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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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Progress Towards a Molecular Vaccine against Malaria

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INTRODUCTION

Malaria is a major cause of human mortality and morbidity in the tropics. In rural Gambia, it has been estimated recently that the malaria mortality rate was 6.3 per 1000 per year in infants and 10.7 per 1000 per year in children aged 1-4 yrs (Greenwood *et al.*, 1987). These figures represent 4% and 25% of the total deaths for the respective age groups. The rationale for the development of immunological approaches to the control of malaria has been previously discussed (Ramasamy, 1987a). Since the time of that review, significant progress has been achieved in characterising protective antigens and in the understanding of the immunology of malaria. This has been particularly apparent in the case of *P. falciparum*, where the need for disease control is greatest, and is partly due to the ability to culture the parasite *in vitro*. Indeed preliminary vaccine trials in man have been performed using vaccines based on synthetic peptides and recombinant proteins. The purpose of this paper is to review recent progress in this area.

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TYPES OF VACCINE

There are three major stages in the life cycle of the parasite that are targets for vaccine development. A vaccine based on sporozoites is designed to prevent infection and therefore the development of the symptoms of the disease. Immunisation with antigens based on the asexual blood stages, while not preventing infection, is expected to reduce or eliminate parasites in the blood which are responsible for most of the pathology of malaria. Vaccination against the sexual blood stages is aimed to interfere with the ability of the parasite to infect mosquitoes and thereby prevent the transmission of disease to uninfected individuals in the population. Ideally, a vaccine against malaria should contain all three features viz., it should be anti-infection, anti-disease and anti-transmission and be based on a number of antigens. Such a 'cocktail' vaccine is likely to be more successful than one based on a single antigen or one directed against a particular stage of the parasite.

NATURE OF A VACCINE

Killed or attenuated pathogens have in the recent past provided some very successful vaccines. The smallpox and polio vaccines are two examples. Even if adequate quantities of malaria parasites can be obtained from *in vitro* culture

for this pupose, the use of such blood derived material for vaccination carries with it the risk of transmitting infectious agents such as the human immunodeficiency and hepatitis viruses. Such a vaccine would also contain large amounts of immunologically irrelevant material that can give rise to undesirable side effects. Some of this extra material may be counter productive in inducing a suppression of the immune response. Synthetic vaccines can be designed to avoid the inclusion of pathogen components that induce autoimmunity (Jahnke *et al.*, 1985). It is for these reasons that a vaccine based on defined molecules (Molecular or Subunit Vaccines) that are capable of inducing the necessary protective immune response are being developed (Ramasamy, 1988).

Molecular vaccines can take a number of forms. Proteins can be produced in large amounts by recombinant DNA techniques in bacteria, yeast or eukaryotic cell lines. The quantities of protein required for a mass vaccination campaign can theoretically be produced by this method. A recombinant vaccine composed of the surface antigen of the Hepatitis B virus produced and purified from yeast by Smith Kline Biologicals has been approved for human use in some countries. This will compete with the currently available plasma-derived vaccine containing the surface antigen. A recombinant protein based on the circumsporozoite (CS) protein of *P. falciparum* has been tested for safety and efficacy as a vaccine in man (Ballou *et al.*, 1987).

It was first demonstrated by Anderer (1963) that peptide fragments from the tobacco mosaic virus can inhibit precipitation of the virus by an antiserum and that immunisation with a hexapeptide fragment of the virus coupled to a carrier could induce the formation of virus neutralising and precipitating antibodies. This work has now been extended by a number of workers to produce neutralising antibodies against several viral pathogens and bacterial toxins. A synthetic peptide vaccine based on the C terminus of the beta subunit of the human chorionic gonadotropin hormone has been subjected to phase 1 clinical trial with encouraging results (Jones *et al.*, 1988). Synthetic peptides based on the repeat region of the *P. falciparum* CS protein (Herrington *et al.*, 1987) and a number of asexual blood stage antigens (Pattaroyo *et al.*, 1988) have been used as a basis for vaccinating man. Because synthetic peptides are often small molecules they are usually made immunogenic by coupling to larger protein molecules referred to as carriers. Such carrier molecules that have been used in human immunisations include tetanus and diphtheria toxoids. The carrier molecule is necessary to stimulate thymus processed lymphocytes or T cells that cooperate with the B lymphocytes which differentiate to produce antibodies to the peptide epitope. If immunity induced by vaccination is to be boosted under natural conditions both T and B epitopes must be included in a molecular vaccine. The use of peptide based vaccines without a carrier therefore necessitates the determination of epitopes recognised by T cells in addition to epitopes recognised by antibodies on candidate antigens. The determination of relevant T cell epitopes is also important if cellular immunity plays a major role in eliminating a malaria infection.

Recombinant vectors, viral or bacterial, carrying the gene for the relevant antigen may be used to deliver an immunising dose of the antigen. The considered viral vector for human use is *Vaccinia,* the virus that was the basis for immunisation against small pox and therefore of which we have considerable experience. *Vaccinia* has the advantage that it can accommodate a fairly large number of genes (Macket *et al.*, 1982; Panicali *et al.*, 1982). A disadvantage with *Vaccinia* is that it is not certain how many effective boosts may be given since an immune response against the virus itself may limit the replication of the virus in a secondary immunisation. Other viral vectors that have been considered for immunisation include *Herpes* (Roizman and Jenkins, 1985), Adenovirus (Davis *et al.*, 1986) and *Varicella*

(Chicken pox virus). *Varicella* has the advantage that it is a naturally spreading vector but, like other vectors, there is the possibility of serious disease in immuno-compromised individuals. Recently the CS protein gene of *P. berghei,* a mouse malaria parasite, was cloned into an attenuated strain of *Salmonella typhinurium* and the recombinant *Salmonella* used to orally immunise mice against *P. berghei* infection, with some success (Sadoff *et al.*, 1988). The use of a similar vaccine against *P. falciparum* sporozoites based on attenuated *S. typhi* may therefore be possible.

The use of anti-idiotypes as a possible vaccine (Finberg and Ertl, 1986) has seen little investigation in malaria. However, these may form the only basis for immunisation against carbohydrate epitopes in parasite antigens (Ramasamy and Reese, 1985; 1986).

The use of defined molecules, as opposed to killed or attenuated pathogens, results in the need for adjuvants to enhance the immune response. Complete Freunds adjuvant is unsuitable for human use because of its severe side effects. Adjuvants suitable for human use include muramyl dipeptide derivatives (Ellousz *et al.*, 1974), alum and immunostimulating complexes (ISCOMS) involving membrane proteins in a matrix of a glycoside Quil A (Morein *et al.*, 1984). More research is needed to develop strong adjuvants that are safe for human use. Recently, it has been shown that incorporation of synthetic peptides representing the CS repeats into hydrophobic proteosomes of meningococcal outer membrane protein via an acyl moiety renders them highly immunogenic (Lowell *et al.*, 1988). These proteosomes are known to be B cell mitogens but have been given to man as components of the group B meningococcal vaccine with no ill effects. One of the advantages of a live expression system for delivering a vaccine is the lack of a need for adjuvant. However, it is interesting to note that a recent human trial, of limited success, using polymers of peptides based on asexual blood stage antigens of *P. falciparum* (Pattaroyo *et al.*, 1988) was performed without either carriers or adjuvants.

