first purified by Miyake *et al.* (1977). It is a glycoprotein with relative molecular mass (Mr) of 34,000 daltons as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It contains 30% carbohydrate (11% sialic acid, 11% hexose, and 8% N-acetyl glucosamine). The peptide portion has 166 amino acids with molecular weight of 18,398 daltons. Human urinary erythropoietin has a specific activity of 74,000 U/mg protein (peptide) or 50,000 U/mg of the total weight of the hormone.

Recombinant human erythropoietin was simultaneously developed for the first time by investigators from two biotechnology firms, Genetics Institute and Amgen (Jacobs *et al.*, 1985; Lin *et al.*, 1985). Recombinant erythropoietin expressed in Chinese hamster ovary cells has a specific activity of 210,000 U/mg protein or 129,000 U/mg total weight (Browne *et al.*, 1986). The differences in specific activity between urinary and recombinant erythropoietin may be due to inactivation of some of the urinary erythropoietin in the process of extraction and collection (Krantz, 1991).

Sialic acid residues on erythropoietin molecule are important for the biological activity of the hormone *in vivo* but not *in vitro* (Goldwasser *et al.*, 1974). It is suggested that an enzyme neuraminidase cleaves the sialic acid residues from the erythropoietin molecules, resulting in reduced half life of the hormone *in vivo*. Desialation exposes the galactose residues on the erythropoietin molecule which binds to

galactose receptors in the liver and so the molecule is quickly metabolised. It is likely that much of the hormone *in vivo* is eliminated through desialation and metabolism in the liver, since little of the radiolabelled desialated erythropoietin is taken up by the kidney or bone marrow or is lost through the urine (Krantz, 1990).

The kidney is the major erythropoietin producing organ in adult life, while the liver produces only 10-15% of the total amount (Erslev, 1987). The liver is, however, a major erythropoietin producing organ in fetal life (Bondurant *et al.*, 1991). Studies in adult mice using RNA and DNA probes demonstrated erythropoietin mRNA in kidney tubular cells or peritubular endothelial cells of renal cortex and outer medulla (Lacombe *et al.*, 1990). The Kupffer cells and hepatocytes in the liver and macrophages in "erythropoietic blood islands" in the bone marrow may also be erythropoietin producing cells (Bauer, 1991; Krantz, 1991).

2.3.4. Measurement of erythropoietin

The definition of a standard unit for measuring erythropoietin had its origin in the work of Goldwasser, in 1958, who was studying the erythropoietic effect of cobalt in fasted male Sprague-Dawley rats (Garcia, 1979). He noted that injection of different doses of cobalt into starved rats produced proportional increase in erythropoiesis which was identical to that produced by erythropoietin. One unit of erythropoietin was set to be equal to the response of 5 millimoles of Cobalt in the fasted rat assay. When sheep plasma erythropoietin extract became available, one batch was set aside as standard A. One Unit of standard A was equivalent to 0.05 mg of erythropoietin preparation. As this material was used up, it was replaced by standard B, a lyophilised crude preparation of human urinary erythropoietin. One unit of standard B was 1.48 mg. This was called the first international reference preparation (First IRP). The World Health Organization (WHO) expert committee on biological standards established a second IRP for erythropoietin in 1971 as a replacement for the first IRP. The international unit (I.U.) for human urinary erythropoietin is now defined as the activity contained in 0.5 mg of the second IRP (Annable *et al.*, 1972).

The initial assays for erythropoietin measured the erythropoietin content of the sample by its effect on haematocrit, haemoglobin concentration, or reticulocyte counts in a recipient animal such as mice or rabbit (Garcia, 1979). Currently, there are three general approaches for measuring erythropoietin, namely *in vivo* bioassay, *in vitro* bioassay and immunoassay.

2.3.4.1. The *in vivo* bioassay: According to Garcia (1979) the *in vivo* bioassay for erythropoietin, measures the percentage of radiolabelled iron (^{59}Fe) incorporated into red cells of polycythemic mice (polycythemic mouse assay) or starved rats (starved rat assay). These animals are starved or rendered polycythemic (with above normal PCV) in order to decrease the endogenous rate of erythropoiesis. This renders the animals more sensitive to exogenous erythropoietin than normal animals. The animals are injected with the test sample (erythropoietin containing material) and its content of erythropoietin is assessed by how much ^{59}Fe is incorporated into erythrocytes. The erythropoietin content of the test sample is calculated from dose-response curves obtained from standardised erythropoietin preparations. The *in vivo* assay is not sensitive enough to measure plasma erythropoietin levels of less than 50 mU/ml, thus it cannot detect normal plasma erythropoietin levels. Moreover it lacks precision, it is time consuming, and it requires the use of large volumes of sample material and large numbers of mice. However, it has the advantage of being specific and it measures only the physiologically active erythropoietin. It remains the major reference assay for erythropoietin.

2.3.4.2. The *in vitro* bioassay: The *in vitro* bioassays for erythropoietin measure the erythropoietin-induced colony formation, proliferation or haeme synthesis by short term cultures of bone marrow, spleen and liver cells (Haga and Falkanger, 1979; Brandon *et al.*, 1981; Krystal, 1983). When grown in methyl cellulose medium *in vitro*, erythroid progenitor cells (CFU-E) divide to form colonies. The number of colonies formed depend on the concentration of erythropoietin in the culture medium. Therefore, the

erythropoietin content of the test material is measured by the number of colonies it induces (Haga and Falkanger, 1979). Erythropoietin stimulates a dose-dependent proliferation, differentiation as well as induction of heme synthesis in erythropoietin responsive cells (ERC). The erythropoietin induced proliferation of ERC can be determined by the rate of DNA synthesis, as measured by the amount of incorporation of ^{3}H-thymidine or ^{125}I-deoxyuridine in DNA (Krystal, 1983). Haeme synthesis which takes place in relatively late non-dividing erythroblasts as well as earlier dividing ones can be measured by the amount of ^{59}Fe incorporated into haeme by ERC (Krystal, 1983). In order to have an optimal proportion of ERC in the cell culture, foetal mouse liver cells or spleen cells from phenylhydrazine treated mice are used. In the foetus, the liver is the major erythropoietic organ, and foetal liver cells taken at 13 days of gestation are used for the assay when the foetal liver contain optimal numbers of ERC (Brandon *et al.*, 1981). Two daily consecutive injections of mice with phenylhydrazine at a dose of 60 mg/kg body weight induces a haemolytic anaemia which in turn stimulates an erythropoietic response in the spleen. Between days 2 to 5 after the last phenylhydrazine injection, the number of cells in the spleen increases up to ten times, of which more than 90% are erythroid precursors (Krystal, 1983). Phenylhydrazine is thought to induce anaemia through immunological mechanisms (Dornfest *et al.*, 1992). Phenylhydrazine may act by causing alterations to the membranes of younger erythrocytes, similar to changes which occur on membrane of aged erythrocytes. It may also act by inducing an increase in the quantity of autologous IgG which recognises aged erythrocytes. This results in opsonisation of the altered erythrocytes by the autologous IgG and their subsequent accelerated removal from circulation by splenic macrophages through Fc receptor recognition of erythrocytes coated with IgG. Spleen cells collected 3 days after the last phenylhydrazine treatment are used for the assay since they are more sensitive to erythropoietin (Krystal, 1983). A defined number of foetal liver cells or spleen cells ranging from 2×10^5 to 4×10^5 are pipetted into each well of a 96 well plate. The test material (serum or plasma), heat inactivated at 56°C for 30 minutes in order to eliminate non-specific inhibitors of erythropoiesis, is added to cell cultures. The erythropoietin

content of the test material is measured by its effect on erythroid incorporation of either ^{59}Fe into haeme, or ^{3}H-thymidine or ^{125}I-deoxyuridine into DNA. *In vitro* bioassays are more sensitive than *in vivo* bioassays because they can detect erythropoietin levels of 1-5 mU/ml. However, these assays measure biologically but not necessarily physiologically active molecules. They thus measure asialoerythropoietin as well, which is active *in vitro* but inactive *in vivo*. *In vitro* bioassays are also more sensitive to non-specific inhibitors and stimulators of erythropoiesis (Krystal, 1983).

2.3.4.3. The immunoassays: The immunoassays that detect erythropoietin are based on the anti-erythropoietin antibody recognition of epitopes on the erythropoietin molecule (Garcia, 1979). Following the production of an antibody which could neutralise the biological activity of erythropoietin, a number of immunoassays were developed (Garcia, 1979). These included the Ouchterlony double diffusion technique, the haemagglutination inhibition test , and the radioimmunoassay for human and sheep erythropoietin. Recently, an enzyme-linked-immunosorbent assay (ELISA) for measuring human erythropoietin was developed (Wognum *et al.*, 1989; Kientsch-Engel *et al.*, 1989).

The radioimmunoassay is based on the competitive binding of the radiolabelled and unlabelled erythropoietin to the anti-erythropoietin antibody. The test material, the radiolabelled erythropoietin and the appropriately diluted specific antibody are mixed and allowed to react at a specified temperature and for a specific period of time. The free and antibody bound radiolabelled erythropoietin are separated using immunosorbents or the "double antibody" technique followed by centrifugation (Chard, 1990). The radioactivity of the antibody bound radiolabelled-erythropoietin in pellet is determined. The erythropoietin content of the test sample is calculated from the degree of inhibition on the binding of the radiolabelled erythropoietin to the antibody.

Following the purification of erythropoietin from urine and later, the production of recombinant erythropoietin and the use of the pure hormone in radioimmunoassay, the

specificity and sensitivity of this assay was greatly improved. In addition, the radioimmunoassay was found to be accurate and reproducible, making it more advantageous than the bioassays (Garcia *et al.*, 1979; Sherwood and Goldwasser, 1979; Egrie *et al.*, 1987). The disadvantage of the radioimmunoassay is its inability to measure the bioactivity of erythropoietin. Thus, it may also measure biologically inactive precursors and/or breakdown products of the erythropoietin molecule (Krystal, 1983).

2.3.5. Erythropoietin titres in health and disease

2.3.5.1. Physiologic erythropoietin titres: The circulating levels of erythropoietin under a normal steady state is sufficient to maintain the erythrocyte production. Normal plasma levels of erythropoietin as measured by radioimmunoassay are 10-25 mU/ml in humans (Cotes, 1982), 40 mU/ml in mice, 25 mU/ml in rats, 20 mU/ml in baboon and 56 mU/ml in monkeys (Garcia *et al.*, 1979). Using the *in vitro* assay, the levels of erythropoietin in dogs was 88.2 $\pm$ 30.7 mU/ml, 55.2 $\pm$ 8.9 mU/ml in horse, 41.7 $\pm$ 10.3 mU/ml in cow and 39.4 $\pm$ 5.4 mU/ml in cat (Ikeda *et al.*, 1990).

In patients with severe anaemia, excluding cases of renal disease and rheumatoid arthritis, there is an overall inverse and exponential correlation between haematocrit and erythropoietin levels. This correlation however becomes less apparent as the haematocrit increases above 33% (Erslev *et al.*, 1987; Erslev, 1991). Although the mean erythropoietin titre shows a relationship to haematocrit, wide variations occur between individuals with the same haematocrit. For example at a haematocrit of 30%, erythropoietin titre could be 50 mU/ml or 500 mU/ml (Erslev, 1991).

2.3.5.2. Erythropoietin titres in non-infectious disorders: Determination of erythropoietin levels can be of diagnostic and therapeutical importance in both polycythemias and anaemia. Polycythemia is a haematological disorder associated with abnormally elevated numbers of erythrocites, haemoglobin concentration and haematocrit. In primary polycythemia or polycythemia vera, the bone marrow acts

autonomously and over produces erythrocytes causing an increase in haemoglobin and haematocrit. This in turn produces tissue hyperoxia and cuts off erythropoietin production. Thus, primary polycythemia is associated with low plasma erythropoietin. Secondary polycythemia occurs due to impaired oxygen transport. The resulting tissue hypoxia generates erythropoietin which in turn stimulate bone marrow to produce more red blood cells. Consequently erythropoietin titres are usually elevated in uncompensated secondary polycythemia. Disorders classified as secondary polycythaemia include carbon monoxide intoxication, diseases associated with high oxygen affinity haemoglobin, high altitude disease, pulmonary disease and congenital heart disease associated with shunting of blood from right to left ventricle (Spivak, 1991). However, in cases where polycythemia compensates for tissue hypoxia, erythropoietin levels return to normal as was observed in cyanotic congenital heart disease (Haga *et al*, 1987). Polycythemia may also be caused by inappropriate production of erythropoietin by certain renal or extra-renal tumours or cysts (Erslev and Caro, 1984). In the absence of evidence of either primary or secondary polycythemia, elevated erythropoietin titre may be suggestive of an erythropoietin producing lesion.

Erythropoietin levels are usually lower in people who are anephric and those suffering from chronic renal disease. Since erythropoietin is primarily produced by the kidney, anaemia in these patients is due to reduced production of the hormone (Erslev and Caro, 1989).

Insufficient erythropoietin production has been reported in patients suffering from anaemia of chronic disease. Despite normal kidney function, patients suffering from rheumatoid arthritis (Baer *et al.*, 1987), various malignancies (Miller *et al.*, 1990), acquired immunodeficiency syndrome (Cazzola *et al.*, 1992) and sickle cell anaemia (Sherwood *et al.*, 1986) appear to have low erythropoietin titres compared to their degree of anaemia. It has been suggested that blunting of renal oxygen sensor or of renal erythropoietin synthesis is in part responsible for these anaemias (Erslev, 1991). Recombinant erythropoietin has been used to treat anaemia in patients with end-stage

chronic renal disease (Estbach *et al.*, 1987), various types of cancer and rheumatoid arthritis (Oster *et al.*, 1990; Pincus *et al.*, 1990).

2.3.5.3. Erythropoietin titres in infectious diseases: Anaemia is a major consequence malaria infection in both humans and rodents (Rencricca *et al.*, 1974). This anaemia is attributed to decreased erythrocyte survival and depressed erythropoiesis (Magio-Price *et al.*, 1985). A number of investigators have tried to establish the cause of inadequate erythropoiesis in murine malaria including studies of the erythropoietin response.

Adequate erythropoietin titres in response to anaemia were observed in several mouse strains infected with different species of Plasmodia. These included virulent *Plasmodium berghei* infections of mouse strains CD-1 (Rencricca *et al.*, 1974) and C57Bl/6J (Magio-Price *et al.*, 1985) or a non-lethal *P. chaubadi adami* infections of Balb/c and Swiss Webster and non-lethal *P. yoeli* infection of Balb/c/B_yC (Weiss *et al.*, 1989).

These infections were characterised by decreased marrow cellularity and a reduction in the colony forming unit spleen (CFU-S), the pluripotent stem cells, and the erythroid progenitors early in infection, even before the development of anaemia. The anaemia was followed by elevation of erythropoietin titres up to one hundred times normal in the malaria infections causing severe anaemia. While in some malaria infections, a progressive decrease in erythropoietic activity was noted, in others reported an enhanced erythropoiesis both in spleen and bone marrow was reported. However, the enhanced erythropoiesis could not compensate for the rate of erythrocyte destruction. Hence, infected mice finally succumbed to the effects of severe anaemia. In those infections, where insufficient erythropoiesis occurred, sufficient erythropoietin titres were produced. Thus, the subsequent insufficient erythropoiesis may have been due to a depletion in erythroid progenitor cells in the bone marrow which occurred early in infection. Alternatively it may have occured due to interference in erythropoietin receptors on erythroid progenitor cells i.e down regulation of erythropoietin receptors caused by cytokines (Faguin *et al.*, 1992).

Very few studies on the pathogenesis of anaemia in ruminants have included erythropoietin. Jatkar and Kreier (1967) investigated the changes in erythropoietin titres following *Anaplasma marginale* infection of calves and *A. ovis* infection of sheep. Anaemia in anaplasmosis is primarily due to erythrophagocytosis and intravascular haemolysis (Jatkar and Kreier, 1967). Calves and sheep responded to infection in a similar way. The erythropoietin titres began to rise following the development of anaemia (when PCV's were between 25 and 38%) and reached a peak when anaemia was most severe (PCV = 16%) or later in the recovery period. The elevation in erythropoietin titres was followed by an increase in reticulocyte counts, a change in myeloid-erythroid ratio in favour of erythrocytes and increase in PCV with subsequent recovery. Similarly, elevation of erythropoietin titres was observed in *Theileria sergenti* infection of calves (Yagi *et al.*, 1992). The anaemia in this disease is caused by reduced survival of both parasitised and non-parasitised erythrocytes (Yagi *et al.*, 1991). There was a surge in the erythropoietin level when the PCV dropped to 17%. The erythropoietin levels peaked (approx 600 mU/ml) when anaemia was most severe (PCV = 12%) then declined rapidly to normal pre-infection levels 5 days later. The elevation of erythropoietin titre was closely followed by increase in MCV, reticulocyte counts and PCV and subsequently, the animals recovered from the disease. On the other hand, a poor erythropoietin response to anaemia followed by a poor erythropoietic response was reported by Igbokwe and Anosa (1989) in *T. vivax* infection of sheep. The *T. vivax* infected sheep developed a progressive anaemia associated with a drop in packed cell volume, number of red blood cells and haemoglobin concentration. The erythropoietic response was characterised by progressive increase in the MCV which attained maximum size 3 weeks after infection and then started declining. Plasma from anaemic sheep drawn 2 and 4 weeks after infection elicited a poor reticulocyte response when injected into mice as compared to the plasma from normal sheep. Plasma drawn three weeks after infection from anaemic *T. vivax* infected sheep elicited a good reticulocyte response in mice. This response was however inferior to that elicited by plasma from sheep with an artificially induced anaemia of the same magnitude and classification.

This suggested low or inadequately increased levels of erythropoietin in *T. vivax* infected sheep.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Setting up a radioimmunoassay for detection of human and bovine erythropoietin

3.1.1. Reagents

3.1.1.1. Binder (antibody)

Bovine erythropoietin has not been purified or cloned and the anti-bovine erythropoietin antibody is not presently available. Therefore, a rabbit anti-recombinant human erythropoietin (anti-R-HuEPO) polyclonal antisera and a rabbit anti-human urinary erythropoietin (anti-U-HuEPO) were used as the binder (antibody). Rabbit anti-R-HuEPO antisera (Genzyme Corporation, Cambridge, USA) was produced by immunizing rabbits with purified recombinant human erythropoietin. The rabbit anti-U-HuEPO antisera (Terry Fox Laboratory, Vancouver, Canada) was prepared by immunizing rabbits with partially purified human erythropoietin (10,000 units/mg) and then boosting at intervals with pure human erythropoietin (80,000 units/mg) before bleeding.

3.1.1.2. Erythropoietin standard (immunogen)

Three erythropoietin preparations were used as standards:

(1) A partially purified human urinary erythropoietin (U-HuEPO)(Terryfox Laboratory) with specific activity of 1,000 units/mg in McCoy's medium containing 1% polyethylene glycol and 1% bovine serum albumin (BSA).

(2) A purified U-HuEPO (Terryfox Laboratory) with specific activity of 80,000 U/mg supplied in 0.3 M potassium phosphate containing 0.06% Tween 20.

(3) A recombinant human erythropoietin (R-HuEPO) with specific activity of 70,000 U/mg and supplied as a filter-sterilised solution of 50% glycerol in 25mM HEPES buffer, pH 7.2 (Amersham International, Buckinghamshire, England). The two U-HuEPO preparations were purified from urine of patients with aplastic anaemia. The R-Hu-EPO was produced by recombinant DNA technology in mammalian cells and purified by sequential chromatography.

3.1.1.3. Tracer

The ^{125}I-labelled recombinant human erythropoietin (^{125}I-R-HuEPO) with specific activity of 3,000-4,000 Ci/mM and supplied in 0.1M sodium acetate, 0.1M sodium chloride containing 0.2% bovine serum albumin (BSA) was used as the tracer (Amersham). The ^{125}I-R-HuEPO was prepared by iodination of R-HuEPO using sodium iodide and chloramine-T (an oxidising agent which converts iodine to a more reactive form) and purified by gel filtration high performance liquid chromatography. Before it was used in the radioimmunoassay, the radioactivity of the stock solution of ^{125}I-R-HuEPO was diluted in 1% BSA in phosphate buffered saline (PBS) pH 7.4 to a radioactivity of 10,000-20,000 counts per minute (CPM) in 100 μl.

3.1.2. The antibody dilution curve

An antibody dilution which binds 50% of the tracer is commonly used for radioimmunoassays (Chard, 1990).

The percentage of the tracer bound is calculated using the following formular:

$$\text{Percentage of tracer bound} = \frac{\text{CPM bound by antibody}}{\text{CPM of of tracer added}}$$

Therefore, dilutions of the two anti-erythropoietin sera (see 3.1.1.1) were tested to determine the dilution which will bind 50% of the tracer added (see 3.1.1.3) as described by Chard (1990). Anti-R-HuEPO sera was tested at dilutions of 1:100, 1:320, 1:1,000, 1:3,200 and 1:10,000. The dilutions tested for Anti-U-HuEPO sera were 1:1,000, 1:10,000, 1:50,000 and 1:100,000. The antibody dilutions tested for each antibody were determined according to the procedure used by Kessler *et al.*(1975). The diluent used was made up of 1% bovine serum albumin (BSA, purity 96%) (BDH Chemicals ltd., Poole, England) in phosphate buffered saline (PBS) pH 7.4. Each antisera dilution was tested in duplicate tubes. One hundred microlitres of the various antisera dilutions and 100 ul of ^{125}I-R-HuEPO containing 10,000 - 20,000 counts per minute (CPM) were mixed in 1.5 ml Eppendorf microtest tubes (Eppendorf-Nethelel-Hunz GmbH, Hamburgh, Germany) and the mixture was allowed to react at 4°C for 4 days. The free and antibody-bound ^{125}I-R-HuEPO in the reaction mixture were separated by adding Tachisorb (goat anti-rabbit IgG conjugated to *Staphylococcus aureus*, Calbiochem, California, USA) according to Egrie *et al.*(1987). Tachisorb is an immunosorbent which crosslinks to and causes the aggregation of immune complexes, rendering them easy to precipitate. Tachisorb was added in the reaction mixture at a ratio of 1.2 times the volume of the reaction mixture in the tube as per the producer's instructions. After an incubation period of 30 minutes at room temperature (22 ^{0}C), the tubes were centrifuged at 10,976 (x g) for 1 minute in an Eppendorf microcentrifuge (Eppendorf). The free ^{125}I-R-HuEPO in the supernatant was aspirated and the radioactivity of the antibody-bound ^{125}I-R-HuEPO in the pellet was determined by using a gamma counter (Beckman, Scientific Instruments Division, California, USA).

