SERUM TUMOUR NECROSIS FACTOR IN CHILDREN SUFFERING FROM *PLASMODIUM FALCIPARUM* INFECTION

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

Dedicated to my husband Nyakundi and all my children.

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LIST OF ABBREVIATIONS

TNFα - Tumour necrosis factor alpha.

LPS - Lipopolysaccharides.

hrTNF - Human recombinant tumour necrosis factor.

BCG - Bacillus calmette-Guerin.

TNS - Tumour necrosis serum.

HRP Horseradish peroxidase.

FBS - Foetal bovine serum.

PBS - Phosphate buffered saline.

BSA - Bovine serum albumin.

IL-3 - Interleukin 3.

rIL-3 - Recombinant Interleukin-3.

GM-csF - Granulocyte macrophage-colony stimulating factor.

OPD - O-phenylenediamine.

Hbss - Hanks balanced salt solution.

EDTA - Ethylenediaminetetraacetic acid

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ABSTRACT

The involvement of tumour necrosis factor (TNF) in human malaria was investigated in children infected with *Plasmodium falciparum* in Kilifi and in uninfected children in the same community. For these investigations, a total of 71 children aged between 4 months and 6.8 years with an average age of 2.9 years were enrolled. Fourteen of these children were uninfected, twenty three had non-severe malaria and thirty four had severe malaria.

TNF levels were determined using both a cytotoxicity assay and an immunoassay (ELISA). Glucose levels were determined by Glucose oxidase method. Parasitaemia was determined from thin blood films stained by Giemsa stain. Formol-ether concentration method for stool was used for the examination of helminth ova and protozoan cysts.

High TNF levels were seen more frequently in severe malaria patients than in either non severe malaria or uninfected children ($p<0.001$ and $p<0.00001$ respectively). TNF levels increased directly with increasing *Plasmodium falciparum* parasite densities ($r=0.54$, $p=0.002$). However TNF levels were equally elevated in children with cerebral malaria and those with other signs of severe malaria. Children with hyperparasitaemia, anaemia and age <3 years had elevated TNF levels on univariate analysis. There was no correlation between the TNF levels and blood glucose of the 34 children suffering from severe *Plasmodium falciparum* infection. In 3 patients suffering from cerebral malaria TNF levels were reduced to normal after 3 days of treatment.

In this study high TNF levels were associated with several manifestations of severe malaria, and the data also indicated that moderate TNF response to *Plasmodium falciparum* infection is not necessarily deleterious to the host.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW.

1.1 GENERAL INTRODUCTION

Malaria is one of the diseases that cause high morbidity and mortality in tropical Africa and other continents. The life cycle of human malaria involves the *Anopheles* mosquito. When a female *Anopheles* mosquito bites it injects sporozoites into the blood stream. The sporozoites develop in the liver cells and undergo schizogony (exo-erythrocytic schizogony) to release merozoites. The merozoites enter the red blood cells where they undergo erythrocytic schizogony. This schizogony releases merozoites which can again enter the red blood cells and repeat the cycle or develop into female and male gametocytes. When a mosquito takes a blood meal from an infected person the gametocytes grow in the stomach of the mosquito to produce gametes. Fertilization of gametes occurs and produces a zygote which develops in the stomach wall. Various stages of development lead to the production of sporozoites in the salivary glands which can again be injected into human blood stream when a mosquito is taking another blood meal. The malaria parasites responsible for human infection belong to the genus *Plasmodium* and there are four species which infect man. *Plasmodium falciparum* causes the most devastating form of human malaria and this occurs in all tropical

regions (WHO, 1989).

Fever is the most common clinical sign in all types of malaria, the length of the incubation period varies from nine to thirty days depending on the parasite species and the patient's immune status.

A typical malaria paroxysm comprises three successive stages: cold stage, hot stage and sweating stage. The cold stage starts with shivering and feeling of intense cold, pulse is rapid but weak, patients may vomit and children often have convulsive seizure. The hot stage which follows the cold stage is marked by a feeling of hotness, nausea and vomiting. In the third stage (sweating phase), the patient feels hot and sweats profusely after which there is a dramatic fall in body temperature. The patients usually fall asleep after the cycle (Bruce-Chwatt 1985). The total duration of a typical attack may be from 8 - 12 hours. This duration is related to the time of rupture of sufficient number of mature schizonts and consequent release of merozoites into the blood stream. The attack is composed of a number of paroxysms. High fever (hyperpyrexia) is a common feature of severe *falciparum* malaria, but classical tertian or subtertian periodicity is seen only if patients are untreated. Persistent spiking fever or a daily paroxysm corresponding with schizogony is more usual. Hyperpyrexia is particularly common in children.

Temperatures above 38.5°C are associated with an increased incidence of convulsions especially in children (Axton and Sieber, 1982; Patel *et* al., 1971).

Pathological effects of malaria include anaemia and the degree of the anaemia depends on the species of invading *Plasmodium*. In *P. falciparum* infections where destruction of erythrocytes may be extensive and rapid, the anaemia is pronounced and is usually greater than would be expected from the proportion of parasitized erythrocytes. Anaemia is an important and commonly life threatening complication of falciparum malaria in children. The rate of development and degree of anaemia also depend on severity and duration of parasitaemia. In some children repeated episodes of otherwise uncomplicated malaria may lead to chronic normochromic anaemia in which dyserythropoetic changes in the bone marrow are prominent (Abdalla *et al.*, 1980). In other children with normal or near normal haemoglobin levels, severe anaemia may develop rapidly in association with a severe *P.falciparum* infection (Mcgregor *et al.*, 1956). In these patients acute destruction of parasitized red cells appears to be the major mechanism.

Severe lesions on the central nervous system are also manifested in *Plasmodium falciparum* infection (Bruch-Chwatt, 1985; Phillips and Warret, 1986). The physiology of the changes observed is complex and there

is no direct relationship between the degree of parasitaemia and the occurrence of cerebral symptoms suggesting that there could be other contributing factors. The definition of cerebral malaria in children is defined as: unrousable coma in *Plasmodium falciparum* malaria in the absence of an alternative or additional cause of altered consciousness. A child with loss of consciousness after a febrile convulsion is not considered to have cerebral malaria unless coma persists for more than half an hour after the convulsion. However, in some cases level of consciousness may be depressed for several hours after a febrile convulsion. In children with cerebral malaria who are admitted to hospital the duration of febrile symptoms is usually short. In a series of 131 patients studied in Malawi the mean length of reported history was 47 hours (range 2 hours to 7 days) (Molyneux *et al.*, 1989). The earliest symptom is usually fever, which is followed by failure to eat or drink. Vomiting and cough are reported in the majority of the cases in Malawi, diarrhoea is an unusual symptom. Convulsions are common before or after the onset of coma.

Hypoglycaemia is also recognised as a complication of *P.falciparum* malaria and its treatment. Hypoglycaemia is associated with severe malaria in adults and children and carries a bad prognosis. In

Malawi 20% of the children with impaired consciousness and *falciparum* malaria were hypoglycaemic before treatment (Taylor *et al.*, 1988), While in the Gambia 32% of a group of children requiring parenteral antimalarial treatment were hypoglycaemic before treatment (White *et al.*, 1987). Pretreatment hypoglycaemia was also reported in Indonesian children (Hoffman *et al.*, 1988).

Tender enlargement of the liver and spleen is a common finding in all human malarias especially in young children and non-immune adults. Low and falling serum albumin is an important index of hepatic dysfunction. Pulmonary oedema is also a fatal manifestation of severe falciparum malaria which may develop suddenly after one or two days' treatment in adults. In parts of the world where *falciparum* malaria is endemic, most severe malaria occurs among children over age of six months, with greatest mortality in those between one and three years of age. Severe malaria is less common in older children and adults because of the acquisition of partial immunity (WHO 1990a). The clinical pathology of malaria is of great complexity, while the malaria parasites initiate the disease process the subsequent events depend on many internal and external factors, including humoral and cellular immune responses as well as the nutritional state of the host. Among the factors

leading to the lower incidence of severe malaria in repeatedly exposed individuals, immunity is considered the most important. The precise mechanisms underlying this immunity in humans are not understood but it is generally agreed that antibody plays a major role. (WHO 1990b). There is evidence that cell mediated immunity is involved against asexual blood stage parasites and this has come largely from animal models and more recently from humans (Hoffman 1992).

Cell mediated immunity to malaria may involve macrophages, the monokines that mediate endotoxicity and rective oxygen species. The parasitized red blood cell (PRBC) combines with antigen presenting cell (APC). This combination of PRBC and APC releases IL-1. IL-1 activates a subpopulation of T-lymphocytes called T-inducer cells. This leads to the activation of T-helper cells. The T-helper cells release Gamma-interferon (macrophage activating factor) and IL-2. While IL-2 activates cytotoxic T-cell, Gamma-interferon has the capacity to activate macrophages. The activated macrophages then produce a variety of factors such as TNFα and oxygen radicals. These factors are responsible for intracellular killing of parasites

There is substantial evidence for activation of the monocyte macrophage system during malaria infection. In mice blood monocytosis is common and increased numbers of macrophages can be attracted from the spleen

and liver particularly in non-lethal infections or vaccine protected mice. There is also an increase in the output of several macrophage products during malaria including plasminogen-activator and reactive oxygen intermediates
(Taverne *et al.*, 1988).

1.2 LITERATURE REVIEW

1.2.1 Discovery of TNF

Tumour necrosis factor (TNF) was discovered when it was demonstrated that sera from mice which had been either severally infected with Bacillus calmette-Guerin (BCG) or immunised with Lipopolysaccharride (LPS) from Gram negative bacteria could cause hemorrhagic necrosis of tumours when injected into tumour bearing animals (Carswell *et al*.., 1975). This hemorrhagic necrosis was characterized by bleeding vessels within the tumour. Under such conditions, the tumour blackened and eventually dried up to form a scab. This necrosis could be induced by tumour necrosis serum but not by endotoxin alone, indicating an indirect effect of endotoxin on tumour cells. It was therefore suggested that endotoxin stimulated the production of a tumour necrosis factor which caused hemorrhagic necrosis when transferred to a tumour bearing animal (Helson *et al*., 1979).

Tumour necrosis factor in endotoxin treated animals is a cytokine produced by monocytes and macrophages which have phagocytosed the bacteria product. TNF is thought to play an important role in the host response to inflammation and infection. (Beutler *et al*., 1985b; Beutler and Cerami, 1987; Nathan *et al*., 1987; Michie *et al*., 1988a).

Lymphotoxin is a cytokine related to TNF and is

primarily produced by T-lymphocytes following stimulation
by an antigen. Functionally the activities of TNF and lymphotoxin are indistinguishable and cloning of the genes for both molecules has revealed a high degree of homology. Other similarities including gene location and organization and the fact that the two molecules complete the same cell surface receptor on target cells have led to the nomenclature in which the original macrophage derived TNF is designated TNFα while lymphotoxin is referred to as TNFß (Matthew and Neale, 1987).

1.2.2 Triggers of TNFα

Although LPS was the first trigger described for TNF release, it is now realized that there are several other substances which can cause release of TNF from macrophages such as antigenic materials from murine malaria parasites (Clark *et al.*, 1981).