A possible disadvantage with synthetic vaccines, especially those based on small peptides, is the capacity to induce auto-immunity. Shared sequences between the pathogen and normal human body proteins have been implicated in serious auto-immune complications (Jahnke *et al.*, 1985; Fujinami and Oldstone, 1985). Small peptides, because of their tendency to assume a number of different conformations in solution, are particularly prone to produce antibodies with a broadly cross-reactive specificity. Therefore, amino acid sequences considered for mass vaccination must be carefully checked against known sequences of human proteins and also tested experimentally for possible auto-immune reactions.

IMMUNISATION AGAINST SPOROZOITES

Evidence for immunity

Malaria infection in man is initiated by the innoculation of hundreds or thousands of sporozoites by the bite of an infected mosquito taking a blood meal. The sporozoites enter the blood stream and are usually cleared within minutes by the spleen and the liver. The sporozoites then multiply within hepatocytes to give rise to the extra erythrocytic forms (EEF). Each sporozoite may ultimately produce several thousand merozoites. These are released by the rupture of the hepatocyte after about one week from the initial infection. In the case of *P. falciparum* the merozoites then enter the blood stream and invade erythrocytes to undergo asexual multiplication. Stage specific, protective immunity against sporozoites has been achieved by the innoculation of relatively small numbers of irradiated sporozoites into rodents, monkeys and humans (Nussensweig and Nussensweig, 1984). This has encouraged investigations into the development of a molecular vaccine against sporozoites.

The circumsporozoite (CS) protein

It has been established that sporozoites of all the malaria species are covered on the outside with a single major coat protein referred to as the circumsporozoite or CS protein. The genes for the CS protein of *P. falciparum* (Dame *et al.*, 1984; Enea *et al.*, 1984), *P. vivax* (Arnot *et al.*, 1985) and *P. malariae* (Lal *et al.*, 1988) have been cloned and their structure determined. Only one copy of the gene is present in the haploid genome of the sporozoite and no introns have been observed. Like many of the parasite proteins that have been sequenced so far the CS protein contains a centrally located region composed of tandemly repeated sequences. In *P. malariae* the sequences NAAG and NDAG are repeated while in *P. vivax* the repeat is basically composed of the sequence GDRADGQPA with some variation and in *P. falciparum* of NANP with the variant NVDP. The repeat region is immunodominant and the bulk of antibodies produced against the CS protein are therefore directed against it. All the strains of *P. falciparum* and *P. vivax* sequenced so far, from different parts of the world contain the same immunodominant repeats, although the number of repeats varies between strains. This is unlike the situation in monkey malarias where significant variation of the repeats between strains has been observed (Sharma *et al.*, 1985; Cochrane *et al.*, 1986). It may be that conservation of the repeat structure is relatively important for parasite function and that the human malaria parasites have had insufficient time to evolve variants that are viable in man.

On the basis of structural predictions and homology analysis, the CS protein can be divided into several domains with possible functional relevance. There are typical signal and anchor sequences at the N and C termini. Two regions of charged residues are found, one immediately next to the N terminus of the repeats and the other closer to the C terminus. There is significant homology between *Plasmodium* strains in these regions indicating possible functional relevance. Indeed a synthetic peptide from the N terminal region immediately preceding the repeats of *P. falciparum* and *P. knowlesi* CS proteins (LKQPGDGNPDP in *P. falciparum and* KLKQPNEGQP in *P. knowlesi)* were reported to have affinities for the human hepatoma cell line HepG2-A16 *in vitro* (Aley *et al.*, 1986), suggesting that this might be a possible liver cell recognition site. Interestingly, a slightly longer *P. falciparum* peptide KPKHKKLKQPGDGNPDP was ineffective in the assay. Other workers have reported that antibodies to a synthetic peptide CKHKKKLKQPGDG was unable to block invasion of *P. falciparum* sporozoites into liver cells *in vitro* (Ballou *et al.*, 1985). A possible explanation is that the maturation of the CS protein, which is known to involve loss of 50-100 amino acids during biosynthesis, may involve cleavage in this region, to produce a molecule with binding activity to liver cells. The repeat region of all the CS proteins are predicted to form beta turns and it is likely that the repeating epitopes are arranged in a regular array on the surface of the sporozoites. It can be calculated that if there are 10^6 CS protein molecules on the surface of the *P. falciparun* sporozoite (Potocnjak *et al.*, 1982), then there are about 3×10^7 NANP repeats on the surface. The immunodominance of the repeat is readily explained by this feature.

A number of single amino acid substitutions and a ten amino acid insertion are found in the non-repeat region of the CS proteins in different strains of *P. falciparum* (de la Cruz *et al.*, 1987; Lockyer and Schwartz, 1987). Since no silent third base changes are observed in the four strains sequenced it is possible that the mutant proteins have been selected for under immunological pressure. Indeed one variant region corresponds to the putative liver cell binding site and others to putative T cell epitopes.

Humoral immunity against sporozoites

An early observation was that when CS proteins are cross-linked on the surface of sporozoites by antibodies, the coat is gradually shed towards

one end of the parasite in a process called the circumsporozoite precipitation or CSP reaction (Vandenberg *et al.*, 1969). Parasites that have shed their coats are no longer infective.

However, the induction of a CSP reaction may not be required for protection. In a murine model, the passive transfer of IgG and Fab fragments of a monoclonal antibody (mab) against the *P. berghei* CS protein repeat were shown to produce some protection in mice against challenge with *P. berghei* sporozoites (Potocnjak *et al.*, 1980). Fab fragments do not induce a CSP reaction since they are unable to cross-link the CS protein. Protection was however dependent on the dose of antibodies and the number of sporozoites used for challenge. 100 μg/mouse of the IgG antibody protected against 10^3 but not 10^4 sporozoites. 300 μg/mouse was needed to protect against 10^4 sporozoites. *In vitro* studies on sporozoite invasion of human hepatoma cells with mabs to *P. berghei, P. vivax* and *P. falciparum* sporozoites suggest that the antibodies inhibit invasion (Leland *et al.*, 1984; Hollingdale *et al.*, 1984) and this might occur through simple steric hindrance of the sporozoite-hepatocyte interaction. However, high concentrations of antibodies are required for efficient inhibition (250 μg/ml). Polyclonal antibodies to the repeat region of *P. falciparum* CS protein were also found to inhibit invasion (Ballou *et al.*, 1985). But, in a study performed in Kenya where the rates of reinfection in drug-cured individuals were followed in an area with intense malaria transmission, no correlation between the levels of anti-sporozoite antibodies and the resistance to re-infection was found (Hoffman *et al.*, 1987).

Role of cellular immunity against sporozoites

In an early study in mice depleted of T and B lymphocytes, it was observed that immunisation with irradiated sporozoites led to protection against *P. berghei* sporozoite challenge in B deficient but not in T deficient mice (Chen *et al.*, 1977). This early observation on the role of T cells was not fully appreciated until more recent work confirmed this observation. In an elegant analysis of factors governing immunity to *P. berghei* sporozoites, Egan *et al.* (1987) showed that immunisation with irradiated sporozoites led to significantly better immunity (in terms of resistance to greater numbers of sporozoites in the challenge inoculum) than immunisation with synthetic peptides or recombinant protein. No correlation was observed between the titres of antibodies as measured by immunofluorescence, CSP reaction or inhibition of hepatoma cells and protection. Protection was not adoptively transferred with B-enriched spleen cells from mice protected with irradiated sporozoites. In contrast, T-enriched spleen cells from the same mice were able to transfer protection. However, the ability of mab (100 μg/mouse) to protect against challenge with relatively small numbers of sporozoites (500) on passive transfer was confirmed.