3.1.3. The standard curve for erythropoietin.

The radioimmunoassay of known concentrations of the erythropoietin standard is used to produce a standard curve. Using the standard curve, unknown concentrations of erythropoietin in test samples can be determined. Standard curves were constructed

using various combinations of erythropoietin standard, binder and tracer in order to select a combination which will produce the most sensitive radioimmunoassay.

Three combinations of anti-erythropoietin antibodies and their dilutions, erythropoietin standard and tracer tested in order to select a combination which will result in a more sensitive erythropoietin radioimmunoassay standard curve (Table 3.1). Anti-recombinant human erythropoietin sera (anti-R-HuEPO) (Genzyme) and anti-human urinary erythropoietin sera (anti-U-Hu-EPO) (Terrfox) were used as binders. Partially purified (impure) human urinary erythropoietin (U-HuEPO) (Terryfox) with specific activity of 1,000 U/mg, pure U-HuEPO (Terryfox) with specific activity of 80,000 U/mg and recombinant human erythropoietin (R-HuEPO) (Amersham) with specific activity of 70,000 U/mg were used as standards and ^{125}I-labelled R-HuEPO (Amersham) was used as the tracer.

Table 1. Combination of binder, erythropoietin standard and tracer used to produce standard curves for erythropoietin radioimmunoassay.

Binder	Binder dilution	Erythropoietin standard	Tracer
anti-R-HuEP	1:2500	impure U-HuEPO	^{125}I-HuEPO
anti-R-HuEPO	1:2500	R-HuEPO	^{125}I-R-HuEPO
anti-U-HuEPO	1:50000	pure U-HuEPO	^{125}I-R-HuEPO

The assay reagents were dissolved in a diluent made up of 1% BSA in PBS pH 7.4. Ten-fold dilutions from 10,000 to 10 mU/ml of a partially purified U-HuEPO and R-HuEPO were made and the anti-R-HuEPO was used at a final dilution of 1:2500 (see 4.1.1). Double dilutions of the pure U-HuEPO from 1,000 to 1.95 mU/ml were made in the same diluent and the anti-U-HuEPO was used at a dilution of 1:50,000 (see 4.1.1). The ^{125}I-R-HuEPO was used as the tracer in all combinations tested. One

hundred microlitres of the diluted erythropoietin and 100 μl of the diluted antibody were pipetted into Eppendorf microtest tubes and the mixture was incubated at 4°C for 72 hours. Thereafter 100 μl of ^{125}I-R-HuEPO containing 10,000 -20,000 CPM were added into each tube. The reaction mixture was incubated for an additional 24 hours. The free and antibody-bound ^{125}I-HuEPO were separated by the addition of Tachisorb and centrifugation (see 3.1.2). The radioactivity of the antibody bound ^{125}I-HuEPO in the pellet was determined using a gamma counter (see 3.1.2). The standard curve indicating the concentration of the erythropoietin standard on the logarithmic scale (X-axis) and the corresponding radioactivity on the linear scale (Y-axis) was plotted on the semi-logarithmic paper.

3.1.4. The radioimmunoassay for human and bovine serum erythropoietins

Human serum was collected from a normal male in a sterile manner and stored at -70 ^{0}C. Bovine serum used was collected from an anaemic calf 24 hours after withdrawal of 50% of its estimated total blood volume (see 3.4.2). The combination of reagents which produced the most sensitive radioimmunoassay i.e. an anti-U-HuEPO as the binder at a 1:50,000 dilution, the pure U-HuEPO (80,000 U/mg) as the standard and the ^{125}I-R-HuEPO as the tracer (see 4.1.2), was used to provide the standard curve for the radioimmunoassay of human and bovine serum erythropoietin. Double dilutions of erythropoietin standard from 1,000 mU/ml to 7.8 mU/ml were made. Human and bovine serum was tested at three dilutions i.e. 1:2, 1:4 and 1:8. One hundred microlitres of of the various erythropoietin standard dilutions were pipetted into Eppendorf microtest tubes. Other tubes were set up with 100 μl of various serum (human or bovine) dilution. One hundred microlitres of a 1:50,000 dilution of anti-U-HuEPO were pipetted into each tube and the mixture was incubated at 4°C for 72 hours. Thereafter 100 μl of ^{125}I-R-HuEPO containing 10,000 -20,000 CPM were added into each tube. The reaction mixture was incubated for an additional 24 hours. The free and antibody-bound ^{125}I-HuEPO were separated by the addition of Tachisorb and followed by centrifugation (see 3.1.2). The radioactivity of the antibody bound ^{125}I-HuEPO in

the pellet was determined using a gamma counter (see 3.1.2). The erythropoietin concentrations in serum samples was determined from the standard curve constructed on the semi-logarithmic paper.

3.2. Development of bioassay for erythropoietin

3.2.1. The standard curve for erythropoietin bioassay

The *in vitro* bioassay for erythropoietin based on the incorporation of ^{3}H-labelled thymidine into spleen cells from phenylhydrazine treated mice as described by Krystal (1983) was modified and used as follows:

Female Balb/c mice between 2 and 3 months old, weighing between 17-22g were used. These mice were maintained in the small animal unit at ILRAD. They were kept in mouse cages (North Kent Plastic Cages Ltd., Dartford, Kent, England) and fed mouse cubes (Unga Feeds Kenya Ltd., Nairobi, Kenya). The mice were made anaemic by two consecutive daily injections (i.e. one injection per day) of a freshly prepared solution of phenylhydrazine hydrochloride (Sigma Chemical Co., St. Louis, Missouri, U.S.A.). The phenylhydrazine hydrochloride solution was prepared by dissolving the phenylhydrazine hydrochloride powder in α-minimum essential media (α-MEM) (GIBCO BRL, Life Technologies, Paisely, U.K.) to produce a concentration of 6 mg/ml. The mice were injected with phenylhydrazine by the intraperitoneal route, at a dose of 60 mg/kg body weight as described by Krystal (1983). Three days after the last phenylhydrazine injection, the mice were killed by cervical dislocation and the spleens were aseptically removed. The spleens were minced on sterile stainless-steel tissue sieve with scissors. Using a plunger from a 5 ml syringe, the minced spleens were gently pressed on the tissue sieve and washed through it with α-MEM. The resultant cell suspension was collected into a sterile beaker. The cell suspension was pipetted up and down with a 10 ml pipette for several times to disaggregate larger clumps of cells. In order to determine the concentration of cells in the spleen cell suspension, a sample was diluted in Turk's solution (to lyse the mature erythrocytes) and the number of viable nucleated cells was counted using an improved Neubauer haemocytometer (Assistant,

Germany). The spleen cell suspension was then centrifuged at 71 (xg) for 5 minutes at 4 ^{0}C in a Sorvall refrigerated centrifuge (E.I. Du pont de Nemaurs & Co., Biomedical Products Division, Newton, U.S.A.), and the cell pellet was resuspended in α-MEM containing 40% foetal calf serum and 2 X 10^{-2} milli Molar mercaptoethanol (Merck-Schuchardt, Munich, Germany) to a concentration of 8 x 10^6 nucleated cells/ml. To each well of a 96-well polystyrene plate (Costar Corporation, MA,. USA) 50 μl of the spleen cell suspension (containing 4 x 10^5 cells) and 50 μl of the standard erythropoietin or plasma/serum to be tested were added. Plasma or serum to be used in the assay was heat-inactivated at 56 ^{0}C for 30 minutes to denature the complement factors. The human urinary erythropoietin, specific activity 80,000 U/mg, double diluted in α-MEM from 2,000 mU/ml to 7.8 mU/ml, was used as a standard. The cell cultures were incubated in a Hereus incubator (W.C. Heraeus GmbH, Hanau, Germany) at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2) in air. After 22 hours of incubation, 20 μl of α-MEM containing 1 μCi of ^{125}I-deoxyuridine (IUDR) (Amersham International, Buckinghamshire, England) from a 50 μCi/ml stock solution of IUDR in α-MEM, was added to each well. The cell cultures were incubated for an additional 2 hours, and the cells were harvested on the glass fibre filters using a Titertek cell harvester (Flow Laboratories, Lier, Norway). The radioactivity collected onto the glass fibre discs was measured using the gamma counter.

3.2.2. Bioassay of erythropoietin in bovine plasma

The erythropoietin concentration in plasma from two groups of calves was estimated using the bioassay. The first group consisted of four calves BJ62, BJ63, BJ64 and BJ65, which were infected with *T. congolense* (see 3.3.2). A total of 38 plasma and serum samples were collected from each animal thrice weekly for the first 85 days post-infection (dpi) and then once weekly up to 116 dpi. Plasma from *T. congolense*-infected animals was tested in both undiluted form (BJ62 and BJ63) and in 1:5 dilution (all calves). Serum samples from BJ 64 were tested in an undiluted form.

The second group consisted of four calves BK45, BK46, BK50 and BH259 which were made anaemic by withdrawal of 50% of their estimated total blood volume. Erythropoietin concentration was estimated in plasma samples collected from each of the four calves at 0, 3, 6, 9, 24, 33, 48, 72, 120, 144, 168 and 336 hours post-bleeding (hpb). Plasma samples were used in the bioassay at 1:5 dilution. In addition, erythropoietin concentrations were estimated in an undiluted, 1:2, 1:4 and 1:8 dilutions of plasma collected 24hpb from BH 259.

3.2.3. Effect of anti-U-HuEPO on the bioassay of erythropoietin in bovine plasma

The effect of anti-U-HuEPO on the bovine plasma-induced IUDR incorporation into spleen cells was examined. The anti-U-HuEPO was heat inactivated at 30 ^{0}C for 30 minutes. In each well of a 96 well plate, 50 μl of spleen cell suspension, 25 μl of 1:300 dilution of antibody and 25 μl of either undiluted, 1:2, or 1:4 dilution of heat inactivated plasma from an anaemic calf, which had 50% of its estimated total blood volume removed 24 hours previously, were mixed and cultured. The IUDR incorporation was determined as explained above.

3.3. Experimental infection of calves with *T. congolense* and measurement of haematological parameters.

3.3.1. Experimental animals.

Four male Friesian calves aged between 8 months and one year were obtained from Naivasha, a trypanosomiasis-free area of Kenya. The calves were fed on hay and concentrates (Unga Feeds Kenya Ltd., Nairobi, Kenya). The animals were slightly anaemic with PCV ranging between 23 and 27 % upon arrival at the ILRAD farm. The calves tested free of antibodies to *Babesia bigemina, B. bovis, Theileria mutans and Anaplasma marginale* using enzyme-linked immunosorbent assays (ELISA) (Katende *et al.*, 1990; Katende, personal communication) and tested free of antibodies to *T. parva* using an indirect fluorescent antibody (IFA) technique (Goddeeris *et al.*,1982). Six

weeks prior to the experimental infection, the calves were treated with a trypanocidal drug, diminazine aceturate (Berenil, Hoechst, Frankfurt, Germany), at a dose of 7mg/kg body weight as a routine sanitive measure. In addition, the animals were dewormed using rafoxanide and thiabendazole (Ranizole, Merck Sharp & Dohme Ltd, Hertfordshire,England), and fed vitamins (Multivitamin, Chanelle Veterinary Ltd, Lougrea, Ireland). The PCV of the calves had improved to values of between 27 and 34% at the time of the experimental infection.

3.3.2. Infection of calves with *T. congolense*

The four Friesian calves were infected by allowing five infected *Glossina morsitans centralis* to feed on their left flank. The tsetse flies had been fed on goat infected with *T. congolense* IL 1180 (Nantulya *et al.*, 1984) and had been verified as infected with *T. congolense* by allowing the infected tsetse flies to probe on glass slides as described by Dwinger *et al.*(1987).

3.3.3. Measurement of haematological parameters in infected calves.

Blood samples were collected for haematological, parasitological analysis, plasma and serum preparation thrice weekly for the first 85 days post infection (dpi) and then once weekly until 164 dpi when the experiment was terminated. Plasma and serum were collected in a sterile manner and stored at -70°C. Parasitemia was determined using the buffy coat/ phase contrast technique (Paris *et al.*, 1982). Haematology was performed on blood samples using standard techniques. The packed cell volume (PCV) was determined using the microhaematocrit centrifuge (Damon/IEC Division, Needham Heights, MA, USA) and a haematocrit reader (Gelman Hawksley Ltd; England). Erythrocyte counts (RBC) and leucocyte counts (WBC) were determined by using a coulter counter (Coulter Electronics, England). Haemoglobin concentration (Hb) was determined by using the haemoglobinometer (Coulter Electronics). The mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC)

were calculated from the corresponding PCV, RBC and Hb values using the following formulae (Jain, 1986):

$$\text{MCV (femtolitres)} = \frac{\text{PCV (\%)} \times 10}{\text{RBC (millions/}\mu\text{l of blood)}}$$

$$\text{MCHC (\%)} = \frac{\text{Hb (g/dl)} \times 100}{\text{PCV (\%)}}$$

The percentage of reticulocytes in 500 red blood cells was determined on thin blood smears stained with brilliant cresyl blue (Sigma) and the absolute numbers of reticulocytes were estimated by multiplying the percentage of reticulocytes by the RBC. Differential WBC were determined on 100 leucocytes on thin blood smears stained with diff quick (Dade Diagnostics Inc. Aguada, Puerto Rico). The absolute numbers of lymphocytes, neutrophils, monocytes and eosinophils were determined by multiplying the WBC by the percentage of each white blood cell sub-population. Three calves were treated with 7mg/kg body weight diminazine aceturate (Berenil, Hoechst, Frankfurt, Germany) when their PCV dropped to 15% on 57, 85 and 106 dpi infection respectively.

3.4. Induction of sudden blood loss in calves

3.4.1. Animals

Four calves, one Boran x Ayrshire calf and three Boran calves weighing between 44.5 and 84 kg and aged between 4-5 months were used. These calves were obtained

from the large animal unit at ILRAD. They were fed hay and concentrates (Unga Feeds Kenya Ltd). The calves were routinely checked for blood and gastrointestinal parasites.

3.4.2. Bleeding of the animals

Calves were subjected to sudden loss of blood equal to 50% of their estimated total blood volume in order to stimulate increased levels of plasma erythropoietin. Plasma collected from these calves was used in the erythropoietin assays. These plasmas were used to study the relationship between changes in erythropoietin levels and erythrocyte parameters following sudden blood loss in cattle.

The calves were bled of half of their blood volume in a single bleeding. The calves were weighed using a weighing scale (L.P. Morris Ltd., Shropshire, England) and the total blood volume was estimated to be 57 millilitres of blood for every one kilogram body weight (Jain, 1982). The bleeding equipment comprised of a 14 gauge needle connected by a 6 millimitre bore plastic tube to a filter flask (Pyrex, France). The filter flask was also connected to a Nalgene hand pump (Neward Enterprises, California, USA). The needle was inserted in the jugular vein of the calf and blood was drawn in the flask by the suction pressure created by the pump.

3.4.3. Haematology and sample collection

The mean PCV ($\pm$ SD) of the calves before the experiment was 35.8 $\pm$ 3.2%. Blood samples were taken from the calves before bleeding and 3, 6, 9, 24, 27, 30, 33, 48, 72, 120, 144 hours and one week after bleeding. On day 14 after bleeding, blood sample for only one animal was collected. Serum and plasma was collected and stored at -70 ^{0}C and haematology was done as described in 3.1.3.

CHAPTER FOUR

4. RESULTS

4.1. The radioimmunoassay for erythropoietin

4.1.1. The antibody dilution curve

The antisera dilution curves for anti-R-HuEPO and anti-U-HuEPO are shown in Fig. 1 and Fig. 2, respectively. The curves show log dilutions of the antisera and their corresponding counts per minute (CPM), indicating the amount of the ^{125}I-R-HuEPO bound at each antibody dilution. There was a proportional decrease in the amount of ^{125}I-R-HuEPO bound to the antibody as the concentration of the antibody decreased. A 1:2500 dilution of anti-R-HuEPO binding 29 % of the tracer and 1:50,000 dilution of U-HuEPO binding 14 % of the tracer were subsquently used for the radioimmunoassay, since they produced the most sensitive assays.

4.1.2. The standard curve for erythropoietin radioimmunoassay

The standard curve was constructed in order to determine the response (inhibition) produced by known concentrations of erythropoietin, hence unknown erythropoietin levels in plasma could be determined from the standard curve (Figs. 3 and 4).

Different combinations of binder, erythropoietin standard and tracer were evaluated in order to select a combination which will produce a more sensitive assay. The detection limits achieved using different combinations of reagents are given in table 2.

Table 2. Sensitivity (detection limits) of three combinations of reagents used to produce the standard curve for erythropoietin radioimmunoassay.

Binder	Erythropoietin standard	Tracer	Detection limit (mU/ml)
anti-R-HuEPO	U-HuEPO	^{125}I-R-HuEPO	100
anti-R-HuEPO	R-HuEPO	^{125}I-R-HuEPO	-
anti-U-HuEPO	U-HuEPO	^{125}I-R-HuEPO	10

The standard curve produced by the anti-R-HuEPO and an impure U-HuEPO (1,000 U/mg) combination is shown in Fig. 3. The standard curve produced by the anti-U-HuEPO and the pure U-HuEPO (80,000 U/mg) combination is shown in Fig. 4. The erythropoietin standard (unlabelled) inhibited the binding of ^{125}I-R-HuEPO to the binder (anti-erythropoietin antibody). This inhibition led to a proportional decrease in the amount of radiolabelled erythropoietin bound to the antibody as the concentration of the erythropoietin in the standard increased. A sigmoid curve was obtained which was linear between 1075 CPM and 2,448 CPM for the anti-R-HuEPO and U-HuEPO (1,000 U/mg) combination (Fig. 3). Thus, the assay could detect erythropoietin concentrations between 100 mU / ml and 10,000 mU / ml. However, when the anti-R-HuEPO and R-HuEPO combination was tested, different concentrations of R-HuEPO failed to show any inhibition in the assay. This combination was therefore unsuitable for construction of a standard curve. The standard curve produced by anti-U-HuEPO and U-HuEPO (80,000 U/mg) combination was linear between 550 CPM and 11,000 CPM. Thus the assay could detect erythropoietin concentrations between 10 mU/ml and 1,000 mU/ml (Fig. 4). Since this assay was more sensitive it was used for measuring erythropoietin in human and bovine serum.

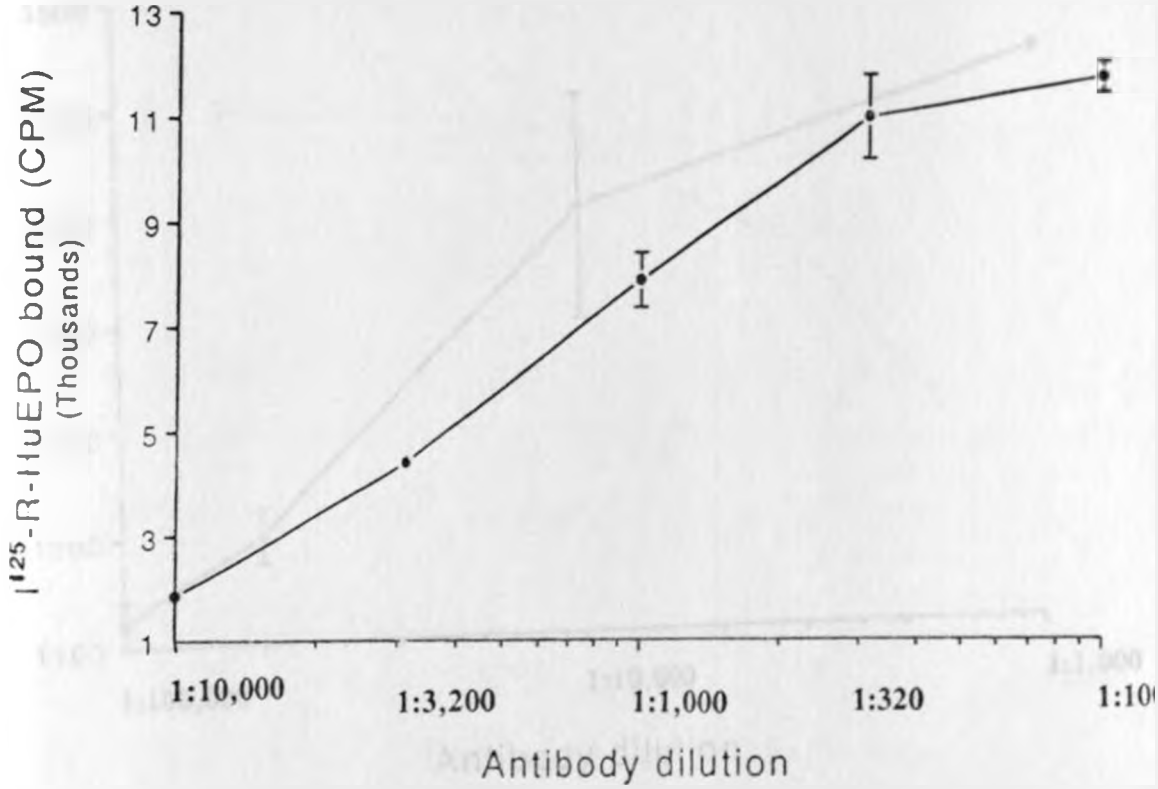

Figure 1. The antibody dilution curve for rabbit anti-human recombinant erythropoietin (anti-R-HuEPO).

