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Note: The editor assumes no responsibility for the statements and opinions expressed by the contributors. This issue has been delayed for reasons beyond our control.

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The Interaction of Drugs and Immunity in Malaria

W. PETERS[1]

INTRODUCTION

This oration* is given every year to perpetuate the memory of Dr Arun Kumar Banerjee, a brilliant young scientist who, in the 1950's, helped to establish the discipline of immunology in this famous Institute but who, sadly, died in 1962 at the premature age of 34 without having time to see his efforts come to fruition. This year, 1980, also commemorates the Centenary of the discovery by the French doctor Charles Louis Alphonse Laveran, working in Algeria of an organism causing malarial fever, an organism that we now call *Plasmodium falciparum*. Eighteen years later, here in Calcutta, Ronald Ross made the first observation of the development of a malarial parasite in a mosquito, in fact a parasite of birds, and the story of the malaria life cycle was all but complete.

Malaria as a cause of human suffering has in this century resolutely defied all efforts at its control and India in particular has been seeing a massive resurgence of this disease in spite of a decade of attempts to interupt its transmission. From a mere 100,000 cases in 1965 the numbers rose as high as 6.4 million by 1976 and still several million Indian people are afflicted by malaria today (Anonymous, 1976).

Over the past decade considerable advances have been made both in our understanding of the immunological responses of the mammalian host to invasion by malaria parasites and in the search for new antimalarial drugs. It seemed to me fitting, therefore, to marry the two topics today and to consider some aspects of the interface between drugs and immunity in relation to future strategy for the control of this devastating disease.

GENERAL NATURE OF IMMUNITY IN MALARIA

Natural Immunity: Natural or innate immunity to malaria is based initially on genetically inherited characteristics both of the parasites and the hosts. Species specificity has long been recognised in the genus *Plasmodium*, the blood stages of *Plasmodium falciparum* of man, for example, not being infective to rhesus monkeys. However, several unexpected facts have emerged from recent studies. Firstly, it is possible under certain circumstances to infect host species other than those normally infected in Nature. Several species of South American monkeys, for example, are susceptible to *P. falciparum* if inoculated with infected human blood. One specie, *Aotus trivirgatus*, has proved of immense value for

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investigations of experimental antimalarial drugs, and of protective vaccines against this parasite. Conversely, man in susceptible to infection with *P. cynomolgi bastianellii* and *P. knowlesi* of the rhesus monkey which may indeed give rise occasionally to zoonotic infections in man in Asia.

Species specificity appears to be stricter in relation to the infection of red blood cells by the erythrocytic stages of *Plasmodium* than is the case with the pre-erythrocytic stages in the liver. It has been shown, for example, that sporozoites of *P. vinckei* of the African tree rat *Thamnomys surdaster* can readily invade the livers of abnormal hosts such as the cotton rat (Bafort, 1971). The liver schizonts mature but the subsequent generation of erythrocytic parasites fail to establish itself in the erythrocytes of the abnormal host. Moreover, hepatic schizonts of *P. berghei* develop (at least partially) in tissue cultures, not only of rodent cells, but even in those of birds or man (Strome *et al.*, 1979).

Erythrocyte specificity is probably related to the presence of appropriate receptors on the erythrocyte surface membrane, receptors which match those of the surface of the merozoite and permit the latter to attach to and invaginate red cells of the correct host species. It has recently been demonstrated that the insusceptibility of people of Negro origin to *P. vivax* is associated with a deficiency of the Duffy antigen on the surface of their erythrocytes (Miller *et al.*, 1976). Experimentally, a line of albino mice was selected by Indian workers that were relatively resistant to *P. berghei*, but the mechanisms of this resistance were not determined (Ramakrishnan *et al.*, 1964).

Apart from these factors, a number of genetically determined characteristics of human blood are believed to bestow some degree of innate resistance against infection with malaria parasites or at least to offer a selective advantage to such individuals. Thus the presence of the sickle-cell trait at high levels in holoendemic areas of malaria in Africa is believed to have been selected for by the paradoxical advantage that this trait provides in the face of heavy and continuous challenge by *P. falciparum*, the trophozoites of which cannot grow as well in HbS as in normal red cells containing HbA. This hypothesis has recently gained support from *in vitro* studies that show how rapidly HbAS containing erythrocytes are sickled in the presence of very young *P. falciparum* trophozoites (Roth *et al.*, 1978). HbF and certain types of G6PD deficiency may also offer a selective advantage in areas highly endemic for *P. falciparum* and it has recently been suggested that a low level of red-cell pyridoxal kinase may be a further advantageous factor in West Africa (Martin *et al.*, 1978). A comparison of the severity of illness in American whites and blacks infected with *P. falciparum* in Vietnam indicated that the latter suffered relatively less for the same level of parasitaemia and responded better to equivalent chemotherapy (Powell *et al.*, 1972). It was suggested that this may have been associated with a relatively low red-cell ATP level in American Negroes. One net result of a relative tolerance by Africans of *P. falciparum* infections may be the failure until recently of chloroquine to select mutants of this parasite which are resistant (Fig. 1) to 4-aminoquinolines (Peters, 1969) and (Hall and Canfield, 1972). This problem is only now beginning to appear in Africa, some 20 years after it was first recognised in Southeast Asia and the New World (Campbell *et al.*, 1979).

Acquired Immunity: While most work on sporozoite vaccination has demonstrated that protective anti-sporozoite antibodies can be induced in animal models and in man, demonstrable levels of such antibodies have not yet been found in man even in a highly endemic part of West Africa (Bray, 1978). It seems likely that acquired immunity to the erythrocytic stages of *Plasmodium* is the most important survival factor in man in Nature, but debate still rages around the relative importance of the roles of cellular and humoral mechanisms in this phenomenon

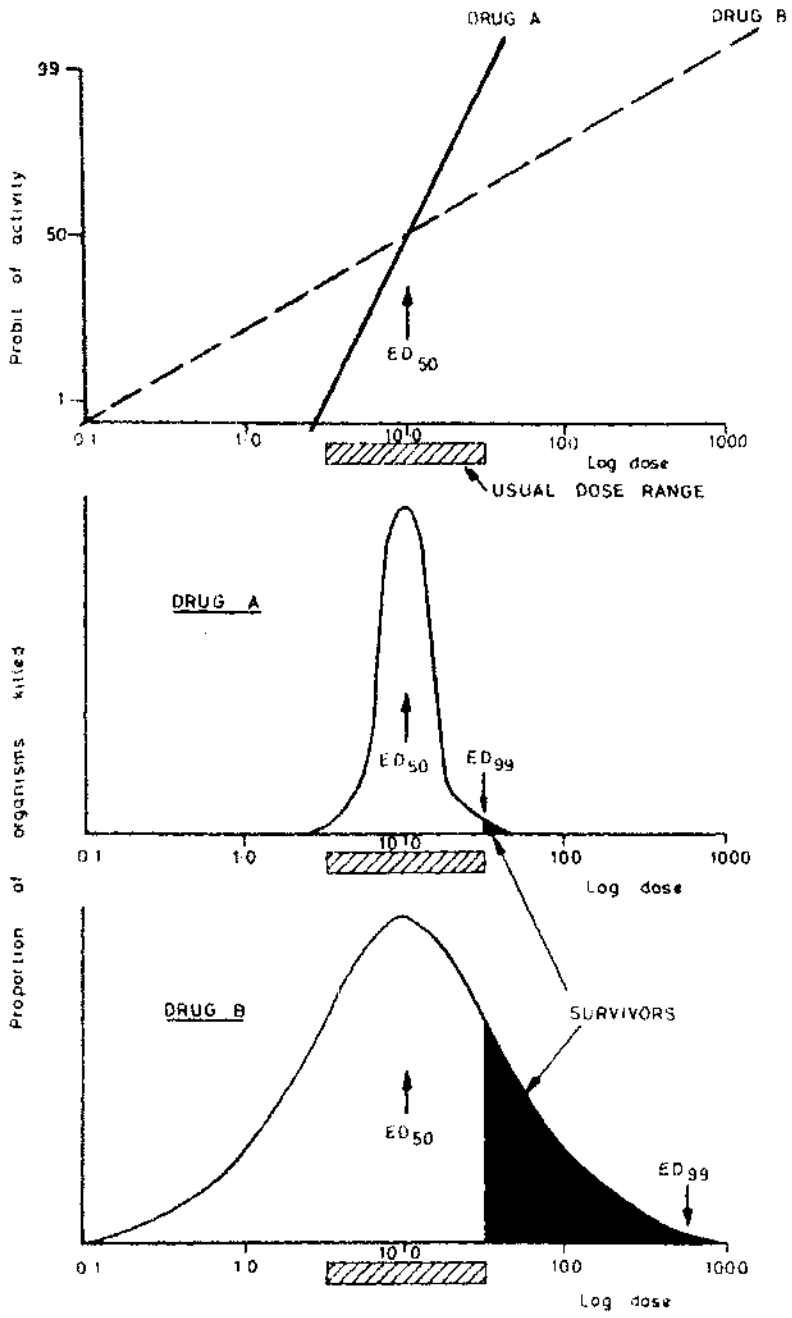

Fig. 1. A schematic comparison of the effect of different dose-activity response curves in two drugs and the effect of these on parasite survival. Drug A is a compound such as chloroquine, Drug B one such as pyrimethamine. Resistance can emerge much more readily to Drug A. (Peters, 1969).

(Cohen, 1979). That both are involved is now quite clear. In rhesus monkeys infected with *P. knowlesi* the levels of which are suppressed by chemotherapy, for example, IgG (and IgM) antibodies develop which are capable of blocking the merozoite invasion of new red cells (Cohen, 1979). Moreover, developing trophozoites in mouse erythrocytes that are already infected with *P. berghei* are killed by serum factors other than immunoglobulin. The nature of these factors which appear to be common to serveral intra-erythrocytic protozoa (e.g. *Babesia*) has not yet been defined although a parallel has been drawn with them and endotoxin (Clark, 1978). Merozoite-inhibitory antibody, on the other hand, is species specific, and its production is facilitated by helper T-cells. Splenectomy increases the susceptibility to malaria infection. Paradoxically the presence of malaria parasitaemia has an immunodepressive effect which may be one factor underlying the high level of susceptibility of the inhabitants of highly malarious areas to intercurrent infections such as measles (Greenwood *et al.*, 1972) and (Williamson *et al.*, 1978). This phenomenon may be caused by the activation of suppressor cells by lymphocyte mitogens released, possibly from infected red cells, during the course of malaria infection. A similar phenomenon has been observed in trypanosomiasis and may be a means for enhancing parasite survival. Nevertheless, a cellular immune response has been clearly demonstrated in malaria by numerous investigators and the phagocytic clearance of merozoites and parasite debris from disrupted schizont-containing erythrocytes is accompanied by an active segregation in the spleen and other reticulo-endothelial tissues of some uninfected, as well as infected intact red cells.

In addition to the serum factor already mentioned there is now good evidence that interferon, too, plays a role in limiting the multiplication both of pre-erythrocytic (Jahiel *et al.*, 1969) and erythrocytic stages (Sauvager and Fauconnier, 1978) of, at least, rodent malaria parasites.

The cumulative result of the various mechanisms that come into operation during the course of naturally acquired human malaria infections is that the survivors among the infected population may be able to resist further natural challenges by mosquitoes infected with the same *Plasmodium* species and strains. Moreover, in such semi-immune people gametocyte formation is suppressed, and anti-gamete antibodies may be present to offer a further hindrance to continuing transmission. However, in the absence of repeated challenge, infection with *P. falciparum* usually dies out within about one year, and with *P. vivax* in about 3 years, a factor which is reflected by a decreasing titre of IgG antibodies (Collins *et al.*, 1968). By contrast, *P. malariae* may persist for decades. A recent longitudinal survey of Nigerians made before, during a period of malaria control by drugs and insecticides, and following the arrest of control operations, showed clearly, compared with unprotected controls, a significant drop in antibody titres by the IFAT against *P. falciparum* at all ages after 16 months of protection, but mainly in the younger age groups and teenagers against *P. malariae* (Brőgger *et al.*, 1978).

Finally, it must not be forgotten that chronic infection with *P. malariae*, if not with other species, may result in immune complex formation resulting in the development of malarial nephrosis or a peculiar type of chronic and massive splenomegaly ("big spleen disease").

MODE OF ACTION OF ANTIMALARIAL DRUGS

Antiparasitic action: The antimalarial drugs (Fig. 2) that are generally available today fall into several distinct groups, each with their own mode of action (Peters, 1970) and (Peters and Howells, 1978). Much credit must be given here to the pioneering studies of Indian investigators at the famous Malaria Institute of India, now of course reformed as the National Institute for Communicable Diseases in Delhi.

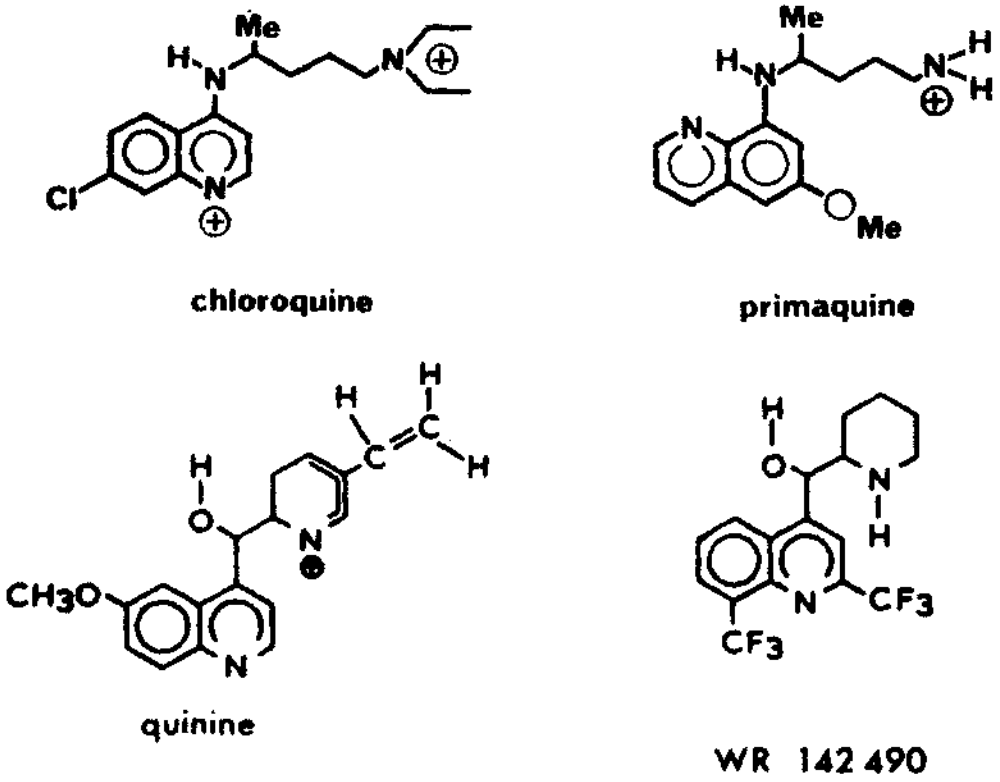

Fig. 2: Structures of some blood schizontocides (WR 142490=mefloquine).

4-aminoquinolines: The most widely used 4-aminoquinoline is chloroquine, a potent and rapidly acting drug that inhibits the growth of, and may eventually kill the asexual intraerythrocytic stages of all human *Plasmodium* species. It also has a limited action on gametocytes of *P. vivax*, *P. ovale* and *P. malariae*, but only on very young gametocytes of *P. falciparum*. Chloroquine and another commonly used 4-aminoquinoline, amodiaquine, have no action on the pre or secondary exoerythrocytic stages in the liver, and are thus neither causal prophylactics nor anti-relapse drugs.

While these compounds possess complex pharmacological properties, their specific mode of action against malaria parasites is still not clear. Within a short time of exposure to chloroquine (30 to 40 minutes) distinct morphological changes may be observed in the parasites, notably a coarsening and then clumping of the fine granules of malaria pigment (haemozoin, a pigment end-product of haemoglobin digestion by the parasite, Fig. 3). This change is associated with a high degree of concentration of the drug within the parasitised erythrocytes by a mechanism that so far eludes our understanding (Warhurst and Thomas, 1975).

Quinine and synthetic quinine analogues: Quinine is the oldest known chemotherapeutic agent and its potent antimalarial properties have been recognised and exploited for some 400 years. The spectrum of action against the different stages and species of the malaria parasite is essentially the same as that of the 4-aminoquinolines. How-

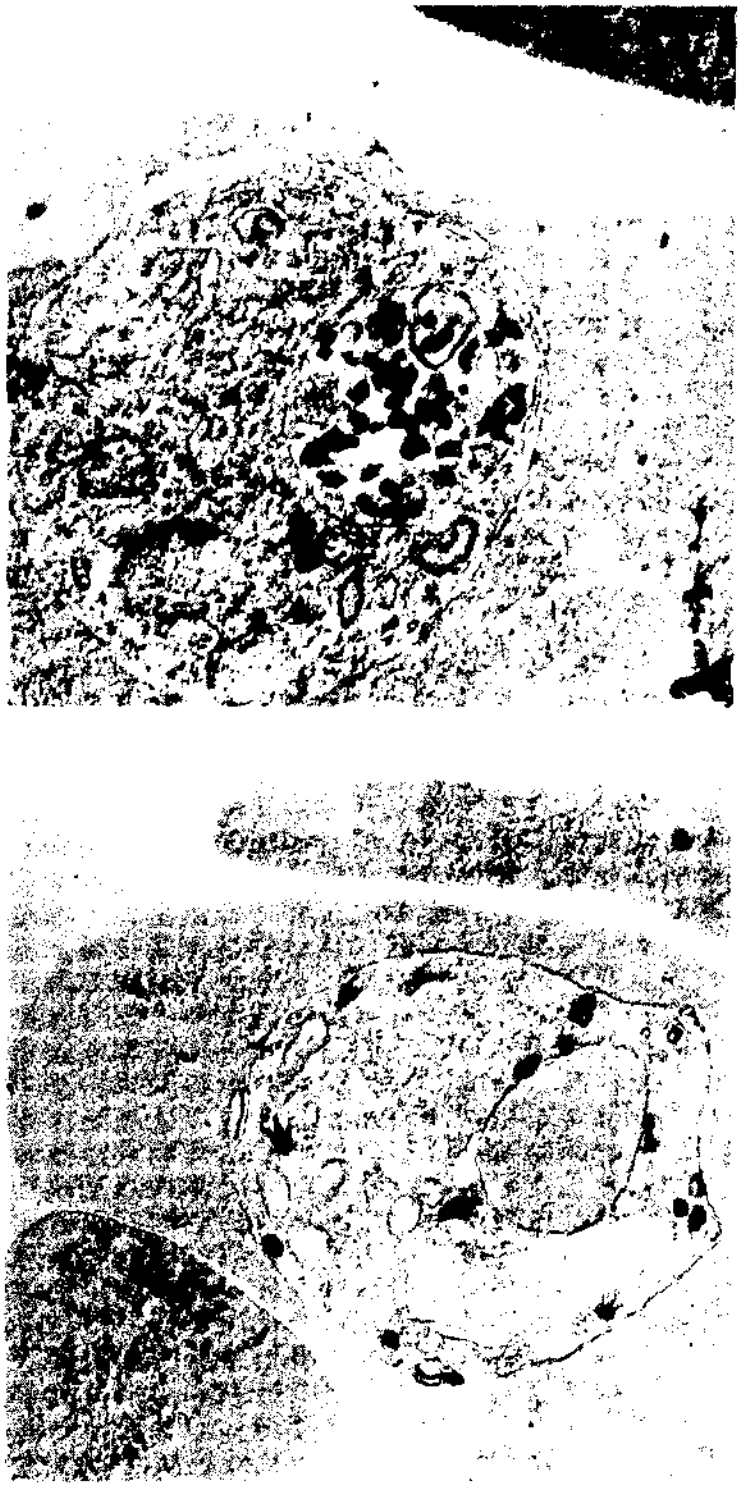

Fig. 3. Action of chloroquine on malarial pigment in *P berghei* (left—untreated control; right—40 minutes after exposure to chloroquine).