In a recent study Ferreira *et al.* (1986) showed that gamma interferon which is produced by activated T cells is toxic to EEF. In a follow up study Schofield *et al.* (1987) showed that antibodies to gamma interferon administered to mice immunised with irradiated *P. berghei* sporozoites abrogated the normally observed protection. Such mice hyperimmunised against irradiated sporozoites were normally resistant to challenge with upto 1.5×10^5 sporozoites. Depletion of CD8+ but not CD4+ lymphocytes (which are surface markers for cytotoxic/suppressor and helper T lymphocytes respectively) in mice eliminated protective immunity in such mice. Some evidence for a synergistic effect between antibodies and T cells in passive transfer of immunity was obtained in these experiments. The importance of CD8+ cells in immunity to sporozoites has also been reported by Weiss *et al.* (1988).

The mechanism of action of gamma interferon and CD8+ cells is not entirely clear. It is known

that gamma interferon inhibits the growth of exoerythrocytic forms (EEF) in the liver (Ferreira *et al.*, 1986). Presumably, the CD8+ T cells recognise processed sporozoite antigen on the surface of hepatocytes, in association with MHC (major histocompatibility complex) Class I molecules on the cell surface, and then elaborate gamma interferon which is toxic to the EEF. An alternative is for the T cells to exert a direct cytotoxic effect on infected hepatocytes consequent to specific recognition of antigen + MHC Class I molecules on the surface. It is known that CS proteins are found in liver cells after invasion and therefore these as well as other sporozoite proteins could be processed for expression on the surface.

Identification of T cell epitopes on the CS protein

In view of the role of T cells in immunity to sporozoites it is clearly important to identify the epitopes recognised by T cells in CS proteins. Using $(NANP)_{40}$ as an immunogen it was shown that only mice bearing I-A b Class 2 histocompatibility antigens were able to produce antibodies. This demonstrates genetic restriction at the level of T cell recognition, with only I-A b bearing mice recognising an epitope based on $(NANP)_{40}$ (Del Giudice *et al.*, 1986). A strong correlation between regions capable of forming amphipathic alpha helices in proteins and T cell epitopes in model proteins has been reported (Delisi and Berzovsky, 1985). Using an algorithm for predicting regions of alpha helical amphipathicity a number of other T cell stimulating epitopes have been identified in non-repeat region of the *P. falciparum* CS protein (Good *et al.*, 1988a; Sinigaglia *et al.*, 1988; Rzepczyk *et al.*, 1988b). One of the predicted T cell epitopes termed Th2R (residues 326 to 343 in the 7G8 strain CS protein sequence) was used to construct a synthetic immunogen Th2R-NP $(NANP)_5$ NA which then elicited antibodies in B10Br and B10.A(4R) mice which were of the I-A k haplotype (Good *et al.*, 1987). Genetic restriction of T cell recognition was also suggested by the limited number of mice strains that produced anti NANP antibodies when immunised with the whole CS protein in recombinant *Vaccinia* (Good *et al.*, 1986).

In a study of the human T cell proliferative response to overlapping peptides of the *P. falciparum* CS protein performed with peripheral blood lymphocytes from donors in Gambia, certain immunodominant regions were located including Th2R (Good *et al.*, 1988a). Interestingly there was a correlation between these dominant T epitopes and the regions of the *P. falciparum* CS protein subject to variation between strains (de la Cruz *et al.*, 1987; Lockyer and Schwartz, 1987) suggesting that immune pressure at the level of T cells may be the driving force for such variation.

Prospects for a sporozoite vaccine

Two groups in the United States have already performed human trials with immunogens based on the CS protein of *P. falciparum*. In one trial a 12 amino acid synthetic peptide $(NANP)_6$ conjugated to tetanus toxoid with aluminium hydroxide as an adjuvant was administered (Herrington *et al.*, 1987). Seroconversion occurred in only 53% and 71% of the recipients of 100 μg and 160 μg of vaccine in an ELISA assay. Three vaccinees with the highest antibody titres were challenged by the bite of five infected mosquitoes (at least one mosquito with >100 sporozoites per paired salivary gland). One individual did not develop malaria while two had a slightly delayed patency. The reason for the poor antibody response in the vaccinees is not clear, but may be due to epitope suppression resulting from prior exposure to tetanus toxoid or inadequate adjuvant effect.

In the second study, a recombinant vaccine $(R32tet_{32})$ composed of MDP $(NANP)_{15}$ NVDP $(NANP)_{15}$ NVDPtet_{32} (where tet_{32} is the first 32 amino acids of a tetracycline resistance gene read out of frame) was administered in alum as an adjuvant (Ballou *et al.*, 1987). In general, antibody response was poor with significant

boosting being observed in only 1/15 individuals and that in a person receiving the highest dose of immunogen (800 μg). Only this individual was protected against sporozoite challenge. Two other vaccinees with relatively good antibody response showed a delayed patency. Because of the data on genetic restriction of the response to $(NANP)_n$ in mice, it is quite likely that the poor antibody response to the $R32tet_{32}$ in man is due to the genetic restriction in the recognition of $(NANP)_n$ and tet_{32} by T cells. In this context the use of the CS repeats in meningococcal outer membrane protein proteosomes, which gives vastly better antibody responses, might be important (Lowell *et al.*, 1988).

Given the observations on the importance of T cell immunity in *P. berghei* murine malaria and the observations on genetic restriction of the response to *P. falciparum* CS protein, it is clear that greater emphasis has to be placed on stimulating T cell immunity to sporozoites for efficient protection. It is noteworthy that alum is a poor inducer of T cell immunity compared to Freunds adjuvant. Antibody at high concentrations probably also has a role in immunity but the required concentration may be difficult to achieve and maintain for long periods. The data from murine studies on vaccination with *P. falciparum* CS repeats and also with *P. vivax* CS protein (Campbell *et al.*, 1987) and the limited human vaccination data with *P. falciparum* suggest that the influence of genetic restriction on the response to peptide vaccines is quite important. Whether individuals in a human population respond in a restricted manner like congenic strains of mice or whether they respond more like outbred mice to molecular vaccines is an important consideration in the design of a vaccine construct. Also recent work suggests that the incorporation of synthetic peptide antigens derived from the CS protein in an emulsion with adjuvant that also contains recombinant Interleukin 2 (IL-2) results in antibody production in normally non-responder mice. This suggests that IL-2 delivered in this manner overcomes the immune response gene (Ir gene) defect present in non-responder T cells in a manner that is not clearly understood (Good *et al.*, 1988b). The result implies that IL-2 delivered with similar peptide vaccines in man may be effective in converting potential non-responders into antibody producing responders thereby inducing some protection.

Two categories of anti-sporozoite vaccines may be considered. One that would require to be effective for relatively short periods is suitable for tourists and military personnel from non-endemic countres going into endemic areas. On the other hand a vaccine that is to be used in endemic areas to prevent malaria will need to be effective for long periods. This may require frequent boosting and one possible way that this can take place is naturally through the innoculation of sporozoites by the bite of infected mosquitoes. For a molecular vaccine to be boosted naturally, the initial immunising antigen must contain T epitopes that are present on the CS protein which will generate memory T cells (both helper and cytotoxic T cells). Recent studies based on the influenza virus system (Scherle *et al.*, 1986) however suggest that Th epitopes present on other sporozoite antigens may be able to provide T cell help for antibody formation against the CS protein.

It is also clear that the epitope recognised by cytotoxic T lymphocytes (CTL) may be an important constituent of a vaccine against sporozoites. An epitope recognised by CTL on the *P. falciparum* CS protein has been recently determined, as being residues 368-390 in the 7G8 sequence, using CTL from mice immunised with *Vaccinia* carrying the CS gene, L cells transfected with the CS gene and a series of synthetic peptides spanning the CS sequence (Kumar *et al.*, 1988). Interestingly this region is also subject to variation in different *P. falciparum* strains (de la Cruz *et al.*, 1987).