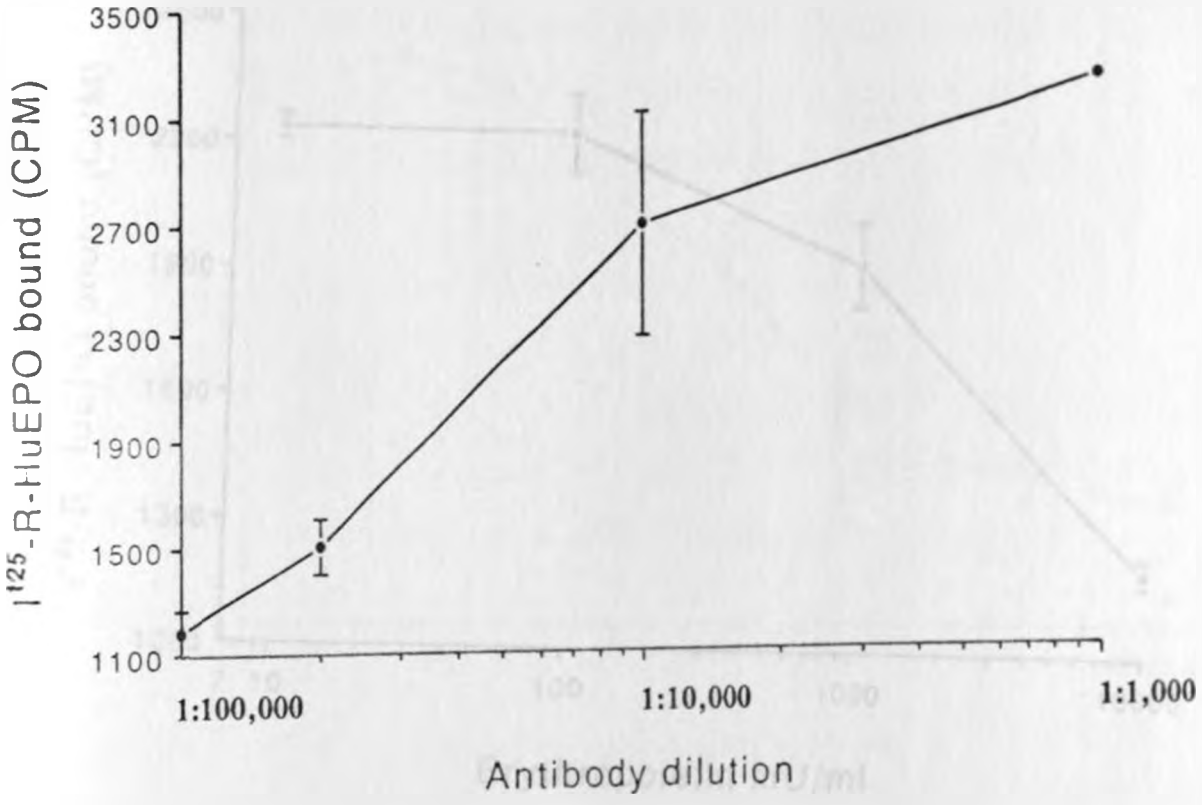

Figure 2. The antibody dilution curve for rabbit anti-human urinary erythropoietin (anti-U-HuEPO) antisera.

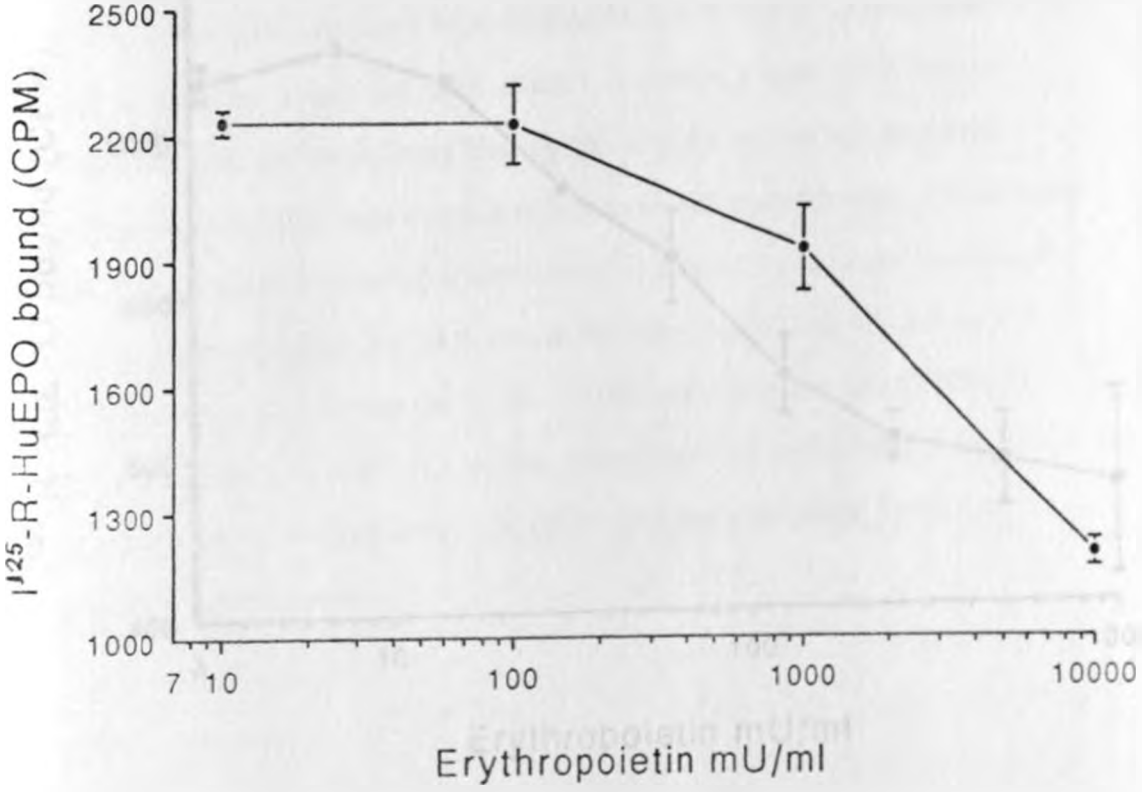

Figure 3. The standard curve for the partially purified human urinary erythropoietin (1,000 U/mg).

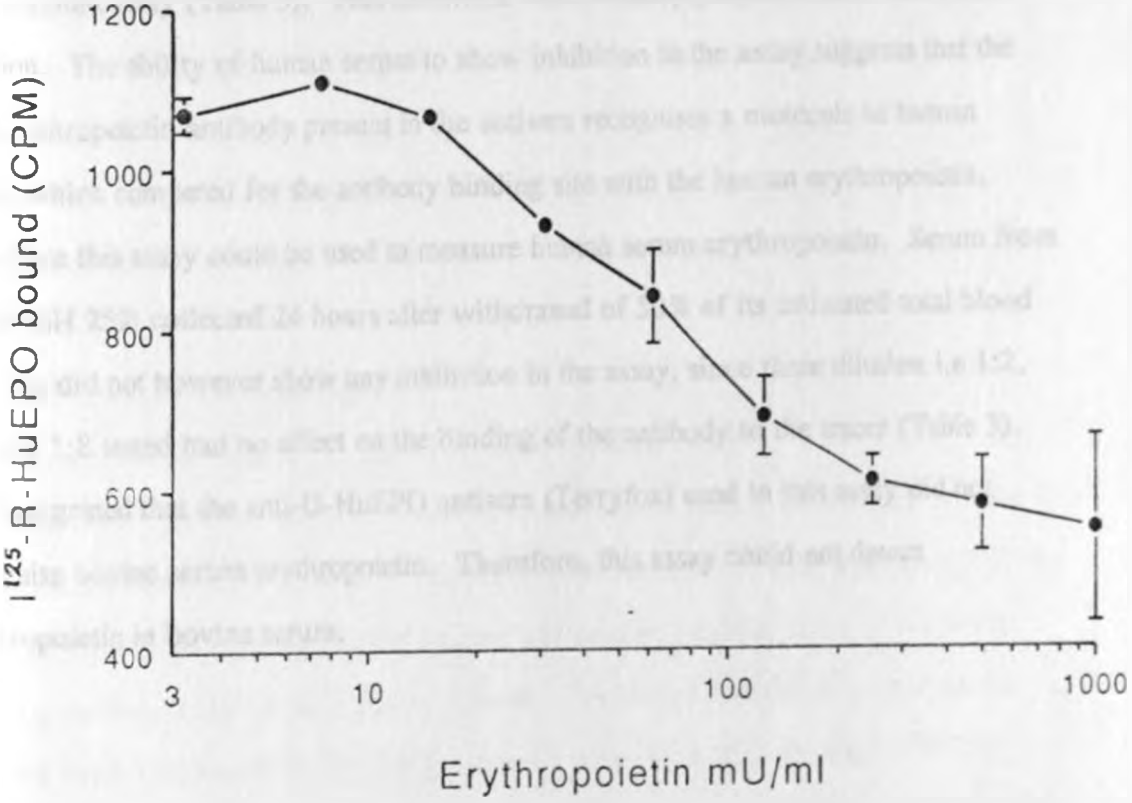

Figure 4. The standard curve for the pure human urinary erythropoietin (80,000 U/mg).

4.1.3. Measurement of erythropoietins in human and bovine serum

Human serum from a non-anaemic person was tested at three dilutions of 1:2, 1:4, and 1:8 by radioimmunoassay using the combination of reagents which resulted in a more sensitive radioimmunoassay (see 4.1.2). The erythropoietin level in the human serum was 50 mU/ml. Human serum, like erythropoietin standard, exhibited an inhibition to the binding of ^{125}I-R-HuEPO to the anti-U-HuEPO in the radioimmunoassay (Table 3). This inhibition was inversely proportional to the serum dilution. The ability of human serum to show inhibition in the assay suggests that the anti-erythropoietin antibody present in the antisera recognises a molecule in human serum which competed for the antibody binding site with the human erythropoietin. Therefore this assay could be used to measure human serum erythropoietin. Serum from a calf (BH 259) collected 24 hours after withdrawal of 50% of its estimated total blood volume, did not however show any inhibition in the assay, since three dilution i.e 1:2, 1:4 and 1:8 tested had no effect on the binding of the antibody to the tracer (Table 3). This suggested that the anti-U-HuEPO antisera (Terryfox) used in this assay did not recognise bovine serum erythropoietin. Therefore, this assay could not detect erythropoietin in bovine serum.

Table 3. Effect of various dilutions of human serum and bovine serum to the binding of ^{125}I-labelled recombinant human erythropoietin (CPM) in the erythropoietin radioimmunoassay.

Dilution	(CPM) Human serum	(CPM) Bovine serum
1:2	940.0 ± 32.0	1424 ± 52.0
1:4	1165.0 ± 9.2	1556 ± 172.0
1:8	1474.5 ± 0	1542 ± 5.6

4.2. The bioassay for erythropoietin

4.2.1. The standard curve for erythropoietin bioassay

Different concentrations of human erythropoietin standard were tested in order to determine the erythropoietin dose-dependent incorporation of ^{125}I-deoxyuridine (IUDR) in spleen cells. The resultant standard curve was used in the calculation of unknown erythropoietin concentrations in bovine plasma. Doubling dilutions of erythropoietin standard from 2,000 to 31.25 mU/ml produced a dose-dependent decrease in IUDR incorporation into spleen cells Fig. 5. The incorporation induced by 2,000 mU/ml was 51,355 ± 728 CPM. The incorporation decreased proportionally as the concentration of erythropoietin standard decreased, reaching the plateau at 3,752 ± 333 CPM, corresponding to erythropoietin standard concentration of 62.5 mU/ml. Further decrease in erythropoietin standard concentration did not induce significant decrease in the CPM. Thus, the assay could not detect erythropoietin levels below 62.5 mU/ml.

4.2.2. Response of the assay to plasma from anaemic calves

In order to determine whether the bioassay for human erythropoietin could be used to measure bovine plasma erythropoietin, different bovine plasma concentrations were tested in the bioassay. The change in IUDR incorporation induced by undiluted, 1:2, 1:4, and 1:8 dilutions of plasma from an anaemic calf with a PCV of 22% which had about 50% of its blood volume removed 24 hours previously is shown in Fig. 5. Bovine plasma produced a dose-dependent change in IUDR incorporation. The incorporation was higher at high concentrations of plasma (undiluted) and decreased proportionally through 1:2, 1:4 and 1:8 dilutions. The IUDR incorporation in spleen cells incubated with various plasma dilutions produced a curve which closely correlated with the standard curve produced by erythropoietin. This suggests that the spleen cells were responding to bovine plasma in similar manner as to human urinary erythropoietin. In addition, the anti-U-HuEPO blocked the biological activity of bovine plasma in the assay (Table. 4). Therefore the bioassay could be used to measure bovine plasma erythropoietin.

Table 4. Effect of anti-human erythropoietin antisera on the stimulation of IUDR incorporation (CPM) into spleen cells from phenylhydrazine-treated mice by plasma from an anaemic calf.

Plasma dilution	Plasma (CPM)	Plasma + Antibody (CPM)
Undiluted	5760 ± 929	403 ± 83
1:2	3994 ± 646	440 ± 53
1:4	2585 ± 679	607 ± 120

4.2.3. Erythropoietin levels in calves following sudden blood loss

Using the bioassay, erythropoietin levels were measured in plasma of four calves which had 50% of their blood volume removed (Fig. 6). The pre-bleeding plasma erythropoietin levels were below the detection limits of the assay. Increased erythropoietin levels of 180 mU/ml were first detected 6 hpb. The erythropoietin levels increased to 587 mU/ml by 9 hpb, followed by a surge in erythropoietin levels which reached a peak of 919 mU/ml by 33 hpb. Thereafter the levels decreased to 270 mU/ml at 48 hpb and was below detection limit of the assay by 72 hpb.

4.2.4. Erythropoietin levels in *T. congolense*-infected cattle

Plasma collected from four *T. congolense*-infected calves over a period of 164 dpi were tested for their erythropoietin content in the bioassay. Plasma collected from calves before infection and up to 13 dpi induced a mean IUDR incorporation into spleen cells equal to 2355 CPM. From 13 dpi onwards, plasma from infected animals (BJ62 and BJ63) exhibited profound inhibition to IUDR incorporation into spleen cells (Fig. 7), resulting in a mean incorporation of 600 CPM only. Serum from the *T. congolense*-infected calf (BJ 63) exhibited a similar suppression in the assay. However, if plasma from infected animals was diluted 1:5 or more before it was used in the assay, the inhibitory activity was ablated. Plasma was therefore used in the assay at a 1:5 dilution since further dilution would have reduced erythropoietin in the samples below the detection limit of the assay.

Figures 9, 11, 13 and 15, show the erythropoietin responses in the four *T. congolense* infected calves BJ62, BJ63, BJ64 and BJ65. Erythropoietin levels increased either during the acute stages of the disease when the PCV was dropping rapidly or during the chronic stages of the disease when the PCV had stabilised between 16-20%. The erythropoietin levels occurred as several distinct peaks, rather than as sustained increase. Increased levels of erythropoietin in BJ62 occurred during the chronic stage of anaemia between 57 and 67 dpi (Fig. 9). This animal attained the highest levels of

erythropoietin compared to other animals ranging between 1225 and 2300 mU/ml. The highest levels of erythropoietin of 2300 mU/ml occurred when the PCV of this animal dropped to 15% on 57 dpi and the animal was treated the same day.

The erythropoietin response in BJ63 occurred during both the acute and chronic stages of the disease (Fig. 11). During the acute stage, three peaks of erythropoietin of between 700 and 925 mU/ml occurred between 7 and 22 dpi. During this period, the PCV dropped from 30% on 7 dpi to 23% on 22 dpi. In the chronic stage, four peaks of erythropoietin ranging between 710 and 1,100 mU/ml occurred between 53 and 85 dpi. During this period the PCV was maintained at 17-16%. The last peak of erythropoietin of 1100 mU/ml, which was the highest for this animal occurred on 85 dpi, when the PCV of the animal dropped to 15% and the animal was treated later the same day.

For BJ64 (Fig. 13), one peak of erythropoietin of 350 mU/ml occurred 25 dpi, during the acute stage of the disease when the PCV had dropped to 21%. Four peaks of between 350 and 1315 mU/ml of erythropoietin occurred in the chronic stages of the disease between 43 and 106 dpi when the PCV was stable between 17 and 18%. The last peak of erythropoietin (750 mU/ml) occurred on 106 dpi, and the animal was treated later the same day, .

Three peaks of erythropoietin were detected in BJ65 , between 29 and 43 dpi ranging between 300 and 765 mU/ml (Fig. 15). The first peak of 765 mU/ml which occurred on 29 dpi, coincided with the end of the acute stage of the disease when the PCV dropped to 19%. The other five peaks occurred when the PCV had stabilised between 18 and 20% until the end of the experiment. The animal was not treated.

4.3. Haematology

4.3.1. *T. congolense* infected animals

T. congolense were first detected in the peripheral blood of all the four infected calves 12 days post-infection (dpi). Parasitaemia reached a peak ($5x10^5$ trypanosomes/ml of blood) 15 dpi and remained at this level until 18 dpi. Thereafter, several waves of parasitaemia were observed, but the peaks became gradually lower

(Fig. 17). The PCV (Fig. 9, 11, 13, 15 and 18), RBC counts (Fig. 19) and Hb (Fig. 20) decreased rapidly from 15 dpi (time of peak parasitaemia) to 39 dpi by which time mean PCV had dropped to 16-17 %. Thereafter the PCV gradually decreased to 15 % in three animals at which time the animals was treated, or stabilised between 16-20 % in one animal. Data of haematological changes in treated animals were not included in the graphs. BJ62, BJ63 and BJ64 were treated on 57, 85 and 106 dpi respectively when their PCV had dropped to 15%. There was an increase in PCV after treatment and the rate at which the PCV recovered was faster the earlier the animal was treated. BJ62 which was treated on 57 dpi, regained its pre-infection PCV value of 30% by 4 weeks after treatment while BJ63 which was treated on 85 dpi had its PCV increased to 26% only (84% of its pre-infection PCV) by 11 weeks after treatment. The PCV in BJ64 was not measured after treatment. One animal (BJ65) maintained PCV values of between 16-20% and was not treated during the period of the study. RBC and Hb values essentially followed the same pattern as PCV.

Concomitant with the fall in PCV, the MCV started to increase on 15 dpi (Fig. 10, 12, 14, 16 and 21). The mean pre-infection value was 35 fl. A maximum value of 50-55 fl was attained between 39 and 76 dpi. Thereafter, the MCV decreased gradually to 40 fl by 144 dpi when the experiment was terminated.

Reticulocytes were detected between 53 and 78 dpi in three out of four infected animals, when the mean PCV was between 16 and 17% (Fig. 9, 11, 13 and 15). The reticulocyte counts were however low, ranging between 5400-6200 reticulocytes /μl (0.2%) and 11,200-12,000 reticulocytes /μl (0.4%). The MCHC gradually decreased from the pre-infection value of 35% on 0 dpi to lowest values of between 26 and 29 % between 48 and 106 dpi (Fig. 10, 12, 14, 16 and 22). Thereafter, the MCHC increased gradually to normal values.

The WBC started to decrease from 13 dpi (pre-infection mean was $9.9 \pm 1.8 \times 10^3/\mu l$) and attained the lowest average values of $6.2 \pm 0.8 \times 10^3/\mu l$ on 15 dpi (Fig. 23). The highest and lowest values of WBC were however within the normal range of total leucocyte counts ranging between 4 and 12×10^3 leucocytes /μl of blood (Jain,

1986). The decrease in leucocyte counts was due to a decrease in neutrophil counts which began to decrease on 13 dpi (pre-infection value was 2360 neutrophils / μl of blood) and maintained lower values of between 600 to 980 neutrophils / μl of blood. Three animals BJ62, BJ63 and BJ64 developed a transient neutropaenia of between 304 and 544 neutrophils per μl of blood on 22 dpi. Otherwise, the neutrophil counts, though decreased, remained for the rest of the experiment within the normal range given for cattle of between 600 and 4,000 neutrophils / μl of blood. Lymphocyte, monocyte, eosinophil and basophil counts remained unchanged. Following treatment, the neutrophil counts and WBC of BJ62 and BJ63 recovered to their pre-infection values.