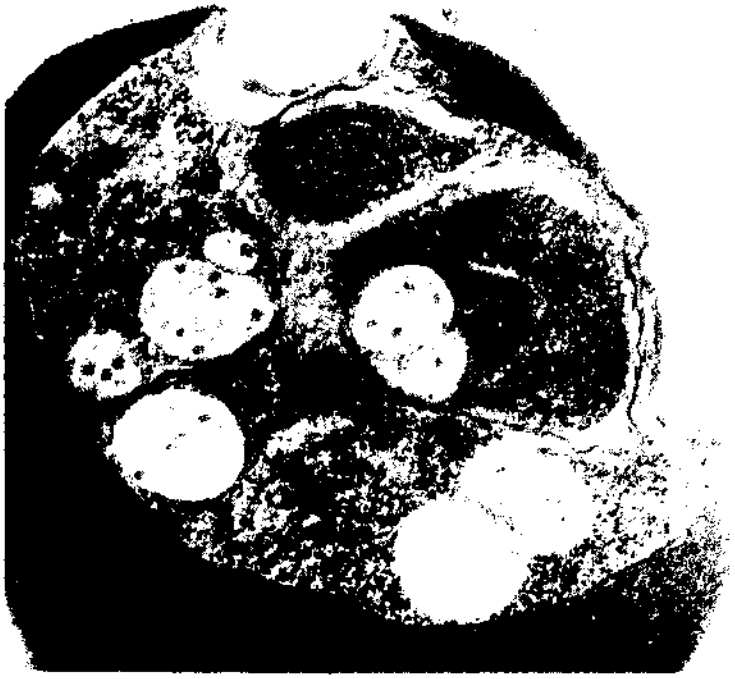

Fig. 4: Action of mefloquine on malarial pigment in *P. berghei*. Note the agglomeration and loss of electron density of the pigment granules.

ever, unlike the latter, quinine and its modern synthetic analogue mefloquine (a 4-quinolinemethanol some hundred times more potent than quinine) produce quite different morphological changes in the parasites. These effects are characterised by a disturbance of intra-parasitic membranes, and a peculiar clumping and apparent dissolution of the haemozoin granules. The main difference, however, between the two types of compounds is that quinine retains its effectiveness against strains of *P. falciparum* that are resistant to chloroquine, and can therefore be used to treat patients who are infected with such parasites (Fig. 4).

8-aminoquinolines: Primaquine, the most widely used of this group, possesses a broad spectrum of activity and indeed has some inhibitory effect on all stages of the parasites in appropriate concentrations. These drugs are, however, toxic and primaquine itself has a very short half-life in the body (Bruce-Chwatt *et al.*, 1981), so that this double handicap prohibits its use for all but two indications. Given over a period of 5 to 14 days, primaquine will effect a radical cure of vivax or ovale malaria in a high proportion of patients. This means that it is able to destroy the stages of the parasite that are responsible for the production of true vivax relapses, the so-called secondary exo-erythrocytic schizonts. Experiments being carried out at this moment by Garnham and his colleagues (Krotoski *et al.*, 1980) indicate that the origin of these forms may in fact be a special type of sporozoite, the "hypnozoite". It is postulated that this type of sporozoite, injected with the original infective inoculum by the *Anopheles* mosquito, remains dormant for months or even

years before commencing development, and giving rise to the next crop successively of exoerythrocytic schizonts, then erythrocyte-invading merozoites. Where such hypothetical "hypnozoites" are located, and what immunological factors may regulate their dormancy and reactivation remains to be clarified. What does seem likely, however, is that primaquine has the ability to kill them, and so stop their further maturation.

The precise mode of action of primaquine against human malaria parasites is unknown. It is speculated that either the parent compound, or an active metabolite may intervene in mitochondrial respiratory activities of the parasites by an action on parasite ubiquinones. The fact that the ubiquinones utilised by *Plasmodium* are different from those that are used by the host may offer a point at which a differential toxicity of primaquine may come into effect. What is known is that primaquine produces marked ultrastructural changes in the mitochondria both of the asexual stages of the rodent parasite *P. berghei*, and the exoerythrocytic merozoites of the avian parasite *P. fallax*. Moreover, a single dose of primaquine renders sterile the gametocytes of *Plasmodium*, a phenomenon that has been shown clearly in *P. yoelii* in mice and *P. falciparum* in man. Interestingly, primaquine does not have a direct action on sporogony in the mosquito, and this has been taken to indicate that it must first undergo transformation to an active metabolite in the mammalian host, but that such activation does not occur in the mosquito (Peters and Howells, 1978).

Inhibitors of purine metabolism (Fig. 5): Malaria parasites, like many bacteria, cannot utilise preformed purines and therefore possess the range of enzymes necessary for their synthesis from basic metabolites. One of the starting materials is *p*-aminobenzoic acid (PABA) which is first converted to dihydrofolate, and thence to

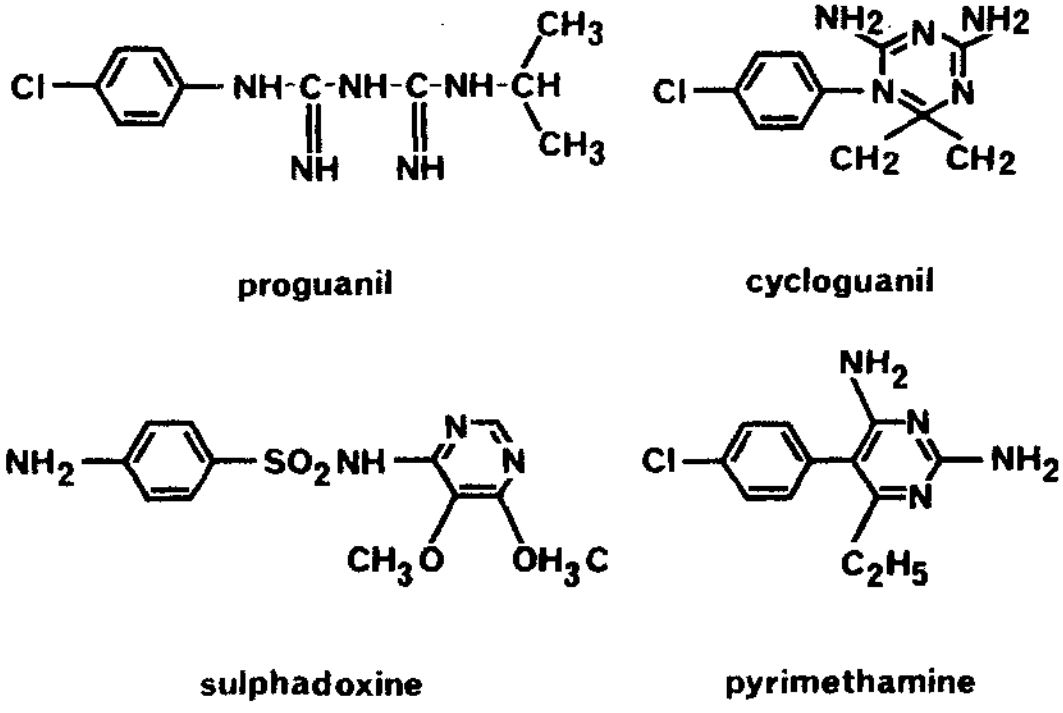

Fig. 5: Structuresof some drugs that interfere with early stages of purine metabolism.

tetrahydrofolate by the mediation of the enzyme dihydrofolate reductase (DHFR). The condensation of PABA with pteridine is antagonised by sulphonamides and sulphones. The antimalarials proguanil and pyrimethamine bind to and inactivate DHFR. Either type of intervention results in an inhibition of purine synthesis which, in turn, blocks nucleic acid synthesis and nuclear division of the parasites at schizogony. Use of both a sulphonamide and a DHFR inhibitor leads to sequential blockage of the two synthetic processes and a very high degree of drug potentiation.

Understandably, therefore, sulphonamides and DHFR inhibitors possess a broad spectrum of activity against the different stages and species of the malaria parasites, and the latter have proved to be highly effective causal prophylactics against many *Plasmodium* species, good but slowly acting blood schizontocides and true sporontocidal drugs. Proguanil itself has little inherent activity but is rapidly converted in the liver to its active triazine metabolite, cycloguanil. The level of sensitivity of the different human malaria parasites to all these agents varies somewhat, *P. vivax* liver stages especially being relatively unresponsive to pyrimethamine, and even more so to sulphonamides.

These compounds used individually may indeed lead to an inhibition but not a complete arrest of development of pre-erythrocytic schizogony. However, the potentiating effect of suitable combinations may be sufficient to overcome this obstacle, provided that the parasites have not developed resistance to one or other component. The failure of Fansidar in Thailand to control *P. vivax* may be due, for example, to the local strains having become resistant to the pyrimethamine component of this combination, while never having been inherently sensitive to the sulphonamide component. The relative failure of sulphonamides may be due to the ability of the developing pre-erythrocytic schizont to bypass the synthetic step which these components block, or to a failure of sulphonamides to penetrate the parasites in sufficiently high concentration. It would be interesting to see whether targetting a sulphonamide by incorporating it in an appropriate lysosomotropic carrier such as a liposome would enhance its activity against the liver stages, as has been shown to be the case with primaquine (Pirson *et al.*, 1979).

The fundamental point to note in relation to all the compounds described above is that they do not, by themselves, cure the patient. It will be shown later that an active immune response on the host's side is an essential component of the elimination of the parasites from the body, and that drugs alone will not cure an immunodeprived host except in unacceptably high dosage. It has been postulated that some drugs, for example chloroquine, may influence the response of the invading merozoites to specific receptors on new erythrocyte host cells, but if this is so it does not appear to be of major significance in the practical application of such chemotherapeutic agents.

Suppression of Cellular Immunity: It has long been known that chloroquine in suitable concentration will depress several types of lymphocyte response to antigenic stimuli such as phytohaemagglutinin or streptolysin (Hurvitz and Hirschorn, 1965). It may also inactivate haemolytic amboceptors and block other autoimmune responses. One mechanism by which this action may be brought about is the stabilising effect that chloroquine has on lysosomal membranes. These properties of chloroquine, other 4-aminoquinolines and mepacrine were exploited in the treatment of a variety of auto-immune collagen diseases in which they produced a similar benefit to corticosteroids, but without the undesirable side effects of the latter. Today, however, these antimalarials are little used in these indications because of their tendency to accumulate in the eye where they may cause retinitis. Recently, it has been shown that primaquine, quinine and the quinine-like mefloquine (Thong

et al.,1979) also inhibit mitogen-induced proliferative lymphocyte responses, but only in concentrations significantly higher than those employed in the treatment of malaria.

EFFECT OF IMMUNOSUPPRESSION ON THE COURSE OF A MALARIA INFECTION

While it might be supposed that a reduction of phagocytic activity by the application of drugs that suppress cellular immunity would result in an enhanced parasitaemia, this has not always proved to be the case. Cortisone may actually potentiate the antimalarial action of chloroquine or mepacrine against *P. gallinaceum* in chicks, and enhance gametogenesis in *P. berghei* in mice (Tandon and Bhattacharya, 1970). Betamethasone, by depressing reticulocytosis in mice, depresses an infection with *P. yoelii* (an obligatory parasite of reticulocytes) but enhances *P. chabaudi* (which lives in mature erythrocytes) by its inhibitory action on phagocytes (Cox, 1974). Conversely, the stimulation of the reticuloendothelial system by steroids, such as diethylstilboestrol, alone (Bliznakov, 1971) or in combination with chloroquine may also increase the survival of mice infected with *P. berghei*. While there is clearly a balance, certainly dose-dependent, between the harmful and the beneficial effects of immuno-depressant agents, the antimalarial dosage of such drugs as chloroquine lies well to the beneficial side of the balance.

CLINICAL INTERACTION OF DRUGS AND IMMUNITY

From early in this century it was recognised that quinine was more effective against *P. vivax* if the patient was allowed to pass through a few paroxysms of fever before he was treated. These observations were confirmed in detailed studies on neurosyphilitic patients receiving malariotherapy (Yorke, 1925), and recently in mice infected with *P. berghei* that were treated with chloroquine (Golenser *et al.*, 1978). Conversely, quinine was shown to be relatively ineffective in *P. knowlesi*-infected rhesus monkeys that had been splenectomised (Nauck, 1934) and in bursectomised *P. gallinaceum*-infected chickens (Taliaferro, 1948). On the other hand, some Italian workers suggested that acquired immunity influenced in a positive way not the intensity of the response of *P. falciparum* but the speed of its response to quinine therapy. Immunosuppressed animals, on the contrary (e.g. *P. chabaudi*-infected mice), need higher doses of drugs to effect a cure (Lwin *et al.*, 1979).

Some doubt still envelops the question of whether immunity to malaria can be a true sterile immunity, or is of the type known as premunity, where a few surviving parasites serve as a continuing low-grade antigenic stimulus. Sulphanilamide treatment of *P. knowlesi* infection in the rhesus, for example, is said to produce a sterile immunity (Coggeshall, 1938). This may also occur in chloroquine treated *P. berghei*-infected mice (Lapierre, 1954), and certainly does in *P. vinckei*-infected mice. The interdependence of the level of acquired immunity and the effective drug dose was clearly shown in *P. gallinaceum* infections of chicks treated with quinine (Taliaferro and Taliaferro, 1949) or a naphthoquinone (Clarke and Theiler, 1948) . Neither drug was found to kill the exo-erythrocytic tissue forms of this parasite, although the latter compound did inhibit their growth. Coulston and Huff as long ago as 1948 (long before the age of liposomes) made what may prove to be a prophetic statement that—"the indication is strong that a really good suppressive may in fact have prophylactic potentialities. It is not inconceivable, if quinine could be placed strategically in the host cells of the pre-erythrocytic stages, that this drug might act as a causal prophylactic in the same sense as sulphadiazine" (Coulston and Huff, 1948).

Recent work at Lucknow has demonstrated the same dependence of chloroquine on acquired immunity to give a radical cure of *P. knowlesi* infections in rhesus monkeys that we saw earlier with quinine (Dutta and Singh, 1978). In mice

the incomplete suppression of *P. berghei* infection by means of a PABA-deficient milk diet permits the active acquisition of immunity (Jacob and Kretschmar, 1962). Similarly, the treatment with chloroquine of patients infected with a mildly chloroquine-resistant strain of *P. falciparum* may permit them to acquire a high level of immunity but not a radical cure, the net result being that they become asymptomatic carriers of asexual parasites and, more importantly, infective gametocytes (Rieckmann, 1970). There is indeed evidence that the continued exposure of such infections to chloroquine may actually enhance the infectivity of these gametocytes to anopheline vectors (Ramkaran and Peters, 1969).

PRACTICAL IMPLICATIONS OF THE LEVEL OF COMMUNAL IMMUNITY FOR CHEMOTHERAPY IN MALARIA CONTROL

Whether we are talking of expatriates incompletely cured of their infections who have acquired a high level of premunity to *P. falciparum*, or indigenous communities who have both inherited and acquired a high degree of tolerance to their parasites, it is well known that a clinical cure can readily be achieved by quite small doses of antimalarial drugs. Thus the standard recommended adult dose of chloroquine for the presumptive treatment of falciparum or vivax malaria in semi-immune adults recommended by WHO (Bruce-Chwatt *et al.*, 1981) is 600 mg base, with correspondingly less for younger age groups. Following a period of malaria control, and as the level of immunity in the population falls (especially in the youngest age groups), a higher drug dosage is needed to achieve clinical cure. This has been noted, for example, with chloroquine in East Africa (Pringle and Lane, 1966) and pyrimethamine in Pakistan (Afridi and Rahim, 1962). Partly anecdotal evidence suggests that the same phenomenon is being observed in parts of India where some physicians fail to achieve complete suppression of acute vivax malaria with a single adult dose of 600mg chloroquine base. Nor can the old routine of giving 5 days' treatment with primaquine yield as high a proportion of radical cures of *P. vivax* infection as in former times (Sharma *et al.*, 1973). A similar problem has been noted in *P. falciparum* presumptive radical therapy with chloroquine in Karnataka State (Roy *et al.*, 1977). In the renewed campaign that has been launched to control malaria in India this problem has not, I feel, received adequate attention.

Table 1: Chloroquine dosage for Tanzanian children (Pringle and Lane, 1966).

Place	Year	CD^{50}
Muheza	1956	20 mg
"	1960	27 mg
Gombero	1965-66	47 mg
"	"	48 mg

(CD^{50} = dose in mg/kg body weight required to clear parasitaemia in 50% of children).

Today we hear much of the possible role of vaccination in bringing malaria under control, but fortunately the wisdom and experience of the surviving fathers of malariology in your country will not permit them to be misled into putting all their eggs in the vaccination basket (Ray, 1977). A total, multipronged attack must be made on the problem, taking into account control of the vectors of the parasites and of the hosts.

Before any vaccine, if and when it becomes available a decade or two hence, is applied to protect the human population, it will be absolutely vital to give much forethought to the integrated role of antimalarial drugs plus the artificial vaccination procedures before launching into the campaign. Perhaps some of the ideas proposed in this paper will help guide our thoughts in the right direction.

SUMMARY

Natural passive immunity to malaria has long been known, but the molecular factors involved are only just being recognised. Within a receptive

host, immunity is actively acquired through a complex interplay of humoral and cellular factors, and an unfortunate combination of these can lead to various immunopathological changes in the host.

Antimalarial drugs in current use interfere with the metabolism of the parasites. Some such as proguanil and pyrimethamine block folate metabolism, resulting in the arrest of maturation of the intra-erythrocytic schizonts. Chloroquine and mepacrine interfere in a manner as yet undefined with the parasites' ability to digest host-cell haemoglobin. Quinine and the new quinine analogue, mefloquine, also interfere with haemoglobin digestion, but by a different mechanism. Primaquine blocks mitochondrial function in all the developmental stages of the parasites. None of these drugs actually kills the parasites at the sort of concentration that they reach in human blood and tissues. It is the interaction of the host's immune processes with the drugs that actually removes the parasites from the circulation and destroys them. In the process antigens are formed which, in turn, serve as the stimulants to enhance host immunity still further. Some of the antimalarials in high doses may actually have an immunosuppressive action.

It can readily be shown than an immunodeprived animal is not cured by antimalarial drugs, or at least needs abnormally high doses to recover. Similarly, a human population such as the younger generation of Indians, growing up in a relatively malaria-free environment, may now require unusually high doses of drugs to cure them when they do become infected with malaria in later life, as during the current epidemic.

Any future strategy for the control of malaria must take into account the need to exploit the possibility of boosting the active acquisition of immunity, for example by the use of vaccines, by the judicious use of appropriate drugs.

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Influence of Dietary Protein Restriction on *Plasmodium berghei* Infection in Rats

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Outcome of malaria infection and immunological response towards *Plasmodium berghei* antigen was studied in protein malnourished (8% protein isocaloric diet fed) or protein calorie malnourished (8% protein low caloric diet fed) rats. Control rats were either given ad libitum diet (18% protein) or were pair fed. None of the animals from protein calorie malnourished group developed patent parasitaemia. The percentage parasitaemia in protein malnourished group animals was relatively lower as compared to pair fed controls. The humoral as well as cellular immune response was found to be altered in the animals fed on protein or protein calorie deficient diets. T & B cells counts decline in animals fed on either 8 percent or 18 percent protein isocaloric after *P. berghei* infection, however, no change in B cells was observed in animals fed on 8 percent protein low calorie diet. We thus feel that the absence or lower parasitaemia in 8 percent protein fed animals was not due to altered immune status of host but due to lack of certain essential nutrients required for growth and metabolism of parasite.