Antigens of the EEF

Advances in *in vitro* culture of the EEF of *P. vivax* (Mazier *et al.*, 1984) and *P. falciparum* (Smith *et al.*, 1984) have permitted a greater exploration of the antigens of these stages which may be of importance for immunisation since they could be the target of a cytotoxic T cell response. Using a human serum with sporozoite and liver stage restricted specificity, a clone corresponding to an EEF antigen was isolated from a genomic expression library of *P. falciparum* DNA (Guerin-Marchand *et al.*, 1987). The antigen was shown to contain a 17 residue repeat. Clearly the identification of more antigens of the EEF and their determination of their ability to be recognised by cytotoxic T cells is an important area for future investigation.

ASEXUAL BLOOD STAGES

Introduction

Considerable progress has been recently made in identifying asexual blood stage antigens that might be useful vaccine candidates. The approaches taken by different groups can be put into three broad categories. In one approach, exploited with great success by the Walter and Eliza Hall Institute group in Melbourne, sera from individuals in a *P. falciparum* hyper-endemic area of Papua New Guinea were used to screen a recombinant cDNA library made from *P. falciparum* blood stages to identify clones coding for parasite antigens (Kemp *et al.*, 1983). The rationale here was that sera from partially immune adult individuals from endemic areas would contain antibodies against protective antigens. Once a clone is identified, DNA sequencing of the insert and full length genomic clones isolated by hybridisation with the insert, rapidly leads to a determination of the amino acid sequence of the antigen. A related approach involves the use of sera from immune *Aotus* monkeys to identify recombinant proteins from a cDNA library (Ardeshir *et al.*, 1985). In both these cases sera from non-immunes provide a control for the specificity of the detected recombinant clones. The characteristics of the antigens corresponding to the identified clones could be studied in the parasite by the use of antibodies affinity purified on the recombinant clone (Crewther *et al.*, 1986). This approach has the advantage that a very large number of clones can be identified quickly. However, it is not certain that most of the antibodies in "immune" sera are protective, since it has been argued that the presence of immunodominant repeats in many parasite antigens and their extensive cross-reactivity is directed towards producing low affinity antibodies against antigens irrelevant to protection (Anders, 1986). The selection of putative protective antigens among the myriad of irrelevant antigens by this method has therefore relied heavily on additional information provided by other approaches.

A second major approach has been to identify potentially protective antigens by the use of a combination of mabs, localisation of the antigen within the parasite by immunofluorescence and immuno-electron microscopy and drawing analogies with protective antigens of murine malaria. For example, it was demostrated very early on that a high molecular weight merozoite surface antigen and antigens in the rhoptry organelles of the merozoites of *P. yoelli* were able to protect mice after immunisation (Holder and Freeman, 1981). Corresponding *P. falciparum* antigens present on the merozoite surface and rhoptries have been identified using mabs and relevant recombinant clones isolated using monospecific polyclonal sera produced against purified antigens (Hall *et al.*, 1984; Certa *et al.*, 1988).

A third type of approach has utilised the specificity of mabs and the ability to assay the effect of the antibodies on parasite growth *in vitro*. Mabs that inhibit parasite growth *in vitro* have been reported not only against the well-studied merozoite surface and rhoptry antigens (Perrin

and Dayal, 1982) but also against antigens in the parasitophorous vacuole (Kara *et al.*, 1988; Horii *et al.*, 1988) and other novel internal parasite antigens (Ramasamy *et al.*, 1988).

Mechanism of immunity against asexual blood stages

Early work with unfractionated *P. falciparum* material obtained from *in vitro* cultures indicated that significant protection could be induced in owl monkeys after immunisation in either Freunds adjuvant or muramyl dipeptide (Siddiqui, 1977; Reese *et al.*, 1978). The sera from immune owl monkeys inhibit parasite growth *in vitro* (Reese and Motyl, 1979) although no direct correlation between *in vitro* inhibition and *in vivo* protection were reported in a study in *Saimiri* (squirrel) monkeys (Fandeur *et al.*, 1984). The ability of antibodies to inhibit the growth of parasites *in vitro* has been confirmed with mabs, although 100% inhibition of invasion has been reported to require antibody concentrations ranging from <2 μg/ml to 250 μg/ml (Perrin and Dayal, 1982; Schofield *et al.*, 1986; Brown *et al.*, 1986). Antibodies to a 140 kDa merozoite surface antigen on the monkey malaria parasite *P. knowlesi* were reported to inhibit reinvasion by agglutinating the merozoites (Miller *et al.*, 1984). Cross-linkage of the surface coat by antibodies (Lyon *et al.*, 1986) may also interfere with the invasion process. Since merozoite release and subsequent reinvasion is a very rapid process it has long been recognised that the rate of binding antibody molecules to merozoite components might be a limiting factor. High concentrations of anti-merozoite antibodies might therefore be required to inhibit invasion (Saul, 1987).

In studies on murine models of malaria using *P. yoelli,* evidence was obtained to suggest that immunity against a merozoite surface antigen was partly cell-mediated (Freeman and Holder, 1983). *P. chabaudi* (a murine equivalent of *P. falciparum)* infections are resolved in B cell deficient mice (Grun and Weidanz, 1981) and immunity can be adoptively transferred with an L3T4+, gamma interferon and IL-2 secreting T cell clone to athymic nude mice (Brake *et al.*, 1988). Studies on murine models have implicated tumour necrosis factor or TNF (Clarke *et al.*, 1987; Bate *et al.*, 1988) and hydrogen peroxide (Ockenhouse and Shear, 1984) produced by activated macrophages in toxicity to intraerythrocytic parasites. Studies on human malaria suggest that T cells from immune individuals in endemic areas produce high levels of gamma interferon and IL-2 in response to stimulation with parasite antigen (Troye-Blomberg *et al.*, 1985). Gamma-interferon activated monocyte-derived macrophages are known to induce crisis (or degenerate) forms of *P. falciparum* blood stages *in vitro* and this is at least partly due to production of hydrogen peroxide (Ockenhouse *et al.*, 1984) and possibly tumour necrosis factor. An elegant study performed in Gambia concludes that recovery from acute malaria in children is at least partly due to parasite toxic products released from monocyte/macrophages activated by soluble factors from antigen activated T cells (Brown *et al.*, 1986). However, parasite inhibitory antibody was also found in the sera of children recovering from malaria, reaching a peak in convalescent children (Brown and Greenwood, 1985). Furthermore, antibody-mediated-immunity was found to be more and cell-mediated immunity less dependent on the strain of parasite (Brown and Greenwood, 1985). Thus one can reach a provisional conclusion that while antibody at high concentration may protect against infection by acting on extra-cellular merozoites, such immunity might be strain specific and exposure to different strains over a long period may be required to develop cross-protective antibodies. Cellular immunity, dependent on T cells acting in concert with monocyte/macrophages may provide immunity against a variety of strains early in life and might be particularly important in resolving an established infection. It should be remembered however that *P. yoelli* unlike *P. falciparum* infects reticulocytes that

have Class I histocompatibility antigens on the surface. Since erythrocytes lack histocompatibility antigens and cytotoxic T cells recognise foreign antigen in the context of Class I molecules, the mechanisms of cellular immunity operative against erythocyte invasive parasite such as *P. falciparum* and *P. chabaudi* may be different from those against *P. yoelli* and *P. vivax*. More extensive investigations are however clearly needed to fully justify these conclusions.

