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SEVERE MALARIA-THE ROLE OF REGULATORY CYTOKINES

A Thesis presented to
The Board of Graduate Studies
University of Ghana, Legon
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Master of Philosophy(M. Phil.)
(Biochemistry)

INTEGRI PROCEDAMUS

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SEPTEMBER, 1999

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DECLARATION

I do hereby declare that except for other people's investigations cited which have been acknowledged, this exercise is a result of my own original research, and that this thesis, either in whole or in part, has not been presented elsewhere for another degree.

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DEDICATION

To the memory of the fallen heroes/heroine of MALARIA across the world, who could not get the chance to make an impact.

Ironically, man who was made in the image of God, harbors in his vital organs various forms of loathsome creatures, which riot in his fluids and consume the very substance of his tissues.

PHILIP HENRY GOSSE

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LIST OF ABBREVIATIONS

CD	Cluster of differentiation
MHC	Major Histocompatibility
IFN-γ	Interferon gamma
TNF-α	Tumour Necrosis Factor
IL	Interleukin
IFAT	Immunoflourescence Antibody Test
Ig	Immunoglobulin
PHA	Phytohaemagglutinin
ELISA	Enzyme Linked Immunosorbent Assay
PCV	Packed Cell Volume
cpm	counts per minute
BFU-E	Burst-forming units of erythroid cells
CFU-E	Colony- forming unit of erythroid cells
EPO	Erythropoietin
PBS	Phosphate buffered saline
^{3}H	Tritium
NMIMR	Noguchi Memorial Institute for Medical Research
Th	T-helper cells
LPS	Lipopolysaccharide
cAMP	Cyclic Adenosine monophosphate
CTLS	Cytolitic T- lymphocytes
PHA	Phytohaemoglobin

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ABSTRACT

An anti-inflammatory cytokine, interleukin 10 (IL-10) suppresses T-helper 1 cells (Th1) route and enhances the Th2 route in mice by boosting the antibody response. The present work studied the effect of exogenous IL-10 in the system of BALB/C mice during the development severe anaemia. BALB/C mice developed severe anaemia within 2-3 weeks after inoculation with 10^6 parasitised RBCs (pRBCs) intraperitoneally. BALB/C mice treated with recombinant IL-10 developed patent parasitaemia of 7% on day 7-post inoculation as compared to 10.5% in controls. Nevertheless, there was no significant difference between the two groups haematologically (parasitaemia $P>0.01$, haemoglobin levels $P>0.01$, reticulocytosis $P>0.01$ and in hematocrit values $P> 0.01$). There was generally a positive correlation between anaemia and the parasite count and reticulocytosis, but negative correlation with haemoglobin levels and the hematocrit levels measured. Cytokine levels *in vivo* and *in vitro* were determined using the ELISA technique. The endogenous IL-10 measured was higher in the IL-10 treated than the controls. However there was no difference statistically between the mortality of the two groups, though it appeared to be slower in the IL-10 treated BALB/C mice. Also, there was no significant difference between the TNF-α measured in both sera and the spleen cell culture supernatants of the two groups. IgG and IgM were measured by means of Immunoflourescence Antibody Test (IFAT) and using whole blood stages of the parasites as antigens. The role of immunoglobulin in erythrophagocytosis was observed because there was enhanced

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erythrophagocytosis between the 7th and 12th day, that was when IgG2a recorded the highest peak value in both test and control mice. On the other hand, IgG3 recorded low levels with a significant increase in TNF-α during the same period. The use of mitogen *in vitro* showed that activation of T cells results in the production of cytokines.

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CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Malaria continues to pose the greatest single infectious disease threat to the health of the world. The tropical areas, mostly developing countries, are the most affected. Within the range of this tropical zone, malaria prevalence varies with different natural and socio-economic conditions. Malaria is characterized by fever and it is caused by sporozoa of the genus *Plasmodium* (Marsh *et al.*, 1991). Infection is initiated by the bite of an infected female mosquito that injects sporozoites into the blood stream of the vertebrate host. Eventually, there is synchrony of schizont formation and red blood cell (RBC) rupture, and this is responsible for the regular repeated bouts of fever, which are almost diagnostic of the infection (Wakelin, 1984).

In Africa, the most common complications of severe malaria are anaemia and cerebral malaria (Marsh *et al*, 1991). Malarial anaemia remains one of the major causes of morbidity and mortality in patients with acute *Plasmodium falciparum* infection, especially in children and pregnant women residing in malaria endemic areas (Bruce-Chwatt, 1980; Weatherall, 1988; Mota *et al.,* 1998). Anaemia in *Plasmodium falciparum* malaria occurs when RBCs are destroyed more rapidly than they are replaced by the erythropoietic process (Miller *et al.*, 1989a). The complete cyclical development of malaria parasites

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can be sustained indefinitely by uninterrupted blood or sporozoite (mosquito) induced transmission in laboratory rodents. Rodent malarias are important tools continually used for all kinds of research with appreciable success. *Plasmodium berghei,* induces an anaemia in its rodent hosts just as *P. falciparum* does, which is in excess of that which can be accounted for solely by direct destruction of erythrocytes by the mature schizonts at the time of merozoite release (Roth & Herman, 1979).

In earlier studies, a number of mechanisms have been postulated as possibly contributing to such excessive anaemia in a variety of malarial infections. These include a possible concomitant infection (Mc Ghee and Loftis, 1968; Ludford *et al.*, 1969), alteration of the erythropoietin mechanisms (McGhee, 1970; Frankenburg and Greenblatt, 1977), erythrocyte membrane alterations and/or hemolysis by the action of parasite products (Cox, 1966; Fife *et al.*, 1972; Holz *et al.*, 1977; Klein *et al.*, 1977), autoimmunity (Zukermann, 1964, 1966) and reticuloendothelial hyperphagocytosis directed against host erythrocytes (Todorovic *et al.*, 1967; Sheagren *et al.*, 1970; Zukermann *et al.*, 1973). Nevertheless, recent studies have focused on ineffective erythropoiesis and immune mediated haemolysis as major underlying factor for these anaemias (Woodruff *et al.*, 1979; Clark and Chaudhri, 1988; Villeval *et al.*, 1990).

Research has so far not identified the detailed pathogenesis of severe malarial anaemia. Whilst it is possible to conduct most malaria research using human

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subjects, certain studies especially immunological interventions cannot be carried out using human subjects for ethical and other considerations. Experimentation with other alternative models of malaria involving non-human hosts has thus become essential for finding answers to the many questions in malaria research. Furthermore, correlation of housekeeping genes, similarities of biochemical (Homewood, 1978; Homewood & Neame, 1980) and genetic processes (Walliker, 1989) between rodent and human malaria parasites have justified the use of rodent parasites for malaria studies. The ease and safety of handling and manipulation of the different stages of the parasites in mice also lends itself to *in vivo* experimentation. A useful model for severe anaemia similar to that in humans is the infection of BALB/c mice with *P. berghei.*

The hemopoietic and the immune systems are now known to be interrelated via a network of interleukin growth factors and cytokines regulating the proliferation and differentiation of cells in the network. These cytokines are regulatory peptides produced by the cells of the immune system (and other tissues). They play a vital role in the interaction between parasites and the immune systems (Miller *et al.,* 1989b; Oppenheim 1994; Paul and Seder, 1994). There are five components involved in cytokine activity: the producer cell, the cytokine itself, a receptor on the target cell, the target cell and the response of the target cell (Oppenheim, 1994). Tumor Necrosis factor (TNF) is a cytokine released as a result of systemic activation of macrophages by malarial parasites and together with other cytokines have been implicated in dyserythropoiesis and increased phagocytosis in experimental malaria (Clark *et al* 1987a; Clark and Chandri,

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1988; Miller *et al* 1989a). It has recently been shown that certain cytokines when inoculated into mice, modulate the immune response by increasing mainly the antibody-mediated Th2 response and accelerating the elimination of malaria parasites from mice (Akanmori *et al.*, 1996)

Severe malarial anaemia is associated with high levels of TNF leading to dyserythropoiesis (Miller *et al.*, 1989a). The effects of TNF are counteracted by the anti-inflammatory cytokine IL-10. Recent studies have shown that low plasma IL-10 is associated with severe malarial anaemia in children in Ghana and Kenya (Kurtzhals *et al.,* 1998; Othoro *et al.,* 1999). In order to test the hypothesis that IL-10 can reverse or prevent TNF-induced dyserythropoiesis and anaemia, we examined the effects of injections of recombinant mouse (rm) IL-10 into mice experimentally infected with *P. berghei* in comparison with untreated mice.

The major objective of the study is to provide more information on the pathogenesis of severe malarial anaemia.

The specific objectives of the study were:

- To determine the haematological, biochemical and immunological changes associated with *P. berghei* infection in BALB/c mice.
- To determine the effects of injections of rmIL-10 on survival, course of parasitaemia, haematological and immunological changes in BALB/c mice infected with *P. berghei.*

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1.2 LITERATURE REVIEW

1.2.1 MALARIA AS A DISEASE

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. It affects vertebrates in which the asexual form of the parasite is transmitted by an invertebrate vector (the mosquito) in which the sexual reproductive phase of the cycle takes place (Perrin *et al.,* 1982; Wakelin, 1984)

More than 100 species of Plasmodia are known, most of which have extremely restricted host ranges. Natural infection is initiated by the bite of an infected female mosquito that injects sporozoites carried in the insect's saliva. The sporozoite enters hepatocytes/ parenchymal cells of the liver shortly after injection. Within the liver cells the sporozoite then divides asexually to form a large pre-erythrocytic schizont. The rupture of the affected parenchymal cell releases thousands of exoerythrocytic merozoites into circulation. After a period of growth, during which the parasite passes through the ring and trophozoite stages there is a schizogony and production of the erythrocytic schizont, with division of cytoplasm and nucleus to form a relatively smaller number (32 or less) of merozoites. The infected cell then bursts, releasing merozoites, and repeated cycles of schizogony and RBC infection ensues, so that a high proportion of available RBC may become infected (illustration of the life cycle is in Appendices 5 & 6). It has been accepted that the rupture of the erythrocytic schizont is associated with the periodic fever of the disease (Perrin *et al.*, 1982; Wakelin, 1984; Bruce-Chwatt, 1980).

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The patterns of pathology differ with changes in the degree of endemicity (Miller *et al.*, 1994). In regions of high endemicity, the greatest suffering is borne by children less than 5 years of age, whereas in areas of low endemicity, the disease affects all age groups (Miller et. al., 1994). The interaction of the malaria parasite and the host system has evolved into various forms of the disease, as stated by Khurtzhals and colleagues (Khurtzhals *et al.* 1998). In all cases, complicated and uncomplicated malaria are recognized. Complicated malaria is further categorized into cerebral and the severe malarial anaemia. Cerebral malaria usually affects non-immune patients and African children (Miller *et al.*, 1994). They further stated that human *P. falciparum* infection is common in areas where severe anaemia is a major problem.

1.2.2. MALARIA PARASITES

The microorganisms causing malaria are commonly referred to as malarial parasites. Malaria parasites belong to the family *Plasmodiidae* within the order *Coccidiidae*, sub-order *Haemosporidiidae*, which comprises various parasites found in the blood of reptiles, birds and mammals. The zoological family of *Plasmodiidae* includes the parasites, which undergo two types of multiplication by asexual division (schizogony) in the vertebrate host and a single sexual multiplication (sporogony) in the mosquito host (Bruce-Chwatt, 1980).

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1.2.2.1 HUMAN MALARIA PARASITES

There are four recognized species of malaria parasites of man in general: *P. malariae, P. vivax, P. falciparum and P. ovale.* Of these human malaria parasites only *P. falciparum* causes significant mortality. This mortality affects children in Africa, especially those under the age of 5 and pregnant women in the high endemic areas (Weatherall, 1988). However in the low endemic areas it affects all ages (Miller *et al.,* 1994). *P. falciparum* occurs in the tropics and sub-tropics. *P vivax* is the most widely distributed parasite occurring in most temperate areas as well as parts of the tropics. *P vivax* uniquely is associated with clinical relapses due to reactivation of hypnozoites in the liver. Differently, is *P. malariae*, one of the human forms, which affects non-human primates especially chimpanzees (Killick-Kendrick, 1978; Bruce-Chwatt, 1980). *P. ovale* however occurs largely in the western parts of Africa and produces a milder form of the disease.

1.2.2.2 RODENT MALARIA PARASITES

There are a number of malaria parasites, which infect wild rodents and are transmitted by mosquitoes. Among these is *Plasmodium berghei. P berghei* was first isolated In the wild tree rats by Vincke and Lips (Vincke and Lips, 1948; Yoeli,1965) in the forest galleries of Democratic Republic of Congo (former Zaire) and has since been used for various research works. This was possible because, various animal species (37 and more) have been found susceptible to *P. berghei* (Yoeli, 1965). A number of clinical patterns were

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observed during the course by infection of *P. berghei* in different hosts. These are grouped as follows:

1. A fulminating and fatal course terminating in death within 7 to 14 days accompanied by high and rising parasitaemia. Example in white mouse and the baby albino rat
2. A more prolonged clinical course, terminating in the death of the host within 2-3 weeks and accompanied by a rising parasitaemia e.g. golden hamster.
3. Chronic and latent course with spontaneous recovery. Rarely, death from the infection and fluctuation in parasitaemia were noticed e.g. adult albino rat.
4. Mild and transient *P. berghei* infection with low parasitaemia and complete recovery.
5. Short-lived survival of inoculated parasites in an alien host, without power of multiplication. Detection of "static" infection was detected when blood was sub-inoculated into susceptible hosts.

It is also reported that *P berghei.* Anka specifically produces neurological syndrome 7-14 days post inoculation in CBA mice whilst it takes 2-3 weeks to produce severe anaemia in BALB/c mice (Schneider, 1968 and Lucas *et al.,* 1997)

The complete cyclical development of malaria parasites can be sustained indefinitely by uninterrupted blood or sporozoic (mosquito) induced transmission in laboratory reared rodents. This advance was realized by Vincke and Lips in 1948 (Vincke and Lips, 1948; Schneider, 1968).

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Later, the classical histochemical research of Yoeli (1965) and other investigators (Laudan and Killick-Kendrick, 1966) led to full visibility of the exoerythrocytic growth stages in the liver of rodents and the sporogonic growth stages in mosquito vectors (Schneider, 1968).

Differences among inbred strains of mice in response to infection with various rodent malaria species have been observed. Greenberg and colleague showed that the morbidity and mortality vary significantly with various inbred strains of mice after infection with *Plasmodium berghei* (Greenberg and Coatney, *1954)*. Nevertheless, the ultimate of such infection with this species of rodent malarial parasite is lethal in all mice (Stevenson *et al,* 1982). Carter *et al* (1973) studied the enzyme variation in rodent malaria parasites and put *P. berghei* in two groups, among the strains of malaria parasites studied; one representing *P. berghei berghei* (*P berghei.* Anka is found in this group) and *P. b. yoelii*, *P. b. killicki* and the Nigeria *P. berghei* in the other group. Carter and colleagues used the term isolate to refer to parasites derived from a single wild host species on a unique occasion whilst strain meant parasites of a single species derived from an isolate.

Rodent malaria are vital tools that are increasingly used for investigating immunity, physiology, metabolism chemotherapy, long acting antimalarial drugs, ultrastructural morphological characteristics and drug resistance strains (Zuckerman and Yoeli, 1954; Fulton and Spooner, 1956; Bowman *et al.*,1960; Hawking and Gammage, 1962; Jacobs *et al.,* 1963; Thompson *et al.,* 1963;

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Zuckerman, 1964; Nagarajan, 1964; Peters, 1965; Jacobs, 1965; Landau and Killick-Kendrick, 1966 and Powell, 1966).

1.2.3.VECTOR

The *Anopheles* belong to the order of *Diptera*, sub-order *Nematocera*, family *Culicidae*, sub-family *Culicinae* and tribe *Anophelini* in the zoological classification. Within the tribe *Anophelini* the genus *Anopheles* has several sub-genera. There are about 400 species of Anopheles mosquitoes throughout the world, but only some 60 species are important vectors of malaria under natural conditions. The Anopheles mosquito is known to transmit the malaria parasites in general. The Anopheles mosquitoes are most frequent in tropical or sub-tropical regions but they are also found in temperate climates and even in the arctic during the summer. As a rule Anopheles are not found at altitudes above 2000-2500 meters.

However, different strains of the Anopheles are known to transmit the parasites to specific hosts. The female Anopheles *gambiae* is the most important vector of human malaria in the African tropical areas (Bruce-Chwatt, 1980), though there are many others based on the twelve epidemiological zones of malaria as classified by Macdonald (Bruce-Chwatt, 1980). Anopheles mosquito transmits rodent malaria parasites. The *P berghei* sporozoite was first isolated in wild sylvatic anopheles *dureni* (Yoeli, 1965). Also the first infection was observed in the tree rat (*Thamnomys surdaster*). Other vectors were tried and Anopheles *stephensi* was found most susceptible to *P berghei*. Cyclical transmission by

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bite was successful through Anopheles *quadrimaculatus* and Anopheles *stephensi*. Nevertheless, blood passage is used in most laboratories since some strains apparently have lost the capacity to produce gametocyte (Wellde, *et al.*, 1966) or unless sporogony work is of importance in a particular research.

1.2.4 MICE

The natural host of *P. berghei* is *Thamnomys surdaster* (tree rats). Apart from its mammalian rodent host (Thamnomys), more than 37 animal species have been found susceptible to *P. berghei*, majority of which belong to the group of *Rodentia* from different parts of the world (Yoeli, 1965).

The rodent parasites were first identified in wild rodents and later adapted to laboratory rodents and specific models of the disease produced. The rodent malaria parasites have become models for studying human malaria. For example *P. berghei* infection in BALB/c mice produces anaemia models and *P. berghei* infection in CBA mice produces neurological syndrome (cerebral malaria). Nevertheless, regardless of the mode of infection, there are two categories identified, based on the mouse strain as well as the parasite strain (host-parasite combination); lethal and non-lethal malaria models: *P. chabaudi chabaudi* in C57BL/6 mice is non-lethal (Akanmori *et al.*, 1996). Also, non-lethal models can be observed in *P. chabaudi chabaudi* in BALB/c x C57BL/6 and *P. yoelii* in the same mouse strain, whilst in *P. berghei* NK65 in CBA mice is lethal (Akanmori *et al.*, 1994) as are *P. yoelii* and *P. berghei* in BALB/c x C57BL/6 (de Souza *et al.*, 1997). Based on these models it is possible to

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design immunological interventions to examine protection from morbidity or early resolution of disease in mice. For this study, the specific pathogen-free female BALB/c mice were purchased from Bomholtegaard, Denmark and used for the work when they were between 6-8 weeks old.

1.2.5 IMMUNITY AGAINST MALARIA

1.2.5.1 CELLULAR IMMUNITY

Many microorganisms live inside host cells where it is impossible for humoral antibody to reach them. In combating intracellular infections by acquired immune responses, T cells exploit two main strategies. The secretions of soluble factors called lymphokines which activate the cells they combine with to enhance their contribution to microbicidal defence mechanisms and the production of cytolitic T cells which kill the infected target cells (Roitt, 1988).

In line with these, there has been considerable evidence in animal experiments to indicate that T-cell mediated immunity is involved in both protective immunity and pathogenesis against malaria (Waki *et al.,* 1992). Evidence from a rodent malaria parasite is fatal to nude or T cell deprived mice irrespective of the virulence of the parasite (Jayawardena *et al.,* 1977; Clark and Allison, 1974; and Waki *et al*, 1982).

