IN VITRO CELLULAR STUDIES ON THE HUMAN IMMUNE

RESPONSE TO PLASMODIUM FALCIPARUM MALARIA

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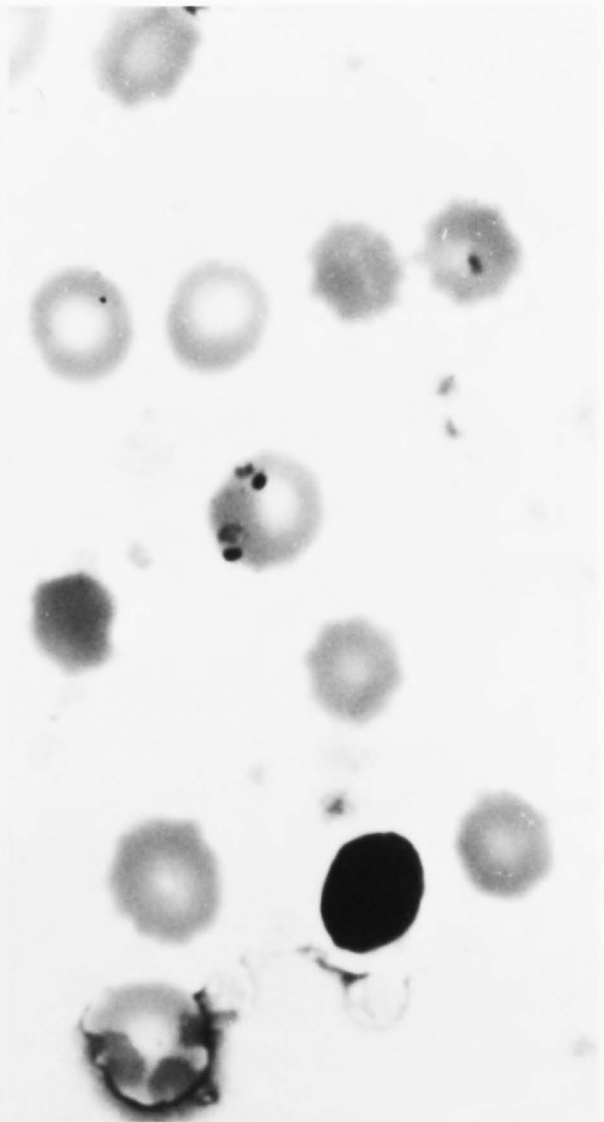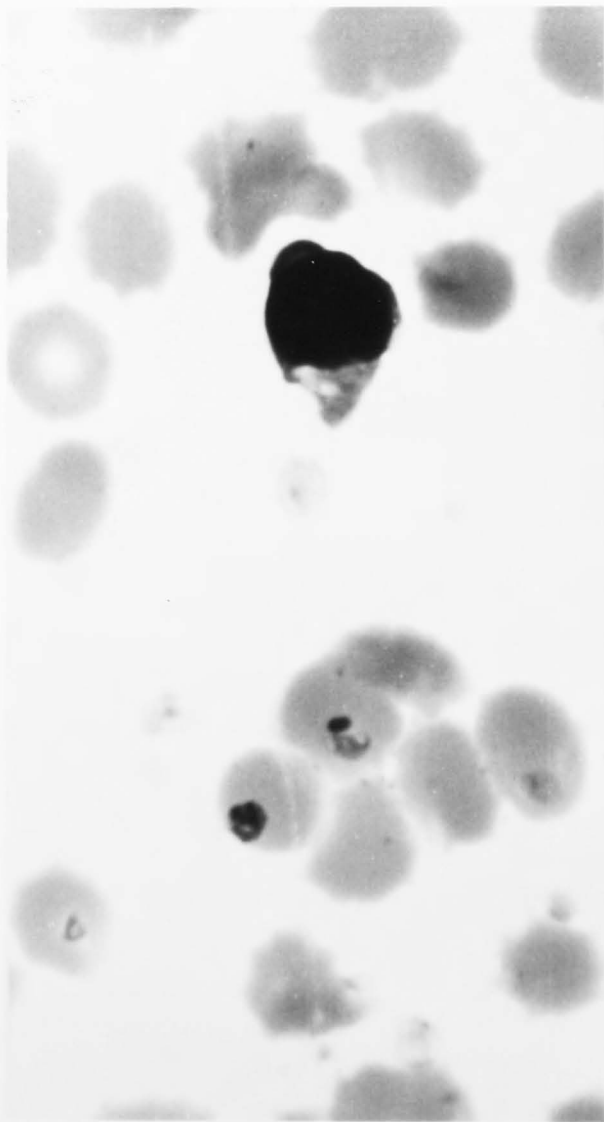of Doctor of Philosophy

May 1983

FRONTISPIECE.

A.   Normal P. falciparum growth in the presence of
mononuclear cells from healthy adult Europeans.

B.   European mononuclear cells cause intra-erythrocytic
parasite death when 'activated' by supernatants of
T cells from children with P. falciparum malaria.
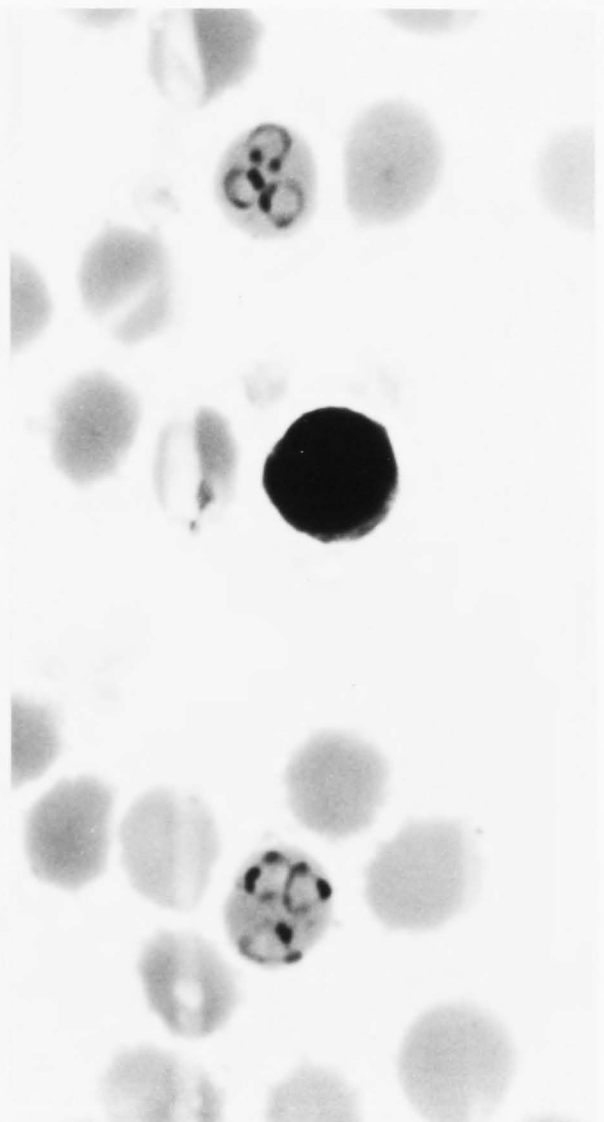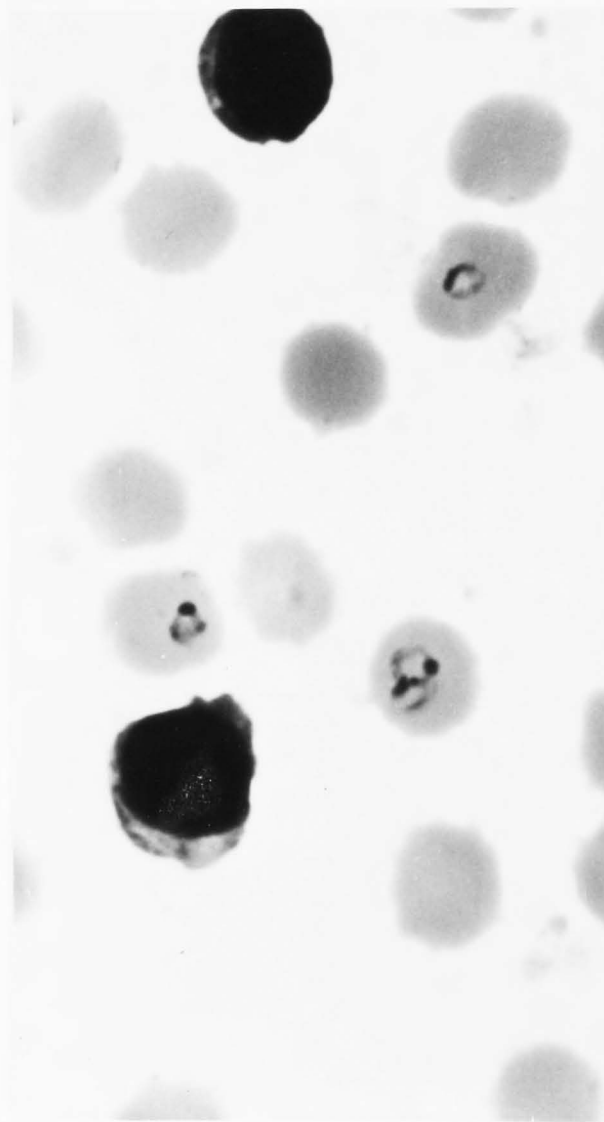
TO JOANNE, LESLEY AND CLAIRE

TO MY MOTHER AND THE MEMORY
OF MY FATHER
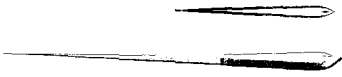
Ye sprightly youths, quite flush with hope and spirit,
Who think to storm the world by dint of merit,
To you the dotard has a deal to say,
In his sly, dry, sententious proverb way!
He bids you mind, amid your thoughtless rattle,
That the first blow is ever half the battle;
That, tho' some by the skirt may try to snatch him,
Yet by the forelock is the hold to catch him;
That, whether doing, suffering, or forbearing,
You may do miracles by persevering.

ON NEW YEAR'S DAY EVENING 1790

Robert Burns.

CONTENTS

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student of Brunel. His unbiased attitude towards my unconventional
academic background was refreshing and despite the distance between
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and for adding new dimensions to my future scientific career.

In vitro cellular studies on the human immune
response to Plasmodium falciparum malaria

by

J. BROWN

ABSTRACT

This thesis reports the results of a large number of experiments which were designed to elucidate the mechanisms whereby Gambian children, suffering from acute Plasmodium falciparum malaria may eventually control their infections. These experiments were carried out in vitro and success or failure of the various test systems was judged by their effect on parasite multiplication.

Early in the course of these investigations it was demonstrated that mononuclear cells from these children could cooperate with antibodies present in their serum to bring about a marked reduction in parasite growth. The efficiency of this antibody-dependent cellular cytotoxicity (ADCC) mechanism was related to levels of parasitaemia in the children, being greater in convalescent children than in those with acute malaria.

Attempts were now made to identify the effector cells in this ADCC. Purified T and B cells were ineffective and although purified adherent cells (A) had an effect, it was much less than that mediated by the undepleted mononuclear cell population. Adherent cells were, however, fully effective in ADCC if they were exposed to the supernatant from T cells non-specifically activated by PHA. Thus cell cooperation leading to activation appears to play an important role in this system.

Finally, experiments were set up to determine whether activated mononuclear cells could exert an inhibitory effect on parasite multiplication which was independent of anti-malarial antibody. It was shown that depression of parasite growth could be achieved by mononuclear cells, either from the children or from Europeans, if these cells were exposed to supernatants of previously stimulated mononuclear cells.

These findings can be assembled to provide a tentative model of the development of protective responses in vivo. Perhaps following phagocytosis of parasite antigens and their presentation on the cell surface, T cells become activated: they may cooperate with B cells to produce parasite specific antibodies; they may also activate other mononuclear cells (non T, non B) to become effector cells. These cells, either alone, or perhaps more efficiently in cooperation with antibody, are able to kill parasites by the release of toxic factors, and the infection is brought under control. Finally, large amounts of specific antibodies of appropriate isotypes are synthesized. Acting as opsonins or by activating complement, they may serve to destroy remaining parasites. Their continued presence, by preventing merozoite penetration, may provide at least a temporary defense against reinfection. It is assumed that Gambian adults who have suffered repeated malaria infections and are now immune are defended by their possession of circulating IgG antibodies and B memory cells of all appropriate specificities.

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1   1.   <u>INTRODUCTION</u>

More than 100 years have elapsed since Laveran first described the causative organism of 'intermittent fever' yet the form of malaria caused by <u>Plasmodium</u> <u>falciparum</u> remains one of the most important communicable diseases of man (Ifediba & Vanderberg 1981). <u>P. falciparum</u> has a life cycle of continuous change (Aikawa 1971) which stimulates immune responses within the host to each stage of its development (Brown, K.N. 1976). However, many of the antibodies which develop to these stimuli have no anti-plasmodial function and the host is protected from the clinical symptoms and pathology of malaria only after immunity is gained to the asexual parasite stages (McGregor 1972).

In endemic areas, malaria is mainly a disease of childhood to which effective immunity is acquired after repeated infection (McGregor 1967). The many years it takes man to develop immunity to malaria is believed to represent the time taken to encounter and overcome the parasite strains and variants present in his environment (Brown, K.N. 1971). One of the first signs of immunity to malaria is the ability of the host to curtail the clinical manifestations of the disease despite the persistence of relatively high parasite numbers in the blood (McGregor 1967). In partially immune children with <u>P. falciparum</u> infection parasitaemia is diminished but it may take years to develop sufficient immunity to completely eradicate the parasites (Brown, K.N. 1979). In the majority of individuals immunity develops during childhood until in adolescence the detection of malaria parasites in the blood is unusual (McGregor 1967). However, many young children do die of <u>P. falciparum</u> malaria despite an obvious immune response to the massive immunological stimulus provided by the parasite (Brown, K.N. 1976). The reasons why some children die of malaria, whilst

others who are infected to the same degree survive, are not precisely known. It has been suggested that on some occasions massive sporozoite inoculation leads to rapid multiplication in asexual parasite numbers and overwhelms the host's natural defense mechanisms before they have had sufficient time to function (McGregor 1972). Another possible reason why some children fail to overcome malaria infection is that they may have a depressed immune state at the time of parasite challenge. This could be due to infections other than malaria or due to loss of immunity during the dry season. In such children, malaria infection is likely to have devastating consequences (Bruce-Chwatt 1980).

Eradication measures based upon vector control and prophylaxis have successfully eradicated malaria in many parts of Europe, Asia and North America (Cohen 1979). However, these measures have proved impracticable in Africa and in other parts of the world where vector resistance to insecticides together with the emergence of parasite strains resistant to antimalarial drugs have resulted in a resurgence of malaria (Wernsdorfer 1980). At the present time one-third of the world's population is exposed to the risk of malaria infection (Cohen 1979). In addition, in those areas where control measures have proved inadequate, epidemic malaria is a dangerous possibility as the natural immunity of populations living in such areas is low as a result of the interruption of malaria trans-mission (Wernsdorfer 1980). Drought has led to reduced exposure to P. falciparum in the Sahel region of Africa. This has re-sulted in decreasing malaria antibody levels in subjects living in The Gambia (McGregor & Williams 1978) and has subsequently led to an increase in the number of cases of acute malaria in age groups normally immune to infection (Brown, J. unpublished data). The effect of malaria control programmes and climatic changes on

protective immunity is not known and there is a need to develop techniques which will measure changes in immunity which are a consequence of vector control and the widespread use of antimalarial drugs.

It is now believed that successful malaria control requires a multi-pronged approach involving vector control, chemotherapy and eventually, immunization (Wernsdorfer 1979). Although successful immunization against sporozoites (Nussenzweig et al. 1967), exoerythrocytic forms (Holbrook et al. 1974), asexual stages (Holder & Freeman 1981) and gametocytes (Gwadz 1976) has been achieved against malaria in experimental animals, progress towards vacci- nation against human malaria has been impeded by our poor under- standing of the mechanisms involved in the acquisition of natural immunity. Until recently, this in part, has been due to our in- ability to maintain P. falciparum in laboratory cultures. Fortu- nately, the method developed by Trager and Jensen (1976) has made possible in vitro studies on the asexual cycle of P. falciparum which is the stage responsible for the clinical symptoms and patho- logy of human malaria (Brown, K.N. 1976; Bruce-Chwatt 1980). Com- plete maturation of P. falciparum gametocytes has also recently been achieved (Ifediba & Vanderberg 1981). However, the formidable obstacles involved in culturing sporozoites and liver stages of P. falciparum have hampered studies on the development and anti- genicity of these stages. Natural immunity and resistance to malaria gained by immunization is largely stage specific, i.e. immunity to pre-erythrocytic stages and gametocytes does not inhibit multiplication of asexual erythrocytic forms (Brown, K.N. 1976; Nardin et al. 1979).

Additional factors which may hinder the acquisition of natural immunity and indeed, the production of a malaria vaccine, are those

of parasite strain differences and antigenic variation. The prolonged period required before man develops immunity to malaria points to the existence of strain differences between P. falciparum isolates. Enzyme patterns unique to individual isolates have been identified in P. falciparum infections (Carter & McGregor 1973). That man eventually acquires immunity sufficient to eliminate the clinical features of malaria is evidence that the number of protective antigens possessed by different P. falciparum strains is finite. However, the number of P. falciparum strains has yet to be determined and it is not known how they might differ in antigenicity and virulence. Antigenic variation is defined as the ability of a single parasite isolate to evade a potent immune response by continually altering its antigenic character. This phenomenon has been recognised in P. knowlesi infections and is thought, by some workers, to be a feature of P. falciparum infection (Brown, K.N. 1977). The detection of parasites in the blood of clinically immune individuals suggests that antigenic variation probably does occur in P. falciparum malaria.

Before a successful P. falciparum vaccine is produced, the number of strains and variants of the parasite against which the vaccine is to be directed requires to be determined and a test which could determine whether vaccinated individuals would be resistant upon exposure to P. falciparum would be necessary and of great value.

1 2. NON-SPECIFIC RESISTANCE TO P. FALCIPARUM

a. Haemoglobin S

Beet (1946) first suggested that sickle cell haemoglobin may have an inhibitory effect on the growth of P. falciparum. Further epidemiological surveys also suggested that the heterozygote AS

genotype also prevented severe clinical malaria (Brain 1952; Allison 1954). More recent investigations have shown that P. falciparum parasites will invade and grow normally in AS and SS haemoglobin containing erythrocytes when cultured under aerobic conditions (Freidman 1978). However, when oxygen tension is reduced to between 1% and 5% malaria parasites inside SS erythrocytes are killed, the infected red cells become physically deformed and are subsequently haemolysed (Freidman 1978). The growth of parasites inside AS erythrocytes is halted at the early trophozoite stage (Pasvol 1978). In vitro experiments have shown that the retarded P. falciparum growth observed in haemoglobin S erythrocytes occurs before the red cell sickles; parasite death possibly being due to its inability to metabolise this type of haemoglobin (Pasvol 1978). There is evidence that such parasitized erythrocytes undergo significant physical changes under reduced oxygen tension before sickling begins (Messer, Hahn and Bradley 1975). Pasvol (1978) has postulated that merozoites will penetrate and grown in well oxygenated haemoglobin S peripheral erythrocytes in vivo and that parasite damage may occur only at the stage of schizont development in the low oxygen atmosphere of the deep tissues. Under such conditions, any increase in parasite multiplication in an HbS individual would be detrimental to the parasite rather than the host as developing parasites may be blocked and killed by increasing vascular stasis and anoxia in deep tissue capillaries.

In malaria endemic areas in Africa as many as 25% of children may possess the AS haemoglobin trait (Edington & Gillies 1969). If the observations of Freidman (1978) and Pasvol (1978) simulate the situation in vivo, sickle cell haemoglobin may indeed be a major non-immunological defense mechanism against P. falciparum malaria, which although failing to prevent infection may yet limit

its clinical severity.

b. G6PD deficiency

Enhanced resistance to malaria is also believed to occur in individuals deficient in glucose-6-phosphate dehydrogenase (Motulsky 1965). G6PD deficiency is the most common defect of red cell metabolism known, affecting 100 million individuals world-wide (World Health Organization 1967) and there is a definite geographic coincidence of this condition and P. falciparum malaria (Motulsky 1965).

G6PD and/or its by-products may be essential components for parasite growth (Pasvol 1978). Similar invasion by P. falciparum occurs in red cells deficient in G6PD as in normal red cells (Pasvol 1978). However, once inside G6PD deficient red cells, para-site growth is severely retarded (Pasvol 1978). Contradictory epidemiological reports on the protective value of G6PD deficiency suggest that more detailed studies are required before any precise role in host resistance to malaria is established.

c. Haemoglobin C and E

Epidemiological evidence of protection against malaria in carriers of HbC and HbE is less convincing than that for HbS and G6PD deficiency. Thus, although lower parasite counts and lower mortality from P. falciparum infection have been found in HbC and HbE carriers by some workers (Edington & Watson-Williams 1965) others have found no differences when normals and HbC and HbE carriers were compared (Kruatrachue et al. 1970). At a cellular level, Pasvol (1978) failed to demonstrate any effect of these haemoglobinopathies on P. falciparum growth in vitro.

d. Thalassaemia

Erythrocytes containing foetal haemoglobin retards the in vitro

growth of both sexual and asexual P. falciparum parasites (Pasvol et al. 1976). This could explain the selective advantage in malaria endemic areas of B-thalassaemia heterozygotes, in whom the normal post-natal decline of haemoglobin F is retarded. Pasvol (1978) has shown that parasites will invade young HbF red cells as readily as HbA red cells of the same age but after entry into the HbF cells parasite growth is seriously affected. In subjects with hereditary persistence of HbF the parasite preferentially invades HbA red cells not because the HbF red cells are more resistant to invasion but because P. falciparum invades younger erythrocytes more readily (Pasvol 1978).

e. The Duffy blood group

The relative insusceptibility of West Africans and American negroes to P. vivax infection is believed to be due to the rarity of the Fya Fyb Duffy blood group determinants in these populations, whilst they are common in other racial groups (Bruce-Chwatt 1980). This suggests that the Fya Fyb determinants may be red cell receptors for P. vivax. However, as Lopez-Antunano and Palmer (1978) have successfully infected Fya Fyb negative Aotus monkeys with P. vivax, other genetic markers on erythrocytes may also be involved in preventing P. vivax merozoites penetrating African Fya Fyb negative red cells.

Recently Pasvol (1982) has demonstrated that human En (a⁻) red cells deficient in glycophorin A and human S⁻ s⁻ U erythrocytes deficient in glycophorin B are relatively resistant to invasion by P. falciparum merozoites. However, once the glycophorin deficient cell has been penetrated the growth of the parasite proceeds normally.

f. Malnutrition and iron deficiency

High parasitaemia and the cerebral complications of P. falciparum infection are rarely seen in malnourished children and it has been suggested that their malnourished state affords them a degree of protection against malaria (Edington & Gillies 1969; Hednrickse et al. 1972). However, there is evidence that clinically, malaria in malnourished children is as severe as in well-nourished children who develop fulminating infections (Brown, J. & Wojciechowski, in preparation).

The clinical severity of low P. falciparum infection in malnourished children may be due to immunosuppression caused by co-existing infections in addition to lowered tolerance caused by metabolic deficiencies. Iron deficiency anaemia, which is a feature of malnutrition, is believed by some workers to afford a degree of protection against malaria. Masawe, Muindi and Swai (1974) showed that with both oral and intramuscular iron replacement, parasite counts in iron-deficient patients with malaria rapidly increased. Murray et al. (1975) observed that patients admitted to hospital for reasons other than malaria commonly develop attacks of malaria. They attributed this to diet and showed increasing serum iron and transferrin levels. However, they also noted dramatic rises in reticulocyte counts and therefore the increase in the younger red cell population could well have accounted for the increases in parasite multiplication. Murray et al. (1975) tried to confirm their studies by using an animal model. They found that 20 rats given intramuscular iron dextran two weeks prior to infection with P. berghei had a more rapidly rising parasitaemia than control rats given normal saline. These data remain inadequate to establish whether iron deficiency decreases susceptibility to P. falciparum infection as neither of these studies included non iron-deficient

controls. Furthermore Pasvol (1978) failed to show any reduction of P. falciparum invasion or retardation of parasite grown in iron deficient erythrocytes in vitro.

g.   Endotoxin

Clark (1978) has described the release of a potentially lethal endotoxin with similar properties to Escherichia coli lipopoly-saccharide (L.P.S.) during Plasmodium infections in mice. However, this endotoxin is also believed, by Clark, to potentiate the release of non-antibody mediators from mononuclear cells which may control parasite multiplication during primary attacks. Clark's hypothesis stems from the fact that the clinical features of malaria are simi-lar to those produced by high concentrations of endotoxin and from experiments which showed that mice were protected from malaria by injections of B.C.G., Corynebacterium parvum and zymosan, all of which induce responsiveness to very low concentrations of L.P.S. (Clark, Allison & Cox 1976). However, Clark (1978) later demon-strated that extremely low doses of L.P.S. were lethal to mice already infected with P. berghei or B. microti whereas uninfected control mice given 10 times the concentration of L.P.S. survived. Endotoxin has also been shown to stimulate B-cell transformation in human malaria (Greenwood 1974), and small quantities of a sub-stance with properties similar to endotoxin have been identified in the serum of patients with P. falciparum malaria. If Clark's hypothesis was verified in human malaria, cell mediated reactions would be implicated to have an extremely important role in host re-sistance. However, if excess endotoxin was already present as a result of bacterial infections, further release of this substance due to co-existing P. falciparum infection might have lethal con-sequences in the host. At the present time, evidence is not avail-able which would support such a view.

1.3. THE IMMUNE RESPONSE TO MALARIA

a. Specific humoral immunity

Coggeshall and Kumm (1937) first demonstrated the existence of protective antibodies in malaria when they observed the beneficial effect of the administration of immune serum to monkeys with experimental malaria infections. Antibody may protect the host by blocking red cell attachment sites on merozoites, which are a primary requirement for blood stage infectivity (Miller et al. 1973) thus preventing penetration of uninfected erythrocytes (Miller, Aikawa & Dvorak 1975). Support for this view is to be found in the studies of Cohen, Butcher and Crandall (1969) who reported that sera from rhesus monkeys, immune to P. knowlesi infection, inhibit the cyclic growth of the parasite in vitro but has no effect on the development of intra-erythrocytic stages.