It is relevant to consider the various antigens that are possible candidates for inclusion in a vaccine against asexual blood stages. They are categorised by location within the parasite where possible since molecules present in the same location may have common features that influence their use in a vaccine.

Antigens on the surface of the infected red cell

Infection with *P. falciparum* results in alterations of the host erythrocyte membrane. The most prominent change morphologically in mature parasites is the appearance of collections of electron dense material underneath the lipid bilayer forming protrusions of the membrane referred to as knobs. Antibodies from immune sera bind largely, if not exclusively, to the outside surface of knobs in trophozoites and schizonts (Langreth and Reese, 1979). This suggests that parasite-derived proteins may be inserted into the host cell membrane and that these might be suitable targets for protective immunity. Antigens on the surface of parasitised red cells are particularly suitable as targets since they are readily accessible to host antibodies over a significant time period (Ramasamy, 1981). A knob associated histine rich protein or KAHRP of Mr 85-10kDa in different isolates which is not found in parasite strains lacking knobs has been desribed (Kilejian, 1979). However the evidence is fairly conclusive that this protein is not exposed on the outside surface of the red cell. A high molecular weight antigen termed the mature parasite infected erythrocyte surface antigen or MESA (Mr 250kDa) associated with the cytoskeleton and showing considerable strain variation has been reported (Coppel *et al.*, 1986). MESA has been reported to be different (Howard *et al.*, 1987) from a strain specific, high Mr antigen exposed on infected erythrocytes on the basis of accessibility to lactoperoxidase catalysed iodination (Leech *et al.*, 1984). The latter termed Pf EMP 1 (EMP for erythrocyte membrane protein) or the cytoadherence protein mediates the cytoadherence of mature parasites to capillary endothelium and leads to the sequestration of mature *P. falciparum* in capillaries. This process may enable the parasite to escape macrophage-mediated killing in the spleen and liver and could play a role in cerebral malaria by blocking capillaries in the brain. The cytoadherence protein is located in the region of knobs but knobs may be produced by parasites that have lost their capacity to cytoadhere to endothelial cell lines *in vitro* (Udeinya *et al.*, 1983). The isolation of a recombinant clone coding for the cytoadherence protein is being pursued intently but to date no sequence information on the protein has been published.

Two reports suggest that the *P. falciparum* endcodes and transferrin receptor of Mr 93-102kDa that is incorporated into the infected erythrocyte membrane (Halder *et al.*, 1986; Rodriguez and Jungery, 1986). An exposed portion of the receptor may be antigenic and therefore a suitable target for immunological attack.

Recent work suggests normal erythrocyte surface components may be modified in mature parasites. The modifications which may occur in the vicinity of knobs may be detected by changes in lectin binding (Sherman and Greenan, 1986; Ramasamy, 1987b). Autoantibodies to senescent antigen present in normal human serum has been reported to bind to knobs and be directed against altered Band 3 or the anion transport protein (Winograd *et al.*, 1987). Caution is therefore needed in interpreting reports on the

presence of parasite-derived antigens exposed on the outside surface of the host erythrocyte.

A 155kDa protein present in the rhoptry-microneme complex of merozoites is deposited on the erythrocyte membrane during invasion and has been termed the ring-infected erythrocyte surface antigen or RESA (Coppel *et al.*, 1984) or alternatively as Pf155 (Perlmann *et al.*, 1984). It is generally agreed that RESA/Pf155 is inserted on the cytoplasmic side of the red cell membrane and not exposed to the outside. A series of conserved 11 amino acid repeats in the middle of the molecule and 4 and 8 animo acid repeats towards the C terminus are a feature of RESA/Pf155 from different isolates. Immunisation of owl monkeys with recombinant proteins containing the repeats in Freunds adjuvant has been reported to induce protection (Collins *et al.*, 1986) and antibodies to RESA/Pf155 to inhibit parasite growth *in vitro* (Udosangpetch *et al.*, 1986). A number of T cell epitopes on RESA/Pf155 recognised by man have now been determined (Rzepcyzk *et al.*, 1988a). RESA/Pf155 therefore appears to be a good candidate antigen for a malaria vaccine although the mode of immunological protection has not been entirely clarified.

Antigens of the rhoptry organelles

The contents of the paired rhoptry organelles of merozoites are extruded during invasion and have been postulated to give rise to the parasitophorous vacuole (Bannister *et al.*, 1986). A complex of proteins of Mr 145, 135 and 104kDa have been identified by immunoprecipitation with mabs (Campbell *et al.*, 1984) and a cDNA clone corresponding to the 104kDa protein sequenced (Coppel *et al.*, 1987). The 104kDa protein probably corresponds to the rhoptry protein reported to be released onto the erythrocyte membrane during invasion (Sam-Yellowe *et al.*, 1988). The three proteins are reported to be structurally unrelated, and a mab which precipitates the complex has been shown to weakly inhibit parasite growth *in vitro* (Cooper *et al.*, 1988). Another complex of proteins of Mr 78,63,42 and 40kDa (Mr varying slightly in reports from different groups) were also identified in rhoptries with mabs (Campbell *et al.*, 1986; Howard *et al.*, 1986). The 63kDa protein is produced from the 78kDa protein by processing while the smaller components are unrelated to the 78kDa protein (Clark *et al.*, 1987; Schofield *et al.*, 1986). A mab directed against the 78kDa component was inhibitory as was another which precipitated the whole complex (Schofield *et al.*, 1986). It has recently been shown that the larger 78kDa component is a neutral serine protease which is activated by a phosphatidyl inositol specific phospholipase C (Braun-Breton *et al.*, 1988). The isolation of a clone for a 41kDa rhoptry protein (presumably the 42 or 40kDa component identified by others) has enabled its sequence to be determined (Certa *et al.*, 1988). Interestingly, the protein appears to be the *P. falciparum* equivalent of the glycolytic enzyme aldolase based on sequence homology. The purified 41kDa protein effectively protects squirrel monkeys on immunisation (Perrin *et al.*, 1985) unlike the immunisation of owl monkeys with antigens of the 145, 135 and 104kDa complex (Siddiqui *et al.*, 1987).

Recently a 255kDa rhoptry antigen was identified in *P. falciparum* (Roger *et al.*, 1988) and this could represent the equivalent of the highly protective 235kDa rhoptry protein of *P. yoelli* (Holder and Freeman, 1981). A 55kDa glycosylated rhoptry protein that probably contains a glycophosphatidyl inositol membrane anchor has been recently cloned (Smythe *et al.*, 1988). The mechanism by which antibodies to rhoptry proteins exert their inhibitory effect is not known with certainty, but this could occur by their binding to proteins secreted during invasion and thereby interfering with the invasion process.

Merozoite surface antigens

Analogous to the protective 230kDa *P. yoelli* merozoite surface antigen (Holder and Freeman, 1981), a 185-200kDa precursor glycopro-

tein that is processed to smaller proteins (predominantly, 83, 42 and 19kDa fragments) that are found on the merozoite surface was characterised in *P. falciparum* (Holder and Freeman, 1984). The sequence of the antigen has been determined by gene cloning (Holder *et al.*, 1985; Mackay *et al.*, 1985). The protein has an N-terminal signal peptide, a hydrophobic C-terminus which may or may not act as anchor sequence, since the membrane form of the protein has a myristylated phosphatidyl inositol moiety (Haldar *et al.*, 1985; Ramasamy, 1987b), and numerous potential glycosylation sites. The oligosaccharide side chains may modulate the antigenicity of the native protein (Ramasamy and Reese, 1985; 1986). There is a short segment of repetitive polypeptide sequence near the N terminus. Analysis of the structure of the protein from several isolates suggests that the protein is coded for by dimorphic alleles, represented by the MAD20 and K1 isolates, that can undergo limited genetic exchange within the N terminal third or so of the molecule (Tanabe *et al.*, 1987; Peterson *et al.*, 19 3).