Waki and his colleagues (Waki *et al.*, 1992) re-emphasized the antagonistic role played by CD4+ and CD8+ T-cells to each other in immunity to murine malaria;

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they showed that CD8+ T cells play a vital role in pathogenesis in *Plasmodium berghei* infection whilst CD4+ T cells are important for the induction of protective immunity to *P. berghei* infection. Immunity to malaria can be transferred adoptively by lymphocytes (e.g. *P. berghei* in rats). Taylor- Robinson and co-workers in 1993 also showed that, a vital role is played by CD4+ T cells in protective immunity against blood stages of malaria however the mechanism was unclear. By adoptive transfer of T-cells they made known that both subsets of T-helper cells (Th1 and Th2) are involved distinctively. However, these subset responses vary according to the type of parasite species concerned. In self- resolving infections of *Plasmodium chabaudi*, a sequential Th1-Th2 response is required for elimination of parasitaemia whilst in mice infected with attenuated non-lethal *P. berghei* or with *P vinckei*, Th1 subset activity appears to be crucial for parasite elimination (de Souza *et al.*, 1997).

Thus, relative importance of individual components of the immune response in protection against malarial parasites is very difficult to assess. Nevertheless it is clear that immunity is T-cell dependent. Also in 1987, vaccine failure taught Hoffman and his colleagues that fighting the parasite with antibodies alone is like trying to stop an elephant with a handgun (Hoffman *et al.*, 1987). The antibodies need help from the other parts of the immune system (the cellular immunity).

1.2.5.2 HUMORAL IMMUNITY

In as much as immunity is T-cell dependent, the vital role of the antibodies cannot be overlooked (Wakelin, 1984) e.g. blocking penetration of cells and

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agglutination/opsonization of infected RBCs. The effectiveness of the humoral arm was demonstrated in the now classical experiment of Cohen, who showed that transfer of immunoglobulins from adults immuned to malaria into infected children was followed by a rapid fall in parasitaemia (Cohen & Waren, 1982).

Also, in general, despite variations between systems, adoptive transfer of B lymphocytes or mixed population of B and T cells, have given the best results and this reinforces the importance of antibody in immunity against malaria (Wakelin, 1984). Studies have shown that there are malarial antigens present on the surface of infected RBCs and also present in the serum of infected hosts. Labeling experiments indicate that surface antigens originate primarily from the developing parasites within the cell, although it is not fully understood how transfer to the surface takes place. Antibodies which react with these antigens also precipitate antigens present in the ring, trophozoite, schizont and merozoite stages (Wakelin, 1984).

Specifically, in rodent malaria Th2 protection is by the accelerated production of specific immunoglobulin G1 antibody (Taylor-Robinson *et al.,* 1993). Other immunoglobulins have been implicated in the protection of rodents from malaria. Akanmori *et al.,* 1996 suggested the involvement of the subclasses of IgG (IgG2a, IgG2b and IgG3) in the humoral immune mechanism against *Plasmodium chabaudi chabaudii* in C57BL/6 mice. The predominant role of IgG2a in immunity against *Plasmodium berghei Nk65* in CBA mice was also noted (Akanmori *et al.*, 1994). IgG2a was reportedly detected on the surface

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membrane of *P. berghei*-infected erythrocytes after incubation with hyperimmune serum (Waki, 1994). IgG3 was significant in protection of BALB/c mice infected with 17x strain of *P yoelii* (Majarian *et al.,* 1984). Murphy and Logie (1984) also supported the view that humoral immunity to *P berghei* may be mediated by more than one soluble factor and effected through at least two different pathways.

1.2.6 IMMUNOPATHOLOGY OF SEVERE MALARIAL ANAEMIA

If the host system is regarded as a complex system of cells trying to maintain a steady state, then an attack of malaria must represent a monumental perturbation. The host responses to this insult are primarily immunoregulatory and protective (Jayarwardena, *et al.,* 1977). Nevertheless, immunopathology is the immune phenomenon occurring in response to infection, which is not protective or is responsible for causing pathology in the immune system.

Anaemia is a major concomitant effect in the pathology of malaria as observed in humans (Jilly and Nkrumah, 1965; Rencricca *et al.,* 1974) and in rodents (Cox *et al.,* 1966; Singer, 1954; Topley *et al.,* 1970). The degree of anaemia observed in many animal models was not in proportion to the destruction of infected red blood cells. Since erythropoiesis could not keep pace with the rate of erythrocyte destruction, severe uncompensated haemolytic anaemia occurred (Miller *et. al.*, 1989a).

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Central to this interaction are cytokines that are released by immunocompetent cells in a highly regulated fashion. One of these cytokines is TNF-α, which has been implicated in the immunopathology of severe malarial anemia. Earlier, Clark and his group had postulated that TNF-α release as a result of the systematic activation of macrophages by malarial parasites, may mediate in part the anemia of malaria through its ability to inhibit erythropoiesis (Clark *et al.,* 1987b). This was accepted by Miller and colleagues and they went on to suggest that, the release of the soluble mediators, (e.g. TNF-α) inhibit erythropoiesis and therefore increase the severity of the anemia (Miller *et al.,* 1989a).

It has been established that during the erythrocytic cycle, soluble products of *Plasmodium* species known as malarial toxins cause the release of pro-inflammatory cytokines such as TNF-α. Also, parasite antigens stimulate T cells to directly secrete or induce production of cytokines from other cells (Miller *et al.,* 1994). TNF-α production is known to increase during infection with malaria, and its production is down-regulated by anti-inflammatory cytokines such as interleukin-10 (Hyde, 1992; Corradin *et al.*, 1993; Ho and Moore, 1994). It has also been shown that low IL-10 level is associated with severe malarial anaemia (Kurtzhals *et al.,* 1998) and its absence causes severe anaemia in mice (Kuhn *et al.*, 1993). Kurtzhals and colleagues also buttressed the point of well-defined mechanisms that it is not clear if severe malarial anaemia represents the extreme of a continuum of pathology or constitutes a separate

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disease entity. The fine balance between cytokines appears to be important in the pathogenesis of cerebral malaria and severe malaria.

This however, requires confirmation, which even though it is not possible in human subjects, can be tested in murine model of malaria. A study has shown that recombinant IL-6 boosts specific serum anti-plasmodial IgG subtype titres and suppresses parasitaemia in *Plasmodium chabaudi chabaudi* infection (Akanmori *et al.,* 1996). It is obvious therefore that anaemia and cytokines which provide Scientists with an important target for research into malaria pathogenesis and the mechanisms are however not fully understood.

1.2.7 CYTOKINES AS REGULATORY FACTORS

Cytokines are regulatory peptides that can be produced by virtually every nucleated cell type in the body and they have pleiotropic regulatory effects in haematopoietic and many other cell types that participate in host defense and repair processes. Constitutive production of cytokines is usually low or absent. Production *per se* is regulated by various inducing stimuli at the level of transcription or translation (Oppenheim, 1994).

In mice, it has been shown that T helper cells may have different physical manifestations as indicated by their profiles of cytokine production and eventual effector functions (Ho and Moore, 1994). As indicated in earlier sections, basically T helper cells are categorized into Th1 and Th2. Th1 cells produce IL-2, IL-12 and IFN-γ and preferentially mediate cellular immunity whilst Th2 cells

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produce IL-4, IL-5, IL-6 and IL-10 and provide superior help for antibody responses. However, the cytokines of interest are; IL-10, IL-6, TNF-α and IFN-γ with respect to the severity of the disease (malaria).

1.2.7.1 INTERLEUKIN 10(IL-10)

IL-10 an anti-inflammatory cytokine was originally described as a cytokine synthesis-inhibiting factor produced by T-cells. Subsequent studies showed that it had pleiotropic activities (Oppenheim, 1994). It has been shown that mouse IL-10 and human IL-10 proteins are 73% identical (Ho and Moore 1994). Also, mouse and human receptors for IL-10 have been identified and characterized (Tan *et al.*, 1993; Ho *et al.*, 1993; Liu *et. al.*, 1994).

IL-10 secreted from Th2 cells suppresses the production of Th1 cells and it has recently been assigned a further cross-regulatory role of inhibiting cytokine synthesis by Th1 cells (Hyde, 1992). It has been known to inhibit several macrophage functions (Owens *et al.*, 1996; Fiorentino,*et al.*, 1991; de Waal Malefyt *et al.*, 1991; Ding and Shevach, 1992).

Also, IL-10 is involved in the stimulation of the growth of mast cells (Thompson-Snipes *et al.*, 1991; Ho and Moore, 1994) and thymocytes (Macneill *et al.*, 1990). It is a potent growth and differentiation factor for B lymphocytes (Go *et al.*, 1990). Cells other than T-cells including B cells, macrophages and keratinocytes have also been shown to produce IL-10 (Owens *et al.*, 1996). In all cases, T cells only produce IL-10 after stimulation with antigen or polyclonal

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activators. Importantly, IL-10 secretion is relatively late compared with other cytokines which may explain why macrophages are able to secrete substantial amount of various cytokines before IL-10 inhibition occurs (Oppenheim, 1994). IL-10 suppresses cytokines synthesis by murine T helper cells (Th1) and macrophages (de Waal Malefyt *et al.*, 1991; Fiorentino *et al.*, 1991). IL-10 and IFN-γ antagonize each other's functions and production in several systems (Chomarat *et al.*, 1993). IL-10 has been reported to inhibit nitric oxide synthesis and microbicidal activity of interferon-γ -stimulated macrophages by preventing the secretion of tumor necrosis factor-α (Corradin *et al.*, 1993).

It has been suggested that IL-10 could be a switch factor for human IgG1 and IgG3 production (Rousset *et al.*, 1992; Defrance *et al.*, 1992; Briere *et al.*, 1994). Nevertheless, IL-10 did enhance the viability of mouse splenic B cells in culture and increased their expression of class 2 Major Histocompatibility Complex -MHC- (Go *et al.*, 1990).

1.2.7.2 INTERLEUKIN-6 (IL-6)

Interleukin-6 (IL-6), a multifunctional and a pro-inflammatory cytokine is produced by both lymphoid and non-lymphoid cells and regulates immune responses, acute phase reactions and haemopoises. IL-6 had been referred to differently by many writers in the 1980s, until the cloning of proteins with names like IFN-β2, 26-kDa protein and BSF-2, which revealed that all these molecules were identical. Therefore it came to be known as IL-6 by the end of 1988 (Oppenheim, 1994).

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IL-6 is a glycoprotein with a molecular mass ranging from 21to 28kDa. Human IL-6 shows a homology with that of mouse by 65% at the DNA level. IL-6 production is induced in T cells or T-cell clones by T-cell mitogens or by antigenic stimulation (Horii *et al.*, 1988; Hodgkin *et al.*, 1988). Also, lipopolysaccharide (LPS) enhances IL-6 production in monocytes and fibroblasts, whereas glucocorticoids inhibit it (Helfgott *et al.*, 1987).

A variety of peptide factors such as IL-1, TNF and various agents causing elevation of intracellular cyclic Adenosine monophosphate (cAMP) levels also induce IL-6 production. However, IL-4 and IL-13 inhibit IL-6 production in monocytes. Anti IL-6 antibody inhibits IL-4 driven IgE production, suggesting that endogenous IL-6 plays an obligatory role in the IL-4 dependent induction of IgE (Vercelli *et al.*, 1989). It has been demonstrated that IL-4 induces IL-6 production in normal human B cells (Smeland *et al.*, 1989). Antigen-specific antibody production by primary murine B cells is dependent on IL-6 but not the secondary murine B cells (Hilbert *et al.*, 1989). IL-6 is also reported to augment the *in vivo* production of anti-s RBC antibodies in mice (Takatsuki *et al.*, 1988).

IL-6 is involved in T cell activation, growth and differentiation. It acts on murine thymocytes to induce proliferation and also induces the differentiation of cytotoxic T lymphocytes (CTLS) in the presence of IL-2 from murine as well as from human, thymocytes and splenic T cells (Oppenheim, 1994).

IL-6 has been found to play a central role in defence mechanisms, the murine response, haematopoiesis and acute-phase reactions. Conversely, deregulated

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expression of the IL-6 gene has been implicated in the pathogenesis of a variety of diseases. IL-6 production is enhanced by total unsaturated fatty acid intake (Grimble and Tapia, 1998).

1.2.7.3 TUMOR NECROSIS FACTOR-α (TNF-α)

Tumour necrosis factor alpha (TNF-α) is one of the many pro-inflammatory cytokines, which occurs rapidly following trauma, or invasion of the body by pathogenic organisms (Grimble *and* Tapia, 1998). TNF-α, a 17 kDa protein is produced by the monocyte, lymphocytes, mast cells, basophils, eosinophils, N K cells, B cells, T cells (Oppenheim, 1994).

TNF-α has been implicated in the mediation of a number of other diseases, one of which is severe malaria and its production has been observed in both human and murine malaria (Clark *et al,* 1987a; Grimble and Tapia, 1998). The pleiotropic nature of TNF-α prevents generalization about whether it is beneficial or injurious. The protective effect of TNF-α has been shown by the inhibition of the multiplication of malaria parasites *in vivo* (Clark and Chaudhri, 1988; Taverne *et al.,* 1987). Clark postulated that the activation of macrophages by malarial parasites leads to excessive production of TNF-α which mediates much of the pathology associated with malaria through its action on a variety of cell types (Clark, 1987). On the other hand, Grau and colleagues (1987) abrogated the cerebral malaria in CBA/ca mice infected with *P. b.* Anka when they treated the mice with anti-TNF serum.

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The biochemistry of TNF-α *in vivo* is definitely not in isolation of that of the entire cytokine network. One of the key biological effects of TNF-α is to trigger the release of a series of other cytokines that amplify and extend the biological activities of TNF-α alone (cytokine cascade). TNF-α triggers the release of IL-6, while IFN-γ and IL-1 enhance TNF-α production (Oppenheim, 1994) with IL-10 acting as a potent inhibitor of macrophage functions including TNF-α (Corradin *et al.*, 1993).

Haemopoietically, TNF-α has been mentioned in the development of the anaemia of chronic diseases. Evidence has also shown that TNF-α is involved in dyserythropoiesis and erythrophagocytosis in malaria infection in mice (Clark *et al.*, 1987).

1.2.7.4 INTERFERON-GAMMA (IFN-γ)

IFN-γ is an important immunomodulatory cytokine that regulates natural, cell-mediated and humoral immunity by eliciting a number of biological responses in many cell types. IFN-γ is a lymphokine that displays no molecular homology with the other interferons but shares some important biological activities.

Two different subsets of mouse Th cells have been described based on the array of lymphokines secreted. IFN-γ is a Th1 cytokine, which is secreted by T-lymphocytes and natural killer cells (NK) and is produced during infection (Rhodes-Feuillette *et al.*, 1985), since it is detected in the serum of patients

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shortly after acute *P. falciparum* infection (Wenisch *et al.,* 1995). Both in humans and mice, IFN-γ synthesis has been observed in T cells of the cytotoxic/suppressor phenotype bearing either the CD8 or the Ly-2 antigen respectively (Oppenheim, 1994). IFN-γ preferentially inhibits the proliferation of Th2 but not Th1 cells. This implies that during an immune response, IFN-γ would stimulate the proliferation of Th1 cells (Gajewski and Fitch, 1988).

IFN-γ indirectly enhances MHC II expression on macrophages as antigen presenting cells for triggering on T-helper cells. For example, intravenous injection of recombinant murine IFN-γ to mice results in an increased expression of these antigens on macrophages and on many other cells (Oppenheim, 1994).

IFN-γ activates macrophages and neutrophils in phagocytosis and secretion of toxic factors because parasite killing is significantly activated when infected macrophages are exposed to IFN-γ (Oppenheim, 1994). IFN-γ *in vivo* inhibits development of exoerythrocytic parasites in the liver stages of *P. berghei* (Mellouk *et al.,* 1991) and also of *P. falciparum* schizonts in *in vitro* cultures of human hepatocytes (Mellouk *et al.,* 1994).

IFN-γ induces the formation and release of TNF-α by macrophages (Philip and Epstein, 1986). Production of reactive oxygen intermediates and secretion of hydrogen peroxide are correlated with the capacity of macrophages to kill intracellular parasites (Murray, 1981, Clark and Hunt, 1983). Nevertheless, the stimulation of the secretion of reactive oxygen intermediates seems to be an

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exclusive function of IFN-γ. IFN-γ can induce production of reactive nitrogen intermediates by macrophages, neutrophils, kupffer cells and hepatocytes (Adams *et al.*, 1990; Green *et al.*, 1990; Liew and Cox, 1991). It has been demonstrated that nitric oxide released by human monocytes can contribute to the ability of these cells to kill malarial parasites (Gyan *et al.*, 1994), though a direct effect of IFN-γ on parasite growth has been reported it had conflicting results (Jones *et al.*, 1989; Orago and Facer, 1993).

IFN-γ is one of the natural B-cell differentiation factors since the addition of anti IFN-γ antibodies to activated T-cell supernatants abrogates the capacity of these supernatants to stimulate B cells into production of antibody-forming cells (Sidman *et al.*, 1984). Studies in gene knockout mice unequivocally show that IFN-γ is essential for the generation of normal IgG2a and to a lesser extent IgG3 response (Huang *et al.*, 1993).

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CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

Chemical	Supplier
Sodium chloride	BDH Laboratory Supplies, England
Diethyl ether	BDH, Chemical Ltd Poole England
Ethanol	BDH Laboratory Supplies, England
RPMI 1640	Sigma Chemical Co. St. Louis USA
Tris hydroxymethylaminomethane	Fluka AG Bachs SG in Switzerland
Ammonium chloride (NH_4Cl)	AnalaR Analytical Reagent
Heparin	Wako Pure Chemical Industries Ltd , Japan
Hydrogen chloride	Wako Pure Chemical Industries Ltd , Japan
Giemsa stain	BDH laboratory Supplies , Eangland
Brilliant cresyl blue	Wako Pure Chemical Industries Ltd , Japan
Disodiumhydrogenphosphate (monobasic)	Wako Pure Chemical Industries Ltd , Japan
Potassiumdihydrogenphosphate (monobasic)	Sigma chemical Co. St. Louis , USA
Sorbitol	Sigma chemical Co. St. Louis , USA
Glycerol	Sigma chemical Co. St. Louis , USA
Potassium cyanide	Wako Pure Chemical Industries Ltd ,Japan
Potassium ferricyanide	Wako Pure Chemical Industries Ltd ,Japan
Methanol	BDH Laboratory Supplies, England
Nonidet	Wako Pure Chemical Industries Ltd Japan
Sterox	Wako Pure Chemical Industries Ltd ,Japan

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Microil immersion oil	BDH Laboratory Supplies , England
β -thymidine	NEN Boston, MA USA
Foetal calf serum	Sigma Chemical Co. St. Louis , USA
Tripan blue	Sigma Chemical Co. St. Louis , USA
Penicillin-streptomycin	GIBCO BRL Life Tech. in Paisley Scotland
L-glutamine	Sigma Chemical Co. Ltd, U.K

ANTIBODIES

Goat anti-mouse Ig G (Fab specific)	Lot 067H4845, Sigma Chemical Company, USA.
Goat anti-mouse Ig G1 (heavy chain specific)	Lot 077H9022, Sigma Chemical Company, USA.
Goat anti-mouse Ig G2a (heavy chain specific)	Pdt.No M8269, Sigma Chemical Company, USA.
Goat anti-mouse Ig G2b (heavy chain specific)	Lot 046H8968, Sigma Chemical Company, USA.
Goat anti-mouse Ig G3 (heavy chain specific)	Lot 086H8886, Sigma Chemical Company, USA.
Goat anti-mouse Ig M (μ - chain specific)	Lot 084H8862, Sigma BioSciences, USA.

2.1.2 EQUIPMENT

CENTRIFUGE

1. Kubota Centrifuge KS- 4000	Kubota Corporation, Japan.
2. Compur Microspin	Bayer Diagnostics Mfg. Ltd., Ireland.
3. Sakuma RSL-O5A	Sakuma Corporation, Japan.
4. Kubota 1120	Kubota Corporation, Japan.