The in vivo parasite inhibitory effect of immune serum has been demonstrated by Kreier et al. (1976) who demonstrated that non-immune mice were protected from P. berghei infection if the parasites were injected together with immune serum. However, the virulence of P. berghei parasites, pre-incubated in immune serum, was not affected if the parasites were washed before being injected into non-immune mice along with normal mouse serum. These experiments added support to the view that immune serum, acting independently, does not affect intra-erythrocytic parasites. In vitro experiments have described the maintenance of low-level B. microti parasite density in the presence of sera from immune hamsters (Batisto & Kreier 1979). However, when immune serum is replaced by serum from non-immune hamsters parasite density rapidly increases. The importance of inhibitory antibody in resistance to malaria can be further supported by the inability of immune mice to overcome P. yoelii infection after the administration of cyclophosphamide which

suppresses mainly B cell transformation (Jayawardena et al. 1975). Furthermore, Butcher and Cohen (1972) have shown that parasite inhibitory antibody levels correlate with the immune status of monkeys challenged with P. knowlesi. These findings have been confirmed in studies of P. falciparum in owl monkeys (Reese & Motyl 1979) and in studies of the effect of immune human sera on the in vitro growth of P. falciparum (Wilson & Phillips 1976). However, Wilson and Phillips (1976) observed varying degrees of parasite inhibition by serum from Gambian adults immune to malaria; individual sera from these subjects inhibited some parasite isolates more than others. Repeated challenge by P. falciparum isolates sharing common protective antigens should have resulted in comparable susceptibility of each parasite isolate to a single immune serum. This was not the case and it is possible that these reactions reflected parasite strain differences. This view finds support in the work of Mahoney, Redington and Schoenbechler (1966) who demonstrated that when autologous P. falciparum parasites from subjects are used as antigen, fluorescent antibody titres of these individuals are higher than when antigen prepared from heterologous P. falciparum parasites is used. The existence of P. falciparum strains with major antigenic differences would be an important factor in interpreting tests of immunity and infectivity as polyvalent antigens may be required to ensure the detection of all important antibodies.

b.   Methods of measuring antibodies

Malaria infection stimulates a particularly high rate of immunoglobulin synthesis but only a small proportion of this antibody is anti-malarial and even less is protective (Brown K.N. 1976). Nevertheless, antibodies against asexual P. falciparum parasites can be detected in the serum of patients soon after parasitaemia is established (Tobie et al. 1966). Reactions between serum and malaria

antigens have been used as measures of protective immunity and as indicators of recent and existing infections.

Malaria antibodies, measured by fluorescent (IFA) and haemagglutination (HA) techniques using homogenized placental schizonts or cultured schizonts as antigen, are detected in serum during P. falciparum malaria and their concentration follows the pattern of infection (Tobie et al. 1966; Voller, Meuwissen & Goosen 1974). Correspondingly, as parasitaemia decreases IFA and IHA antibody titres gradually fall (McGregor 1965). Although IFA and IHA malaria antibodies are found in high concentrations in adults, their detection in children gives no indication of the level of acquired immunity (McGregor 1965). That antibodies measured by these techniques can be detected using antigen from single parasite isolates, indicates that all infected individuals develop antibody to a determinant common to the majority of P. falciparum strains. Although not protective on its own, this antibody may be important in the stimulation of other effector mechanisms. An enzyme-linked immunosorbent assay (ELISA) is also available for the detection of malaria antibody, and it is analogous to the IFA test except that an enzyme is used to label the antiglobulin instead of fluorescein; a colourimetric estimation of the enzyme by means of a chromogenic substrate replaces the microscopical estimation of fluorescence in the IFA test. Malaria antibodies can be detected by the ELISA technique within a week of the establishment of patent parasitaemia (Voller, Meuwissen & Verhave 1980).

All of the above techniques are extremely useful as serodiagnostic tools in malaria but their use should be limited to epidemiological surveys. For the diagnosis of clinical infections microscopic identification of malaria parasites remains the most reliable method. As the protective antigens in human malaria have

as yet not been identified IHA, IFA and ELISA techniques fail to give any indication of protective immunity.

c.   Measurement of protective antibody

Precipitins

In 1918, Pewny first suggested that malaria antibodies may combine with antigen to form precipitates.   A direct correlation between the detection of precipitating malaria antibodies and protective immunity has since been described in human malaria (Wilson et al. 1969) and in P. berghei malaria in rats (Zuckerman et al. 1969).   McGregor (1972) has shown that the prevalence of precipitating antibodies in human malaria increases with age.   Zuckerman et al. (1969) demonstrated that rats, challenged with P. berghei parasites, developed precipitating antibodies 3-17 days after infection was established.   Only one rat out of 46 did not develop precipitating antibodies and this rat died of a fulminating infection.

Parasite growth inhibition assays

The direct action of serum from infected and immune animals on malaria parasites in vitro using isotope labelling techniques can also be used as a means of measuring protective immunity (Butcher & Cohen 1972) and has been developed for use in human malaria (Wilson & Phillips 1976).   The results of tests of immunity using this method must be interpreted with caution.   In addition to the possible existence of antigenic variance, it must also be established whether or not the patient is receiving anti-malarial treatment at the time of testing.   Even the effect of immune adult serum may result in only a reduction in parasite numbers during one schizogony rather than total parasite death and morphologically damaged parasites may continue to incorporate isotope. Parasite multiplication in childrens' sera which may not be entirely

inhibitory, may be a measure of some degree of immunity as long as parasite growth is inhibited more than in European control serum.

Although useful for measuring protective immunity in adults or on a population basis neither of these methods are of any practical value as indicators of resistance or susceptibility to P. falciparum in children.

d.   Opsonising antibodies

In addition to preventing merozoite penetration of erythrocytes, malaria antibody stimulates phagocytes to ingest malaria parasites (Kreier 1976).   Merozoites are rarely phagocytosed in the absence of specific antibody (Brooks & Kreier 1978).   Effective phagocytosis may require the activity of both cytophilic antibody and opsonic antibody.   Cytophilic antibody (IgG1) and opsonic antibody (IgG2) are both present in the sera of rats immune to P. berghei (Green & Kreier 1978).   Cytophilic antibody binds to macrophages before they can bind to antigen.   In P. berghei infections in rats this combination is only weakly phagocytic until opsonic antibody is present;   phagocytosis of merozoites then greatly increases (Green & Kreier 1978).   However, in the absence of specific antibody, macrophages may bind and ingest schizonts (Brooks & Kreier 1978).   This has been demonstrated in P. berghei infections in mice (Chow & Kreier 1972).   Brown, K.N. (1971) has suggested that host resistance in malaria may be greatly enhanced by non-specific as well as specific activation of phagocytosis.

The importance of phagocytosis in malaria infections can be demonstrated by the greater protection conferred, on rats challenged with P. berghei, by immune serum plus macrophages (which have previously been incubated with free merozoites) when compared with the protective effect of immune serum alone (Gravely & Kreier 1976).

Although phagocytosis undoubtedly plays a part in host resistance to human malaria the significance of this mechanism in the overall immune response is poorly understood and the conditions which facilitate phagocytosis of P. falciparum parasites requires investigation.

e.   Immunoglobulin class of anti-malarial antibody

Both in experimentally induced infections (Tobie et al. 1966) and in naturally acquired immunity (Turner & McGregor 1969) malaria antibody activity has been found in IgG, IgM and IgA serum fractions.   Furthermore, a direct correlation between serum concentrations of IgG and IgM and malaria precipitins has been noted in individuals in endemic areas (McGregor et al. 1970).   IgM antibodies can be detected in the sera of children soon after malaria parasites are observed in the blood (McGregor 1972).   Subsequently, IgG production follows and parallels a drop in IgM levels (McGregor 1972).   Although IgM has been shown to block merozoite invasion of red cells in monkeys immune to P. knowlesi (Cohen & Butcher 1970), as yet, no definite protective role has been established for IgM or for IgA in human malaria.   However, IgG from immune adults (Cohen, McGregor & Carrington 1961) and IgG from the cord blood of new-born Nigerian infants (Edozin, Gillies & Udiozo 1962) protects children with acute P. falciparum infections, although this protection has no lasting effect.

f.   Antigens involved in antibody/antigen reactions

More than 30 malaria antigens have been identified, by gel precipitation, which may be classified as S (stable), R (resistant) and L (labile) on the basis of their susceptibility to heating at $100^{o}C$ for 5 minutes (Wilson et al. 1969).   Antibodies to S antigens are rarely seen in the sera of new-born infants and young

children.   They tend to follow an age-related pattern being
detected more often in the sera of immune adults (McGregor 1972).
Antibodies to S antigens are found equally in IgM and IgG serum
fractions and appear briefly in the sera of children heavily
infected with P. falciparum (Wilson et al. 1969).   These anti-
bodies tend to persist for only short periods, even in the sera of
immune adults and it is believed that S antigens are poorly immuno-
genic as there is no epidemiological evidence that antibodies to S
antigens function importantly in any protective role (McGregor 1972).
Conversely, there is strong evidence to suggest that L antigens
stimulate the production of protective antibody.   Antibodies to
L antigens are found exclusively in IgG (McGregor 1972).   In
endemic areas, the incidence of specific malaria antibody is high at
birth, then proceeds to fall to lower levels during the first two
years of life after which antibody levels steadily increase and
reach peak levels during adolescence (McGregor 1967).   The only
immunoglobulin which crosses the placenta in other than trace
amounts and follows the described pattern is IgG (Bruce-Chwatt
1980).   The distribution of antibodies to L antigens throughout
life is similar to the pattern described for IgG (Wilson et al.
1969).   Despite testing many hundreds of infected bloods, McGregor
(1972) has isolated R antigens only twice.   This had hindered
further study on their role in malaria immunity.

Recently, great advances have been made towards the identifi-
cation of protective antigens and antibodies in malaria using
hybridoma technology.   Monoclonal antibodies have been produced
which will inhibit the in vitro growth of P. falciparum (Perrin et
al. 1981) and in animal malaria monoclonal antibodies have been
identified which will protect against sporozoite challenge (Yoshida
et al. 1980); and yet another has been developed which prevents

malaria transmission by blocking gametocyte fertilization (Rener et al. 1980). A monoclonal antibody against P. yoelii merozoite antigens confers passive protection on non-immune mice when challenged by the parasite (Freeman et al. 1980). This antibody has since been used to identify the antigen involved which has been found to have a molecular weight of 235,000, being associated with an internal merozoite organelle. This antigen when inoculated into mice in Freund's adjuvant produced high levels of merozoite specific antibody which protected mice from death following parasite challenge although high parasitaemia persisted for 16 days (Holder & Freeman 1981). A second monoclonal antibody reacted with schizonts, but was not protective on passive transfer. This antibody identified a surface antigen of M.W. 230,000 which was present in early schizonts and occurred also on merozoites (Holder & Freeman 1981). Mice immunized with this antigen developed high titres of schizont antibodies and were extremely resistant to parasite challenge. Monoclonal antibodies may themselves inhibit parasite growth but the results of Holder and Freeman (1981) showed that the best protective immunity in mice was in response to an antigen whose monoclonal antibody failed to provide passive protection. This protective antigen could have stimulated cell mediated immunity which may have been the important mechanism involved in terminating the acute infections in these mice. Research on human malaria is entering an exciting era as a result of the opportunities provided by the use of monoclonal antibodies in the identification of protective antigens. However, the immune processes involved in the acquisition of immunity to human malaria are as yet poorly understood; we do not know how many strains of P. falciparum there are or how they differ in virulence and there is no means of measuring changes in protective

immunity.   These problems must be overcome before vaccination against human malaria is achieved.

1   4.   <u>CELL MEDIATED IMMUNITY</u>

It is now customary to apply the term 'cell-mediated immunity' (CMI) to denote those biological phenomena which result from the interaction of sensitized mononuclear cells and specific antigen in which no obligatory role can be found for the classical activity of antibody.   Thymus-derived lymphocytes (T-cells), lymphocyte sub-populations and macrophages, but not B-cells, are the effector cells in CMI.   The main functions of mononuclear cells in CMI are:

1.   The mediation of cytotoxic reactions directed against foreign cells and organisms.

2.   The secretion of soluble, non-antibody products which acti-vate other lymphocytes and/or macrophages.

a.   <u>CMI in malaria</u>

Evidence supporting an important role for CMI in malaria is at present mainly restricted to results of experiments on animals, but it is unlikely that the massive mononuclear cell proliferation seen in human malaria (Charmot & Bastin 1979) occurs solely for the purpose of antibody production.   The independent action of antibody in malaria is responsible for interrupting the cyclic growth of the parasite by preventing the penetration of red cells (Cohen, Butcher & Mitchell 1977) and the combined activity of phagocytes and antibody results in ingestion of free merozoites and mature schizonts (Kreier 1976).   However, neither of these mechanisms can account for the frequent observation of 'crisis forms' (damaged intra-erythrocytic parasites).   Taliaferro (1949) suggested the existence of a cytotoxic mechanism directed

specifically against these stages. This was a far-sighted observation as parasitized red cells have since been shown to have altered permeability which might enhance the entry of various products of immune reactions (Homewood 1977).

Rhesus monkeys vaccinated against P. knowlesi will eliminate a massive homologous challenge, but fail to clear a different Plasmodia which is present as a concomitant infection (Butcher, Mitchell & Cohen 1978). Damaged forms of the second parasite appear during the elimination of P. knowlesi, perhaps indicating the activity of a non-specific cytotoxic agent. Passive transfer of immunity by antisera and mononuclear cells to rats with P. berghei infection is more effective than the administration of immune serum alone (Phillips 1970). The importance of CMI in animal malaria can also be demonstrated by the ability of B-cell deficient mice to overcome P. chabaudi infection (Grun & Weidanz 1981). Clonal expansion of T lymphocytes may occur in malaria by the clustering of lymphocytes around macrophages which are actively digesting malaria antigen. This observation has been made in P. berghei infections in rats (Herman 1977). It may be that both humoral and cell-mediated reactions to malaria are required for a fully expressed immune response.

b. Effector cells

Natural killer lymphocytes

Strictly speaking natural killer lymphocytes (NK cells) should be included in the section dealing with non-specific resistance as NK cells kill their target in the absence of any known antigenic stimulation and in the absence of antibody. However, as NK cells are believed to be immature T-cells, it was more relevant to include them in this section dealing with mononuclear cells. Recently, increased NK cell activity has been demonstrated in

patients with acute P. falciparum malaria (Ojo-Amaize et al. 1981).

These patients also had raised interferon levels and their mono-nuclear cells were able to kill virus infected tumour cells in the absence of antibody. In addition, the degree of NK cell activity correlated positively with parasitaemia. Furthermore, NK cell activity directed against malaria parasites, has been dis-played by lymphoid spleen cells from Kra monkeys infected with P. knowlesi (Langhorne et al. 1978).

T-cells

Specific T-cell cytotoxicity is unlikely to be an operative effector mechanism in malaria as cytotoxic T-cells must recognise products of the major histocompatability system (MHS) expressed on the target cell surface before killing can occur. As parasi-tized erythrocytes do not express MHS determinants, killing by cytotoxic T-cells is unlikely to take place. Nevertheless, in addition to their 'helper' role in humoral immunity, T-cells most likely function supportively in cell-mediated reactions in malaria.

K-cells

There is scant information supporting an important role for CMI in human malaria. However, there is indirect evidence to support such a view. Antilymphocytotoxic antibodies have been found in the sera of Thai adults with malaria (Wells et al. 1979) and large numbers of hyperbasophilic mononuclear cells are observed in the blood of children with acute P. falciparum infections (Charmot & Bastin 1979). Increases in non-rosette forming lympho-cytes (null cells) in children with acute malaria parallels a decrease in T-cell numbers but the proportion of B-cells remains relatively the same as in uninfected children (Wyler 1976). Greenwood, Oduloju and Stratton (1977) demonstrated that haemolysis

of antibody-coated chicken red cells was more effective in the presence of mononuclear cells from children with acute malaria than in the presence of their convalescent mononuclear cells. These workers attributed their findings to the increased number of null cells found in these children during the acute phase of their infections and postulated that the non-rosetting mononuclear cells were K-cells. Antibody-dependent cellular killing of malaria parasites has also been demonstrated in vitro and in vivo in experimental animals (Coleman et al. 1975; Clark, Allison & Cox 1976). In both these studies K-cells were cited as effector cells although macrophages were also believed to have functioned as cytotoxic cells.

Activated macrophages

Macrophages, activated by T-cells or T-cell products have been shown to be cytotoxic to certain tumour cells and in experimental animal malaria macrophages are believed to function similarly by secreting substances which destroy asexual parasites in the intra-erythrocytic location (Clark, Allison & Cox 1976). Detailed cellular studies on P. yoelii infected mice have revealed that

cells of myeloid origin are extremely effective in killing asexual parasites whereas lymphocytes from the same animals had no effect on parasite multiplication (Taverne, Dockrell & Playfair 1982). These authors concluded that the effector cells belonged to the monocyte-macrophage series and although they were insensitive to antimacrophage serum they were yet phagocytic.

However, these cells were also shown to be cytotoxic in the presence of antibody. Cytotoxicity was greatly enhanced by the addition of supernatant fluid from Concanavalin A stimulated spleen cells from P. yoelii infected mice. In recent years soluble non-antibody products of antigen stimulated monocytes (interleukin 1)

and lymphocytes (interleukin 2) have been shown to participate in various cell-mediated reactions. These substances can be identified and differentiated both by their structural and functional characteristics. The clinical importance of interleukin 1 and interleukin 2 has yet to be fully established. However, Kreier and Green (1980) have suggested that they may play a part in killing intra-cellular asexual malaria parasites. Other authors have postulated that these substances may be involved in regulating immune responses to malaria by restricting antibody production by B-cells (Cohen 1976) and by inducing lymphocyte and macrophage suppressor cell activity (Feldman 1973; Wyler, Oppenheim & Koontz 1979).

Theories based on results of experiments on experimental animals, such as those described above, strongly suggest that cell-mediated immunity is indeed an essential component of host resistance to malaria. However, mononuclear cell cytotoxicity directed specifically against malaria parasites has yet to be demonstrated in adults immune to malaria or in children with acute P. falciparum infections.

5. AIMS OF THIS STUDY

Up until the time this study was begun no information was available on the direct action of human leucocytes on the growth of P. falciparum. In particular, no studies had been carried out to investigate the possibility of cellular cytotoxicity in human malaria despite the knowledge of such mechanisms being operative in malaria of experimental animals (Coleman et al. 1975; Clark, Allison & Cox 1976). In the light of these findings and there being no satisfactory explanation for the observation of intra-erythrocytic parasite death, this study set out to investigate the possibility of cell-mediated immunity in human

malaria which could enhance our knowledge of the cellular involvement in the human immune response to the disease. The availability of established asexual P. falciparum parasite lines provided an opportunity to develop in vitro methods for the study of interactions between P. falciparum and human leucocytes. To examine the reactions fully an in vitro technique which would accommodate the proliferation of asexual parasites as well as leucocytes was a necessary and important pre-requisite of this study. The method which was eventually used was adapted from a micro-method employed for the maturation of P. falciparum gametocytes (Smalley 1976). The micro culture technique is described in detail in a later chapter.

Thus, the aims of the study were as follows: -

1. Firstly, to determine whether or not human leucocytes killed or inhibited the growth of P. falciparum.

2. To establish whether or not cellular and/or humoral parasite inhibition was related to parasitaemia or protective immunity.

3. To identify the cell or combination of cells responsible for any observed parasite inhibition.

4. To establish whether or not such reactions were antibody-dependent and to classify the antibody involved in positive reactions.

5. To investigate the possible release of lymphokines and monokines from activated mononuclear cells and to examine their in vitro activity.

It was with these objectives in mind that this study commenced at the beginning of the rainy season in 1979.

CHAPTER 2

2 1. ESTABLISHING 'WILD' P. FALCIPARUM PARASITE ISOLATES IN CON-TINUOUS CULTURE

The corner stone of this entire study was the availability of stock laboratory strains of P. falciparum parasites. The handling and maintenance of long established P. falciparum parasite lines is a relatively simple, although time-consuming procedure (Trager & Jensen 1976). However, establishing fresh P. falciparum isolates, from infected blood, in continuous culture in laboratories in malaria endemic areas presents several logistical problems which may not be fully appreciated by those using this technique in more developed countries. Having some experience in establishing optimal growth conditions for P. falciparum in the tropics, I decided to devote a small section of this study to describe exactly how a parasite line is grown and established from infected blood.

a. Laboratory conditions

A major obstacle to be overcome before attempting to cultivate P. falciparum is that of erratic electricity supply. A back-up electricity supply is essential as P. falciparum growth is seriously affected if incubator temperature drops below $37^{o}C$ for more than a few hours. An alarm system with an extremely loud klaxon connected to the incubator should ensure that emergency electricity is connected soon after a mains failure. Such a contingency plan is essential for successful P. falciparum cultures.

b. Sterility

Sterility of media and other culture materials is also of prime importance. The inclusion of antibiotics in the final growth medium greatly assists in this respect but does not affect contamination by yeast and fungi, the levels of which are extremely

high in the tropics especially during the dry season.

As anti-fungal drugs adversely affect in vitro P. falciparum growth, good sterile technique is the only way to ensure the exclusion of yeasts and fungi from cultures. Maintenance of sterility is made easier if the area where manipulations are to take place is kept exclusively for malaria cultures. If culture media cannot be sterilized using an enclosed micro-filtration system (Nalgene, Sybron Corp., New York), autoclavable RPMI 1640 medium is now available which satisfactorily supports P. falciparum growth (Nissui Seiyaku Co., Japan).

c. Culture sera

The most important factor in culturing P. falciparum parasites is the availability of regular supplies of freshly frozen, non-immune European sera which are free from antimalarial drugs. Sera must be sent by air on dry-ice, aliquoted and stored at -20°C on arrival and defrosted only immediately before use. Individual serum pools (collected from one individual on several occasions) have varying parasite growth properties. Some sera will support growth when diluted in medium to a concentration of 5% whilst others require to be used at concentrations of up to 20%. Several serum pools may require to be tested before a suitable growth supporting serum is found. The use of sera containing high lipid concentrations is to be avoided as they are often impossible to filter-sterilize even when diluted to a concentration of 5%. Recent experiments carried out in this laboratory have shown that non-immune plasma also supports the growth of P. falciparum in continuous culture. Reconstituted freeze-dried serum or plasma may also be used as alternatives and are particularly useful in areas where constant freezer temperatures are not possible.

d. Erythrocytes for malaria culture

To avoid agglutination of red cells in culture, the use of AB serum may be necessary. However, group A serum is more easily obtained and as long as group O erythrocytes are used as the standard culture red cells, agglutination should not occur except when commencing cultures from group B and group AB infected blood. P. falciparum parasites which begin their existence in culture in incompatible host erythrocytes will in fact grow normally and re-invade erythrocytes compatible with the culture serum. This is an important observation which permits the establishment of most para-site isolates irrespective of red cell compatibility with the stan-dard culture serum.   Jensen and Trager (1978) advocate the use of outdated blood bank erythrocytes. However, as a general rule, if red cells do not show any sign of haemolysis on storage at 4^oC in acid citrate-dextrose, they are suitable for P. falciparum culture.

e. Culture medium

The standard culture medium for P. falciparum growth, RPMI 1640, can be prepared in deionized water; reconstitution of the medium in glass distilled water is not essential. Stock RPMI con-taining 20 mM Hepes buffer will store for up to 6 weeks at 4^oC. Medium for washing cells consists of the stock medium to which 4.2 mls of 5% $NaHCO_3$ and 250 µl of gentamicin (10 mg/ml) per 100 ml medium are added prior to use. The final culture medium consists of the washing medium containing the appropriate concentration of serum; usually between 10%-15%. Both the washing medium and final culture medium will store at 4^oC for up to 7 days.

f. Gassing conditions

Many of those using the Trager and Jensen culture method suggest the use of bottled gas mixtures which give a culture

atmosphere of 7% CO_2; 5% O_2; 88% N_2.   After many comparative tests, this laboratory has not found any improved P. falciparum growth using commercially obtained gas mixtures when compared with the low oxygen conditions created by the simple candle-jar technique (Jensen & Trager 1978).

g.   Cryopreservation of P. falciparum parasites

Cryopreservation of malaria parasites is not always possible in the tropics but is strongly advised where regular supplies of liquid nitrogen are available to prevent the loss of long established parasite lines due to contamination or altered culture conditions.

Washed packed parasitized red cells are preserved in liquid nitrogen in equal volumes of a cryoprotectant solution which contains 38 gm glycerol, 3 gm sorbital, 0.6 g NaCl in 100 ml of distilled water.   Parasites may be retrieved from liquid nitrogen by resuspending thawed P. falciparum in a hypertonic solution prior to reconstituting in the final culture medium (Rowe et al. 1968) or by a much longer procedure employing the gradual addition of decreasing concentrations of sorbital (Wilson, Farrant & Walter 1977).   The former method results in approximately 50% haemolysis of erythrocytes.   The method of Wilson et al. (1977) although tedious, does produce much higher yields of viable parasites. However, using either method, only ring forms can successfully be retrieved from liquid nitrogen storage and these stages may take several weeks before they multiply at a similar rate to that attained before freezing.

h.   Modifications of the Trager and Jensen culture method

Blood from patients with P. falciparum infections is collected in heparin (10 iu/ml blood) and parasitaemia established.

Parasitaemia is taken as the number of parasitized red cells, not the number of parasites seen, as only one schizont will develop from multiple infected erythrocytes. After washing twice in RPMI 1640 (pH 7.2), infected red cells are diluted in uninfected group O erythrocytes to a starting parasite density of between 1.0%-2.0%, not 0.1% as advocated by Trager and Jensen (1976). After handling in excess of 300 fresh P. falciparum isolates, I have rarely been able to establish a parasite line which was begun in culture at less than 1.0%. A culture haematocrit of 6.0%, made up in the growth medium, is used in this laboratory as more rapid parasite growth has been observed at this red cell concentration than at higher haematocrits. Cultures are dispensed in 1.5 ml amounts into 35 mm plastic petri dishes and incubated at 37°C in the low oxygen atmosphere created by allowing two white candles to burn the oxygen inside the candle jar. Coloured candles often release gases which are toxic to the parasite.