INTRODUCTION

In recent years, there has been an upsurge of interest in studying the effect of various dietary insufficiencies on immune responsiveness of host. It has become increasingly evident that nutritional state of the host plays an important role in its susceptibility to many infections including parasitic infections. The exacerbation of these infections in malnourished host has been related to the depressed state of immunity. However, in malaria infections, contradictory reports are available. Though malaria infection has been observed to be severe in immunodepressed rats (Brown *et al.*, 1968; Stechschulte, 1969 and Spira *et al.*, 1970) lesser parasitaemia and lower or delayed mortality has also been observed in immunodepressed mice and hamsters infected with *Plasmodium berghei* (Sheagren and Monaco, 1969; Vinayak *et al.*, 1981 and Wright, 1968). In clinical practice cerebral malaria has been found to be less prevalent in malnourished children than well nourished ones (Edington, 1967 and Hendrickse *et al.*, 1971). Some workers tried to elucidate the effect of dietary components on outcome of malaria (Ramakrishanan, 1954; Rafaat and Bray, 1953 and Platt *et al.*, 1960). They observed that milk diet or low protein diets reduced malaria infection. Recently, Edirisinghe *et al.* (1981) demonstrated that outcome of malaria infection is directly proportional to the protein in diet. In their experiments the low protein diets were started on the day the animals were inoculated with *P. berghei* infection. The present investigations have thus been carried out to ascertain (a) the effect of chronic dietary protein insufficiency or chronic protein calorie insufficiency on the outcome of malaria infection and (b) the effect of protein or protein calorie deficient diets on the immune response of the host to *P. berghei* infection.

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MATERIAL AND METHODS

Animals and Parasite: Wistar strain of albino rats of 4-6 weeks age were employed for the present study. The animals were housed in separate cages and were pair fed where necessary. They were given a known amount of diet and water ad libitum. The animals were infected with 1×10^7 parasitized red blood cells (PRBC). The percentage parasitaemia was checked daily in Giemsa stained tail blood films.

Diets: Two types of diets containing 18 percent protein (casein) or 8 percent protein were prepared. Protein in diet was supplemented with starch to make it isocaloric. Animals were fed on 18 percent protein or 8 percent protein isocaloric or 8 percent protein low caloric diet. Diet formulae were as below:

	18% protein	*8% protein*
Casein	180 gm	80 gm
Starch	569 gm	670 gm
Salt mix.	40 gm	40 gm
Vitamin mix.	10 gm	10 gm
Cotton seed oil	80 gm	80 gm
Sugar	100 gm	100 gm
Cod liver oil	20 units	20 units

Vit. mix. Thiamine 100 mg, riboflavin 200 mg, pyridoxine HCl 200 µg, Ca Pentothenate 800 mg, niacin 800 ng, folic acid 40 mg, biotin 4 mg, Vit. B-12 0.4 mg, Vit. K 100 mg, Vit. E (50%) 2.0 g, Inositol 5.0 g, PABA 2.0 g made upto 200 g with starch and used in the diet at 1.0% level.

Maximum consumption of diet in the rats (4-6 weeks old) was 10 gms/rat/day. Thus, the animals were initially kept on 10 gms diet/rat/day/in isocaloric groups. Thereafter the diet left over was reduced in gms from the diet the following day. Animals fed on low caloric diet were given 5 gm of low protein or adequate protein diet/rat/day. Six weeks after their maintenance on a particular diet, animals were infected. The same diets were continued till the end of experiments.

Groups of animals: The animals were divided into various groups according to the type of the diet they received.

Group I (control) (18% protein diet ad libitum): The animals (n = 40) of this group were fed on diet containing 18 percent casein and water ad libitum for six weeks prior to infection. The same diet continued after the infection till the end of the experiment.

Group II (8% protein isocaloric): Animals (n = 25) of this group were fed on diet containing 8 percent casein. Each animal received 10 gms of diet at the start of the experiment. The left over diet was weighed daily and reduced in gms. from the diet the following day.

Group III (Pair fed): The animals (n = 25) group were pair fed with the animals of group II, with the only difference that the diet provided to these animals contained 18 percent casein.

Group IV (Protein calorie malnutrition or PCM): The animals (n = 25) of this group were fed on diet containing 8 percent casein (protein) and the amount of the diet was reduced to half as compared to control i.e. each animal received 5 gm diet/day.

Group V: The animals of this group were fed on 18 percent protein diet and were pair fed with group IV.

Assessment of malnutrition: The status of malnutrition in animals was checked by daily weight gain/loss, change in fur and weekly changes in serum protein and albumin.

Assessment of immune status: The immune responses of the animals were checked before inoculation of parasite and thereafter at weekly intervals post infection. The humoral immune responses were checked by indirect haemagglutination test (IHA) and counter-current-immunoelectrophoresis (CIEP). The cellular immune response was checked by leucocyte migration inhibition (LMI) test. In addition, T and B cells counts were also done.

Indirect haemagglutination test (IHA): The IHA

was performed by the basic techniques of Meuwissen (1974) with a few modifications. The test was performed in 12×75 mm tubes using 2.5 percent suspension of sheep red blood cells. Antigens were prepared from *P. berghei* infected erythrocytes (test antigen) or from uninfected erythrocytes (control antigen). Both of these antigens were prepared by an identical method i.e. freezing and thawing by the technique of Lunde and Powers (1976). The highest dilution *of serum showing 2* + agglutination with specific antigen (antigen prepared from infected erythrocytes) was taken as an end titre. Control antigen did not react with any serum.

Counter-current immune electrophoresis (CIEP): CIEP was performed in 0.75 percent agarose in veronal buffer pH 8.2. Agarose was poured on 70 × 25 mm microscopic slides. A current of 5 m A/slide was applied for one hour thirty minutes. The slides were washed, dried and stained with 0.1 percent amido black.

Lymphoid cell preparation: The spleens of animals were removed, mashed and washed in medium 199. The lymphocytes were collected from these suspensions on lymphoprep (1.07 sp. gr). Lymphocytes were finally suspended in MEM so as to contain 2×10^6 viable cells/ml.

Determination of T cells: T cells were determined by the technique of Mysliwski *et al.* (1977) with slight modification. Briefly spleen cells sensitized with PHA (10ug/ml) were incubated with 0.50 percent SRBC suspension. The suspensions were kept at 4°C overnight. Rossettes were enumerated in a haemocytometer and expressed as percentage of the total cell number.

Determination of complement receptor lymphocytes (CRL's): CRL's were determined by the techniques of Gravely *et al.* (1976). Briefly it consists of incubating the lymphoid cells with 5 percent suspension of SRBC(E) sensitized by rabbit antibody, or Frossman antigen (A) and mouse complement (C). Rossettes were enumerated in haemocytometer and expressed as percentage of the total cell number.

Leucocyte migration inhibition test (LMI): LMI was performed by following the basic techniques of Coleman *et al.* (1976) with a few modifications. Erythrocytes in spleen cell suspensions were lysed with 0.85 percent ammonium chloride. The antigens prepared from infected or uninfected erythrocytes by the technique of Lunde and Powers (1976) were employed.

RESULTS

The weight gain or loss by the animals of groups I, II and III is given in Fig. 1. Animals of all these three groups gained weight, but the weight gain was significantly higher in group I ($P < 0.05$). No significant difference in weight gain was obtained ($P<0.05$) in groups II or III. Animals of group IV had a significant fall ($P < 0.05$) in body weight but animals of group V gained weight (Fig. 2). There was a significant decrease ($P < 0.05$) in serum albumin and protein in 8 percent protein fed animals (group II and IV) but hardly any change in control groups (I, III and V).

The fur of the animals on low protein diet or low protein low caloric diet became soily and loose. Fifty percent of the animals of group IV (PCM) showed loss of fur also.

Parasitaemia: The percentage parasitaemia developed by animals is shown in Fig. 3. The prepatent period in groups I, II, III and V was identical i.e. 2 days. The animals of group I developed a peak parasitaemia of 31±6 percent and of group III 28±5 percent. The peak parasitaemia of group II and V was 18±4 percent and 20±2.3 percent respectively. The parasitaemia levels in animals amongst group II remained at lower levels as compared to animals of groups I, III and V throughout the period of patency. However, parasitaemias in these groups of animals was not significantly ($P>0.05$) different.

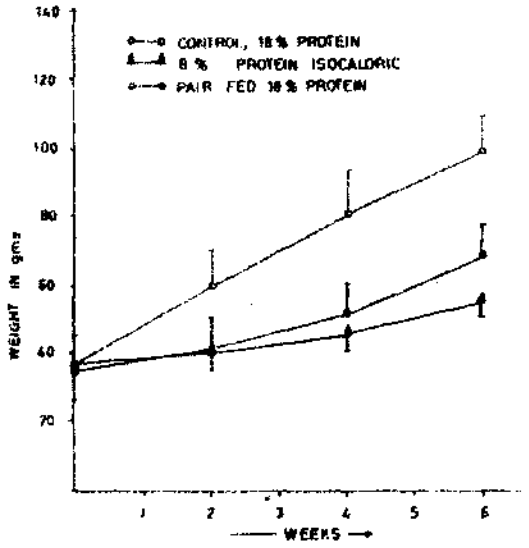

Fig. 1: Change in body weight in animals of group I, II and III.

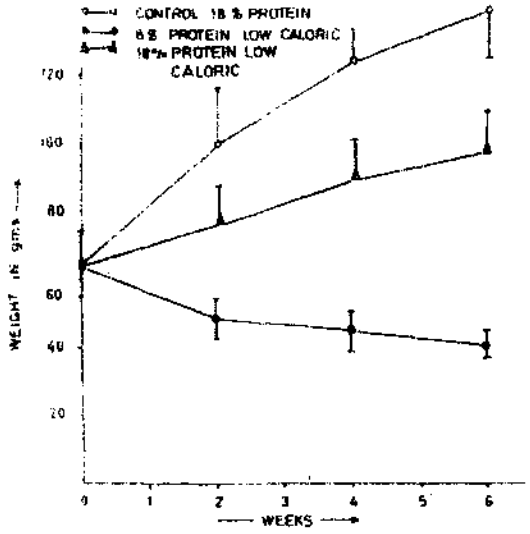

Fig. 2: Change in body weight in animals of group I, IV and V.

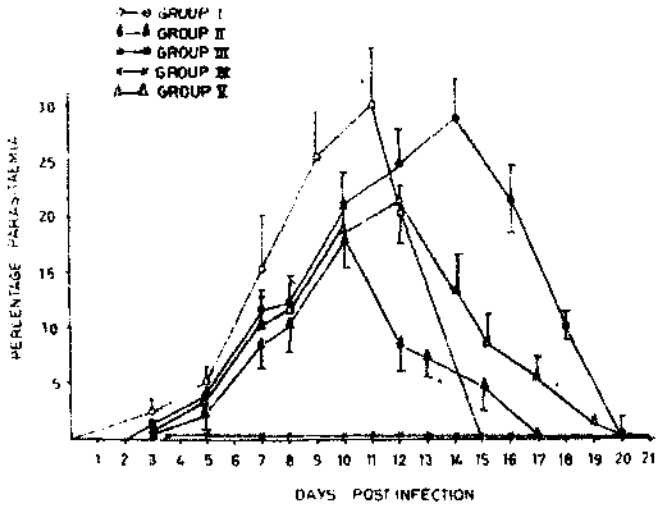

Fig. 3: Percentage parasitaemia in animals of group I to V.

The parasite remained patent for 14 days, 17 days, 21 days and 19 days respectively in groups I, II, III and V. None of the animals of group IV (PCM) showed patent parasitaemia during the course of infection.

Humoral Immune Response: The humoral immune response as demonstrated by IHA and CIEP is given in Table 2. The animals of groups I, II, III and V had increased haemagglutinin titres with the increase in parasitaemia. This relation was maintained upto a certain limit. The decline of parasitaemia had no effect on the antibody levels. Comparing the anti-malaria antibody levels amongst animals of groups I, II, III and V, it was obtained that animals of group II had tendency to have lowest levels of antibodies. Interestingly, none of the animals of group IV (PCM) had haemagglutinin in serum. The precipitins also behaved similarly (Table I).

Cellular Immune Response: The cellular immune status of all the groups as measured by LMI is given in Table 2. No significant difference ($P>0.05$) in percent LMI was observed in group I, III and V. The animals of groups II and IV had significantly lower percent LMI ($P<0.05$) during the course of infection and also after the clearance of infection from blood.

The T and B cell counts of animals of all the groups is shown in Table 2. In the group I the mean T and B cell counts of the uninfected animals were 41±7 and 33±6 respectively. Groups II and IV showed decreased T cell counts prior to infection as compared to group I ($P<0.05$). No significant difference in T or B counts in groups I, III and V was observed prior to inoculation of parasite ($P>0.05$).

The counts of T as well as B cells decreased gradually with increasing parasitaemia and had a tendency to increase after the clearance of parasite in blood. This was observed in all groups but group IV animals, in which none of the animals developed patent parasite.

The T cell counts of group IV infected animals were significantly lower as compared to infected animals of groups I and V during the course of infection. However, these animals had higher B cell counts as compared to groups I and V animals.

DISCUSSION

The course of infection with *P. berghei* had been reported to be altered by age (Zuckerman and Yeolli, 1954) sex and species of animals. It is fatal in mice and younger hamsters and self limiting in rats. However, Kamboj *et al.* (1981) reported that adult hamsters had a tendency to develop chronic malaria infection which was found to be due to age related resistance. The *P. berghei* infection was found to be lethal in young hamsters. The outcome of infection has been related with immune status of host. Thymectomized or ALS treated showed increased rate of infection (Brown *et al.*, 1968; Stechschulte, 1969 and Spira *et al.*, 1970) while depressed state of immunity lowered the mortality rate in mice (Sheagren and Monaco, 1969; Vinayak *et al.*, 1981; Wright, 1968 and Wright *et al.*, 1971). Protein deficiency is known to depress the cell mediated immunity but its effects on B cells have been found to vary (McFarlane and Hamid, 1973; Puri *et al.*, 1980 and Reddy *et al.*, 1977). Frequency of T cells in peripheral blood was much reduced in malnutrition and this reduction paralleled the severity of weight loss, impaired cutaneous DTH and T cell proliferative response to PHA (Chandra, 1974; Smythe *et al.*, 1971). The percentage of B cell peripheral blood in malnutrition is normal or increased (Chandra, 1977).

In our experiments, we found that protein or protein calorie malnutrition results in decrease in T cell counts without effecting B cell counts in uninfected animals. However, following *P.*

Table 1: Humoral response of the animals at weekly intervals.

Group	Geometric mean of IHA titre Day PI				*CIEP*			
	7	14	21	28	7 NP/NS	14 NP/NS	21 NP/NS	28 NP/NS
Group I	180	570	752	542	8/8 (100)	8/8 (100)	6/8 (75)	3/8 (37.5)
Group II	25	111.4	32	16	0/4 (0)	2/5 (40)	2/6 (33.3)	1/4 (25)
Group III	128	721	721	361	3/5 (66)	4/5 (80)	3/4 (75)	2/5 (40)
Group IV	–	–	–	–	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)
Group V	92	448	448	256	3/5 (66)	3/5 (66)	2/4 (50)	1/4 (25)

Figures in parenthesis represent percentage of animals showing positive test.
NP = Number of animals positive NS = Number of animals studied PI = Post infection, measured by indirect haemgglutionation test (IHA) and counter current immunoelectrophoresis (CIEP).

Table 2: Cellular immune response of the animals at weekly intervals

Group	Day PI % LMI				% T cells					% B cells				
	7	14	21	28	0	7	14	21	28	0	7	14	21	28
I	53.5±4.5	70±2	79.5±6.5	59.5±5.5	41±7	21±2	17±1.5	17±2	20±2	33±6	21±2	16.5±4	16.2±5	20±2
II	11±4	16±2	22±5	15±7	32±4	25±3	18±3	22±2	21±2	34±6	27±6	20±3	20±2	22±2
III	53±8	62±4	70±8	49±5	42±8	24±3	16±2	18±2	18±2	33±7	27±4	20±2	20±2	22±2
IV	15±3	15±3	12±2	15±5	24±6	22±3	23±3	21±2	20±2	34±4	28±4	25±4	28±5	30±2
V	46±8	51±7	60±8	41±2	37±5	41±3	40±1	40±2	42±1	35±3	22±4	23±2	32±2	23±3

PI = Post infection, measured by percentage leucocyte migration inhibition (% LMI) % T cell and % B cells.

berghei infection a decrease in T as well as B cell counts was observed in all but group IV. This could be due to the fact that animals of all the groups developed patent parasitaemia, while none of the animals in group IV had patent parasitaemia in blood. We feel that the observed depression in T and B cell counts in infected animals of group IV represented the alterations due to malnutrition only and that subpatent parasite had little effect on T and B cell counts of these animals.

Interestingly, the protein malnourished animals had lower parasitaemia as opposed to their pair fed controls or those fed on ad libitum diet. Such observations have already been made by some workers, Ramakrishnan (1954), Rafaat and Bray (1953), Platt *et al.*, (1960). Recently Edirisinghe *et al.* (1981) observed that *P. berghei* infection was directly proportional to protein content in diet. Our results indicate that besides protein, the protein calories in diet are equally important in expression of parasitaemia in blood. This fact is based on our observations that PCM animals did not develop the patent parasite at all. In a clinical study by Lunn *et al.* (1966) on volunteers who were infected with sporozoite of *P. vivax* followed by chloroquine therapy, anti malaria antibodies could not be demonstrated till the chloroquine therapy continued. The chloriquine therapy inhibited invasion of erythrocyte by the parasite and no patent parasitaemia developed which consequently led to non demonstrability of antibodies. After the chloroquine was stopped, the patent parasitaemia developed which led to demonstrable levels of anti malaria antibodies. Because of non-availability of essential nutrient, essentially PABA for the survival and multiplication of malaria, in the diet given to animals of group IV, the parasite did not develop to patency. This precisely is the reason for non demonstrable levels of antimalarial antibodies in any of these animals.

In our experiments, the protein or protein calorie deficient uninfected animals showed depressed T cell counts but no change in B cell counts. The CMIR as well as the humoral immune response was depressed towards the parasite antigen in animals as compared to the normal diet fed animals. It will not be out of place to mention here that the antiplasmodial antibody levels in protein or protein calorie deficient diet fed animals were low inspite of the normal B cell counts. This could be due to the fact that the antibody response to malaria antigen might be T dependent. Since the T cell counts are decreased in protein or protein calorie malnutrition, hence the T helper cells are also decreased in turn affect T-B cells cooperation to form antibodies.

The low levels of parasitaemia in low protein or low protein calorie diet fed animals in our experiments may be due to protein or protein calorie deficiency itself, or due to the secondary effects produced by the protein or protein calorie deficient diets. In our experiments (unpublished) we found that addition of PABA increased the levels of parasitaemia in rats but could never bring the levels to normalcy. We did not suggest that protein or protein calorie deficiency produces resistance to *Plasmodium berghei* infections. The reduction of parasite may be due to the lack of certain essential nutrients like PABA required for the growth and metabolism of the parasite. Similar observations have been made by Hawking (1954) and Ferone (1977). Reduction of the parasite to subpatent levels or alternatively persistence of the parasite at low levels in the tissues in low protein calorie diet fed animals would lead to strong immunity to parasite on rechallenge.

This could probably explain the findings of Edirisinghe *et al.* (1982) where protein deficient animals could develop immunity to rechallenge. Detailed studies in this regard are currently being carried out in our laboratory.

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Experimental Malarial Infection I. *Plasmodium berghei* Infection in Normal and T-cell Deficient Mice

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The course of lethal infection with *P. berghei* was examined in normal and T-cell deficient swiss albino mice. Markedly increased parasitaemia and mortality were observed in deficient animals compared to normal controls during both primary and secondary infections. These results establish the requirment of T-cells for recovery during primary infection as well as for immunity against a rechallenge infection. It is not clear whether T-cells are required as helper cells for antibody production or for the development of cell-mediated immune response.

INTRODUCTION

The mechanism of immunity against the erythrocytic stages of malarial infection remains a matter of controversy. The weight of evidence supports that the humoral factors are playing a dominent role in protection (Briggs *et al.*, 1966; Brown and Phillips, 1974; Brown *et al.*, 1971; Bruce Chwatt and Gibson, 1955; Coggeshall and Kumm, 1937; Cohen *et al.*, 1961; Diggs abd Osler, 1969; Jayawardena *et al.*, 1978). However, by employing thymectomised animals, it has been shown that cell mediated immune responses may also be required in the development of immunity (Brown *et al.*, 1968; Spira and Silverman, 1969, Spira *et al.*, 1970; Stechschulte, 1969(a); Stechschulte, 1969(b); Weinbaum *et al.*, (1976). In several infections it has been shown that the mechanism of immunity against a rechallenge infection may not be the same as the one that is responsible for recovery from a primary infection (Jayawardena *et al.*, 1979; Rank and Weidanz, 1976; Roberts and Weidenz, 1979). In the present paper the course of primary and secondary experimental malarial infections, in normal and thymectomised mice are compared.