MICROSCOPY

1. Olympus CH-2, model CHT-G	Olympus Optical Company Ltd. Japan.

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2. Olympus BX6DF-3	Olympus Optical Company Ltd. Japan.
SPECTROSCOPY	
1.Photic-100	Erma Inc.,Tokyo Japan.
COULTER	
β-Coulter counter	PACKARD A Canberra Company Australia
CELL HARVESTER	
Filtermate 196	PACKARD A Canberra Company Australia
Micromate 196	PACKARD A Canberra Company Australia
INCUBATOR	
1.CO_2 incubator (10-0211 type)	
ikemotoRika kogyo	Ikemoto Scientific Technology Co. Ltd Japan

2.Te-Her cubic incubator (HT-120) Hirasawa-Japan

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2.2 MICE

The specific pathogen-free female BALB/c mice purchased from Bomholtegaard, Denmark were kept in the animal house of the Noguchi Memorial Institute for Medical Research (NMIMR). They received food and water *ad libitum*. All mice were six to eight weeks old at the commencement of the experiments.

2.3 MALARIA PARASITES

The rodent malaria parasite *Plasmodium berghei berghei* Anka strain from a frozen stock kept in liquid nitrogen in the immunology unit of the NMIMR was used after at least 20 passages through BALB/c mice. The strain was obtained from the Department of Infectious Diseases, Copenhagen University Hospital, Denmark. It has since been stored frozen in liquid nitrogen with occasional passage through mice. The mice were inoculated intravenously with a suspension of 10^6 parasitized red blood cells.

2.4 PROPAGATION OF *P. BERGHEI BERGHEI* ANKA IN BALB/C MICE

For continuous *in vivo* maintenance of rodent malaria parasites in mice, blood passages were done every 6-8 days. An infected mouse with high peripheral parasitaemia (30-80% parasitaemia) was anaesthetised with diethyl ether. Blood was collected through cardiac puncture and the total number of red blood cells was counted in a Neubauer counting chamber microscopically. The inoculum was adjusted to 10^6 parasitized RBCs by diluting in Phosphate

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buffered saline (PBS) and injected intraperitoneally into 6-8 week old BALB/c naive mice.

2.5 TREATMENT OF BALB/C MICE WITH rm-IL-10

Recombinant mouse IL-10 (rmIL-10), lot number 066H6712 (Sigma Chemical Company St. Louis USA), with biological activity of 0.7ng/ml determined in a cell proliferation assay using mc/9 cells, of a mouse mast cell line was used. Each of the test groups of 15 BALB/c mice was injected intraperitoneally with 0.167μg of rm-IL-10 in 0.1ml saline, a day before and after parasite inoculation. Control mice of 15 in number were simultaneously injected with saline.

2.6 HAEMATOLOGY

2.6.1 ESTIMATION OF HAEMATOCRIT IN P.BERGHEI ANKA PARASITIZED MICE

Haematocrit level was monitored in two groups of 5 mice, IL-10 recipient mice and corresponding control non-recipient mice. The infected mice were bled from the caudal vein by making a tiny incision on the tail-tip and blood taken into heparinised micro capillary tubes on days 3,5,7,14 and 21. This was put in the Compur microspin and spun at maximum speed of the centrifuge for 3 minutes (auto stop). The haematocrit level in each tube was read with an in-built haematocrit reader (Compur microspin, Bayer Diagnostics mfg. Ireland).

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2.6.2 ESTIMATION OF HAEMOGLOBIN LEVEL

Haemoglobin levels were determined for 5 rmIL-10 recipient mice and a corresponding number of control non-recipient BALB/c mice. Blood samples were collected with a micropipette from the caudal vein through a tiny incision made at the tip of the tail. These were diluted with Drabkin solution (.0.5g potassium cyanide, 0.2g potassium ferricyanide, 0.14g potassium dihydrogen phosphate and 0.5ml Nonidet in 1L of distilled water). The diluted blood samples were allowed to stand at room temperature between 10 and 30 minutes, after which the absorbances were read at 540nm with a photic-100 spectroscope. The haemoglobin levels in grams per litre were determined from a standard curve of absorbance- concentration curve.

2.6.3 ESTIMATION OF RETICULOCYTES

The percentage reticulocytes were determined for 5 of each of the rm-IL-10 recipient mice and non-recipient mice on the following designated days (3,5,7,14 and 21). Five microlitres (5μl) of caudal vein blood were placed in a test tube containing a 0.04% brilliant cresyl blue in PBS at pH7.4. Later, an improved Neubauer chamber was filled with the diluted blood and observed under the microscope with x40 objective. Reticulocytes appeared bluish whilst normocytes appeared pinkish (Appendix 7). The percentage reticulocytes were determined with the formula below;

$$\text{\% Reticulocytes} = \frac{\text{number of reticulocytes} \times 100}{\text{total RBCs count}}$$

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2.7 DETERMINATION OF PARASITAEMIA

The course of parasitaemia was monitored in 5 rm-IL-10 recipient mice and a corresponding number of control non- recipient mice. Blood smears were made on days 3,5,7,14 and 21, post-inoculation with parasites. These were deemed adequate based on previous trials where blood smears taken daily were compared to smears taken on the designated days. From a drop of caudal vein blood placed on a clean microscope slide, a thinly spread blood film was prepared, air-dried, fixed with methanol and air dried again before staining for 10 minutes with a 1:10 dilution of Giemsa stain (stock solution) to 0.1M phosphate buffer, pH 7.2. After complete staining, the slide was washed with distilled water, dried and examined under the microscope with the oil immersion objective.

2.8 ANTIBODY MEASUREMENT

The antibody levels against *P berghei berghei* ANKA blood stage antigens in the various blood samples were determined with the Immunoflourescent antibody test (IFAT) technique. The procedure involved initial preparation of antigen slides as described below and the actual IFAT.

2.8.1 PREPARATION OF ANTIGEN SLIDES

Blood was taken from *P berghei* ANKA infected-BALB/c mice into heparinised tubes. The erythrocytes were washed five times with sterile saline. Centrifugation was done at 4000rpm for 10minutes. A suspension of

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erythrocytes containing 10^6 parasites in 0.2ml saline was injected into non-infected BALB/c mice and parasitaemia monitored as indicated in earlier sections. When average parasitaemia rose to about 20% on day 5, the mice were anaesthetized with diethyl ether and blood was sampled through cardiac puncture. The blood was pooled and three drops placed on a clean microscope slide, a thinly even spread blood film was prepared, air-dried, fixed with acetone and air-dried again. About 250 prepared slides were wrapped individually in a tissue paper and groups of five, sealed in plastic bags and repacked in the glass slide boxes. These were preserved in the –80°C freezer until required for IFAT.

2.8.2 ANTIGEN-WELL PREPARATION

The antigen coated–slides were removed from the –80°C freezer and allowed to thaw at room temperature. Islands of antigens (wells) were created by wiping strips of the blood film with an alcohol wetted tissue rod. Equal wells were created (a total of 16 wells) by carefully tracing the crevices with a non-soluble pen and then allowing them to dry for a few minutes.

2.8.3 IMMUNOFLOURESCENT ANTIBODY TEST (IFAT) FOR TITRATION OF *ANTI-PLASMODIAL* IgG SUBTYPES AND IgM

The indirect IFAT was performed as described elsewhere (Akanmori *et al.*, 1996). Briefly four-fold serial dilution of sera obtained from both IL-10 recipient and non-recipient mice in phosphate buffered saline (PBS; dilution range 1:4 to

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1: 16384) were applied to wells of antigen coated slides in duplicate. After 30 minutes incubation period at 37°C, the slides were washed and goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 or IgM monoclonal antibodies were added. The slides were incubated for another 30 minutes. After washing the slides, a fluorescent- conjugated rabbit anti-goat IgG was added and the slides were incubated for a further 30 minutes. After a final wash, mountant (50% glycerol) was applied and the slides were examined under a type BX6DF-3 incident light illuminating fluorescent microscope (Olympus, Japan). Each sample was tested in duplicate.

2.9 SPLEEN CELL CULTURES

Spleen cell cultures were set up and the supernatants collected for cytokine assays.

2.9.1 PREPARATION OF MONONUCLEAR CELLS FROM THE SPLEEN

Two mice each were sacrificed from the two groups, IL-10 recipient and non-recipient, on the designated days (3,5,7,14 and 21). The spleen was removed from anaesthetized mice by making an incision at the back and pulling gently to expose the spleen. The spleen was suspended in RPMI 1640 medium in petri dishes, crushed with the ends of two frosted slides and mixed well to separate cells and enable the removal of clumps. The cells were kept on ice until all the spleens had been mixed. The suspension was centrifuged at 1500 rpm for 5min and the supernatant discarded. The pellets were resuspended in 5ml of 0.83% ammonium chloride (NH_4Cl) in Tris for 2min. RPMI 1640 was then added before centrifuging at 1500rpm for 5mins and the supernatant was

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discarded. The cells were washed three more times using RPMI 1640 before finally being resuspended in 3ml of complete medium (2ml penicillin in streptomycin, 4ml L-glutamine, 5% v/v foetal calf serum all in 500ml RPMI 1640).

2.9.2 CELL CULTURES

For viable cell count, 0.44% tripan blue was added to an equal volume of cell suspension. This enabled the cell suspension to be adjusted to a population of 10^6 per volume. The cell suspension was aliquoted at 150μl per well in a round bottom tissue culture plate. To each well 20μl each of the mitogen (phytohaemaglobin (PHA)) or the antigen (*P. berghei.* ANKA) was added in triplicate. The plate was incubated for 24 hours in a CO_2 incubator (10-0211 type Ikemoto Rika kogyo) at 37°C. After incubation, supernatants were collected from each well. The culture was then spiked with ^{3}H-thymidine (1mCi per well) and the volume added up to 170μl per well with complete medium. The plate was further incubated for 24 hours at 37^0C in a CO_2 incubator and the cells were harvested with S.F filtermate 96-cell harvester. The filter paper was dried in an oven at a temperature range of 50- 90^0C. The nuclei adhered to the surface of the filter paper were counted using a β-counter to see how the labelled thymidine was utilised by DNA for the mitotic processes. The ^{3}H utilised is proportional to the extent of proliferation by the cells which had imbibed the β-thymidine.

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2.10 CYTOKINE MEASUREMENTS IN THE SERUM AND SPLEEN CELL - CULTURE SUPERNATANTS

Interleukin-10 (IL-10), Tumour necrosis factor-alpha (TNF-α) and Interferon-gamma (IFN-γ) were measured by commercial enzyme linked immunosorbent assay (ELISA) kits from R& D systems (Minneapolis, MN, USA). The assays detect both recombinant and natural mouse cytokines.

Briefly, the assay employs the quantitative sandwich enzyme immunoassay technique as described by the manufacturers of the kits used. An affinity purified polyclonal antibody specific for mouse IL-10, TNF-α or IFN-γ had been pre-coated onto separate microplates.

Standards, controls specific for the cytokines and samples were pipetted into the wells and incubated for 2 hours at room temperature to allow binding to the immobilised antibody. After washing four times with buffer (25x concentrated solution of a buffered surfactant, with preservatives) to remove any unbound substances, an enzyme- linked polyclonal antibody specific for mouse IL-10, TNF-α or IFN-γ was added to the wells. The plates were further incubated for 2 hours at room temperature and washed to remove any unbound antibody-enzyme reagent. A substrate solution (equal volume of stabilised hydrogen peroxide and stabilised tetramethylbenzidine) was added to the wells and the plates were incubated for 30 minutes at room temperature in the dark. A stop solution (diluted hydrochloric acid solution) was added and the optical density determined.

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The optical density was measured at 450nm with a correction factor at 570nm on a microplate reader (Corona Electric, Japan). The intensity of the colour measured was in proportion to the amount of mouse IL-10, TNF-α and IFN-γ bound in the initial step. The concentration of cytokine in the samples was then calculated from the standard curve.

STATISTICAL ANALYSIS

Student 's t test was performed to determine statistically significant differences between groups. $P < 0.01$ was considered significant.

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CHAPTER 3

3.0 RESULTS

3.1 HAEMATOLOGICAL MEASUREMENTS

To study the effect of exogenous IL-10 on haematological, parasitological and immunological parameters, it was justified through repeated passages that the individual mice in a group were comparable to each other by measuring parasitaemia, haemoglobin levels (Hb), reticulocytes and packed cell volume (PCV). Figure 1 illustrates the justification to use the mean of the data from the individual mice in the group.

IL-10 recipient mice and controls were then infected with 10^6 *P. berghei* Anka. This was followed by an injection of an equal amount of rmIL-10 into the IL-10 recipient mice and the course of infection followed. Figures 2,3,4 and 5 illustrate the profiles of mean parasitemia, Hb, reticulocytes and PCV observed in rmIL-10 recipient and control mice (5 in each group), after inoculation with *P berghei* Anka.

3.1.1. PARASITAEMIA (THE COURSE OF INFECTION)

Mean patent parasitaemia in IL-10 recipient mice on day 5 was 0.2%, rising to 7% on day 7 and followed by a further rise sharply to 29.7% on day 12 (see figure 2). This tapered to 32.5% on day 14. All mice died before day 21 (100% mortality on day 19). The control mice developed a similar pattern of parasitaemia. The onset of patent parasitaemia of 0.2% occurred on day 5, 10.6% on day 7, 21.0% on day 12 and a maximum of 31.4% on day 14 (100% mortality by day (Table of results in Appendix 1)

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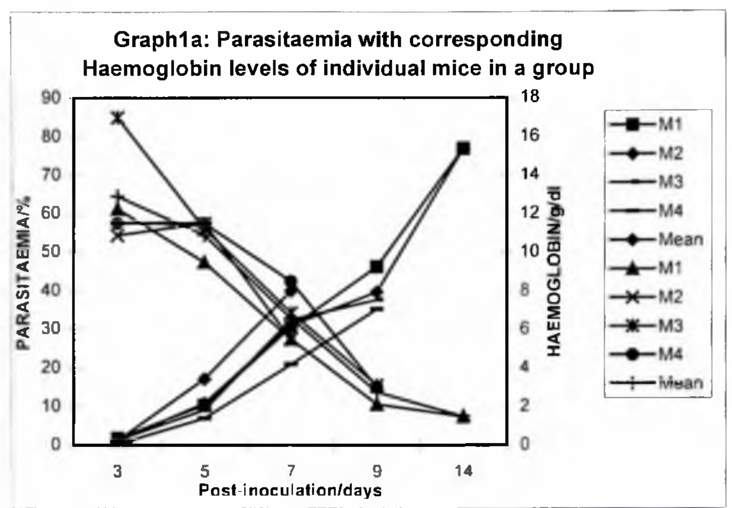
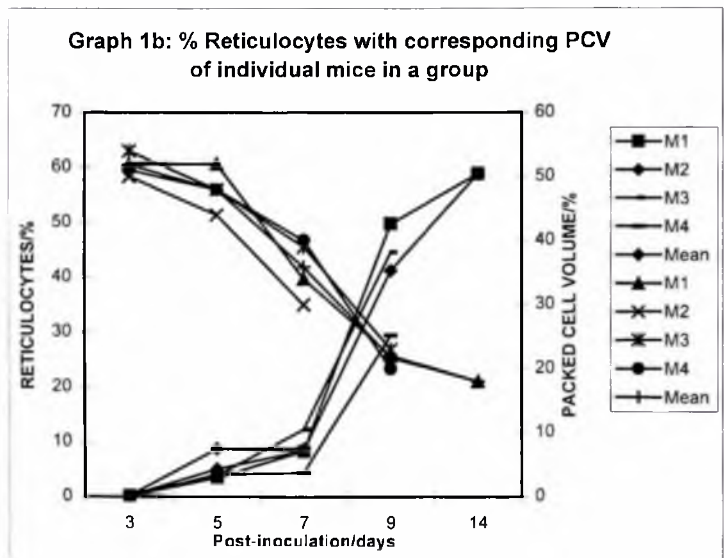
FIGURE 1a:Graphs to justify that the mice are comparable in a group





KEY:
M1,M2,M3 & M4 - Individual mice
In graph 1a: the ascending lines denote % Parasitaemia rise and the descending lines also denote fall in Haemoglobin levels over a period

In graph 1b: the ascending lines denote % Reticulocytes and the descending lines also denote fall in % Packed Cell Volumes over a period

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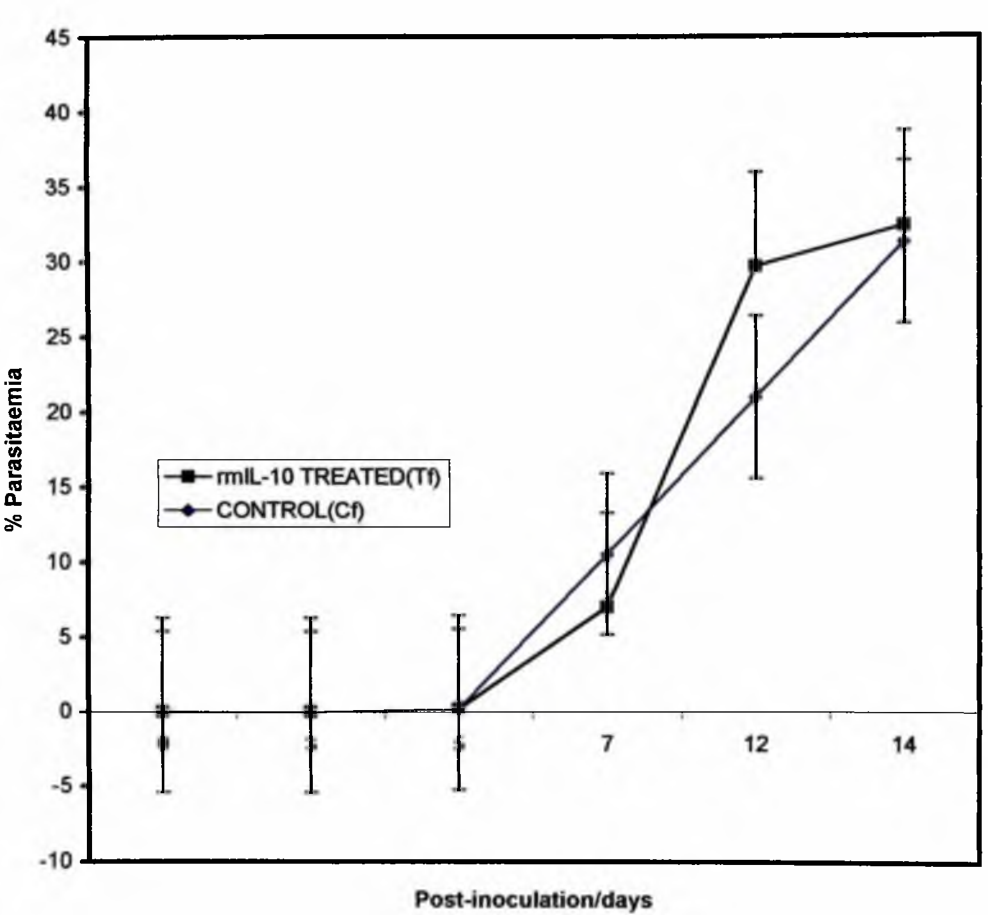
Figure 2:Graph of Parasitaemia for rmIL-10 treated and control balb/c mice



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19). A point worth mentioning is that on day 7, parasitaemia was 10% in controls as compared to 7% in rmIL-10 recipient.

3.1.2 ANAEMIA

3.1.2.1 HAEMOGLOBIN LEVELS

Controls were not significantly more anaemic than the IL-10 recipient mice (P>0.01). Haemoglobin levels remained stable in both groups until day 7. It then decreased sharply to approximately 6.00g/dl on day 12 and 14. From figure 3 it was clear that significant difference occurred on day 5, however there was no significant difference by day 14. The normal haemoglobin level measured in the animal unit of the NMIMR for similar mice was 12.0 ±2 g/dl.