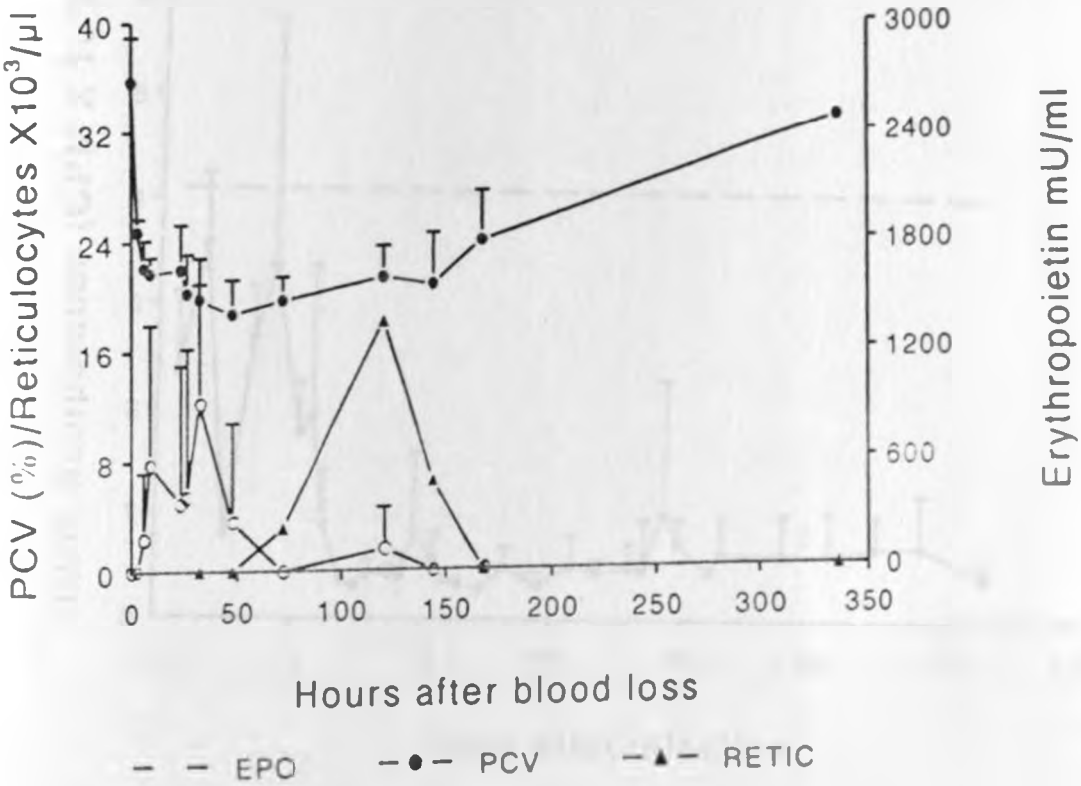

Figure 6. Changes in the mean (±SD) packed cell volume (PCV), erythropoietin levels (EPO), and means of reticulocyte counts (RETIC) following sudden blood loss in four calves.

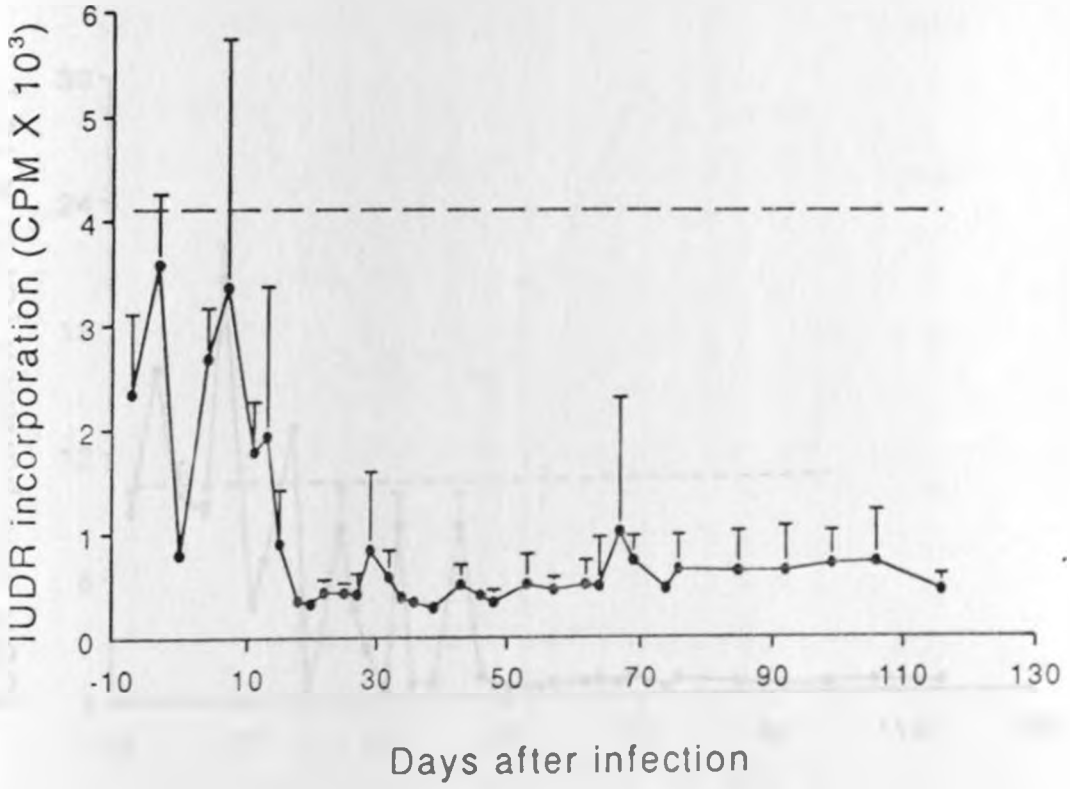

Figure 7. Effect of plasma from two calves (BJ 62 and BJ 63) infected with *T. congolense* IL 1180 on the incorporation of ^{125}I-labelled deoxyuridine (IUDR) into spleen cells from phenylhydrazine treated mice. The broken line indicates background incorporation.

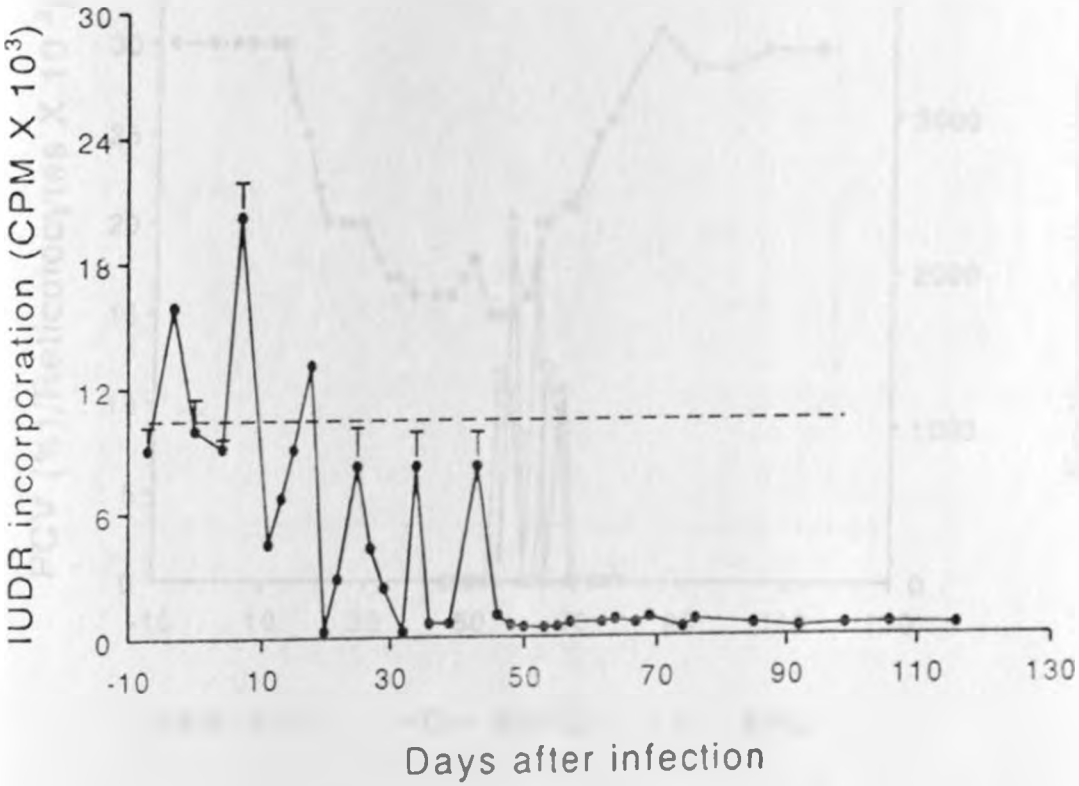

Figure 8. Effect of serum from a calf (BJ 64) infected with *T. congolense* IL 1180 on the incorporation of ^{125}I-labelled deoxyuridine (IUDR) into spleen cells from phenylhydrazine treated mice. The broken line indicates background incorporation.

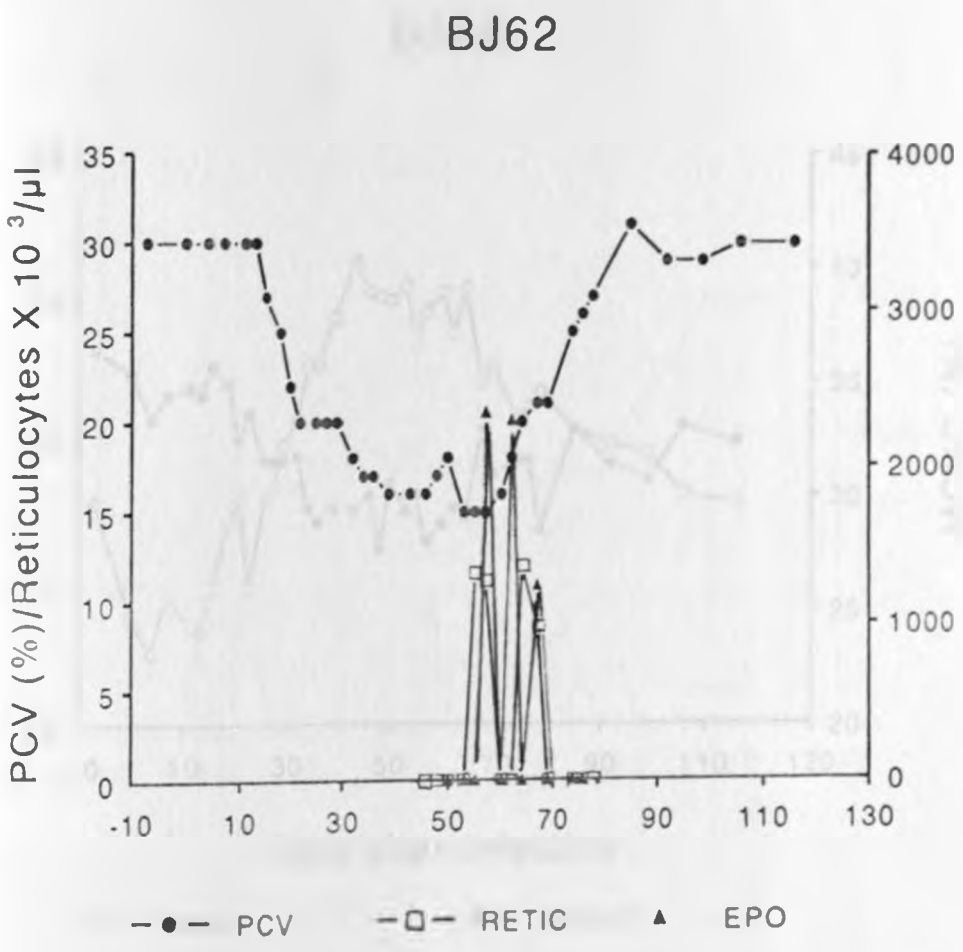

Figure 9. Changes in packed cell volume (PCV), erythropoietin (EPO) levels and reticulocyte counts (RETIC) in a Friesian calf (BJ62) during a *T. congolense* IL 1180 infection.

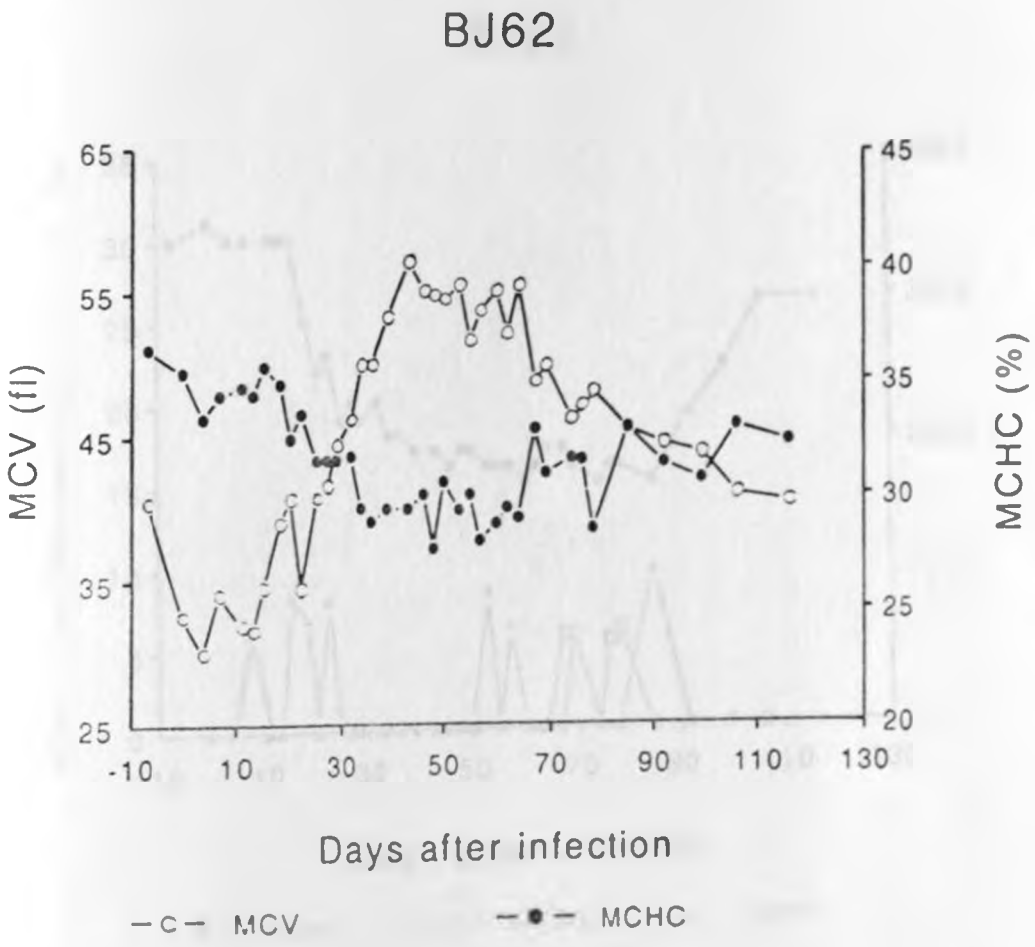

Figure 10. Changes in mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) of a Friesian calf (BJ62) during a *T. congolense* IL 1180 infection.

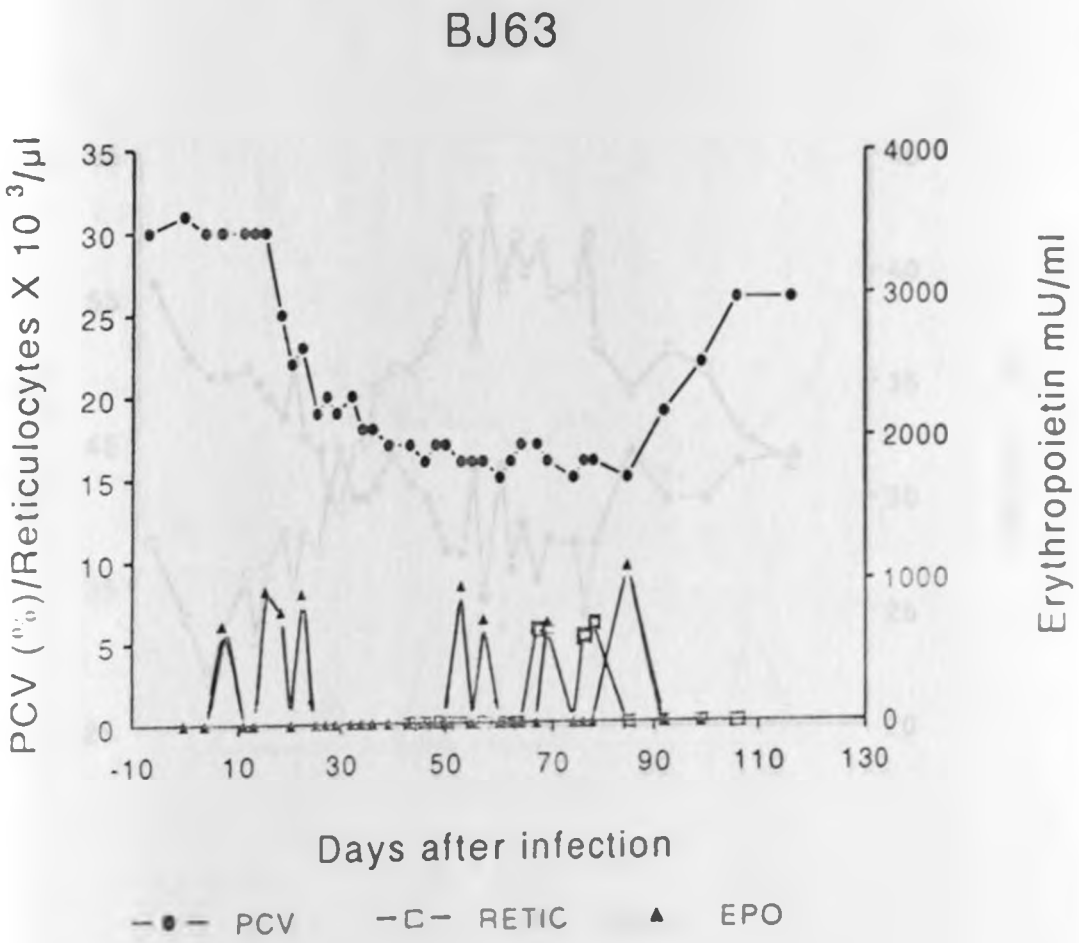

Figure 11. Changes in packed cell volume (PCV), erythropoietin (EPO) levels and reticulocyte counts (RETIC) in a Friesian calf (BJ63) during a *T. congolense* IL 1180 infection.

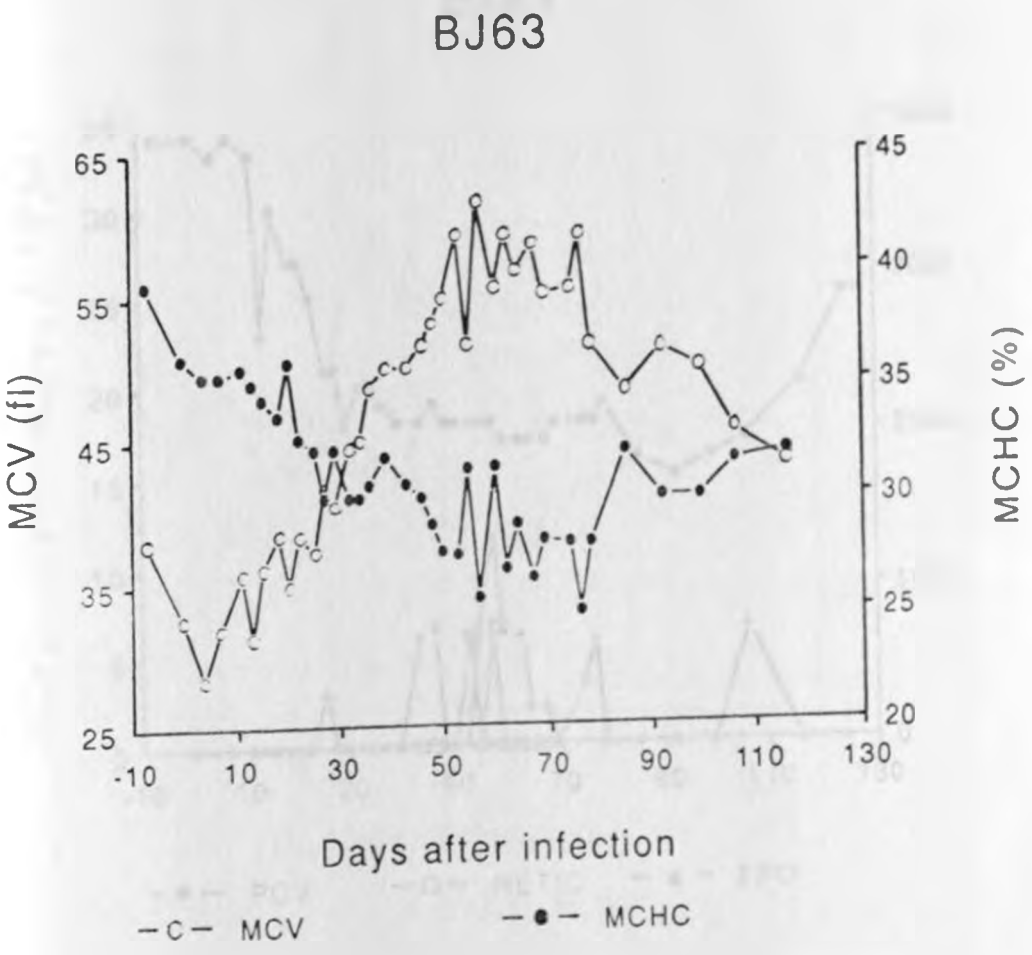

Figure 12. Changes in mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) in a Friesian calf (BJ63) during a *T. congolense* IL 1180 infection.