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MATERIAL AND METHODS

Animals: Randomly bred adult swiss albino mice weighing 20-25 g were used in all experiments. In individual experiments, animals of only one sex and of same weight were used.

Haffkine strain of rabbits of either sex were used to raise the antisera against mouse thymocytes. Mice were maintained on laboratory standard diet and rabbits on green vegetables, germinated grams and water *ad libitum*.

Parasite: A strain *of P. berghei* obtained from the National Institute of Communicable diseases, India, was used in all experiments. It was maintained routinely by intraperitoneal inoculation of 1.5×10^8 infected RBCs into adult mice.

Infection of the Animals: Intravenous inoculation of 10^5 *P. berghei* infected RBCs in 0.2 ml into adult mice usually produced a lethal infection lasting for about 3-4 weeks with a peak parasitaemia (average about 40%) just before mortality. In order to study the course of secondary infection, mice were cured of their primary infection at 10-15% parasitaemia with chloroquine, 320 mg/l in drinking water for 7 days.

Mortality, Parasitaemia and Packed Cell Volume (PCV): The course of infection was followed by studying mortality, parasitaemia and PCV.

All experimental mice were observed twice daily for mortality. Parasitaemia was determined twice a week by Leishman stained thin smears prepared from tail blood. Degree of parasitaemia was recorded by counting the number of infected RBCs out of 10,000 RBCs. Animals were considered negative if no parasite could be detected in thin smears after examining at least 40 oil immersion fields.

Tail blood was allowed to run into heparinised micro-haematocrit capillaries which were then sealed and spun at 15,250 g or 5 min on a micro-haematocrit centrifuge and PCV was measured.

Preparation of T-cell Deficient Mice: Thymectomised mice were used as a model of T-cell deficiency. Thymectomy was performed at the age of 3-4 weeks by suction under light anaesthesia (Miller, 1960). Sham operated mice were used as controls.

Anti-thymocyte serum (ATS) was prepared in rabbits as described elsewhere (Levy and Medawar, 1966). Pooled ATS was precipitated with ammonium sulphate and the anti-thymocyte globulin (ATG) was stored at −70°C until used.

Ten days after thymectomy, mice received 15 daily injections of 0.25 ml ATG and thereafter two injections a week till the end of the experiment.

Characterization of T-cell deficiency: Cytotoxicity of ATG was assessed by trypan blue exclusion technique (James, 1973) and the titre of ATG was 1:512. Thymectomised and ATG treated mice were tested for T-cell deficiency by a) skin allograft rejection pattern (Billingham and Medawar, 1951; Theones, 1969), b) immunofluorescent staining for B-cells (Holborow, 1970) using the IgG fraction of fluorescein isothyocyanate (FITC) conjugated rabbit antimouse gammaglobulin antibody (Cappel Laboratories, USA) at a dilution of 1:20 and c) histological examination of lymphnode and spleen sections.

Statistical analysis: Each experiment was repeated atleast two times. Values shown are the mean ± standard deviation/error of the total number of animals of a representative experiment. The significance of the differences was calculated by following unpaired students T test

RESULTS

Establishment of T-cell deficient mice: Anti-thymocyte globulin (ATG) was employed following thymectomy as an agent to prepare T-cell deficient mice. The skin allograft in deficient mice survived for 71 ± 9.8 days, whereas it survived only for 11 ±0.66 days in normal mice. The spleen of deficient mice had a higher proportion (90 ± 2.42%) of surface Ig positive cells (B-cells) while in normal spleen the number was 48.5 ± 0.85% Histological examination showed a marked depletion of lymphocytes from T-dependent area in both lymphnode and spleen in thymectomised—ATG treated mice, however, normal cellularity was retained in B-dependent areas. In control animals, both T-and B-dependent areas were intact. These observations suggest that the ATG treated mice were deficient in T-cells and its function.

Table 1. PCV following infection with *P. berghei*

Days after infection	Mean PCV ± SD	
	Normal mice	T-cell deficient mice
0	56.87 ± 4.28 (10)	51.32 ± 1.70 (10)
7	40.43 ± 4.95 (9)	32.91 ± 9.10 (7)
14	28.20 ± 8.93 (7)	20.65 ± 7.6 (4)
21	14.37 ± 1.79 (1)	

Figures in parentheses indicate the number of animals tested.

Course of primary infection: Groups of normal and T-cell deficient mice received intravenously 10^5 *P. berghei* infected RBC and the PCV was measured at weekly intervals (Table 1). Both the groups of animals developed progressive anaemia. However, deficient mice developed anaemia faster than normal mice. Table 2 depicts the mortality figures in these groups of mice. *P. berghei* induced an uniform lethal infection in both groups, but deficient mice died much earlier than normal mice. All through the course of infection, the deficient mice maintained a higher level of parasitaemia in comparison to normal animals (Table 3).

Course of secondary infection: Since the primary infection was fatal, it was necessary to cure it before a second infection. The primary infection in normal and T-cell deficient mice was cured with chloroquine at about 10-15% parasitaemia. Ten days after chloroquine treatment the animals were rechallenged with 10^5 infected RBC. Primary infection induced some immunity against a secondary infection in normal mice. The degree of immunity, however, varied from animal to animal. The mortality data during secondary infection is compared with that of primary infection in Table 4. After primary infection both normal and T-cell deficient mice,

Table 2. Mortality following infection with *P. berghei*

	Days after infection	
	Normal mice (10)	T-cell deficient mice (10)
Mean mortality rate	18.4 ± 6.2	12.4 ± 5.6
50% mortality	19	13
100% mortality	25	20

Figures in parentheses indicate the number of animals tested

Table 3. Parasitaemia following infection with *P. berghei*.

Days after infection	Mean Parasitaemia 10^4 RBC		P value
	T-Cell deficient mice	Normal mice[a]	
4	504.44 ± 130.91 (9)[b]	79.8 ± 32.88 (10)	< 0.001
8	691.85 ± 190.20 (7)	331.1 ± 66.6 (9)	< 0.001
12	1824.42 ± 496.40 (7)	877.75 ± 116.02 (8)	< 0.001
16	2615.00 ± 916.19 (3)	1441.43 ± 204.55 (6)	< 0.02
20	2617.00 (1)	2346.25 ± 221.10 (4)	NS[c]
21	NT[d]	3263.00 ± 206.56 (4)	

a Groups of normal and T-cell deficient mice received i.v. 10^5 *P. berghei* infected RBC.
b Parentheses indicate the number of animals.
c Mean differences are not significant.
d Animals were not available for testing.

if left uncured, died within four weeks. Following secondary infection, almost 1/3rd of normal mice survived more than four weeks. By contrast, the T-cell deficient mice showed 100% mortality within 4 weeks after secondary infection. However, even in normal mice there was no absolute immunity, because all the animals, during secondary infection, finally died in about 90 days.

The parasitaemia during secondary infection in normal animals was highly variable, hence the data cannot be presented as mean. Therefore, for the sake of simplicity the animals were divided into three groups:

1. Animals that died within 2 weeks
2. Animals that died between 2—4 weeks
3. Animals that survived more than 4 weeks

Table 4. Mortality following primary and secondary infections with 10^5 *P. berghei* infected RBC

	Normal mice		T-cell deficient mice	
	Primary infection (10)	Secondary infection (63)	Primary infection (10)	Secondary infection (10)
Died within 2 weeks	4*	29	7	6
Died between 2–4 weeks	6	14	3	4
Survived more than 4 weeks		20	...	

Figures in parentheses indicate total number of animals tested.
*Number of animals dead.

The Parasitaemia in each mouse at different periods following rechallenge with 10^5 infected RBC is shown in Figures 1, 2 and 3 respectively. Mice that died within 2 weeks had least evidence of immunity (Fig. 1). The parasitaemia, even though highly variable, mostly clustered around the mean of parasitaemia during primary infection as shown by horizontal bars in the figures. Animals that died between 2-4 weeks (Fig. 2) and that survived more than 4 weeks (Fig. 3) had a definite evidence of immunity. The parasitaemia in all these mice, though variable, was less than mean parasitaemia during primary infection. The above data show clear difference in parasitaemia between primary and secondary infection in normal mice.

By contrast, the T-cell deficient mice showed uniform progressive parasitaemia after secondary infection (Fig. 4). The parasitaemia in these animals was less variable. There was no difference after primary and secondary infection.

DISCUSSION

There has been a greater emphasis on humoral mechanisms of immunity in malaria (Briggs *et al.*, 1966; Brown and Phillips, 1974; Cohen *et al.*, 1961). However, investigations into the role played by cellular components have not been completely neglected. Thus thymectomised animals, anti-lymphocyte serum (ALS) treated animals, nude mice, thymectomised and X-irradiated animals have all been shown to suffer from a markedly exacerbated infection following challenge with different strains of plasmodia that infect rodents (Jayawardena *et al.*, 1978; Weinbaum *et al.*, 1976; Clark and Allison, 1974; Jayawardena *et al.*, 1977).

In the present study also, the primary infection with *P. berghei* in thymectomised mice was found to be more serious than in the normal animals, as evidenced by increased mortality, higher parasitaemia and more pronounced anaemia. These observations agree with the earlier reports (Brown *et al.*, 1968; Stechschulte, 1969a).

Since mechanism of immunity during primary and secondary infections may be different (Jayawardena *et al.*, 1979; Rank and Weidanz, 1976; Roberts and Weidanz, 1979), it was considered worthwhile to investigate how the thymectomised animals would behave towards a rechallenge infection. In order to study this, it was necessary to cure the primary infection, since the strain of *P. berghei* used in experiments produced a lethal infection.

Mice that were allowed to recover from blood induced *P. berghei* infections by drug treatment have been shown to develop resistance to rechallenge infection (Cox, 1957; 58; 59; 62; 64; Poels *et al.*, 1977; 1977a). The degree of resistance, however, depended upon the duration for which parasitaemia was allowed to continue during the primary infection. The longer the period, the greater was the degree of immunity developed. In the present study, therefore, it was decided to cure the animals of the primary infection at about 10-15% parasitaemia. Interrupting the infection earlier than this may interfere with induction of sufficient immunity and any further delay may lead to high mortality.

The normal animals showed a very definite evidence of immunity against reinfection. The degree of immunity, however, varied markedly from animal to animal. Some animals died early while others survived much longer than noticed during primary infection. The animals that died during first two weeks had but little immunity, as their parasitaemia was mostly clustered around the mean parasitaemia during primary infection. The animals that survived longer had less parasitaemia, than that during primary infection. Still, it is worth noting that there was no absolute immunity as all the animals died after prolonged survival in about 90 days. The above observations are consistant with similar studies carried out earlier (Cox, 1957; 58; 59; 62; 64; Poels *et al.*,

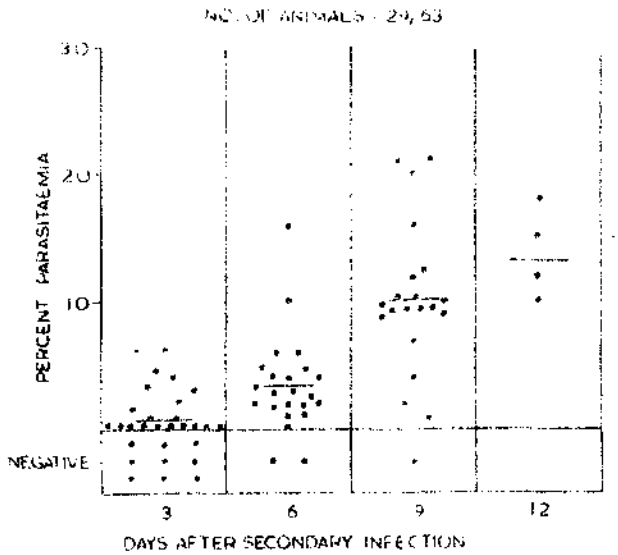

Fig. 1: Course of secondary infection in normal mice following i.v. infection with 10^5 *P. berghei* infected RBC. (Animals that died in two weeks) Horizontal bars indicate the mean parasitaemia of primary infection.

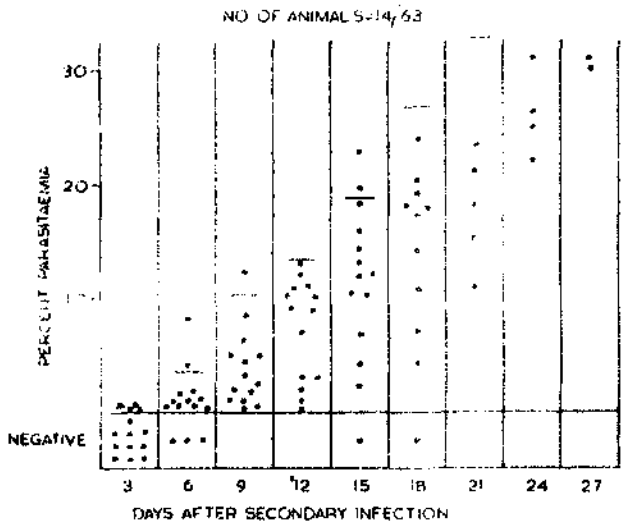

Fig. 2: Course of secondary infection in normal mice following i.v. infection with 10^5 P. berghei infected RBC. (Animals that died between two-four weeks). Horizontal bars indicate the mean parasitaemia of primary infection.

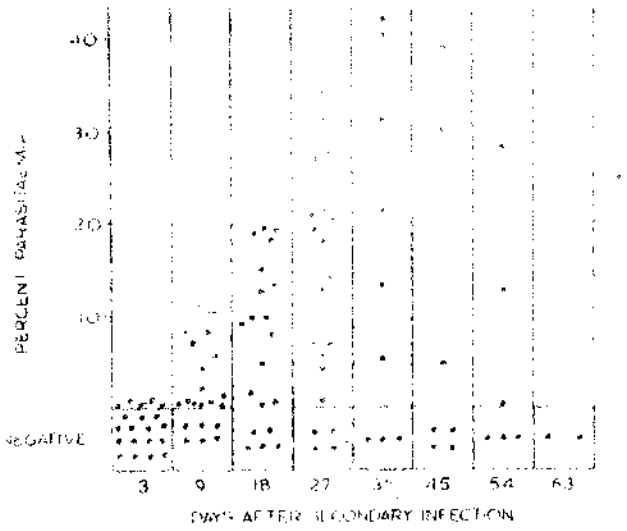

Fig. 3. Course of secondary infection in normal mice following i.v. infection with 10^5 *P. berghei* infected RBC (Animals that survived more than four weeks). Horizontal bars indicate the mean parasitaemia of primary infection.

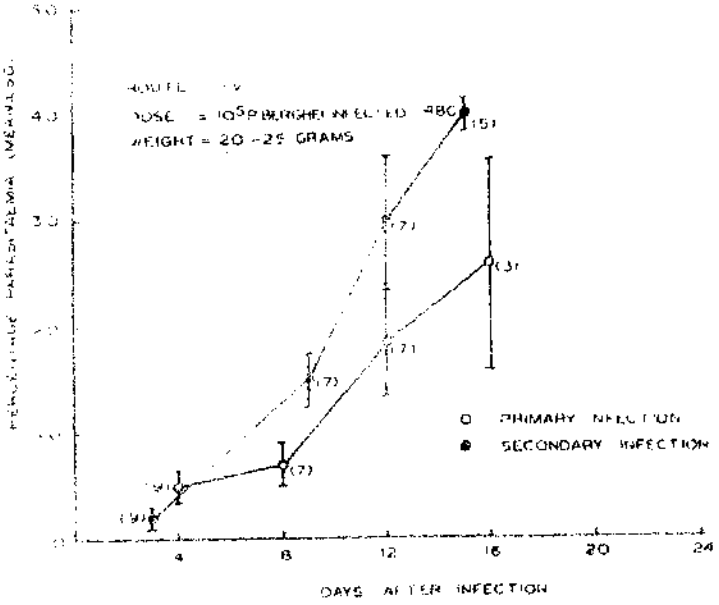

Fig. 4. Course of secondary infection in T-cell deficient mice following i.v. infection with 10^5 *P. berghei* infected RBC.

1977; 1977a; Box and Gingrich, 1958; Lapierre, 1954).

In contrast to the normal control animals, the course of secondary infection with *P. berghei* in T-cell deficient animals was in no way different from the primary infection.

It is concluded that T-cells are required for the development of immunity during primary infection as well as during reinfection. Conflicting evidence is available concerning the effects of thymectomy on the antibody response in malarial infection. There is some evidence that the production of specific class of immunoglobulin is selectively inhibited (Jayawardena *et al.*, 1979; Jayawardena *et al.*, 1977) which has been refuted by others (Stechschulte, 1969a). In view of these findings, the importance of thymus dependent antibody production is uncertain. Therefore it cannot be commented whether T-cell requirement is for the production of thymus dependent antibodies or for the induction of cell mediated immunity. Further experiments are necessary before resolving this issue.

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Isolation of *Plasmodium berghei* by Density Gradient Centrifugation on Histopaque

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Treatment of *Plasmodium berghei* infected erythrocytes with hemolysin resulted in the release of intracellular parasites. The parasites were isolated from this lysate by density gradient centrifugation on Histopaque. The method was found useful for isolating a comparatively pure preparation of *P. berghei* antigen. Subsequent testing of antigen preparation did not appear to show any contamination from host erythrocytes.

INTRODUCTION

The removal of leucocytes from parasitized blood is an important procedure for isolating *Plasmodium berghei* in pure form. McEwen *et al.* (1971) used centrifugal elutriation for the removal of leucocytes. Filtration of blood through a column of Sephadex G-25 and SE-cellulose (Nakao *et al.*, 1973) has also been used for the same purpose. For the release of parasites from infected erythrocytes, saponin was used by Christophers and Fulton (1939), while Martin *et al.* (1971) used ammonium chloride. Heidrich *et al.* (1979) have described the usefulness of free flow electrophoresis for separating parasites from host erythrocyte fragments. Similarly, differential and density gradient centrifugations have been used for purifying parasite preparations by Prior and Kreier (1972a) and Eisen (1977).

This communication describes the procedure for removing leucocytes from parasitized blood by passing it through a column of α-cellulose and microcrystalline cellulose (Beutler *et al.*, 1976). The parasite were released by treating infected red blood cells with hemolysin. The parasite preparation was purified by means of density gradient centrifugation on Histopaque.

MATERIAL AND METHODS

A group of 80 mice (Hissar strain, India) was infected with *P. berghei* (NK-65 strain). Each animal was inoculated intraperitoneally with an aliquot of blood containing 2 x 10^6 parasitized erythrocytes. Blood with 60-80% parasites was directly obtained from the heart in a tube containing Acid Citrate Dextrose (ACD) anticoagulant.

Equal amounts of α-cellulose (Sigma) and microcrystalline cellulose (Sigma) were suspended in excess of 0.154 M sodium chloride solution and left overnight at room temperature. This suspension was packed upto a height of 2 cm in a plastic syringe. The parasitized blood in ACD was applied to the column and the leucocyte free blood eluted with normal saline at a flow rate of 15 ml/hour.

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The RBC suspension obtained after removal of leucocytes was lysed by the addition of pyocyanin free, heat stable hemolysin (titre 1 : 800, pH 7.0) isolated from strain I of *Pseudomonas aeruginosa* (Liu, 1957). This lysate was washed three times and suspended in 0.154 M NaCl, 0.01 M sodium phosphate, pH 7.2 (PBS). The above suspension was layered on equal volume of Histopaque (Sigma) and centrifuged at 400 g for 30 minutes. The brown layer formed at the interface was withdrawn (Fig. 1). The parasites were disrupted by ultrasonication at 9 Kc for 8 mts.