When mouse peritoneal macrophages were incubated with erythrocytes infected with *Plasmodium yoelii* or with *Plasmodium berghei* in the presence of polymyxin B (to exclude the effect of any contaminating endotoxin), they secreted a cytotoxic factor into the supernatant that was shown to be tumour necrosis factor (Bate *et al.*, 1980; Clark *et al.*, 1981).

More recently Taverne *et al* (1990a) showed that two soluble antigens of *Plasmodium falciparum* induced TNF

release from macrophages. These soluble antigens derived from malaria parasites acted like toxins in stimulating the production of TNF. It is therefore possible that immunization with antibodies against such antigens may diminish TNF secretion and consequently affect many of the clinical manifestations of the disease. (Bate *et al.*, 1989; Vander Meer *et al.*, 1988). It has also been indicated that human and rodent blood-stage parasites liberate heat-stable soluble antigens that induce the release of TNF by activating macrophages both *in vitro* and *in vivo* (Taverne *et al.*, 1989; Taverne *et al.*, 1990b).

Early experiments in mice suggested that TNF has cytotoxic effect on asexual blood stage malaria parasites. This was based on the striking activity of tumour necrosis serum (TNS) on the stages both *in vitro* and *in vivo*. The parasite-killing recombinant TNF does not have this cytotoxic effect *in vitro*, while its anti-parasitic activity *in vivo*, though significant, is not as strong as that of an equivalent amount of TNS. Thus it appears TNS contains another cytotoxic molecule and that TNF itself may act indirectly *in vivo* perhaps by activating an effector cell (Playfair *et al.*, 1987).

TNF has other biological activities. It enhances the expression of HLA antigens on endothelial cells and fibroblasts (Collins *et al*, 1986) as well as on T-cells

(Scheurich *et al.*, 1987), it activates some neutrophil functions (Shalaby *et al.*, 1985), it induces the release of colony stimulating factors (Munker *et al.*, 1986), it induces prostaglandin E_2 and collagenase release in fibroblasts and synovial cells (Dayer *et al.*, 1985).It has been described as a potent antiviral agent (Mestan *et al.*, 1986). TNF has also been proposed as a major mediator of cachexia and derangements observed in endotoxin-induced acute toxicity in experimental animals (Beutler and Cerami 1986) However, the *in vivo* physiologic role of TNF still remains to be well understood (Old 1987).

1.2.3 Possible roles of TNFα in host defenses:

Several studies have shown raised levels of TNFα in some parasitic infections especially in leishmaniasis.(66.6%) and in malaria (70%). It has hence been suggested that TNF plays a role in host defences (Scuderi *et al.*, 1986; Ghiara *et al.*, 1987)

Specific binding sites for TNFα have been shown on the membrane of some T cell tumour lines but not on a B cell tumour line, suggesting that only T cells among the lymphocytes might be responsive to TNF (Scheurich *et al.*, 1987). It has been reported that interaction of hurTNFα with its receptors on activated normal T lymphocytes leads to an increase in the number

of IL-2 receptors. Another possible consequence of the interaction of TNF with helper T cells could be the release of soluble factors that can affect B cell proliferation or differentiation. TNF has been shown to release *in vitro* of IFN-B2 (Van Damme *et al.*, 1987). This molecule is identical to the B cell stimulating factor-2 (Billiau 1986), a helper T cell derived lymphokine that has been shown to induce the final maturation B cell into antibody secreting cells(Hirano *et al.*, 1986). TNF has been reported to induce IL-1 synthesis and release both *in vitro* and *in vivo* (Libby *et al.*, 1986), thus the presence of circulating IL-1 after injection of hurTNFα cannot be excluded and may play an important role in mediating TNF induced immunostimulation (Ghiaria *et al.*, 1987).

Preyron *et al* (1990a) measured plasma levels during a longitudinal survey of eighty-four subjects living in an endemic area of malaria and found that mean plasma TNFα was found at it's highest level during malaria transmission peak.The levels however went down to normal during the dry season. Elevated TNFα levels were reported in African children (Grau *et al.*, 1989b) and European travellers (Kern *et al.*, 1989) with severe *Plasmodium falciparum* infections. Kwiatkowski *et al* (1990), found that fever in Gambian children suffering from uncomplicated *Plasmodium falciparum* infection, was associated with moderately elevated TNFα

levels. Clinical and *in vitro* data suggests that schizont rupture stimulates bursts of TNF production that mediates paroxysms of malaria fever and that fever could possibly be beneficial to the host (Traverne *et al.*, 1984; Playfair *et al.*, 1987). It was further shown that serum containing TNF was toxic to human malaria. When varying concentrations of this serum were added to cultures of *Plasmodium falciparum* morphological deterioration of parasite was manifested. These observations suggested that a non-antibody mediator of parasite killing could play a role in human malaria. It was also indicated that human malaria parasites *(Plasmodium falciparum)* were susceptible to killing *in vitro* by macrophage secretory products such as TNF and O2 radicals, hence these findings strongly suggested that such macrophage secretory products played an important role in the immunity to malaria. (Wozencraft *et al.*, 1984; Clark 1978).

When mice infected with *Plasmodium chabaudi adami* were given hrTNF released from intraperitoneal osmotic pumps at a rate of 6 x 10^3 u/hr, degeneration of intraerythrocytic parasites were observed. These suggested that TNF is involved in the killing of parasites inside circulating cells (Clark *et al.*, 1987b; Stevenson and Chadirlan, 1988).

When rabbit sera containing tumour necrosis factor were assayed *in vitro* for killing activity against

Plasmodium falciparum, 73% inhibition of parasite metabolism was achieved. The inhibition was determined by measurement of incorporation of radiolabelled nucleic acid precursors. Furthermore sera from Sudanese residents from malaria-endemic areas were tested for their effect on *in vitro* metabolism of parasite and were shown to cause an inhibition of 95% compared to normal serum. These observations suggested that TNF was apparently not responsible for the induction of intraerythrocytic parasites but instead was responsible in the inhibition of metabolism (Carlin *et al.*, 1985; Jensen *et al.*, 1985).

The multiplication of two strains of *Plamodium falciparum* in culture as measured by {3H} hypoxanthine incorporation was inhibited in a dose dependent manner by granule proteins secreted by purified eosinophils obtained from patients with hypereosinophilic syndrome (Walter *et al.*, 1987). Morphological examination revealed the presence of abnormal parasites inside erythrocytes indicating that they were killed *in situ* and the latter stages of the development cycle were found to be most susceptible to these toxic effects. A monoclonal antibody against eosinophil cationic protein partially blocked the inhibitory effect, suggesting that it was caused by more than one of the eosinophil granule proteins. Thus some of the anti-malaria effects of TNFα which activates eosinophils may be

mediated through the enhanced production of eosinophil secretion products (Walters *et al.*, 1987).

1.2.4 The role of TNFα in the pathology of malaria

It is now evident that the illness and pathology associated with malaria are not entirely caused directly by parasite products but that they may also be caused by normal components of the host's immune responses particularly cytokines when produced in excess. Such cytokines are released from the host's monocytes and macrophages in response to stimulation by parasite products (Clark *et al.*, 1989). It can further be deduced that most of the associated tissue injury is not entirely caused by mechanisms such as parasite competition for host's nutrients or the direct effect of toxins from the parasites but rather by proteins which the patient's cells secrete in response to the presence of the malaria parasites. TNFα and functionally related proteins such as interleukin 1 (IL-1), γ-interferon and IL-6 which are normal and essential components of the hosts immune response, have been particularly incriminated. It is believed that such proteins, if produced excessively, they may be toxic giving rise to an illness such as is associated with malaria (Clark *et al.*, 1989).

When infected mice were treated with rabbit antibodies to murine rIL-3 or Gm-csf separately there was no

protective effect and when both anti-Gm-csf and anti-rIL-3 antibodies were injected together, the occurrence of neurological damage was prevented in 90% of the cases. The rise in serum TNFα was prevented and macrophage accumulation in the spleen was significantly reduced. Hence the murine cerebral malaria appears to involve a cytokine cascade in which IL-3 appears and Gm-csF lead to
the accumulation of TNF-releasing macrophage *in vivo* (Grau *et al.*, 1985).

TNF and neopterin were investigated in 37 Brazilian patients with uncomplicated *Plasmodium falciparum* malaria and in a group of 15 healthy individuals. It was found that the levels of the two proteins markedly increased in the plasma of patients and remained slightly elevated after chemotherapy. The high plasma levels of TNF and neopterin indicated excessive release of these molecules by activated macrophages and other cellular immune mechanisms during the infection (Kremsher *et al.*, 1990; Peyron *et al.*, 1990b). A Similar observation was made in Solomon islands by Butcher *et al* (1990).

Kwiatkowski (1990) studying the association of TNFα and severity of malaria in Gambian children, found that TNF levels were ten times higher in fatal cerebral malaria than in uncomplicated malaria thus providing support for the hypothesis that excessive TNF

production contributed to the pathology in severe malaria. Clark *et al* (1987a) working with the mouse and *Plasmodium vinkei* showed that a single intravenous injection of recombinant tumour necrosis factor produced the essential changes seen in terminal *Plasmodium vinkei* malaria in the mouse. These changes included hypoglycaemia and an accumulation of neutrophils in pulmonary vasculature. In addition plasma lactate was increased as occurs in terminal disease. All of these changes occur in severe falciparum
malaria. It was also noted that animals with sub clinical malaria required 20-30 times less TNF than did normal mice to pfoduce the same degree of changes. These effects by TNF had long ago been noted by Sadun *et al* (965), while working with the mouse and *Plasmodium berghei*.

Studies in the systemic pathology caused by TNFα have also been done in dogs (Tracey *et al.*, 1987). These workers found that inflammatory cells were present in pulmonary vessels and that lactate levels were increased in dogs injected with recombinant TNF. Such dogs suffered from hypertension, hemorrhagic lesions, adrenal medullary necrosis and acute renal tubular necrosis, which had long been reported in human malaria.

It was further reported that a small (1.5-5.0 mg)

intravenous dose of recombinant human TNF caused abortion in infected 16 day pregnant mice that were carrying low densities of *Plasmodium vinckei*. In contrast 50 mg recombinant human TNF did not cause foetal death or abortion in uninfected 16 day pregnant mice. Endogenous TNFα, which was not detectable in plasma of low parasitaemia animals, whether pregnant or not was present (1.6+0.9 ng/ml) in samples from malaria pregnant mice on day 17 when parasitaemia was high and the first signs of impending abortions were evident. No TNFα was detectable in the plasma of uninfected mice at day 17 of pregnancy (Dalfer *et al.*, 1985; Bachwich *et al.*, 1986; Clark and Chaudhri, 1988a).

Interestingly TNF has been used in clinical trials as possible treatment for tumours. These trials produced appreciable information on the systemic toxicity of TNFα as it gave rise to conditions similar to the illness seen in human clinical malaria (Michie *et al.*, 1989b; Phillips *et al.*, 1986a; Serby *et al.*, 1987)

Miller *et al* (1989) showed that TNFα released by macrophages in response to malaria infections contributed in part to the anaemia associated with malaria possibly through its ability to inhibit erythropoiesis. Similarly TNF has been shown to cause anaemia in malaria by causing dyserythropoiesis and erythrophagocytosis. This was demonstrated by

injecting hrTNFα intravenously into mice made susceptible to this monokine by low density infection with mouse malaria *(Plasmodium vinkei)* (Phillip *et al.*, 1986b. Clark and Chaudhri, 1988b).