When culturing fresh isolates, parasites will be more easily established if culture medium is replaced twice daily until consistent, prolific parasite growth occurs. There is no fixed time schedule for sub-culturing in fresh red cells. However, during the first week of their culture existence P. falciparum may grow rapidly and may require to be sub-cultured every 2 days. On sub-culturing, parasite density should not be reduced to less than 1.5% during this early period. However, after the first week parasites fail to multiply so rapidly and may only attain twice the starting parasite density over a 4 day period. In such circumstances parasitized cells should only be diluted with an equal volume of uninfected red cells. If the culture has viable parasites yet is barely retaining the starting parasite density, only sufficient red cells to make up the original culture

Continuous cultivation of P. falciparum
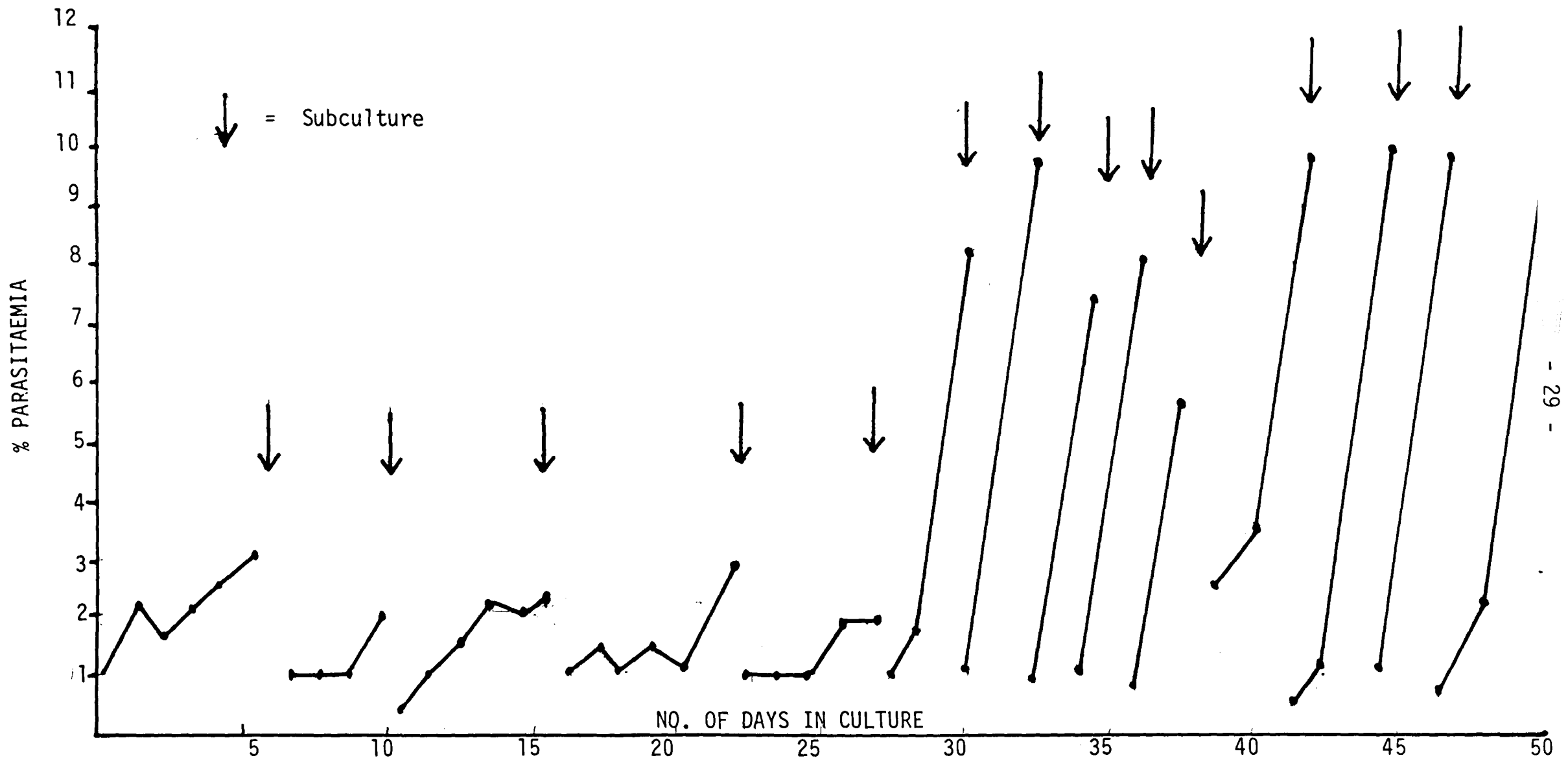
FIG. 1   Establishing a 'wild' isolate of P. falciparum in continuous culture.   Medium changed twice daily until day 28.

haematocrit should be added. This sluggish growth pattern may continue for 3 to 4 weeks during which time sub-culture may only be necessary every 4-7 days as sub-culturing more frequently will result in depleting the numbers of viable parasites. This is a most critical stage in the establishment of a parasite line and daily parasite counts on smears from the culture are necessary to monitor its progress. Under optimal culture conditions P. falciparum parasites are extremely resilient organisms but, during the initial stages of culture growth few parasites may be seen. However, perseverance on the part of the worker usually results in a successfully established P. falciparum parasite line. Figure I provides an example of the early growth pattern of a P. falciparum line which has now been in culture for more than 9 months. Once a parasite culture is established, media need only be replaced once daily and sub-cultured every fourth day. Under such conditions parasite multiplication from 1%-10% normally occurs.

CHAPTER 3

- 3   ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN HUMAN MALARIA

3   1.   INTRODUCTION

Effective host resistance to malaria is thought to involve
a cell mediated phase (Phillips 1970; Phillips et al. 1970;
Weinbaum, Evans & Tigelaar 1976) during which antibody-dependent
cellular cytotoxicity (ADCC) by monocytes and lymphocyte sub-
populations may play an important role (Coleman et al. 1975;
Greenwood, Oduloju & Stratton 1977; McDonald & Phillips 1978).

Specific killing of P. berghei infected red blood cells by
mononuclear cells in the presence of specific antibody has been
demonstrated in mice (Coleman et al. 1975), yet evidence of ADCC
in human malaria is limited to the results of a non-specific assay
in which chicken erythrocytes were haemolysed in the presence of
rabbit anti-chicken RBC antibody by lymphocytes from children with
acute malaria (Greenwood et al. 1977).

The introduction by Trager and Jensen (1976) of continuous
culture of the asexual cycle of P. falciparum has allowed the
development of a simple microassay, used in this study, for investi-
gation of ADCC in human malaria.

3   2.   SUBJECTS AND METHODS

Subjects

a.   Fifteen healthy uninfected adult Gambians aged between 25
     and 50 years, wholly or partially immune to P. falciparum
     malaria.

b.   Twenty-five Gambian children with clinical malaria aged
     between 3 months and 6 years, with parasitaemias from 1%
     to 6%.

c. Two adult Gambians aged 20 and 21 years with low-grade

P. falciparum infections (<0.25%).

Methods - micro-cultivation method for ADCC

Six ml of blood was taken, with parental consent, from each subject.   Serum from 1 ml of blood was used as a supplement for the culture medium and for subsequent malaria antibody determination.   The remainder of the blood was heparinized (10 i.u/ml blood) and mononuclear cells were separated on a lymphoprep gradient after centrifugation at 1800 r.p.m. for 15 minutes. Cells were washed twice in RPMI 1640 and counted. An average of 98% of cells were viable when assessed by the trypan blue dye exclusion test.

An adaptation of the micro-cultivation technique used by Smalley (1976) was employed in this and all subsequent studies. A single asynchronous P. falciparum isolate which had been established in continuous culture for several months was used as the target cell in cell experiments (Trager & Jensen 1976). Parasites were adjusted to a starting density of 0.3% by dilution in European group O erythrocytes in RPMI 1640 (containing 20 mM Hepes and 0.25 ml Gentamicin per 100 ml) to give a final concentration of approximately 3.0×10^4 parasitized erythrocytes per culture. Parasite suspensions were then dispensed in 100 µl aliquots into a 96-well, sterile microtitre tray. A further 100 µl of medium containing the appropriate number of mononuclear cells was added and duplicate cultures were supplemented with 10% test or control serum (previously heated to 56^OC for 30 minutes). Cultures were then incubated at 37^OC for 48 hours in an atmosphere of 5% O_2; 7% CO_2 using the candle jar method of Jensen and Trager (1977), the medium being replaced daily.   Films were taken every second

day and stained with Giemsa.

The systems tested were: -

1. Mononuclear cells + autologous serum.

    Control - Autologous serum without mononuclear cells.

2. Mononuclear cells + non-immune European serum.

    Control - European serum without mononuclear cells.

Mononuclear cell:parasite ratios of 10:1, 20:1 and 50:1 were examined.

The degree of ADCC was determined by assessment of the rate of parasite multiplication in the presence of mononuclear cells and serum, compared with parasite growth in serum alone. Ten thousand erythrocytes were counted in each one.

Malaria antibody was measured against a schizont antigen grown from a single P. falciparum isolate using a double-diffusion gel technique (McGregor & Williams 1978).

The statistical method used was Wilcoxon's rank sum test. Parasite growth in this chapter is represented by mean multiplication ratios (on a log scale) over the starting parasite density of 0.3% (i.e. log 1).

3 3. RESULTS

To ensure that P. falciparum was not killed merely by un-favourable culture conditions created by high mononuclear cell numbers, target parasites were cultivated in stock European control serum with mononuclear cells from 7 non-immune Europeans. At an effector cell:target ratio of 50:1 (1.5×10^6 mononuclear cells: 3×10^4 parasites/culture) no inhibition of P. falciparum growth occurred (Table 1). Thus, any observed parasite death caused by mononuclear cells from infected children at this ratio

TABLE 1

The effect of non-immune European mononuclear cells on P. falciparum growth

No.	Serum control	Mononuclear cells $(1.5 \times 10^6 + \text{serum})$
L62	5.0	4.3
L63	5.0	7.0
L64	4.7	3.7
L65	5.3	5.0
L66	5.0	5.0
L82	3.0	4.7
L83	3.0	3.3

Mean parasite multiplication rates of duplicate cultures after 48 hours in European serum and European serum plus mononuclear cells (effector cell:target ratio 50:1).

CHILDREN WITH ACUTE P.falciparum MALARIA
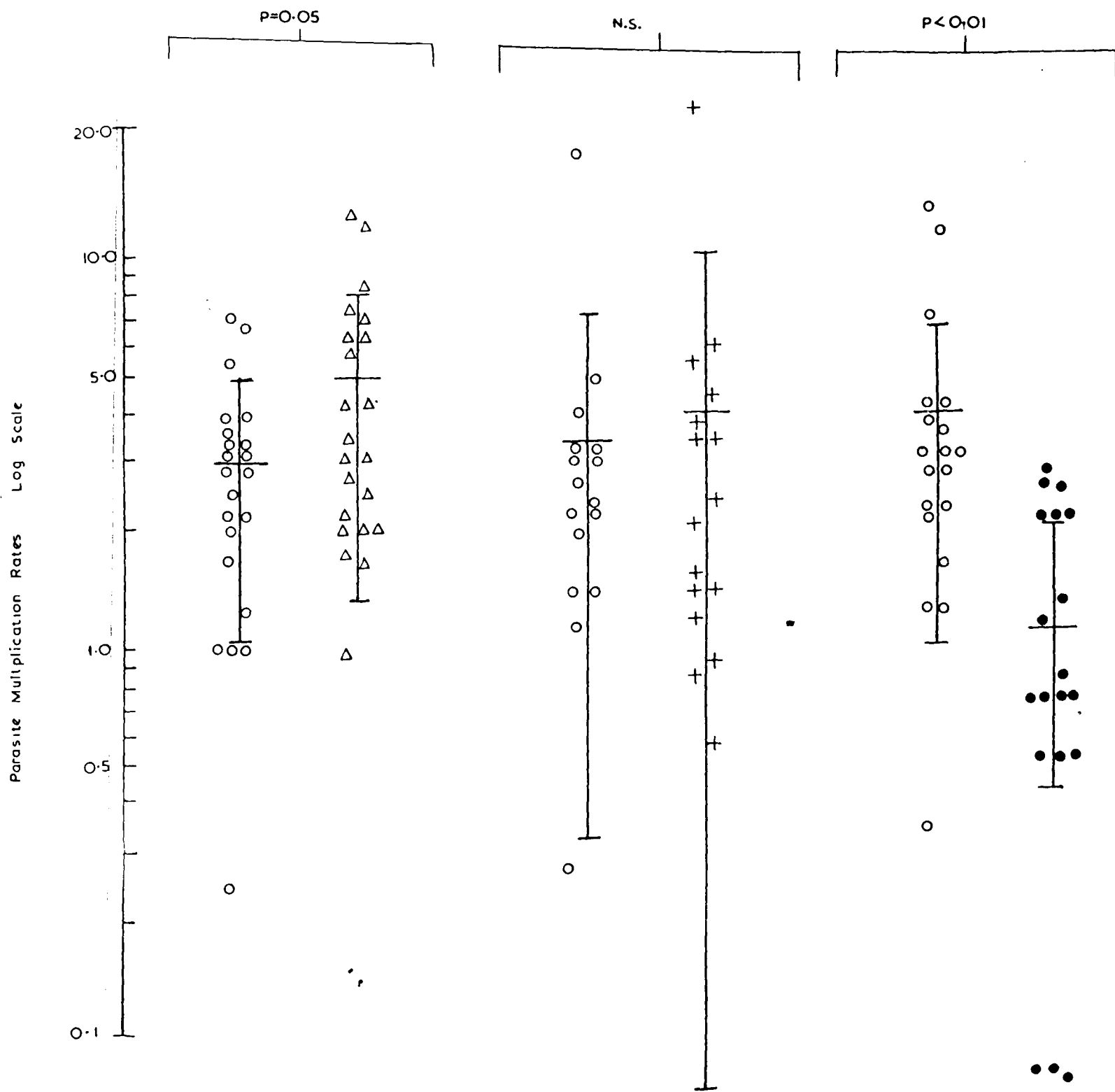
FIG. 2   Children with acute P. falciparum malaria.

Parasites in autologous serum - 2 day cultures.

(o) Serum only; mononuclear cell:parasite ratios

(△) 10:1 (+) 20:1 (●) 50:1

1.0 = starting parasitaemia (0.3%)
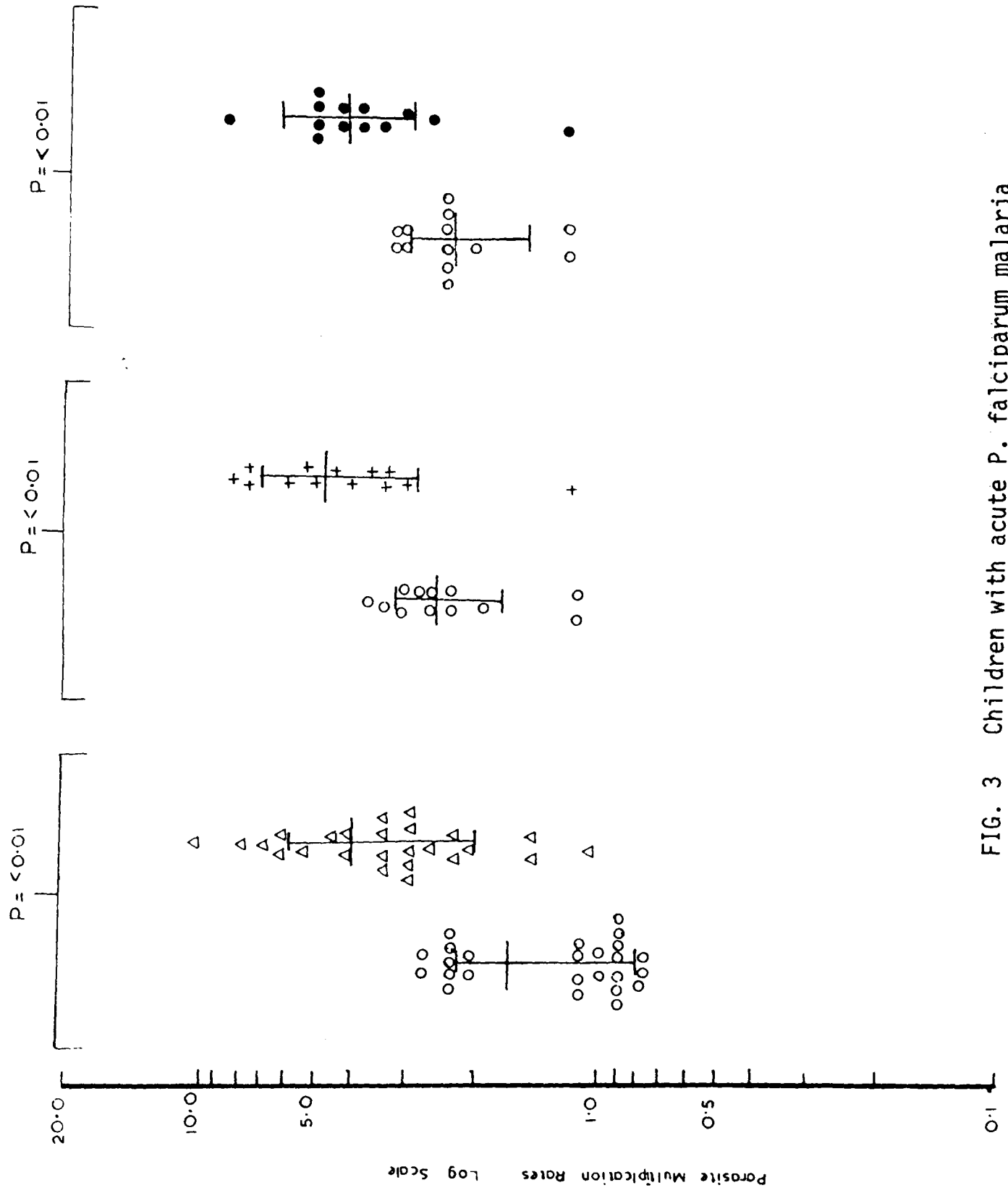
FIG. 3   Children with acute P. falciparum malaria

FIG. 3   Children with acute <u>P. falciparum</u> malaria

Parasites in non-immune European serum.

2 day cultures.

(o) Serum only

Mononuclear cell:parasite ratios: ( ) 10:1

(+) 20:1 (o) 50:1

1.0 = starting parasitaemia (0.3%)
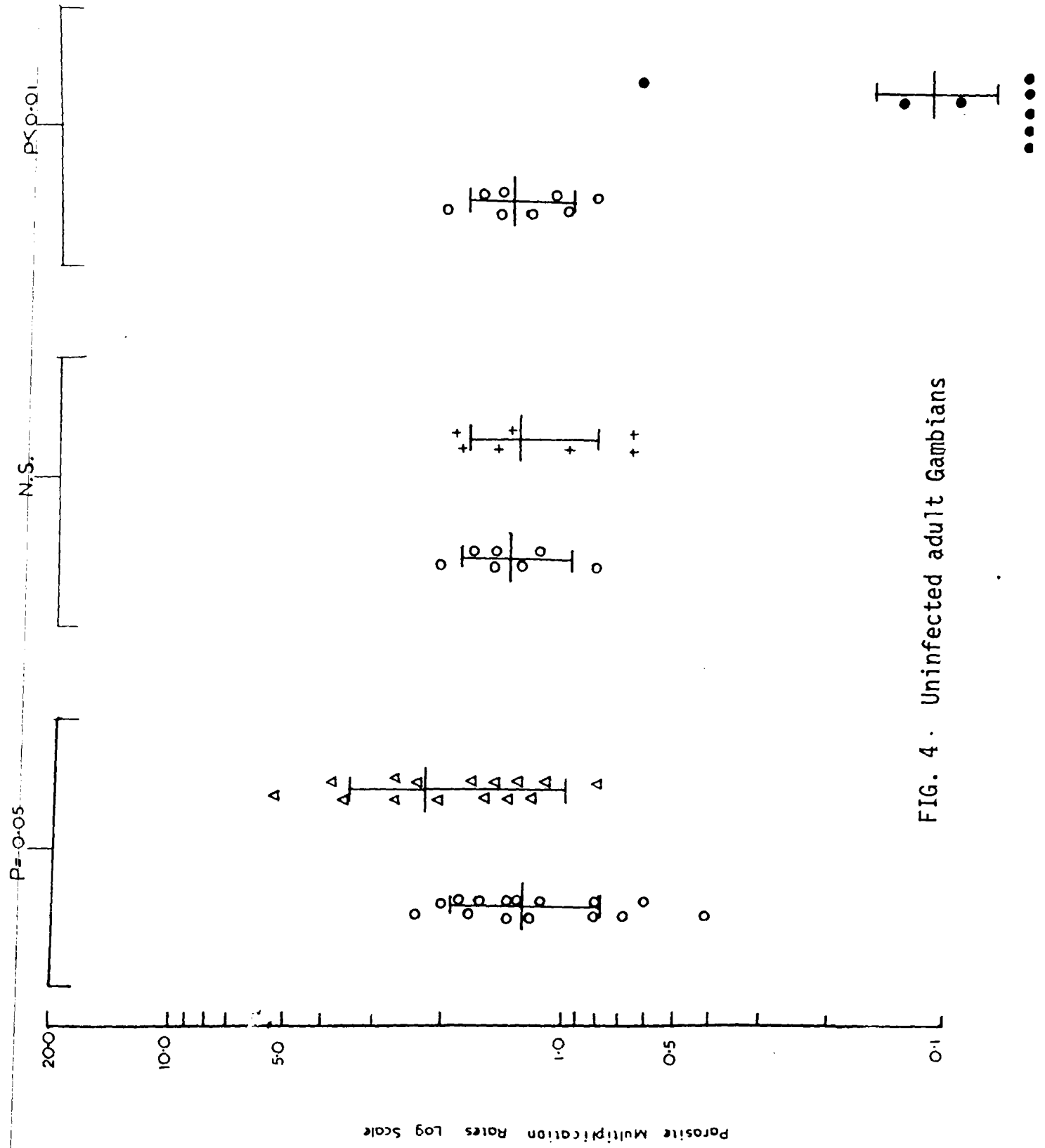
FIG. 4 · Uninfected adult Gambians

FIG. 4   Uninfected adult Gambians

Parasites in autologous serum.

2 day cultures.

(o) Serum only

Mononuclear cell:parasite ratios: ( ) 10:1

(+) 20:1 (o) 50:1

1.0 = starting parasitaemia (0.3%)
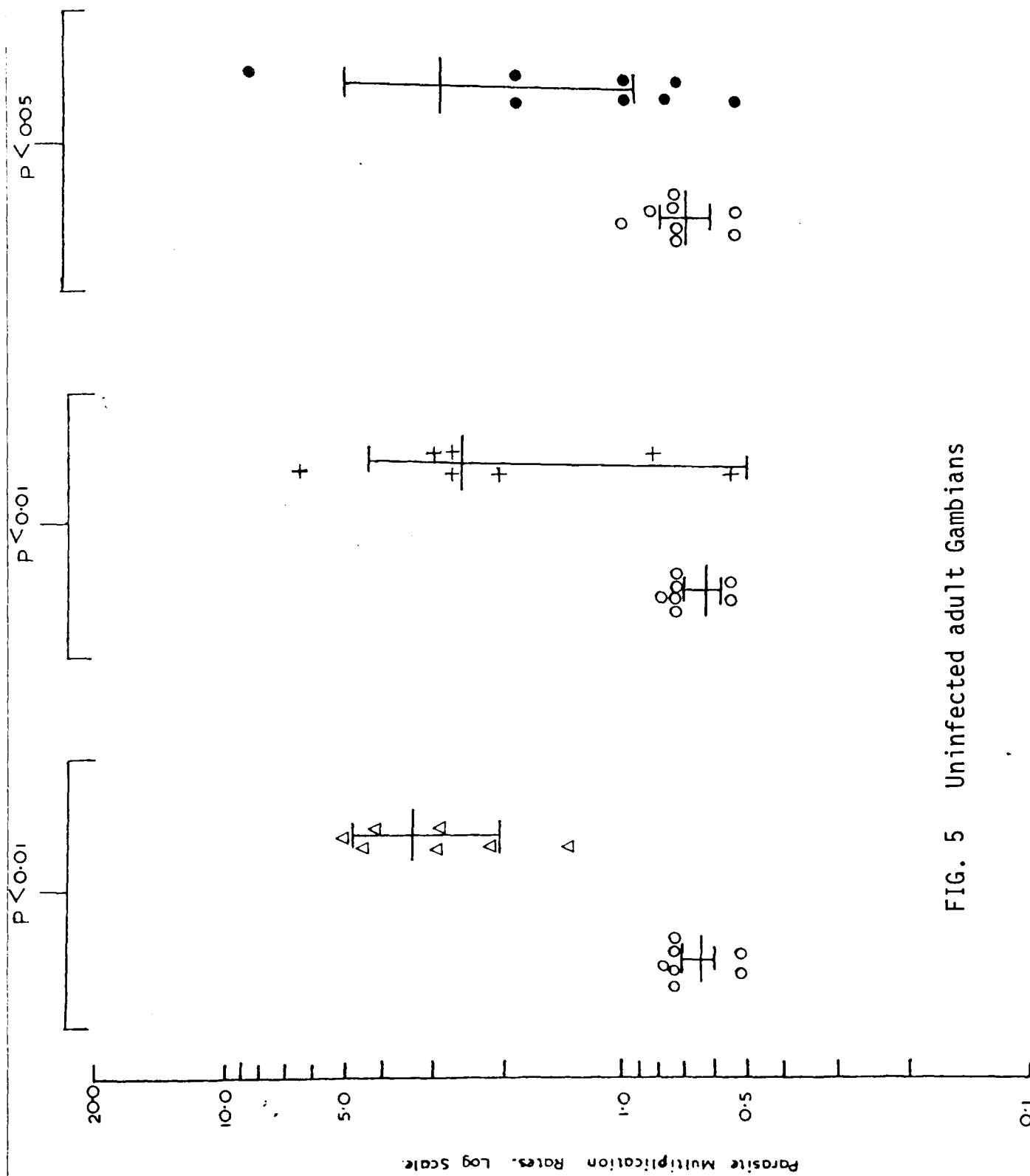
FIG. 5   Uninfected adult Gambians

FIG. 5   Uninfected adult Gambians

        Parasites in non-immune European serum.
        2 day cultures.
        (o) Serum only
        Mononuclear cell:parasite ratios ( ) 10:1
        (+) 20:1 (o) 50:1
        1.0 = starting parasitaemia (0.3%)

was likely to be the result of a recognised immunological reaction rather than depletion of nutrients in the culture medium.

Children

Figure 2 shows that at a mononuclear cell:target ratio of 10:1 in autologous serum there was a significant increase in growth of the parasite at the 5% level compared with the serum control. There was no difference in growth at a ratio of 20:1, but at a ratio of 50:1 mononuclear cells from children with malaria produced a significant decrease in parasite growth in autologous serum ($P<0.01$) compared with growth in serum only.

In non-immune European serum, a significant increase in parasite growth was observed at mononuclear cell:target ratios of 10:1, 20:1 and 50:1 ($P<0.01$ in each case) compared with the serum control (Fig. 3).

Gambian adults

When mononuclear cells from uninfected immune Gambian adults were grown in autologous serum (Fig. 4) at an effector cell:target ratio of 10:1 there was a significant increase in parasite growth over the serum control ($P<0.05$). At a ratio of 20:1 there was no difference but at 50:1 there was significant inhibition of growth ($P<0.01$).

When mononuclear cells from immune adults were cultured in non-immune European serum there were significant increases in growth at effector cell:target ratios of 10:1 ($P<0.01$), 20:1 ($P<0.01$) and 50:1 ($P<0.05$) compared with the European serum controls (Fig. 5).

Table 2 shows that after 6 days parasite growth in childrens' sera without mononuclear cells was 14.3 times the starting

parasite density, whereas there was no increase on day 2 and an inhibition of growth on day 4 when mononuclear cells were added at a 50:1 effector cell:target ratio. In adults there was no increased growth on day 2, a slight increase on day 4 and a reduction on day 6 in autologous serum only. The addition of fifty mononuclear cells per parasite produced marked killing of P. falciparum on day 2.

Table 3 shows that there was effective ADCC at an effector cell:parasite ratio of 10:1 in both individuals during infection. However, after recovery 2 weeks later, killing of the parasite was evident only at a ratio of 50:1.