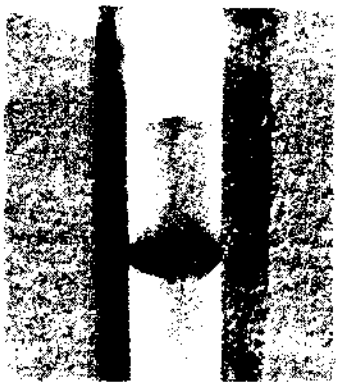

Fig 1. Tube after density gradient centrifugation showing parasite band at the interface.

The purity of the above preparation was checked by allowing it to react against normal mouse red blood cell antiserum. The antiserum was raised by injecting rabbits, intravenously, with 0.2 ml of 1% normal mouse RBC suspension on alternate days for 3 weeks. Mouse RBC antiserum and the parasite preparation were used in the immunodiffusion and counterimmunoelectrophoresis tests for checking the RBC contaminants.

RESULTS

We were able to remove about 99.9% leucocytes and 85% platelets on passing the parasitized blood through α-cellulose microcrystalline cellulose column. The treatment of infected RBC with hemolysin resulted in the recovery of intact parasites (Fig. 2). The material recovered from the interface layer, formed after density gradient centrifugation on Histopaque, did not appear to contain any mouse RBC contaminants. This was confirmed immunologically by using the above material against normal mouse RBC antiserum in immunodiffusion and counter-immunoelectrophoresis tests.

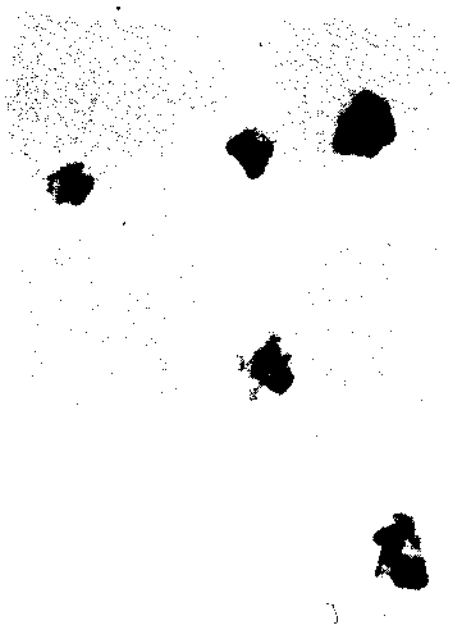

Fig 2. Photomicrograph showing parasites obtained from the interface after density gradient centrifugation on Histopaque. Original magnification 2,000 X.

DISCUSSION

The removal of leucocytes by α-cellulose-microcrystalline cellulose column gave us comparatively better results as compared to the various procedures described earlier. The treatment of parasitized erythrocytes with hemolysin was successfully employed for liberating the parasites in an intact and free form. Heidrich *et al.* (1979) have opined that most commonly encountered problem in isolating the parasites from

infected RBC is that of removing red cell ghost contaminants. Another important host cell contaminant is the red blood cell material ingested by the growing parasites. Diggs (1966) has pointed out that such material is usually found localized within the phagocytic vacuoles of the parasite. It has always been a formidable task to remove the contaminants from such parasite preparations. The procedure adopted by the present investigators seems to have a definite advantage over many earlier attempts, as no contaminating bands were immunologically detectable. The antigen extract which we obtained appeared to be considerably free from some of its inherent contaminants. The use of Histopaque density gradient yields a comparatively more refined product than those described earlier by Prior and Kreier (1972a), Eisen (1977) and Heidrich *et al.* (1979).

In the antigen obtained by us, no host cell contaminants were detectable within the sensitivity limits of the tests employed for this purpose. The isolated antigen was double checked by reacting it against host red cell antiserum. No immune reaction was obtainable either in immunodiffusion or counterimmunoelectrophoresis runs. Further, the parasite antigen prepared as above gave correspondingly better results in various biochemical immunological studies in our laboratory.

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Mosquitoes of Nainital Terai (U.P.)

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Twenty one mosquito species belonging to 8 genera viz. *Anopheles, Aedeomyia, Aedes, Armigeres, Coquillettidia, Culex, Mansonia* and *Mimomyia* were collected from the six tehsils of U.P. terai (Nainital) during mosquito fauna survey carried out in Sept. 1980, May-June 1981, Sept.-Oct. 1981 and Jan.-Feb. 1982. Three species of genus Anopheles i.e. *A. aitkenii, A. lindesayi* and *A. kochi* were collected for the first time from this belt. *A. karwari* and *A. minimus* previously reported from this belt were absent. The seasonal prevalnce of some important anophelines was studied.

INTRODUCTION

Information on the mosquito fauna of terai belt of Uttar Pradesh is mainly based on studies carried out in connection with the epidemiology and control of malaria. Robertson (1909), Cameron (1921) and Phillips (1924) recorded a few anophelines. In subsequent studies Clyde (1931), Srivastava (1950), Srivastava and Diwan (1951), Issaris *et al.* (1953) and Rahaman *et al.* (1956) recorded the existance of about 10-17 anopheline species. After a lapse of about a decade Wattal *et al.* (1967) recorded 4 anopheline species 6 species of *Culex* and 1 of *Aedes*. For about last two decades no further observations were made on the mosquito fauna of this region.

In order to update the information on the mosquito fauna of this region studies were undertaken during 1980-82 in six tehsils of U.P. terai. Results of these studies are briefly summarised in this paper.

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MATERIAL AND METHODS

Area: Nainital district of U.P. has an area of 6792 sq. km., of which 1243 sq. km. has been classified as hilly, 3214 sq. km. as forest, 298 sq. km. Bhabhar, 45 sq. km urban area and 1997 sq. km. as terai region. Terai region streches from Sarda river on the East to Kashipur in the West, lying between 28°53″ and 29°26″ North latitude and between 78°53″ and 80° East longitude. The belt comprises six tehsils viz. Kashipur, Bazpur, Gadarpur, Kichha, Sitarganj and Khatima. Paddy is one of the major crops and covers, 1,12,175 hac. in the Nainital district, of which terai region has 94,581 hac. (83.3%). The area is heavily irrigated and has 1,573 km. length of canals out of which 1,136 km. (72.21%) run in the terai region.

Intensive and extensive mosquito fauna surveys were done in Sept. 1980, May-June 1981, Sept.-Oct. 1981 and Jan.-Feb. 1982 in six tehsils viz., Kashipur, Bazpur, Gadarpur, Kichha, Sitarganj and Khatima. In May-June 1981, Sept.-Oct 1981 and Jan.-Feb. 1982, 74 villages were surveyed. While in Sept. 1980 only 38 villages were surveyed (Fig. 1).

Fig. 1: Map showing the collection sites in U.P. Terai.

Adult mosquitoes were collected with the help of suction tube from different resting places viz. cattle sheds, human dwellings, mixed dwellings paddy crops, forests, tree crevices and other man made structures. Mosquitoes were also collected by pyrethrum-space-spray. Immatures were collected from ponds, canals, pools, streams, pits, wells, tree crevices etc. and kept in field laboratory until adult emergence. All freshly emerged mosquitoes were killed with ether and packed in cellophene papers. All collections were brought to the MRC Delhi laboratory for further studies and preservation. Mosquitoes were identified with the key of Christophers (1933), Barraud (1934) and catalog of Knight and Stone (1977).

RESULTS AND DISCUSSION

A total of 20,463 mosquitoes collected in four surveys of U.P. terai were identified into 29 species of 8 genera viz., *Anopheles, Aedeomyia, Aedes, Armigeres, Coquillettidia, Culex, Mansonia* and *Mimomyia*. The genus *Anopheles* comprised of 18 species (1288 specimens), genus *Aedeomyia* 1 species (1 specimen), genus *Aedes* 2 species (31 specimens), genus *Armigeres* 1 species (25 specimens), genus *Coquillettidia* 1 species (16 specimens), genus *Mansonia* (72 specimens) and genus *Mimomyia* (1 specimen). Total number of mosquitoes collected from six tehsils of terai, and the number of genera and species encountered in each Tehsil are given in Tables 1 and 2 respectively. Tehsilwise average collection of each species is also given in Table 2. Surveys revealed that *A. subpictus* dominated the anopheline collections during September 1980 and September-October 1981 accounting for 29.6% and 53.3% respectively. This was followed by *A. culicifacies* 24.96% and 21.03% and *A. fluviatilis* 19.66% and 8.02% respectively. However, during May-June 1981 survey *A. culicifacies* dominated (42.5%) followed by *A. subpictus* (30.7%) and *A. annularis* (17.0%). During the Jan.-Feb. 1982 survey, *A. fluviatilis* was the most prevalent species (53.62%) followed by *A. splendidus* (25.06%) and *A. culicifacies* (8.09%). *Culex quinquefaciatus* was the most prevalent species (87.25%) among the culicinae collections in all the 4 surveys. This was followed by *Cx. tritaeniorhynchus* (11.38%) and *Cx. vishnui* (1.16%).

Two species viz., *A. karwari* and *A. minimus*, recorded in the earlier surveys by Issaris *et al.* (1953) were not found in this survey. *A. aitkenii, A. lindesayi* and *A. kochi* are further additions to the fauna of this region. The seasonal prevalence of some important anophelines are given in Table 3. It was revealed that *A. culicifacies* and *A. fluviatilis* were distributed throughout the terai belt. While *A. culicifacies* exhibited two seasonal peaks, i.e. in May-June and Sept.-Oct., there was only one peak of *A. fluviatilis* during Jan.-Feb. contrary to the findings of Issaris *et al.* (1953) who had reported one seasonal peak of *A. culicifacies* (October-November) and two peaks of *A. fluviatilis* (October-November and March-May). These changes may have occurred due to the development of an intensive irrigation net-

Table 1. Occurrence of Mosquito Genera and their Species in Terai

Sl. No.	Mosquito genera Collected	No. of Species Collected					
		Kashipur	Bazpur	Gadarpur	Kichha	Sitarganj	Khatima
1.	*Anopheles*	10	11	9	11	12	17
2.	*Aedeomyia*	1	0	0	0	0	0
3.	*Aedes*	2	1	1	0	0	0
4.	*Armigeres*	1	0	0	1	1	1
5.	*Coquillettidia*	1	1	0	0	0	0
6.	*Culex*	4	2	3	3	3	3
7.	*Mansonia*	1	1	0	1	0	0
8.	*Mimomyia*	0	0	0	0	1	0

Table 2. Mosquitoes Collected from Terai

S.No.	Species Collected	Numbers collected from each tehsil							
		Kashipur	Bazpur	Gadarpur	Kichha	Sitarganj	Khatima	Average	Collection
1.	*Anopheles aconitus* Donitz, 1902	54	32	5	3	7	104	34.16 ±	39.50
2.*	*A. aitkenii* James, 1903	0	0	0	0	0	1	0.16 ±	0.40
3.	*A. annularis* Vander Wulp, 1884	197	53	259	589	60	207	227.50 ±	195.70
4.	*A. barbirostris* Vander Wulp, 1884	12	3	5	8	4	25	9.50 ±	8.26
5.	*A. culicifacies* Giles, 1901	494	178	656	1497	305	252	563.60 ±	489.30
6.	*A. fluviatilis* James, 1902	752	271	572	118	11	463	364.30 ±	281.90
7.	*A. gigas* Giles, 1901	0	1	0	0	0	0	0.16 ±	0.40
8.*	*A. kochi* Donitz, 1901	0	0	0	0	0	12	2.00 ±	4.89
9.*	*A. lindesayi* Giles, 1900	0	0	0	0	0	3	0.50 ±	1.22
10.	*A. maculatus* Theobald, 1901	0	1	0	3	1	49	9.00 ±	19.60
11.	*A. nigerrimus* Giles, 1900	267	25	1	17	42	14	61.00 ±	101.82
12.	*A. pallidus Theobald* 1901	0	0	0	0	1	1	0.33 ±	0.51
13.	*A. splendidus* Koidzumi, 1920	242	133	139	51	98	401	177.30 ±	126.40
14.	*A. stephensi* Liston, 1901	0	0	0	0	4	3	1.10 ±	1.80
15.	*A. subpictus* Grassi, 1899	1546	240	446	633	126	736	621.10 ±	507.80
16.	*A. umbrosus* (Theobald), 1903	0	0	0	0	0	1	0.16 ±	0.40
17.	*A. vagus* Donitz, 1902	4	0	0	4	0	6	2.33 ±	2.65
18.	*A. varuna* Iyengar, 1924	42	11	51	1	15	20	21.66 ±	20.85
19.	*Aedeomyia venustripes* (Skuse), 1889	1	0	0	0	0	0	0.33 ±	0.51
20.	*Aedes scatophagoides* Theobald, 1901	14	1	0	6	6	0	2.50 ±	5.60
21.	*Ae. vittatus* (Bigot), 1861	15	0	1	0	0	0	2.66 ±	6.05
22.	*Armigeres Kuchingensis* Edgards, 1915	3	0	0	3	13	6	4.16 ±	4.83
23.	*Coquillettidia crasspes* (vander wulp), 1891	14	2	0	0	0	0	2.66 ±	5.60
24.	*Culex fuscanus* wiedman, 1820	15	0	0	0	0	0	2.50 ±	6.12
25.	*Cx. quinquefaciatus* Say, 1823	2878	394	443	2222	203	604	1124.00 ±	1131.14
26.	*Cx. tritaeniorhynchus* Giles, 1901	226	60	149	177	117	170	149.83 ±	56.73
27.	*Cx. vishnui* Theobald, 1901	25	0	25	6	3	12	11.83 ±	10.94
28.	*Mansonia annulifera* Theobald 1901	51	15	0	6	0	0	12.00 ±	19.98
29.	*Mimomyia huzonensis* (Ludlow), 1905	0	0	0	1	0	0	0.16 ±	0.40
	Total	6852	1420	2752	5338	1011	3090	×	

*Recorded for the first time.

Table 3. Seasonal prevalence of some important Anophelines in U.P. Terai

Species	Kashipur	Bazpur	Gadarpur	Kichha	Sitarganj	Khatima
September 1980						
A. annularis	4 (1.47)	4 (16.66)	1 (1.44)	—	5 (17.85)	—
A. culicifacies	1 (0.36)	2 (8.33)	16 (21.18)	—	—	3 (14.23)
A. fluviatilis	2 (0.73)	—	—	—	—	—
A. splendidus	—	—	—	—	—	21 (37.5)
A. subpictus	14 (5.61)	16 (66.66)	49 (71.01)	29 (96.66)	13 (64.28)	24 (42.85)
May-June 1981						
A. annularis	120 (7.28)	3 (1.30)	168 (27.90)	551 (25.20)	3 (7.89)	19 (4.96)
A. culicifacies	266 (16.15)	83 (36.08)	352 (58.47)	1343 (61.43)	10 (26.31)	109 (28.45)
A. fluviatilis	1 (0.06)	1 (0.43)	4 (0.66)	1 (0.04)	—	1 (0.26)
A. splendidus	5 (0.30)	1 (0.43)	1 (0.16)	7 (0.32)	—	—
A. subpictus	1242 (75.4)	125 (54.34)	77 (12.79)	265 (12.12)	25 (65.78)	251 (65.55)
Sept.-Oct. 1981						
A. annularis	26 (4.18)	13 (7.92)	45 (7.25)	27 (5.72)	8 (4.25)	72 (7.99)
A. culicifacies	169 (27.21)	37 (22.56)	207 (33.38)	91 (19.27)	32 (17.02)	88 (9.76)
A. fluviatilis	80 (12.88)	5 (3.04)	50 (8.06)	8 (1.69)	11 (5.85)	84 (9.32)
A. splendidus	1 (0.16)	—	—	—	—	9 (0.99)
A. subpictus	290 (46.69)	99 (60.36)	313 (50.48)	338 (71.61)	83 (44.14)	460 (51.05)
Jan.-Feb. 1982						
A. annularis	47 (4.36)	33 (6.21)	45 (5.33)	11 (4.66)	44 (10.47)	116 (12.01)
A. culicifacies	58 (5.38)	56 (10.54)	81 (9.60)	63 (26.69)	24 (5.71)	47 (4.90)
A. fluviatilis	671 (62.30)	265 (49.90)	518 (51.44)	109 (46.18)	239 (56.90)	373 (39.45)
A. splendidus	236 (21.91)	132 (24.58)	138 (16.37)	44 (18.64)	98 (23.33)	371 (38.72)
A. subpictus	—	—	7 (0.83)	1 (0.42)	—	1 (0.10)

% in parenthesis

Table 4. Average seasonal collection of some important Anophelines in U.P. Terai

Species	Kashipur	Bazpur	Gadarpur	Kichha	Sitarganj	Khatima
A. annularis	49.25 ± 50.32 (102.17)	13.25 ± 13.91 (104.90)	64.75 ± 71.89 (111.02)	147.25 ± 269.39 (182.94)	15 ± 19.44 (129.6)	51.75 ± 52.56 (101.56)
A. culicifacies	123.5 ± 117.86 (95.43)	44.51 ± 34.04 (76.49)	164 ± 148.3 (90.42)	374.25 ± 646.69 (172.79)	16.5 ± 14.27 (86.48)	61.75 ± 46.87 (75.90)
A. fluviatilis	188.5 ± 323.78 (171.76)	67.75 ± 131.5 (194.09)	143 ± 251.02 (175.53)	29.5 ± 53.11 (180.03)	62.5 ± 117.78 (188.44)	114.5 ± 176.77 (154.38)
A. splendidus	60.5 ± 117.01 (193.40)	33.25 ± 65.83 (197.98)	34.75 ± 68.83 (198.07)	12.75 ± 21.09 (165.41)	24.5 ± 49 (200.00)	100.25 ± 180.70 (180.24)
A. subpictus	386.5 ± 585.75 (151.55)	60 ± 61.32 (102.2)	111.5 ± 137.37 (123.2)	158.25 ± 168.46 (106.45)	30.25 ± 36.61 (121.02)	164 ± 215.83 (117.29)

Coefficient of variation is given in parenthesis.

work to increase agricultural production. *A. culicifacies* and *A. fluviatilis* have been incriminated as vectors in terai by Choudhury and Co-workers (1983) and the region has become endemic to malaria with high parasite load in the community.

Table 4 exhibits average seasonal collection and coefficient of variation. From the table it is observed that throughout the studies maximum variability in mosquito population is found in the case of *A. splendidus* while minimum variability in *A. culicifacies* except in Kichha tehsil, where it is 172.79. In *A. fluviatilis* maximum coefficient of variability (194.09) is found in Bazpur while minimum in Khatima (154.38).

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Karyotypic Studies on *Anopheles fluviatilis*

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Somatic chromosomes from neurogonial cells of *Anopheles fluviatilis*, an important vector of malaria, were karyotyped and measured to construct the idiogram. The diploid complement consists of six chromosomes which align into 3 pairs with distinguishable length and morphology. Both the autosomal and sex chromosomal pairs were found to be metacentric. The sex chromosomes were homomorphic in females and heteromorphic in males.

INTRODUCTION

A. fluviatilis, an important vector of malaria in the Indian subcontinent, belongs to the group *Myzomia* of the subgenus *Cellia*. It has a wide distribution throughout India and in parts of the eastern Oriental region. It occurs throughout the year in the plains and foothills and its seasonal prevalences depend more on the availability of breeding sites than on the atmospheric conditions. In general, density of *A. fluviatilis* builds up significantly soon after monsoon (Vishwanathan, 1950).

A. fluviatilis is an efficient vector throughout Western Ghats and Nilgiris. It is a vector of medium efficiency in the east-central India, Assam and Himalayan foot hills while its lowest efficiency has been recorded in the plains (Rao, 1981). Recently a sporozoite rate of 1.4-6.2% in *A. fluviatilis* was recorded in the followup entomological studies in the Gadarpur PHC (D.S. Choudhury, personal communication).

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Cytogenetical studies in *A. fluviatilis* were undertaken since the earlier reports on meiotic and mitotic chromosomes (Sharma and Choudhury, 1968 and Chowdaiah and Seetharam, 1975) were brief in their descriptions. The present study deals with a detailed karyotypic analysis of *A. fluviatilis*.