Organ impairment in human malaria has been shown to be correlated with the amount of circulating cytokine levels of TNFα. Thus imbalances of the cytokine network in untreated *Plasmodium falciparum* infection could serve as a marker of severity of the disease (Tracey *et al.*,1988; Kern *et al.*, 1989).

Some degree of liver injury has often been reported in animal and human malaria. The readines of TNF/cachetin to produce hypotension (Tracey *et al.*, 1986) offers credence to the suggestion that low hepatic flow could contribute.

Lesions are also produced in the lungs. This is due to accumulation of neutrophils in pulmonary venules (Clark *et al.*, 1987).

Plasmodium falciparum is known to cause the adhesion of knob-bearing infected red cells to vascular endothelium. This adherence when prevalent in small blood vessels in the brain is sufficient to cause cerebral malaria by restricting blood supply. Thrombospondin (glycoprotein) is responsible for the increase in attractiveness of the cerebral vascular endothelium to parasitized red cells. There is evidence of increased messenger ribonucleic acid for

thrombospondin and its increased production in human endothelial cells exposed to TNF. Thus if thrombospondin is indeed involved in cerebral malaria it could be enhanced by TNFα during acute infection. Clark *et al.*, 1989; Nawroth and Stern, 1986).
It has been shown that TNFα plays a central role in experimental cerebral malaria which is an acute and lethal neurological syndrome induced by *Plasmodium berghei* infection in CBA mice (Grau *et al.*, 1987; Roberts *et al.*, 1985; Rock *et al.*, 1988). Treatment with antibodies against tumour necrosis factor protected these mice from cerebral complications of *Plasmodium berghei* infection (Beutler *et al.*, 1985c).

1.3 JUSTIFICATION

Although the clinical aspects of the human disease caused by infection with *Plasmodium falciparum* are well documented, much of its pathogenesis remains obscure. The changes seen in its severe forms are complex and not directly attributable to presence of protozoa inside erythrocytes. It has therefore been proposed that soluble mediators released from mononuclear phagocytes, including TNFα could cause many of these changes. Studies therefore need to be carried out to investigate the pathophysiologic relationship between this cytokine and the various manifestations of severe malaria. The prognostic significance of high cytokine levels also need to be established. Malaria is by far the major single cause of death in hospitalized children. It has been considered a major contributor to the failure both in Kilifi district as well as in other districts in the country to reduce childhood mortality over the last 30 years (WHO 1986). Hence the results of this study would clarify the possible use of TNFα levels for prognostic purposes and use of this cytokine as immunotherapy during *Plasmodium falciparum* infections. If TNF is the cause of the pathology that is manifested in severe malaria, then immunization with antibodies against this cytokine would reduce the pathological effects.

1.3.1. AIM AND OBJECTIVES.

AIM

To determine the role of TNFα in children suffering from *Plasmodium falciparum* infection.

OBJECTIVES

1. Determine the TNFα levels in children with mild and severe *Plasmodium falciparum* infection and compare them to those observed in uninfected children in the same community.
2. To investigate the correlation between TNFα levels in *Plasmodium falciparum* malaria and:-
   (i) Parasitaemia.
   (ii) Glucose levels.
   (iii) Haemoglobin levels.
   (iv) Age.
3. To relate TNFα levels to the rate of recovery of the patient.

CHAPTER TWO

MATERIALS AND METHODS

The blood samples used in this work were from malaria patients from Kilifi district in Coast province of Kenya. The Coastal province was chosen on the basis of malaria endemicity and the fact that *Plasmodium falciparum* is the most common species. In this study area, malaria was known to be the commonest cause for new attendance of children at the health facilities in Kilifi district. Furthermore surveillance of all paediatric admissions to Kilifi District hospital had been maintained since 1989. Between November 1990 and October 1991 there were 1591 admissions from the study area. Of these, 1482 were non neonatal admissions of which 620 (42%) were malaria.

Children between age four months to seven years were studied and three different groups were defined in the study.

These were:

1. Severe malaria children.

These were children with *Plasmodium falciparum* parasites on blood film, with cerebral malaria, severe anaemia (HB<5g/100ml) or other major complication of malaria. Severe cases were divided into cerebral malaria and non-cerebral malaria.

2. Mild malaria children.

Children with *Plasmodium falciparum* parasites on

blood film without severe malaria, and no evidence of another cause of fever.

3. Control group.

These were Uninfected children in the same community.

In choosing severe malaria cases the WHO guidelines on severe and complicated malaria were observed. These definitions for severe malaria have been revised by the KEMRI/Oxford University Research program currently working on the epidemiological studies in Kilifi. A child was considered to have severe malaria if he/she:-

1. Had *Plasmodium falciparum* infection and was admitted to Kilifi hospital paediatric ward.

2. Had one or more of the following:-

(a) Cerebral malaria coma.

(b) Prostrated (unable to sit/stand unaided or unable to drink.)

(c) Two or more generalized convulsions in the past 24 hrs.

(d) Anaemia (Hb<5g/100ml).

(e) Hyperparasitaemia {>20%}.

(f) Renal failure.

(g) Pulmonary oedema.

(h) Circulatory collapse.

(i) Spontaneous systemic bleeding.

Severe cases were then matched with mild malaria cases and community control cases. The mild malaria

cases were picked from the out patients department of Kilifi General hospital. The uninfected children were recruited from the community. In both groups age and sex were matched and recruitment was done at the same time.

For severe cases in the ward blood samples (1ml) were obtained at various times (i.e. prior to treatment, 16hrs, 24hrs, 60hrs and 72hrs after start of treatment). For the mild and control cases blood was collected once. After centrifugation serum samples were kept in sterile
tubes at -70°C awaiting analysis.

TNFα levels were determined using two methods:

1. Immunoassay (ELISA).
2. Cytotoxicity assay.

2.1 Immunoassay (TNFα ELISA)

The BIOKINE TNF (T Cell Sciences) test kit was used for the determination of TNFα in the sera. The immunoassay was established by Yamazaki *et al* (1986) and modified by Meager *et al* (1987). Mouse antihuman TNF monoclonal antibody was first absorbed onto polystyrene microtitre wells using PBS (pH 7.2) as a coating buffer. After coating, the plates were incubated for 48 hours. 300μl of blocking buffer (BSA 1% in PBS tween 20) was added to each well and incubated for 2 hours. At the end of the incubation

period the blocking buffer was removed by inverting the plate over a sink and washed 3 times with PBS plus surfactant solution (washing buffer). Serum samples and TNF Standards were then added (Dilution of 1:2). The arrangement of plates was as shown in Table 1. The plates were then incubated for 2 hours and at the end of the incubation period a wash step as above was done. 100μl of anti-TNF antibody (HRP conjugated murine monoclonal antibody to human TNF) was added and incubated for 2 hours. After the incubation period, the plates were washed and OPD substrate solution was added and then incubated uncovered for 30 minutes at room temperature. After 30 minutes 50μl of stop solution (2N H_2SO_4) was added and absorbance read at 490nm. A standard curve was prepared from 5 TNFα standards and unknown values were determined from the standard curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Bl	3	3	11	11	19	19	27	27	35	35
B	S1	S1	4	4	12	12	20	20	28	28	36	36
C	S2	S2	5	5	13	13	21	21	29	29	37	37
D	S3	S3	6	6	14	14	22	22	30	30	38	38
E	S4	S4	7	7	15	15	23	23	31	31	39	39
F	S5	S5	8	8	16	16	24	24	32	32	40	40
G	1	1	9	9	17	17	25	25	33	33	41	41
H	2	2	10	10	18	18	26	26	34	34	42	42

Table 1. TNF ELISA microtitre plate arrangement.

Bl. - Blank well

S1-S5. - Standards (Known TNFα concentrations)

Nos. 1-42 - Serum samples (All done in duplicates)

- Two plates were set and arrangements were as shown above.

2.2 Cytotoxicity assay

In this assay the mouse L-929 cell line was used. This is an adherent cell line which when treated with inhibitors of replication such as actinomycin D, is particularly susceptible to TNFα (Matthews and Neale, 1987; Espevik and Nissen-Meyer 1986).

Reagent preparation for the cytotoxicity assay

1. Hanks balanced salt solution.

This was used to arrest cell division. About 5% less deionized distilled water than desired total volume of medium was measured. Powdered medium was then added to water with gentle stirring at room temperature. 0.35g $NaHCO_3$ per litre of medium was added and a pH of 7.2 obtained. This was then sterilized by membrane filtration.

2. Trypsin/EDTA

This was used for dislodging cells. 5mls of 0.25% Trypsin (DIFCO 1:250) solution and 0.4μl of 1% versene solution was added to 20μl of PBS without calcium or magnesium.

Growth and maintenance of L929

The murine-tumour L929 was grown and maintained in the laboratory before the assay was done.

The steps in growing and maintaining the cell line in the laboratory were as follows:-

- The cells were cultured in RPMI 1640 containing

$NaHCO_3$ (7.5%), hepes salt, penicillin/streptomycin (5000 units) and 10% FBS (Gibco laboratories, Grand Island NY).

- They were kept in culture flasks at 37°C with 5% CO_2 to grow.
- Changes of culture medium were carried out every 2-3 days.
- The growth was monitored and when fully grown they formed a monolayer at the bottom of the culture flask until eventually the bottom of the flask was covered.
- To subculture, a medium was poured off and about 10μl of Hbss added for about 3-5 minutes.
- Hbss was then poured off and 2mls of Trypsin/EDTA were added and the flask was kept at 37°C for 5 minutes.

(The trypsin dislodges the cells).

- Complete medium was then added and the cells aliquoted into different culture flasks to continue growing.

In the actually assay after trypsinization of the cells, they were washed twice and then counted. They were put in 50μl centrifuge tubes and were incubated with actinomycin D at a concentration of 2 micro grams/ml at 37°C and 5% CO_2 for 1-2 hours.
After the incubation period cells were plated at a concentration of 5 x 10^4 cells/well. 96well flat-bottomed plates were used. Arrangement was as shown in

Table 2.

- The plates were then put at 37°C and 5% CO_2 for 4 hours to allow adherence of the cells onto the bottom of the wells.

- Samples and standards were added and incubated for 18-24 hrs. Assessment of cytotoxic/cytolytic effects of TNFα were made by phase contrast microscopy and objective measurement of cytotoxity was made by spectrophotometric measurement of vital dye (crystal violet). The staining process was as follows:-

- Medium was removed with a multichannel pipette set at 200ml.

- Then the plate was washed with PBS plus Sodium azide. In the wash step 200μl of the washing media were added to each well using a multichannel pipette and then removed gently.

- About 50μl of crystal violet were added and left to stain at room temperature for 30 minutes.

- Stain was removed by inverting the plate over a sink and then the plate washed with distilled water.

- The plate was air dried for about 30 minutes and absorbance was read at 620nm with ELISA reader.

The control wells denoted by C (Table 2) were negative control wells in which cells remained untreated throughout the incubation period of the assay.

1	2	3	4	5	6	7	8	9	10	11	0
A											
B	C	S62	S500	S4000	1	4	7	10	13	16	
C	C	S62	S500	S4000	1	4	7	10	13	16	
D	S15	S125	S1000	S10000	2	5	8	11	14	17	
E	S15	S125	S1000	S10000	2	5	8	11	14	17	
F	S31	S250	S2000	S20000	3	6	9	12	15	18	
G	S31	S250	S2000	S20000	3	6	9	12	15	18	
H											

Table 2: cytotoxicity assay microtitre plate arrangement.