Experiments carried out to compare ADCC in 25% serum compared with 10% serum showed no difference.

Three children (13%) showed increased ADCC at the 10:1 ratio, eleven (65%) at 20:1 and fourteen (93%) at 50:1 (Table 4). Each child showing increased killing at these levels had detectable malaria antibody. Eighteen children (72%) who had antibody failed to show ADCC at 10:1. Four children (24%) who had malaria antibody failed to show killing of the parasite at 20:1. Four children (17%) who had no demonstrable malaria antibody failed to show activity at a mononuclear cell:parasite ratio of 10:1, two (12%) failed to show killing at 20:1 as did one (17%) at 50:1.

In immune adults without malaria, none of the subjects tested showed ADCC at 10:1, although all had malaria antibody. Of seven adults tested at 20:1, four (57%) showed killing of the parasite and three (43%) did not, although all had malaria antibody. At a ratio of 50:1 all eight adults (100%) tested demonstrated ADCC and all had malaria antibody.

TABLE 2

Parasite multiplication in serum versus serum plus mononuclear cells

	Day 2	Day 4	Day 6
Children			
Serum only (n=11)	2.6 ± 1.5	6.6 ± 3.8	14.3 ± 5.5
Serum + mononuclears (n=16)	1.0 ± 0.75	0.3 ± 0.2	Dead
Adults			
Serum only (n=13)	1.0 ± 0.5	1.4 ± 1.4	0.47 ± 0.67
Serum + mononuclears (n=9)	0.15 ± 0.3	0.1 ± 0.4	Dead

Mean parasite multiplication rates over 6 days in homologous serum and homologous serum plus mononuclear cells (mononuclear:parasite ratio 50:1) in adult Gambians and Gambian children with acute malaria.

TABLE 3

ADCC during and after infection

Patient	Serum control	Mononuclear-parasite ratios		
		10:1	20:1	50:1
During infection				
K.C.	2.5	0.4	<0.01	<0.01
K.D.	3.3	0.16	<0.01	<0.01
After infection				
K.C.	1.0	1.7	1.0	0.66
K.D.	1.75	2.7	1.7	0.2

Parasite multiplication rates after 2 days in two young Gambians, during and after infection with P. falciparum. Parasites in homologous serum.

TABLE 4

Mononuclear cell killing of P. falciparum - dependence on malaria antibody

n Monocuclear parasite ratio	No. tested	No. showing killing		No. showing no killing	
		With AB	Without AB	With AB	Without AB
Children					
10:1	25	3	0	18	4
20:1	17	11	0	4	2
50:1	15	14	0	0	1
Adults					
10:1	15	0	0	15	0
20:1	7	4	0	3	0
50:1	8	8	0	0	0

ADCC in relation to the presence of malaria antibody in uninfected adult Gambians and Gambian children with acute malaria.   Two-day cultures.

3   4.   DISCUSSION

Significantly increased ADCC to malaria parasites was demonstrated at a mononuclear cell:parasite ratio of 50:1 in uninfected adult Gambians and in children with acute malaria.   However, individuals from each group showed increased ADCC at ratios of 10:1 and 20:1.   Killing was without exception, dependent on the presence of malaria antibody.   Control cultures containing similar numbers of non-immune European mononuclear cells allowed normal parasite growth.   Thus, it is unlikely that adverse culture conditions were responsible for the marked parasite death observed in test cultures.

Transient protection against malaria by passive transfer of immune serum has been shown in humans (Cohen, McGregor & Carrington 1961) and in rats (Phillips & Jones 1972).   However, the most effective passive transfer of immunity in murine malaria requires both serum and cells (Phillips 1970; Cottrell, Playfair & De Souza 1978).   In these experiments, the parasite killing effect of adult serum was evident by day 6; with addition of mononuclear cells enhanced killing was demonstrable by day 2. In children, however, the addition of mononuclear cells seemed to be a more important factor in killing the parasite, as parasite replication was reduced from 14.3 times the starting parasite density after 6 days to 0.3 times the starting density on day 4 when the appropriate number of mononuclear cells were added.

It is possible that those children who exhibited increased ADCC at ratios of 10:1 and 20:1 possessed higher levels of malaria immunity than those who reacted only at the 50:1 effector cell: target ratio.   Such a view is supported by the observation that

at a ratio of 50:1 cells from immune adults showed a significantly greater killing capacity than did cells from infected children (P<0.01). It may be that the level at which ADCC occurs in an individual is an indication of protective immunity. Further studies comparing individuals with varying levels of parasitaemia, employing a more detailed dose response curve, will demonstrate whether or not this assay is a measure of protective immunity in human malaria.

T lymphocytes with IgG Fc receptors and non-T and non-B lymphocytes (null cells) have been described as effector cells in ADCC against chicken erythrocytes (Li Shen et al. 1979) and against P. berghei infected RBC's (Coleman et al. 1975). However, Phillips and his co-workers (1970) demonstrated that T lymphocytes had no cytotoxic effect on erythrocytes containing malaria parasites. Brown, K.N. (1971) suggested that the role of T lymphocytes in malaria immunity was related to the production of protective antibody. Measurement of malaria antibody levels on culture supernatants at the beginning of a culture and after parasite death due to ADCC with a T-cell depleted culture control, could possibly confirm this.

Monocytes (McDonald & Phillips 1978) and polymorphonuclear neutrophil leucocytes (PMN) (Brown, J. & Smalley 1981)have also been shown to inhibit the growth of malaria parasites. It is unlikely that PMN acted as cytotoxic cells in the experiments described here, since the killing of P. falciparum infected erythrocytes by neutrophils in vitro has been found to be independent of antibody (Brown, J. & Smalley 1981). Therefore, non-specific killing of the parasite by PMN would also have occurred in non-immune European serum. Since monocytes accounted for at least 3% of mononuclear cells some contribution to killing by monocytes

could not be discounted.

Greenwood et al. (1977) described increased non-specific ADCC in children with acute malaria associated with increased numbers of 'null' cells. After recovery, a decrease in ADCC was accompanied by a return to normally low 'null' cell levels. Two adult Gambians in my experiments, studied during and after P. falciparum infection, showed five times more killing during infection than on recovery. These observations agree with those of Greenwood et al. (1977) and suggest that increased ADCC against P. falciparum infected erythrocytes may be due to increased numbers of 'null' cells which could contribute to the elimination of blood-stage P. falciparum parasites. It appears that serum on its own containing adult levels of malaria antibody is more effective in killing P. falciparum than sera from children and that in immune adults, mononuclear cells merely hasten inevitable parasite death. However, in infected adults far fewer effector cells are required to kill the parasite than in infected children.

At mononuclear cell:parasite ratios of 10:1, 20:1 and 50:1 in European serum, in both adults and children, and at a 10:1 ratio in autologous serum, there were significant increases in parasite growth over controls. The reasons for this phenomenon remain unknown and require further investigation. Nevertheless, this may prove to be an important finding in that the isolation of a soluble mononuclear cell product promoting P. falciparum growth could prove invaluable in the in vitro production of high yields of asexual parasites.

Finally, since it is unlikely that the children and adults studied had past experience of only one strain or variant of P. falciparum and while mononuclear cells from a majority of

subjects tested were yet able to kill the target parasite used in this study, a degree of cross-protection between P. falciparum strains and/or variants seems likely.

CHAPTER 4

4   THE ROLE OF ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN RESISTANCE
     TO PLASMODIUM FALCIPARUM MALARIA.

4   1.   INTRODUCTION

In man, repeated attacks of malaria are necessary for the
development of protective immunity (McGregor 1967). Effective
immunity to malaria is associated with high levels of specific
antibody which is believed to protect the host by preventing
merozoite invasion of erythrocytes (Miller, Powers & Shiroishi
1977) and by inducing macrophages to ingest schizonts (Celada,
Cruchaud & Perrin 1982). Acute P. falciparum infection in
infants also stimulates the production of specific antibody but
little of the immunoglobulin produced is believed to be protective
(Brown, K.N. 1976). Such acute malaria sera do not prevent parasite
multiplication in vitro (Chapter 3) and the sera of individuals
recovering from a primary attack of malaria fail to induce phago-
cytosis of schizonts (Celada et al. 1982). Nevertheless, most
children do recover from malaria despite their apparent lack of
protective antibody, suggesting that additional mechanisms may be
involved in resistance to acute P. falciparum infection. Evi-
dence from work on animal models suggests that cell-mediated
reactions are involved in recovery from acute malaria (Phillips
1970; Clark, Allison & Cox 1976).

A frequent observation during malaria infection is the appear-
ance of 'crisis-forms' (Taliaferro 1949). Jensen, Boland and
Akood (1982) have recently shown that such damaged parasites can
be induced in culture by the activity of hyperimmune sera from a
malaria endemic area.

In contrast, previous studies have shown that in the presence

of sera from children with acute malaria, dramatic increases in P. falciparum growth can occur (Chapter 3). In mice with malaria, Clark et al. (1976) attributed the appearance of 'crisis-forms' to the activity of cytotoxic mononuclear cells and more recently, early cells of the monocyte-macrophage series have been implicated as effector cells against P. yoelii infections in mice (Taverne, Dockrell & Playfair1982). The potential importance of cell-mediated immunity in recovery from acute malaria can also be demon-strated by the ability of B-cell deficient mice to overcome P. chabaudi infection (Grun & Weidanz 1981) and by the greater protec-tion conferred on rats with P. berghei infections by the admini-stration of immune serum plus immune mononuclear cells when com-pared with the lesser protective effect of immune serum alone (Phillips 1970).

In human malaria, antibody-dependent cellular cytotoxicity (ADCC), by mononuclear cells from a majority of children with malaria and immune adults, was shown to kill P. falciparum in vitro (Chapter 3). As ADCC was more effective in immune adults than in acutely infected children, it was suggested that such measure-ments made in vitro may reflect levels of protective immunity.

This study attempts to correlate the ability of mononuclear cells to kill blood stage P. falciparum parasites in vitro, with parasitaemia in malarious Gambian children and to assess the possible importance of such a mechanism in host resistance to acute infection.

4   2.   SUBJECTS AND METHODS

Subjects

1.   Fifty-one children with P. falciparum malaria whose parasi-taemias ranged from 0.1% to 10.0% were studied.   Of these, 24

children aged between 12 months and 12 years had acute infections of >2.0% with a short history of fever of 2-4 days. Their mean Hb was - 9.6 ± 1.2g%. The remaining 27 children aged between 12 months and 14 years had a history of fever for at least 10 days with a parasitaemia <2.0% and all were anaemic with mean Hb being 7.2 ± 1.2g%. None of these children had received antimalarial treatment before attending the clinic at Fajara.

2. Sixteen children aged between 8 months and 4 years were studied during acute malaria and also 2-4 weeks after treatment with chloroquine (150 mg). During acute infection, parasitaemias ranged between 2% and 12%; after treatment none had malaria parasites in the blood.

3. Eleven children, aged between 8 months and 14 years who had no recent history of malaria and had no malaria parasites in the blood, were studied at the end of the dry season when there is little or no malaria transmission.

4 3. LABORATORY METHODS

Cytotoxicity assays

Mononuclear cells were examined for ADCC against a stock laboratory isolate of P. falciparum as described in Chapter 3. Each test culture contained approximately 3×10^4 parasitized red blood cells diluted in normal group 0 erythrocytes giving a final culture haematocrit of 3%. Mononuclear cells were added in numbers sufficient to test effector cell:target ratios of 10:1, 20:1 and 50:1. Cultures were set up in duplicate and incubated at 37°C for 48 hours in an atmosphere of 5% O_2 and 7% CO_2.

Serum inhibition assays

In these experiments, the independent inhibitory effect of

sera on the growth of established autologous parasites and 'wild' heterologous P. falciparum isolates was examined. As 'wild' isolates were used as target cells on the day blood was taken, synchronization of cultures was not necessary. However, to obtain synchronous ring stages from established parasite lines concentration of schizonts by Plasmagel (Roger Belon, France) sedimentation was performed.

All established cultures to be used as target cells were washed twice in RPMI and resuspended in an equal volume of RPMI containing 20% foetal calf serum. The parasite suspension was then mixed thoroughly with an equal volume of Plasmagel and incubated at $37^{o}C$ for 15 minutes. The supernatant (containing schizonts) was removed and washed twice in RPMI. The centrifuged deposit contained more than 70% parasitized red blood cells of which at least 80% were infected with P. falciparum schizonts. The schizont deposit was diluted in normal group 0 erythrocytes to a parasite density of 1.0%, returned to stock culture and allowed to complete one schizogony before ring stages were harvested for use in the test.

All target parasites used in the serum inhibition assay were washed twice before use, resuspended in RPMI and dispensed in 100 µl aliquots into a sterile, flat-bottomed, 96-well microtitre tray. Cultures each contained 3×10^4 parasitized erythrocytes diluted in normal group 0 red blood cells to give a culture haematocrit of 3%. Fifteen microlitres of test or control serum were added to each culture before incubation at $37^{o}C$ for 48 hours in an atmosphere of 5% O_2 and 7% CO_2. Stock European serum was used as the parasite growth control. The degree of parasite reinvasion was assessed on Giemsa stained blood films taken from duplicate cultures by counting the number of parasitized

erythrocytes seen in 10^4 red blood cells.

Data were analysed by Spearman's correlation coefficient or by the two-way paired or unpaired student's 't' test. Parasite growth in the figures in this chapter are represented by Log_{10} multiplication ratios, with the starting parasite density of 0.3% shown as $Log_{10}0$. This means of display was necessary to demonstrate the distribution of individual ADCC results which fell below the starting parasite density. Results in Tables 5 & 6 are presented simply as parasite multiplication ratios with a ratio of 1 representing the starting parasite density of 0.3%.

4   4.   RESULTS

Relationship between parasitaemia and ADCC

At a 50:1 effector cell:target ratio ADCC showed an inverse correlation (r_s = -0.69; P<0.001) with parasitaemia in children with clinical malaria (Fig. 6). Parasite growth was inhibited more by mononuclear cells plus serum (at the 50:1 ratio) than by serum alone in children with parasitaemias >2.0% (P<0.001), but ADCC did not occur at ratios of 10:1 or 20:1 (Fig. 7). However, children with parasitaemias <2.0% displayed significantly greater ADCC at the 50:1 cell:parasite ratio (P<0.001) than did children with higher parasitaemias. In addition, children with parasitaemias <2.0% also showed ADCC at the 20:1 effector cell:target ratio.

Figure 7 also shows that sera from children with parasitaemias <2.0% inhibited the growth of P. falciparum more than sera from children with parasitaemias >2.0% (P<0.005).

Children studied during and after acute malaria

Figure 8 shows that a mixture of mononuclear cells and serum from children with acute malaria inhibited parasite growth at a cell:parasite ratio of 50:1 (P<0.02), but not at ratios of

Serum + cells
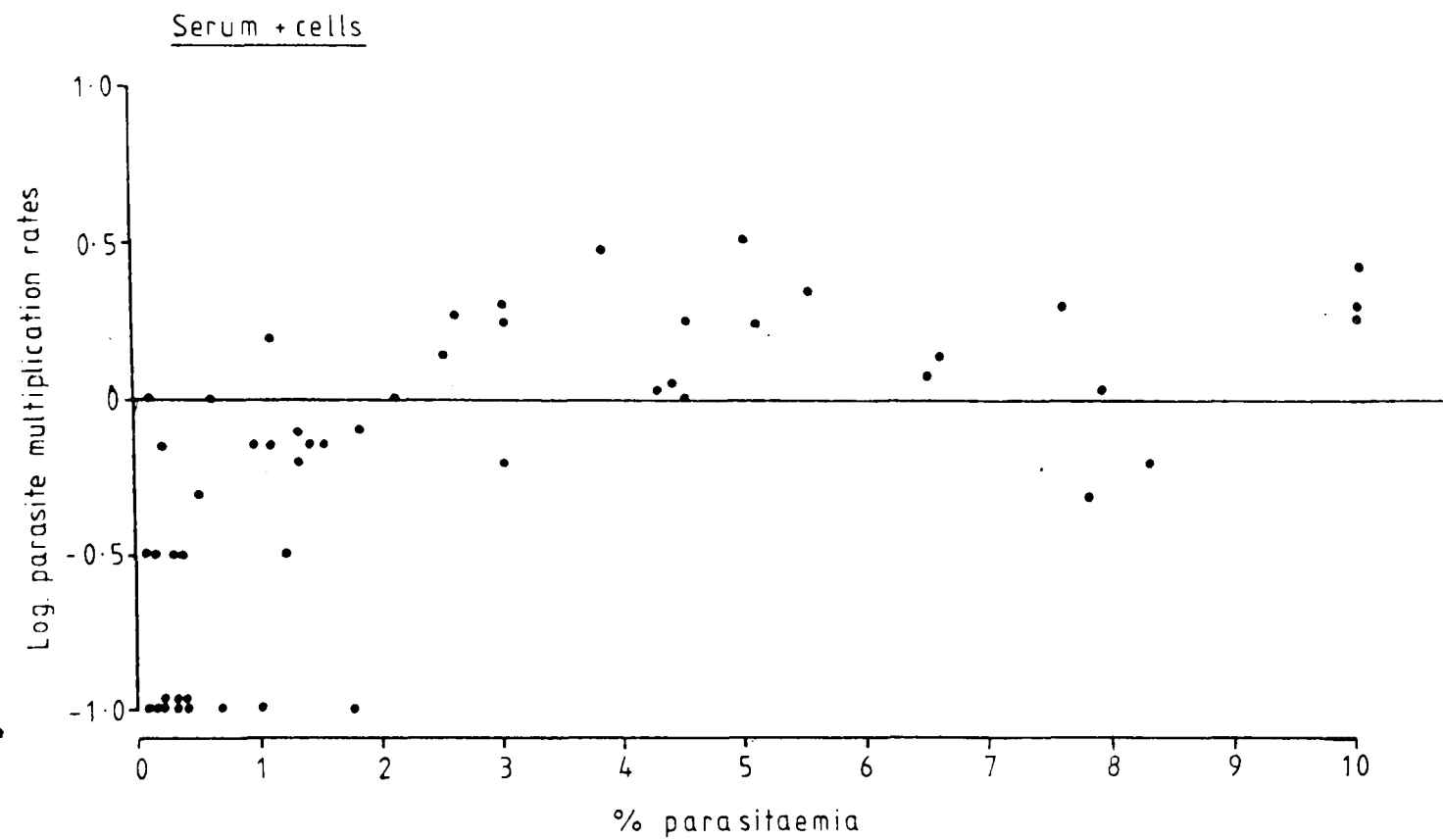
FIG. 6   Children with P. falciparum malaria

Log10 parasite multiplication ratios (at a 50:1 mononuclear cell:parasite ratio in autologous serum) in relation to parasitaemia in children. 2 day cultures. Log10 0 represents the starting parasitaemia (0.3%).
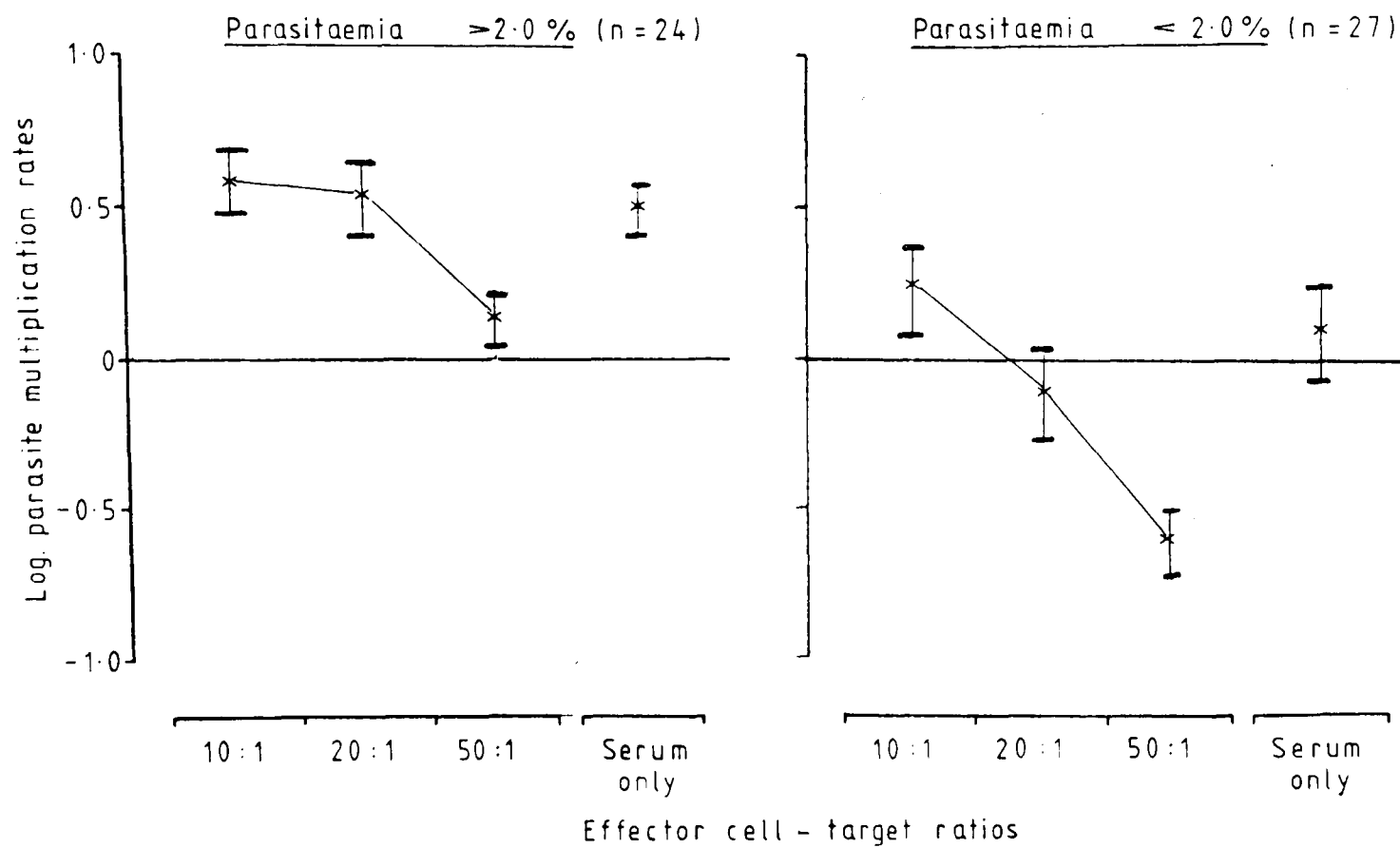
FIG. 7   Children with P. falciparum malaria

Mean Log$_{10}$ parasite multiplication ratios (± S.E.). Parasites in autologous serum with or without mononuclear cells. 2 day cultures. Log$_{10}$0 represents the starting parasitaemia (0.3%).
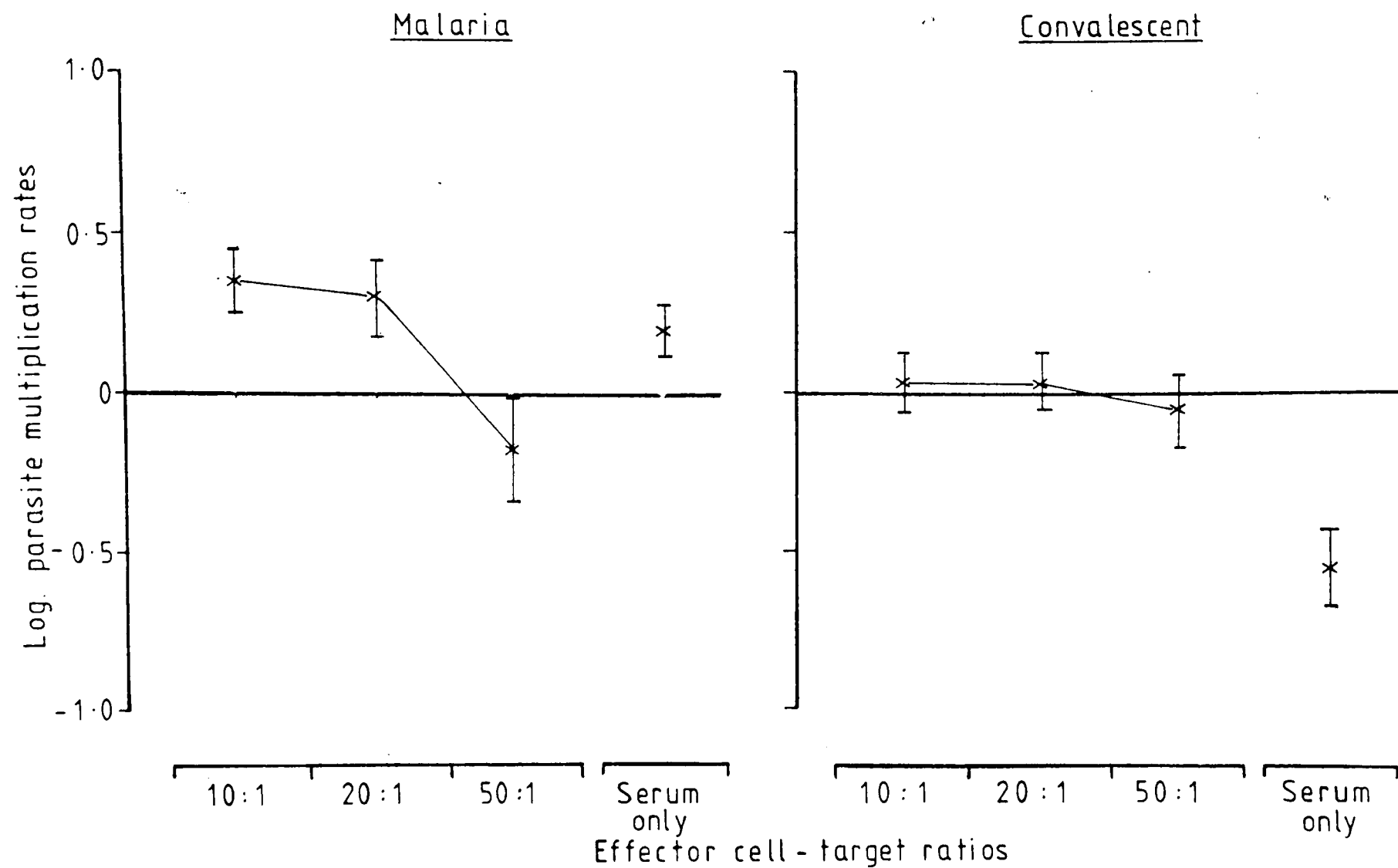
FIG. 8   Children during and after P. falciparum malaria.