3.1.2.2 RETICULOCYTES

The normal percentage reticulocyte count measured in the animal unit of the NMIMR was 0.5% ± 0.5.

The reticulocyte counts were performed as a confirmation to the anaemic situation in the internal environment of the BALB/c mice. The percentage of reticulocytes in the blood as against the normal RBCs was an indication of synthesis of mature RBCs lagging behind the destruction of infected RBCs in the spleen. There was no difference between IL-10 recipient and control mice (P>0.01 for the means of percentage reticulocyte count). In both groups, the reticulocyte count fluctuated between 1.5 and 3.5% on day 5. It started rising on day 7 and rose drastically to over 25% on days 12 and 14. (See Figure 4)

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Figure 3: Graph of Haemoglobin level for rmIL-10 Treated and control balb/c mice

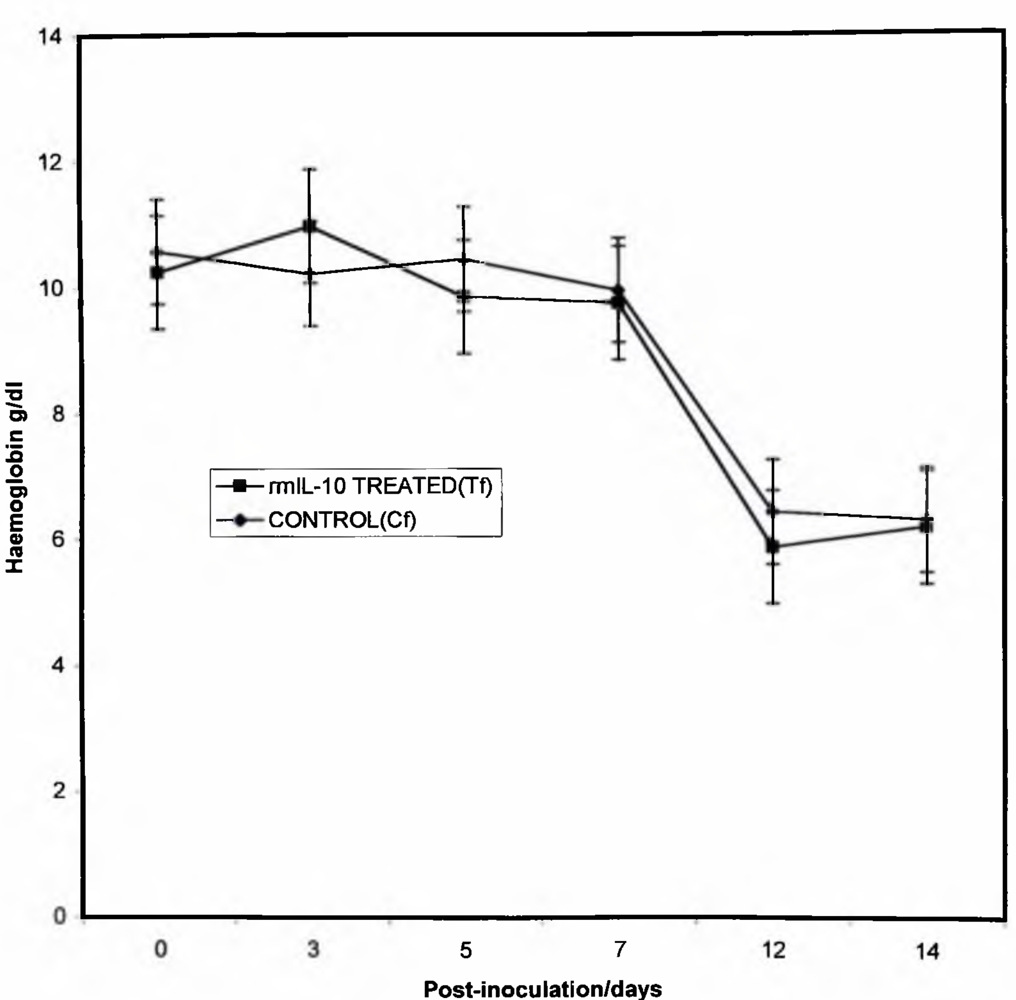

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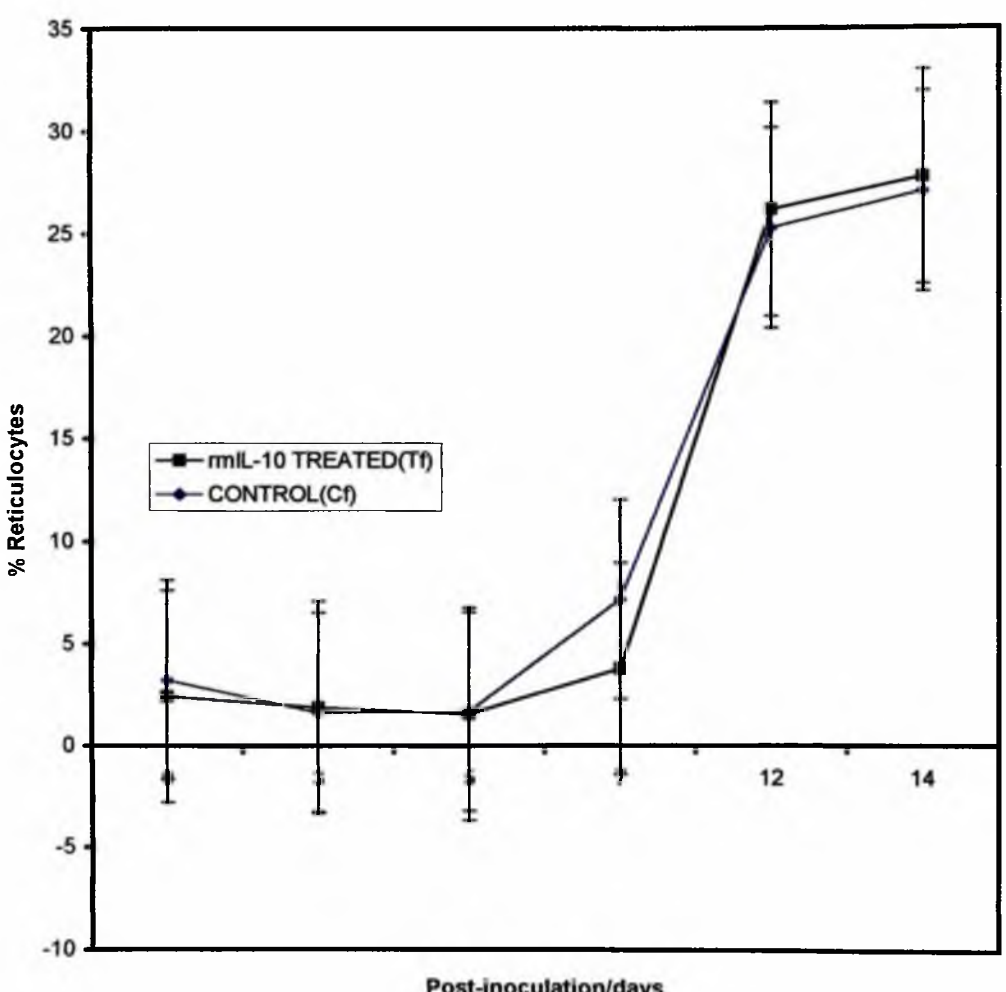
Figure 4: Graph of % Reticulocytes in peripheral blood of rmIL-10 treated and control untreated balb/c mice



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3.1.2.3 PACKED CELL VOLUME (PCV)

For further confirmation on the anaemic status in the BALB/c mice the packed cell volume was determined. Here again, the patterns were almost identical in the two groups (Fig.5).

3.2 MORTALITY

It is known that *Plasmodium berghei* Anka has a predilection for the immature rbcs (reticulocytes) put into circulation compensatedly as a result of early onset of anaemia. This anaemia progresses and eventually terminates the life of the mice. The mortality of the BALB/c mice was determined from a group of 5 mice each; the IL-10 recipient mice and the controls after the inoculation of *P. berghei* Anka. Both IL-10 recipient mice and control started dying after day 7. On day 12, percentage survival dropped sharply from 100% to 60% for the IL-10 recipient mice, a further drop to 40% on day 14 and by day 19, all the mice were dead. There was identical pattern for control mice however with slight differences in value. Percentage survival was 40% on day 12, which dropped to 20% for day14 and stayed constant till day 18. All the mice were also dead by day 19. (Fig.6). Any significant difference in mortality started beyond day 7 and before day 18. Speculatively, any critical observation must lie beyond day 7 since meaningful differences occurred here ($P \leq 0.01$).

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Figure 5: Graph of % PCV for rmIL-10 treated and control balb/c mice

% PCV

60
50
40
30
20
10
0

0 3 5 7 12 14

Post-inoculation/days

rmIL-10 TREATED(Tf)
CONTROL(Cf)

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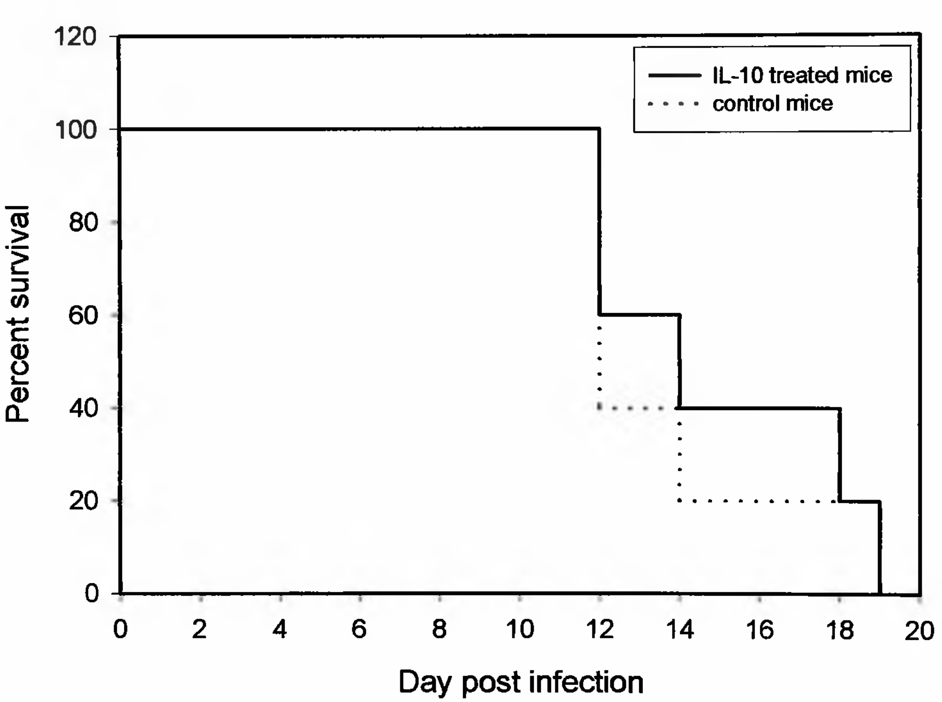
Figure 6: Kaplan-Meyer survival graph for rmIL-10 treated and control mice



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3.3 TITRES OF ANTI-PLASMODIAL IgG SUBTYPES AND IgM

IgG subtypes and IgM were measured on day 3,5,7 and 14 by means of IFAT and using whole blood stages of the parasites as antigen.

During the course of infection, *anti-Plasmodial* IgM titre rose to 8704 on day 3 which decreased to 4096 on day 5 and dropped drastically on day 7 to 160. However there was a transient increase of 256 by day 14 for the IL-10 non-recipient BALB/c mice (control). In contrast, rmIL-10 recipient mice recorded almost twice as much for the IgM titre on day 3, which also traced the same graph pattern in the controls. This is as shown in fig. 7f.

With the *anti-Plasmodial* whole IgG, it rose to an average value of 10240 maintained over a period of 3 days, and decreased to a value of 4096 by day 14 in the rmIL-10 recipient mice whereas in controls, the peak was 16384 on day 5, and took a plunge to 4096 by day 7 This fell further to 256 on day 14 as it was on day 3 (Fig. 7a) and Appendix 3.

In addition, there was a progressive rise in *anti-Plasmodial* IgG1 from 256 to 640 to 1024 on days 3,5 and 7 respectively. This rose sharply to 16384 on day 14. With controls, average titres fluctuated from 1024 on day3 to 2080 on day 5 and then decreased to 1024 on day 7 and by day 14 it was 64 (Figure 7b).

The picture painted by *anti-Plasmodial* IgG2a in rmIL-10 recipient was not anything different from the controls. The titre rose from 2080 on day 3 to 16384 on day 5 maintained till day 14 when it dropped to 4096. In controls, peak value

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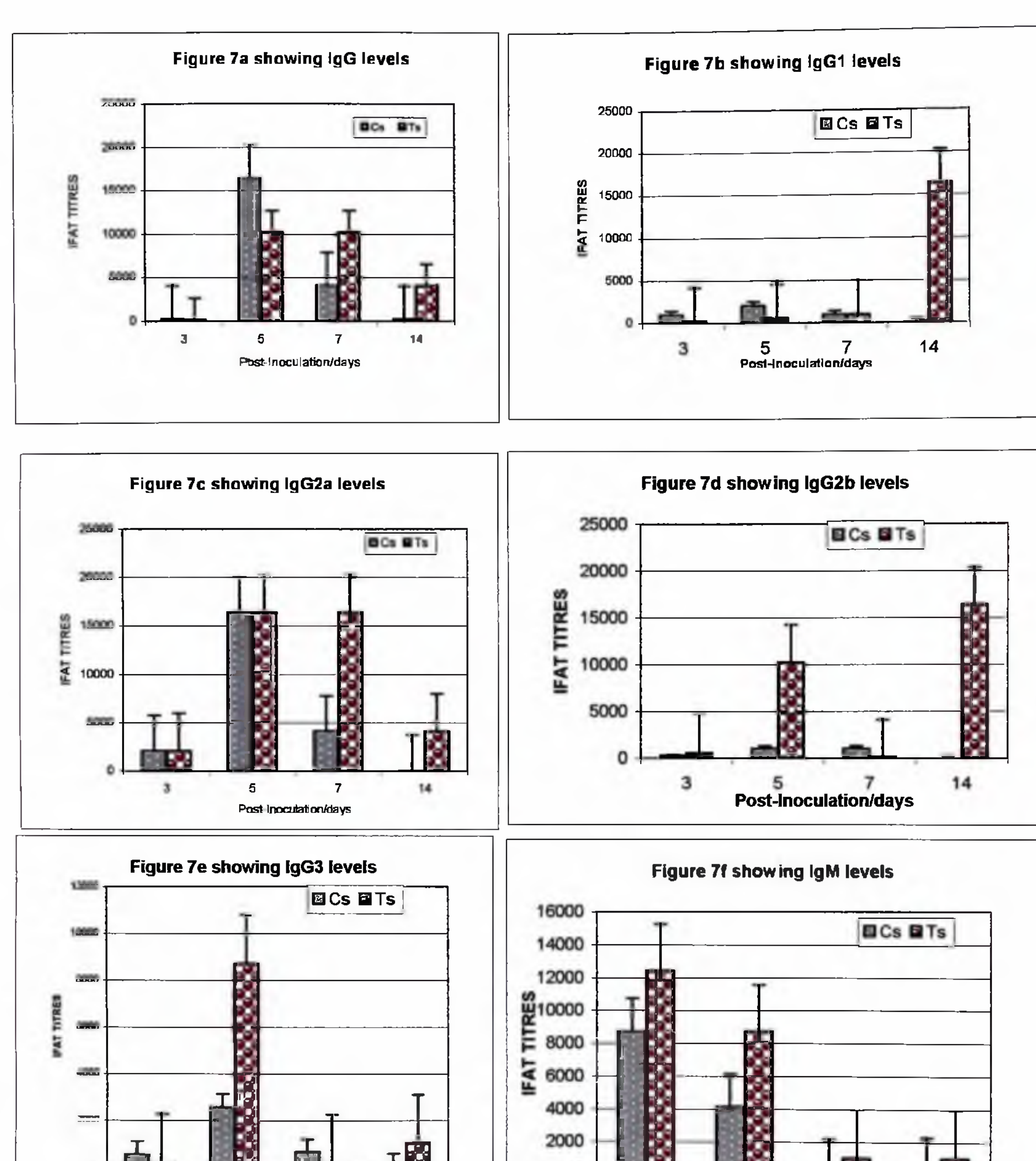

FIGURE 7:

IFAT GRAPHS FOR rmIL-10 TREATED(Ts) AND CONTROL(Cs) MICE SHOWING THE VARIOUS IgG'S & IgM

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of 16384 was on day 5 only, which dropped quickly to 4096 on day 7 and a final drop to 64 by day 14 (Figure 7c).

Anti-Plasmodial IgG2b fluctuated in the rmIL-10 recipients, which reached 544 on day 3 and rose to 10240 on day 5 and then dropped sharply to 2176 on day 7, this rose again to the highest value of 16384 by day 14. Comparatively, the values recorded by the controls were lower. It rose from 256 on day 3 to 1024 on days 5 and 7 and dropped to the titre value of 16 (Figure 7d).

Anti-Plasmodial IgG3 recorded the minimum peak value of 8704, which occurred on day 5. By day 7, it had gone down to 160 and rose to 1024 by day 14. The control had its peak also on day 5 but with a lesser titre value of 2560, which dropped to 64 on day 7, and dropped further to 16 by day 14. These are as shown in Fig. 7e. (see appendix 3 for Table of Results)

To summarize, anti-*Plasmodial* IgM had the highest peak on day 3. On day 5 it was anti-*Plasmodial* IgG2a which maintained the peak to day 7. However, day 14 saw IgG1 and IgG2b recording the maximum titre value of 16384 for the rmIL-10 recipient mice (Fig 9b). With the control, the anti-*Plasmodial* IgM recorded the highest peak (8704) on day 3 and on day 5 it was IgG and IgG2a. This was maintained on day 7 but with different peak value (4096). By day 14 all titre values had decreased to the minimum with IgG and IgM recording the highest of 256 (Fig 7).

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3.4 SPLEEN CELL PROLIFERATION

The splenic lymphocytes from the BALB/C mice were prepared with the following treatments: -

(i) Ts- treated with IL-10 and challenged with parasites and sacrificed

(ii) Cs- not treated with IL-10 but challenged with parasites and sacrificed

(iii) Cw- not treated with IL-10 and not challenged with parasites but sacrificed

were cultured in triplicate at 37°c in a CO_2 incubator for 24hrs for cytokine release and was spiked with β-thymidine at a cell population of 10^5 for cell proliferation and incubated for a further 24 hours.

With the rmIL-10 recipient mice (Ts), the mitogen-driven (phytohaemagglutinin-PHA-) culture recorded 690 counts (cpm) whilst the antigen-driven (*Plasmodium* infected RBCs-pRBCs-) culture was 216 cpm. However, the unstimulated cells had a count of 500cpm on day 3. On day 5, unstimulated cells count was 221 cpm and that for PHA dropped to 210 whilst pRBCs was 538cpm. Day 7 had the stimulated cell categories recording higher than unstimulated cell category. This was at 409cpm for unstimulated 426 cpm for PHA and 462 cpm for pRBCs. These values dropped to 213 for unstimulated, 189 cpm for PHA and 215cpm for pRBCs on day 14. (Details in Appendix 4).

For the Cs-category, the unstimulated cells counts were 270, 189, 376 and 195 cpm for days 3,5,7 and 14, respectively. The mitogen-driven proliferative response was 674, 293, 359 and 266 cpm for days 3,5,7 and 14 respectively.