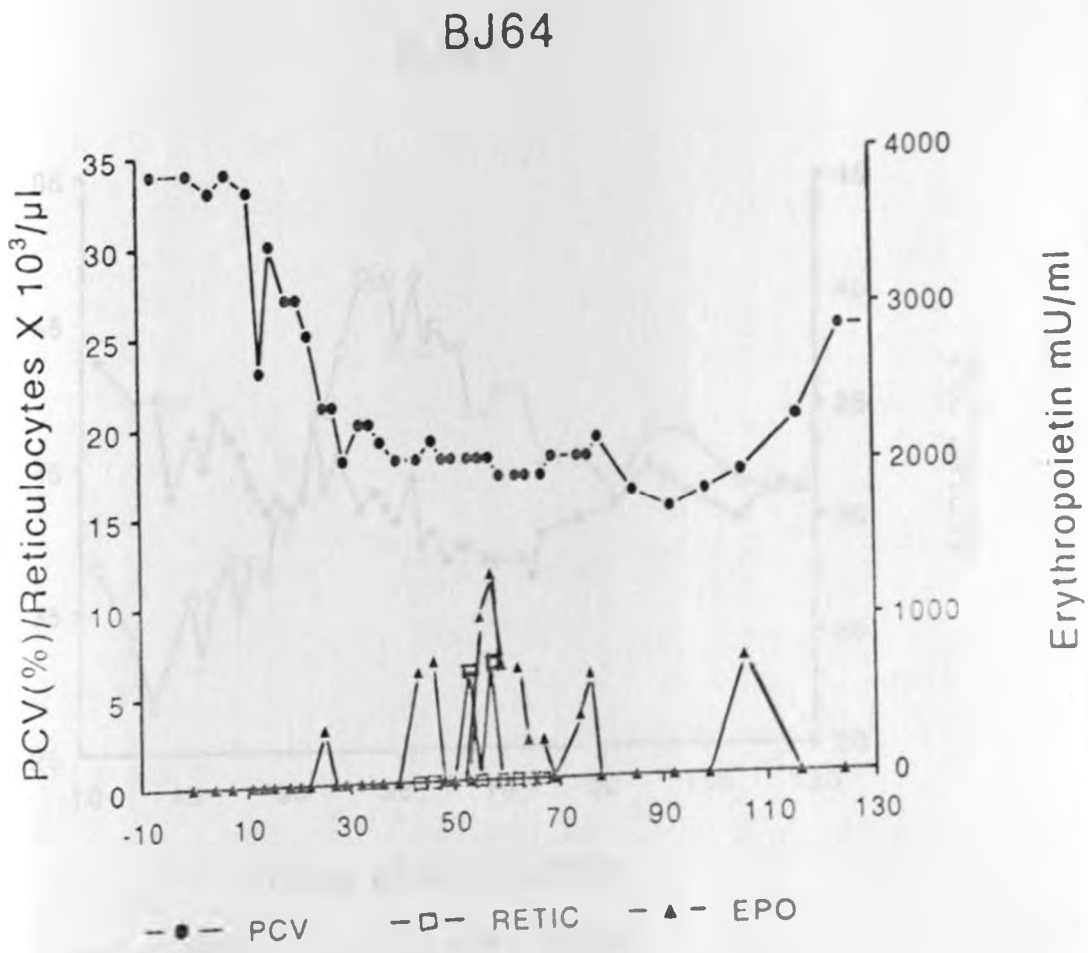

Figure 13. Changes in packed cell volume (PCV), erythropoietin (EPO) levels and reticulocyte counts (RETIC) in a Friesian calf (BJ64) during a *T. congolense* IL 1180 infection.

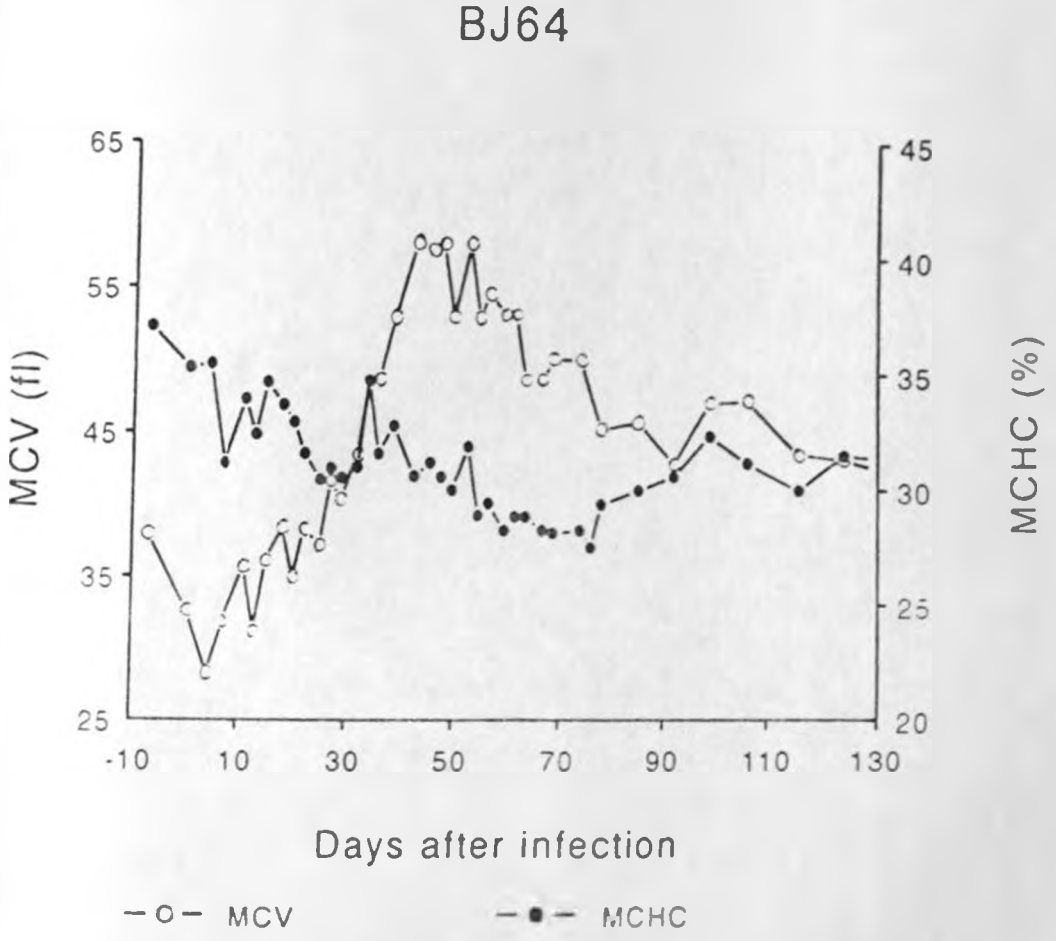

Figure 14. Changes in mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) in a Friesian calf (BJ64) during a *T. congolense* IL 1180 infection.

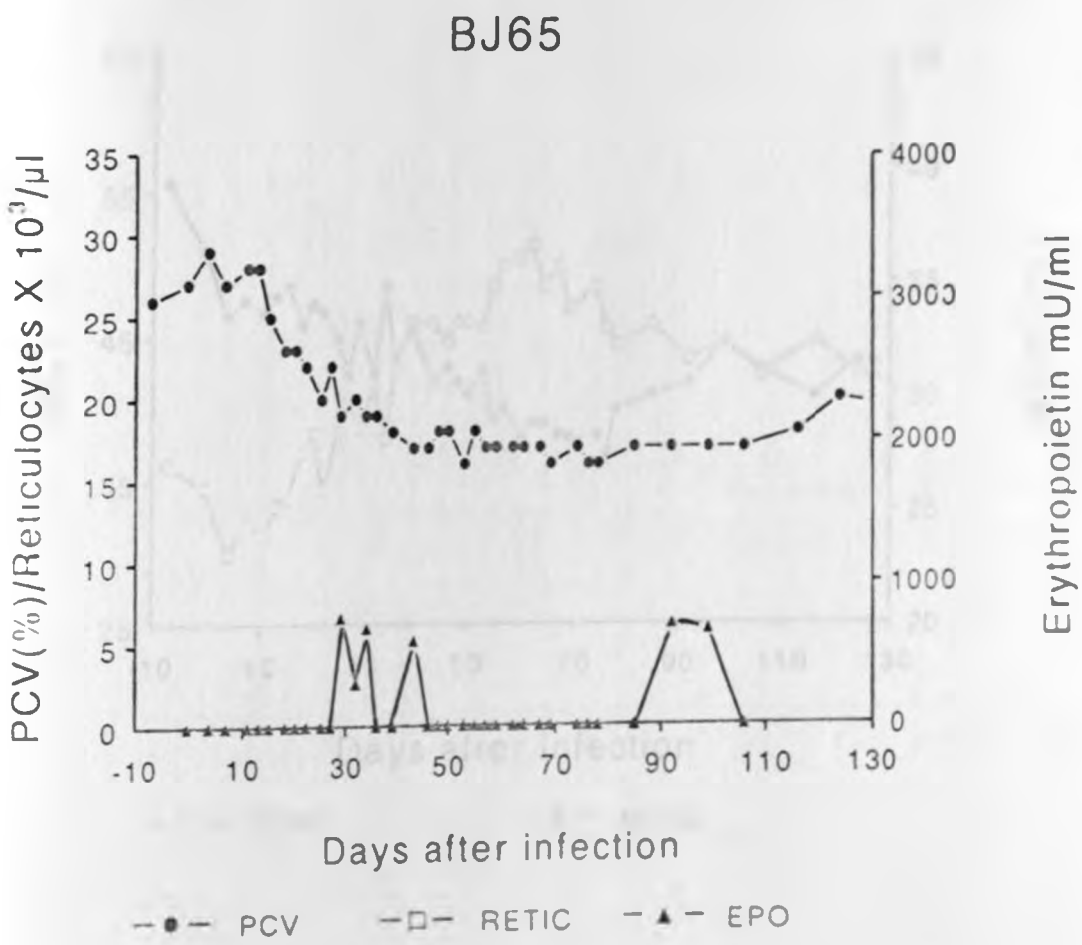

Figure 15. Changes in packed cell volume (PCV), erythropoietin (EPO) levels and reticulocyte counts (RETIC) in a Friesian calf (BJ65) during a *T. congolense* IL 1180 infection.

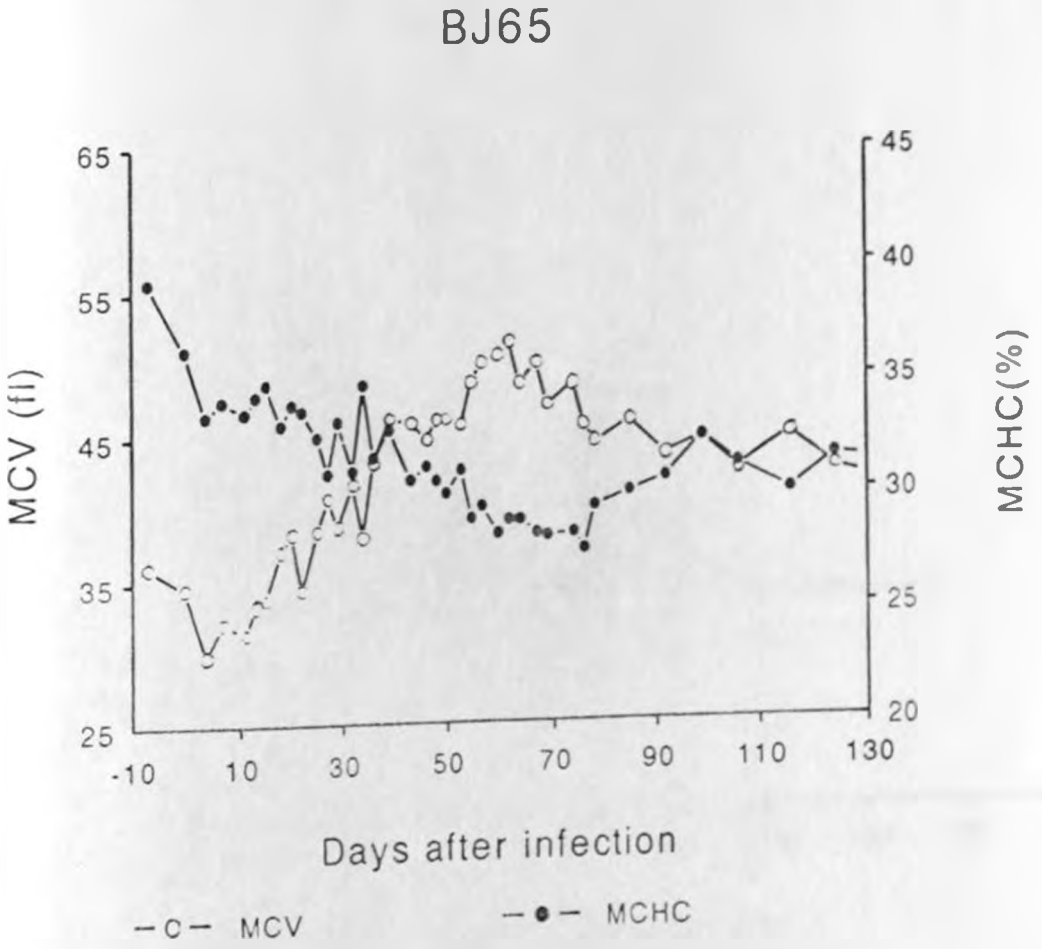

Figure 16. Changes in mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) in a Friesian calf (BJ65) during a *T. congolense* IL 1180 infection.

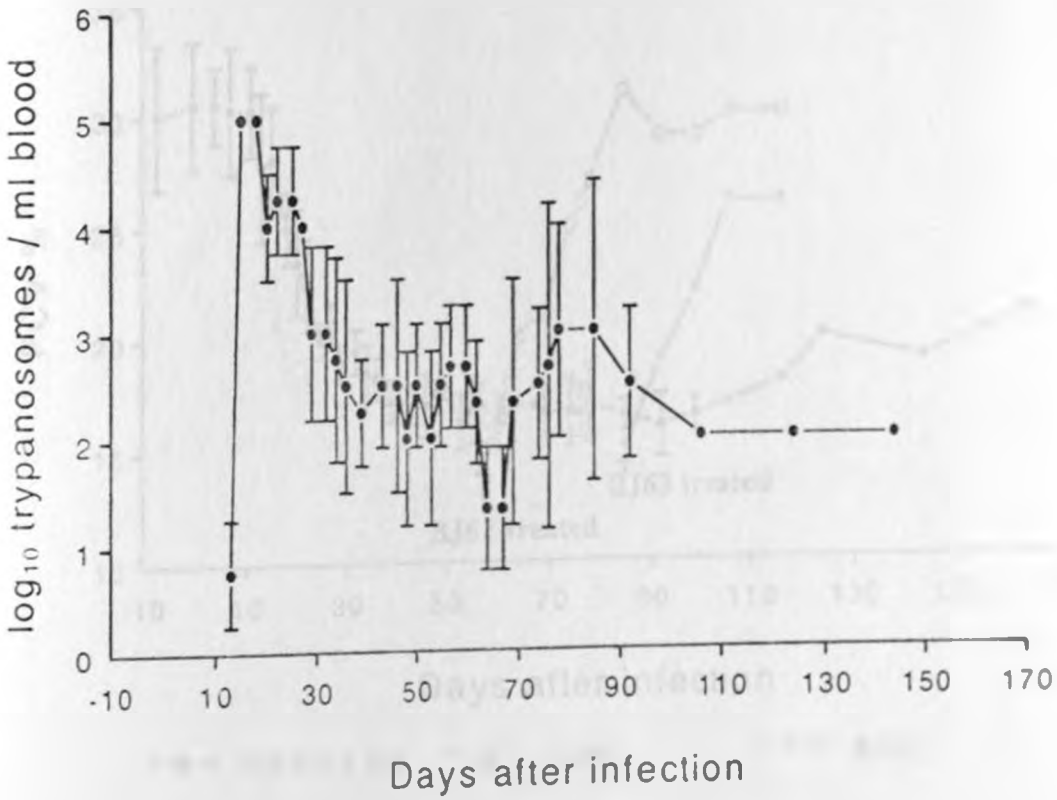

Figure 17. Changes in mean (± SD) parasitaemia during infection with *T. congolense* IL 1180 in four Friesian calves.

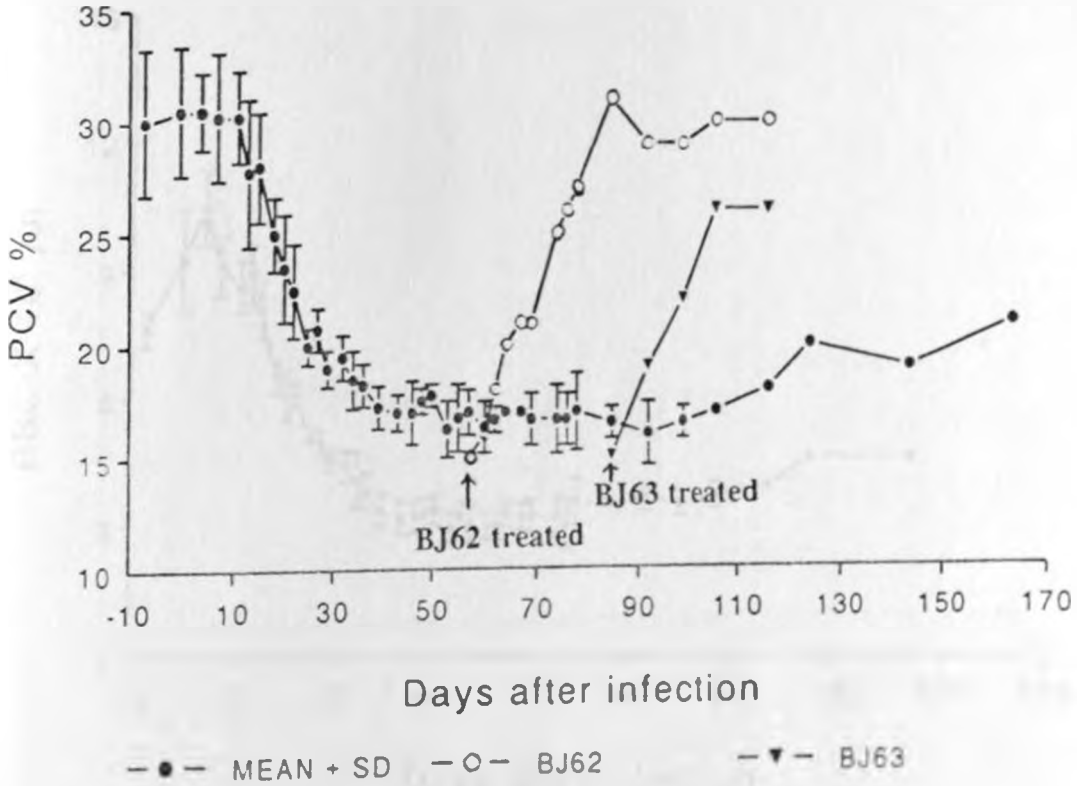

Figure 18. Changes in the mean (±SD) packed cell volume (PCV) in four Friesian calves following infection with *T. congolense* IL 1180 and changes of PCV in two calves BJ62 and BJ63 following treatment with Berenil at a dose of 7 mg/kg body weight.

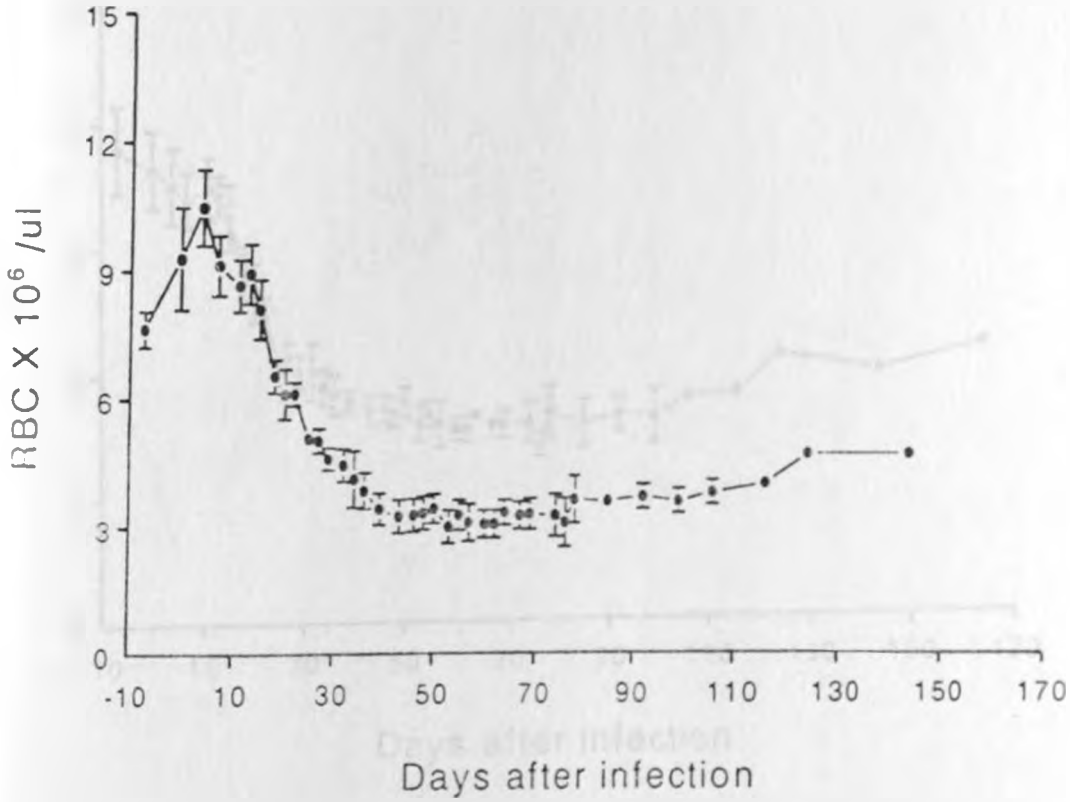

Figure 19. Changes in mean (± SD) red blood cell counts (RBC) following infection with *T. congolense* IL 1180 in four Friesian calves.