Parasites in autologous serum with or without mononuclear cells. 2-day cultures
$Log_{10}0$ represents the starting parasitaemia (0.3%). Mean (± S.E.) of 16 paired
samples are shown.
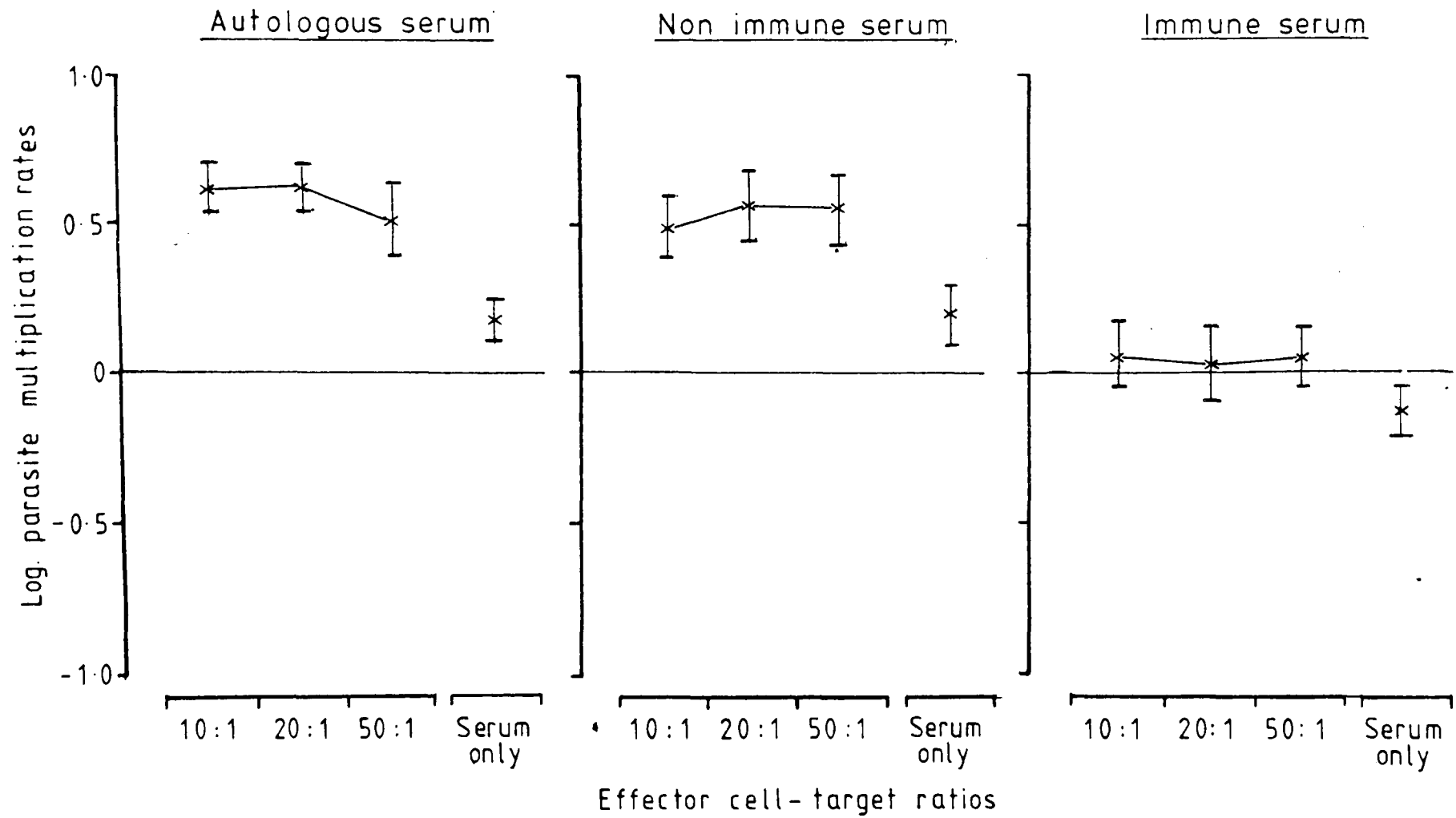
FIG. 9   Uninfected children tested during dry season.

Mean Log_{10} parasite multiplication ratios (± S.E.).   2 day cultures.   Mononuclear cells from each child tested in autologous serum, non-immune European serum and immune serum. $Log_{10}0$ represents the starting parasitaemia (0.3%).   11 children tested.

10:1 or 20:1; their sera alone had no inhibitory effect on P. falciparum growth. However, 2-4 weeks later, sera from these children inhibited P. falciparum growth (P<0.001) but now the addition of autologous convalescent mononuclear cells did not enhance this effect (Fig. 8). Control sera from 10 non-immune, adult European volunteers tested 2 weeks after chloroquine administration (300 mg) did not affect parasite multiplication.

ADCC in children without malaria

Mononuclear cells from uninfected children studied during the season of minimal malaria transmission did not inhibit P. falciparum growth in the presence of either autologous serum or non-immune European serum (Fig. 9). In addition, the inhibitory effect of immune serum, obtained from an adult Gambian, was not enhanced by the addition of mononuclear cells from uninfected children.

Specificity of ADCC

The specificity of ADCC during acute malaria was examined by testing mononuclear cells (at the 50:1 ratio) from children with malaria against their autologous parasite and also against a 'wild' heterologous P. falciparum isolate. Table 5 shows that in each child, ADCC was similar against both parasite isolates. Those children who showed effective ADCC against their infecting parasite strain displayed similar killing of the heterologous parasite. Conversely, those children whose mononuclear cells failed to kill their autologous parasite in vitro also showed ineffective ADCC against the heterologous P. falciparum isolate. These results suggest that ADCC is not strain specific.

Specificity of parasite inhibitory antibody

The inhibitory effect of acute and convalescent sera was examined by cultivating parasites isolated from 16 children and 7

TABLE 5

Children with malaria - Specificity of ADCC

No.	Autologous parasite +		Heterologous parasite +	
	Cells + Serum	Serum only	Cells + serum	Serum only
L41	1.0	3.3	0.3	6.0
L42	0	1.0	0	1.0
L43	2.7	2.7	3.7	3.3
L44	0	2.0	0	1.3
L45	2.7	2.7	2.7	1.7
L46	2.0	2.7	1.7	2.7
L47	1.0	3.0	1.0	2.7
L48	2.7	4.0	2.3	2.0

Parasite multiplication ratios in: -

Serum + cells = Autologous serum + autologous mononuclear cells at cell:
               parasite ratio of 50:1.

Serum only = Autologous serum without mononuclear cells.

Ratio of 1.0 = Starting parasite density (0.3%).

               Culture period 48 hours.

TABLE 6

Children during and after acute malaria - specificity of parasite inhibitory antibody

	Autologous P.f.	P.f.A	P.f.B	P.f.C	P.f.D	P.f.E	P.f.F	P.f.G
Acute serum	2.0 ± 0.25	2.4 ± 0.15	1.7 ± 0.1	2.8 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	2.7 ± 0.3	2.0 ± 0.3
	P<0.001	P<0.05	P<0.01	NS	NS	NS	NS	NS
Convalescent serum	0.4 ± 0.05	1.6 ± 0.1	1.1 ± 0.1	2.3 ± 0.3	0.9 ± 0.15	0.7 ± 0.15	2.2 ± 0.3	2.4 ± 0.4

P. falciparum growth in 16-paired serum specimens - multiplication ratios ± standard error shown. Parasite growth in acute or convalescent serum.

Ratio of 1.0 = starting parasite density of 0.3%

P.f = P. falciparum isolate. Culture period - 48 hours.

fresh heterologous P. falciparum isolates for 48 hours in paired
serum samples obtained from these children during and after acute
malaria.   Two parasite isolates, PfD and PfE (Table 6) were
inhibited by a majority of acute-phase sera.   Individual conva-
lescent sera showed increased inhibition of their autologous para-
site isolate.   PfA and PfB were also inhibited by convalescent
sera.   No increased inhibition of the remaining 5 P. falciparum
isolates was recorded.

4   5.   DISCUSSION

ADCC against P. falciparum was found to vary with parasitaemia
in children with malaria; mononuclear cells from children with
low parasitaemia were more effective than those from children
with acute infections.   As children with low parasitaemias had a
long history of fever and were anaemic, it is likely that they
had infections of relatively long duration.   Also, inhibitory anti-
body was detected only in the sera of children with low parasite
densities which suggests that they were recovering naturally from
their infections.   The greater parasite killing displayed by mono-
nuclear cells from children recovering from malaria may have been a
measure of protective immunity.

Mononuclear cells from only 7 of 24 acutely infected children
prevented parasite multiplication.   These 7 children may have
been more able to overcome P. falciparum infection than those whose
mononuclear cells failed to prevent parasite multiplication.
Nevertheless, in all infected children, parasite numbers were
always less, at the end of the culture period, in the presence of
mononuclear cells and autologous serum than in the presence of
autologous serum alone, indicating that ADCC might be a primary
protective mechanism against acute malaria.   This view can be

supported by the comparison made between cellular and humoral parasite growth inhibition during acute malaria and 2-4 weeks after treatment. During acute malaria P. falciparum was killed by acute-phase mononuclear cells and serum but not by acute-phase sera alone, whereas in convalescence, parasite multiplication was greatly reduced by convalescent sera acting independently. Mononuclear cells from convalescent children did not enhance this effect. It is unlikely that the inhibitory effects of convalescent sera were due to the presence of chloroquine, as sera from European volunteers, tested two weeks after chloroquine administration, failed to inhibit P. falciparum growth.

In The Gambia, P. falciparum malaria is prevalent for only 6 months of the year. This could explain why sera from only 2 of 11 uninfected children displayed minimal parasite inhibition; humoral immunity in the majority of uninfected children may have waned due to several months free from parasite challenge. Cohen et al. (1961) have shown that West Africans begin to lose malaria immunity after only a few months residence in the United Kingdom.

In convalescent children, parasite inhibitory antibody increased against their autologous infecting P. falciparum strain and against some, but not all heterologous parasite isolates tested. As antigenic diversity of P. falciparum is now known to occur in The Gambia (McBride & Brown, J. unpublished data), this result could represent a strain-specific humoral response by the host (a hypothesis previously put forward by Brown, K.N. (1976)). Conversely, ADCC during acute malaria was not strain related. Thus, as measured by this assay, ADCC appears to be a first-stage mechanism of resistance to malaria which preceeds the conventional humoral response to the infecting parasite strain.

In this study, no attempt was made to characterise the effector cell or the means by which parasite numbers were reduced (this is investigated in Chapter 5).   However, microscopic examination revealed that the majority of parasites were killed at the late trophozoite or early schizont stage by a mechanism other than phagocytosis.   Charmot and Bastin (1979) have described a population of hyperbasophilic mononuclear cells present in the blood of children with malaria which probably belong to the elevated 'null' cell population reported by Wyler (1976).   It has been postulated that such cells may act as cytotoxic effector cells during acute malaria (Greenwood et al. 1977).   As cytotoxicity was antibody dependent, 'K' cells could be implicated as effector cells but the ill-defined 'null' cell population could also include cytotoxic monocytes/macrophages similar to those described by Taverne et al. (1982).

In conclusion, the results of this study add support to the view that cell-mediated immunity during acute malaria is an important protective mechanism which can be measured by the ability of mononuclear cells to kill P. falciparum in vitro.

CHAPTER 5

5 EFFECTOR CELLS INVOLVED IN ANTIBODY-DEPENDENT CELLULAR KILLING OF PLASMODIUM FALCIPARUM

5 1. INTRODUCTION

For many years it was assumed that antibody-mediated phago-cytosis was the major effector defense mechanism against malaria (Taliaferro 1929). Since then, however, several studies using animal models have failed to prove that phagocytosis plays a decisive role in recovery from acute infection (Lucia & Nussenzweig 1969; Cantrell, Elko & Hopff 1970; Playfair 1979). Allison and Eugui (1982) have recently suggested that a major protective func-tion of macrophages in acute malaria may lie in their ability to become cytotoxic to blood stage parasites. This hypothesis has been supported by the findings of Taverne et al. (1982) who des-cribed a population of mouse myeloid cells which killed P. yoelii before being fully differentiated as macrophages.

As yet, there are no reports of cytotoxic T cells acting as effector cells in malaria, but both NK cells (Eugui & Allison 1979) and K cells (Coleman et al. 1975; Greenwood et al. 1977) have been cited as potential killer cells in both murine and human malaria. NK cell activity and interferon production have both been shown to increase during acute P. falciparum infection (Ojo-Amaize et al. 1981), but the proposal that NK cells were important in killing intra-erythrocytic parasites (Eugui & Allison 1979) was not supported by the results of experiments which deliberately reduced NK cell activity (Wood & Clark 1982). In a recent review, Allison and Eugui (1982) conceded that the cell, thought previously to be an NK cell, which killed malaria parasites (Eugui & Allison 1979) may in fact, be a cell of the monocyte/macrophage series.

Coleman et al. (1975) concluded that enhanced intra-erythro-
cytic parasite killing by lymphocytes from infected and normal
mice, in the presence of specific antibody, was an indication of
K cell activity. ADCC by cells from children with acute malaria,
reported in Chapter 3 could also have been due to the activity
of K cells. As, however, the mononuclear cell preparations in-
cluded monocytes and possibly immature macrophages, killing of P.
falciparum by such cells could not be discounted.

This study attempts to identify the effector cell(s) involved
in the destruction of P. falciparum parasites in vitro.

5   2.   SUBJECTS AND METHODS

Subjects

Sixty-three Gambian children with clinical malaria, aged between
one and 15 years with parasitaemias ranging from 0.2% to 12% were
tested.

Methods

Seven ml of blood was taken with parental consent from each
subject. Mononuclear cells were harvested and tested for ADCC
against a stock laboratory strain of P. falciparum and compared
with the individual and combined ability of T cells, adherent
cells and B cells, from the same individual, to kill P. falciparum.

Cell separations

1.   T cells

T cells were separated from lymphoprep-harvested mononuclear
cells by sheep red cell (SRBC) rosetting. One volume of 5% neura-
minidase treated SRBC's in RPMI-10% FCS was mixed with 4 volumes
of washed mononuclear cells suspended in the same medium, at a
concentration of 5×10^6 cells/ml. This suspension was incubated
at 37°C for 10 minutes in a waterbath, centrifued at 1000 r.p.m.

for 5 minutes, then incubated at 4°C on ice for 1 hour. The supernatant was then removed, the cell button gently resuspended in 3 ml of RPMI-FCS and the suspension layered onto a lymphoprep gradient which was centrifuged at 1800 r.p.m. for 15 minutes. All non-rosetting cells were removed from the interface and kept for purification of adherent cells and B cells. The rosetted pellet was again suspended in 3 ml RPMI-FCS and passed through a further lymphoprep gradient until at least 95% of the final preparation consisted of rosetted T cells. SRBC were removed from T cells by incubation at 37°C for 10 minutes in 10 ml 0.8% Tris-ammonium chloride lysis buffer. Purified T cells were washed twice in RPMI without FCS before being dispensed in the appropriate numbers in the final autologous serum-medium mixture.

2. Adherent cells

Mononuclear cells removed from the gradient interface above the SRBC rosetted cells were washed twice in RPMI-FCS, counted and the proportion of monocyte-macrophages determined by a differential count on a Giemsa stained cytospin preparation. Two hundred micro-litre aliquots of the T cell depleted suspension in RPMI-FCS, containing the requisite number of monocyte/macrophages were dispensed into the wells of a sterile, flat-bottomed, polyvinyl micro-titre tray which was then incubated at 37°C for 1 hour to enhance cell adherence. After the incubation period all non-adherent cells were washed vigorously from the wells and pooled for subsequent purification of B cells. Two hundred µl of RPMI containing 3% normal group 'O' red blood cells of which 0.3% were infected with P. falciparum parasites (3×10^4/culture) were added to each well now containing only adherent cells and supplemented by the addition of 10% autologous serum. Cultures were then handled in the manner described in Chapter 3.

3. B cells

Mononuclear cells, depleted of T cells and adherent cells were incubated on a nylon fibre column to enrich the yield of B cells. Nylon fibres were soaked overnight in 0.2 mol/L HCL, rinsed thoroughly in distilled water and dried. Treated nylon fibre was wrapped in aluminium foil in 0.1 gm amounts and sterilized by auto-claving. For use, sterile nylon fibre was teased apart and placed in the barrel of a sterile 1 ml plastic syringe. The column was then rinsed with 10 ml sterile PBS, followed by 10 ml RPMI-FCS pre-warmed to 37°C. Both ends of the syringe were sealed with parafilm and the column incubated at 37°C for 30 minutes. Following incubation an 18G syringe needle was attached to the syringe, the lymphocyte suspension in 0.5 ml RPMI-FCS was applied to the column and excess medium allowed to escape through the needle end. The syringe was resealed and incubated at 37°C for 30 minutes in a horizontal position. After incubation, 10 ml of medium at 37°C was passed through the column and discarded. An 18G needle was again attached to the column which was placed in a sterile, 10 ml plastic centrifuge tube containing 3 ml of medium at 22°C and the syringe plunger replaced. B cells were eluted by vigorous movement of the plunger without packing the nylon wool. The re-sultant suspension was counted and adjusted to the appropriate cell concentration in RPMI-autologous serum for use in the cyto-toxicity assay. B cell purity was found to be >90% when tested by fluorescence for the presence of surface immunoglobulin.

Culture supernatants from activated cells

T cell supernatants. Purified T cells from children with clinical malaria were harvested by the method previously described, adjusted to a concentration of 1 x 10^6/ml in RPMI-10% FCS and dispensed in 1.5 ml amounts into a sterile 24 well Linbro plate (Flow Labs.).

Phytohaemagglutinin (PHA) was added to half of the wells to give a final concentration of 1.0 µg/ml. To each remaining well 45 µl of packed red blood cells, which were infected with 0.3% P. falciparum parasites, were added and the plate incubated at 37°C for 5 days using the candle jar method (Jensen & Trager 1977). After the culture period, supernatants were removed and stored at -20°C in 100 µl aliquots.

Adherent cell supernatants. T cell depleted mononuclear cells from children with clinical malaria were dispensed into a 24 well, sterile Linbro plate in RPMI-10% FCS in sufficient numbers to give 5 x 10^5 monocyte/macrophages per ml. After a one hour incubation period at 37°C all non-adherent cells were removed and 1.5 ml RPMI-10% FCS containing 3% erythrocytes infected with 0.3% P. falciparum parasites was added to each well. The plate was then incubated at 37°C for 5 days using the candle jar method (Jensen & Trager 1977). After the culture period supernatants were removed and stored at -20°C in 100 µl aliquots.

Cytotoxicity assays

T cells and B cells were tested for ADCC at an effector cell: target ratio of 20:1 (i.e. 6 x 10^5 cells/200 µl culture); adherent cells were tested for ADCC at the 10:1 ratio (i.e. 3 x 10^5 cells/ 200 µl culture). These were found to be the mean proportions of each cell population at the 50:1 cell:parasite ratio. The ability of separated mononuclear cells to kill P. falciparum in autologous serum was compared with ADCC by autologous undepleted mononuclear cells and with parasite growth in autologous serum without cells.

Combinations of T cells + B cells at a 40:1 cell:parasite ratio (20:1T + 20:1B); B cells + adherent cells at a 30:1 ratio (20:1B + 10:1 adherent); and T cells + adherent cells at a 30:1

ratio (20:1T + 10:1 adherent) were tested in the same way.   In addition, the ability of T cells (at a 20:1 cell:parasite ratio) plus adherent cell supernatant; and adherent cells (at a 10:1 ratio) plus T cell supernatant to kill P. falciparum was compared with ADCC by autologous undepleted mononuclear cells and with parasite growth in autologous serum without cells (with or without cell supernatants).

Cytotoxicity assays were set up as described in Chapter 3. In experiments where T cell and adherent cell supernatants were tested, 15% v/v of cell supernatants were added at the start of the culture together with 200 µl RPMI-10% autologous serum, this mixture being replaced after 24 hours.   At the end of the 48 hour culture period, the number of viable parasites in 10^4 red blood cells were counted on Giemsa stained films taken from each well.

Due to limited cell recovery, it was not possible to control the effect of mononuclear cells and mononuclear cell subpopulations on parasite growth in the presence of non-immune European serum.   However, experiments in which the target parasite did not grow to at least twice the starting density in RPMI-10% European serum (without cells) were discarded.   Where cell yields permitted, cultures were set up in duplicate.

Data were analysed using the two-way paired student's 't' test.   Parasite growth in these experiments is expressed simply as percentage parasitaemia.

5   3.   RESULTS

All the experiments carried out in this section of the work contained two control groups: the negative control was provided by autologous serum alone (AS); and the positive control consisted of "undepleted cells", (U) suspended in autologous serum.   In the

negative controls, parasite numbers always increased during incubation, mainly 3-4 fold; in the positive controls (U), there was never an increase in parasite numbers, but decreases amounting to between 1/3 to 1/2 of numbers at the start. Because undepleted cells (U), effective at a 50:1 cell:target ratio, contained on average 40% T cells, 40% B cells and 20% adherent cells (A), these constituent cell populations were tested at cell:target ratios of 20:1, 20:1 and 10:1 respectively.

Figures 10, 11 and 12 show clearly that neither T cells nor B cells are effective in ADCC assays if present alone in appropriate cell:target ratios and although adherent cells (A) and autologous serum (AS) did cause some inhibition of growth mainly due to phagocytosis (P<0.01) this effect was far less than the marked parasite death which occurred due to the activity of undepleted cells (U) and serum (AS) from the same children. This can only mean that cooperation between the populations must occur in order to produce effective killing. The results shown in Figures 13 and 14 indicate that mixtures of T and B cells, and A and B cells cannot substitute for undepleted cells, but Figure 15 shows that a mixture of appropriate numbers of A and T cells can do so.

It is commonly found that with cell-cell cooperation in immune effector systems, one cell population represents the potential effector cells, while the second population provides an essential activation signal for these potential effectors. Accordingly, experiments were set up to determine whether T cells provided the activation signal for potentially effector A cells or vice versa. A comparison of Figures 16 and 17 leaves no doubt that the first alternative is correct. A cells treated with supernatant from PHA activated T cells are just as effective

CHILDREN with P. falciparum MALARIA

AS = Autologous serum only

T = T cells (20:1)

U = Undepleted cells (50:1) ⎤ + Autologous serum
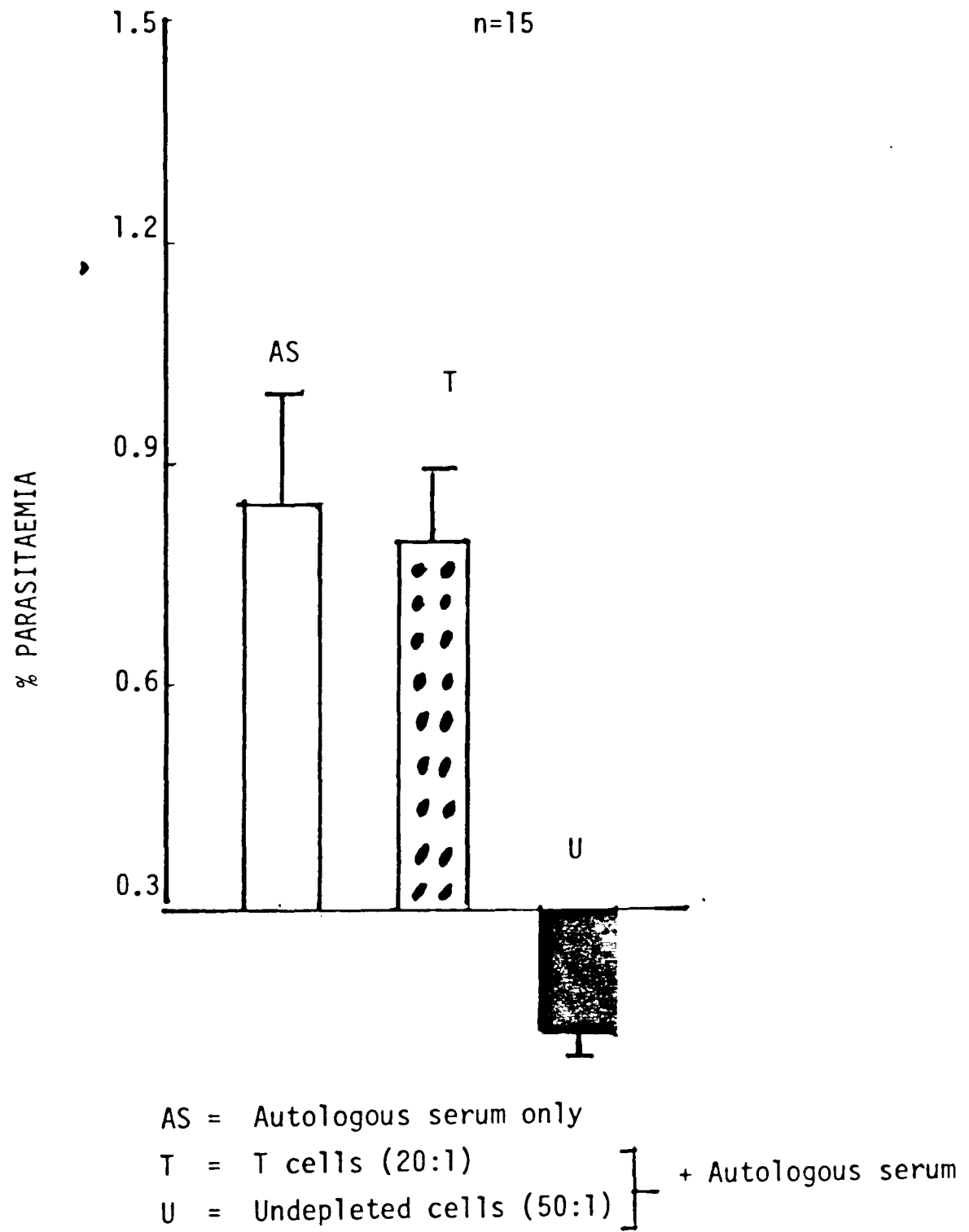
FIG. 10 ADCC - T cells vs undepleted cells

Mean P. falciparum growth ± standard error.
48 hr cultures. 0.3% = starting parasitaemia.