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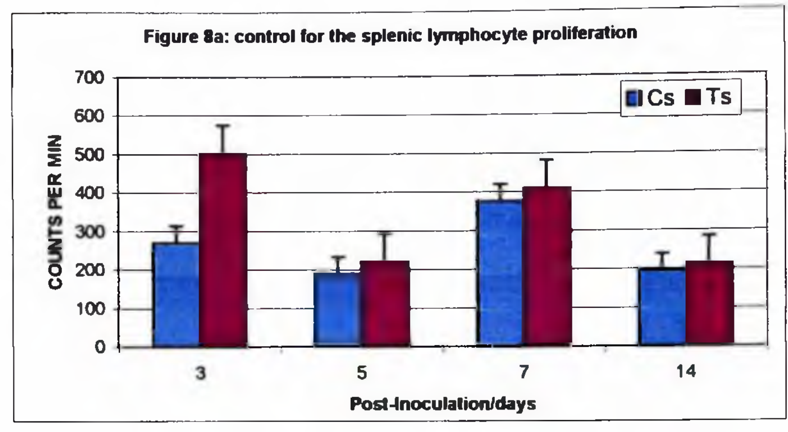

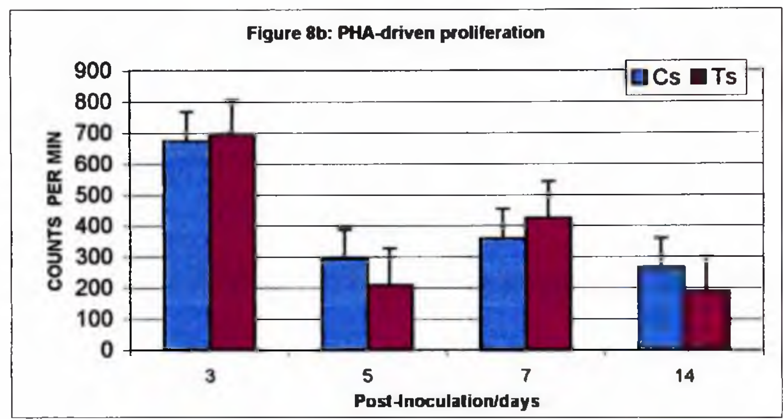

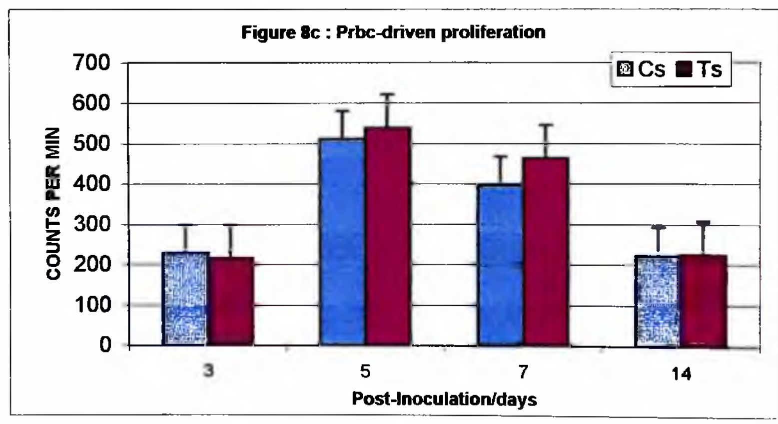

FIGURE 8: Graph of mean spleen lymphocytes proliferation of IL-10 Treated (Ts) and Controls (Cs)

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That for antigen-driven was 228cpm on day 3 and recorded the highest of 510 cpm on day 5. This dropped to 396 cpm on day 7 and again to 222 cpm day 14. The proliferative response induced in cells from the Cw- category was higher for plasmodium-driven at 311 cpm followed by PHA- driven at 164 cpm and 139 cpm for unstimulated cells. These are shown in Figure 8. Comparatively, induction of proliferative response was higher for plasmodium-driven than PHA-driven (fig. 7) because they had more ability to incorporate β Thymidine.

In general, the proliferative induction of splenic lymphocytes of rmIL-10 treated BALB/c mice was not statistically different from that of the control (P> 0.01). Also, the PHA-driven response was highest on day 3 for both Ts and Cs categories with a value of 690 and 674cpm respectively. That of plasmodium-driven occurred on day 5 for both Ts and Cs categories of 538 and 510cpm, respectively.

3.5 CYTOKINES MEASUREMENT

3.5.1 INTERLEUKIN-10

The serum IL-10 measured by means of ELISA was higher in rmIL-10 recipient mice than the non-recipient mice. Remarkably there was no detectable level of IL-10 in both groups of mice on day 3, but 48 hours later, the serum IL-10 was 16.38pg/ml in test mice and 12.48pg/ml in controls. This level rose drastically to 255pg/ml in test mice on day 7 whilst the level in control mice dropped to

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10pg/ml. There was a further rise to 400pg/ml in test mice as against 64.04pg/ml in controls on day 14. This is as shown in Figure 9.

With the supernates from culture, it showed that the cells were viable and had the capacity to induce IL-10 production. The response was more effective in the IL-10 non-recipient mice cells (both in mitogen-driven and plasmodium-driven culture). In order of days 3, 5, 7 and 14, the mitogen-driven IL-10 induction was 2.5,' 50, 90 and 40pg/ml for test mice and 1150, 170, 670 and 48.5pg/ml for controls, whilst, 333, 100, 36 and 773pg/ml for test in plasmodium culture and 340, 1150, 9.98 and 590pg/ml for controls were measured. Figure 10. (See tables in Appendix 4)

3.5.2 TUMOUR NECROSIS FACTOR-ALPHA (TNF-α)

TNF-α levels in IL-10 recipient mice were almost the same as in the controls on days 3,5 and 7 However there was a drastic rise of TNF-α in the test mice to 625pg/ml as against 117pg/ml in controls on day 14. Mortality was observed after day 7 when there was appreciable detectable level of TNF-α in both test and control mice.

The high level of serum TNF-α on day 14 in test mice may account for the mortality after day 14, though it appeared the controls were dying earlier than the test mice. This is shown in Fig. 11

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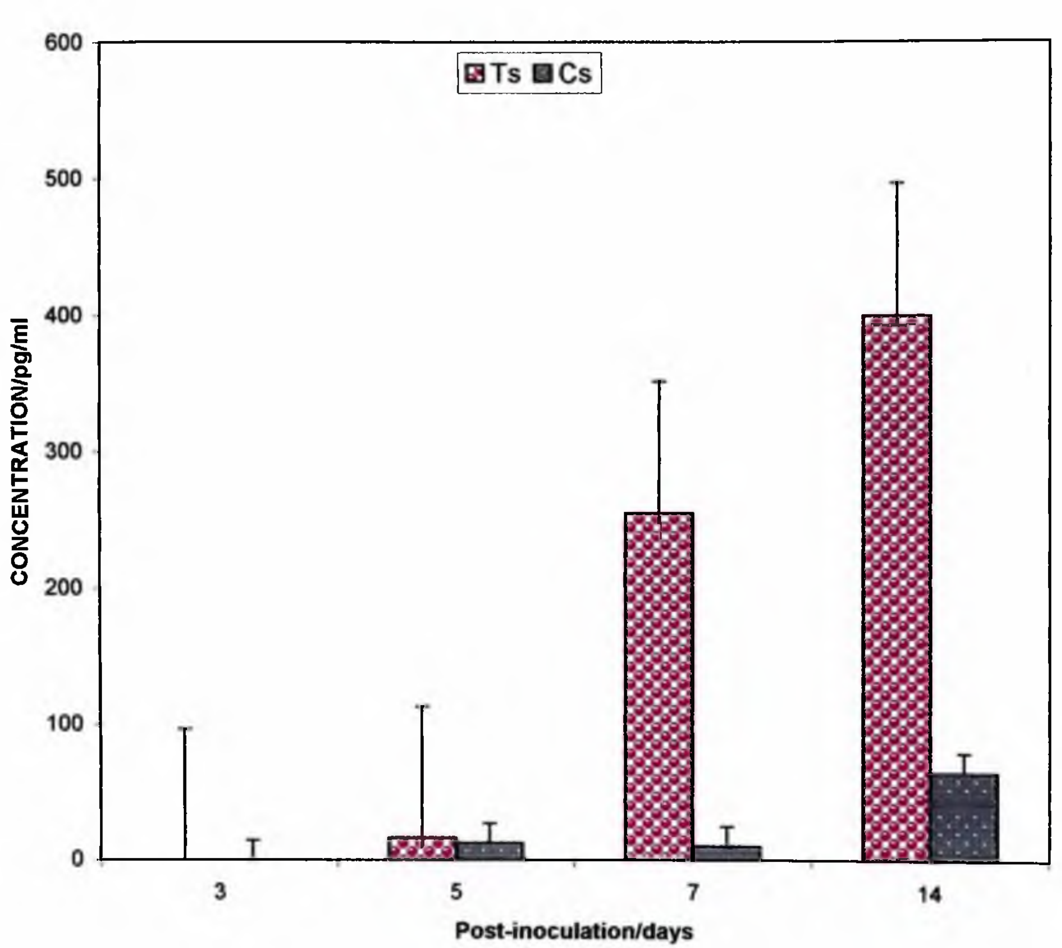
Fig. 9 IL-10 LEVELS IN SERA OF rmIL-10 TREATED (Ts) AND CONTROL (Cs) BALB/C MICE



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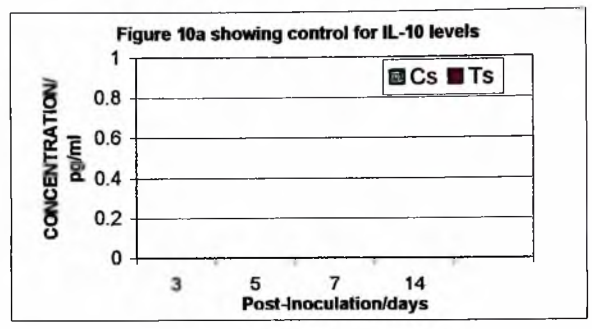

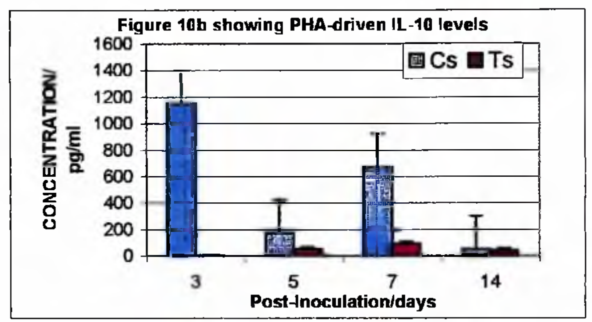

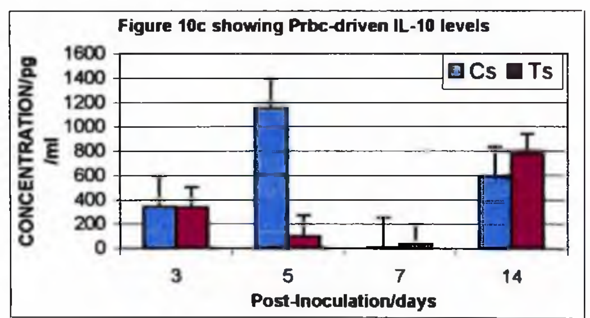

FIGURE 10: Graph of IL-10 LEVELS IN SPLEEN CELL CULTURE SUPERNATES FROM IL-10 TREATED (Ts) & CONTROLS (Cs)

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With the supernates it was observed that the plasmodium-driven culture had the capacity to induce TNF-α especially on days 3 and 5. However low levels of TNF-α were measured in the serum on days 3 and 5. Effective response was from plasmodium- driven culture. For both test and control mice, 3000pg/ml was measured on day 3 and 2525pg/ml on day 5 for control mice. The rest of the measurements were below 100pg/ml, except mitogen-driven induction of 230pg/ml for controls on day 3. This is shown in Fig.12.

3.5.3 INTERFERON-GAMMA (IFN-γ)

The measured values of IFN-γ in both test and control seemed to be very low; 2.21pg/ml for test mice and 5.25pg/ml for controls on day 3. However an increase to 25pg/ml for test mice and 6.64pg/ml for controls were observed on day 5. A sharp rise to 510pg/ml for the test mice whilst as low as 4.7pg/ml for controls on day 7 were measured. This figure dropped sharply to 9.68pg/ml in test mice with a slight increase to 28pg/ml in controls on day 14. Though IFN-γ is known to induce TNF-α there seem not to be any correlation between the two cytokines(Figures 11 & 13).

The inductive response was effective in both mitogen-driven and plasmodium-driven culture, but there was induction of IFN-γ in the culture controls. Needless to say there was no appreciable detection of IFN-γ in cells from non-inoculated mice. For the plasmodium- driven culture, it was 1075, 880, 1205 and 570pg/ml in test mice and 1205,725,50 and 880pg/ml for controls on days 3, 5, 7and 14

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respectively. For the mitogen-driven culture, the levels were 130, 925, 1205 and 410pg/ml for test mice and 405, 42, 133 and 880pg/ml for controls on days 3, 5, 7 and 14 respectively. Culture controls for rmIL-10 recipient were 305, 250, 552 and 12pg/ml and that for non-recipient were 434, 45,50 and 16pg/ml for days 3, 5, 7 and 14 respectively. This is shown in Fig. 14.

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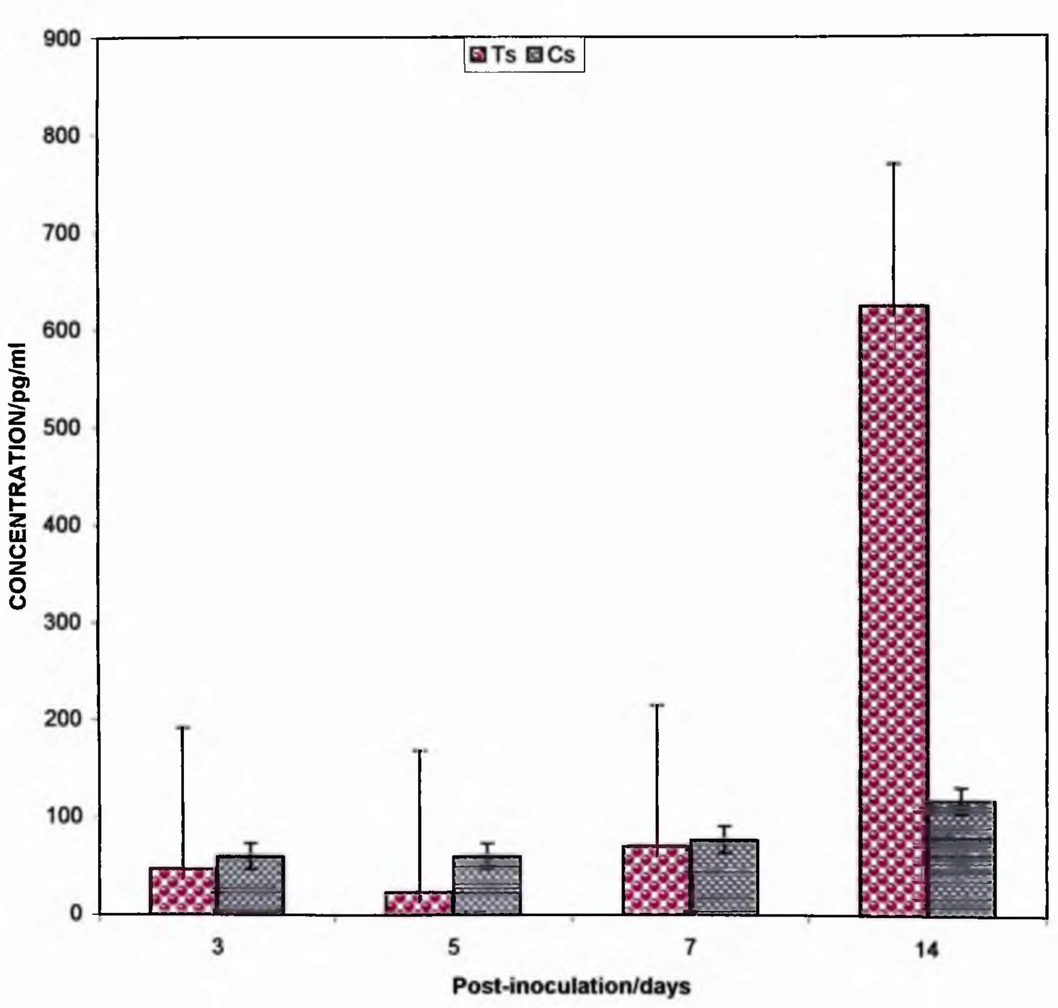
Fig. 11: TNF-alpha LEVELS IN SERA OF rmIL-10 TREATED(Ts) AND CONTROL (Cs) BALB/C MICE



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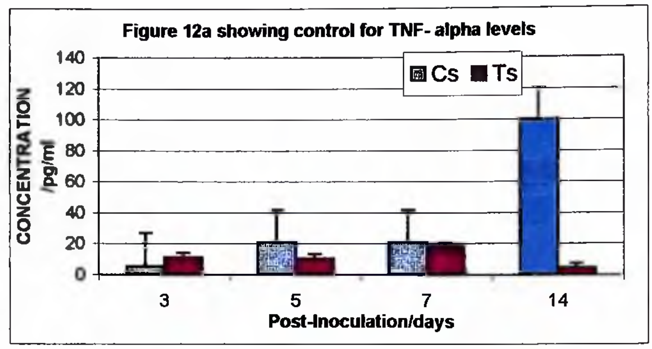

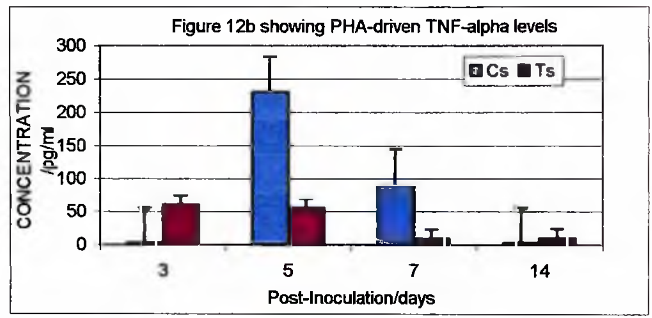

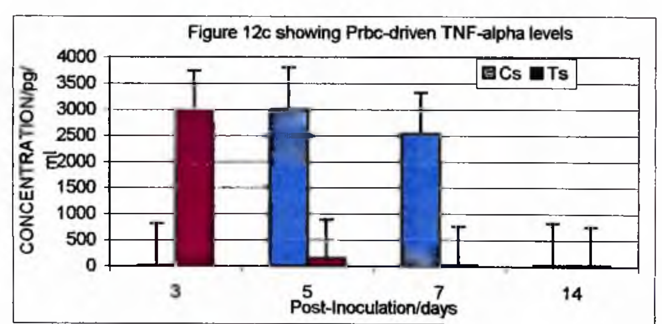

FIGURE 12: TNF-alpha LEVELS IN SPLEEN CELL CULTURE SUPERNATES FROM rmIL-10 TREATED(Ts) AND CONTROLS (Cs)