C Control (Cells and media alone)

S standard (known TNFα concentrations).

Numbers 1-18 serum samples (All done in duplicates)

Two plates were set and the arrangement was as shown above.

The standard wells (S) contain known TNFα standards from the lowest (S15 to the highest S20,000 units/ml). Measurements for the dilution series of the standard and test samples were plotted graphically.

2.3 Glucose determination

Glucose levels were determined using glucose oxidase method. The instrument for measurement uses a polarographical oxgyen electrode that measures the rate of oxgyen consumption after the sample is added to a solution containing glucose oxidase. In this method glucose is broken down by glucose oxidase to produce hydrogen peroxide. The hydrogen peroxide is broken down by catalase present in some preparations of glucose oxidase as shown below:

$$H_2O_2 + C_2H_5OH \xrightarrow{\text{catalase}} CH_3CHO + 2H_2O$$

ethanol acetaldehyde

Glucose oxidase is put in a thin layer of resinous material and sandwiched between two membranes. When a buffered sample is introduced, glucose diffuses through the first polycarbonate membrane and reacts with the enzyme to produce H_2O_2. This diffuses through the second smaller-pore cellulose acetate membrane and is oxidized at a platinum anode. The current generated is directly proportional to the glucose concentration in diluted sample. The circuit is completed at a silver cathode, where oxgyen is reduced to water.

2.4 Parasitemia determination

Parasitemia was determined from thin blood films stained by Giemsa stain. In the preparation of thin

films a small drop of blood from a finger, toe (of children) or ear lobe was brought in contact with one end of a microscope slide and was immediately drawn out into a smooth film one-cell thick with the smooth edge of another slide. The films were allowed to dry thoroughly before staining. The thin films were then fixed in absolute methyl alcohol for two to three minutes. Then the slides were immersed for a period of ten to thirty minutes in the stain (Giemsa stain). The excess stain was then washed off and the slides dried in the air before observation under the microscope.

2.5 Stool examination

Stool examination was done for all the severe and non-severe cases. Formol-ether concentration method for stool was used for the examination of helminth ova and protozoa. Each stool sample was homogenised in a mortar using pestle with 10% formol saline. The homogenate was filtered through gauze (8 ply) into a 15ml centrifuge tube. 2mls of diethylether was added into the same tube and shaken vigorously for about 1 minute. The tubes were then centrifuged at 1500g for 5 minutes. The supernatant was decanted and a drop of iodine solution was added to the bottom of the tube before observation for ova and cysts under the microscope. Those that were positive were not included in the study.

CHAPTER THREE

RESULTS

3.1 TNFα levels by ELISA method and Cytotoxicity assay.

(a) By ELISA method.

Calibration curve for TNFα standards by ELISA method is shown in figure 1. The distribution of TNFα in the clinical groups is as shown in figure 2. Both the severe malaria patients and non-severe malaria patients had levels that were significantly higher than those of uninfected children ($P<0.05$ by ANOVA followed by multiple range test). Non-severe malaria children did not have levels that were significantly different from severe malaria children. The ELISA method gave values that numerically exceeded those of the bioassay (Table 3 and 4).

(b) By cytotoxicity assay.

The cytotoxicity assay standard curve is shown in figure 3. In the growth of L929 they formed a monolayer which eventually covered the bottom of the culture flask (Plate 1, 2 and 3). After tripnization the cells were dislodged as shown in Plate 4. TNFα in the serum samples lyses the L929 cells in the wells and the colour intensity after staining indicates the amount of lysis. High colour intensity gives a high absorbance and hence low TNF present (Plate 5). The distribution of the TNFα levels in the clinical groups

is shown in figure 4. The mean TNFα levels of the 3 different groups are shown in Table 4. Children with severe malaria had significantly higher TNFα concentrations than uninfected children and those with non-severe malaria (P=0.00001 and P=0.001 respectively).

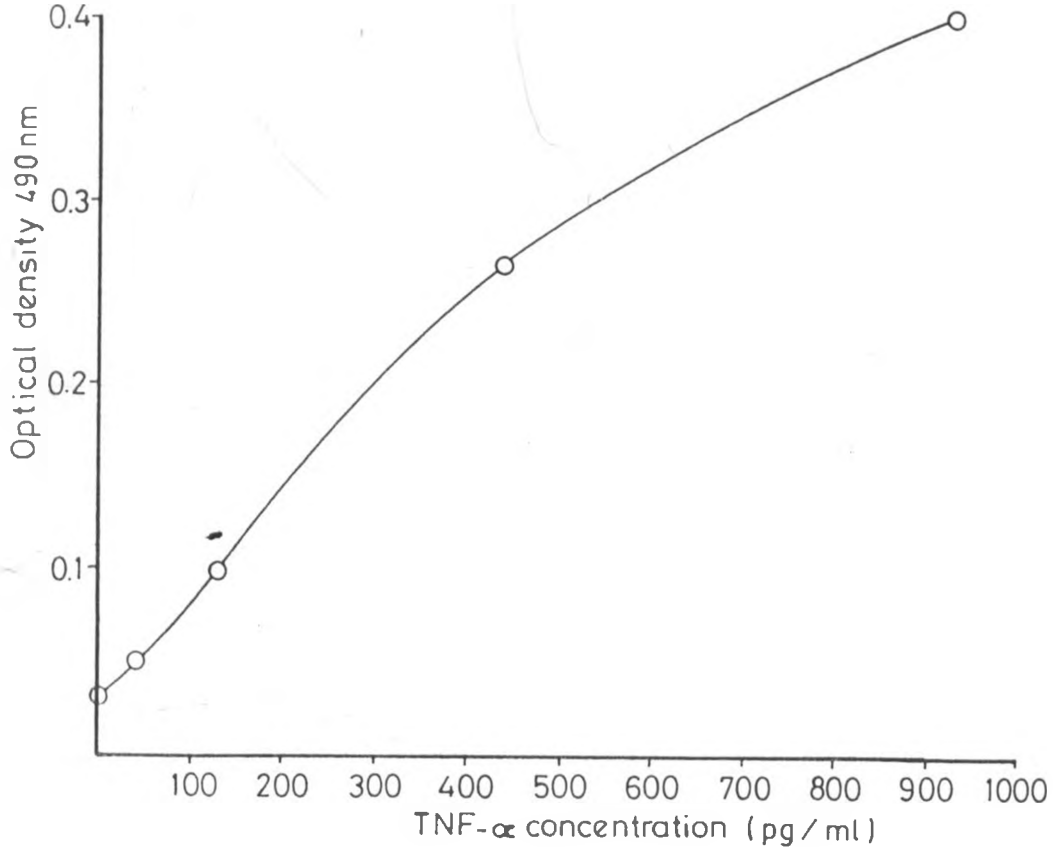

Figure 1: ELISA calibration curve for TNF standards.

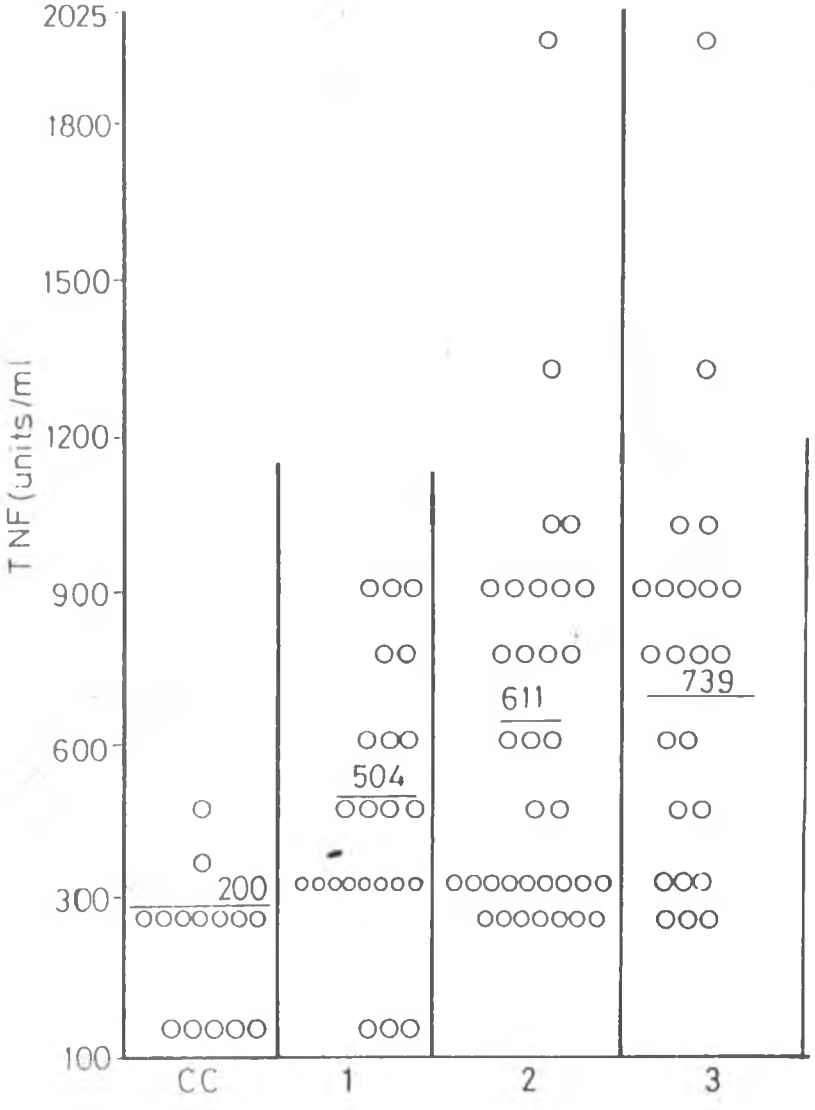

Figure: 2 Distribution of TNFα levels in the clinical groups by ELISA method, horizontal bars show mean TNFα level.

KEY

CC. Community control

1. Non severe malaria

2. All severe malaria

3. Cerebral malaria.

Each circle represents TNFα levels for one patient.

Group	no. of subjects	mean TNF level (units/ml)	Standard error(SE)	95% CI	Range min.-max.
Normal children	14	200	65	182-471	100-600
Non-severe malaria	23	504	42	476-653	400-900
Severe malaria	34	611	51	453-664	200-1300

Table 3: Mean TNF levels (Standard error 95% Confidence interval and Range) in different clinical groups (Uninfected children, non-severe malaria and severe malaria) by ELISA method.

Group	no. of subjects	mean TNF level (units/ml)	Standard error(SE)	95% CI	Range min.-max.
Normal children	14	6.2	0.9	4.1-8	5.0-15
Non-severe malaria	23	21	4.2	11.8-29	5.0-80
Severe malaria	34	178	32	113-243	50-800

Table 4: Mean TNFα levels. (Standard error 95% confidence interval and range) in different clinical groups (Uninfected children, Non-severe malaria and severe malaria) by cytotoxicity assay.

The non-severe patients did not have significantly higher levels than the uninfected children. Children with cerebral malaria did not have higher levels than with non-cerebral but severe malaria (P=0.3).