MATERIAL AND METHODS

A. fluviatilis strain utilized in the study was collected from Gadarpur village in U.P. and was colonized at the Malaria Research Centre following the general procedures of mosquito colonization. Somatic chromosomes were prepared from the neural ganglia dissected out from the fourth instar larvae using squash technique of Breland (1961) with some modifications. The larvae were placed in 0.01% colchincine for 1-1½ hours and the brain tissue was removed stained in 2% lacto-aceto-orcein for 10-20 minutes and squashed. To ascertain the diploid number, metaphases from both the sexes were scored. Chromosomes were measured on photographic prints of twenty well spread metaphase plates of each sex to construct an idiogram. The chromosomes have been classified following Levan *et al.* (1964) nomenclature.

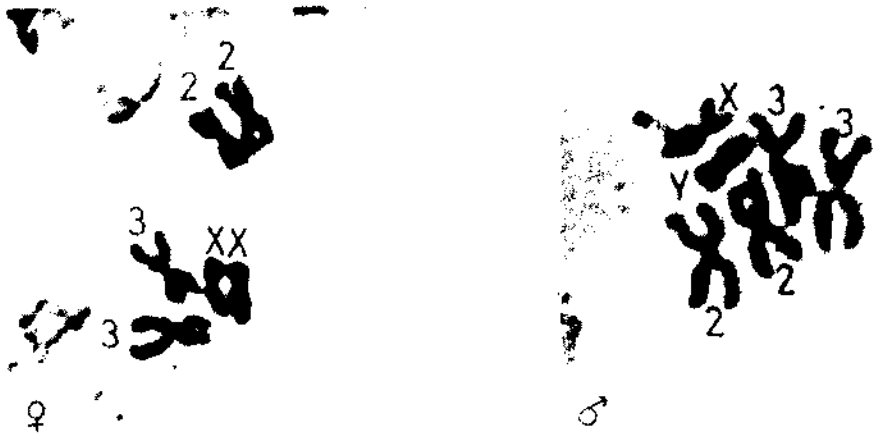

Fig. 1: Mitotic chromosomes from the neurogonial cells of female (XX) and male (XY) *Anopheles fluviatilis*.

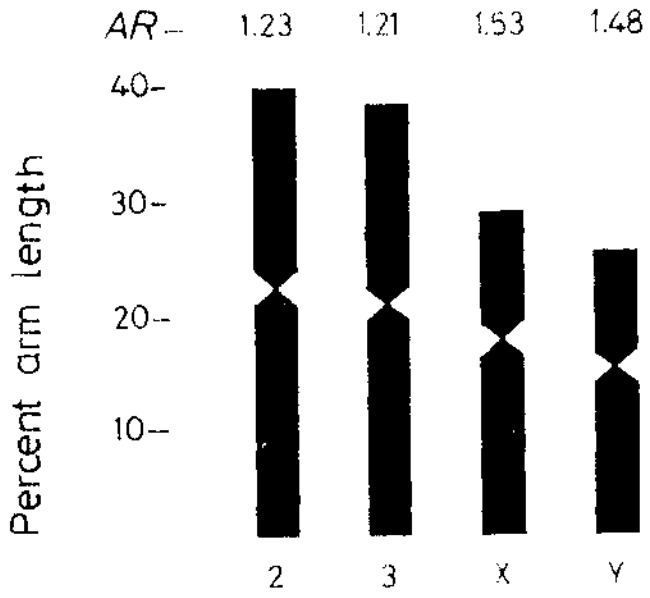

Fig. 2: A composite idiogram of *Anopheles fluviatilis* AR = Arm ratio.

RESULTS AND DISCUSSION

A total of 560 well spread metaphases from 30 females and 25 males were screened to establish the diploid number of the complement and to study the Karyotype (Table 1). The chromosome complement of *A. fluviatilis* consists of two pairs of autosomes and one pair of sex chromosomes, XX in females and XY in males. The karyotype is shown in Fig. 1.

Table 1. Analysis of *A. fluviatilis* karyotype (Gadarpur, Terai)

Chromosomes	% TCL	Arm ratio
Chromosome 2	37.86	1:1.23
Chromosome 3	35.05	1:1.29
X-chromosome	28.07	1:1.53
Y-chromosome	25.86	1:1.48

The autosome 2 was the largest pair constituting 37-38% of total chromosome length (TCL) followed by autosome 3, the second largest pair comprising 34-36% of the TCL. Sex-chromosome pair was the smallest. The X-chromosome varied from 25-28% of the TCL while the Y-chromosome contributed around 26% of the TCL. Both autosome and sex chromosomal pairs were metacentric with the arm ratios 1: 1.23; (autosome 2); 1: 1.2 (autosome 3); 1 : 1.53 (X-chromosome) and 1 : 1.48 (Y-chromosome), Fig. 2.

In *A. culicifacies* complex, another member of the group *myzomia*, autosome 2 is metacentric (Vasantha *et.al.*, 1982) or submetacentric (Saifuddin *et al.*, 1978) while autosome 3 is metacentric as in this species. However, the sex chromosomes of the two taxa differ. In *A. culicifacies* species A, X-and Y-chromosomes are submetacentric while in species B, X-chromosome is submetacentric and Y-chromosome is acrocentric (Vasantha *et al.*, 1982)

Further, it was found that the genome size in terms of the per cent TCL, for the X-and Y-chromosomes, was more or less similar in both the species i.e. about 28% of TCL for X-and 26% TCL for Y-chromosome respectively. Thus the difference in the centromeric position in the sex-chromosome pair in *A. fluviatilis* may be due to a chromosomal rearrangement, probably a pericentric inversion during the course of speciation.

ACKNOWLEDGEMENT

We wish to thank Drs. S.K. Subbarao, Rekha Chaturvedi and K. Vasantha for their suggestions in analysing the results of this study. We are also, grateful to Miss Meenu Chawla, artist, for her help in graphics.

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Mosquitoes of Coastal Orissa

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A total of 32 species of mosquitoes belonging to six genera i.e., *Anopheles, Aedes, Aedeomyia, Armigeres, Culex* and *Mansonia* were collected during the surveys carried out in December 1980, September 1981 and June-August 1982 in Coastal districts of Balasore, Cuttack, Puri and Ganjam. *Anopheles minimus* and *A. pulcherrimus* were recorded for the first time from this region. Four anopheline species i.e., *A. jeyporiensis, A. maculatus, A. sundaicus* and *A. theobaldi* earlier reported from this region were absent.

INTRODUCTION

Fry (1912) appears to have been the first to study the mosquito fauna of coastal Orissa and Chilka Lake areas which revealed the presence of 5 anopheline species. Subsequently, Sarathy (1932) reported 15 anopheline species from Puri district. Senior White and Adhikari (1939) reported the occurrence of *Anopheles sundaicus* for the first time of a total of 20 anopheline species based on larval collection from Chilka Lake. The collections also included four additional records. The study of Covell and Singh (1942) revealed the existence of 17 anopheline species which contained one additional record and three *Anopheles* recorded by Senior White and Adhikari (1939) were absent. Since then there is no published record dealing on the mosquito fauna of coastal Orissa. Because of extensive ecological changes that have taken place as a result of insecticidal pressure as well as developmental changes in agriculture, industry etc., considerable changes might have taken place in the mosquito fauna. Therefore, the present faunistic studies were initiated. The results of observations made during the three surveys carried out in coastal Orissa are summarised in this communication.

MATERIAL AND METHODS

Topography: The state of Orissa is located on the East coast of India in between 17° 48' and 22° 34' North latitude and 81° 24' and 87° 29' East longitude. Touching the Bay of Bengal, it stretches along the sea beach to about 482 km. The alluvial belt runs along the sea coast of the state and consists of rugged tracts with jungle and clad hills of eastern ghats, occasionally broken by ravines and deep valleys. Broadly, the territory of the state is divided into four physiogrpahic regions i.e., eastern plateau, the central river basin, the eastern hill and the coastal belt. The coastal belt comprises of four districts, i.e., Balasore, Cuttack, Puri and Ganjam (see Fig. 1).

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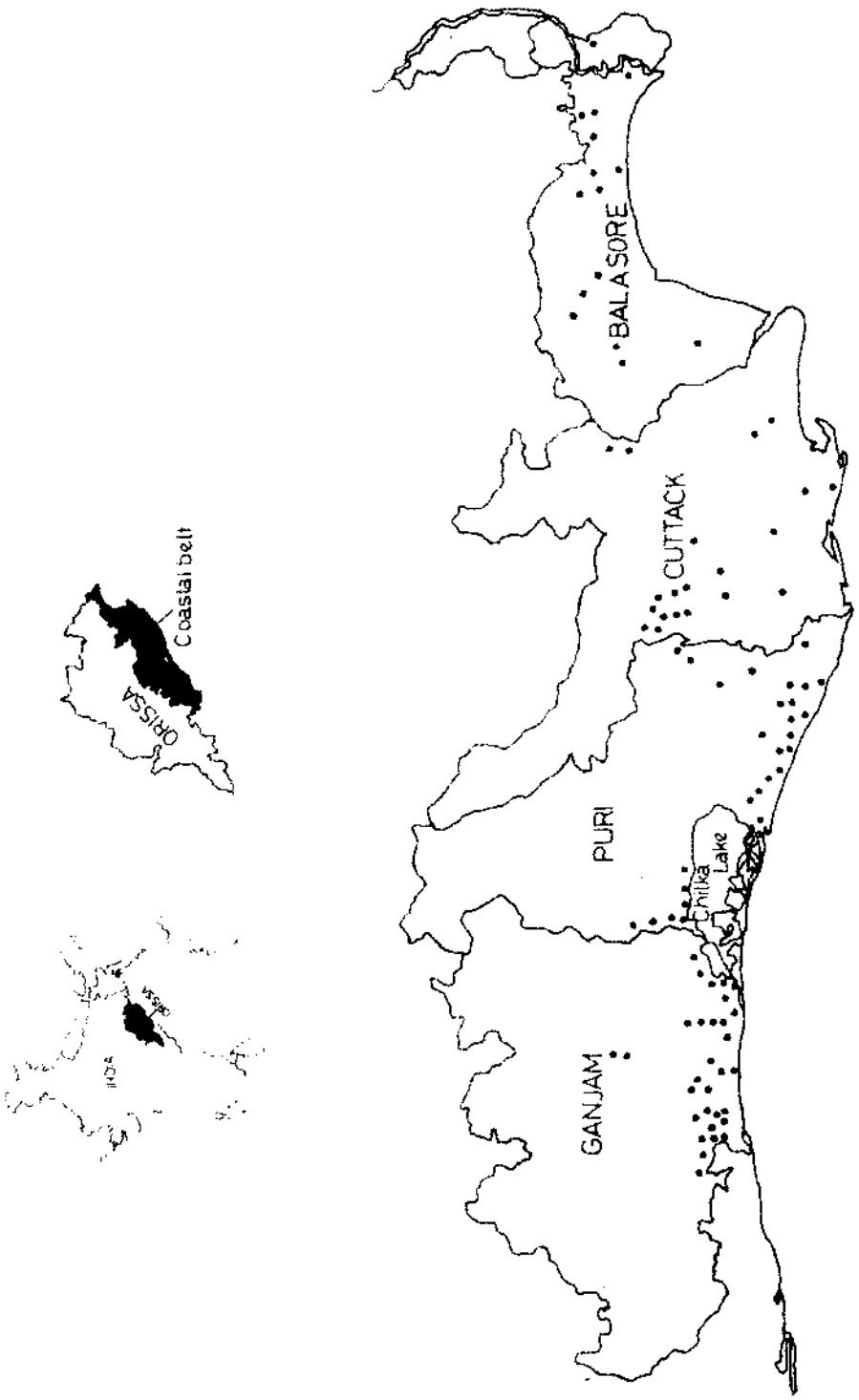

Fig. 1. Map showing the collection sites in Coastal Orissa.

Adult mosquitoes that were resting indoor, outdoor and biting man and cattle were collected by suction tube method both during dawn (5 AM to 10.30 AM) and dusk (5.30 PM to 9.30 PM). Collections were made from cattle sheds, human dwellings, mix dwellings occupied by man and cattle including other man made structures. Out doors resting adult mosquitoes were collected from the shrubs around the cattle sheds and human dwellings, paddy fields, forests, tree holes and bus depots. In each survey 98 villages were covered in 4 districts of coastal Orissa viz. Balasore, Cuttack, Puri and Ganjam. Mosquito collections were made using suction tube from a minimum of 3 cattle sheds and 5 human dwellings from each village, in addition to this total catches were made from one or two huts. These villages were also searched for the larval breeding sites and immatures were collected from Chilka Lake, pits around the Chilka Lake, paddy fields, ponds, pools, streams, margins of canals, wells and tree crevices etc. Larvae were also collected from the water stored inside houses and cattle sheds and from the discarded tyres. All larvae were kept in field laboratory until adults emerged. Emerging adult mosquitoes were killed with ether and packed in cellophene papers. All mosquitoes collected during the surveys were brought to the Centre for identification and further study. Mosquitoes were identified using the key of Christophers, 1933 and Barraud, 1934. The collection sites (villages) are shown in Fig. 1.

RESULTS AND DISCUSSION

A total of 30,347 mosquitoes belonging to thirty two species of 6 genera viz, *Anopheles, Aedeomyia, Aedes, Armigeres, Culex* and *Mansonia* were collected in 3 surveys. Indentification of mosquitoes has revealed that: (i) genus *Anopheles* consisted of 19 species (27,432 specimens) (ii) genus *Aedes* consisted of 1 species (77 specimens) (iii) genus *Aedeomyia* consisted of 1 species (7 specimens) (iv) genus *Armigeres* consisted of 2 species (105 specimens) (v) genus *Culex* consisted of 7 species (1891 specimens) and (vi) genus *Mansonia* consisted of 2 species (835 specimens) (Table 1).

Table 1. List of mosquitoes collected from Coastal Orissa.

Sl. No.	Species collected	Total mosquitoes collected
	Genus *Anopheles*	
1.	*Anopheles aconitus* Doniz, 1902	1457
2.	*A. annularis*, Van der Wulp, 1884	6548
3.	*A. barbirostris*, Van der Wulp, 1884	288
4.	*A. culicifacies* Giles, 1901	1902
5.	*A. fluviatilis* James, 1902	3
6.	*A. jamesii*, Theobald, 1901	80
7.	*A. karwari* James, 1902	5
8.	*A. minimus* Theobald, 1901	40
9.	*A. nigerrimus*, Giles, 1900	4010
10.	*A. pallidus*, Theobald, 1901	111
11.	*A. philippinensis* Ludlow, 1902	6
12.	*A. pulcherrimus* Theobald, 1902	30
13.	*A. ramsayi* Covell, 1927	646
14.	*A. splendidus* Koidzumi, 1920	2
15.	*A. stephensi* Liston, 1901	14
16.	*A. subpictus* Grassi, 1899	3905
17.	*A. tessellatus* Theobald, 1901	9
18.	*A. vagus* Doniz, 1902	8283
19.	*A. varuna* Iyengar, 1924	93
	Genus *Aedeomyia*	
1.	*Aedeomyia venustipes* (Skuse), 1889	7
	Genus *Aedes*	
1	*Aedes aegypti* Linnaeus, 1726	77
	Genus *Armigeres*	
1.	*Armigeres kuchingensis*, Edwards, 1915	83
2.	*Ar. theobaldi* Barraud, 1934	22
	Genus *Culex*	
1.	*Culex bitaeniorhynchus* Giles, 1901	29
2	*Cx. quinquefasciatus* Say, 1823	728
3	*Cx. minutissimus* Theobald, 1907	23
4.	*Cx. sinensis* Theobald, 1903	19
5.	*Cx. tritaeniorhynchus* Giles, 1901	623
6.	*Cx. vishnui* Theobald, 1901	427
7	*Cx. whitmorie* Giles, 1904	42
	Genus *Mansonia*	
1	*Mansonia annulifera* (Theobald), 1901	719
2.	*Ma. longipalpis* Van der Wulp, 1892	116

Table 2: Details of *A. minimus* collected from coastal Orissa

Name of the village	Date of collection	No. of specimens collected from different habitats						
		CATTLE SHEDS		HUMAN DWELLINGS		MIXED DWELLINGS		TOTAL
		Dawn Hrs.	Dusk Hrs.	Dawn Hrs.	Dusk Hrs.	Dawn Hrs.	Dusk Hrs.	
Phulnokhara (Cuttack)	14.12.1980 21.09.1981 23.07.1982	1	2	—	1	-	3	7
Gotiotpatna (Cuttack)	15.12.1980	1	--	--	--	1	--	2
Bidibatae (Cuttack)	15.12.1980 22.09.1981 26.07.1982	3	-	2	-	3	--	8
Bagadharapur (Cuttack)	16.12.1980 22.09.1981 25.07.1982	2	4	1	1	3	2	13
Rupsa (Balasore)	20.12.1980	2	1	1	---	2	-	6
Bahapal (Balasore)	22.12.1980	1	-	1		2	--	4

Among the anophelines, the most prevalent species was *A. vagus* (30.19%) followed by *A. annularis* (23.6%) and *A. nigerrimus* (14.61%). Among culicines the most dominant genus was *Culex*. In this genus the most prevalent species was *Culex quinquefaciatus* (38.4%) followed by *Culex tritaeniorhynchus* (32.94%) and *Culex vishnui* (22.58%).

A. sundaicus is an important malaria vector although its zone of influence is confined to Coastal Orissa which includes Chilka Lake area and parts of Ganjam district as well as parts of Vijayanagaram district of Andhra Pradesh (Rao, 1955; Covell and Singh, 1942; Senior White and Adhikari, 1939). During the present study, *A. sundaicus* was conspicuous by its absence in the areas where adults and larvae of this species were found in abundance in the earlier studies. This would suggest that *A. sundaicus* might have disappeared from its erstwhile strong holds of Coastal Orissa.

The other three anophelines viz *A. maculatus*, *A. jeyoporinsis* and *A. theobaldi* which were reported by Covell and Singh (1942) were not found in this survey. Further, two new additions to the fauna of this region are *A. minimus* and *A. pulcherrimus*.

A. minimus is an efficient vector of malaria and it is likely that in some parts of Orissa it may be transmitting malaria. The focus of this vector appears to be limited and an effective spraying can eliminate *A. minimus* from Orissa before it becomes abundant and also resistant to the commonly used insecticides. During the present study 40 specimens of *A. minimus* were collected, 30 from Cuttack and 10 from Balasore district (Table-2).

A total of 30 specimens of *A. pulcherrimus* were collected from 5 villages of Puri district (5 from cattle sheds, 7 from human dwellings and 18 from mixed dwellings). Records show that the distribution of this species appears to have been confined to North and North West (Punjab and U.P.) and Western regions (Gujarat) of India. *A. pulcherrimus* does not play any role in the transmission of malaria in India. However, it is of epidemiological importance in northern Afghanistan (Onori, 1975) and southern parts of USSR (Covell, 1944).

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Role of Cyclophosphamide in Modulating Behaviour of *P. berghei* Infection in Mice

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Acute malaria was produced experimentally in an outbred strain of Swiss mice using a standard inoculum of parasitized red cells. Effect of cyclophosphamide (CY) treatment on the degree of parasitemia, percentage of T and B cells and thymic and liver weights was studied longitudinally. Results indicate that CY, in the dosage of 150 mg/kg given 48 hours prior to the parasite challenge, results in a significant prolongation of the prepatent period and a statistically significant decrease in parasitemia for the first 12 days. It is associated with marked atrophy of thymus and spleen during the first four days along with lowering of both the subpopulations of lymphocytes. The spleen weight pick up in due course. The transitory protection observed in experimental animals is linked with a marked depression of reticulocyte count. After the reticulocyte counts are normalized, parasitemia in the two groups becomes comparable. Acute malaria is also associated with a decrease of NBT and acid phosphatase activity of peritoneal macrophages although a transitory rise is observed during the first forty eight hours.