CHILDREN WITH P. falciparum MALARIA

n = 40
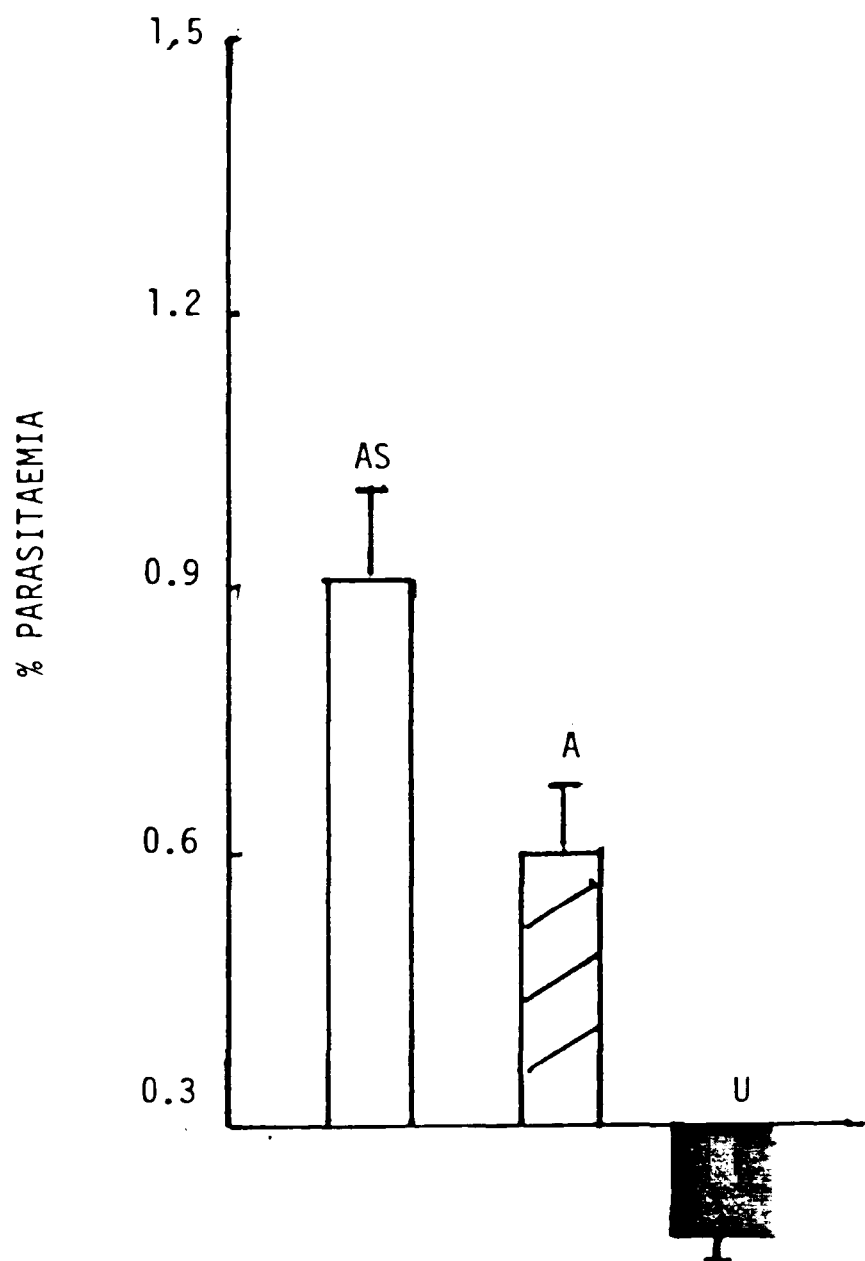
AS   = Autologous serum only

A   = Adherent cells (10:1) ⎤
                              ⎥   + Autologous serum
U   = Undepleted cells (50:1)⎦

FIG. 11   ADCC - Adherent cells vs Undepleted cells

Mean P. falciparum growth ± standard error.   48 hour
cultures.   0.3% = starting parasitaemia.

CHILDREN WITH P. falciparum MALARIA

n = 9
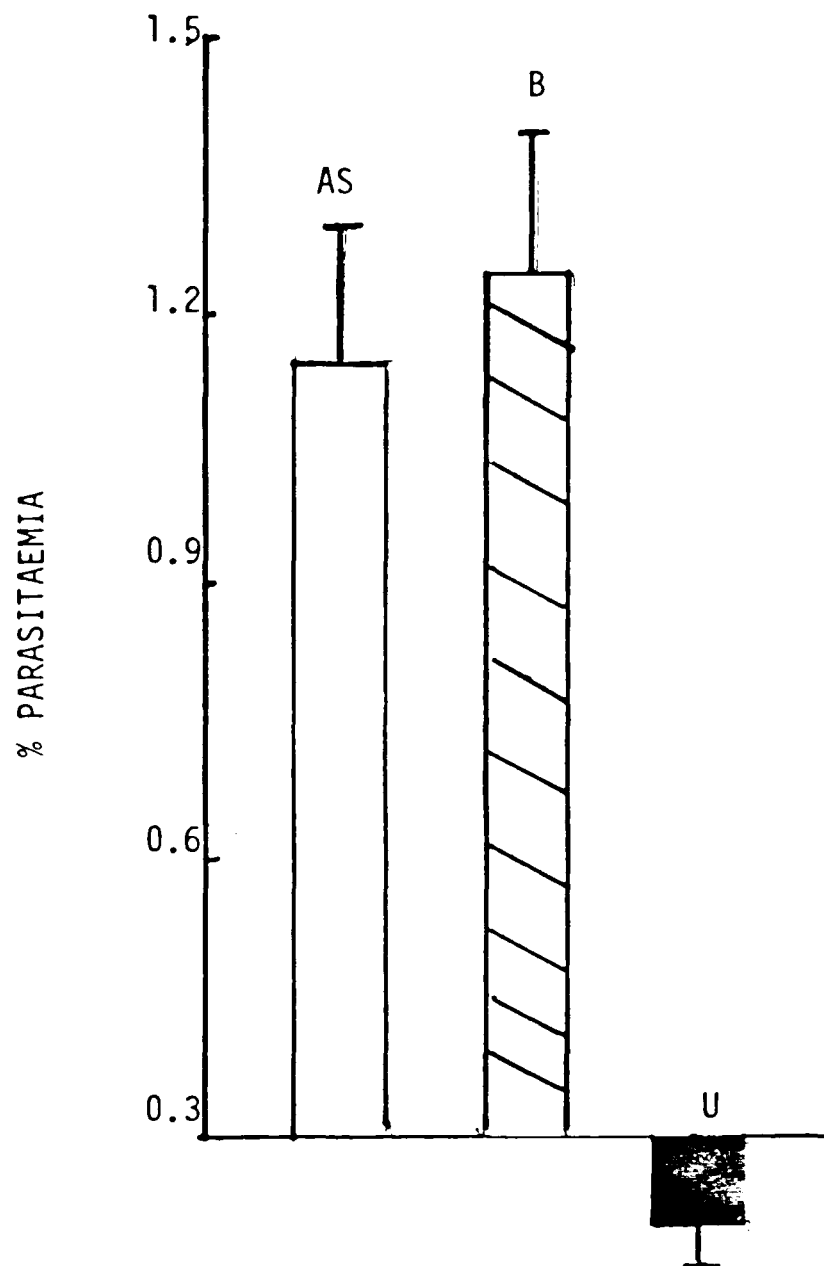
AS   = Autologous serum only

B   = B cells (20:1)

U   = Undepleted cells (50:1)   + Autologous serum

FIG. 12   ADCC - B cells vs Undepleted cells

Mean P. falciparum growth ± standard error.   48 hour
cultures.   0.3% = starting parasitaemia.

CHILDREN WITH P. falciparum MALARIA

n = 24
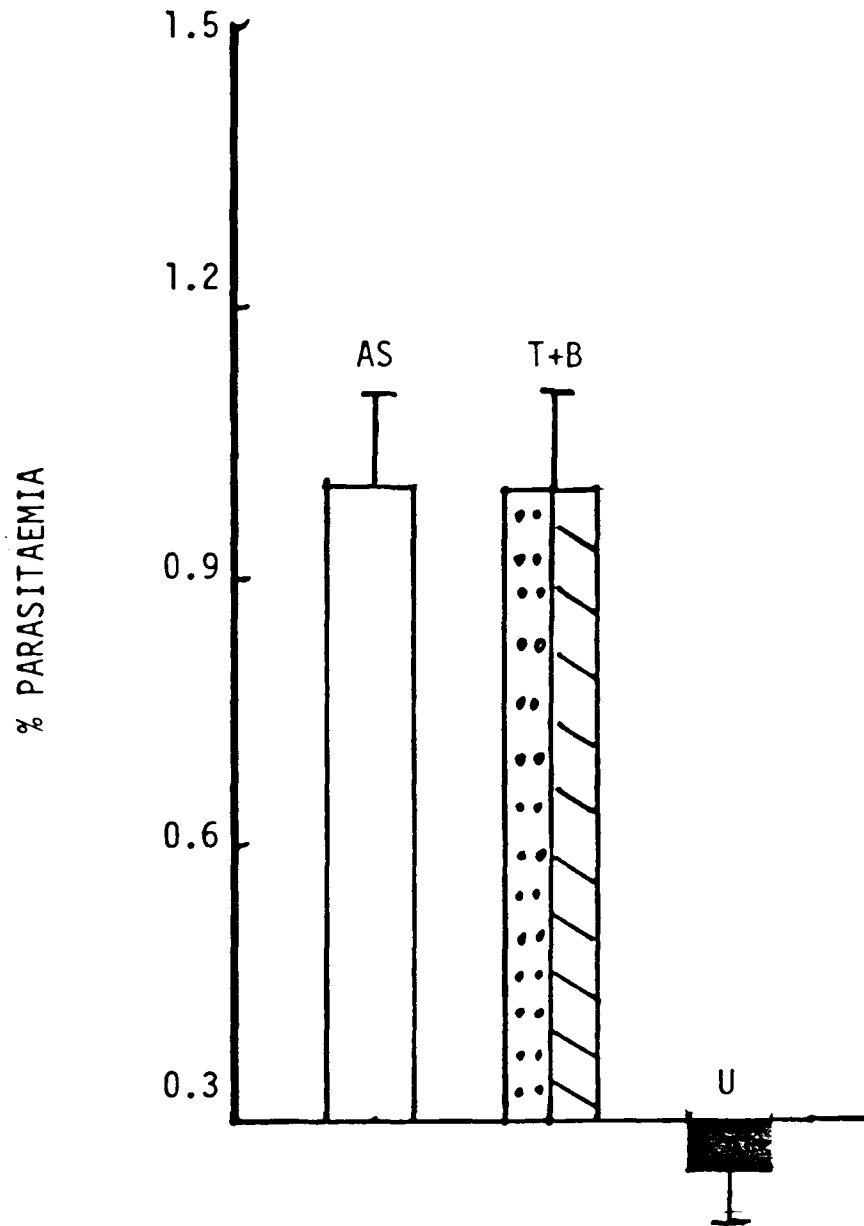
AS   = Autologous serum only

T+B = T cells (20:1)
           +
       B cells (20:1)   ] + Autologous serum

U   = Undepleted cells (50:1)]

FIG. 13   ADCC - T cells + B cells vs Undepleted cells

Mean P. falciparum growth ± standard error.   48 hour
cultures.   0.3% = starting parasitaemia.

CHILDREN WITH P. falciparum MALARIA

n = 10
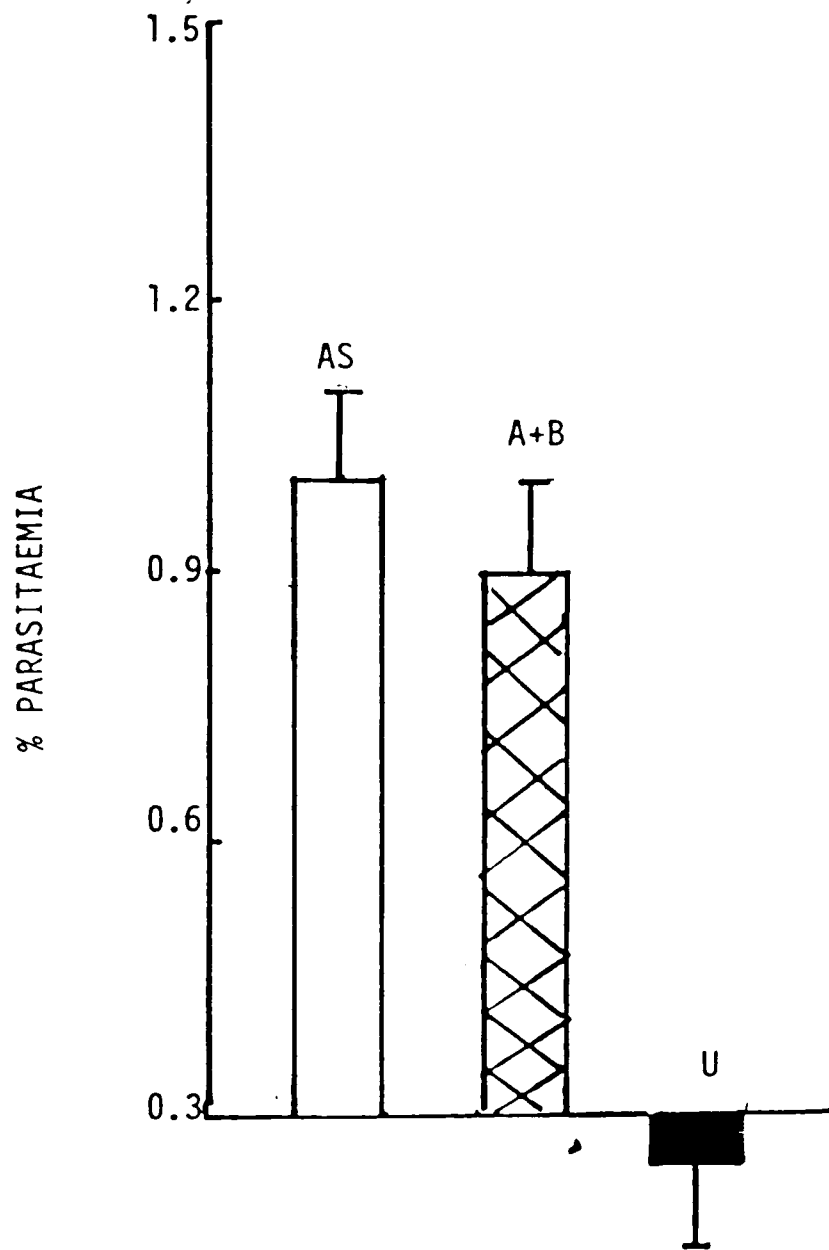
AS   = Autologous serum only

A+B   = Adherent cells (10:1)
         +
         B cells (20:1)

U   = Undepleted cells (50:1)

+ Autologous serum

FIG. 14   ADCC - Adherent cells + B cells vs Undepleted cells

Mean P. falciparum growth ± standard error. 48 hour
cultures. 0.3% = starting parasitaemia.

CHILDREN WITH P. falciparum MALARIA

n = 15
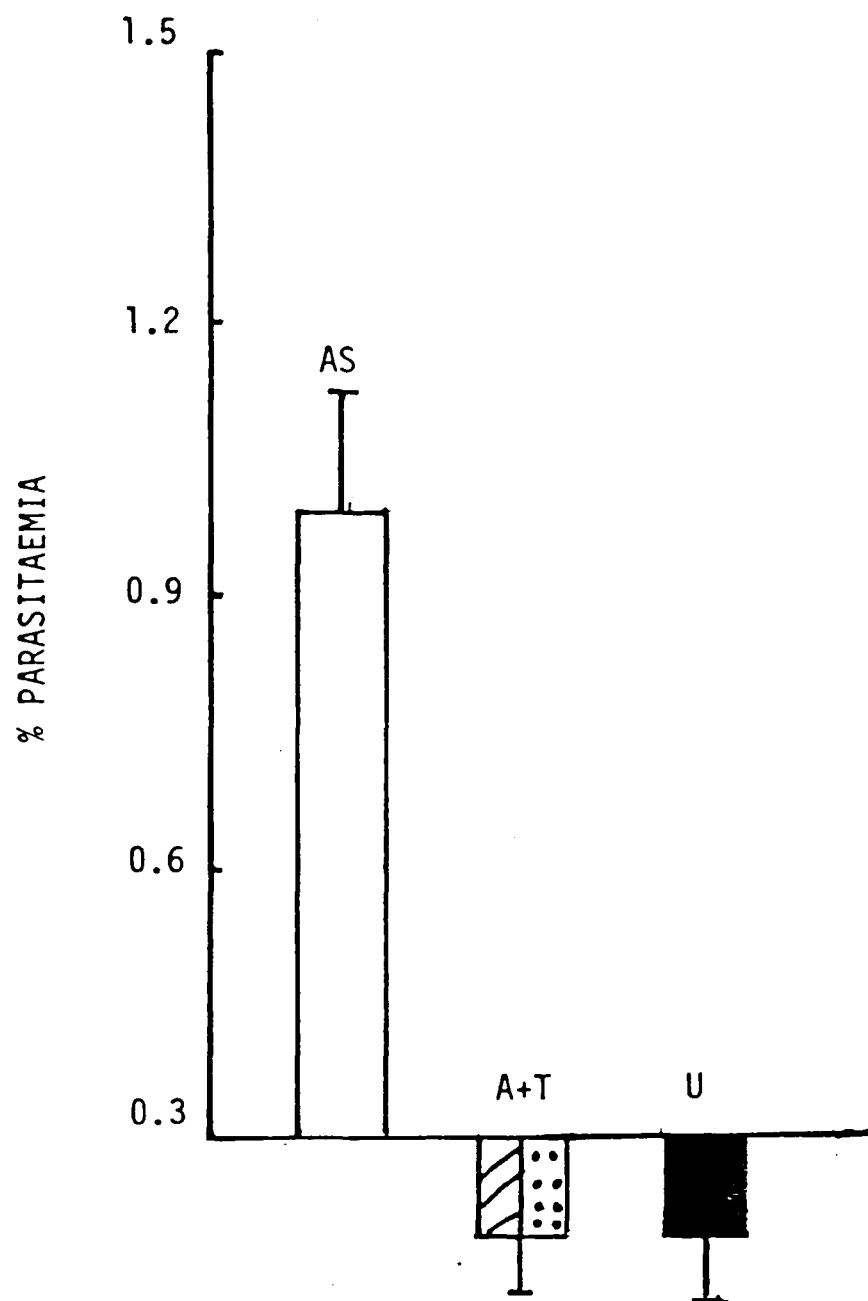
AS   = Autologous serum only

A+T = Adherent cells (10:1)
           +
         T cells (20:1)   + Autologous serum

U   = Undepleted cells (50:1)

FIG. 15   ADCC - Adherent cells + T cells vs Undepleted cells

Mean P. falciparum growth ± standard error.   48 hour
cultures.   0.3% = starting parasitaemia.

CHILDREN WITH P. falciparum MALARIA

n = 8

AS   = Autologous serum only

A SUP = Adherent cell supernatant

T   = T cells (20:1)

U   = Undepleted cells (50:1)
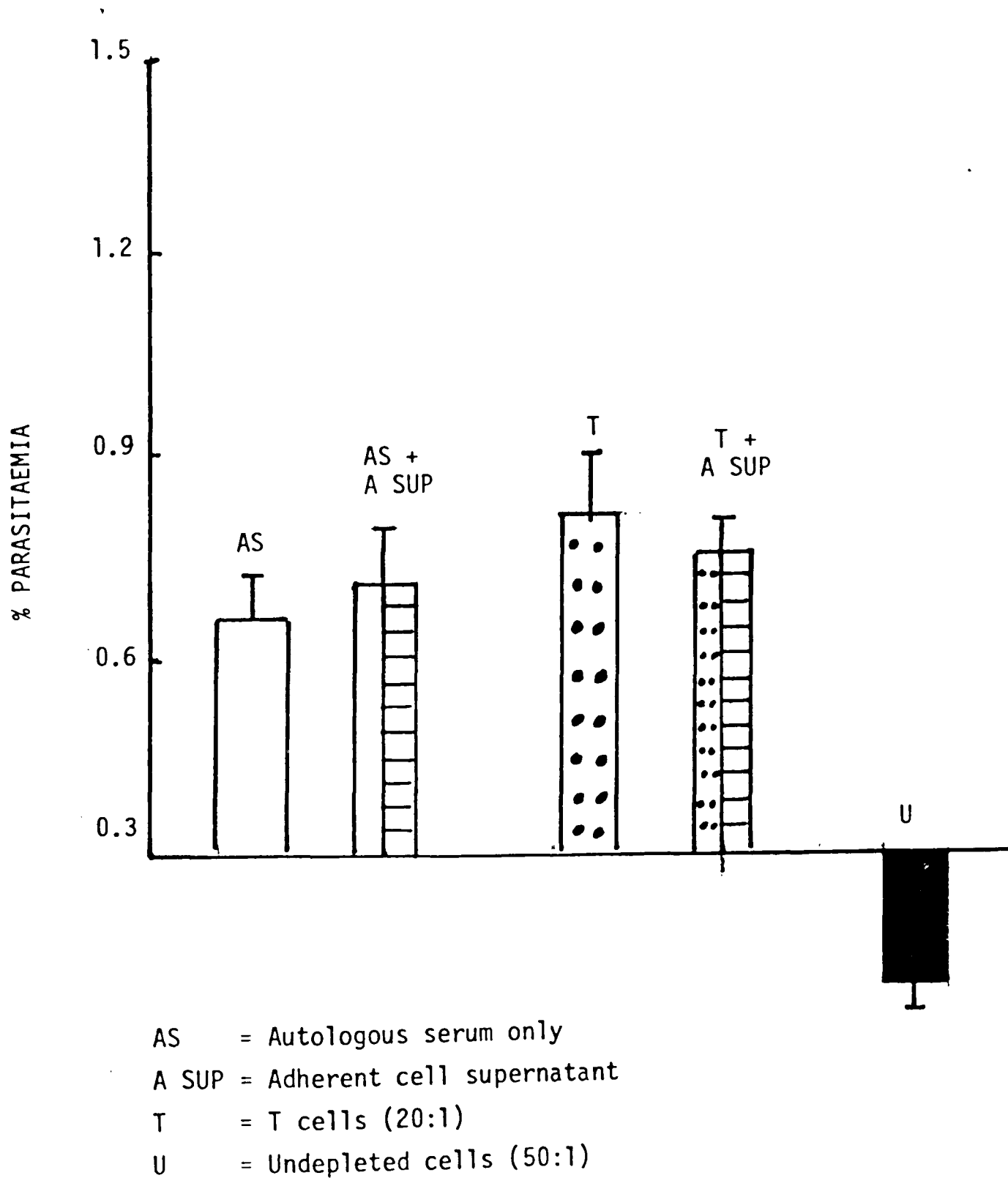
FIG. 16   ADCC - T cells + Adherent cell supernatant
vs Undepleted cells

Mean P. falciparum growth ± standard error.
48 hour cultures. Autologous serum used
throughout. 0.3% = starting parasitaemia.

CHILDREN WITH P. falciparum MALARIA
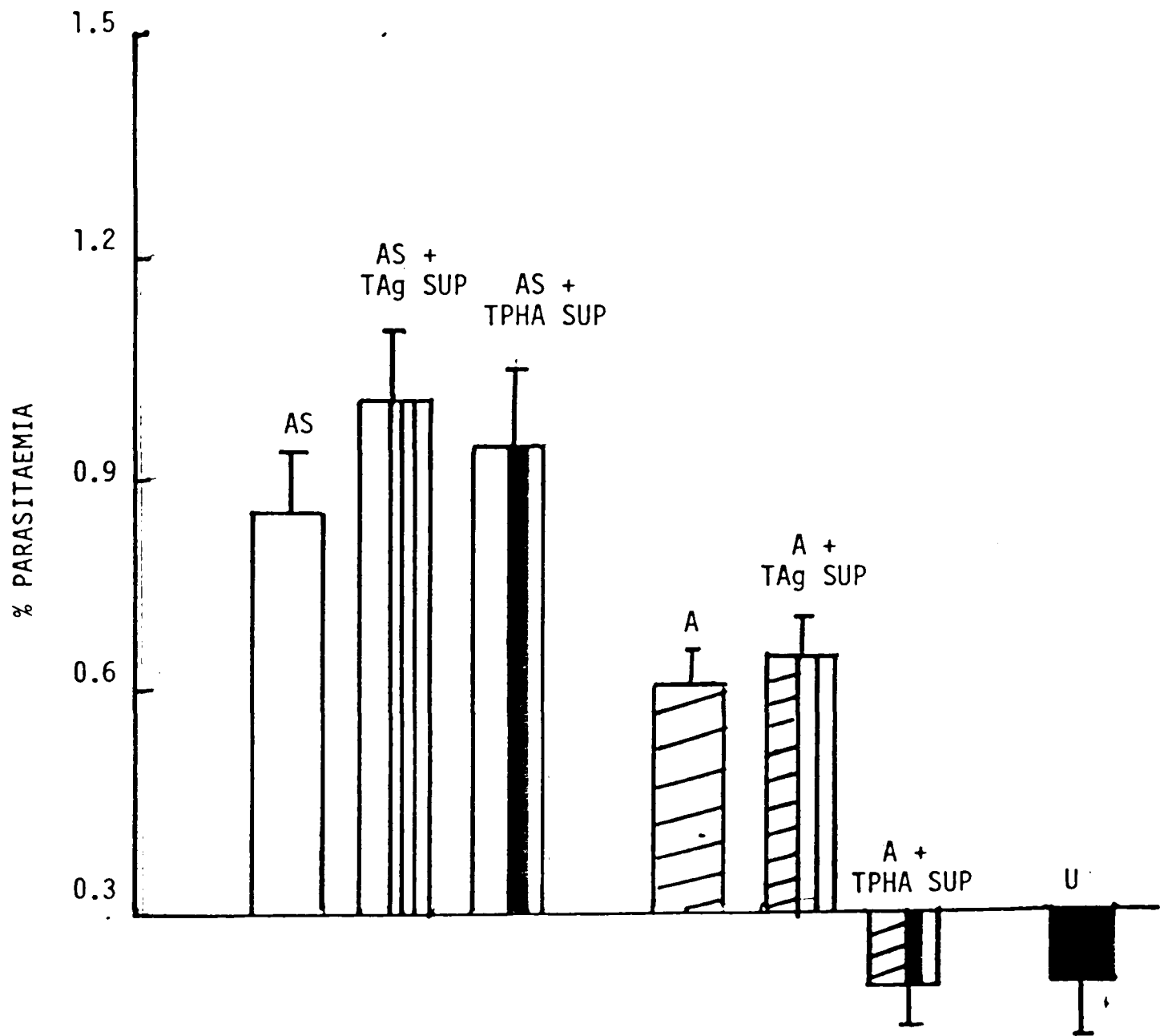
AS	= Autologous serum only
A	= Adherent cells (10:1)
TAg SUP	= T cell supernatant (antigen stimulated)
TPHA SUP	= T cell supernatant (PHA stimulated)
U	= Undepleted cells (50:1)

FIG. 17   ADCC - Adherent cells + T cell supernatants
           vs Undepleted cells.

           Mean P. falciparum growth ± standard error.
           48 hour cultures. Autologous serum used
           throughout. 0.3% = starting parasitaemia.

as undepleted cells; T cells treated with supernatant from "activated" A cells are not effective. A majority of dead parasites were inside red cells which had not been ingested.

It should be noted that only the supernatant of PHA stimulated T cells provided activation; supernatant from T cells incubated with parasitized cells was ineffective. The reasons for this finding are discussed in the next section.

5 4. DISCUSSION

The results of the in vitro assays reported in this section indicate that children with clinical malaria are probably required to develop an extremely complex immune response in order to control parasitaemia. The specific antibody required in the ADCC system is almost certainly produced as a result of cooperation between T and B lymphocytes, and accessory cells which may be a sub-population of cells of the mononuclear phagocyte system. The exact nature of cooperating cells and the nature and specificity of signals passing between them are still important goals for much research in fundamental cellular immunology.