3.2 TNFα and characteristics associated with malaria

When sampled on the first day of admission, mean parasitaemia levels were lower in non severe malaria children than in severe malaria. (P=0.000007, two tailed wilcoxon rank test) (Table 5). TNFα concentrations increased directly with increasing *Plasmodium falciparum* parasite densities (r=0.54 p=0.002) (Figure 5). The mean haemoglobin levels of the severe cases were significantly lower than those of uninfected children and non-severe children (p=0.000006 by students t-test, two tailed) (Table 6). The mean hemoglobin levels of the non-severe malaria children were not significantly different from those of uninfected children (Table 6) Using univariate analysis of severe malaria by t-test on logarithmically transformed data, children with parasitaemia > 5% had significantly higher TNFα levels than those with parasitaemia < 5% (p=0.0002 Table 7). Children with haemoglobin < 5g/100ml had significantly higher TNFα levels than those with hemoglobin > 5g/100ml (p=0.0362) Table 7). Those aged < 3 yrs had higher levels of TNFα than children aged > 3 yrs (p=0.0006) Table 7).

Group	no. of subjects	mean parasitaemia level	Standard error
Uninfected children	14	0.01	0.005
Non-severe malaria	23	1.55	0.5
Severe malaria	34	8.9	1.4

Table 5: Mean parasitaemia levels and standard error.

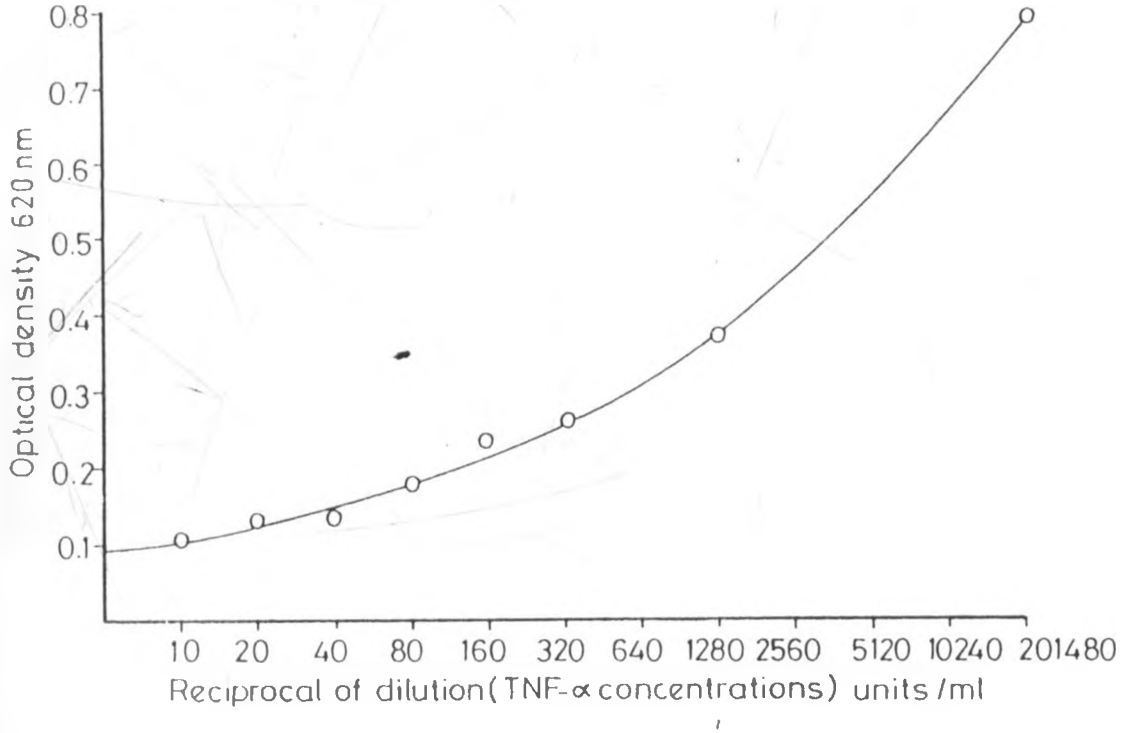

Figure 3: Bioassay standards curve for TNF standards. Each point represent the optical density at 620nm of each well of the microtitre plate.

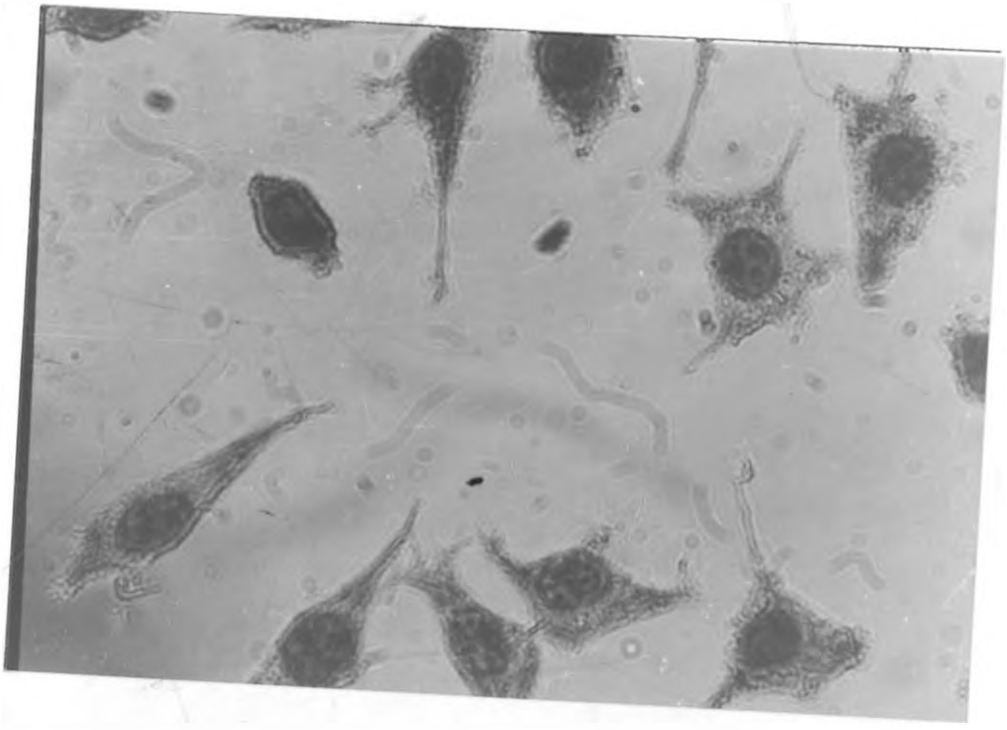

Plate 1: L929 cells starting to form a mono layer at the bottom of a culture flask.

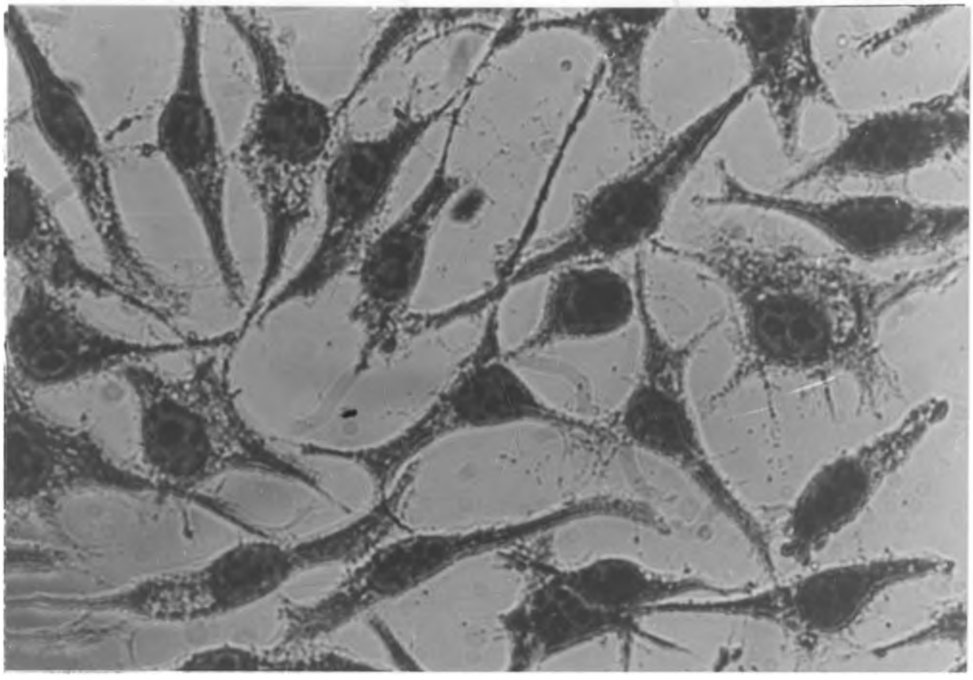

Plate 2: L929 cells monolayer formed at the bottom of the culture flask.

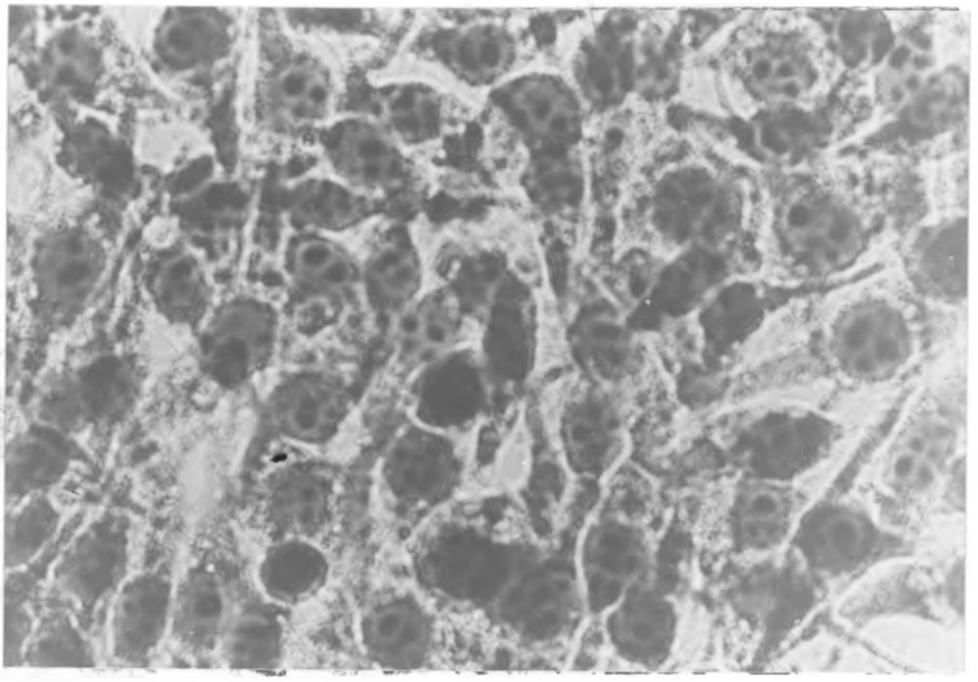

Plate 3: L929 cells monolayer at the bottom of culture flask ready for tripsinisation.