INTRODUCTION

The intricate and reciprocal relationship between immunosupression and malaria has intrigued workers for the past several years. On the one hand, immunosupression as a result of fulminant malaria is a well established fact (McGregor *et al.*, 1962; Greenwood *et al.*, 1972). On the other hand immunosupression as an important predisposing factor for lethal outcome of malarial infection has also been documented (Clark and Allison, 1964). However, there is little or no direct evidence in the literature as yet to indicate an important effective role for cell mediated immunity. At present, one could only postulate that T cells might complement or supplement the all important humoral immunity.

Cyclophosphamide (CY) causes profound alterations of the immune system of the host. Further, there are serious controversies regarding immunosupression caused by cyclophosphamide. Earlier studies indicated that the major burnt was on the 'T' cells (Berenbaum, 1965; Starlz *et al.*, 1971 and Albright and Albright 1976) while subsequent observations suggested that the 'B' cell compartment is the hardest hit. Linna *et al.* (1976) offered convincing evidences that CY could produce a chemical bursectomy in new born chicks. The thymic lymphoid tissue, if at all, showed only a transient setback. Similarly, Marbroock and Baguloy (1976) reported that CY treatment produces a mouse which is severely depleted of B cells. Stockman *et al.* (1973) corroborated these observations by morphological studies. Paradoxically enough, stray reports also hint at protection induced by CY in malarial infections (Finerty and Edward, 1976). The present study was therefore designed to investigate the effect of CY on the sub-populations of lymphocytes and on *Plasmodium berghei* infection in an outbred strain of Swiss mice.

MATERIAL AND METHODS

Animals: Four to six weeks old outbred (VRC) Swiss mice weighing 20-25 gms were selected for the study and fed ad libitum. Material parasite:

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(Vinvkei & Lips, 1948) strain of *P. berghei* obtained from NICD New Delhi was used in this experiment. It was maintained by weekly serial intraperitoneal passages in the Swiss mice.

Acute malaria was induced experimentally in 30 control animals by using 1×10^3 malaria infected red blood cells intraperitoneally as the standard inoculum.

In the experimental group of 40 animals, cyclophosphamide was given intraperitoneally (150/kg body wt.) 48 hours prior to parasite challenge. Both the groups were observed daily for the extent of parasitemia (Parashar *et al.*, 1977) and mortality. Random batches of mice from each group were sacrificed at 48 hours intervals and weights of spleen and thymuses recorded carefully and splenict and B cells estimations carried out.

Serial samples of peritoneal macrophages were obtained at 48 hours interval during the course of infection and acid phosphatase estimated according to the method of McBride *et al.*, (1974). A qualitative Nitroblue tetrazolium dye reduction test was also conducted (Segal and Levi 1973).

Splenic sub-population of lymphocytes: Spleens were taken out, teased in cold minimum essential medium (MEM) and then passed through a 60 mesh gauze. The cells were centrifuged at 1500 rpm at 40°C for 10 minutes and washed thrice. The viability was tested with trypan blue, cells were counted and adjusted at 2×10^6/ml viable cells.

T cells: These were estimated by the modified method of Amysliwski *et al.* (1977). A suspension of 2×10^6 cell were treated with 1 μg of phytohaemagglutinin P (Difco Laboratories) at 37°C for half an hour. These were washed twice with MEM, readjusted and incubated with an equal volume of 0.5% washed sheep erythrocytes (SRBC) for 15 minutes at room temperature. The mixture was centrifuged at 200 rpm for 5 minutes and kept at 4°C overnight. A total of 500 cells were counted for assessing the percentage of rosette forming cells.

EAC Rosettes: The method of Bianco *et al.* (1970) was used. Briefly, a 5% suspension of SRBC was incubated with a 1 in 1000, dilution of amboceptor for ½ hour at 37°C and suspended in Hanks balanced Salt Solution (HBSS). EA cells were treated with an equal volume of 1 in 10 fresh mouse serum at 37°C for ½ hour. A 1:5 dilution of EAC was further incubated with an equal volume of 5×10^6/ml of lymphocytes for ½ hour at 37°C. The mixture was centrifuged at 500 rpm for 3 minutes and rosetting cells counted after addition of 0.6 ml of HBSS. Serial reticulocyte counts were done both in the control and in CY treated animals.

RESULTS

During acute malaria, in the control animals the thymus weight gradually dropped from a mean of 50.6 mg ± 16.7 to a mean of 8.4 mg ± 2.07 by the 10th day after which the weight remained stationary up to the 14th day of observation. The splenic weight on the contrary continued to rise slowly till the 8th day after which it revealed a sudden sharp increase and by the 14th day, the mean weight was 1,000 mg against a normal mean of 100 mg (Fig. 1).

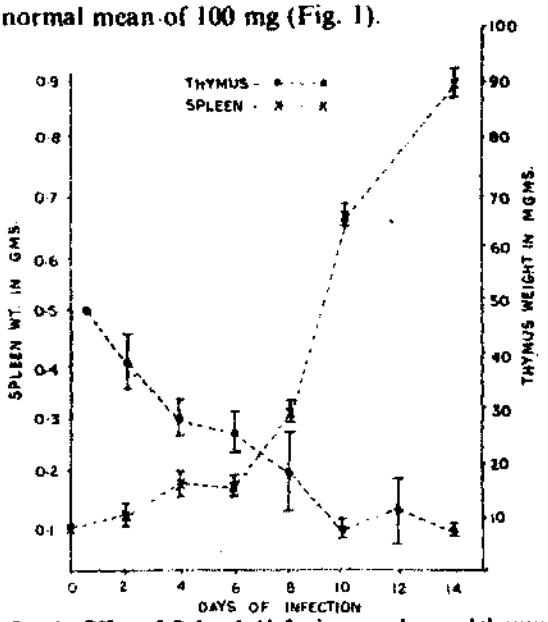

Fig. 1—Effect of *P. berghei* infection on spleen and thymus weights.

Effect of CY treatment: (i) *Parasitemia*: The prepatent period in the control group was 2 days after which there was a progressive rise of parasitemia with a peak parasitemia of 5% by day 10 (Figs. 2 and 3).

The prepatent period in the CY treated animals was clearly prolonged by at least 5 days. On the 10th day of CY administration i.e., 8th day of parasite challenge it was barely 4.88 ± 3.7% while in the control group it was 42 ± 5.4% on the 8th day of parasitic challenge. Thus the parasitemia was significantly greater in the control group on days 8, 10 ($p < .001$) and 12 ($p < .001$) of observation. The differences being highly significant. After 14 days, the CY treated animals showed a comparable degree of parasitemia.

(ii) *Spleen weight*: in the animals given CY the spleen weight dropped drastically during the first 4 days, the mean weight being 35.4 mg ± 5.98 on day 2 of parasite challenge compared with a control spleen weight of 100 mg ± .44. On the 6th day of cyclophosphamide, the spleen weight had increased to 55.4 mg ± 15.67 and by the 8th day it revealed a rebound phenomenon attaining a mean wt. of 300 mgs ± 81 which was higher than the corresponding normal at that time 190 ± 27. The spleen weights gradually increased during the subsequent course of the infection (Fig. 2).

(iii) *Thymus weights*: The CY treated animals likewise showed a more drastic drop of the thymus weights as compared to that of the controls. The resting mean weight was 50.6 mg ± 6.7. The mean weight at the end of 48 hours being 10.2 mg ± 1.9 and 96 hours after CY treatment it was 12.75 mg ± 4.5 when the thymus weight in the control was 19.25 ± 7.88 mg, the difference being statistically significant ($p < .01$). Thereafter, the difference became insignificant (Fig. 3).

(iv) *T & B cells*: The result of modified E and EAC rosettes indicates that under the effect of CY at a dosage of 150 mg/kg body wt., both the sub populations of lymphocytes show a distinct decline in the spleen although it was more marked in the case of T cells (Fig. 4). The control

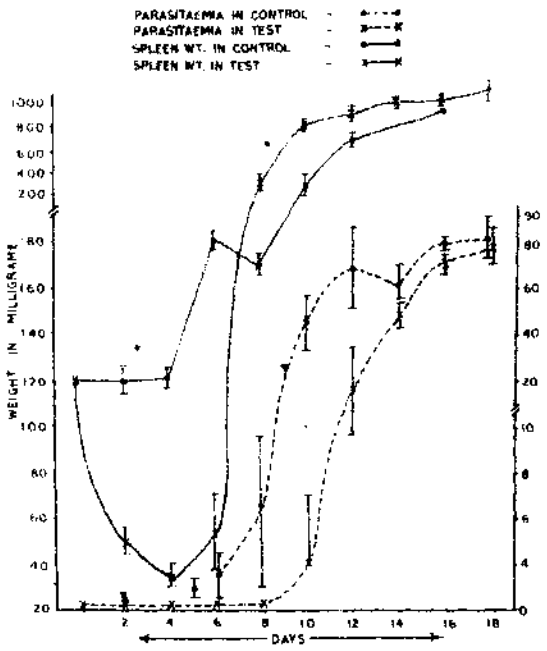

Fig. 2 —Effect of cyclophosphamide on spleen weight during *P. berghei* infection.

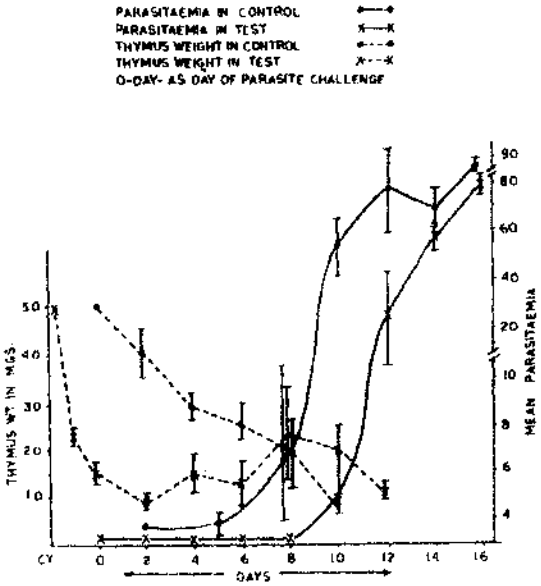

Fig. 3 —Effect of cyclophosphamide on thymus weight during *P. berghei* infection.

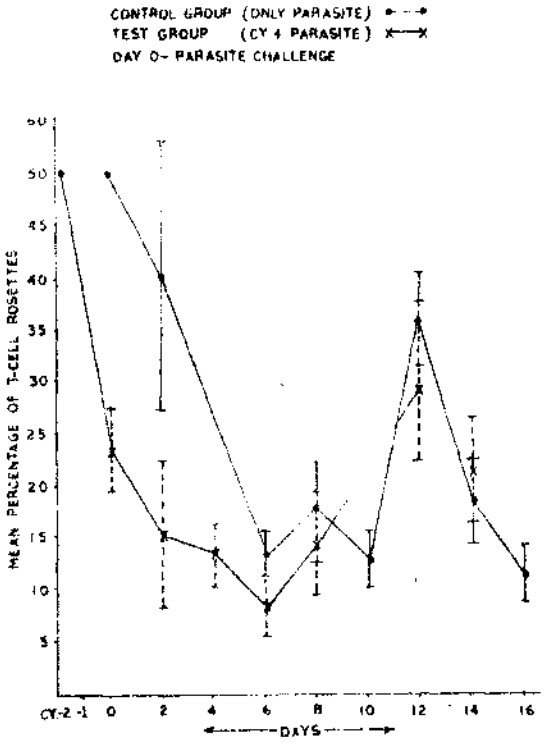

Fig. 4 Effect of cyclophosphamide and *P. berghei* infectio on 'T' cell population of the host.

animals also revealed a fall in splenic T cells but it was less marked that in the CY treated animals. Further, the B cells revealed a rebound after an initial dip when by day 10, the mean value was 24.8 ± 5.006 as opposed to the control figure of 10.25 ± 4.34. Eventually the B cells declined as the parasitemia progressed (Fig. 5). Acid phosphatase activity of the splenic macrophages increased from 2.2 K.A.U. to 8.6 by the 2nd day and to 12.6 by the 4th day. But on the 6th day, the activity was only 1.13 K.A.U. The activity declined further on 10th day of observation. Results of the NBT test also revealed a similar trend, during the first 48 hours it rose from 10-15% to 35 ± 15% and thereafter steadily declined till the 8th day of observation.

Reticulocytes: The reticulocyte count steadily increased to a peak of 52 ± 12.5% before death in the control group and paralleled closely the extent of parasitemia. In the CY treated animals on the other hand, no significant reticulocyte response could be discerned till the 8th of parasite challenge while the control group revealed a mean of 4.7 ± 2%. On the day 10, the difference was still more marked; in the control group the reticulocyte response was 38.3 ± 7.2 while in the CY treated group it was barely 8 ± 3.2. The difference was statistically significant ($p < .001$) Thereafter the difference became insignificant.

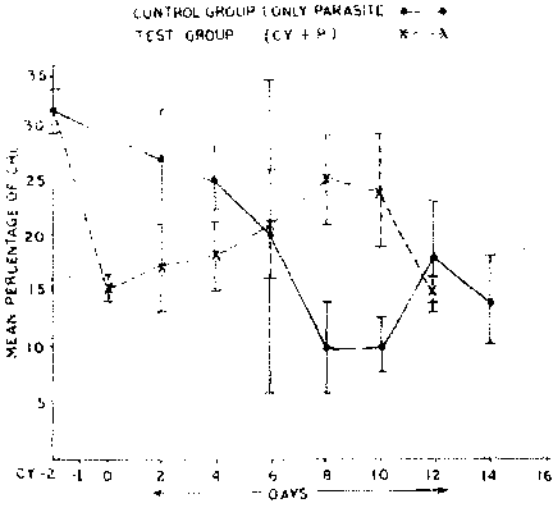

Fig. 5 Effect of cyclophosphamide and *P. berghei* infection on 'B' cell population of the host.

DISCUSSION

The purpose of the present study was to investigate the possible mechanisms of protection (if any) induced by CY against malaria. The results of the experiment indicate that CY treatment causes a distinct delay in the appearance of parasitemia with prolongation of the prepatent period alongwith a statistically significant lowering of parasitemia on days 8, 10 and 12 after the parasite challenge.

If selective B cell depletion was so conspicuous a feature of CY treatment as suggested by Linna *et al.* (1976), Wells *et al.* (1977), and Stockman *et al.* (1973) one would expect enhancement of parasitemia since humoral factors play a dominant

role in passive immunity against malarial infection. Marbook and Baguley (1976) in their study indicated that CY selectively eliminates SRBC plaque forming clones, when the B cell depression was abrogated by antilymphocyte serum (ALS) or steroids suggesting suppression by T cells to be a contributing factor. But studies of Finerty and Edward (1976) and Spira *et al.* (1968) clearly indicate protection induced by CY in *P. berghei* infection. Finerty and Edward (1976) however, stressed that the main mode of action was through augmentation of the cell mediated immunity. In fact, a somewhat similar mechanism has been postulated by Wyler *et al.* (1976) who reported definite augmentation of cell mediated immunity associated with protection against plasmodium infection after CY. It is interesting to note that in our study maximum protection was observed during a period, when there was almost dissolution of spleen and an acute involution of thymus. Further, it is during this phase that both the subpopulations of lymphocytes showed a marked decline (Figs. 4 & 5). From the present study it appears that thymic involution is much more drastic under the effect of cyclophosphamide than due to malarial infection *per se*, splenic T cells 48 hours after CY being 13.75 ± 3.86 while in the control, the mean value was 40 ± 1.37. In fact, earlier studies by Berenbaum (1963) and Starlz *et al.* (1971) indicated suppression of 'T' cell activity in man and mice as judged by prolongation of homograft survival after CY. On the other hand, Albright and Albright (1976) in their adoptive transfer studies concluded that CY selectively kills or supresses the supressor T cells and that CY treated animals exhibit vigorous antibody response. Their results do not corroborate findings of Linna *et al.*, (1976). Wells *et al.* (1977) or Stockman *et. al.* (1973). Observations of Albright and Albright are however, corroborated by findings of Poulter and Turk (1972) and Askanese *et al.* (1975) who claimed that helper T cells were resistant to the action of CY. Distant thymic atrophy alongwith marked shrinkage of splenic size shortly after CY in our study suggests knocking of multiple functions of T cells pool. B cells may be effected suigeneris or via the helper T cells and the ultimate outcome in a given situation is dependent on the dose, time of drug administration and the degree of relative insults to the subpopulations of lymphocytes.

In fact, Lagrange *et al.* (1974) summarised that enhanced susceptibility of B cells to CY was due to a higher turnover and that activated T cells could be equally sensitive to the action of CY.

In the present study one can envisage neither 'T' cell nor 'B' cell-dependent mechanisms affording transitory protection in *P. berghei* infection mice since there was a universal depression of the bone marrow. Since *P. berghei* has a predilection for the reticulocytes, it seems logical to conclude that CY depletes the host of the requisite cell.

In the present study, the protection was confined to a period when the reticulocyte count was low. After the effect of the drug waned off and reticulocyte response picked up, there was a parallel increase in the parasitemia. Therefore, it is likely to be a spurious protection rather than true protection. Furthermore, it is apparent that mere estimation of parasitemia may not be a fool proof indicator of protection.

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Suppressive Action of an Interferon Inducer (6 MFA) on Blood Induced Rodent Malaria.

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Effect of 6-MFA, an interferon inducer, on the course of *Plasmodium berghei* infection in random bred Swiss mice has been studied and it has been found to produce a significant suppression of parasitaemia during the early stage of infection (between days 4-7 post inoculation) and also a significant extension of mean survival time of infected mice compared to the controls.

INTRODUCTION

Interferon and interferon inducers are well known to provide a non-specific protection against viruses (Gupta, 1978; Stewart, 1981). Statolon an inducer of interferon isolated from *Penicillium stoloniferum* (Powell *et al.*, 1952; Kleinschmidt and Probst, 1962) and helenine produced from *P. funiculosum* (Shope, 1953) are known to exhibit antiviral activity against Semliki forest and MM viruses. In addition, 6MFA an interferon inducer from *Aspergillus ochraceus* has been found to possess broad spectrum antiviral properties (Maheswari *et al.*, 1971; 1977; 1978; Rama Kant and Gupta, 1976; Lal *et al.*, 1980). Limited studies conducted so far suggest that interferon inducers like West Nile virus (WNV) and New Castle disease virus (NDV) (Huang *et al.*, 1968; Schultz *et al.*, 1968; Jahiel *et al.*, 1968; 1969) and other interferon inducers like Statolon (Jahiel *et al.*, 1969; Bliznakov, 1980) can exert suppressive action against malaria. The antimalarial activity of 6-MFA which is a strong inducer of interferon, has not been evaluated so far. The present communication reports the suppressive action of 6-MFA on blood induced rodent malaria.

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MATERIAL AND METHODS

Random bred Swiss mice (weighing 20/g) from the CDRI colony, were used for malaria infection and antiviral assay.

6-MFA was prepared in the laboratory from *Aspergillus ochraceus* (ATCC-28706) according to the method of Maheshwari *et al.* (1977). Stock solution of 6-MFA in P.B.S. (pH 7.2) was adjusted to contain 1.5 mg/ml of the product which induces 3000 IU of interferon per ml of mouse serum (titrated against vesicuar stomatitis virus in L-cell system; Personal communication from Dr. Maheshwari, unpublished data). The preparation was frozen at -20°C and aliquotes of each sample were assayed for interferon production in mice against Semliki Forest Virus, where it gave 90% or more protection by inoculating 1ml of 6-MFA introperitoneally 24 hours before challenge with the virus (Lal *et al.*, 1980).

In order to assess the protective effect of interferon induced by 6-MFA in *Plasmodium* infection, the mice were divided into four groups (I to IV) which were administered 6-MFA by i/p route. Group I received 1 ml × 3 doses of 6-MFA i/p (day-1, +4, +8); Group II received 1 ml. + 3 doses, i/p (day-1, +4, +8); Group III received 1ml. i/p (day-1); and Group IV received 1 ml i/p (1 hr before infection). The control group as well

as 6-MFA trated groups of mice were infected with *P. berghei* (1+10⁶ parasitized RBC)by i/p route. The course of parasitaemia was followed by examining Giemsa stained smears daily. The mortality in control and experimental groups was recorded.