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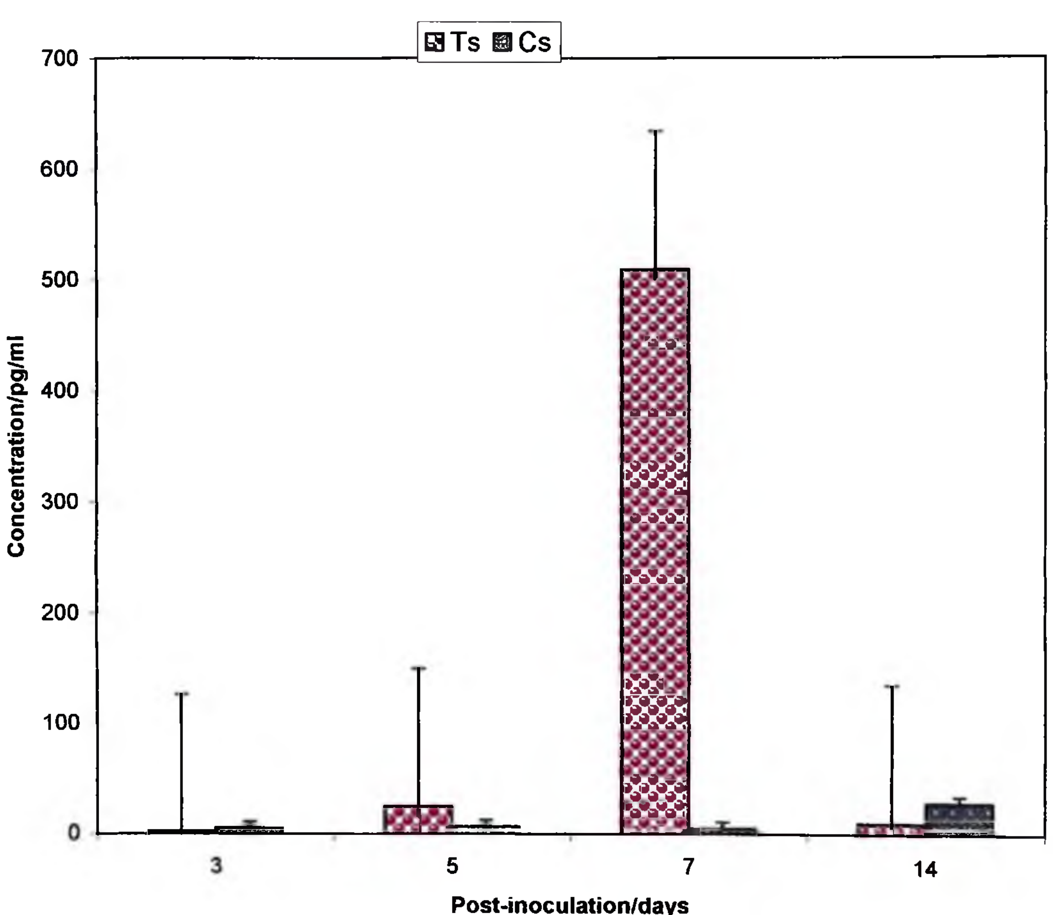
Fig. 13: IFN-gamma LEVELS IN SERA OF rmIL-10 TREATED (Ts) AND CONTROL (Cs) BALB/C MICE



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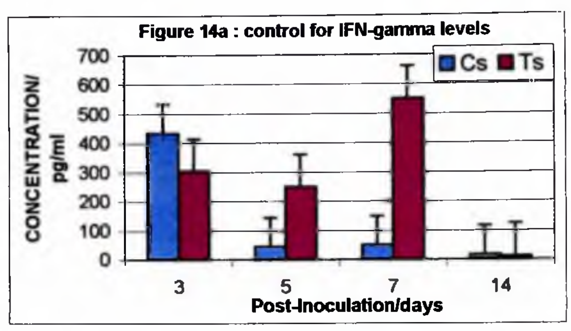

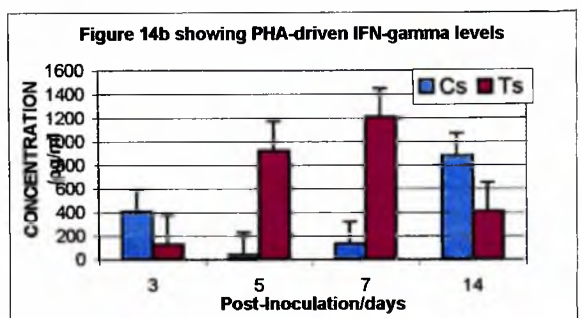

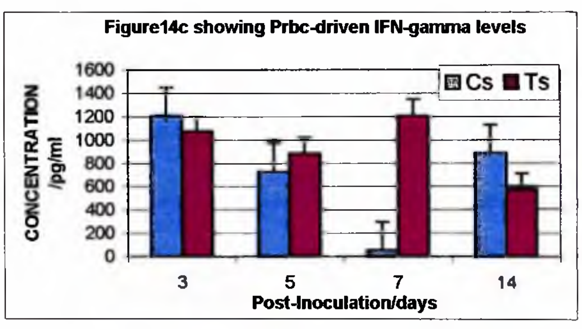

FIGURE 14: IFN-gamma IN SPLEEN CELL CULTURE SUPERNATES FROM rmIL-10 TREATED (Ts) AND CONTROLS (Cs)

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CHAPTER 4

4.0 DISCUSSION AND CONCLUSION

4.1 DISCUSSION

Malaria has been the bane of developing countries in the tropics and in as much as scientists try to arrest it once and for all, it has rather developed uncompromising clout and delivered the deadliest blow cumulatively ever to have received from any other disease. One of its hallmarks is anaemia.

The pathogenesis of this form of severe malaria is not fully understood. A suitable model of severe anaemia is associated with BALB/c mice infected with *P. berghei* Anka. The mice develop a rapid increase in parasitaemia and die off by the 2nd or 3rd week (Schneider, 1968; Lucas *et al.*, 1997). This situation was shown repeatedly in the present study (figure 1) before the final experiment was carried out. The progressive anaemia associated with malaria in mice begins on day 7 (Roth and Herman, 1979). This is detectable by the measurement of these haematological parameters: reticulocytes, haemoglobin levels and haematocrit (Roth and Herman, 1979; McGuire Jr. and Haines, 1985) in addition to parasitaemia. This is consistent with the present work, where the parasitaemia was 10.5% in the controls and 7% in the IL-10 recipient BALB/C mice on day 7, after this there was a sharp rise to 29.7% and 21% for IL-10 recipient and controls respectively on day 12. This occurred with a corresponding sharp rise in

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reticulocytes and sharp falls in PCV and haemoglobin levels (Figures, 2, 3, 4 & 5).

Generally, the anemia has a positive correlation with the parasite count and reticulocytosis, but a negative correlation with haemoglobin and the haematocrit levels, as was observed in the study. However with the injection of rmIL-10, an anti-inflammatory cytokine, it was expected that a significantly delayed mortality would occur in these mice, as a result of suppressed parasitaemia, enhanced antibody mediation, stable levels of haemoglobin, haematocrit and low levels of the pro-inflammatory cytokines implicated in the pathology of malaria. Nevertheless, there appeared to be no significant change of the parameters measured between the IL-10 recipient and the control mice. It may be that rmIL-10 injected into the mice was not enough to cause a significant observable change in the parameters measured between IL-10 recipient and control mice. The sharp rise of' the mean parasite count and reticulocytosis with their corresponding sharp fall in haemoglobin levels and that of haematocrit indicate that *P. berghei* has a predilection for immature RBCs. The appreciable rise of parasitaemia on day 5 compelled the bone marrow to respond (Rencricca *et al.,* 1974; Maggio-Price *et al.,* 1985) to the destruction of infected erythrocytes in the spleen (splenic phagocytosis). Therefore the sharp rise of the reticulocytes with a cumulative rise in the parasite count could be because of the affinity of *P berghei* for immature Rbcs, with the ripple effect in the other parameters (that is the sharp rise in reticulocytes which accompanied the losses in PCV and haemoglobin). This is supported by the observation that TNF-α caused an

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increase in immature erythroid cells (BFU-E), but inhibited later-stage of erythropoiesis (CFU-E in the marrow) (Johnson *et al.,* 1988). The decreases in PCV and haemoglobin levels seem to follow increases in TNF-α. This cytokine has been shown to cause dyserythropoiesis (Clark and Chaudhiri 1988). But it appeared there was no bone marrow suppression because the reticulocyte population did not fall. Of course one would have to measure the erythropoietin levels to confirm this. Erythropoietin is produced by the kidney in response to anaemia and induces bone marrow response (Erslev *et al*, 1989). The occurrence of a clearly excessive anemia as seen early in *P. berghei* infection is not observed in mice with *P. chabaudi* infection (Akanmori *et al.,* 1994). Such an early onset of anemia associated with *P berghei* could be an adaptive phenomenon. This phenomenon is one of the strain specific characteristics, which can be understood when they are looked at concurrently in relation to the internal milieu of cytokines.

Plasma IL-10 was higher in the recipient than the control, however there seem not to be any significant difference in the mortality between the two groups although it appeared mortality was delayed in the IL-10 recipients. IL-10, an anti-inflammatory cytokine is known to have a positive feedback down regulation on the pro-inflammatory cytokine TNF-α (Hyde, 1992; Ho and Moore, 1994). Yet a high TNF-α was measured in the IL-10 recipient. This supports the observation that TNF-α increases in production during malaria infection (Miller *et al.,* 1994) and it could not be regulated by IL-10. Speculatively, IL-10 secretion is relatively late compared with other cytokines (Oppenheim, 1994), therefore before effective

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regulation could be done, the mice died. Also, another possible reason was that it was not secreted near the cells producing TNF-α since IL-10 acts locally.

Moreover, TNF-α is known to be protective when an appreciable level is detected (Clark and Chaudhri, 1988). This was the case in the first 7 days of inoculation but the situation changed when large amount of TNF-α was produced far exceeding the anti-inflammatory IL-10. This was when the mice started dying. This is in line with Clark and his colleagues' observation (Clark and Chaudhri, 1988).

Whether TNF-α has a positive association with the severity of malarial anaemia (Miller *et al.,* 1989a) could not be substantiated in this study. Erythropoietin (EPO) has been reported to prevent the decrease in hematocrit observed after TNF-α treatment of mice for five days (Johnson *et al.,* 1990). The negative correlation of TNF-α with hematocrit in this work supports that (figure 5 & 11). It appears therefore that for a well-defined mechanism of anemia in malaria, a spectrum of parameters should be determined simultaneously.

The role of immunoglobulin (constitutive and/or induced) in erythrophagocytosis was suggested as cause of anaemia during malaria infection (Roth and Herman, 1979). It was obvious that enhanced erythrophagocytosis occurred between the 7th and 12th day because Ig coated parasite-infected erythrocytes are readily ingested by activated macrophages (Shear *et al.*, 1979; Tosta & Wedderburn, 1980). That was when IgG2a recorded the highest peak value both in the test

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and control mice. This is consistent with work done by Waki (1994); IgG2a was detected on the surface membrane of *P. berghei*-infected erythrocytes after incubation with hyperimmune serum. Though, parasitaemia suppression by *anti-Plasmodial* IgG subtypes has been studied it is contrary to our finding. Whilst, IgG2a is reported to play a protective role in *Plasmodium berghei* NK65 infection in CBA mice (Akanmori *et al.,* 1994), its involvement in pathology of *P. berghei* Anka infection in BALB/c mice cannot be overlooked. The interest is centered between day 7 and 12 because that was the period appreciable mortality was observed (Fig. 6).

In addition, Bouharoun-Tayoun and Druilhe reported on the protective nature of IgG1 and IgG3, the two cytophilic isotypes and the non protective role of IgG2 and IgM in children and adults who have sustained a primary malarial attack (Bouharoun-Tayoun and Druilhe, 1992). In the rat, IgG1 is most efficient for opsonisation of erythrocytes for phagocytosis by macrophages, whilst IgG2a is more efficient in mediating antibody-dependent cytotoxicity (Miklos *et al.,* 1993)

Nevertheless, in the mouse, the profile seems not to be clear as different isotypes of antibody are involved in the process of phagocytosis (Ralph *et al.*, 1980). IgG3 was significant in the protection of BALB/c mice infected with 17X strain of *P. yoelii* (Majarian *et al.,* 1984). Hence the low level of IgG3 measured between day 7 and 12 with a significant increase in TNF-α in the BALB/c mice in both test and control tend to give credence to that work. This is consistent with a reported case in West Africa patients with severe malaria (Sarthou *et al.,* 1997).

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Likewise, IgM measured in both test and control, though higher in test animals, seems to follow the normal pattern: highest on day 3 and lowest on day 14. This was in agreement with Eling, (1980) that the spleen-dependent immuno-pathological response was involved in the mortality and morbidity of a *P. berghei* infection in mice. These thymus-dependent host responses to infection have been demonstrated by other authors, when blood monocyte response was depressed in infected T-cell depleted mice. They suggested that activation of T-cells during infection may enhance the differentiation of stem cells into monocytes which are then recruited by the spleen (Jayawardena, 1977). Again, Wyler and Gallin (1977) have demonstrated the presence of a monocyte-chemotactic factor in the spleens of immunologically intact mice infected with *P. berghei*. Though the spleen plays a vital role in malaria by contributing to innate resistance and acquired immunity yet both test and control mice died off almost at the same period regardless of the presence of IL-10.

Moreover, spleen cells were cultured to see the extent of proliferation. The proliferative response initially was higher for mitogen-driven culture than the antigen-driven culture. However, on day 3 the control response was higher than antigen-driven response. It suggests that the cultured spleen cells might have encountered the malarial antigen *in vivo*, so alone in *in vitro* culture they induced response. With time, antigen-driven gave a response equaling that of the mitogen-driven response. This reiterates the fact that there was development of memory T cells, which are important in subsequent infections. Also, the results

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showed that the T cells were viable because of their ability to incorporate β-thymidine and underscores the importance of the spleen in the induction of antimalarial immunity (Figure 8).

The IFN-γ measured in both test and control mice, seemed to be higher in test than control. This observation agrees with the TNF-α concentration measured. It means the relationship between antibodies (immunoglobulins) and cytokines should be well exploited. It would have been useful to measure levels of other anti-inflammatory cytokines in order to verify the effect of exogenous IL-10 on these anti-inflammatory cytokines. Again, the erythrophagocytosis with the exogenous IL-10 or combination with other cytokine should be looked at again. It is likely that the severe malarial anaemia might have a specific pathology as suggested (Kurtzhals *et al.*, 1997).

4.2 CONCLUSION

- The effect of injections of rmIL-10 on survival, course of parasitaemia, haematological and immunological changes in BALB/c mice infected with *P. berghei* provided the following observations:
- IL-10 was higher in the recipient than the control mice
- There was no difference in the parasitaemia or hematological parameters measured in the two groups' (reticulocyte counts, haemoglobin, PCV).
- There was no difference in the mortality between the two groups though it appeared to be delayed in the rmIL-10 recipient mice than control.

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- IL-10 is known to have a positive feedback down regulation on TNF-α but a high TNF-α was measured in the rmIL-10 recipient mice.
- High measurement in rmIL-10 recipient confirms that TNF-α increases in production during malaria infection.
- This confirms the initial hypothesis that inadequate IL-10 an anti-inflammatory cytokine is responsible for the anaemia in acute murine malaria.

The observations suggest that further work should be done with a higher dose of rmIL-10 and the simultaneous measurement of other pro- and anti-inflammatory cytokines in order to fully determine the pathogenesis of severe malarial anaemia.

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REFERENCES

Adams, L. B., Hibb, J. B., Taintor, R. R. and Krahenbuhl, J. L. (1990). Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. J. Immunol. **144:** 2725-2729.

Akanmori, B. D., Kawai, S., Suzuki, M. (1996). Recombinant mouse IL-6 boosts specific serum *anti-plasmodial* IgG subtitres and suppresses parasitaemia in *Plasmodium chabaudi chabaudi* infection. Parasite Immunol. **18:** 193-199.

Akanmori, B. D., Waki, S., Suzuki, M. (1994). Immunoglobulin G2a isotype may have a protective role in *Plasmodium berghei NK65* infection in immunized mice. Parasitol. Res. **80:** 638-641.

Bouharoun-Tayoun, H. and Druilhe, P (1992). *Plasmodium falciparum* malaria: evidence for an isotype imbalance which may be for delayed acquisition of protective immunity. Infect. Immun., **60(4):** 1473-1481.

Bowman, J. B. R., Grant, P. T. and Kermack, W. O. (1960). The metabolism of *Plasmodium berghei* 1. Preparation of the erythrocyte forms of *p. berghei* separated from the host cell. Exp. Parasitol. **9:** 131-136.

Briere, F., Servet-Delprat, C., Bridon, J-M., Saint-Remy, J-M. and Banchereau, J (1994). Human Interleukin-10 induces naïve sIgD+ B cells to secrete IgG1 and IgG3. J. Exp. Med. **179:** 757-762.

Bruce-Chwatt, L. J. (1980). Essential Malariology. 1st edition, William Heinemann Medical Books ltd., pp. 31-32.

Carter, R. (1973). Enzyme variation in *Plasmodium berghei* and *Plasmodium vinckei*. Parasitol. **66:** 297-307.

Chomarat, P., Rissoan, M.-C., Banchereau, J. and Miossec, P (1993). Interferon gamma inhibits interleukin 10 production by monocytes. J. Exp. Med. **177:** 523-527.

Clark, I. A. and Allison, A. C. (1974). *Babesia microti* and *Plasmodium berghei yoelii* infections in nude mice. Nature, **252:** 328-331.

Clark, I. A. and Chaudhri, G. (1988). Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. Br. J. Haematol. **70:** 99-103.

Clark, I. A. (1987). Cell mediated immunity in protection and pathology of malaria. Parasitol. Today **3:** 300-305.

University of Ghana http://ugspace.ug.edu.gh

Clark, I. A. and Hunt, N. H. (1983). Evidence of reactive oxygen intermediates causing hemolysis and parasite death in malaria. Infect. Immun. **39:** 1-6.

Clark, I. A., Cowden, W. B., Butcher, G. A. and Hunt, N. H. (1987a). Possible role of tumour necrosis factor in the pathology of malaria. Am. J. Pathol. 129-135.

Clark, I. A., Hunt, N. H. and Cowden, W. B. (1987b). Immune responses in parasitic infections: Immunopathology of malaria. In: Soulsby, E.J.L (Ed.) Immunology, Immunopathology and Immunoprophylaxis of Parasitic Infections. CRC press. London pp.1-34.

Cohen S. and Warren, K. S. (1982). The Immunology of Parasitic Infections". 2nd Ed. Blackwell Scientific, Oxford. pp 368-396.

Corradin, S. B., Fasel, N., Buchmuller-Rouiller, Y., Ransijn, A., Smith, J. and Mauel, J. (1993). Induction of macrophage nitric oxide production by interferon-γ and tumour necrosis factor-α is enhanced by interleukin-10. Eur. J. Immunol. **23:** 2045-2048.

Cox, H. W., Schroeder, W. F. and Ristic, M. (1966). Hemagglutination and erythrophagocytosis associated with the anemia of *Plasmodium berghei* infections in rats. J. Protozool. **14:** 327-332.

Cox, H. W. (1966). A factor associated with anemia and immunity in *Plasmodium knowlesi* infections. Milit. Med. **131(suppl.):** 1195-1200.

de Souza, B. J., Williamson, K. H., Otani, T and Playfair, J. H. L. (1997). Early gamma interferon responses in lethal and nonlethal murine blood-stage malaria. Infect. Immun. **65(5):** 1593-1598.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M.G., te Velde, A., Figdor, C.,Johnson, K., Kastelein, R., Yssel, H and de Vries, J. E. (1991). Interleukin-10 and viral IL-10 strongly reduces antigen-specific human Tcell proliferation by diminishing the antigen-presenting capacity of monocytes via down regulation of class-II major histocompartibility complex expression. J. Exp. Med. **174:** 915-919.

Defrance, T., Vanbervliet, B., Briere, F., Durand, I., Rousset, F. and Banchereau, J. (1992). Interleukin-10 and transforming growth factor β cooperate to induce anti-CD40- activated naïve human B cells to secrete imunoglobulin A. J. Exp. Med. **175:**671-682.