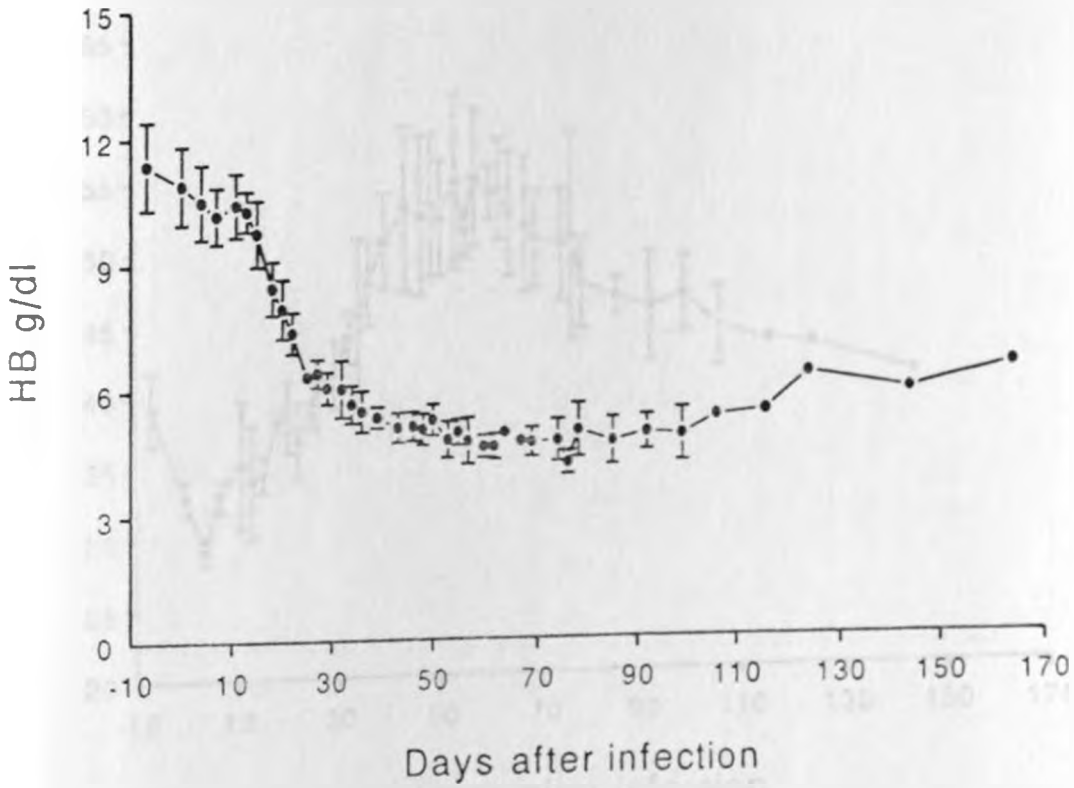

Figure 20. Changes in mean (± SD) haemoglobin concentration (HB) following infection with *T. congolense* IL 1180 in four Friesian calves..

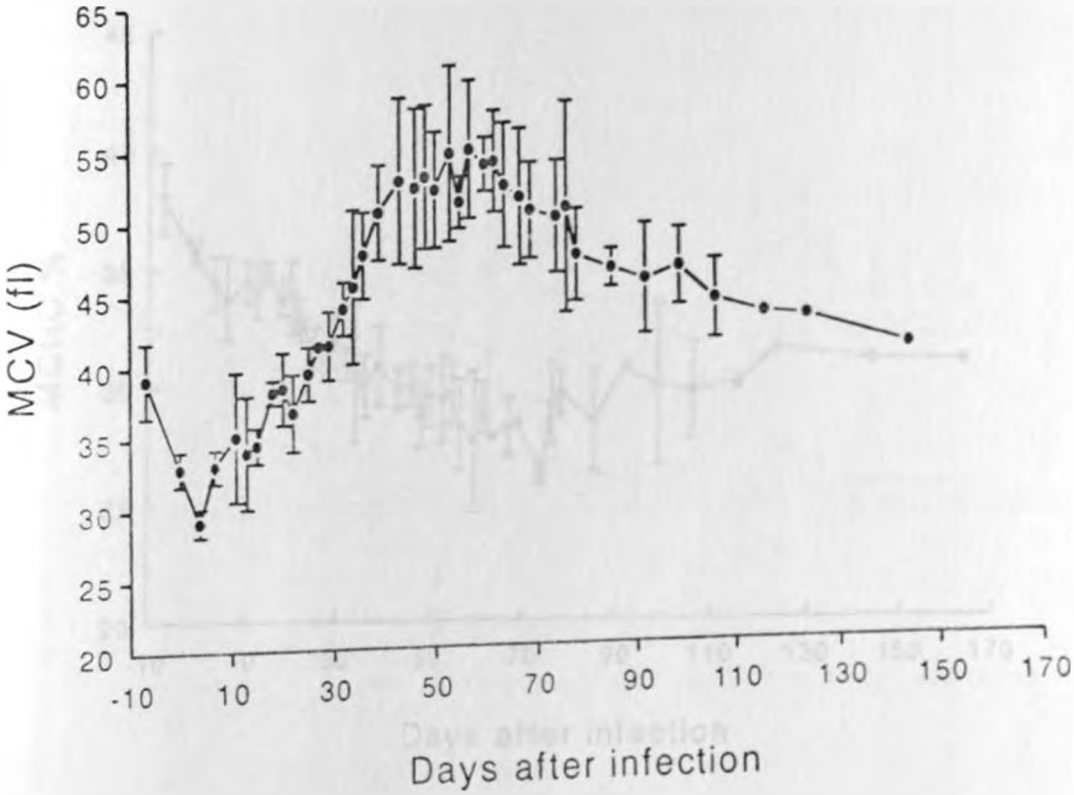

Figure 21. Changes in mean (± SD) mean corpuscular volume (MCV) following infection with *T. congolense* IL 1180 in four Friesian calves.

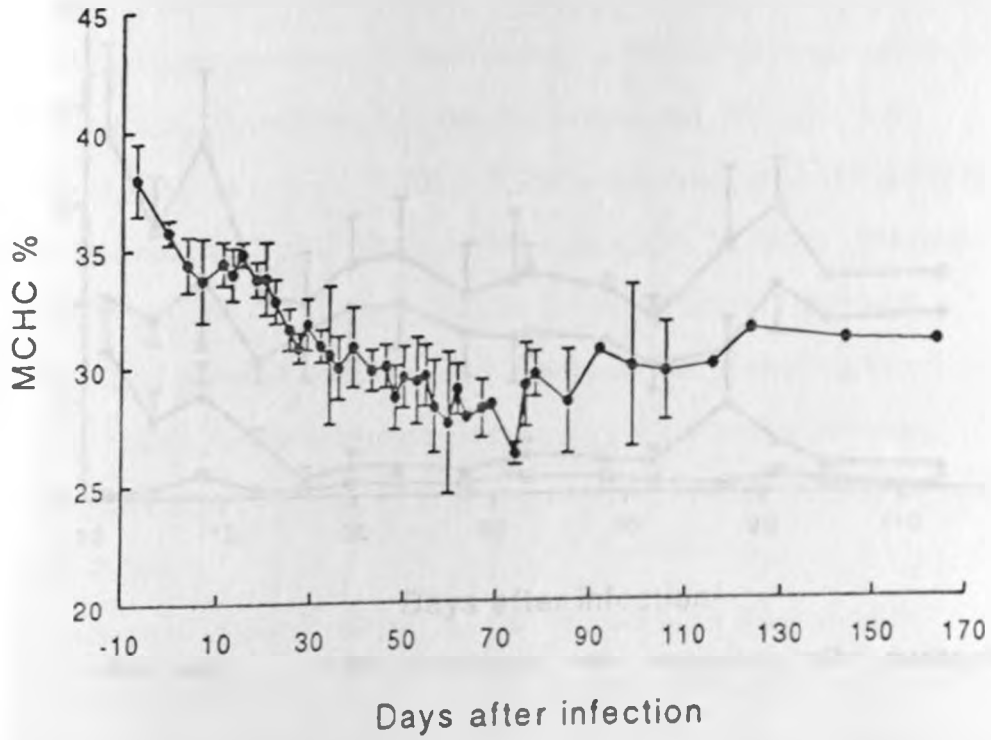

Figure 22. Changes in mean (± SD) mean corpuscular haemoglobin concentration (MCHC) following infection with *T. congolense* IL 1180 in four Friesian calves.

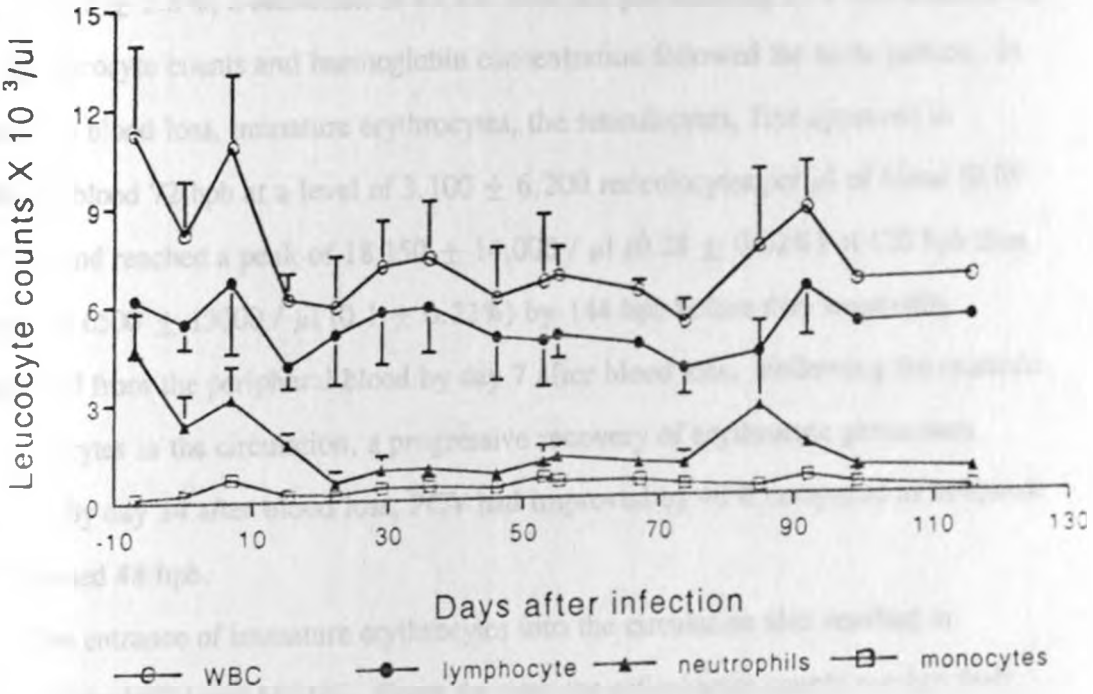

Figure 23. Changes in mean (± SD) total leucocyte counts (WBC), lymphocytes, neutrophils and monocyte counts in four Friesian calves infected with *T. congolense* IL 1180..

4.3.2. Haematology of calves made anaemic by sudden blood loss

Table 5 shows the changes in erythrocyte parameters in four calves following a sudden blood loss of approximately 50% of their blood volume in a single bleeding. Blood loss resulted in reduction of 30.8% in the mean PCV i.e. from the pre-bleeding PCV of 35.8 ± 3.2 % to 24.8 ± 1.0%, three hours after blood loss. The PCV continued to decrease progressively through 6, 9, 24, 27, 30, 33 hpb. The lowest PCV level of 18.8 ± 2.5%, a reduction of 47.8% from the pre-bleeding PCV was attained 48 hpb. Erythrocyte counts and haemoglobin concentration followed the same pattern. In response to blood loss, immature erythrocytes, the reticulocytes, first appeared in peripheral blood 72 hpb at a level of 3,100 ± 6,200 reticulocytes per μl of blood (0.05 ± 0.1%) and reached a peak of 18,150 ± 14,000 / μl (0.28 ± 0.02%) at 120 hpb then declined to 6500 ± 13000 / μl (0.1 ± 0.22%) by 144 hpb before they eventually disappeared from the peripheral blood by day 7 after blood loss. Following the entrance of reticulocytes in the circulation, a progressive recovery of erythrocyte parameters occured. By day 14 after blood loss, PCV had improved by 40% compared to its lowest value attained 48 hpb.

The entrance of immature erythrocytes into the circulation also resulted in changes of the MCV and MCHC. From the time the reticulocyte counts reached their highest levels 120 hpb, the MCV increased from 33.0 ± 1.8 fl to 41.3 fl while the MCHC decreased from 36.05 ± 0.7% to 32.7% by 14 days after blood loss. The progressive increase in MCV and the corresponding decrease in MCHC suggests that anaemia was morphologically progressing towards a macrocytic hypochromic type.

Table 4.4. Changes in mean (± SD) packed cell volume (PCV), red blood cell counts (RBC), haemoglobin concentration (HB), mean corpuscular volume (MCV), reticulocyte counts (RETIC) and mean corpuscular haemoglobin concentration (MCHC) in four calves following blood loss of 50% of their estimated total blood volume.

Hour After Blood Loss	PCV (%)	RBC ($x10^6$/ul)	HB (g/dl)	MCV (fl)	RETIC. ($X\ 10^3$/ul)	MCHC (%)
0	35.75 (± 3.23)	11.53 (± 0.63)	12.88 (± 1.08)	30.81 (± 1.82)		36.07 (± 1.79)
3	24.75 (± 0.96)	7.18 (± 0.49)	8.58 (± 0.73)	34.63 (± 2.99)		34.64 (± 2.52)
6	22.13 (± 2.02)	6.88 (± 0.39)	8.03 (± 0.46)	32.24 (± 3.21)		36.4 (± 2.52)
9	21.75 (± 1.71)	6.38 (± 0.58)	7.7 (± 0.79)	34.23 (± 1.19)		35.44 (± 2.99)
24	22.0 (± 3.27)	6.68 (± 0.99)	7.8 (± 1.06)	33.12 (± 3.36)		35.55 (± 2.04)
27	20.25 (± 2.78)	6.5 (± 0.96)	7.43 (± 0.81)	31.34 (± 3.61)		36.81 (± 1.65)
30	19.5 (± 3.42)	6.18 (± 1.49)	6.73 (± 0.79)	32.13 (± 3.79)		34.74 (± 1.94)
33	19.83 (± 3.01)	5.67 (± 0.76)	7.0 (± 1.04)	35.01 (± 2.33)		35.35 (± 2.71)
48	18.75 (± 2.5)	5.7 (± 1.01)	6.68 (± 0.62)	33.29 (± 4.34)		35.75 (± 1.48)
72	19.75 (± 1.71)	5.9 (± 0.41)	6.95 (± 0.48)	33.52 (± 2.59)	3.1 (0.05%) (± 6.2)	35.24 (± 1.24)
120	21.38 (± 2.29)	6.5 (± 0.88)	7.7 (± 0.78)	33.0 (± 1.75)	18.15 (0.28%) (± 1.4)	36.05 (± 0.74)
144	20.83 (± 3.75)	5.87 (± 0.85)	7.47 (± 1.1)	35.38 (± 1.72)	6.5 (0.1%) (± 13)	36.0 (± 1.43)
168	24.0 (± 3.61)	6.36 (± 0.77)	8.13 (± 0.83)	37.64 (± 1.18)		34.06 (± 1.75)
336	33.0	8.0	10.8	41.25		32.73

CHAPTER FIVE

5. DISCUSSION

Two assays for erythropoietin, a radioimmunoassay and an *in vitro* bioassay utilising spleen cells from phenylhydrazine treated mice were evaluated to assess their suitability to measure erythropoietin in serum/plasma of calves made anaemic by exsanguination and in *T.congolense*-infected calves. The aim of these studies was to ascertain whether the poor erythropoietic response associated with anaemia of chronic bovine trypanosomiasis is due to low levels or lack of erythropoietin. Erythrocyte parameters were also monitored in exsanguinated and infected calves. Antibody raised against human erythropoietin detected erythropoietin in human serum by radioimmunoassay but failed to detect erythropoietin in serum from an anaemic calf. The *in vitro* bioassay was able to detect erythropoietin in plasma from an anaemic calf and was subsequently used to monitor the levels of the hormone in phlebotomised and *T. congolense*-infected calves.

Increased erythropoietin levels in exsanguinated calves was detected 6hpb, reached peak 33hpb and was undetectable 72hpb. The anaemia in exsangunated calves during recovery from blood loss was macrocytic hypochromic with appearence of reticulocytes in the circulation, and thus considered to be responsive. Distinct peaks of elevation of erythropoietin occurred during both the acute and chronic stage of *T. congolense* infection of calves. Throughout the acute stage of the disease, the anaemia was classified as responsive characterised by a progressive increase in MCV. The peak erythropoietic response occurred in the early stages of the chronic disease (between 39-76dpi) when the anaemia was macrocytic hypochromic with few circulating reticulocytes. However from 76dpi, despite increased erythropoietin levels and low PCV, the anaemia progressively became normocytic normochromic with no

reticulocytes, indicating that the bone marrow was unresponsive to the erythropoietin stimulus.

The human based radioimmunoassay failed to detect erythropoietin in serum from an anaemic calf. This observation suggests that differences exists in immunological reactivity between the human and bovine erythropoietin. Similar observation was reported by Sherwood and Goldwasser (1979) who attempted to detect erythropoietin in bovine sera using an erythropoietin radioimmunoassay based on human derived reagents. However, there exists similarities in amino acid sequences between erythropoietin molecules derived from different species for instance the human erythropoietin has a 79% and 94% homology with mouse and monkey erythropoietin respectively (Goldwasser,1989). Likewise, immunological cross-reactivity between erythropoietin from various animal species has been reported. An anti-R-HuEPO was produced by Genzyme corporation which could bind mouse and rabbit erythropoietin and an antibody against U-HuEPO was produced which could bind sheep, rabbit, rat, mice, baboon and monkey erythropoietin in a radioimmunoassay (Garcia, 1979). However other studies indicated that while an anti-U-HuEPO exerted full inhibition of the biological activity of human urinary erythropoietin, it weakly neutralized sheep erythropoietin (Sherwood and Goldwasser, 1979). Such differences may account for the failure of the radioimmunoassay using human derived reagents to detect bovine erythropoietin. Owing to these intra-species and inter-species differences in immunological reactivity between various erythropoietin preparations, a successful species-specific erythropoietin radioimmunoassay should preferably use reagents derived from the same species and stock of erythropoietin. Consequently, a good bovine erythropoietin radioimmunoassay will depend on purification or cloning of the bovine hormone.

Since the radioimmunoassay using human derived reagents failed to detect erythropoietin in bovine serum, a bioassay which responds to erythropoietin in a non-species specific manner was developed. Unlike *in vitro* bioassays, *in vivo* bioassays are less sensitive and require the use of large numbers of mice and large volumes of plasma samples (Garcia, 1979). However, *in vitro* bioassays are known to respond to asialated

erythropoietin which is active *in vitro* but inactive *in vivo*, and in addition, they are said to be sensitive to non-specific inhibitors of erythropoiesis (Krystal, 1983). The *in vitro* bioassay utilising spleen cells from phenylhydrazine treated mice is as sensitive as the radioimmunoassay, with a detection limit as low as 2-5mU/ml of erythropoietin (Krystal, 1983). The latter bioassay was therefore tested and found suitable for the detection of erythropoietin in bovine plasma.

Using the *in vitro* bioassay, different concentrations of plasma from an anaemic calf produced a dose-dependent response which was similar to that produced by the erythropoietin standard. These observations suggest that spleen cells were responding to a substance in bovine plasma which had similar bioactivity to human erythropoietin. It was confirmed that the substance in bovine plasma which stimulated spleen cells was erythropoietin since an antibody to human erythropoietin blocked the stimulatory effect of bovine plasma to the spleen cells. These results are in agreement with observations that the bioactivity of erythropoietin is not species specific (Jain, 1986) and suggest that the anti-U-HuEPO possesses the ability to bind bovine erythropoietin which contrast with the results obtained using the radioimmunoassay. From the results in a bioassay, it is likely that there exists in the anti-U-HuEPO antisera, a population of low concentration antibodies with the capacity to bind bovine erythropoietin and these antibodies were able to bind bovine erythropoietin in the bioassay because the anti-U-HuEPO was used at low dilution (1:300). Since in the radioimmunoassay, the anti-U-HuEPO was used at higher dilution (1:50,000), the concentration of antibodies which could bind bovine erythropoietin were much diluted and could not exhibit any significant binding.

The detection limit for the *in vitro* bioassay was 62.5 mU/ml of erythropoietin. Thus the assay was less sensitive than that developed by other workers who could detect a minimum of 2mU/ml of erythropoietin using spleen cells from (C57BL/6J X C3H/HeB)F1 (Krystal, 1983). The difference in the detection limit may have been due to differences in the strains of mice used.

From 13dpi onwards, plasma and serum from *T. congolense*-infected calves suppressed IUDR incorporation into spleen cells. This confims observations that *in vitro* bioassays are sensitive to inhibitors of erythropoiesis in the plasma (Krystal, 1983). The nature and mechanism of action of the suppressive substance remains to be established. But since the suppression started 13dpi when parasitaemia was detected, the suppressive substance may have originated from the trypanosomes themselves or be a host factor produced as a result of the infection. Mediators of inflamatory response such as IL-1, TNF and γIFN have been shown to inhibit erythropoiesis both *in vitro* and *in vivo* and the suppressive effect of γIFN is mediated through the down regulation of erythropoietin receptors on erythroid progenitors (Means and Krantz, 1991). Increased concentration of these cytokines in plasma produced by T-lymphocytes and macrophages, which are stimulated due to trypanosome infection, may have contributed to the suppressive effects produced by plasma from the *T. congolense*-infected calves.