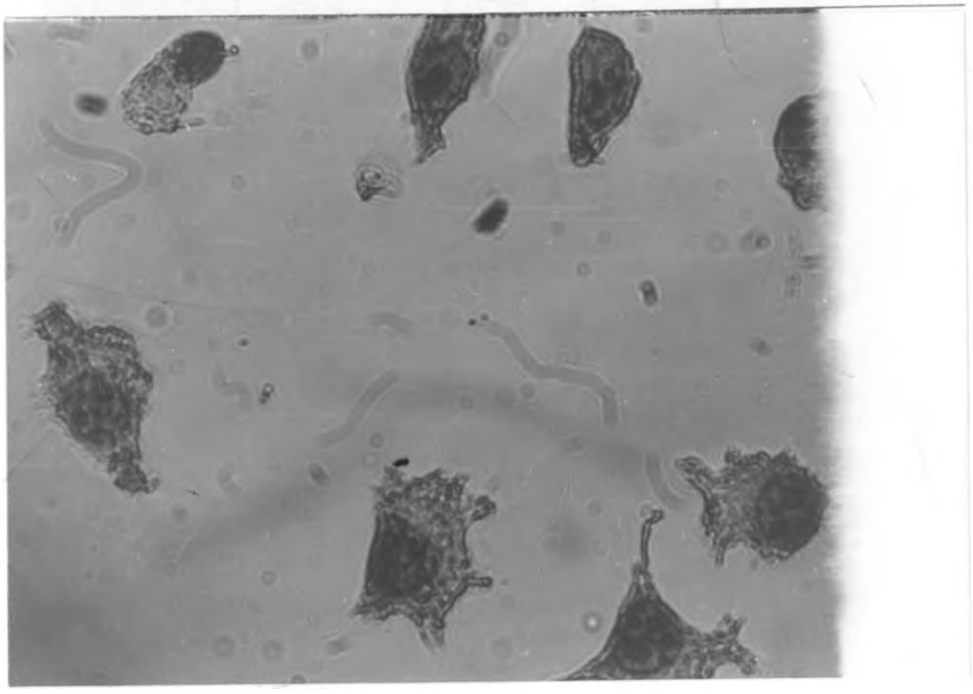

Plate 4: L929 cells immediately after tripsinisation in a culture flask.

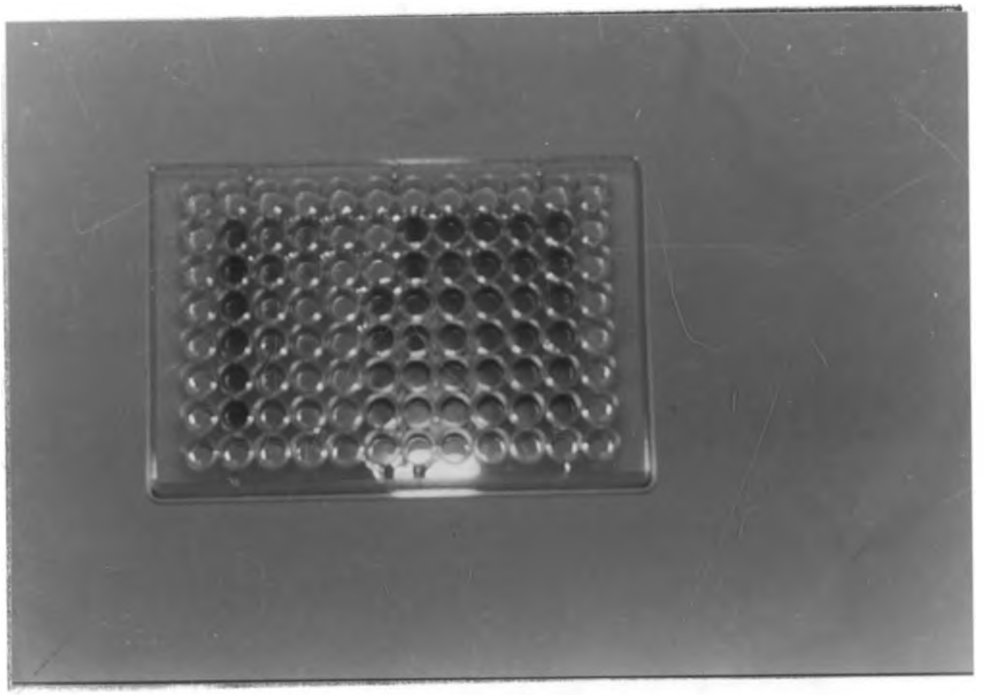

Plate 5: Crystal violet-stained microtitre plate of L929 cytotoxicity assay. All outer wells were not used. Refer table 2, for plate arrangement. Line 2 B and C -contain negative control (untreated cells). The remaining lines (line 2- line 5) represent duplicate titrations of cytotoxicity of different TNF standards. Refer table 2 Line 6- line 11 have duplicate titrations of different serum samples.

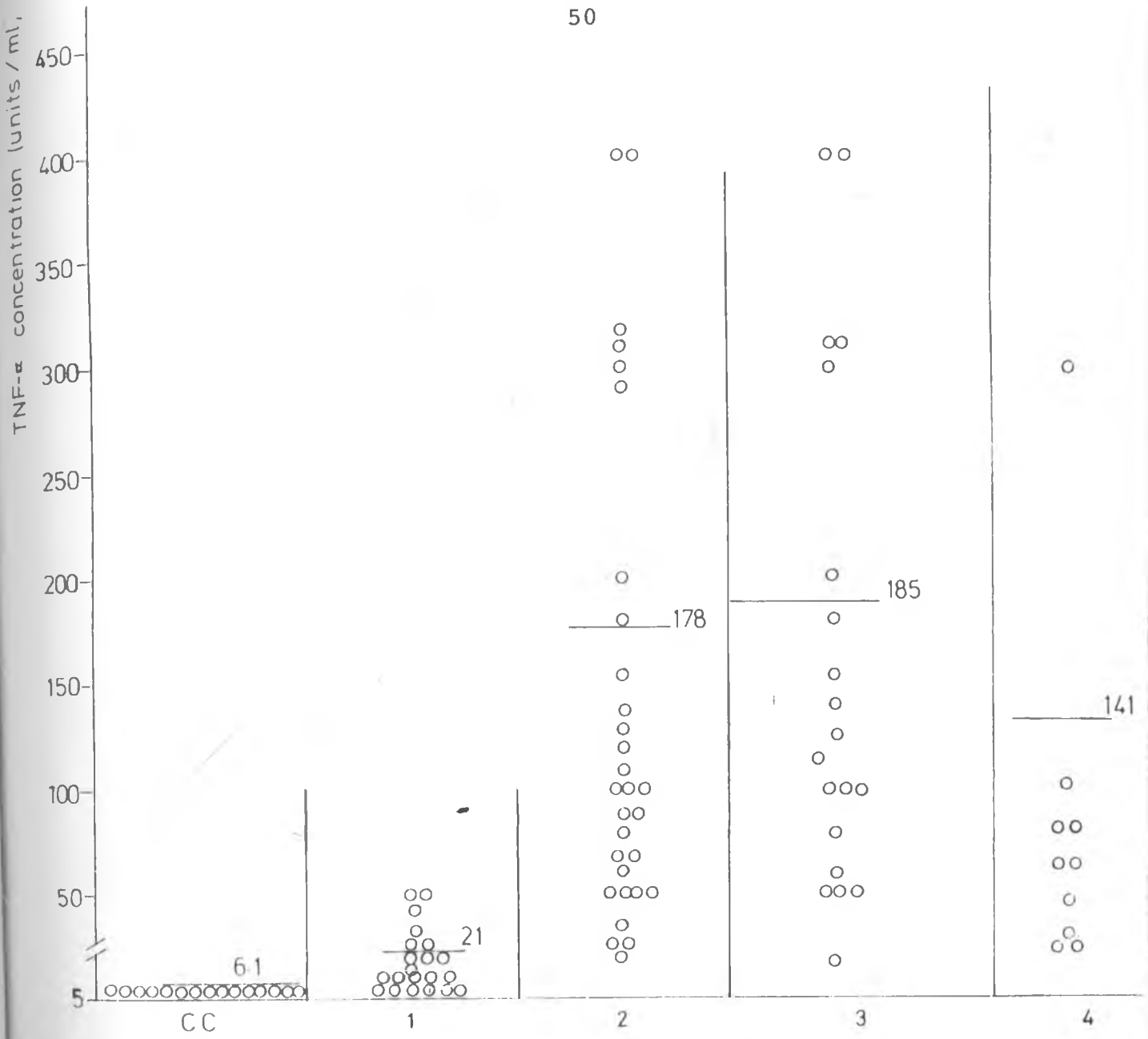

Figure 4: Distribution of TNFα levels in the clinical groups by Cytotoxicity assay. Horizontal bars show mean TNFα levels.

CC Community control.

1. Non severe malaria.
2. All severe malaria.
3. Cerebral malaria.
4. Other signs of severe malaria.

Each circle represents TNFα level for one patient.

Children with glucose levels >2.2 mmols/1 did not show any difference in TNFα levels compared with those with glucose levels <2.2 mmols/1 (p=0.662) (Table 7). In the severe malaria cases, those children with high parasitaemia, had low haemoglobin levels and high TNFα levels. The TNFα levels of three patients with severe malaria were reduced after 72 hours of treatment to similar levels as those of uninfected children in the community (Figure 6, 7, and 8).

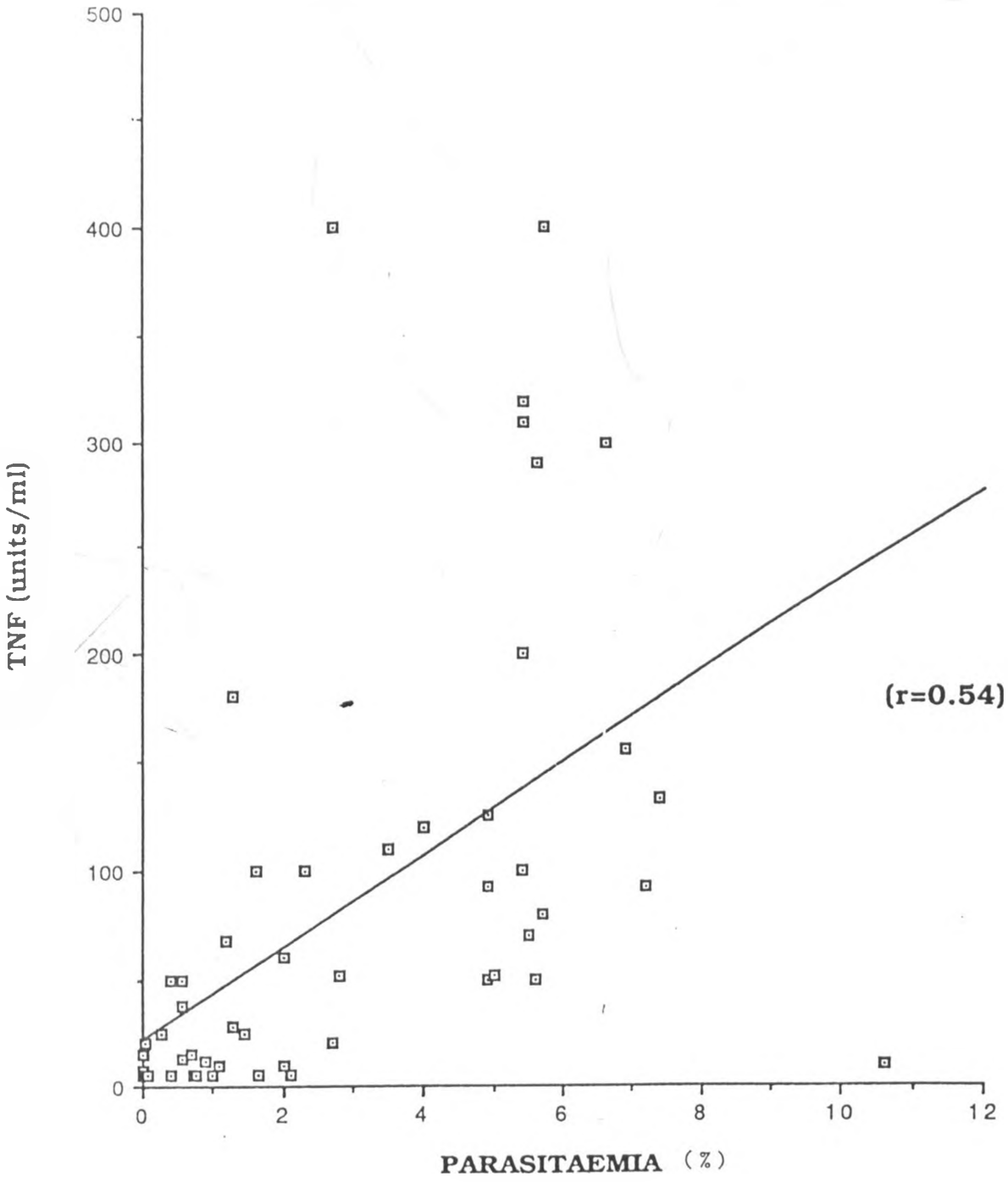

Figure 5: Relation of plasma TNFα concentration and parasitaemia (r=54, p=0.002.)