It is believed that the processed fragments of the protein exist as a complex on the merozoite membrane (McBride and Heidrich, 1987) with the glycosylated 42 and 19 kDa proteins being anchored to the membrane. The 42 and 19kDa molecules represent the C terminus of the precursor protein. Only the 19kDa fragment is found in ring stage parasites and it is reasonable to suppose that the others are shed as the merozoite coat during invasion (Aikawa, 1978).

Immunisation with this antigen which is also called the precursor to the major merozoite surface antigens (PMMSA) or synthetic peptides derived from the sequence protect both owl and squirrel monkeys against malaria (Hall *et al.*, 1984; Perrin *et al.*, 1984; Cheung *et al.*, 1986; Siddiqui *et al.*, 1987). A recent study using recombinant clones identified both T and B cell epitopes within a non-polymorphic region of PMMSA (Crisanti *et al.*, 1988). Non-polymorphic T epitopes recognised by Melanesians from Papua New Guinea and the Solomon Islands have also been identified (Rzepcyzk *et al.*, 1988c).

A 45kDa glycosylated and myristilated smaller surface antigen or GYMSSA that is unrelated to PMMSA has been identified (Ramasamy, 1987c). Mabs against this antigen inhibit parasite growth *in vitro* and epitopes recognised by the mabs have been identified (Epping *et al.*, 1988; Ramasamy *et al.*, 1990). The complete amino acid sequence of the antigen has been determined and shown to contain a signal peptide, a hydrophobic anchor and a possible consensus sequence for attachment of a glycophosphatidyl inositol moiety (Smythe *et al.*, 1988). While GYMSSA shows some antigenic variation (Ramasamy, 1987c) conserved regions will clearly be of value for immunisation. T epitopes on the molecule recognised by man have been successfully identified (Rzepcyzk *et al.*, 1988c).

Antigens of the parasitophorous vacuole

A 22kDa antigen that shares an epitope with the CS protein (Hope *et al.*, 1984) based on the CS repeat sequence has been sequenced (Coppel *et al.*, 1985; Simmons *et al.*, 1987). The protein termed exp-1 has been shown to be located in the parasitophorous vacuole membrane and in vescicles and inclusions in the cytoplasm of the host red cell (Simmons *et al.*, 1987; Kara *et al.*, 1988). A mab directed to a linear epitope on the antigen inhibits parasite growth *in vitro* (Kara *et al.*, 1988). Amino acid sequence data is also available on a 126kDa parasitophorous vacuole antigen (Delplace *et al.*, 1987; Horii *et al.*, 1988) against which inhibitory mabs have been identified (Banyal and Inselburg, 1985). This antigen is proteolytically cleaved and shed into the culture medium during merozoite release (Delplace *et al.*, 1987).

The mechanism by which antibodies to parasitophorous vacuole antigens produce inhibition of reinvasion is not clear, but it is conceivable that the antibodies bind to the antigens after the

schizont ruptures and then inhibit a vital parasite process. An alternate possibility, that antibodies can enter the mature parasite by crossing the red cell membrane, to produce inhibition of parasite functions, is intriguing, and has been reported (Rodriguez and Jungery, 1986). However, this concept needs to be further investigated.

Other target antigens

At least two other antigens, which may have at least a transient location in the parasitophorous vacuole, have been reported to be recognised by inhibitory mabs. These are the S antigen (Saul *et al.*, 1984) and a 26kDa antigen for which partial amino acid sequence has been obtained (Ramasamy *et al.*, 1988). Again the mechanism of inhibition by antibodies is not clear, but in the case of the 26kDa antigen, at least, may involve reaction with the merozoite surface or a physical blockade by the formation of an immunoprecipitate around the newly released merozoites.

The invasion of red cells by merozoites probably involves the specific recognition of red cell surface molecules by receptors on the merozoites. The blockade of such receptors by specific antibodies might be expected to inhibit invasion and such molecules are therefore candidates for vaccine development. 155kDa and 130kDa parasite proteins that bind to red cell glycophorin have been identified and the sequence of the 130kDa protein determined (Kochan *et al.*, 1986). The protein contains a 50 amino acid sequence repeated 11 times and antibodies to the repeat inhibit parasite growth *in vitro*. RESA/Pf155 has also been reported to have an affinity for glycophorin (Udomsangpetch *et al.*, 1986). Other lectin-like proteins present on parasites, of Mr 140, 70 and 35kDa, recognising N-acetyl glucosamine containing glycophorins have been reported but no sequence data is available (Jungery *et al.*, 1983). A 175kDa *P. falciparum* protein that binds only to parasite susceptible red cells and which might act as a bridge between erythrocytes and merozoites in a strain-specific manner has been reported (Camus and Hadley, 1985). No sequence data is available on this potentially important molecule. Unlike in *P. falciparum*, the invasion of red blood cells by *P. vivax* and *P. knowlesi* apparently has an obligatory requirement for the presence of the Duffy blood group antigen on the cells (Miller *et al.*, 1975). It appears therefore that different plasmodia have evolved different red cell recognition mechanisms. It has been reported that parasites within a given species may also retain some degree of plasticity in invasion process by employing several receptor molecules (Perkins and Holt, 1988).

Prospect for an asexual blood stage vaccine

Several examples have been given above where purified antigens have been used to immunise monkeys against falciparum malaria with considerable success. Three out of four squirrel monkeys were protected against fulminant infection when immunised with a 42 residue peptide derived from the N-terminal region of the 83kDa merozoite surface antigen (Cheung *et al.*, 1986). Using sequence data obtained by N-terminal sequencing 55kDa and 35kDa *P. falciparum* antigens to synthesise small peptides and a peptide from the N-terminus of the 83kDa processed antigen of PMMSA, Pattaroyo *et al.* (1987) were able to obtain significant protection in owl monkeys after immunisation with peptides coupled to bovine serum albumin in Freunds adjuvant. Immunisation with a combination of the three peptides led to sterilising immunity in six out of six animals. After this encouraging result, a synthetic disulphide linked polymer (Mr 150kDa) based on the three peptides and the CS repeat was prepared and used to immunise volunteers without the use of adjuvant. Four out of five individuals were protected by the immunisation (Pattaroyo *et al.*, 1988). A polymer based on the CS repeat, the Th2R epitope of the CS protein and the 11 amino acid repeat of Pf155/RESA was considerably less

effective or ineffective (Pattaroyo *et al.*, 1988). These results are clearly encouraging from the point of view of developing an asexual stage vaccine. There are at the present time several other candidate antigens, whose sequences are known, and for some of which epitopes recognised by inhibitory mabs have been characterised. This information can be used to design synthetic vaccines.