Antibodies, alone, are however insufficient to control parasite multiplication; cells which will cooperate with antibodies in an ADCC system appear also to be required. The work reported here strongly implicates adherent cells (A), presumably of the monocyte-macrophage lineage as being the effector cell population. But these experiments also suggest that A cells must become "activated" before they are effective, and that the necessary activation signals are provided by T cells.

In these in vitro assays, activation of A cells occurred when a mixture of A cells and T cells were exposed to 'antigen' in the form of parasitized red blood cells, or when A cells were

exposed to a supernatant containing presumed T cell factors consequent on exposure of these cells to PHA. Supernatant from purified T cells exposed to 'antigen' failed to activate A cells. How can the results of these in vitro assays be reconciled with the situation in vivo where PHA is certainly not present!

Evidence gathered from many and diverse studies can now be interpreted to mean that T cells are only activated immunologically by antigen which is present on cell surfaces and is recognised in conjunction with Class I or Class II antigens of the MHS. Almost alone of body cells, mature erythrocytes have neither Class I or II MHS antigens on their surfaces. Thus, parasite antigens on erythrocyte surfaces will not stimulate T cells to become cytotoxic nor to synthesize and release activation factors. This explains the failure of supernatants of T cell parasitized erythrocyte mixtures to stimulate A cells. Exposure of T cells to PHA directly stimulates the cells to produce activation factors, by-passing normal requirements.

The conclusions of this study are consistent with the findings of several studies on animal models of malaria where increased activity of both T cells and macrophages were thought to be associated with recovery from infection. T cells have been shown to be essential in recovery from normally non-lethal P. berghei (Jayawardena et al. 1977) and B. microti (Roberts et al. 1977) infections in mice and B cell deficient mice remain resistant to reinfection with P. berghei as long as their primary infections are treated (Roberts & Weidanz 1979). Whilst such observations show quite clearly that in animals at least, T cells play a vital role in recovery from malaria, their precise protective function has not been clearly defined. Similarly, increases in absolute monocyte numbers during P. berghei infections in mice

correlates with useful immunity (Lelchuk et al. 1979). No evidence is available, however, which positively links phagocytosis to recovery from acute malaria. Recovery from non-lethal P. yoelii in mice, in fact, parallels a decrease in the antibacterial phagocytic activity of macrophages (Murphy & Lefford 1979).

The immediate rise in R.E.S. activity which follows a recrudescence of malaria infection is thought to be a consequence of macrophage activation by antigen sensitized T cells (North & Mackeness 1973) and a chemotactic factor, possibly of T cell origin, has been shown to trigger the accumulation of macrophages in the spleens of malaria infected mice (Wyler & Gallin 1977). Such results support the requirement of T cells in the activation of human macrophages reported here and as T cells are known to activate macrophages to become killer cells in other disease systems (Evans & Alexander 1972) cooperation between these cells might provide an important protective mechanism in human malaria.

Lymphocytes from animals immune to P. berghei cluster around macrophages which have ingested P. berghei antigens and undergo blastogenesis possibly due to immune stimulation by antibody and activated macrophages (Herman 1977). The requirement of antibody in experiments described here, therefore, may have been to provide a linkage between T cells and phagocytes which had ingested blood-stage parasites.

This raises the question of whether the adherent cell population in the blood of these infected children was homogeneous. Many of the adherent cells seen in cultures where P. falciparum was killed were not typical peripheral blood monocytes yet were obviously phagocytic. Taverne et al. (1982) reported two distinct

mouse phagocytic cells within a mononuclear cell suspension which killed P. yoelii; both were phagocytic, but only immature macrophages were believed to be cytotoxic. Thus, the 'null' cell population, which can constitute as much as 25% of peripheral blood mononuclear cells during malaria infections in humans (Wyler 1976) probably includes macrophages capable of killing blood-stage parasites.

In conclusion, whilst it is recognised that the ultimate goal of a successful immune response to malaria is probably the production of potent inhibitory parasite antibodies, the results reported here suggest that non-inhibitory antibodies combine with macrophages and T cells to provide protection to acute malaria by killing asexual P. falciparum parasites.

CHAPTER 6

6 NON-SPECIFIC KILLING OF PLASMODIUM FALCIPARUM BY HUMAN MONONUCLEAR
CELLS AND MONONUCLEAR CELL PRODUCTS

6 1. INTRODUCTION

Both specific antibody and T cells were shown to contribute
to P. falciparum death caused by adherent cells from children with
malaria (Chapters 3 & 5). However, the function of specific anti-
body in ADCC and the process which led to intra-erythrocytic para-
site death by T cell activated adherent cells were not determined.

Acute malaria stimulates increased production of both specific
IgM and specific IgG antibodies. Thus, during the early stages of
infection, specific antibodies could assist cellular cytotoxicity
in one or both of the following ways:

a. By agglutinating parasitized red cells, making them more
    susceptible to attack by effector cells.

b. By locating appropriate antigens on the surface of accessory
    cells, thus inducing immune stimulation of T cells.

During recovery, however, IgM levels decline whilst specific IgG
levels continue to rise; therefore, ADCC could be enhanced and
gradually superseded by developing humoral responses against the
infecting parasite strain.

In chapter 5, T cells or their products (released after
exposure to PHA) activated adherent cells to kill P. falciparum.
As products of antigen-stimulated T cells failed to do so, it was
concluded that T cells might require prior contact with antigen-
coated accessory cells (probably mononuclear). Such cell-cell
contact can occur in the absence of specific antibody if T cells
recognise MHS determinants on the surface of accessory cells.
Clonal expansion of T cells bearing antigen receptors can then

Missing pages are unavailable

Subjects

1.   Twenty-two children aged between one and 12 years with clinical malaria whose parasitaemias ranged from 0.2% to 15% were studied.

2.   Thirteen uninfected non-immune European adults aged between 25 and 45 years were also studied.

Methods - Cytotoxicity assays

Children

Six ml of blood was taken with parental consent, from each child and mononuclear cells and serum separated in the manner described in Chapter 3.   In these experiments undepleted mononuclear cells were tested for their ability to kill P. falciparum non-specifically, in RPMI-10% FCS with or without supernatant of undepleted mononuclear cells from children with malaria, which had been exposed to either P. falciparum parasites or PHA for 6 days.   Parasites in autologous serum without cells served as positive growth controls and parasites cultivated in autologous serum and autologous undepleted mononuclear cells (without supernatant) provided positive parasite killing controls.   An effector cell:target ratio of 50:1 (i.e. 1.5 x 10^6 cells/culture) was used throughout.   Supernatants were added to give a final concentration of 15% v/v per culture.   Cultures were incubated at 37°C for 48 hours in a low oxygen atmosphere and parasite survival assessed on Giemsa stained blood films taken from each well.

Europeans

Mononuclear cells from uninfected adult European volunteers were harvested from a lymphoprep gradient and tested for their ability to kill P. falciparum in cultures containing either supernatant of European undepleted mononuclear cells or similar

cells from children with malaria which had been exposed to PHA for 6 days. European serum free from antimalarial drugs was used as the standard culture serum throughout. Therefore, test cultures were free from malaria antibody. The effect of cells, serum and supernatant mixtures on parasite growth was compared with parasite growth in European serum alone and in European serum plus European undepleted mononuclear cells. Assays were set up with or without 15% v/v supernatant for 48 hours as previously described. Mononuclear cells were tested at an effector cell: target ratio of 50:1.

T cell transformation by adherent cell supernatants

T cells, adherent cells and adherent cell supernatants from children with malaria, were harvested from whole blood in the manner described in Chapter 5. Negative controls consisted of T cells cultured in medium without supernatants and with supernatant from European mononuclear cells stimulated with P. falciparum parasites for 6 days. T cells, at a concentration of 1.0×10^6/ml, were dispensed in 200 µl amounts into the wells of a sterile, flat-bottomed microtitre tray. Thirty µl of each adherent cell supernatant was added to 5 wells to give a concentration of 15% v/v per culture. Four supernatants of cells from infected children and one control supernatant were tested and cultures were incubated at 37°C for 6 days in a low oxygen atmosphere. On day 5, 50 µl of ^{3}H thymidine (10 µci/ml) was added to each well and allowed to react for 24 hours, before being deposited on glass fibre discs by a Titretek microharvester. Discs were then dried and transferred to glass vials containing 10 ml scintillation fluid (Aqualuma, LUMAC, AG) and counted for one minute on a liquid scintillation counter. Results were

expressed as the mean CPM ± standard deviation of 5 samples.

Estimation of intra-erythrocytic parasite death

Intra-erythrocytic parasite death was determined by growing a stock laboratory strain of P. falciparum under optimal growth conditions after parasites had been exposed to fluids taken from mononuclear cell cultures in which P. falciparum had been killed and from cultures in which mononuclear cells had been ineffective. Fluids were taken from ADCC cultures in which a 50:1 effector cell:target ratio had been used. Two-hundred μl of fluid was removed from each culture and dispensed into the wells of a sterile, flat-bottomed, 96-well microtitre tray. Six μl of group 0 erythrocytes infected with 0.3% asexual P. falciparum parasites were added to each well and cultures were then incubated at $37^{o}C$ for 6 hours in a low oxygen atmosphere. Supernatants were then removed and replaced by 200 μl of standard culture medium supplemented with 10% v/v stock European serum. Cultures were incubated at $37^{o}C$ in a low oxygen atmosphere for a further 42 hours, the medium-serum mixture being replaced after 18 hours. Controls were provided by parasites grown under standard conditions which had not been in contact with cell culture supernatants. The number of viable parasites was assessed on Giemsa stained blood films taken from each well.

Results of parasite growth inhibition assays are expressed simply as per cent parasitaemia.

6   3.   RESULTS

Results obtained in previous chapters indicated that adherent cells (probably cells of the mononuclear phagocyte series) could cooperate with parasite specific antibody in an ADCC system. Furthermore, there was good evidence that the effector cells

required activation by T cells.

Parasite killing by mononuclear cells in the absence of antibody

The first experiment described in this section was designed to demonstrate whether the need for anti-parasite antibody could be by-passed in the killing system. The results are shown in figure 18 which is presented in two sections. The left-hand section indicates that although undepleted cells from children with malaria (U) will control parasite numbers in the presence of autologous serum (AS), they fail to do so in the presence of foetal calf serum (FCS). However, the right hand part of the figure indicates that undepleted cells can control parasite multiplication in the presence of FCS (i.e. without specific antibody) if the system is supplemented with supernatants from either antigen-stimulated undepleted cells (not purified T cells) or PHA-stimulated undepleted cells. The supernatants alone were not effective. Supernatants of antigen stimulated undepleted cells stimulated the effector cell population to kill P. falciparum, whereas antigen stimulated supernatants of purified T cells were previously shown to be ineffective. This indicated that coope-rating T cells within an undepleted mononuclear cell population may have undergone blastogenisis as a result of contact with antigen presented on the surface of accessory cells, in order to release the factor necessary for activation of effector cells.

To test whether such might be the case or whether accessory cell products could stimulte T cells, supernatants of antigen 'activated' adherent cells were added to purified T cells and allowed to react for 6 days. Table 7 shows that T cells from 3 out of 4 children with malaria underwent blastogenisis up to 10 times over controls when exposed to supernatants of adherent

CHILDREN WITH P. falciparum MALARIA

n = 12
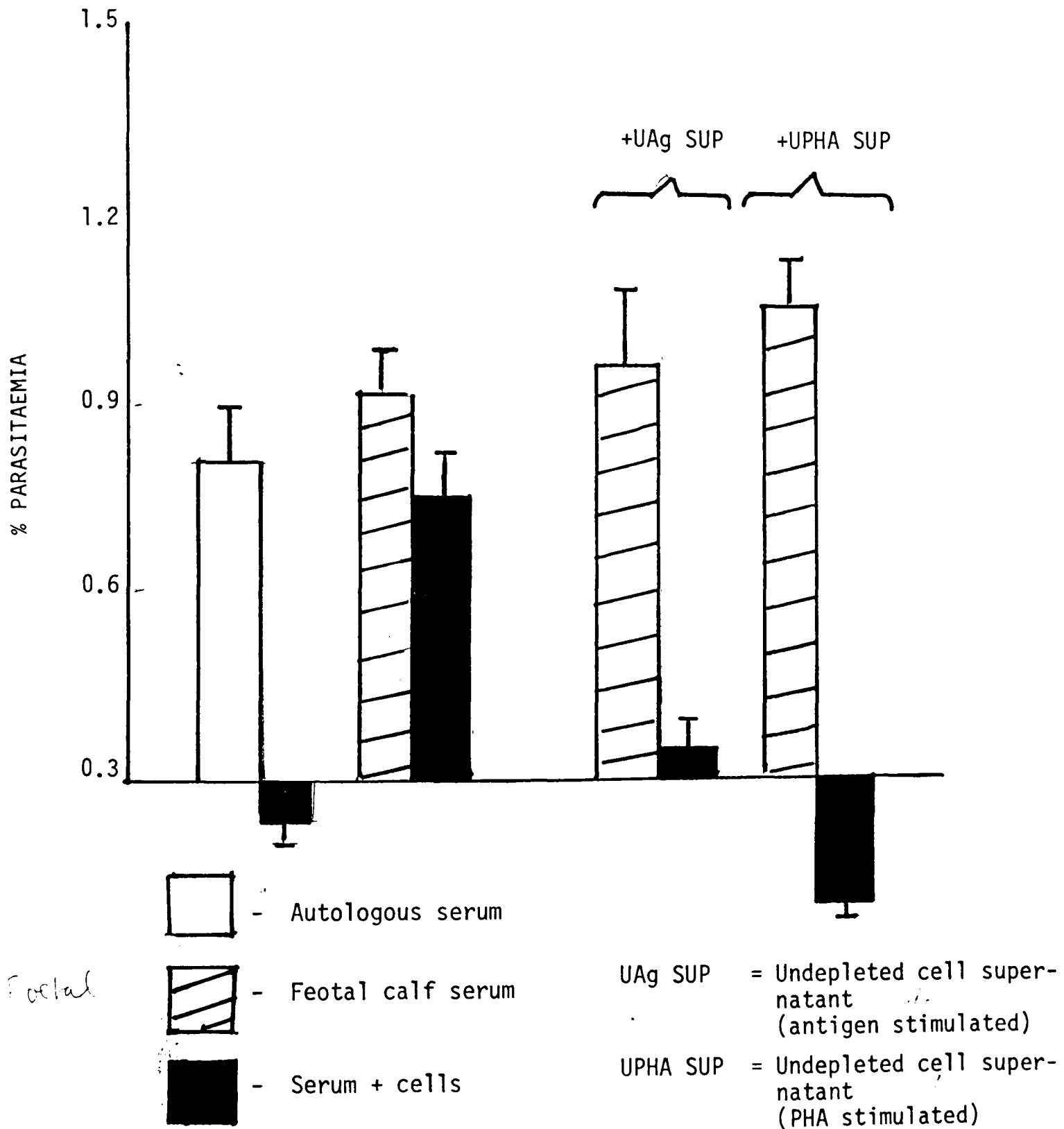
FIG. 18   Non specific cellular cytotoxicity

Mean P. falciparum growth ± standard error.
48 hour cultures. 0.3% = starting parasitaemia.

TABLE 7

Children with malaria - T-cell transformation by Adherent cell
supernatants

Adherent cell supernatants

No.	(No. Sup) Control	+M36	+M37	+M38	+M39	+M5 (European)
T52	238 ± 82	670 ± 156	3374 ± 1153	3324 ± 643	3999 ± 568	783 ± 99
T53	409 ± 76	2164 ± 262	1800 ± 88	1891 ± 614	1247 ± 215	633 ± 112
T54	416 ± 80	419 ± 167	319 ± 123	473 ± 66	500 ± 114	327 ± 77
T58	851 ± 78	2619 ± 181	1667 ± 237	7518 ± 848	3476 ± 922	456 ± 87

T-cells

Mean CPM of 5 samples per culture

TABLE 8

ADCC in relation to T cell transformation

| No. | % Parasitaemia - 48 hour cultures | | |
	Serum only	Serum + cells (50:1)	T cell transformation
U52	0.9	0.2	transformed
U53	0.8	0.1	transformed
U54	1.9	0.8	not transformed
U58	1.1	Dead	transformed

Starting parasitaemia - 0.3%

cells from other infected children. Similar supernatant from European adherent cells (M5) had no stimulatory effect. T cells from one child (T54) did not transform; undepleted mononuclear cells (U54) from this child failed to inhibit multiplication of P. falciparum in vitro (Table 8). The results presented in Tables 7 and 8 suggest that antigen activated adherent cells and/ or their products can transform T cells and that the magnitude of the T cell response may influence the cytotoxic activity of the eventual effector cell.

All experiments to date had been carried out with mononuclear cells from malarious children. It was now decided to attempt to develop a parasite killing system using mononuclear cells from non-malarious Europeans. A summary of the results of these experiments is presented in figure 19. Neither European serum (ES) nor European undepleted mononuclear cells in ES (EU) had any effect on parasite growth, but if EU were supplemented by supernatant from PHA-stimulated EU, a reduction in growth was achieved. An even greater reduction was achieved when supernatant from PHA-stimulated mononuclear cells from malarious Gambian children was added to European cells. Neither of these supernatants had a direct effect on parasite growth.

Figure 20 shows that P. falciparum growth was not affected by exposure to fluids from non-inhibitory European mononuclear cell cultures. Fluids from inhibitory ADCC cultures (i.e. mono-nuclear cells + serum from infected children) however, had a marked inhibitory effect on parasite growth. Similarly, fluids from non-specific cytotoxic cultures (i.e. European cells and serum + PHA supernatant of cells from malarious children) also greatly inhibited P. falciparum growth. As the latter combination
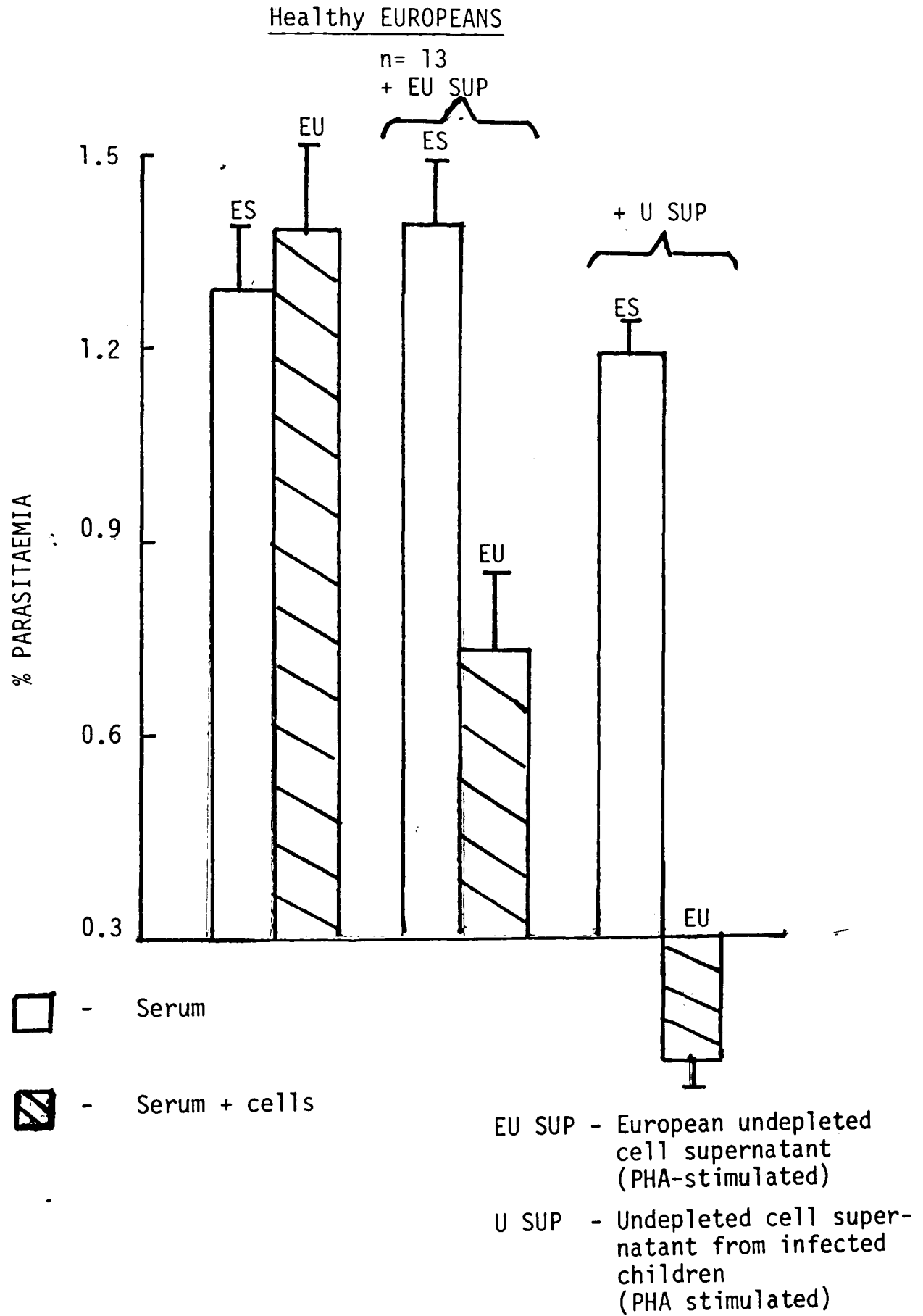
FIG. 19   Cytotoxicity by European cells.

Mean P. falciparum growth ± standard error.
48 hour cultures. 0.3% = starting parasitaemia.

Intra-erythrocytic parasite death
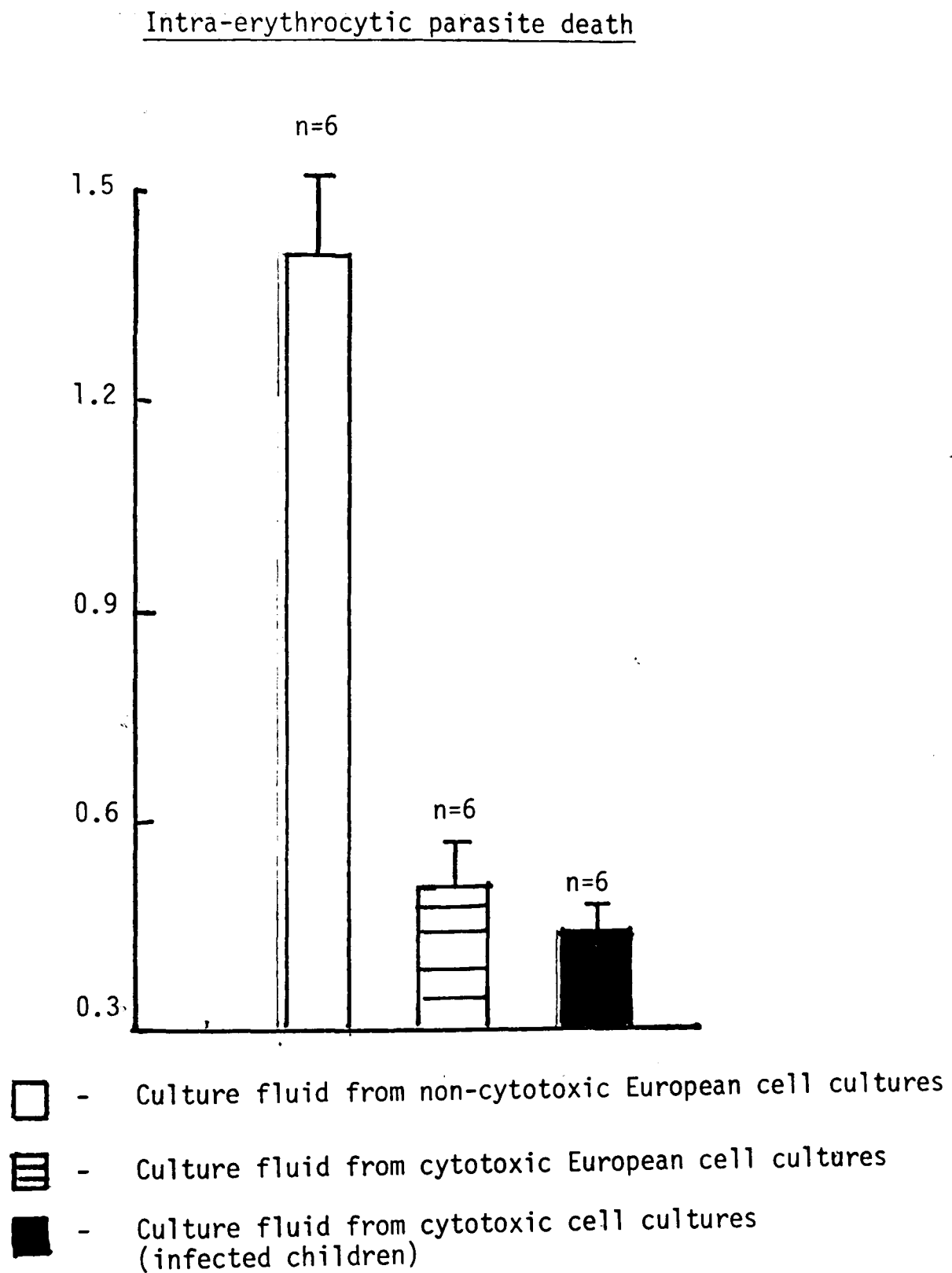
FIG. 20   Inhibition of parasite growth by cytotoxic culture fluids.

Mean P. falciparum growth ± standard error of a single
established isolate after exposure to culture fluids.
48 hour cultures.   0.3% = starting parasitaemia.

contained no specific antibody, parasite death was concluded to have occurred due to the activity of a cytotoxic product, possibly of macrophage origin, which was present in the culture fluid.

6   4.   DISCUSSION

The results of this study indicate that both antibody-dependent and non-specific mechanisms play ancillary roles in the intra-erythrocytic destruction of P. falciparum parasites by cells of the monocyte-macrophage series.

The normal requirement of specific antibody in the ADCC system can be by-passed, as long as effector cells are suitably activated. This was demonstrated when mononuclear cells from children with malaria, which were activated by products of either PHA or antigen stimulated mononuclear cells (also from children with malaria), killed P. falciparum in the absence of malaria antibody (Fig. 18).   Malaria antibody therefore, probably partici-
   with monocytes
pates/in the stimulation of T cells, but once blastogenesis is initiated and effector cells are activated, they may kill parasites non-specifically. Specific antibody may, however, enhance this form of parasite killing by making targets more accessible to effector cell attack.