Ding, L. and Shevach, E. M. (1992). IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage co-stimulatory function. J. Immunol. **148:** 3133-3139.

University of Ghana http://ugspace.ug.edu.gh

Eling, W. M. C. (1980). Role of spleen in Morbidity and Mortality of *Plasmodium berghei* infection in mice. Infect. Immun. **30(3):** 635-641.

Erslev, A. J., Schuster, S., and Caro, J. (1989). Erythropoietin and its clinical promise. Eur. J. Haematol. **43:** 367-373.

Fife, E. H. Jr., Von Doenhoff, A. E. Jr., and D'Antonio, L. E. (1972). *In vitro* and *in vivo* studies on a lytic factor isolated from *Plasmodium knowlesi.* Proc. Helminthol. Soc. Washington **39(special issue):** 373-382.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. H. and O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. J. Immunol. **147:** 3815-22.

Frankenburg, S. and Greenblatt, C. L. (1977). Cellular changes in the bone marrow of *Plasmodium berghei* infected mice. J. Protozool. **24:** 66A (Abstract).

Fulton, J. D. and Spooner, D. F (1956). The *in vitro* respiratory metabolism of erythrocytic forms of *Plasmodium berghei.* Exp. Parasitol.**5:** 59-76.

Gajewski, T. F., and Fitch, F.W. (1988). Anti-proliferative effect of IFN- gamma in immune regulation. 1. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J. Immunol. **140:** 4245-4252.

Go, N. F , Castle, B. E., Barrett, R., Kastelein, R., Dang, W., Mosmann, T. R., Moore, K. W. and Howard, M. (1990). IL-10, a novel B cell stimulatory factor: unresponsiveness of X-chromosome-linked immunodeficient B cells. J. Exp. Med. **172(6):** 1625-1631.

Grau, G. E., Fajardo, L. F., Piguet, P F., Allet, B., Lambert, P. H. and Vassalli, P. (1987). Tumor necrosis factor (cachetin) as an essential mediator in murine cerebral malaria. Science **237:** 1210-1212.

Grimble, R. F. and Tapia, P S. (1998). Modulation of pro-inflammatory cytokine biology by unsaturated fatty acids. Z Ernahrungswiss(XTU). **37(suppl.):** 57-65.

Green, S. J., Mellouk, S., Hoffman, S. L., Meltzer, M. S. and Nacy, C. A. (1990). Cellular mechanism of non specific immunity to intracellular infection: cytokine-induced synthesis of toxic nitrogen oxides from L-arginine by macrophages and hepatocytes. Immunol. letters **25:** 15-20.

Gyan, B., Troye-Blomberg, M., Perlmann, P. and Bjorkman, A. (1994). Human monocytes cultured with and without interferon gamma inhibit *Plasmodium falciparum* parasite growth *in vitro* via secretion of reactive nitrogen intermediates. Parasite Immunol. **16(0):** 1-5.

University of Ghana http://ugspace.ug.edu.gh

Hawking, F and Gamnage, K. (1962). Chloroquine resistance produced in *Plasmodium berghei*. Trans. Roy. Soc. Trop. Med. Hyg. **56:**263-267.

Helfgott, D. C., May, L. T., Sthoeger, Z., Tamm, I., Sehgal, P. B. (1987). Bacterial lipopolysaccharide (endotoxin) enhances expression and secretion of beta 2 interferon by human fibroblasts. J. Exp. Med. **186:** 1300-1309.

Hilbert, D. M., Cancro, M. P., Scherle, P. A., Nordan, R. P., Van Snick, J., Gerhard, W. and Rudikoff, S. (1989). T cell derived IL-6 is differentially required for antigen- specific antibody secretion by primary and secondary B cells. J. Immunol. **143:** 4019-4024.

Ho, A. S.-Y., and Moore, K. W. (1994). Interleukin-10 and its receptor. Therap. Immunol. **1:** 173-185.

Ho, AS-Y., Liu, Y., Khan, T. A., Hsu, D-H., Baszan, J. F and Moore, K. W. (1993). A receptor for Interleukin-10 is related to interferon receptors. Proc. Natl. Acad. Sci. USA. **90:** 11267-71.

Hodgkin, P. D., Bond, M. W., O'Garra, A., Frank, G., Lee, F., Coffman, R.L., Zlotnik, A. and Howard, M. (1988). Identification of IL-6 as a T cell-derived factor that enhances the proliferative response of thymocytes to IL-4 and phorbol myristate acetate. J. Immunol. **141:** 151-157.

Hoffman, S. L., Oster, C. N., Plowe, C. V., Woollett, G. R., Beier, J. D., Wirtz, R. A., Hollingdale, M. R. and Mugami, M. (1987). Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications. Science 237(4815): 639-642.

Holz, G. G., Jr., Beach, D. H. and Sherman, P. W. (1977). Octadecanoic fatty acids and their association with hemolysis in malaria. J. Protozool. **24:** 566-574.

Homewood C. A. 1978 In: Killick- Kendrick and Peters W. (Eds.) Rodent Malaria. Academic Press pp. 345-386.

Homewood C. A. and Neame, K. D. (1980). Biochemistry of malaria parasites, In: Krier, J. P. (Ed.) Malaria (Vol. 1) Academic Press pp. 345-386.

Horii, Y., Muraguchi, A., Suematu, S., Matsuda, T., Yoshizaki, K., Hirano, T. and Kishimoto, T. (1988). Regulation of BSF-2/IL-6 production by human mononuclear cells. Macrophage- dependent synthesis of BSF-2/IL-6 by T cells. J. Immunol. 141(5): 1529-1535.

Huang, S., Hendriks, W., Althage, A., Hemi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R. M. and Aguet, M. (1993). Immune responses in mice that lack the interferon gamma receptor. Science **259:** 1742-1745.

University of Ghana http://ugspace.ug.edu.gh

Hyde, M. R. (1992). Immunology 2nd edition Harwal publishing. pp. 74.

Jacobs, R. L. (1965). Selection of strains of *Plasmodium berghei* resistance to quinine, chloroquine and pyrimethamine. J. Parasitol **51:**481-482.

Jacobs, R. L., Alling, D. W. and Cantrell, W. F. (1963). An evaluation of antimalarial combination against *Plasmodium berghei* in the mouse. J. Parasitol. **49:**920-925.

Jayawardena, A. N., Targett, G. A. T., Carter, R., Leuchars, E. and Davies, A. J. S. (1977). The immunological responses of CBA mice to *P yoelii* 1. General characteristics of the effects of T cell deprivation and reconstitution with thymus grafts. Immunology **32:** 849-856.

Jilly, P., and Nkrumah, F. K. (1965). A survey of anemia in children in the Korle Bu Hospital, with special reference to malaria. Trop. Dis. Bull. **62:** 133-134.

Johnson, C. S., Chang, M. J. and Furmanski, P. (1988). *In vivo* hematopoietic effects of TNF in normal and erythroleukemic mice: characterization and therapeutic applications. Blood **72 :** 1875-1880.

Johnson, C.S., Cook, C.A. and Furmanski, P. (1990). *In vivo* supppression of erythropoiesis by TNF: reversal with exogenous EPO. Exp. Hematol. **18:** 109-113.

Jones, K. R., Contrel, B. J., Targett, G. A. T. and Playfair, J. H. L. (1989). Killing of *Plasmodium falciparum* by human monocyte-derived macrophages. Parasite Immunol. **11:** 105-116.

Killick-Kendrick, R and Peters, W. (1978). Rodent malaria. Academic press, London and New York. pp 125-145, 167-189.

Klein, R. A., Laser, H., Kemp, P., Miller, N. and Lander, D. (1977). Biochemical mechanisms in malaria. J. Protozool. **24:** 39A-40A (Abstract).

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. (1993). Interleukin 10-deficient mice develop chronic entericolitis. Cell **75:** 263-274.

Kurtzhals,J. A, Adabayeri, V., Goka, B Q., Akanmori, B. D., Oliver-Commey, J. O., Nkrumah, F. K., Behr, C., Hviid, L. (1998). Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. Lancet **351:**1768-1772.

Laudan, I. and Killick-Kendrick, R. (1966). Rodent plasmodia of the Republique Centrafricaine: The sporogony and tissue stages of *Plasmodium chabaudi* and *P. berghei* Yoelii. Trans. Roy. Soc. Trop. Med. Hyg. **60:**633-649.

University of Ghana http://ugspace.ug.edu.gh

Liew, F W. and Cox, F E. G. (1991). Non-specific defense mechanism: The role of nitric oxide. In Ash, C. and Galagher, R. B. (eds) Immunoparasitology Today, Elsevier trends, Cambridge p 17-21.

Liu, Y., Wei, SH-Y., Ho, AS-Y., de Waal Malefyt, R. and Moore, K. W. (1994). Expression cloning and characterization of a human Interleukin-10 receptor. J. Immunol. **152:** 1821-1829.

Lucas, R., Juillard, P., Decoster, E., Redard, M., Burger, D., Donati, Y., Giroud, C., Monso-Hinard, C., De Kesel, T., Buurman, W. A., Moore, M. W., Dayer, J.M., Fiers, W., Bluethmann, H. and Grau, G. E. (1997). Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malarial. Eur. J. Immunol. **27:** 1719-1725.

Ludford, C. G., Corwin, R. M., Cox, H. W. and Sheldon, T. A. (1969). Resistance of ducks to a *Plasmodium sp.* induced by a filterable agent. Milit. Med. **134(suppl):** 1276-1283.

Macneill, I., Suda, T., Moore, K., Mosmann, T. and Zlotnik, A. (1990). Interleukin10: a novel growth factor for mature and immature thymocytes. J. Immunol. **145:** 4167-4177.

Maggio-Price, L., Brookhoof, D., Weiss, L. (1985). Changes in hematopoietic stem cells in bone marrow of mice with *Plasmodium berghei* malaria. Blood **66:** 1080-1085.

Majarian, W. R., Daly, T. M., Weidanz, W. P. and Long, C. A. (1984). Passive immunization against murine malaria with an IgG3 monoclonal antibody. J. Immunol. **132(6):** 3131-3137.

Marsh, K., Newton, R. J. C. C., Winstanley, P A., Were, J. B. and Fenelle, K. (1991) In: Targett, G. A. T. (Ed.) Malaria waiting for the vaccine, Wiley pp.31-43.

McGhee R. B. and Loftis W. E. (1968). A filterable, proliferating factor simulating autoimmunity in malarious and nonmalarious ducklings. Exp. Parasitol. **22:** 299-308.

McGhee, R. B. (1970). Avian malaria In: Jackson, J. G; Herman, R. and Singer, I. (Eds). Immunity to parasitic animals (Vol. 2). Appleton-Centory-Crofts. New York.pp 331- 369.

McGuire Jr., R. W. and Haines, H. B. (1985). *Plasmodium berghei*: The influence of blood volume changes on the malaria-induced anemia in Balb/c mice. Parasitenkunde **71:** 305- 312.

University of Ghana http://ugspace.ug.edu.gh

Mellouk, S., Green, S. J., Nacy, C. A. and Hoffman, S. L. (1991). IFN-gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. J. Immunol. **146:** 3971-3976.

Mellouk, S., Hoffman, S. L., Liu, Z.-Z., De La Vega, P., Billiar, T. R. and Nussler, A. K. (1994). Nitric oxide-mediated *antiplasmodial* activity in human and murine hepatocytes induced by gamma interferon and the parasite itself: enhancement by exogenous tetrahydrobiopterin. Infect. Immun. **62:** 4043-4046.

Miklos, K., Tolnay, M., Bazin, H. and Medgyesi T. (1993). Rat IgG subclasses mediating binding and phagocytosis of target cells by homologous macrophages. Mol. Immunol. **30:** 1273-1278.

Miller, K. L., Schooley, J. C., Smith, K. L., Kullgren, B., Mahlmann, L. J. and Silverman, P. H. (1989a), Inhibition of erythropoiesis by a soluble factor in murine malaria. Exp. Haematol.**17:** 379-385.

Miller, K. L., Silverman, P. H., Kullgren, B. and Mahlmann, L. J. (1989b). Tumor necrosis factor alpha and anemia associated with murine malaria. Infect. Immun. **57(5) :** 1542-1546.

Miller, L. H., Good, M. F. and Milon, G. (1994) Malaria Pathogenesis. Science **264:** 1878-1883.

Mota, M. M., Brown, K. N., Holder, A. A. and Jarra, W. (1998). Acute *Plasmodium chabaudi chabaudi* malaria infection induces antibodies which bind to the surfaces of parasitized erythrocytes and promote their phagocytosis by macrophages in vivo. Infect. Immun.. **66(9):** 4080-4086.

Murphy, J. R. and Logie, P S. (1984). Host defenses in murine malaria: humoral immunity to *Plasmodium berghei* in mice. Am. J. Trop. Med. Hyg. **33(3):** 347-356.

Murray,H. W. (1981). Susceptibility of leishmania to oxygen intermediates and killing by normal macrophage. J. Exp. Med. **153:** 1302-1315.

Nagarajan, K. (1964). Pyruvate and lactate levels in relationship to the nicotinamide-adenine dinucleotide levels in malarial parasites *(plasmodium berghei*). Biochimica et Biophysica Acta**. 93:**176-179.

Oppenheim. J. J. (1994) In: Angus Thomson (Ed.) The Cytokine Handbook, Academic Press, pp 145-168, 223-237,265-318.

Orago, A. S. S. and Facer, C. A. (1993). Cytokine-induced inhibition of *Plasmodium falciparum* erythrocytic growth *in vitro*. Clin. Exp. Immunol. **91:** 287-297.

University of Ghana http://ugspace.ug.edu.gh

Othoro, C., Lal; A. A., Nahlen, B., Kolch, D., Orago, S. S., and Udhayakumar, V (1999). A low Interleukin-10 Tumor Necrosis Factor-α ratio is associated with malaria anemia in children residing in a holoendemic malaria region in Western Kenya. J. Infect. Dis. **179:** 279-282.

Owens, J. M., Gallagher, A. C. and Chambers, T. J. (1996). IL-10 modulates formation of osteoclasts in murine hemopoietic cultures. J. Immunol. **157:** 936-940.

Paul, W. E. and Seder, R. A. (1994). Lymphocyte responses and cytokines. Cell **76:** 241-251.

Peters, W. (1965). Drug resistance in *Plasmodium berghei vincke* and Lips, 1948. I. Chloroquine resistance. Exp. Parasitol.**17:** 80-89.

Perrin, L. H., Mackey, L. J. and Miescher, P. A. (1982). The haematology of malaria in man. Semin. Hematol. **19:** 70-82.

Philip, R., and Epstein, L. B. (1986). Tumour necrosis factor as an immunomodulator and mediator of monocytes cytotoxicity induced by itself, IFN-γ and IL-1. Nature **33:** 86-89.

Powell, R. D (1966). The chemotherapy of malaria. Clin. Pharmacol. Therap. **7:**48-76.

Ralph, P., Nakoinz, I., Diamond, B. and Yelton, D. (1980). All classes of murine IgG antibody mediate macrophage phagocytosis and lysis of erythrocytes. J. Immunol. **125:**1885-1888.

Rencricca, N. J., Stout, J. P. and Coleman, R. M. (1974). Erythropoietin production in virulent malaria. Infect. Immun. **10(4):** 831-833.

Rhodes-Feuillette, A., Bellosguardo, M., Druihle, P., Ballet, J. J., Chousterman, S., Canivet, M. and Peries, J. (1985). The interferon compartment of the immune response in human malaria. II. Presence of serum interferon gamma following the acute attack. J. Interf. Res. **5:** 169-178.

Roitt, I. M. (1988). Essential Immunology, 3rd edition, Blackwell Scientific Publication. Oxford. pp 26-30.

Roth, R. L. and Herman R. (1979). *Plasmodium berghei*: Correlation of in vitro erythrophagocytosis with the Dynamics of early onset Anemia and Reticulocytosis in mice. Exp. Parasitol. **47:** 169-179.

Rousset, F., Garcia, E., Defrance, T., Peronne, C., Hsu, D-H, Kastelein, R., Moore, K. W, and Banchereau, J. (1992). IL-10 is a potent growth and

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differentiation factor for activated human B lymphocytes. Proc. Natl. Acad. Sci. USA **89:**1890-1893.

Sarthou, J. L., Angel, G., Aribot, G., Rogier, C., Dieye, A., Toure-Balde, A., Diatta, B., Seignot, P. and Roussilhon, C. (1997). Prognostic value of anti-*Plasmodium falciparum* –specific immunoglobulin G3, cytokines, and their soluble receptors in West Africa patients with severe malaria. Infec. Immun. **65:**3271-3276.

Schneider, M. D. (1968). Characteristics and cross-resistance patterns of chloroquine-resistant *Plasmodium berghei* infections in mice. Exp. Parasitol.**23:** 22-50.

Sheagren, J. N., Tobie, J. E., Fox, L. M. and Wolff, S. M. (1970). Reticuloendothelial system phagocytic function in naturally acquired human malaria. J. Lab. Clin. Med. **75:** 481-487.

Shear, H. L., Nussenzweig, R. S. and Biance, C. (1979). Immune phagocytosis in murine malaria. J. Exp. Med. **149:** 1288-1298.

Sidman, C. L., Marshall, J. D., Shultz, L. D., Gray, P W. and Johnson, H. M. (1984). Interferon gamma is one of several direct B cell maturing lymphokines. Nature **309:** 801-804.

Singer, I., (1954) The course of infection with *Plasmodium berghei* in inbred CF 1 mice. J. Infect. Dis **94:** 237-240.

Smeland, E. B, Blomhoff, H. K., Funderud, S., Shalaby, M. R. and Espevik, T. (1989). Interleukin 4 induces selective production of interleukin 6 from normal human B lymphocytes. J. Exp. Med. **170(4):** 1463-1468.

Stevenson, M. M., Lyanga, J. J. and Skanmene, E. (1982). Murine malaria: Genetic control of resistance to *Plasmodium chabaudi*. Infect. Immun. **38 (1):** 80-88.

Tan, J. C., Indelicato, S., Narula, S. K., Zavodny, P. J. and Chou, C. C. (1993). Characterization of IL-10 receptors on human and mouse cells. J. Biol. Chem. **268:** 21053-9.

Takatsuki, F., Okano, A., Suzuki, C., Chieda, R., Takahara, Y., Hirano, T., Kishimoto, T., Hamuro, J. and Akiyama, Y. (1988). Human recombinant IL-6/B cell stimulatory factor 2 augments murine antigen secific antibody responses *in vitro* and *in vivo*. J. Immunol. **141:** 3072-3077.

Taverne, J., Tavernier, J., Fiers, W. and Playfair, J. H. L. (1987). Recombinant tumour necrosis factor inhibits malaria parasites *in vivo* but not *in vitro*. Clin. Exp. Immunol. **67:**1-4.

University of Ghana http://ugspace.ug.edu.gh

Taylor-Robinson, A. W., Philips, R. S., Severn, A., Moncada, S. and Liew, F Y. (1993). The role of Th1 and Th2 cells in a rodent malaria infection. Science, **260:**1931-1934.

Thompson, P. E., Olszewski, B. J., Elslager, E. F and Worth, D. F. (1963). Laboratory studies on 4,5-diamino-1-(p-chlorophenyl)-1,2-dihydro-2,2-dimethyl-5-triazine pamoate(Cc1501) as a repository antimalarial drug. Am. J. Trop. Med. Hyg. **13:** 209-213.

Thompson-Snipes, L., Dhar, V., Bond, M. W., Mosmann, T. R., Moore, K. W. and Rennick, D. M. (1991). Interleukin-10: a novel stimulatory factor for mast cells and their progenitors. J. Exp. Med. **173:** 507.