The suppressive effect of plasma was decreased by diluting the plasma five fold before it was used in the assay. However, dilution also reduced erythropoietin concentration below the detection limit of the assay in some samples. In addition, the presence of suppressive substance in the plasma may have resulted in the failure to detect erythropoietin below an unknown threshhold. Furthermore, the presence of suppressive material in plasma of *T. congolense* infected cattle may mean that the absolute values of erythropoietin in infected animals may be higher than was indicated by the assay. These shortfalls make this assay less than optimal for measuring erythropoietin in trypanosome infected animals. Thus an assay, probably an immunoassay which will respond specifically to erythropoietin molecules in plasma will be suitable for the detection of erythropoietin.

A sudden blood loss of 50% of the estimated total blood volume in calves, led to a decrease in PCV, RBC and HB. However, blood samples collected 3hpb from the calves revealed only a 31% reduction in PCV instead of the expected 50%. This discrepancy is suggested to be brought about by the partial replacement of erthrocytes in

the circulation from the marginal reserve in the spleen (Schalm, 1984). Although no further massive bleeding was done on the calves, the erythrocyte parameters observed on 3hpb continued to decrease progressively until lowest values were attained 48hpb. This fall in erythrocyte parameters was possibly caused by the dilution effect as the result of movement of extravascular fluid into the circulation to compensate for the decreased blood volume (Jain, 1986; Schalm, 1984). These observations agree with similar experiments in man where following sudden blood loss, the erythrocyte parameters continue to fall progressively and reach the lowest values between 20 - 60hpb at which time, the initial blood volume is restored (Hillman, 1977).

Following the fall in PCV in exsanguinated calves, increased erythropoietin levels were detected by 6hpb, the levels peaked at 33hpb and dropped to baseline levels by 72hpb although the PCV was still low. Immature erythrocytes, the reticulocytes, were first detected in circulation at 72hpb. The reticulocyte numbers peaked by 120hpb and were undetectable by 168hpb (7 days). Following the entrance of reticulocyte into circulation at 72hpb, the MCV increased and the MCHC decreased resulting in a macrocytic hypochromic anaemia, which indicates an active bone marrow response. Eventually, by 336hpb (2 weeks), the PCV had attained 93% of its pre-bleeding level.

The sequential erythropoietic changes which followed blood loss in calves suggest that erythropoietin might have been involved in the recovery of calves from anaemia. A surge of erythropoietin levels as occurs following sudden blood loss stimulates the rapid differentiation of BFU-E to CFU-E to proerythroblasts, resulting in the expansion of the latter two erythroid compartments and the release of reticulocytes in circulation (Papayannopoulou and Abkowitz, 1991). The elevation of erythropoietin peaks around 24 hpb and then return to normal even before the PCV increases (Baer, 1991). However, the CFU-E and the pro-erythroblasts are more sensitive (one thousand times) to erythropoietin than BFU-E (Sawyer *et al.*, 1990; Papayannopoulou and Abkowitz, 1991). Thus, once the CFU-E and the pro-erythroblast compartments are expanded, the basal erythropoietin levels are sufficient for their differentiation to mature erythrocytes. Reticulocytes are macrocytic and thus increased numbers of reticulocytes

in circulation leads to an increase in MCV (Finch, 1982) as was observed in this study. Similary, increses in erythropoietin (Yagi *et al.*, 1992), CFU-E (Kaaya and Maxie, 1984), reticulocytes and MCV (Schalm, 1984; Yagi *et al.*, 1992) were observed in experiments involving serial bleeding of calves. Similarly, increase in erythropoietin was observed in haemolytic anaemia following *Anaplasma* infections of cattle and sheep (Jatkar and Kreier, 1967) and increase in erythropoietin, reticulocytes and and MCV were observed in haemolytic anaemia following *T.sergenti* infection of cattle (Yagi *et al.*,1992). The macrocytic hypochromic type of anaemia which occurs following blood loss in calves, as was observed in this experiment, and by others (Schalm, 1984) and following *T.sergenti* infections of cattle (Yagi *et al*, 1992) and *Anaplasma* infection of cattle (Schalm, 1984) confirm observations that this type of anaemia occurs during recovery from anaemia due to sudden blood loss or haemolytic anaemia (Jain, 1986)

The anaemia which developed in four Friesian calves infected with *T.congolense* was characterised by a rapid drop in PCV from 15dpi (peak parasitaemia). The drop in PCV continued until 39dpi when PCV of 16-17% were reached. The PCV remained at this level and only increased after treatment in BJ62, BJ63, and BJ64 or during the spontaneous recovery in BJ65. Erythropoietin peaks occurred in both the acute and chronic stages of this *T. congolense* infection. In the acute stage, erythropoietin peaks occurred in three animals BJ63, BJ64 and BJ65 between 7-35dpi, when the PCV was dropping rapidly. In the chronic stage, the highest erythropoietin response ocurred between 39-76dpi characterised by many peaks of erythropoietin. In some individuals e.g. BJ62 high levels up to 2300 mU/ml were detected. A number of erythropoietin peaks were also detected after 76dpi, these peaks were observed when the PCV dropped to 15% in the two animals BJ63 and BJ64 which were treated on 85 and 106dpi respectively. In BJ65 which was not treated, peaks of erythropoietin occured between 92-99dpi when the PCV was 16%. The anaemia which developed in the acute stage of the infection was responsive, characterised by progressive increase in MCV. The peak MCV response occurred in the chronic stage of the disease between 39-76dpi when reticulocytes appeared and anaemia was macrocytic hypochromic. However, the

erythropoietic response began to decrease from 76dpi and was characterised by an absence of reticulocytes in the circulation and a normocytic normochromic anaemia. The increase in erythropoietin following the drop in PCV in both the acute and chronic stages of the disease confirms that erythropoietin is stimulated by hypoxia. In addition, the erythropoietic response which reached peak in the chronic stage between 39-76 dpi is in conformity with erythropoietin bone marrow stimulation. Thus the erythropoietin response in the early stages of the disease may have been responsible for bone marrow erythroid hyperplasia and change in M:E ratio in favour of erythroid cells, increased plasma iron turn over and increased red cell iron utilization observed by other workers (Valli *et al.*,1978; Dargie et al., 1979; Wellde et al., 1989; Anosa *et al.*, 1992). The MCV in BJ62 increased in the acute stage as in other animals but no erythropoietin response was detected. Since the increase in MCV is due to erythropoietin bone marrow stimulation, the increase in MCV in this animal might have been caused by increased levels of erythropoietin which were too low to be detected by the assay.

Although the anaemia was responsive in the acute stage as shown by increase in MCV, early erythropoietin peaks in BJ63, BJ64 and BJ65 did not result in detectable reticulocytosis until later in the infection and it is known that erythropoietin causes the appearence of reticulocytes in circulation 72 hours after bone marrow stimulation (Schalm,1984). In addition, the peak erythropoietic response observed in this experiment did not result in an increase in PCV. These observations suggest that the erythropoietic response though increased was inadequate to compensate for the low PCV. The possible cause of the failure to restore PCV is rapid removal of erythrocytes from circulation or insufficient bone marrow production of erythrocytes or both. Erythrophagocytosis by the MPS is suggested to be the major cause of anaemia in the acute stages of bovine trypanosomiasis (Murray et al., 1979; Dargie et al ., 1979). In addition, it was observed that CFU-E decreased early in *T.congolense* infections of Boran cattle even before the anaemia developed and only increased later in the chronic stage (Andrianarivo *et al.*, 1992). This suggests that the bone marrow response to erythropoietin stimulation in the acute stages of the disease may not be optimal.

The reticulocyte response was very poor in both anaemia due to blood loss (0.28%) and due to *T. congolense* infection (0.4%). In cattle, under normal circumstances, while reticulocytes are found in the bone marrow, they are practically absent in the peripheral blood (Jain, 1986). This suggests that bovine reticulocytes undergoes their maturation entirely in the bone marrow. In an attempt to study what degree of blood loss is sufficient to cause a marked reticulocyte response in cattle, sudden blood loss of up to 40% resulted in a poor reticulocyte response (0.5%) (Schnappauf *et al.*, 1967), agreeing with our observations in this experiment where reticulocyte response of only 0.28% occurred following blood loss of 50% of the estimated total blood volume in calves. A marked reticulocyte response (14%) was stimulated in cattle only after a sudden withdrawal of two third of total blood volume in cattle (Schnappauf *et al.*, 1967). In an experiment where equal degrees of anaemia were induced in cattle either by serial blood loss or by *T. sergenti*-infection (causes haemolytic anaemia), reticulocyte and MCV response was relatively poor in exsanguinated cattle as compared to reticulocyte and MCV response in cattle recovering from haemolytic anaemia (Yagi *et al.*, 1992). These results suggested that the erythropoietic response in cattle is more intense in haemolytic anaemias as compared to blood loss anaemias. The possible reason for a poor erythropoietic response to blood loss anaemia is that while in haemolytic anaemias, products of erythrocyte breakdown e.g haemoglobin remains in the body and can be easily recirculated for the production of new erythrocytes, in blood loss anaemia, the erythrocytes are removed and the animal is depleted of some resources for production of new erythrocytes. However, it has been observed that haemolytic anaemias due to *Anaplasma* and *Babesia* infections elicits a better reticulocyte response when compared to haemolytic anaemias following trypanosome infections. Anosa and Isoun (1980) observed that sheep which had earlier succumbed to haemolytic anaemia due to *Anaplasma ovis* and *Babesia ovis* infection responded with a marked reticulocyte response (29%) while the same animals had a poor or no reticulocyte response to haemolytic anaemia due to *T. vivax* infections. Owing to the good reticulocyte response to haemolytic anaemias in ruminants (reviewed by

Igbokwe, 1989), the poor reticulocyte response in *T. vivax* infected sheep and our observations in this experiment and by others (reviewed by Murray and Dexter, 1988) suggests that trypanosome infections causes a suppression of bone marrow erythropoietic response.

It was observed in these studies that the erythropoietic response declined in *T. congolense*-infected calves from 76dpi despite a persistent anaemia and elevated erythropoietin levels. It has been suggested that the bone marrow's unresponsiveness to anaemia in chronic trypanosomiasis is due to retention of iron in the MPS, thus depriving the bone marrow of iron which is essential for erythropoiesis (Dargie et al., 1979; reviewed by Murray and Dexter,1988). The retention of iron in the MPS has been suggested to be the cause of wide spread haemosiderin deposits throughout the body, hypoferraemia and development of microcytosis in chronic and long standing trypanosome infections of cattle (reviewed by Murray and Dexter, 1988). Anaemia of chronic trypanosomiasis is suggested to be analogous to anaemia of chronic disorders in man which is associated with a blunted erythropoietin response to anaemia (Dargie *et al.*, 1979; Suliman and Feldman, 1989). These authors suggested that lack of erythropoietin response may be a contributory factor to anaemia of chronic trypanosomiasis. However, in this experiment, the decrease in erythropoietic response, as judged by decreasing MCV, occurred from 76dpi onwards despite of a number of erythropoietin peaks which occured in three animals BJ63, BJ64 and BJ65. Therefore, the erythroid unresponsiveness noted as judged by lack of MCV response was not due to lack of erythropoietin but rather the failure of the bone marrow to respond to erythropoietin stimulus.

However it should be noted that the bone marrow retained some responsive potential, since the infected animals despite continuing erythrophagocytosis, managed to maintain their PCV above 15% for longer periods of time. Furthermore, it was observed in *T.congolense* infection of Boran cattle that the CFU-E increased after treatment (Andrianarivo, personal communication). In addition, results from this experiment showed that the PCV of treated animals increased to normal levels. This

shows that the presence of parasites in circulation played a significant role in keeping the PCV at low levels. Probably the animals were treated before they reached a stage where response to trypanocidal drug treatment is minimal or absent as was reported by Murray and Dexter (1988). Despite the increase in PCV after treatment, the MCV did not increase although all the animals which were treated exhibited high erythropoietin titre at the time of treatment. This suggests that a certain degree of residual suppression of bone marrow erythropoietic response remains after treatment. It was indeed noted that the erythroid unresponsiveness may be progressive with time after infection since the animal which was treated earlier on 57dpi recovered its pre-infection PCV 4 weeks after treatment while the animal which was treated later on 85dpi had only 84% of its pre-infection PCV 11 weeks after treatment.

The cause of the failure of the bone marrow to respond to elevated erythropoietin levels in trypanosome-infected cattle as observed in this experiment is unknown. Ineffective bone marrow response to elevated erythropoietin levels has also been observed during *Plasmodia*-induced anaemia in mice. The anaemia due to *plasmodia* infections in mice (murine malaria) is attributed to increased erythrocyte destruction and due to ineffective erythropoiesis (Silverman *et al.*, 1987). Despite elevated erythropoietin response to anaemia during murine malaria, ineffective bone marrow erythropoietic response persists suggestively due to suppression of differentiation or proliferation of erythroid precursors or their differentiation from stem cells (Rencrica *et al.*, 1974). Increased TNF levels has been observed during both human and murine malaria and it is thought to play a role in ineffective erythropoiesis (reviewed by Miller *et al.*, 1989). Infusion of recombinant murine TNF in mice mimicked the malaria-induced ineffective erythropoiesis and when *Plasmodium berghei* infected mice were given antiserum against TNF, erythropoiesis was partially restored, suggesting that TNF plays a role in anaemia of malaria infections (Miller *et al.*, 1989). Tumor necrosis factor (TNF) and other cytokine mediators of the inflammatory responses e.g. IL-1, and τ-IFN inhibit erythropoiesis both *in vivo* and *in vitro* (Means and Krantz, 1991). The inhibitory effect of τ-IFN to erythropoiesis is suggested to be induced through the down

regulation of erythropoietin receptors on erythroid progenitor cells. Increased concentration of these cytokines produced by T-lymphocytes and macrophages, which may be stimulated by the massive numbers of trypanosomes in circulation may block the stimulatory effect of erythropoietin on the bone marrow erythroid progenitors, thus leading to ineffective bone marrow erythroid response during trypanosome infections.

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APPENDIX

Appendix 1

Antibody dilution curve for anti-recombinant human erythropoietin polyclonal antisera (anti-R-HuEPO).

Antibody dilution	CPM 1	CPM 2
1:100	11633	12062
1:320	10495	10618
1:1,000	7535	8276
1:3,200	4486	4376
1:10,000	1869	-

Appendix 2

Antibody dilution curve for anti-human urinary erythropoetin polyclonal antisera (anti-U-HuEPO).

Antibody dilution	CPM 1	CPM 2
1:1,000	3148	3342
1:10,000	2403	2994
1:50,000	1579	1432
1:100,000	1126	1245

Appendix 3

Standard curve for A partially purified human urinary erythropoietin (U-HuEPO) and anti-R-HuEPO.

Erythropoietin (mU/ml)	CPM 1	CPM 2
10	2,255	2,212
100	2,163	2,297
1,000	1,859	2,003
10,000	1,226	1,179

Appendix 4

Standard curve for pure human U-HuEPO and anti-U-HuEPO.

Erythropoietin (mU/ml)	CPM 1	CPM 2
7.800	1,119	1,102
15.625	1,070	1,064
31.250	931	931
62.500	801	884
125.000	656	726
250.000	632	588
500.000	622	539
1,000.000	424	675

Appendix 5

Radioimmunoassay of human and bovine serum.

	Bovine serum	
Dilution	CPM 1	CPM 2
1:2	1461	1387
1:4	1435	1678
1:8	1546	1538

	Human serum	
Dilution	CPM 1	CPM 2
1:2	968	913
1:4	1123	1110
1:8	1454	1595

Appendix 6

Standard curve for erythropoietin bioassay.

Erythropoietin (mU/ml)	CPM 1	CPM 2	CPM 3
62.5	5061	4204	4181
125.0	4682	4121	3899
250.0	5490	5110	5199
500.0	7336	6406	7572
1,000.0	16714	15838	12838
2,000.0	66779	51870	50840

Bioassay of bovine plasma.

Serum dilution	CPM 1	CPM 2	CPM 3
undiluted	52994	52713	52029
1:2	21562	23000	23199
1:4	9939	11669	11451
1:8	5178	5438	5724

Appendix 7

Erythropoietin levels in calves following blood loss.

HPB	BH259	BK50	BK46	BK45
0	UD	UD	UD	UD
3	UD	UD	UD	UD
6	720	UD	UD	UD
9	1600	750	UD	UD
24	1560	UD	UD	UD
27	1620	725	1500	UD
33	1225	950	UD	UD
48	1080	UD	UD	UD
72	UD	UD	UD	UD
120	460	UD	UD	UD
144	UD	UD	UD	UD
168	UD	UD		
336	UD			

Key
UD = undetectable
HPB = Hours post-bleeding

Appendix 8

Reticulocyte counts (X 10^3) after blood loss in four calves

HPB	BH259	BK50	BK46	BK45
0	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0
6	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0
33	0.0	0.0	0.0	0.0
48	0.0	0.0	0.0	0.0
72	12.4	0.0	0.0	0.0
120	25.0	32.4	15.0	0.0
144	26.0	0.0	0.0	0.0
168	0.0	0.0	0.0	0.0
336	0.0	0.0	0.0	0.0

Appendix 9

Haematological parameters following blood loss in calves.

		BK45			
HPB	PCV (%)	RBC(x 106/μl)	HB (g/dl)	MCV(fl)	MCHC(%)
0	38.50	12.10	13.60	31.82	35.32
3	26.00	6.80	8.60	38.24	33.08
6	24.50	7.10	8.50	34.51	34.69
9	24.00	7.00	9.00	34.29	35.83
24	26.00	7.00	9.00	37.14	34.62
27	24.00	6.90	8.50	34.78	35.42
30	24.00	6.20	7.70	32.43	32.08
33	23.00	6.20	7.40	37.10	33.48
48	22.00	6.00	7.40	36.67	33.64
72	22.00	6.30	7.40	34.92	33.64
120	23.00	7.50	8.30	30.67	36.09
144	23.00	6.20	8.00	37.10	34.78
168	25.00	6.60	8.40	37.88	33.60

Key
HPB = Hour post-bleeding

Haematological parameters following blood loss in calves.

BK46

HPB	PCV	RBC	HB	MCV	MCHC
0	38.00	12.00	14.00	31.67	36.84
3	25.00	7.60	9.60	32.89	38.40
6	23.00	6.50	8.20	35.38	35.65
9	21.00	6.40	8.10	32.81	38.57
24	22.00	7.30	8.30	30.14	37.73
27	20.00	6.10	7.50	32.79	37.50
30	18.00	5.20	6.60	34.62	36.11
33	19.50	6.00	7.50	32.50	38.46
48	18.00	5.00	6.60	36.00	36.67
72	20.00	5.50	7.20	36.36	36.00
120	22.50	6.80	8.20	33.09	36.44

Haematological parameters following blood loss in calves.

BK50

HPB	PCV	RBC	HB	MCV	MCHC
0	31.50	11.20	12.00	28.12	38.10
3	24.00	6.70	8.00	35.82	33.33
6	20.00	6.60	8.00	30.30	40.00
9	20.00	5.60	7.20	35.71	36.00
24	18.00	5.20	6.60	34.62	36.67
27	17.00	5.40	6.60	31.48	38.82
30	16.00	4.60	5.80	34.78	36.25
33	17.00	4.80	5.80	35.42	34.12
48	16.00	4.80	5.90	33.33	36.88
72	18.00	5.60	6.30	32.14	35.00
120	18.00	5.40	6.60	33.33	36.67
144	16.50	4.90	6.20	33.67	37.58
168	20.00	5.50	7.20	36.36	36.00

Haematological parameters following blood loss in calves.

BH259

HPB	PCV	RBC	HB	MCV	MCHC
0	35.00	10.80	11.90	32.41	34.00
3	24.00	7.60	8.10	31.58	33.75
6	21.00	7.30	7.40	28.77	35.24
9	22.00	6.45	6.90	34.11	31.36
24	22.00	6.45	6.90	30.56	33.18
27	20.00	7.60	7.10	26.32	35.50
30	20.00	7.50	6.90	26.67	34.50
48	-	-	-	-	-
72	19.00	6.20	6.90	27.14	35.79
120	22.00	6.30	7.70	30.67	36.32
144	23.00	6.50	8.20	34.92	35.00
168	27.00	6.99	8.80	38.63	32.59
336	33.00	8.00	10.80	41.25	32.73

Key
HPB = Hour post-bleeding

Appendix 10

Erythropoietin levels in *T. congolense* - infected calves.

DPI	Erythropoietin (mU/ml)
BJ62	
57	2350
62	2300
67	1225
BJ63	
7	700
15	945
22	925
53	965
57	730
69	710
85	1100
BJ64	
25	350
43	700
46	765
55	1040
57	1315
59	730
62	710
64	250
67	250
74	400
76	660
106	750
BJ65	
29	765
32	300
34	690
43	600
92	715
99	675

Key
DPI = Day post-infection

Appendix 11

Absolute reticulocyte counts in *T. congolense*- infected calves.

DPI	Reticulocyte counts/μl
BJ62	
55	11600 (0.4%)
57	11200 (0.4%)
64	12000 (0.4%)
67	8600 (0.2%)
BJ63	
67	5800 (0.2%)
69	5800 (0.2%)
76	5400 (0.2%)
78	6200 (0.2%)
BJ64	
53	6200 (0.2%)
57	6600 (0.2%)

Appendix 12

Stimulation of 125I-deoxyuridine into spleen cells by plasma from *T. congolense* infected cattle.

		BJ62	
DPI	CPM 1	CPM 2	CPM 3
-7	3270	2800	2513
-3	3737	4370	3618
0	807	887	
4	2348	2840	2151
7	832	1127	3372
11	2489	1152	1673
13	4447	1604	1572
15	1311	1113	1428
18	386	302	490
20	326	261	287
22	315	518	606
25	591	408	426
27	349	386	766
29	302	314	276
32	306	519	787
34	416	382	376
36	350	321	466
39	208	305	256
43	309	379	642
46	417	352	488
48	273	421	495
53	414	300	505
57	366	670	397
62	380	337	647
64	252	225	238
67	273	361	528
69	853	1010	846
74	449	556	493
76	755	633	1161
85	541	387	380
92	387	472	497
99	1100	911	268
106	316	1392	1128
116	426	352	416

Stimulation of 125I-deoxyuridine into spleen cells by plasma from *T. congolense* infected cattle.