Group	no. of subjects	mean Hemoglobin level (g/100ml)	Standard error
Uninfected children	13	9.3	0.3
Non severe malaria	23	8.5	0.3
Severe malaria	34	5	0.25

Table 6: Mean haemoglobin levels and standard error. Haemoglobin for 1 patient in uninfected children was not determined.

Characteristic on admission	no. of subjects	Geometric mean TNF(units/ml)	95% CI	P value
Parasitemia				
>5%	22	199	24-1659	0.0002
<5%	12	43	6-312	
Glucose mmols/l				
>2.2	24	125	8-1893	0.662
<2.2	10	102	10-1011	
Age (yrs)				
>3	14	39	5-328	0.0006
<3	20	181	21-1537	
Hemoglobin/100ml				
>5	7	44	3-658	0.0362
<5	27	141	14-1479	

Table 7: Characteristics associated with elevated plasma TNFα in severe children.

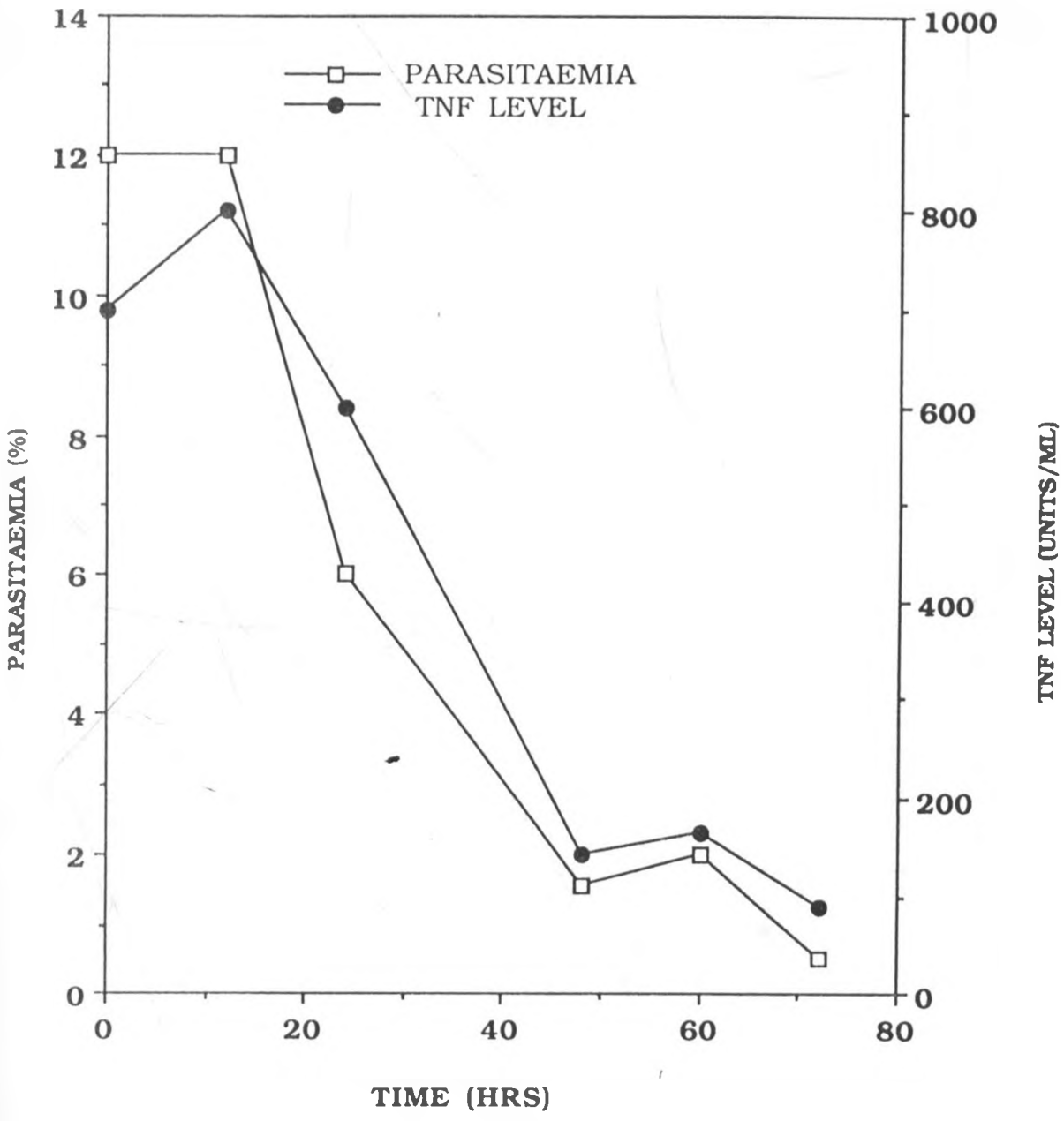

Figure 6: TNFα levels in a severe malaria patient, prior to treatment and at various times after start of treatment. (patient i)

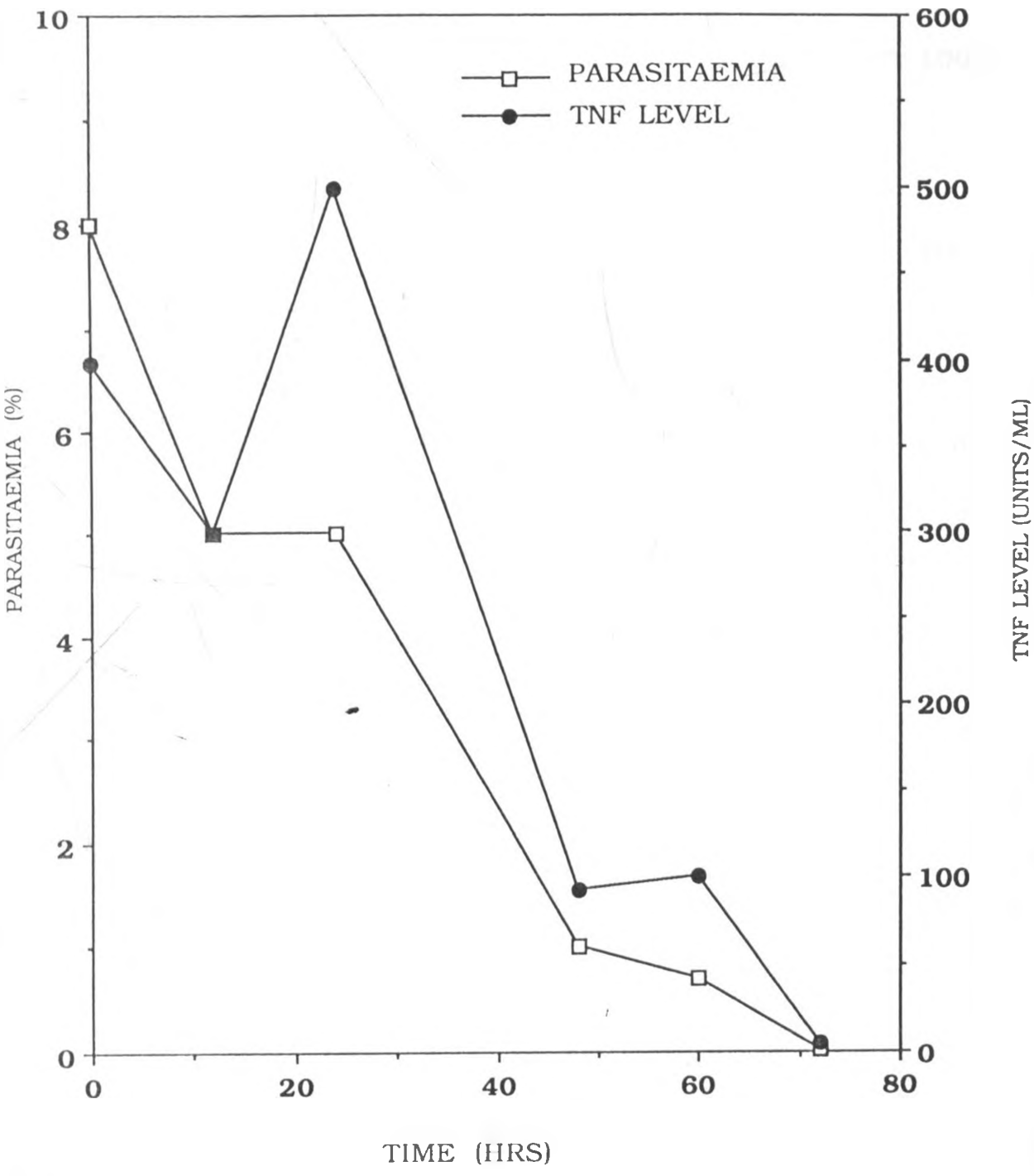

Figure 7: TNFα levels in a severe malaria patient, prior to treatment and at various times after start of treatment. (patient ii)