Todorovic, R., Ferris, D and Ristic, M. (1967). Roles of the spleen in acute p*lasmodial* and *babesial* infections in rats. Exp. Parasitol. **21:** 354-372.

Topley, E., Bruce-Chwatt, L. J. and Dorrell, J. (1970). Haematological study of a rodent malaria model. J. Exp. Med. Hyg. **73:**1-8.

Tosta, C. E. and Wedderburn, N. (1980). Immune phagocytosis of *Plasmodium yoelii*-infected erythrocytes by macrophages and eosinophils. Clin. Exp. Immunol. **42:** 114-120.

Vercelli, D., Jabara, H. H., Arai, K., Yokota, T. and Geha, R. S. (1989). Endogenous interleukin 6 plays an obligatory role in interleukin 4-dependent human IgE synthesis. Eur. J. Immunol. **19(8):** 1419-1424.

Villeval, J-L., Lew, A. and Metcalf, D. (1990). Changes in hemopoietic and regulator levels in mice during fatal and non fatal malarial infections. 1. Erythropoietic populations. Exp. Parasitol. **71:** 364-374.

Vincke, I. H., and Lips, H. 1948. Un nouveau plasmodium d'un rongear sauvage due Congo Plasmodium berghei n. sp. Annales de societe belge de Medicine Tropicale 28: 97-104.

Wakelin, D. (1984). Immunity to parasites (How animals control parasitic infections). Castlefield Press. East KilbrideScotland. 1st ed. pp33.

Waki, S. (1994). Antibody-dependent neutrophil-mediated parasite killing in non-lethal rodent malaria. Parasite Immunol. **16:** 587-591.

Waki, S., Tamara, J., Imanaka, M., Ishikawa, S. and Suzuki, M. (1982). *Plasmodium berghei:* Isolation and maintenance of an irradiated attenuated strain in the nude mouse. Exp. Parasitol. **53:** 335-340.

Waki, S., Uehara, S., Kanabe, K., Ono, K., Suzuki, M. and Nariuchi, H. (1992). The role of T cells in pathogenesis and protective immunity to murine malaria. Immunology, **75:** 646-651.

University of Ghana http://ugspace.ug.edu.gh

Walliker, D. (1989). Genetic recombination in malaria parasites. Exp. Parasitol. **69:** 303-309.

Weatherall, D. J. (1988). In: Wernsdorfer, W. H. and McGregor, I. (Eds). The anaemia of malaria. In "Malaria, Principles and Practice of Malariology" Churchill Livingstone, London. pp 735-751.

Wellde, B. T., Briggs, N. T. and Sadun, E. H. (1966). Susceptibility to *Plasmodium berghei*: Parasitological, biochemical and haematological studies in laboratory and wild mammals. Milit. Med. **131(suppl.):** 839-869.

Wenisch, C., Parschalk, B., Narzt, E., Looareesuwan, S. and Graniger, W. (1995). Elevated serum levels of IL-10 and IFN-γ in patients with acute *Plasmodium falciparum* malaria. Clin. Immunol. Immunopathol. **74:** 115-117.

Woodruff, A. W., Ansdell, V. E. and Pettitt, L. E (1979). Causes of anaemia in malaria. Lancet **1:** 1055-1057.

Wyler, D. J. and Gallin, J. I. (1977). Spleen-derived mononuclear cell chemotactic factor in malaria infections: a possible mechanism for splenic macrophage accumulation. J. Immunol. **118:** 478-484.

Yoeli, M. (1965). Studies on *Plasmodium berghei* in nature and under experimental conditions. R. Soc.Trop.Med.Hyg. (ordinary meeting). pp255-271.

Zuckerman, A. (1964). Autoimmunization and other types of indirect damage to host cells as factors in certain protozoan diseases. Exp. Parasitol. **15:** 138-183.

Zuckerman, A. (1966). Recent studies on factors involved in malaria anemia. Military medicine **131(suppl.):** 1201-1216.

Zuckerman, A. and Yoelii, M. (1954). Age and sex factors influencing *Plasmodium berghei* infections in intact and splenectomised rats. J. Infect. Dis. **49:**225-236.

Zuckerman, A., Spira, D. T. and Ron, N. (1973). A quantitative study of phagocytosis in the spleen of rats infected with *Plasmodium berghei*. In: Zuckerman, A and Weiss, D.W (Eds.). Vol.1 Academic Press. N.Y pp79-115.

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APPENDIX 1

TABLE 1

AVERAGE HAEMATOLOGICAL DATA FOR THE IL-10 INJECTED MICE AND CONTROL

I.D	DayPost Inocula-tion	Parasita-emia %	Hb mg/l	Retics %	PCV %
Tf-210800	0	0	10.25	2.4	52
Tf-240803	3	0	10.976	1.86	50.2
Tf-260805	5	0.178	9.852	1.54	49.4
Tf-280807	7	6.998	9.754	3.76	49.2
Tf-020912	12	29.73	5.876	26.2	36.33
Tf-040914	14	32.465	6.19	27.8	37
Cf-210800	0	0	10.576	3.22	49.8
Cf-240803	3	0	10.226	1.62	48.6
Cf-260805	5	0.182	10.45	1.66	49.2
Cf-280807	7	10.552	9.954	7.16	44.6
Cf-020912	12	21.025	6.437	25.3	41
Cf-040914	14	31.365	6.31	27.1	36

TABLE 2 **PERCENTAGE SURVIVAL FOR IL-10 TREATED AND CONTROL MICE %**

Post Inoc.	0	3	5	7	12	14	18	19
Sur.Time(Cf)	100	100	100	100	40	20	20	0
Sur.Time(Tf)	100	100	100	100	60	40	20	0

KEY:

Sur Time (Cf) - Survival time for Control Mice

Sur Time (Tf) - Survival time for Treated Mice

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APPENDIX 2

TABLE 3				
Mean splenic lymphocytes proliferation/# of cells with Beta uptake counted per min				
Days	**I.D**	**Control**	**PHA**	**Prbc**
3	Ts-3	503	690	216
5	Ts-5	221	210	538
7	Ts-7	409	426	462
14	Ts-14	213	189	225
3	Cs-3	270	674	228
5	Cs-5	189	293	510
7	Cs-7	376	359	396
14	Cs-14	195	266	222
	Cw-	139	164	311

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APPENDIX 3

TABLE 4

MEAN IFAT TITRE VALUES FOR IL-10 TREATED AND CONTROL MICE

I.D	code	IgG	IgG1	IgG2a	IgG2b	IgG3	IgM	P.I/DAYS
Ts-240803	Ts-3	160	256	2080	544	160	12384	3
Ts-260805	Ts-5	10240	640	16384	10240	8704	8704	5
Ts-280807	Ts-7	10240	1024	16384	160	160	1024	7
Ts040914	Ts-14	4096	16384	4096	16384	1024	1024	14
Cs-240803	Cs-3	256	1024	2080	256	544	8704	3
Cs-260805	Cs-5	16384	2080	16384	1024	2560	4096	5
Cs-280807	Cs-7	4096	1024	4096	1024	640	160	7
Cs-040914	Cs-14	256	64	64	16	4	256	14

I.D	code	IgG	IgG1	IgG2a	IgG2b	IgG3	IgM	P.I/DAYS
Ts-240803	Ts-3	160	256	2080	544	160	12384	3
Cs-240803	Cs-3	256	1024	2080	256	544	8704	3
Ts-260805	Ts-5	10240	640	16384	10240	8704	8704	5
Cs-260805	Cs-5	16384	2080	16384	1024	2560	4096	5
Ts-280807	Ts-7	10240	1024	16384	160	160	1024	7
Cs-280807	Cs-7	4096	1024	4096	1024	640	160	7
Ts040914	Ts-14	4096	16384	4096	16384	1024	1024	14
Cs-040914	Cs-14	256	64	64	16	4	256	14

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APPENDIX 4

		E L IS A DATA	(pg/ml)			
TABLE 5						
CYTOKINE LEVELS IN BOTH SERA AND CELL CULTURE						
SUPERNATANT SAMPLES						
SERA SAMPLES						
DAY(P.I)	I.D	IL-10	TNF-alpha	IFN-gamma		
3	Ts-1	0	46.8	2.21		
5	Ts-3	16.38	23.4	25		
7	Ts-5	254.76	70.2	510		
14	Ts-7	400	625	9.68		
DAY(P.I)	I.D	IL-10	TNF-alpha	IFN-gamma		
3	Cs-1	0	60	5.25		
5	Cs-3	12.48	60	6.64		
7	Cs-5	10	76.9	4.7		
14	Cs-7	64.06	117	28		
0	gative cont	0	10.05	3.87		
CELL CULTURE SUP	**IL-10 LEVELS(pg/ml)**					
I.D	CONTROL	PHA	Prbcs			
Ts-1	0	2.5	333			
Ts-3	0	50	100			
Ts-5	0	90	36.06			
Ts-7	0	40	773			
Cs-1	0	1150	340			
Cs-3	0	170	1150			
Cs-5	0	670	9.98			
Cs-7	0	48.5	590			
CTL	0	1.66	0			
CELL CULTURE SUPERNATES TNF-alpha LEVELS(pg/ml)						
I.D	CONTROL	PHA	Prbcs			
Ts-1	11.37	60	3000			
Ts-3	10.7	55	150			
Ts-5	17.38	9.36	22.73			
Ts-7	4.68	10	18.72			
Cs-1	20.73	230	3000			
Cs-3	20.73	90	2525			
Cs-5	100	3.34	22.06			
Cs-7	5.35	2.67	13.4			
Control	35	4.68	19.4			
CELL CULTURE SUPERNATES IFN-gamma LEVELS(pg/ml)						
I.D	CONTROL	PHA	Prbcs			
Ts-1	305	130	1075			
Ts-3	250	925	880			
Ts-5	552	1205	1205			
Ts-7	12	410	570			
Cs-1	434	405	1205			
Cs-3	45	42	725			
Cs-5	50	133	50			
Cs-7	16	880	880			
Control	3.32	1.4	0			

APPENDIX 4

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Contd **APPENDIX 4**

TABLE 6 THE VARIOUS STANDARD CURVE VALUES						
IL-10 STANDARD VALUES			Absorb. at 450nm	Conc. pg/ml		
1st reading	2nd readin	mean	mean			
0	-0.004	0	0	0		
0.035	0.04	0.0375	0.0375	15.6		
0.075	0.064	0.0695	0.0695	31.2		
0.13	0.13	0.13	0.13	62.5		
0.252	0.273	0.2625	0.2625	125		
0.489	0.475	0.482	0.482	250		
0.957	1	0.9785	0.9785	500		
1.648	1.786	1.717	1.717	1000		
TNF-alpha STANDARD VALUES			Absorb. at 450nm	Conc. pg/ml		
1st reading	2nd readin	mean	mean			
0	0.004	0.002	0.002	0		
0.035	0.035	0.035	0.035	23.4		
0.063	0.065	0.064	0.064	46.9		
0.124	0.12	0.122	0.122	93.8		
0.23	0.22	0.225	0.225	187.5		
0.449	0.439	0.444	0.444	375		
0.781	0.812	0.7965	0.7965	750		
1.436	1.414	1.425	1.425	1500		
IFN--gamma STANDARD VALUES						Conc. pg/ml
FOR SERUM			FOR SUPERNATANTS			
1st readinc	2nd readin	mean	1st readinc	2nd readin	mean	
-0.001	-0.01	0	-0.002	-0.003	0	0
0.039	0.03	0.0345	0.017	0.017	0.017	9.4
0.071	0.073	0.072	0.048	0.052	0.05	18.8
0.131	0.132	0.1315	0.104	0.104	0.104	37.5
0.236	0.221	0.2285	0.204	0.203	0.2035	75
0.394	0.4	0.397	0.404	0.412	0.408	150
0.724	0.755	0.7395	0.789	0.794	0.7915	300
1.381	1.362	1.3715	1.519	1.468	1.4935	600

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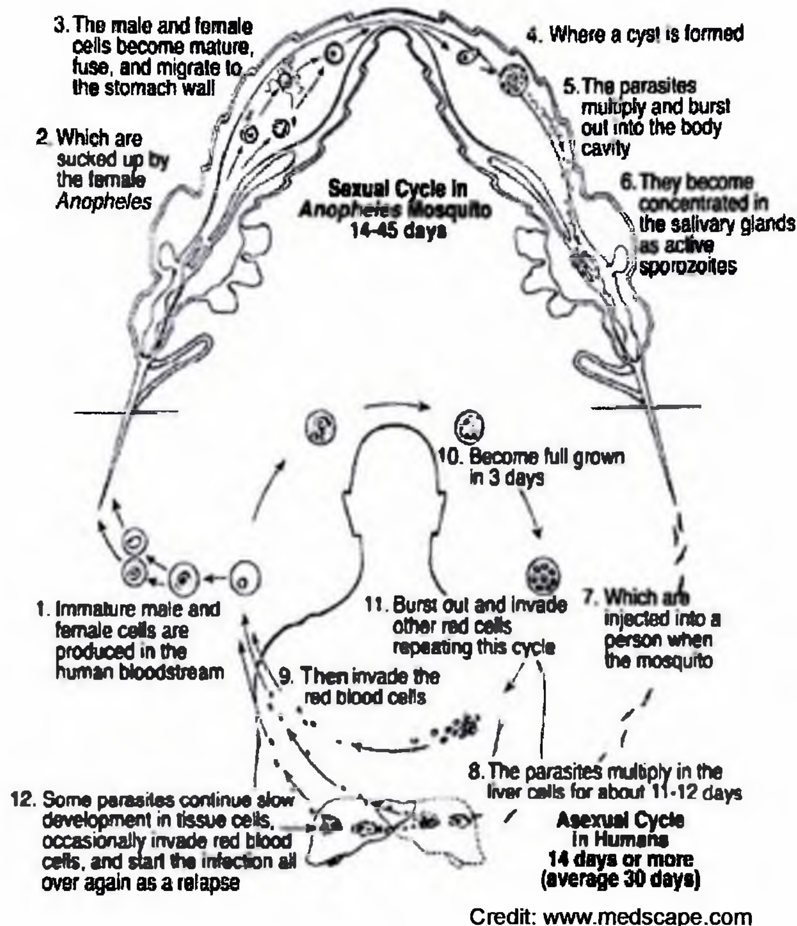
Appendix 5



Life Cycle of *Plasmodium* species

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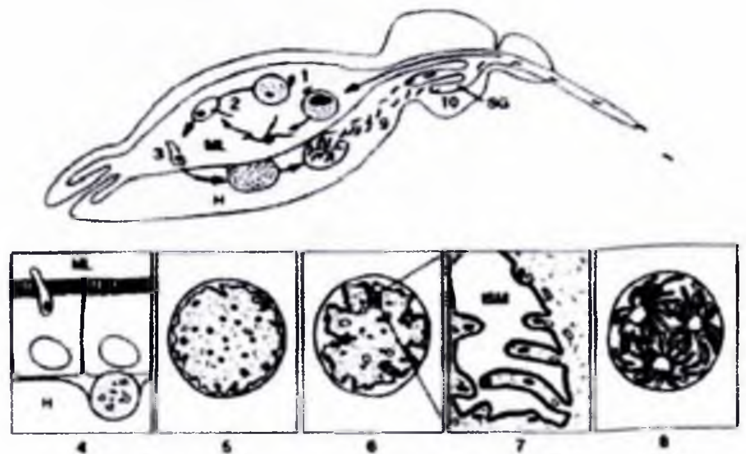
Appendix 6:
Developmental Cycle of *P. berghei* in *Anopheles stephensi*



The developmental cycle of *Plasmodium berghei* in *Anopheles stephensi*. (1) Shortly after the mosquito ingests an infected blood meal, male and female gametocytes are liberated from the red blood cells within the midgut lumen and mature into gametes. (2) Fertilization occurs rapidly, generating a zygote (1-5 h). (3) The zygote then elongates to become an ookinete (10-24 h). (4) The motile ookinete penetrates between or within midgut epithelial cells (24-40 h) and moves to the space between epithelial cells and the basal lamina where it transforms into an oocyst (30-40 h) which protrudes into the mosquito haemocoel (body cavity). The growth of the oocyst over the next 6-14 days ends with the release of 5-10,000 motile sporozoites. (5) The young oocyst undergoes nuclear division while its plasma membrane retracts from the capsule and gives rise to invaginations and subcapsular vacuoles. (6) Vacuoles coalesce into large clefts that penetrate and divide the cytoplasm, forming the sporoblast. (7) Sporozoites evaginate along the surface of the CS-bearing membranes (red line) of the sporoblast (beginning on day 10), a nucleus and various cytoplasmic components of the sporoblast are transferred into the sporozoite during the budding process. (8) The mature oocyst filled with needle-shaped sporozoites releases sporozoites from day 11. (9) Sporozoites migrate to and invade salivary glands (beginning on day 12). (10) Sporozoites contained within the acinar cells of the glands are injected into the vertebrate host during subsequent feedings (beginning on day 13). ML, midgut lumen; H, haemocoel; SG, salivary glands; ISM, intersporozoite matrix.

Credit: www.lifesci.ucla.edu/hhmi

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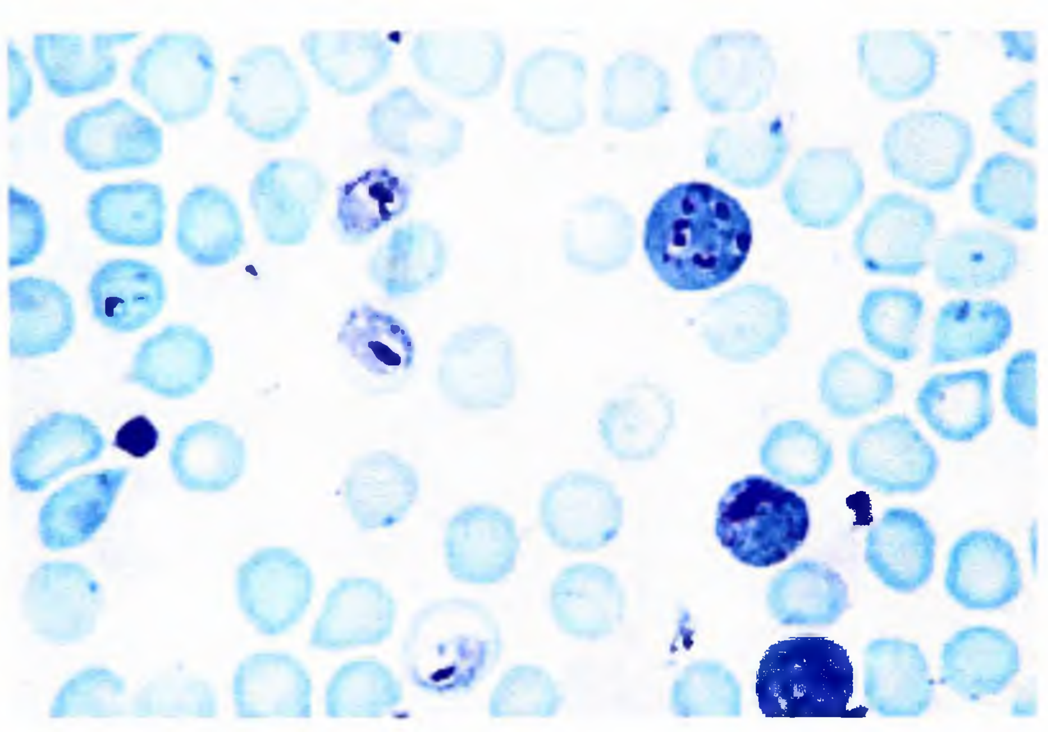
APPENDIX 7:
Blood smear of *P. berghei*



Credit: www.ncbi.nlm.nih.gov/malaria/rodent

Ring, trophozoite and schizont stages of *P. berghei* in mouse host normocytes and reticulocytes (darkly stained)