RESULTS AND DISCUSSION

The data on mean survival time of 6-MFA treated and control mice following *P. berghei* infection, are given in Table 1. All the 6-MFA treated groups showed significant extension of mean survival time (Range 9.80— 16.5 days) compared to the controls (8.3 days).

The results presented in Table 2 show that in all the four groups (I-IV) of mice treated with 6-MFA by intraperitoneal route, there was significant suppression of parasitaemia between day 4 to 7 of infection as compared to the control group. After 7th day, the parasitaemia increased in all groups resulting in 100 per cent mortality.

Yoeli *et al.* (1955) first reported the suppressive effect of WNY inoculation against blood induced *P. berghei* infection in mice. Protection against sporozoite induced infection was reported by Jahiel *et al.* (1968) who observed a marked protection when NDY was given 16-24 hr. after the sporozoite inoculation. Parasitaemia developed in only 13 out of 40 mice. However, when virus was given 48 hrsafter sporozoite inoculation, the protection was less marked. They postulated that the main action of interferon was on the late primary exoerythrocytic stages of the parasites.

Schultz *et al.* (1968) used NDV against blood induced *P. berghei* infection in mice and demonstrated a slight degree of protection, limited to an increase in survival time from 7 to 9.2 days, when virus was given either 2 hrs. before or 12-15 hrs. after malaria infection.

Jahiel *et al.* (1969) demonstrated the antimalarial activity of three interferon inducers namely NDV, Statolon and the double stranded polyribocytidytic and polyriboinosinic acid and observed the highest protection, as evidenced by prevention of parasitaemia and survival of mice against sporozoite induced infection, while protection afforded by NDV or Statolon (given 16-24 hrs. after infection) against blood induced infection was partial and was limited to only suppression of parasitaemia between days 5-7. The use of 6-MFA in the present study against blood induced *P. berghei* infection has shown results which are in close agreement with those of Jahiel *et al.* (1969) who employed NDV and Statolon as inducers. We have observed in 6-MFA treated mice, a partial but significant suppression of parasitaemia on day 4, 5, 6 and 7 post-inoculation. In addition, our study has shown a significant extension of mean survival time in all the groups of mice treated with 6-MFA, as compared to the controls.

Interferon inducer (Statolon) has also been used in conjuntion with chloroquine to study their effect on malaria and it was breifly reported that pretreatment with statolon and chloroquine

Table 1. Effect of 6-MFA on the survival time of mice infected with *P. berghei*.

Group	6-MFA treatment No. of doses (on days)	Route	No. of mice	Mean Survival time ± SD (Days)
CONTROL	--		25	8.3 ± 1.79
6-MFA	I. 1 ml × 3 (day-1, +4, +6)	i/p	15	12.6 ± 5.06
	II. 1 ml × 3 (day-1, +4, +8)	i/p	16	16.56 ± 5.32
	III. 1 ml × 1 (day-1)	i/p	20	10.95 ± 3.41
	IV 1 ml × 1 (hr-1)	i/p	20	9.80 ± 3.50

Mice were infected with 1 × 10⁶ parasitized RBC on day 0

Table 2. Effect of 6-MFA on the Parasitaemia in Mice.

Days	Control % parasitaemia ± S.D.	6-MFA treated mice: Group I % parasitaemia ± S.D.	P. value	Group II % parasitaemia ± S.D	P. value	Group III % parasitaemia ± S.D	P. value	Group IV % parasitaemia ± S.D	P. value
3	0.02			×		×		×	
4	0.89 ± 0.91	0.03 ± 0.74	< .001	0.03 ± 0.10	< .001	0.14 ± 0.27	< .001	0.04 ± 0.10	< .001
5	1.27 ± 1.96	0.16 ± 0.39	< . 01	0.02 ± 0.05	< .001	0.83 ± 1.63	NS	0.49 ± 0.91	NS
6	5.50 ± 5.49	1.62 ± 3.29	< .001	0.62 ± 0.92	< .001	1.88 ± 3.78	< .01	1.72 ± 1.60	< .001
7	7.95 ± 6.70	4.5 ± 5.26	NS	3.38 ± 3.15	< .005	4.47 ± 5.5	NS	3.39 ± 2.1	< .005
8	7.15 ± 3.05	4.67 ± 2.75	< . 01	1.46 ± 1.27	< .001	7.03 ± 4.70	NS	4.22 ± 2.2	< .001
9	12.67 ± 11.62	5.89 ± 4.33	< . 01	2.36 ± 1.85	< .001	2.36 ± 1.80	NS	6.88 ± 6.76	NS
10	5.66 ± 2.08	9.4 ± 5.6		6.78 ± 5.24		10.53 ± 8.54		3.07 ± 1.97	
11	12.66 ± 9.01	9.0 ± 2.0		7.5 ± 4.1		6.66 ± 4.02		4.5 ± 2.08	
12	18.0	9.75 ± 3.59		7.4 ± 7.0		7.75 ± 7.13		8.5 ± 4.50	
13		7.50 ± 4.2		5.3 ± 3.8		12.25 ± 9.4		6.33 ± 1.52	
14		7.0 ± 2.70		4.62 ± 2.56		10.5 ± 3.69		14.50 ± 9.19	
16		9.33 ± 3.05		8.66 ± 10.26		8.0		21.0 ± 12.72	
17		22.25 ± 19.18		4.0 ± 4.0		12.0		14.5 ± 0.70	
18		17.0 ± 15.87		7.66 ± 6.65		10.0		20.0	
19		21.66 ± 7.63		7.66 ± 7.5		19.0			
20		20.0 ± 2.82		14.0 ± 15.09		22.0			
21									
22									
23									

increased the survivorship as well as prolonged the survival time of mice infected with *P. berghei* (Bliznakov, 1980).

Donahoe and Huang (1973) have suggested that interferon and its inducers could exert antiprotozoal effect by stimulating phagocytosis.

The present study with blood induced rodent malaria in mice suggest that 6-MFA exerts a suppressive action on malaria which is probably linked to interferon production and consequent non-specific immuno-modultory effect on the host. Since the resultant infection in all the 6-MFA treated mice was fatal, it is certain that neither inducer (6-MFA) nor interferon exerts a specific antiparasitic action on the malaria parasite which could eradicate the parasitaemia from the host completely.

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SHORT NOTES

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Detection of Sporozoites in *Anopheles subpictus* in Bastar District, Madhya Pradesh

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In India, occurrence of oocysts and sporozoites in *Anopheles subpictus* was first reported in 1938 from Ennore-Nellore and Pattukottai of Madras Presidency in Tamil Nadu (Russel *et al.*, 1939). In recent years, it has been incriminated as a vector in Maldive Islands and a suspected vector in Lakshadweep Islands (Roy *et al.*, 1978) and a vector in coastal villages of south-east India (Panicker *et al.*, 1981). In view of the persistence of malaria in Bastar district, Madhya Pradesh, role of anopheline mosquitoes including *A. subpictus* in the epidemiology of malaria was investigated. Adult anophelines were collected routinely at fortnightly intervals between January 1980 and January 1982 from five topographically different areas of the region. The collections were made from various resting habitats and while biting man and animals. The wild-caught females were identified and dissected for oocysts and sporozoites.

Out of the 12,107 females of *A. subpictus* dissected, 3 females (0.025%) showed the presence of sporozoites in the salivary glands. The positive females were collected one on 23rd February, and other two on 14th July 1981, inside mixed dwellings from localities about 6 kms. from Jagdalpur town. These localities are surrounded by plains with paddy cultivation. The length and breadth of the sporozoites were measured and compared with the sporozoites found in the known vector species *viz.*, *A. culicifacies* and *A. fluviatilis* also collected during the study (Table 1). All were found similar to each other. It was also observed that the size and morphology of sporozoites were close to the human *Plasmo-*

Table 1. Comparative measurements of sporozoites of three species of *Anopheles* (in microns)

	Length			Breadth			Numbers
Species	Min.	Max.	Avg.	Min.	Max.	Avg.	measured
A. culicifacies	9.00	11.50	10.18	1	1.12	1.05	50
A. fluviatilis	9.00	11.00	10.40	1	1.50	1.15	35
A. subpictus	9.35	11.55	10.62	1	1.10	1.05	55

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dium (Garnham, 1966; Boyd, 1969). This finding is further supported by data obtained on density, host preference and longevity as detailed below.

The species was found in high densities during February to early March (Per man hour 19-48) and again from late June to August (PMH range 66-84). The ovariole dissections also indicated that the species lived up to 2 weeks in July. The anthropophilic index of 35.6% based on the precipitin test of 213 blood meals, also indicates that under certain ecological conditions, *A. subpictus* may feed on man and transmit the malaria parasites. However, there is a need to undertake long term intensive studies to establish its potential as a vector in Bastar district.

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Anopheles culicifacies: Mitotic Karyotype of Species C

K. VASANTHA[1], SARALA K. SUBBARAO[1], T. ADAK[1] and V.P. SHARMA[1]

Anopheles culicifacies which was earlier reported to be a complex of two sibling species (Green and Miles, 1980) is now found to be a complex of three sibling species (Subbarao *et al.*, 1983). These three sibling species—species A, species B and species C were identified with the help of fixed paracentric inversions in the polytene chromosomes. The inversion genotypes are $X+^{a}+^{h}$ $2+^{g^1}+^{h^1}$, xab $2g^1+^{h^1}$, and xab $2+^{g^1}h^1$, respectively, of species A, species B and species C. In addition to the differences in the rearrangements in the polytene chromosomes, morphological differences in the Y-chromosomes were observed in the males of species A and species B (Vasantha *et al.*, 1982). In species A, the Y-chromosome was metacentric while it was acrocentric in species B.

In order to study the mitotic karyotype in species C, twenty five metaphases each from female and male larval neurogonial cells were karyotyped. Species C colony was established from *A. culicifacies* populations from Singalkanch, a village in Surat distt., Gujarat. Preparation of mitotic chromosomes is described by Vasantha *et al.*, (1982).

The diploid number, as in other anophelines, was found to be six. Chromosome 2 was the largest pair constituting approximately 37.05 to 39.04% of total chromosome length (TCL). Chromosome 3 comprises of about 31.86 to 33.15% TCL. The arm ratios of chromosome pairs 2 and 3 were 1:1.4 and 1:1.1 respectively. The TCL of the X-chromosome ranged from 28.16% to 29.50% with an arm ratio of 1:2.25. Following Levan *et al.*, (1964) classification, both the autosomes were designated as metacentric and sex-chromosomes as submetacentric (Plate 1). With respect to total chromosome length and the position of centromere, autosomes of all the three sibling species are almost similar. Likewise, the X-chromosomes of all the sibling species are similar in being submetacentric while the Y-chromosome of species C which is submetacentric resembles that of species A and not of species B which is acrocentric (Vasantha *et al.*, 1982).

On the whole, the species C mitotic complement seems to bear a closer resemblance to the species A karyotype rather than to the species B karyotype. It may be pointed out that species C differs from species B by two fixed paracentric inversions on chromosome arm 2 while it differs from species A by three fixed inversions, two in the X-chromosome and one on the chromosome 2 arm.

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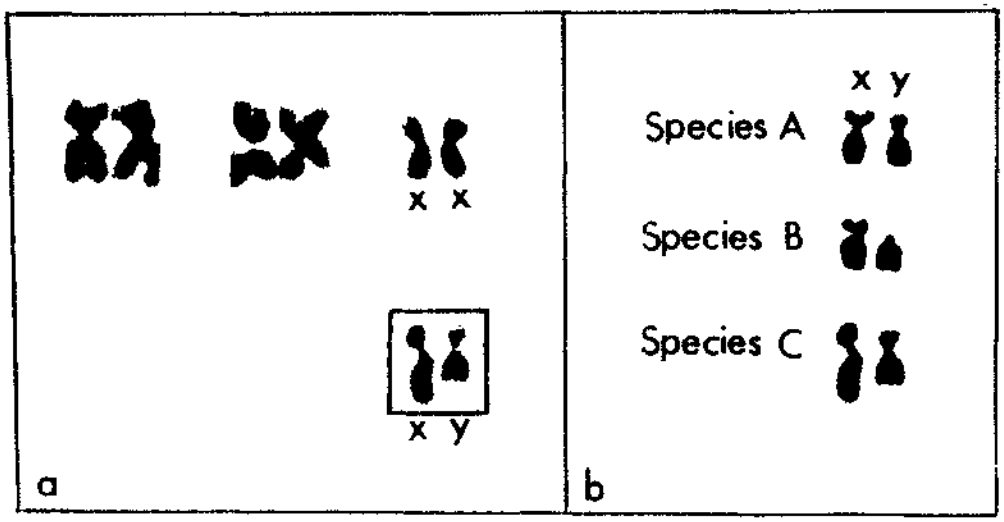

Plate 1 (a) Mitotic karyotype of species C.
(b) Variations in the mitotic Y-chromosome of the sibling species.
Magnification: X 1200

It may be observed that there is a great deal of variation in the TCL of the Y-chromosome between the three sibling species which varies from 20 to 28%. Such variations in the total genome of the Y-chromosome are not uncommon and have been known to occur both within and between closely related species.

Species A, species B and species C which are morphologically alike can now be distinguished either by the larval salivary polytene chromosomes or by the adult ovarian polytene chromosomes or by the male mitotic chromosomes.

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Malaria Epidemic in Shahjahanpur (U.P.)

R.K. CHANDRAHAS[1] and V. P. SHARMA[1].

Following 253 deaths in 28 villages of Negoyi PHC and 96 deaths from 10 villages of Tilar PHC of Shahjahanpur district of Uttar Pradesh during August to October 1983, an investigation was carried out by the Malaria Research Centre during 3rd week of October to determine the cause of these unusual deaths.

These PHCs are located at about 25 Km North of Shahjahanpur town. The data collected from the state health authorities revealed that these unusual deaths started occuring from first week of August and continued till the third week of October 1983 with maximum number of deaths during mid September.

The affected villages were situated in canal irrigated zone and had innumerable seepages and rain water collection sites.

The team carried out survey of fever cases in 8 out of the 28 affected villages in Negoyi PHC. Two villages located outside the affected area were also surveyed. Of a total of 377 blood smears collected and examined, 284 were found positive for malaria parasites giving the slide positivity rate of 75.33. Of the total positives, 273 (96.1%) were due to *P. falciparum*. Blood smears were also collected from the family members of the deceased in 5 villages. Out of 396 blood smears collected, 214 were positive for malaria, the SPR being 54.04. *P. falciparum* was found in 204 out of 214 positives (95.32%), Tables 1 and 2. It may be noted that a few days before this investigation was undertaken, the U.P. Health Department had completed administration of Mass Radical Treatment (MRT) in all the affected villages by the end of September 1983. The administration of Fever Radical Treatment (FRT) was in progress at the time of investigations during the third week of October 1983. Therefore, it was surprising to find such a high rate of falciparum malaria even after these treatments. There were 38 deaths among infants out of a total of 349 deaths (10.8%) in 11 out of 38 affected villages in both the Primary Health Centres. Only two infant blood smears were collected during the fever survey of which the one collected from Parsona village was positive for *P. falciparum*.

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Table 1. Results of fever survey

Village	Population	Number of blood smears collected	Number positive				Age group (yrs)											
							0—11*		1—4		5—9		10—14		15—19		20—24		25+	
				Pv	Pf	Mx	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Bentigontia	65	39	19	0	19	0	0	0	2	1	1	2	3	1	2	0	0	1	2	4
Dhandia	209	12	8	0	8	0	0	0	0	0	0	0	2	0	0	0	1	0	4	1
Jahanpur	561	49	35	0	34	1	0	0	5	0	2	1	3	2	1	1	1	0	9	10
Khaleelpur	420	27	13	0	13	0	0	0	0	0	2	1	1	0	0	0	1	0	4	4
Marakka	479	4	3	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1
Mohammedpur	640	87	77	1	76	0	0	0	11	2	5	8	3	3	1	1	2	0	26	16
Parsona	820	52	47	4	43	0	1	0	4	0	2	3	4	1	2	3	2	0	13	8
Vikrampur	860	107	82	2	77	3	0	0	4	2	5	6	5	2	5	1	3	1	30	10
	4054	377	284	7	273	4	1	0	26	5	15	21	21	10	11	6	10	2	89	60
Dhulia (vo)	587	120	24	3	21	0	0	0	1	1	2	3	3	1	1	1	1	0	5	2
Zindpur	482	131	30	10	20	0	0	0	2	0	2	1	1	2	1	1	3	0	5	2
	1069	251	54	13	41	0	0	0	3	1	4	4	4	3	2	2	4	0	10	4

*0 - 11 months, Pv - *Plasmodium vivax*, Pf - *P. falciparum*, Mx-Mixed infection
vo —Villages located outside the epidemic zone, M - Male, F - Female.

Table 2. Results of blood smear examination of the family members of the deceased

Village	Population	No.of blood smears collected	Number positive				Age groups (yrs.)													
							0--11*		1—4		5—9		10—14		15—19		20--24		25 +	
				Pv	Pf	Mx	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Mohammedpur	640	94	47	6	41	0	0	0	3	1	5	2	3	3	1	1	1	1	12	8
Parsona	820	57	24	0	24	0	0	0	2	0	1	3	1	1	2	1	1	3	3	6
Vikannapur	860	128	63	0	63	0	0	0	3	4	2	5	1	4	4	2	3	1	13	21
Jahanpur	561	89	59	3	55	1	0	0	7	4	7	4	7	3	2	0	1	0	8	12
Dhandia	209	28	21	0	21	0	0	0	0	1	1	4	5	0	2	0	2	0	5	1
Total	3090	396	214	9	204	1	0	0	15	10	16	18	17	11	11	4	8	5	41	48

*0--11 months

Pv—*Plasmodium vivax*, Pf—*P. falciparum*, Mx—Mixed infection M—Male and F—Female.

The investigations revealed an epidemic outbreak of falciparum malaria in these villages. It is quite reasonable to surmise that some of the unusual deaths recorded earlier were due to falciparum infection. The investigations also revealed that there was a complete breakdrown of surveillance in these areas and acute shortage of anti-malarials and microslides with the Multipurpose Workers (MPW) and other supervisory staff.

The Shahjahanpur episode is of great public health importance. It highlighted the value of good surveillance, insecticidal spraying targeted to achieve at least 75 to 80% coverage and improved laboratory services in fulfilling the objectives of the Modified Plan of Operations (MPO) of the National Malaria Eradication Programme (NMEP).

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INDIAN JOURNAL OF MALARIOLOGY

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Books/Monographs

Rao, T.R. (1981). *Anophelines of India* (W.Q. Judge Press, Bangalore).

Landau, I. and Y. Boulard (1978). In *Rodent Malaria*, edited by R. Killick-Kendrick and W. Peters (Academic Press Inc., London): 53-84.

Paper presented Symposium/Conference

Subbarao, S.K. *Cytoplasmic incompatibility in mosquitoes*. Paper presented to the International symposium on recent developments in the genetics of insect disease vectors, Bellagio, Italy, 20-24 April, 1981.

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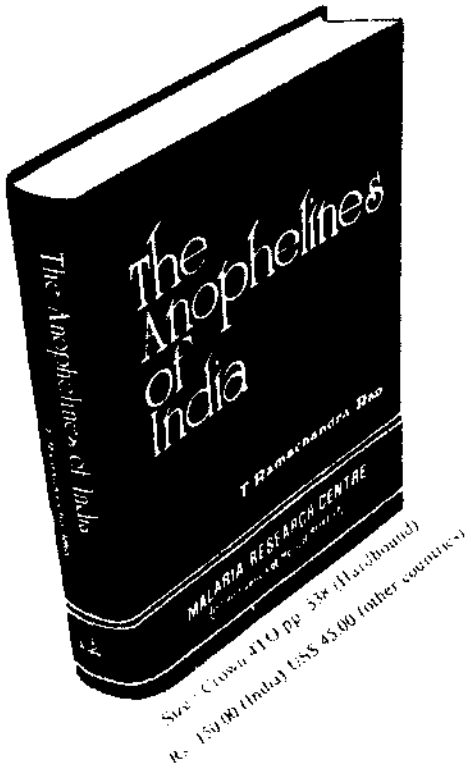

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