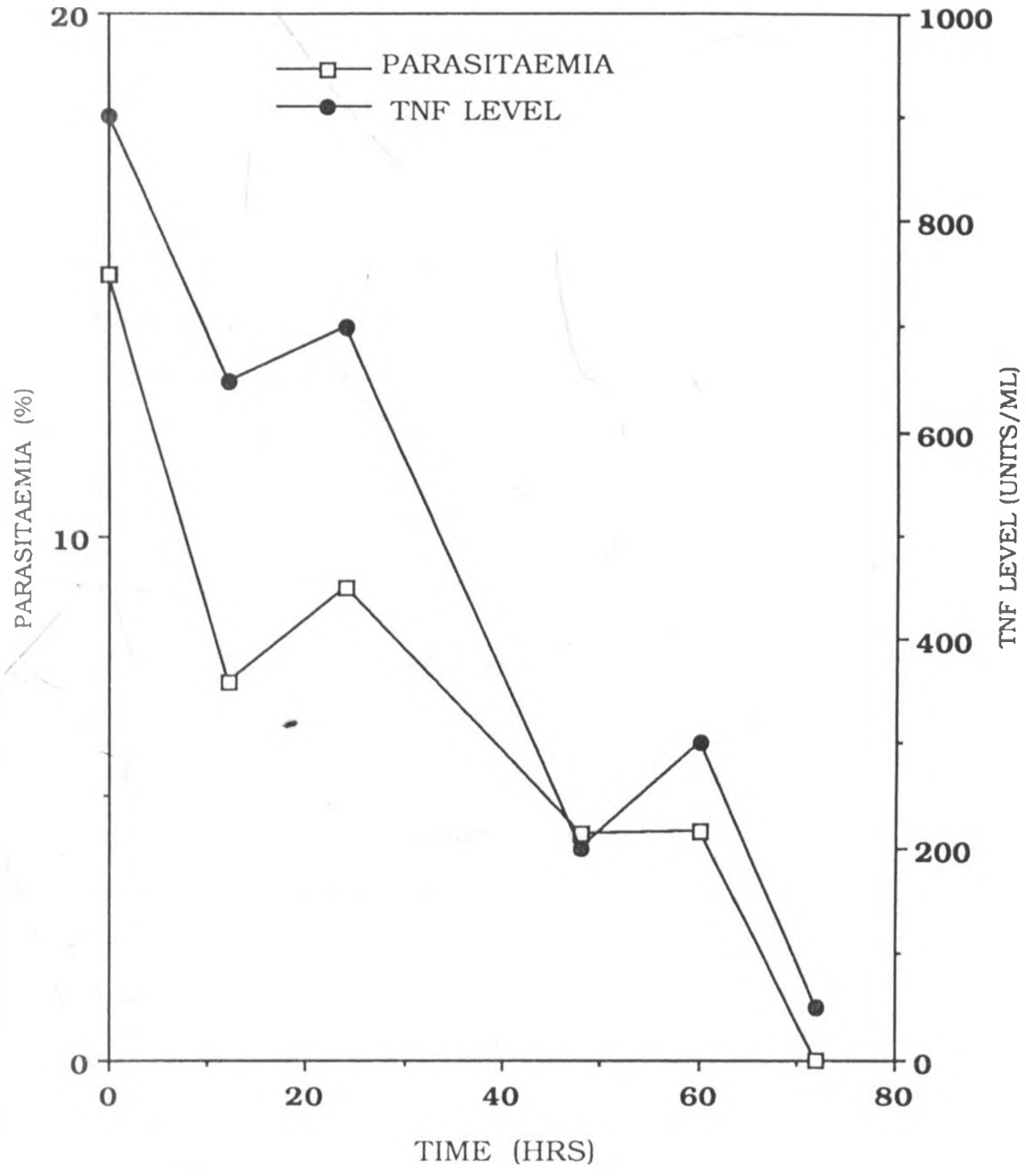

Figure 8: TNFα levels in a severe malaria patient, prior to treatment and at various times after start of treatment. (patient iii)

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

Severe malaria is a multifactorial clinical syndrome with numerous manifestations. It is not known why for instance some children progress more rapidly to cerebral malaria, why others develop severe anaemia or hyperparasitaemia. It has been postulated that host immunological responses including those mediated through the production of TNFα and other cytokines may be important determinants of disease severity (Clark *et al.*, 1989).

4.1 TNFα levels by ELISA method and cytotoxicitu assay

The ELISA and cytotoxicity assay used in the detection of TNFα levels showed slight correlation (r=0.34). A probable explanation being that immunoassays are capable of detecting both biologically active TNFα as well as inactive denatured or aggregated TNFα molecules (Peterson and Moller, 1988). Hence levels of TNFα estimated by the immunoassay tend to exceed those found in cytotoxicity assays which are only capable of detecting biologically active TNF molecules. Both methods gave results that were qualitatively the same. Since the focus of the study was on active TNFα, the cytotoxicity assay results were used for analysis of the data.

(a) By ELISA METHOD.

The ELISA method was found to be reliable as a means of detecting TNFα levels in malaria as shown by the fact that non severe malaria and severe malaria children had TNFα levels that were significantly different from those of uninfected children in the community.

Immunoassays are also advantageous in that they give a higher degree of precision, reproducibility and shorter assay time.

(b) By cytotoxicity assay.

In the presence of TNFα the susceptible cells (L929) seeded as sub-confluent monolayers, slowly deteriorated and died. For optimal performance in the assay, it has been found that certain experimental conditions are critical. Several factors including the inoculation density, the incubation temperature and the incubation period have been shown by other workers to be important (Yamazaki *et al.*, 1986; Meager *et al.*, 1989). For example highly sensitive assays were obtained if cells were taken from the period of maximum growth and seeded in 96-well microtitre trays at a relatively low density ($5x10^4$cells/well) (Flick and Gifford, 1984). Similar factors were also observed in the present study. The cytotoxicity assay was performed in a dilution medium containing the metabolic inhibitor Actinomycin D (AMD). Actinomycin D is a

transcriptase blocker and when present at a concentration of 1-2mg/ml. the sensitivity of L929 to TNFα was increased and the assay period was shortened to between 18-24 hours from 72 ours in the absence of AMD.

4.2 TNFα concentration in different clinical groups.

The present study has shown that TNFα is produced during malarial infections. This is due to the fact that non severe malaria and severe malaria children gave significantly higher levels of the cytokine than uninfected children from the same community. The results also indicate that children with severe malaria, had higher levels of TNFα than those with non severe malaria and also the uninfected group. There was an association between serum levels of TNFα on admission and the severity of the illness such that higher levels of TNFα were found on admission as compared to levels done 72 hours after start of treatment. Hence TNFα could also be playing a role in the host defence mechanism. These findings were consistent with the study done in Zaire by Shaffer *et al* (1988), who found that moderate levels of TNFα production were protective to the host, while high levels cause pathological damage. Similar observations have also been made in the mouse model where for instance malaria infected mice, even those with 5-10% parasitaemia were much more susceptible to the harmful

effects of TNFα than controls. The simplest explanation was that injected TNFα supplemented the amount produced endogenously thus attaining the level of TNFα that causes damage to the host (Clark *et al.*, 1987a).

4.3 TNFα and the characteristics associated with severe malaria.

In this study TNFα has been shown to increase with parasitaemia. Children with parasitaemia of greater than 5% had higher cytokine levels than those with parasitaemia less than 5%. *Plasmodium falciparum* antigens can cause release of TNFα from macrophages (Taverne *et al.*, 1990). Hotez *et al* (1984), found that lysed malaria parasites would induce macrophages to release a substance with cachectin like activity. Also spleen cells and serum of malarial mice have been reported to acquire, as infection progresses cytotoxicity toward TNF-sensitive (but not TNF-resistant) lines of L929 cells (Taverne *et al.*, 1986). In each case cytotoxicity was abolished by IgG directed toward recombinant mouse TNF/Cachectin. It had also been shown that a single injection of TNFα protects mice against the cerebral signs and pathology of *Plasmodium berghei* malaria (Grau *et al.*, 1987). From these findings it can be suggested that higher parasite densities stimulate more production of TNFα from

macrophages.

TNFα has also been shown in the present study to be associated with haemoglobin levels. Children with haemoglobin less than 5g/100ml had higher TNFα levels than those with haemoglobin greater than 5g/100ml. Anaemia is an inevitable consequence of severe malaria. In African children severe anaemia is a common presenting feature of malaria (WHO 1990). It has been reported that TNFα may contribute to the anaemia of malaria by inhibiting erythropoiesis and causing erythrophagocytosis (Clark and Chaudhri, 1988b).

In the present study, children with cerebral malaria had higher TNFα levels than non-severe malaria children and uninfected children (P=0.01 and P=0.0001 respectively). The TNFα levels of cerebral malaria children were not significantly different from the levels of those with other signs of severe malaria such as hypoglycaemia, hyperparasitaemia and anaemia (P=0.3). This could be due to the fact that all the cerebral malaria cases studied were non-fatal. This was not consistent with study done by Kwiatkowski *et al* (1990) in Gambian children. In their study they demonstrated that fatal cerebral malaria children had significantly higher
TNFα levels than children with other signs of severe malaria. A recent study by Udeinya *et al* (1981),

stressed the presence of numerous endothelial projections extending into the lumen of cerebral vessels of patients with acute falciparum malaria. These authors suggest that the projections might have made the vessels more vulnerable to clogging by *Plasmodium falciparum* parasitised erythrocytes which already have a tendency to adhere to endothelium. It is proposed that these projections and the associated tissue damage in malaria may be part of a broad spectrum of changes induced by TNFα .

Severe hypoglycaemia and its resultant hyperinsulinaemia has been demonstrated in human *P.falciparum* malaria, though the differences in the glucose levels among the clinical groups were not statistically significant in the present study. It has been suggested by Spooner *et al* (1979) that TNFα could be indirectly responsible for these hyperinsulinaemia through its inhibition of lipoprotein lipase. Insulin normally up-regulates this enzyme but can not do so even in pharmacologic doses when TNF is present. A compensatory hyperinsulinaemia and thus hypoglycaemia could be expected. A broad range of TNFα values was observed within the clinical groups (Figure 3 and 4). This variability may reflect differences in the timing of sample collection, host factors such as age, prior exposure, immunity and prior treatment.

Little is known of the feedback control mechanisms that

regulate levels of TNFα , the relation between circulating TNFα levels and localized tissue levels, or individual variations in sensitivity and tolerance. It has recently been suggested that TNFα can up-regulate
specific host receptors (Berendt *et al.*, 1989) and that paroxysmal release can occur with schizont rupture (Kwiatkowski *et al.*, 1989). These findings were confirmed by TNFα levels of three patients with severe malaria who had several fluctuations in their levels within three days of treatment before they returned to normal values after 72 hours of treatment (Figure 6, 7 and 8).

Field studies such as the one undertaken to investigate the relationship of TNFα and acute *Plasmodium falciparum* infection in humans may be limited to several factors. These include problems in the standardization of specimen collection, the limited number of observations that can be made of a cytokine with a short half life (Beutler *et al.*, 1985a) during the course of a rapidly evolving, life threatening illness and potential difficulties in obtaining well defined comparison groups. Furthermore studies of a single cytokine may not reflect adequately the complex *in vivo* relationship between TNFα and other soluble mediators and receptors (Grau *et al.*, 1988; Grau *et al.*, 1989a: Dinarello *et al.*, 1987: Le *et al.*, 1987).

4.4 CONCLUSIONS

1. Children suffering from severe *P.falciparum* infection had TNFα levels that were significantly higher than those with non severe malaria and uninfected children.
2. Children suffering from non severe malaria did not have significantly higher levels than the uninfected children by cytotoxicity assay.
3. Among the severe malaria children, those with cerebral malaria did not have higher TNFα levels than with non cerebral malaria. Hence high TNFα levels are not specific to cerebral malaria alone but to all severe malaria characteristics.
4. The TNFα concentration increased directly with *P. Falciparum* parasite densities.
5. The severe malaria children with low haemoglobin (<5g/100ml) had high TNFα levels.
6. The TNFα levels were significantly higher in patients under three years of age than in older children.
7. Though not statistically significant, children with severe malaria had lower glucose (<2.2 mmols/1) levels than those with non severe malaria and uninfected children.
8. By three days after start of treatment, the concentrations of TNFα in three severe malaria patients were again within the range observed in healthy children.

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