TRANSMISSION BLOCKING IMMUNITY

Male and female gemetocytes ingested with blood undergo gametogenesis in the mid-gut of the mosquito. Within 10 mins the gametes become extracellular and undergo fertilisation. The zygotes that are formed become motile ookinetes and penetrate between the cells of the midgut wall to form oocysts. The oocysts mature to produce sporozoites that migrate to the salivary glands to continue the cycle of infection. It has been known for some time that antibodies in the host to antigens on extracellular gametes can block the infectivity of *Plasmodium* gametocytes to mosquitoes (Huff *et al.*, 1958; Gwadz, 1976). This phenomenon termed transmission blocking immunity has been shown to occur in *P. falciparum* (Renner *et al.*, 1983) and *P. vivax* (Munesinghe *et al.*, 1986). The development of *in vitro* cultivation systems for the sexual stages of *P. falciparum* has greatly facilitated biochemical studies (Ponnudurai *et al.*, 1983). A complex of 260, 59 and 53kDa proteins (Mr varying somewhat in the hands of different groups) recognised by transmission blocking mabs have been identified on the surfaces of both male and female gametes (Rener *et al.*, 1983; Kumar and Carter, 1984; Quakyi *et al.*, 1982). After fertilisation new surface antigens appear in the zygote and the ookinete. Antibodies to a 25kDa surface antigen on zygotes and ookinetes also block transmission (Vermeulen *et al.*, 1985). The gene for the 25kDa antigen has been cloned and sequenced (Kaslow *et al.*, 1988). An interesting feature of its structure is that it contains four tandem, cysteine rich domains with homology to similar domains in the epidermal growth factor (EGF). Such regions may be the binding site to mosquito gut epithelial cells. However, a vaccine based on the antigen may induce autoimmunity due to cross reaction with EGF and will therefore require careful monitoring. There is now evidence to suggest that T cell-mediated immunity against circulating *P. yoelli* gametocytes may provide longer lasting transmission blocking immunity than antibodies (Harte *et al.*, 1985). However it must again be remembered that the situation in *P. falciparum* may be different because infected erythrocytes, unlike infected reticulocytes, do not have surface histocompatibility antigens to facilitate T cell recognition.

A vaccine based on the sexual stage antigens while not directly protecting the vaccinated individual will serve to reduce the spread of disease. Also when used in conjunction with anti-sporozoite and anti-asexual stage vaccines, it might serve to control the emergence of resistant mutants. However a serious drawback with transmission blocking vaccines is the observation that depending on the concentration of antibodies used, enhancement of transmission can sometimes be observed (Pieris *et al.*, 1988). It may not be easily possible to control immunisation schedules in a population to ensure that blocking is favoured over enhancement.

Use of mosquito antigens for immunisation

The successful development of a pathogen in the mosquito is closely related to the normal physiology of the mosquito. Refractiveness of anophelines to *Plasmodium* is known to be determined by a single recessive gene (Collins *et al.*, 1986.) Recently it has been shown in novel experiments that antibodies against mosquito components reduce the fecundity of mosquitoes (Ramasamy *et al.*, 1988a;b). Antibodies to mosquito mid-gut also interfere with the infection of mosquitoes with both alpha and flaviviruses taken up in a blood meal (Ramasamy *et*

al., 1990a). The effect of anti-midgut antibodies on the infectivity of *P. berghei* to *Anopheles farauti* was investigated and a significant reduction in oocyst numbers was observed in the presence of the antibodies in the blood meal (Ramasamy *et al.,* 1990b). The reduction in trasmission may be due to antibodies to midgut receptors for the pathogens interfering with the passage of the pathogen through the midgut. Such target molecules in the midgut of anopheline mosquitoes may also be considered as potential candidates for a transmission blocking vaccine.

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Incrimination of *Anopheles dirus* as a Vector of Malaria in Dibrugarh District, Assam

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Anopheles nigerrimus, A. vagus, A. dirus, A. karwari and *A. kochi* were the most abundant species among 12 anopheline species collected during the course of entomological studies carried out from July to September, 1988 in an area endemic for malaria under Tengakhat PHC of Dibrugarh district, Assam. Sporozoites were detected in the salivary glands of one specimen of *A. dirus*, Peyton & Harrison (*A. balabacensis*) which constituted about 18.11 per cent of the total anopheline collection.

INTRODUCTION

Anopheles minimus and *A. balabacensis (=dirus)* had been considered as important malaria vectors in the Northeastern region of India (Misra and Dhar, 1955; Misra, 1956; Sen *et al.*, 1973; Rajagopal, 1976; Bhatnagar *et al.*, 1982; Das and Baruah, 1985; Dutta and Baruah, 1987).

In Assam, survey for anophelines was carried out firstly by Challam (1923) in Kamrup district. In Cachar district, a few infected specimens of *A. kochi* were found by Strickland (1929) and Ramsay (1930). Later studies showed that *A. minimus* was the predominant vector species in Assam although natural infections were detected in some specimens of *A. annularis* (Anderson and Viswanathan, 1941; Viswanathan *et al.*, 1941). In a study conducted simultaneously by Clark and Choudhury (1941), *A. balabacensis (=dirus)* was incriminated from Digboi area and subsequently, from Nowgong district (Rajagopal, 1979). Since then, no study on vector incrimination has been reported from Assam except a few mosquito survey reports (Sarkar *et al.*, 1981; Kareem *et al.*, 1983; Kareem *et al.*, 1985; Nagpal and Sharma, 1987).

In the present study, an observation on vector incrimination made in malaria endemic areas under Tengakhat PHC of Dibrugarh district, Assam during July to September, 1988 has been reported.

MATERIAL AND METHODS

The study area which is 75 kms away from Dibrugarh town is a heterogeneous terrain with foothills, riverbeds, tea-gardens, paddy fields and on one side there is a large forest range (Charaipung Forest range). Climate is hot and humid with high rainfall. The meteorological

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data recorded from the Tea Research Station, Dikom (Dibrugarh) during the study period was as follows—average temperature, Max. 29.42°C and Min. 24.12°C; average relative humidity 94.33% (morning) and 74% (evening) and average rainfall 551.8 mm.

The collection of mosquitoes was done by suction tubes between 1800 hrs to 2400 hrs at night from human dwellings and cattlesheds and also by operating CDC light-traps from dusk to dawn i.e., from 1800 hrs to 0500 hrs in cattlesheds and human dwellings. The collected, mosquitoes were transported to the laboratory and identified. The fresh mosquitoes were dissected to detect gland and gut infections.

Table 1. Light-traps and suction tube night collection from cattlesheds and human dwellings during July to September, 1988 and results of dissection

S. No.	Species	Light-trap collection		Suction tube collection		Total collection	No. dissected*	Positive		Parity %
		CS	HD	CS	HD			Gland	Gut	
1.	*A. aconitus*	0	2	1	0	3 (0.70)	2	0	0	0.00
2.	*A. annularis*	0	0	9	0	9 (2.11)	8	0	0	48.36
3.	*A. dirus*	12	25	1	49	77 (18.11)	74	1	0	57.24
4.	*A. barbirostris*	12	1	2	0	15 (3.52)	0	0	0	0.00
5.	*A. karwari*	47	4	16	0	67 (15.76)	0	0	0	0.00
6.	*A. kochi*	13	5	28	3	49 (11.52)	40	0	0	41.85
7.	*A. maculatus*	10	0	0	0	10 (2.35)	9	0	0	40.08
8.	*A. minimus*	0	1	2	0	3 (0.70)	2	0	0	26.18
9.	*A. nigerrimus*	38	4	42	2	86 (20.23)	0	0	0	0.00
10.	*A. philippinensis*	3	12	8	3	26 (6.11)	21	0	0	44.78
11.	*A. tessellatus*	0	0	0	1	1 (0.23)	0	0	0	0.00
12.	*A. vagus*	0	3	75	1	79 (18.5)	0	0	0	0.00

Figures in parentheses are percentage values.
CS = Cattleshed; HD = Human dwelling.
* Only fresh mosquitoes dissected.