		BJ63	
DPI	CPM 1	CPM 2	CPM 3
-7	1476	1660	1188
-3	2503	3673	2789
0	750	745	767
4	3401	2669	2665
7	5543	5927	5294
11	1901	1725	1784
13	932	1128	884
15	758	811	1000
18	281	317	273
20	295	469	515
22	416	331	510
25	342	388	446
27	268	318	654
29	1784	1530	1638
32	905	398	407
34	352	409	304
36	264	309	308
39	312	354	419
43	771	445	308
46	421	360	389
48	240	270	265
53	327	1012	371
57	430	439	595
62	316	855	1250
64	426	1321	112
67	3313	605	423
69	432	555	424
74	494	338	391
76	347	358	383
85	558	1314	687
92	1403	445	851
99	597	674	728
106	300	480	439
116	718	341	533

Appendix 13

Stimulation of 125I-deoxyuridine into spleen cells by serum from *T. congolense* infected cattle.

DPI	CPM 1	BJ64 CPM 2	CPM 3
-7	9822	8267	7853
-3	15764	16019	15953
0	8896	11050	10244
4	8780	9428	7116
7	19026	21356	20709
11	4384	4646	5633
13	6762	6635	7627
15	9050	9005	10816
18	12785	13393	13601
20	300	298	690
22	3042	2596	2791
25	9536	6905	7589
27	4478	4120	4601
29	2211	2545	2226
32	286	271	366
34	7032	9376	9325
36	697	638	1702
39	617	717	607
43	7032	9376	9325
46	1107	983	1118
48	427	694	421
53	309	630	357
57	407	489	586
62	485	488	461
64	627	708	823
67	633	722	571
69	804	808	955
74	644	676	628
76	892	923	1736
85	352	490	319
92	846	718	873
99	508	609	471
106	402	411	1312
116	512	521	439

Appendix 14

Haematology of *T. congolense* infected Friesian calves.

	PCV			
	ANIMAL NUMBER			
DPI	BJ62	BJ63	BJ64	BJ65
-45	24.00	24.00	27.00	23.00
-38	25.00	27.00	28.00	23.00
-35	27.00	26.00	30.00	24.00
-17	28.00	29.00	34.00	25.00
-14	28.00	30.00	33.00	26.00
-10	29.00	30.00	34.00	27.00
-7	30.00	30.00	34.00	26.00
0	30.00	31.00	34.00	27.00
4	30.00	30.00	33.00	29.00
7	30.00	30.00	34.00	27.00
11	30.00	30.00	33.00	27.00
13	30.00	30.00	23.00	28.00
15	27.00	30.00	30.00	25.00
18	25.00	25.00	27.00	23.00
20	22.00	22.00	27.00	23.00
22	20.00	23.00	25.00	22.00
25	20.00	19.00	21.00	20.00
27	20.00	20.00	21.00	22.00
29	20.00	19.00	18.00	19.00
32	18.00	20.00	20.00	20.00
34	17.00	18.00	20.00	19.00
36	17.00	18.00	18.00	19.00
39	16.00	17.00	18.00	18.00
43	16.00	17.00	18.00	17.00
46	16.00	16.00	19.00	17.00
48	17.00	17.00	18.00	18.00
50	18.00	17.00	18.00	18.00
53	15.00	16.00	18.00	16.00
55	15.00	16.00	18.00	18.00
57	15.00	16.00	18.00	17.00
60	16.00	15.00	17.00	17.00
62	18.00	16.00	17.00	17.00
64	20.00	17.00	17.00	17.00
67	21.00	17.00	17.00	17.00
69	21.00	16.00	18.00	16.00
74	25.00	15.00	18.00	17.00
76	26.00	16.00	18.00	16.00
78	27.00	16.00	18.00	16.00
85	31.00	15.00	16.00	17.00
92	29.00	19.00	15.00	17.00
99	29.00	22.00	16.00	17.00
106	30.00	26.00	17.00	17.00
116	30.00	26.00	20.00	18.00
124	--	--	25.00	20.00
144	--	--	25.00	19.00

Haematology of *T. congolense* infected Friesian calves.

		RBC		
DPI	BJ62	BJ63	BJ64	BJ65
-45	11.00	12.20	11.00	12.40
-38	8.20	7.50	8.50	7.70
-35	9.00	11.80	9.00	12.00
-17	8.00	8.10	8.30	8.30
-14	8.00	8.80	9.80	8.30
-10	8.00	8.70	10.10	8.20
-7	7.40	7.90	8.10	7.20
0	9.20	9.50	10.70	7.80
4	10.00	10.60	11.70	9.70
7	8.80	9.40	10.00	8.40
11	9.40	8.40	8.00	8.90
13	9.50	9.60	8.30	8.40
15	7.80	8.30	9.00	7.40
18	6.40	6.50	7.10	6.20
20	5.40	6.30	6.80	6.00
22	5.80	6.00	6.30	6.40
25	4.90	5.10	5.10	5.20
27	4.80	4.80	5.10	5.40
29	4.50	4.70	4.30	4.90
32	3.90	4.50	4.60	4.80
34	3.40	4.00	4.10	5.00
36	3.40	3.70	3.90	4.40
39	3.00	3.40	3.40	3.90
43	2.80	3.40	3.10	3.70
46	2.90	3.10	3.30	3.80
48	3.10	3.20	3.10	3.90
50	3.30	3.10	3.40	3.90
53	2.70	2.70	3.10	3.50
55	2.90	3.10	3.30	3.70
57	2.80	2.60	3.30	3.40
60	2.90	2.70	3.20	3.30
62	3.32	2.70	3.20	3.30
64	3.60	3.00	3.50	3.50
67	4.30	2.90	3.50	3.40
69	4.20	2.90	3.60	3.40
74	5.40	2.70	3.60	3.40
76	5.50	2.70	--	3.50
78	5.60	3.10	4.20	3.60
85	6.80	3.10	3.50	3.70
92	6.50	3.70	3.50	3.90
99	6.60	4.40	3.40	3.80
106	7.30	5.70	3.60	4.00
116	7.40	6.00	4.60	4.00
124	--	--	5.80	4.70
144	--	--	6.10	4.70
164	--	--	--	7.50

Haematology of *T. congolense* infected Friesian calves

HB

DPI	BJ62	BJ63	BJ64	BJ65
-45	9.20	9.10	9.60	9.10
-38	10.00	9.90	10.60	8.90
-35	10.60	10.30	11.00	9.30
-17	9.90	9.50	11.20	9.10
-14	9.50	10.70	11.00	9.40
-10	10.20	10.40	11.80	9.50
-7	10.90	11.80	12.60	10.20
0	10.60	11.20	12.00	9.80
4	10.00	10.60	10.60	9.70
7	10.30	10.60	10.60	9.20
11	10.40	10.70	11.20	9.40
13	10.30	10.50	10.70	9.70
15	9.60	10.50	10.40	8.70
18	8.70	8.40	9.10	7.60
20	7.20	7.90	8.90	7.80
22	6.70	7.50	7.90	7.40
25	6.30	6.10	6.40	6.50
27	6.30	6.00	6.50	6.80
29	6.30	6.10	5.50	6.30
32	5.70	6.00	6.20	6.20
34	5.00	5.40	5.60	6.60
36	4.90	5.50	5.50	6.00
39	4.70	5.40	5.30	5.90
43	4.70	5.30	5.20	5.20
46	4.80	4.80	5.50	5.30
48	4.70	4.90	5.00	5.50
50	5.50	4.70	5.40	5.40
53	4.40	4.40	5.20	5.10
55	4.50	5.0	5.10	5.20
57	4.20	4.10	5.10	5.00
60	4.60	4.70	4.30	4.80
62	5.30	4.30	4.60	4.90
64	5.80	4.90	5.00	4.90
67	6.90	4.50	4.90	4.80
69	6.50	4.50	5.10	4.50
74	7.90	4.20	5.20	4.80
76	8.20	4.00	--	4.40
78	7.70	4.50	5.70	4.70
85	10.20	4.80	4.30	5.10
92	9.10	5.80	4.60	5.20
99	8.90	6.60	4.40	5.30
106	9.90	8.20	4.80	5.30
116	9.70	8.30	6.30	5.40
124	--	--	7.40	6.30
144	--	--	7.40	6.30
164	--	--	--	6.50

Haematology of *T. congolense* infected Friesian calves

MCHC

DPI	BJ62	BJ63	BJ64	BJ65
-45	38.333	39.917	35.556	39.565
-38	40.00	36.667	37.857	38.696
-35	38.545	38.868	36.667	38.750
-17	33.929	32.759	32.941	36.40
-14	33.329	35.667	33.333	36.154
-10	35.172	34.667	34.706	35.185
-7	36.333	39.333	37.059	39.231
0	35.333	36.129	35.294	36.296
4	33.333	35.333	35.455	33.448
7	34.333	35.333	35.455	33.448
11	34.667	35.667	33.939	33.571
13	34.333	35.00	32.424	34.286
15	35.556	34.333	34.667	34.800
18	34.800	33.600	33.704	33.043
20	32.427	35.909	32.963	33.913
22	33.500	32.609	31.600	33.636
25	33.500	32.105	30.476	32.500
27	31.500	30.000	30.952	30.909
29	31.500	32.105	30.556	33.158
32	31.667	30.000	31.000	31.000
34	29.412	30.000	28.000	34.734
36	28.824	30.556	28.947	31.579
39	29.375	31.765	29.444	32.778
43	29.375	30.588	28.889	30.588
46	30.000	30.000	28.947	31.176
48	27.647	28.824	27.778	30.556
50	30.556	27.647	30.000	30.000
53	29.333	27.500	28.889	31.875
55	30.000	31.250	28.333	28.889
57	28.000	25.625	28.333	29.412
60	28.750	31.330	25.294	28.235
62	29.444	26.875	27.059	28.824
64	29.000	28.824	29.412	28.824
67	32.857	26.471	28.824	28.235
69	30.952	28.125	28.333	28.125
74	31.600	28.000	28.889	28.235
76	31.538	25.000	--	27.500
78	28.519	28.125	30.000	29.375
85	32.903	32.000	26.875	30.000
92	31.379	30.526	30.667	30.558
99	30.690	30.000	27.500	32.353
106	33.000	31.923	28.235	31.176
116	32.333	31.923	31.500	30.000
124	--	--	29.600	31.500
144	--	--	32.400	31.053
164	--	--	--	30.952

Haematology of *T. congolense* infected Friesian calves

MCV

DPI	BJ62	BJ63	BJ64	BJ65
-45	21.818	19.672	24.545	18.548
-38	30.488	36.000	32.941	26.870
-35	30.556	22.458	33.333	18.750
-17	35.000	35.802	40.719	30.120
-14	35.000	34.091	33.673	31.325
-10	36.250	34.483	33.663	32.927
-7	40.541	37.975	41.975	36.111
0	32.609	32.632	31.667	34.615
4	30.000	28.302	28.205	29.897
7	34.091	31.915	34.000	32.143
11	31.915	35.714	41.250	31.461
13	31.579	31.250	39.759	33.333
15	34.615	36.146	33.333	33.784
18	39.063	38.462	38.028	37.097
20	40.741	34.921	39.706	38.333
22	34.483	38.333	39.683	34.375
25	40.816	37.255	41.176	38.462
27	41.667	41.667	41.176	40.741
29	44.444	40.426	41.860	38.776
32	46.154	44.444	43.478	41.667
34	50.000	45.000	48.780	38.000
36	50.000	48.649	48.718	43.182
39	53.330	50.000	52.941	46.154
43	57.143	50.000	58.065	45.946
46	55.172	51.613	57.756	44.737
48	54.839	53.125	58.065	46.154
50	54.545	54.839	52.941	46.954
53	55.556	59.259	58.065	45.714
55	51.724	51.613	52.941	48.649
57	53.571	61.538	54.545	50.000
60	55.172	55.556	53.124	51.515
62	52.217	56.259	53.125	51.515
64	55.556	56.667	48.571	48.571
67	48.837	58.621	48.571	50.000
69	50.000	55.172	50.000	47.059
74	46.296	55.556	50.000	48.571
76	47.273	59.259	--	45.714
78	48.214	51.613	45.238	44.444
85	45.588	48.387	45.714	45.946
92	44.615	51.351	42.857	43.590
99	43.939	50.000	47.059	44.737
106	41.096	45.614	47.222	42.500
116	40.541	43.333	43.478	45.000
124	--	--	43.103	42.553
144	--	--	40.984	42.553

Haematology of *T. congolense* infected Friesian calves

TOTAL LEUCOCYTE COUNT (WBC)

DPI	BJ62	BJ63	BJ64	BJ65
-14	9.000	11.800	10.300	7.900
-10	10.600	10.500	8.500	7.500
-7	14.900	9.700	11.600	8.700
-3	10.100	10.400	8.880	9.20
0	6.000	9.400	9.500	7.900
4	12.300	10.700	10.600	7.500
7	12.500	12.800	10.100	8.100
11	8.900	8.800	8.100	6.900
13	6.500	7.100	6.700	6.00
15	7.000	6.700	5.300	5.800
18	7.000	7.000	5.400	4.600
22	6.800	7.300	3.800	5.900
25	9.800	8.500	5.000	5.900
27	6.300	6.000	6.500	6.800
29	8.400	7.900	5.200	7.200
32	8.200	7.600	5.000	6.300
34	7.400	6.000	4.100	6.900
36	9.000	8.800	5.600	6.300
39	7.500	5.800	4.500	6.200
41	7.400	5.400	4.400	6.900
43	8.800	6.400	5.000	5.800
46	8.200	5.900	4.500	6.100
48	8.500	5.500	5.500	7.200
50	9.700	5.000	4.700	6.600
53	9.000	5.100	4.700	7.700
55	7.900	6.000	6.200	7.100
60	10.900	--	5.300	5.500
64	5.800	4.900	5.000	4.900
67	11.700	6.000	6.600	6.300
69	9.700	6.500	6.700	5.500
74	12.600	4.600	5.900	5.500
76	12.200	5.200	--	5.700
78	11.500	6.500	6.600	6.300
85	18.600	10.600	5.900	9.300
92	8.900	8.000	9.800	7.800
99	9.300	7.900	6.500	6.700
116	11.900	11.800	11.300	6.700

Haematology of *T. congolense* infected Friesian calves

ABSOLUTE EOSINOPHIL COUNTS

DPI	BJ62	BJ63	BJ64	BJ65
-14	0.000	0.000	0.309	0.158
-10	0.000	0.105	0.425	0.000
-7	0.000	0.485	0.348	0.087
-3	0.000	0.104	0.000	0.000
0	0.000	0.470	0.000	0.079
4	0.000	0.321	0.212	0.000
7	0.000	0.256	0.303	0.324
11	0.000	0.264	0.081	0.138
13	0.000	0.142	0.067	0.000
15	0.000	0.000	0.159	0.058
18	0.014	0.070	0.054	0.000
22	0.000	0.146	0.114	0.059
25	0.196	0.340	0.100	0.000
27	0.315	0.060	0.000	0.408
29	0.000	0.000	0.000	0.288
32	0.082	0.076	0.000	0.000
34	0.148	0.180	0.164	0.069
36	0.080	0.000	0.056	0.441
39	0.000	0.058	0.090	0.000
41	0.000	0.000	0.090	0.069
43	0.000	0.064	0.100	0.464
46	0.000	0.236	0.135	0.061
48	0.000	0.110	0.110	0.288
50	0.000	0.150	0.000	0.264
53	0.090	0.204	0.000	0.077
55	0.079	0.180	0.124	0.000
60	0.000	0.000	0.000	0.000
64	0.000	0.049	0.000	0.000
67	0.351	0.060	0.066	0.189
69	0.000	0.000	0.268	0.061
74	0.252	0.046	0.177	0.055
76	0.244	0.052	0.000	0.342
78	0.575	0.065	0.132	0.189
85	0.558	0.318	0.295	0.186
92	0.267	0.400	0.294	0.234
99	0.093	0.237	0.130	0.134
116	0.238	0.590	0.339	0.402

Haematology of *T. congolense* infected Friesian calves

ABSOLUTE MONOCYTE COUNTS

DPI	BJ62	BJ63	BJ64	BJ65
-14	0.000	0.354	0.309	0.000
-10	0.000	0.000	0.000	0.300
-7	0.447	0.000	0.000	0.000
-3	0.606	0.520	0.089	0.522
0	0.240	0.376	0.095	0.237
4	0.246	0.535	0.742	0.450
7	0.750	1.024	0.606	0.486
11	0.712	0.264	0.405	0.414
13	0.130	0.071	0.134	0.540
15	0.000	0.402	0.106	0.406
18	0.280	0.070	0.054	0.460
22	0.272	0.146	0.114	0.295
25	0.098	0.340	0.050	0.354
27	0.315	0.062	0.000	0.408
29	0.252	0.316	0.416	0.576
32	0.164	0.152	0.100	0.315
34	0.148	0.060	0.369	0.276
36	0.810	0.440	0.280	0.063
39	0.150	0.232	0.090	0.434
41	0.148	0.270	0.132	0.552
43	0.000	0.192	0.300	0.464
46	0.492	0.413	0.000	0.427
48	0.170	0.275	0.440	0.432
50	0.194	0.250	0.224	0.594
53	0.810	0.459	0.188	1.001
55	0.553	0.240	0.434	0.710
60	1.199	0.000	0.212	0.660
64	0.348	0.539	0.350	0.392
67	0.468	0.360	0.528	0.567
69	0.388	1.040	0.402	0.915
74	1.890	0.230	0.236	0.605
76	1.952	0.260	0.000	0.855
78	1.265	0.650	0.264	0.567
85	1.302	0.742	0.236	0.279
92	0.623	0.640	0.490	0.624
99	0.558	0.316	0.065	0.603
116	0.833	0.1062	0.791	0.134

Haematology of *T. congolense* infected Friesian calves

ABSOLUTE NEUTROPHIL COUNTS

DPI	BJ62	BJ63	BJ64	BJ65
-14	2.250	3.304	4.017	2.923
-10	4.452	2.835	3.315	2.700
-7	6.258	3.686	3.108	3.404
-3	2.828	3.536	3.108	3.404
0	1.140	2.350	3.420	2.528
4	3.567	2.675	3.498	1.950
7	2.125	4.096	3.939	2.511
11	2.225	2.552	2.835	2.691
13	0.975	1.917	2.144	1.800
15	2.100	1.742	1.272	1.914
18	0.840	0.910	0.756	0.690
22	0.544	0.365	0.304	1.062
25	1.274	1.530	0.600	1.062
27	1.134	0.540	0.975	1.088
29	0.840	0.632	0.676	1.584
32	0.574	0.836	0.600	1.071
34	1.036	0.840	0.615	0.897
36	0.990	1.232	0.840	0.756
39	1.200	0.696	0.585	0.992
41	0.666	0.810	0.704	1.104
43	0.000	0.000	0.000	0.000
46	0.902	0.708	0.540	0.915
48	1.530	0.770	0.660	1.080
50	1.164	0.700	1.008	0.396
53	1.350	0.612	0.752	1.694
55	1.264	0.900	1.054	1.775
60	3.597	0.000	0.848	1.045
64	1.276	1.176	1.200	0.980
67	3.978	0.840	1.320	0.945
69	1.746	0.910	0.871	0.854
74	1.638	0.920	0.649	1.375
76	2.806	1.144	0.000	0.570
78	2.185	1.430	1.188	1.197
85	7.626	6.996	0.885	4.557
92	2.403	2.640	1.470	1.638
99	3.999	3.239	0.975	0.737
116	2.499	4.602	1.808	0.737

Haematology of *T. congolense* infected Friesian calves

ABSOLUTE LYMPHOCYTE COUNTS

DPI	BJ62	BJ63	BJ64	BJ65
-14	6.030	7.906	5.665	4.819
-10	6.148	7.560	5.270	4.500
-7	8.195	5.529	6.844	4.176
-3	6.262	6.240	5.683	5.244
0	4.620	6.204	5.985	5.056
4	4.487	6.955	6.148	4.950
7	9.500	7.424	5.252	4.779
11	5.963	5.720	4.860	3.657
13	5.395	4.970	4.288	3.660
15	4.900	4.556	3.763	3.422
18	5.740	5.950	4.536	3.450
22	5.984	6.643	3.268	4.484
25	8.134	6.290	4.250	4.484
27	4.851	5.400	5.460	5.236
29	7.308	6.952	4.108	4.752
32	7.380	6.536	4.300	4.914
34	6.068	4.920	2.952	5.658
36	7.020	7.128	4.424	5.040
39	6.150	4.814	3.735	4.774
41	6.216	4.320	3.520	5.175
43	0.000	0.000	0.000	0.000
46	6.806	4.543	3.780	4.636
48	6.800	4.345	4.290	5.400
50	8.342	3.900	4.312	5.346
53	6.750	3.825	3.760	4.928
55	6.004	4.680	4.588	4.615
60	6.104	0.000	4.240	3.795
64	4.176	3.136	3.450	3.528
67	6.903	4.740	4.686	4.599
69	7.566	4.550	5.159	4.270
74	8.820	3.404	4.838	3.465
76	7.198	3.744	0.000	3.933
78	7.475	4.355	5.016	4.347
85	9.114	2.544	4.484	4.278
92	5.607	4.320	7.448	5.304
99	4.650	4.108	5.330	5.226
116	8.211	5.428	8.249	5.427