It was previously shown that T cells would not respond to malaria antigen in the absence of accessory cells and under such conditions T cell supernatants failed to induce adherent cells to become cytotoxic (Chapter 5). This result was verified when P. falciparum growth was severely affected by mononuclear cells which had been activated by supernatants from undepleted mono-nuclear cells previously exposed to malaria antigen (Fig. 18). This suggests that activity by accessory cells of mononuclear

phagocyte lineage, was necessary before clonal expansion of appropriate T cells could take place. T cell mitogenisis due to contact with antigen-bearing accessory cells could have occurred in the absence of specific antibody by cell-cell recognition of MHS determinants, but malaria antibody was previously shown to induce phagocytosis of parasites by adherent cells (Chapter 5). As the latter conditions would provide a more efficient system of increasing contact between T cells and accessory cells, it is likely that expansion of T cells occurs in this way. This is perhaps the major function of specific antibody in the ADCC system.

The important role of T cells in ADCC, reported in Chapter 5, was further illustrated when mononuclear cells from one child with malaria, whose T cells were unresponsive to mitogenic stimulation, failed to inhibit P. falciparum in the presence of autologous serum (Tables 7 & 8). T cells from 3 other infected children showed ten-fold transformation when exposed to supernatants of 'activated' adherent cells; mononuclear cells from these children killed P. falciparum in the presence of autologous serum. As T cells from these children underwent blastogenisis in the absence of any cell-cell contact, non-specific transformation of T cells during acute malaria must be considered.

The sera of children with acute malaria contain high interferon levels (Ojo-Amaize et al. 1981). Interferon stimulates macrophages to release IL-1, which in turn, induces the release of IL-2 from T cells; IL-2 causes T cells to transform with subsequent release of non-specific macrophage activating factors (MAF). MAF in supernatants of mononuclear cells from infected children, could explain why such fluids induced European mononuclear cells to kill P. falciparum. In contrast, supernatant of PHA-stimulated European mononuclear cells caused merely a

limited reduction in parasite multiplication when added to European mononuclear cells. This suggests that malaria infection stimulates non-specific defense mechanisms. There is also a possibility that cellular cytotoxicity during malaria infection is serum-dependent rather than antibody-dependent and that parasite killing in vitro by serum and cells from infected children is totally non-specific. Small serum samples from children with malaria prevented the effect of removal of specific antibody on cytotoxicity to be tested, so this remains an important question to be answered. However, results of malaria antibody determinations presented in Chapter 3, revealed a definite association between specific antibody and cellular cytotoxicity. It is likely that both specific and non-specific cytotoxic mechanisms provide an integrated response to acute malaria, the effectiveness of which may influence the outcome of infection, in the absence of protective humoral immunity.

In all ADCC experiments, intra-erythrocytic parasite death was assessed microscopically. Consequently, this method gave no indication of how P. falciparum was actually killed. Results presented in the previous section indicate that parasites were killed by exposure to soluble non-antibody products, probably released from activated adherent cells (Fig. 20). As fluids from cytotoxic cultures, involving mononuclear cells from children with malaria, contained acute-phase serum, parasite death could have been due to factors already present in their sera rather than those released by cytotoxic mononuclear cells during the culture period. This is unlikely to have been so, as parasites were killed just as effectively by exposure to fluids from cytotoxic cultures which contained European serum; these cultures contained European mononuclear cells artificially stimulated by

products of PHA-stimulated cells from infected children.

It would appear then that intra-erythrocytic parasite death by products of mononuclear cells occurs in human malaria as well as in malaria of experimental animal models. The nature of these products and the factors which induce their release from adherent cells are currently being investigated. However, valuable guidelines on the characterisation of these substances have been provided by recent studies in murine malaria (Taverne et al. 1981; Dockrell & Playfair 1983; Clark & Hunt 1983).

It is suggested that during malaria infection P. falciparum parasites are killed inside red blood cells by soluble products released by cells of the monocyte-macrophage series. However, before cytotoxicity occurs, effector cells require prior activation by T cells which have undergone transformation, probably after stimulation by accessory cells or their products. In addition, both the mediation and execution of this system may benefit greatly from the participation of specific antibody. Finally, it is possible that this form of parasite destruction provides the primary defense mechanism during acute P. falciparum infection.

CHAPTER 7

7 GENERAL DISCUSSION AND SUGGESTIONS FOR FUTURE WORK

7 1. INTRODUCTION

This thesis provides strong evidence that killing of blood-stage parasites by cytotoxic mononuclear cells plays an important role in recovery from acute P. falciparum malaria in man. Such a conclusion permits a reappraisal of many studies on the immune response to malaria, which previously could not be interpreted to support such a view. As the results have already been discussed in detail in individual chapters, this section concentrates on presenting a general overview of how these findings relate to existing knowledge of malaria immunity.

7 2. NATURAL RECOVERY FROM ACUTE P. FALCIPARUM INFECTION

Recovery is the usual outcome of P. falciparum in the majority of children living in malaria endemic areas. Immunity acquired from each attack however, does not render the host resistant to the pathology associated with high parasitaemia in subsequent infections until numerous successive attacks have been experienced. This indicates that malaria immunity in early childhood is either highly strain specific or extremely short lived. In either instance, immunity is inadequate, as infants remain at risk to the fatal consequences of this disease for many years.

Massive sporozoite challenge with resultant rapid increases in blood-stage parasite numbers provide conditions which probably overwhelm the host's residual immunity. In such circumstances, it is likely that rapid stimulation of all the host's available defense mechanisms is necessary for death to be averted. Recovery from acute malaria perhaps requires the effective func-tioning of an integrated protective pathway which must include a

mechanism capable of controlling exponential increases in parasite numbers which may be separate yet complimentary to the means of acquiring protection against reinfection. Cytotoxic cells may provide primary protection during the acute stages of infection, and may assist conventional humoral immunity to develop and predominate once malaria parasitaemia is controlled. Immunity gained from each infection however, may provide little or no protection against unencountered parasite strains and until sufficient immunity is accumulated to prevent the symptoms of acute malaria, the ability of mononuclear cells to kill P. falciparum may be the factor which decides the outcome of each attack.

The results presented in Chapters 3 and 4 provide good evidence to support this hypothesis, as the only potential protective mechanism which could be measured in children during the acute stages of their illness, was the ability of their mononuclear cells to inhibit P. falciparum growth in culture. The degree of parasite killing by mononuclear cells rose during the period of recovery, as did levels of parasite inhibitory antibody, with the latter reaching a peak in convalescence when autologous parasites were inhibited by serum alone. Killing of P. falciparum by mononuclear cells plus serum during infection was not strain related whereas inhibition of parasite growth by convalescent serum was usually greater against the most recent infecting parasite strain. As parasite inhibitory antibody was not detected in the sera of a majority of children tested during the season of minimal malaria transmission, acquired immunity may have declined to a level inadequate to prevent severe malaria on subsequent P. falciparum challenge.

HYPOTHESIS
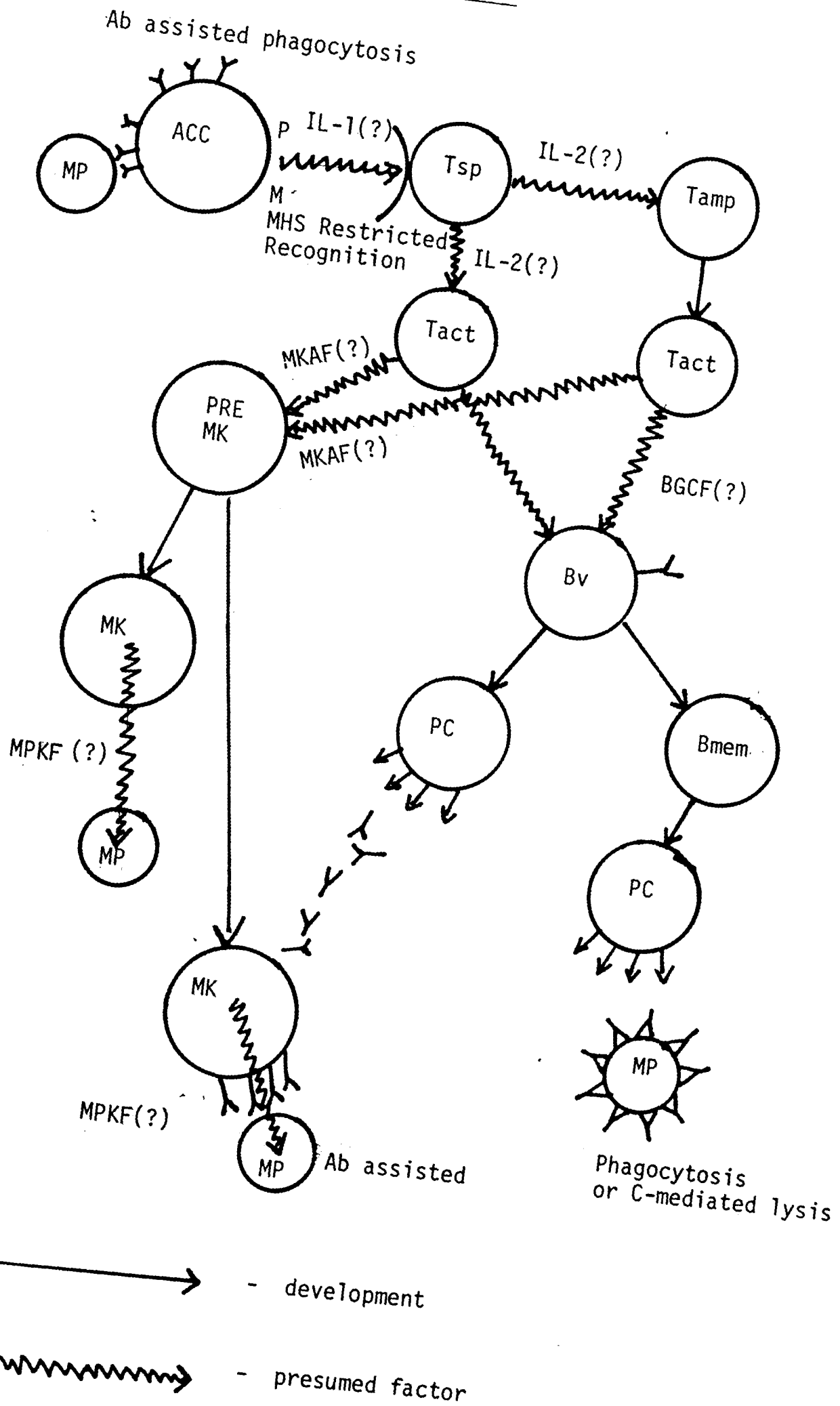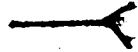
FIG. 21   Model of the cellular response to malaria.

FIGURE 21

CELLS

Ab	=	Specific antibody
ACC	=	Accessory cell presenting parasite antigen (P) in association with MHC coded antigen (M)
Tsp	=	Specific T cell carrying out recognition of PM
Tamp	=	Amplifier T cell
Tact	=	"Activated" T cells
Bv	=	Virgin B lymphocyte with receptor for P
Bmem	=	Memory B lymphocyte with receptor for P
PC	=	Plasma cell secreting P specific Ab
PRE-MK	=	Pre-killer cell
MK	=	Malaria killer cell
MP	=	Malaria parasite (free or within erythrocyte)

FACTORS

IL-1(?)	=	Interleukin 1 (?)
IL-2(?)	=	Interleukin 2 (?)
BCGF(?)	=	B cell growth factor (?)
MKAF(?)	=	Malaria killer cell activation factor (?)
MPKF(?)	=	Malaria parasite killing factor (?)
—<	=	Specific anti-malaria parasite antibody

(Possibly IgM > IgG from Bv stimulation; IgG > Igm from B memory stimulation).

7 3. <u>AN IN VITRO MODEL OF THE CELLULAR RESPONSE TO ACUTE MALARIA</u>

Whilst accepting the need for caution in their extrapolation, it is nevertheless, appropriate to assemble the results of cellular studies presented in this thesis in the form of an hypothesis. This hypothesis is illustrated in figure 21 and is discussed in the order in which the sequence of immune reactions is postulated to occur. It was necessary to apply certain terms and abbreviations to the various activating factors and cellular mechanisms referred to in the text, but it is emphasized that these are largely presumed rather than confirmed by results in previous chapters.

<u>Mediation of cellular cooperation: Accessory cells</u>

Intra-erythrocytic parasite death is probably dependent on a cascade of immune events involving accessory cells, specific antibody, soluble mediators and T cells whose combined activity results in the activation of effector cells which kill their target by the release of cytotoxic products (Chapters 5 & 6).

The first link in this chain of immune reactions is most likely to be the stimulation of accessory cells which are probably mononuclear phagocytes. Macrophages are thought to act as accessory cells during malaria infection by presenting processed antigen to T cells and by releasing soluble products (probably IL-1) which nurture the proliferation of antigen-specific T cell clones (Kreier 1980). This process is believed to stimulate specific B cells to proliferate and transform into antibody-producing cells (Kreier 1980). This view of the development of natural immunity inadvertently adds support to the first section of the hypothesis shown in figure 21 which proposes that it is entirely possible that accessory cell-T cell

cooperation may also lead to the activation of cytotoxic effector cells during malaria infection in man.

Mononuclear cell phagocytes may adopt an accessory cell role when stimulated in a number of different ways. Antibody-induced phagocytosis of P. falciparum parasites by adherent cells from infected children, though insufficient to cause elimination of parasites, may yet provide sufficient antigen to allow accessory cells to enlist T cell cooperation (Chapter 5). However, as mouse macrophages have been shown to ingest P. berghei parasites in the absence of specific opsonising antibodies (Chow & Kreier 1972; Brooks & Kreier 1978), both antibody-dependent and anti-body-independent phagocytosis should be examined as possible sources of accessory cell activity. Additional factors which must also be viewed as potential stimulators of accessory cells during malaria infection are: immune complexes, interferon and macrophage activating factor (MAF), all of which are capable of inducing macrophages to release the T cell activating factor Interleukin I.

The potential importance of accessory cells in the ADCC system was observed when products of antigen-stimulated European mononuclear cells failed to induce European mononuclear cells to kill P. falciparum (Chapter 6). This observation was more likely to have reflected an inactive accessory cell-T cell mechanism rather than a lack of effector cells, as mononuclear cells from these same Europeans included cells which, when activated by similar products of cells from infected children, became cytotoxic to P. falciparum. The discovery of a specially recruited monocyte population, which when removed from circulating mononuclear cells rendered ADCC ineffective, would

suggest that accessory cell activity is an essential component of this type of parasite killing.

As normal European mononuclear cells were capable of killing P. falciparum when suitably activated, the adherent effector cell referred to in Chapter 5 is probably a normal constituent cell of human peripheral blood and not the atypical monocyte observed in the blood of children with malaria; this cell may have been engaged in an accessory cell role.

Further investigations aimed at identifying the accessory cell alleged to be present during malaria infection are therefore desirable and should include tests directed towards establishing optimal conditions required for its activation, as well as the parameters which result in its maximum cooperation with T cells. Future experiments should also pursue the role of accessory cells in the production of malaria antibody as postulated in figure 21. Any defect in the accessory cell-T cell mechanism could possibly prolong recovery from malaria by affecting both parasite killing by effector cells and by retarding the production of protective antibody.

The role of specific antibody in ADCC

The need for specific antibody in the cytotoxicity system was established during the early part of this study, but the precise function of specific antibody in ADCC has yet to be determined. However, as effector cells were shown to kill their targets in the absence of malaria antibody, provided these cells were appropriately activated, the major role of antibody must be in the mediation of cellular cytotoxicity; although specific antibody may also enhance this reaction by rendering targets more easily identified.

One of the objectives of this study which proved difficult
to establish, was the immunoglobulin class which participates
in ADCC (Chapter 5). Preliminary results of ongoing experi-
ments however, have found that ADCC activity is found exclusively
in the IgM fraction of serum of some children with acute malaria,
but this antibody has been found to have no direct inhibitory
effect on P. falciparum growth (Brown, J. and Greenwood 1982).
These findings are consistent with results presented in Chapter
3 which demonstrated that mononuclear cells from children with
acute malaria were ineffective against P. falciparum unless
cultured with autologous serum which contained malaria antibody,
although the sera of these children had no direct inhibitory
effect on parasite growth. As sera from only 5 children have so
far been tested in these experiments, further investigations
are required before a definite link between specific IgM and
cellular cytotoxicity during acute malaria can be confirmed.
However, confirmation of these results would assign a protective
role to IgM antibodies during malaria infection and could
explain why these antibodies appear in serum during the early
stages of this disease.

Activation and function of T cells

When stimulated by malaria antigen, mononuclear cells from
children with malaria were shown to release products which
activated cells from normal, healthy Europeans to kill P.
falciparum (Chapter 6). In contrast, products of similarly
stimulated European mononuclear cells failed to do so. This
observation indicates that in addition to possessing antigen-
specific T cells which stimulate B cells to proliferate and
produce specific antibody (Brown, K.N. 1979), children with
malaria probably possess T cells which are specifically primed

to participate in cytotoxic reactions with effector cells. In addition, as mononuclear cells from infected children which killed autologous parasites were also able to kill heterologous parasites (Chapter 4) their T cells may have been primed to respond to antigen (S) common to many P. falciparum strains.

Malaria induces clinical immunity in children associated with persistent low-level infection.   In addition, merozoite immunization of laboratory animals does not achieve sterilizing immunity unless vaccines are administered in conjunction with adjuvants (Kreier 1980). And as adjuvants stimulate monocyte-macrophage activity, their inclusion in vaccines may enhance accessory cell-T cell cooperation (as well as effector cell activity) thus, increasing the efficiency of cell mediated reactions as well as specific antibody production. Adjuvants may cause the immunized host to respond quickly to parasite challenge by enhancing accessory cell responsiveness which in turn may lead to early activation of T cells by inducing them to release IL-2 (Fig. 21).   An example of the protective potential of adjuvants can be found in the work of Clark (1978), who showed that BCG, C. parvum and zymosan when administered to mice, protects them from both Babesia and Plasmodia infections.

Although this thesis has shown that T cells are involved in the promotion of intra-erythrocytic parasite death by cytotoxic adherent cells the sole function of T cells in malaria immunity was for a long time believed to be their involvement in the production of protective antibody. Transformed T cells may also amplify the proliferation of B cells which become producers of non-specific antibody or malaria antibody which has no protective function (Brown, K.N. 1979). To prevent the complications associated with over-production of antibody, B cell proliferation

may require to be regulated by suppressor T cells or their products as lack of control over antibody production may result in dangerous side effects, such as auto-immune anaemia, splenomegaly and in some cases Burkitt's lymphoma.

Recent studies in this laboratory have found that children with acute malaria probably have a defect of their suppressor T cells which permits the continual proliferation of their own B cells after these cells have been exposed to Epstein-Barr virus (Whittle & Brown, J. 1983). T cell control over transforming B cells is regained when children recover from malaria. These preliminary results imply that during acute malaria, whilst host defenses are committed to eradicating blood-stage parasites, polyclonal proliferation of B cells due to various stimulants may proceed unchecked and overwhelm control by suppressor T cells. Furthermore, as parasitaemia is brought under control former effector cells such as mononuclear phagocytes may adopt a suppressor role and proceed to 'switch off' mechanisms previously aimed at parasite elimination (Weidanz 1982). At this stage of infection, continued proliferation of helper T cells could also inadvertently contribute to immunosuppression against other infections by stimulating suppressor macrophages other than those required for reducing responses to malaria.

Helper and suppressor T cell function is an area of research in human malaria which as yet has received little attention but contrary to previous conclusions, the role of T cells in malaria immunity is unlikely to be confined to assisting specific antibody production. This thesis concludes that T cells play a vital role in recovery from malaria and in the acquisition of clinical immunity. Thus, further knowledge of T cell function and the factors which stimulate them such as those proposed in figure 21

are crucial to our understanding of the human immune response to P. falciparum malaria.

Effector cells

The cell responsible for P. falciparum death in the ADCC system is undoubtedly a cell of monocyte-macrophage lineage which as previously mentioned, is probably present in normal peripheral blood. The cytotoxic cell appears to kill its target by releasing substances which terminate parasite growth within red blood cells (Chapter 6). At the present time, it would be purely speculative to assign an identify to the product(s) which caused P. falciparum death in my experiments, but fluids from cytotoxic cultures must be screened for tumour necrosis factor, superoxides and other products of activated human monocytes which could halt parasite growth. One approach towards identifying such products would be to introduce substances into the culture system which inhibit their activity. Any resultant reverse in cytotoxicity would aid the identification of such cytotoxic substances.

Europeans were shown to lack the means of activating their own mononuclear cells to become cytotoxic yet these cells were capable of killing P. falciparum when suitably activated. This is an important observation which suggests that normal individuals possess the effector mechanism required for killing malaria parasites. Mononuclear cells from many children with acute malaria however, failed to kill P. falciparum in vitro. It is not known whether such results were due to ineffective mediation mechanisms or whether these children had a defect of their cytotoxic effector cells. These observations are being pursued by examining cell supernatants from infected children

for their ability to stimulate effector cells of known cytotoxic capability. This approach is also being used to identify lack of effector cell function by stimulating these cells with supernatants known to induce parasite killing by standard effector cells.

As factors released from mononuclear cells during malaria infection have been shown to activate non-specific effector cells (Chapter 6), it is possible that similar factors released as a result of co-existing infections may also assist parasite destruction. Endotoxin released during malaria infection in mice is believed to mediate parasite killing by macrophages (Clark 1978). However, if animals are given additional substances with similar properties such as bacterial LPS, parasite killing by macrophages increases, but the symptoms of severe malaria may develop and persist (Clark 1978). A similar situation may prevail in malnourished children who display the symptoms of severe malaria despite having low parasitaemias. In such children, effector mechanisms may already be active due to infections other than malaria at the time P. falciparum parasites appear in the blood and although parasitaemia may be controlled, release of malaria endotoxin in addition to bacterial toxins may yet result in a clinical picture of acute malaria. Although such complications may occur only in a minority of cases of malaria, further investigations using animal models should examine how cytotoxic mechanisms may be regulated to eliminate parasites without precipitating serious side effects in the host.

7 4. APPLICATIONS OF THE CYTOTOXICITY ASSAY: MODIFICATIONS OF THE BASIC TECHNIQUE

Future studies should benefit from a modification of the cellular cytotoxicity assay which was introduced towards the end

of the experimental section of this thesis.   Instead of assessing parasite death by microscopy the number of surviving parasites are now determined by measuring P. falciparum incorporation of tritiated hypoxanthine. To avoid isotope incorporation by mononuclear cells, target parasites are labelled with 1 µci/ml of the isotope 24 hours before being used in the ADCC test, excess hypoxanthine being washed from infected red cells before the beginning of the test culture period. Using this method, parasite survival is assessed after 24 hours using a liquid scintillation counter, but tests must be monitored microscopically as dying parasites have been found to incorporate hypoxanthine (Brown, J. unpublished data). This method could be extended to include investigations of accessory cell and T cell response to natural infection or malaria vaccination by culturing mononuclear cells with malaria parasites and measuring specific T cell transformation by incorporation of tritiated thymidine as this marker would not be incorporated by the parasite. It is felt that these modifications of the original microculture method form major improvements which should allow ADCC to be measured more objectively on a larger number of subjects.

Monoclonal antibodies

One of the major advances in malaria immunology which has developed since this thesis began, is the use of monoclonal antibodies for the identification of parasite antigens. As however, extremely few P. falciparum isolates have as yet been characterised antigenically, the number of protective antigens shared between parasite strains is unknown and until such information becomes available, vaccination against P. falciparum will not be feasible. An important first step towards determining the number of protective antigens has been accomplished by McBride, Walliker and

Morgan (1982) who have demonstrated antigenic diversity between
P. falciparum isolates using more than 30 monoclonal antibodies
bearing various stage and strain specificities. This work has
recently been extended to examine differences in the virulence of
various P. falciparum strains in the ADCC system and results to
date confirm the findings of Chapter 4 which showed that ADCC was
unlikely to be strain specific (Brown, J. & McBride, unpublished
data).

Monoclonal antibodies have also been used to monitor antigenic
changes of P. falciparum in continuous culture but so far no spon-
taneous antigenic variation has been observed in more than 20 iso-
lates grown under optimal growth conditions (McBride & Brown, J.
unpublished data). The possibility that partially effective ADCC
might induce antigenic variation of P. falciparum due to immuno-
logical pressure is also being investigated using monoclonal anti-
bodies.

All published studies to date have utilised monoclonal anti-
bodies against P. falciparum which were raised in mice. As anti-
bodies raised against P. falciparum in an unnatural host may
differ in their antigenic specificity from those produced in a
totally human system, human monoclonal antibodies have now been
produced using a modification of the method used for the study of
B cell proliferation in Burkitt's lymphoma (Whittle & Brown, J.
1983). Briefly, the rationale of the technique involves pulsing
peripheral blood B cells from children with malaria with Epstein-
Barr virus which induces these cells to undergo proliferation.
B cells are then subjected to limiting dilution and left in cul-
ture until clones of proliferating cells emerge. Each well
showing B cell transformation is screened for malaria antibody by
fluorescence using a P. falciparum antigen containing a wide

range of stage and strain specificities. Clones positive for malaria antibody are recloned and tested for both stage and strain specificity when prolific B cell growth is again established. Whilst much work remains to be done to improve the efficiency of the cloning procedure and in increasing yields of specific antibody, it is hoped that human monoclonal antibodies to P. falciparum may eventually be tested for their protective abilities in the ADCC system and in inhibitory antibody assays. Apart from obvious advantages in the selection of protective antigens for the prevention of malaria by vaccination, human monoclonal antibodies with potent inhibitory effects on P. falciparum could possibly be developed for use in the therapy of drug resistant malaria.

7   5.   CONCLUSIONS

It is difficult to conceive of a disease of industrialised nations which today would still claim the lives of an estimated 1-1.5 million children annually, as does P. falciparum malaria in Africa. Malaria eradication by insecticides has failed in most parts of the world and the emergence of chloroquine and pyrimethamine resistant malaria in Africa (Bruce-Chwatt 1982) as well as in other endemic areas, is exacerbating an already dangerous situation. Thus, there is an urgent need for other methods of malaria control, the most important of which, may be vaccination.

Recently, progress has been made towards vaccination against blood-stage malaria in laboratory animals (Holder & Freeman 1981) and it is hoped that P. falciparum antigens with similar protective properties for man may also soon be identified. However, an effective human malaria vaccine remains in the unforeseeable future, as the response of laboratory animals to malaria

vaccination may be quite different to that of children living in endemic areas, who frequently display multiple pathology. In addition, as the range of protective antigens which must constitute a human malaria vaccine are not yet known, successful immunization against a single P. falciparum strain may not necessarily protect the host against other strains. Despite these and many other obstacles, vaccination against P. falciparum must be vigorously pursued and should remain the goal of malaria immunology.

Sufficient evidence has now accumulated from work on animal models and from the results of this thesis to conclude that cellular cytotoxicity is an important component of the host's defenses against acute malaria. Thus, a means of activating this mechanism within the host may be an essential element of vaccination against blood-stage malaria in man. Consquently, in addition to the measurement of parasite inhibitory antibody, the efficacy of such a vaccine may also require to be assessed by the ability of the host's cytotoxic effector cells to kill intra-erythrocytic parasites.

Finally, it is hoped that the results of the studies presented here will stimulate further investigations on the cellular basis of host-parasite relationships in human malaria.

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