RESULTS AND DISCUSSION

The study area is malarious with high incidence of *Plasmodium falciparum* malaria. The transmission season is from June to October. In a house to house survey carried out during the period from July to September, 1988 covering a population of about 3000 for detection of fever cases, a total of 546 blood slides of fever cases were examined and out of these 88 cases were malaria positive (68 cases of *P. falciparum* and rest of *P. vivax*) showing Slide Positivity Rate (SPR) and Slide falciparum Rate (SfR) of 16.11 and 12.45% respectively.

Record of anopheline collection from the study area during July to September, 1988 has been shown in Table 1. It is seen from the table that a total of twelve species of anophelines have been detected in collection at night by light-traps as well as suction tubes from cattlesheds and human dwellings. *A. nigerrimus, A. vagus, A. dirus, A. karwari* and *A. kochi* were the prevalent species forming 20.23, 18.58, 18.11, 15.76 and 11.52 per cent of the total collection respectively. All the fresh mosquitoes were dissected and the results of dissection reveal the presence of sporozoites in the salivary glands of one specimen of *A. dirus* captured from human dwellings, the sporozoite rate being 1.35% for this period.

In Assam, *A. balabacensis(= dirus)* was incriminated as vector from Digboi area (Clark and Choudhury, 1941) and Nowgong district (Rajagopal,1979). The present study has again established the role of this species in transmission of malaria in Tengakhat PHC area of Dibrugarh district, Assam. Further, this species has been collected more from the human dwellings, thus disclosing specifically its nature of endophagy as well as anthropophily.

In this survey, *A. minimus* was also encountered but its density was considerably low. This is similar to earlier reports that *A. minimus* which was believed to have disappeared from the Northeastern region of India after the spray of DDT residual insecticide, is still present and is acting as a vector of malaria in this region specially in the areas of poor spray coverage (Bhatnagar *et al.,* 1982; Das and Baruah, 1985; Nagpal and Sharma, 1987; Dutta and Baruah, 1987).

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Studies on Malaria and Filariasis Vectors in Kamorta and Great Nicobar Islands

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A total of 1401 adult mosquitoes mainly dominated by *Anopheles sundaicus* (40.18%) and *Culex quinquefasciatus* (57.31%) were collected from Kamorta and Great Nicobar Islands. It was revealed from human bait collection that both species prevailed almost all through the night (density 8.1/man hour) with a peak around midnight. Mixed breeding of *A. sundaicus* with *A. vagus, Cx. quinquefasciatus, Cx. bitaeniorhynchus* and *Cx. tritaeniorhynchus* was observed at many places. Adult *A. sundaicus* were found highly susceptible to DDT and larvae to Temephos (Abate). *Cx. quinquefasciatus* was resistant to DDT (31% mortality). High parity of *A. sundaicus* (40.42%) is an indication of the vectorial potential of this species to sustain malaria transmission in these islands.

INTRODUCTION

Ecological imbalance caused by progressive development, deforestation and reclamation of land in coastal areas with residual DDT spray has changed the biting behaviour and biology of moquito vectors, to the detriment of public health. This even leads to simian origin of malaria zoonosis in Great Nicobar Island (Kalra, 1980). Presence of *Anopheles sundaicus* and *A. barbirostris* in Nicobar group of islands was first recorded by Krishnan and Bhatnagar (1968), though these species were reported earlier from Andaman group of islands (Christophers, 1933). After a decade, Kalra (1981) defined the susceptibility status of *Anopheles* and *Culex* mosquitoes of South and Little Andaman against DDT, Dieldrin and HCH. Das *et al.* (1981) undertook the treatment of malaria cases at Campbell Bay, however, no investigation was carried out on vectors responsible for active transmission of malaria. Recently, Nagpal and Sharma (1983) collected 11,000 mosquitoes mainly dominated by *A. vagus* from Andaman group of islands and recorded the morphological variations of the species. It is of immense importance to study the changing behaviour of mosquitoes in relation to transmission of vector-borne diseases especially in the fragile island ecosystems. In view of the above, an extensive mosquito survey was carried out in Great Nicobar and Kamorta Islands.

MATERIAL AND METHODS

Great Nicobar Island is the largest (1045 sq km) among the Nicobar group of islands and is located on 7°N and 93°5' E. Kamorta is a comparatively smaller island situated on 8°08' N and

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$93^{o}03'$E. Wild vegetation dominated by tall trees covers almost 90% of the island. The climate is hot and humid and the temperature ranges between 23^{o} and 31^{o}C. The islands were mainly inhabited by Nicobarese and Shompen tribes.

Adult mosquitoes were collected from cattle-sheds, human habitations and goat cabins with the aid of a 6 volt battery operated CDC miniature light-trap, from 1800 hrs to 0500 hrs. Simultaneously, adult mosquitoes were also collected on human baits with the help of suction tubes, to determine the peak biting hours. Larvae were collected from various breeding habitats with standard dippers, ladles and glass droppers. Immature stages were reared for emergence of adults in the laboratory for species identification. Susceptibility tests were conducted as per standard WHO technique with common insecticides against adults and larvae. Natural breeding water was used to make different concentrations for larval susceptibility test (Kalra, 1981). Live adult mosquitoes were dissected to determine their physiological age and vectorial status. The study was conducted during dry season in the month of March, 1986.

RESULTS AND DISCUSSION

Altogether 1401 mosquitoes belonging to 8 species under 4 genera were collected in Kamorta and Great Nicobar Islands (Table 1). Suspected malaria vector *A. sundaicus* and filariasis vector, *Cx. quinquefasciatus* formed the major bulk. It is interesting to note that mosquitoes were attracted more towards human baits in Kamorta than towards CDC light-trap while the reverse was true in Great Nicobar Island. The variation in collection of mosquito fauna in Great Nicobar and Kamorta Islands may be due to differential distribution pattern of mosquitoes in micro habitats. Kondrashin and Kalra (1987) describe this phenomenon in detail under mother and secondary foci distribution of mosquitoes during rainy and non-rainy season.

A total of 449 mosquitoes belonging to 5 species were recorded in 9 trap nights in Great Nicobar Island which was solely dominated by *A. sundaicus* comprising 98.66% of the collection. The density of other species(*A. barbirostris, Cx. tritaeniorhynchus, Cx. quinquefasciatus* and *Cx. malayi)* recorded in the trap collection was very low. In human bait collection, only two species

Table 1. Adult mosquito collection in Kamorta and Great Nicobar Islands

S. No.	Mosquito species	Kamorta		Great Nicobar	
		CDC trap collection	Human bait collection	CDC trap collection	Human bait collection
1.	*Anopheles sundaicus*	—	120	443	—
2.	*A. barbirostris*	—	—	2	—
3.	*Culex malayi*	2	10	2	—
4.	*Cx. quinquefasciatus*	4	779	1	19
5.	*Cx. tritaeniorhynchus*	—	3	1	—
6.	*Cx. vishnui*	2	10	—	—
7.	*Aedes albopictus*	—	1	—	1
8.	*Mansonia indiana*	1	—	—	—

i.e., *Cx. quinquefasciatus* (19) and *Ae. albopictus* (1) were encountered on four human baits in two nights.

In Kamorta Island, only 9 mosquitoes were collected in four trap nights, while a total of 923 mosquitoes of six species were encountered on two human baits in 8 nights. *Cx. quinquefasciatus* was the most dominant species at 84.39% of the total collection followed by *A. sundaicus* (13%), *Cx. malayi* (1.08%) and *Cx. vishnui* (1.08%). Density of the mosquitoes recorded was 8.1 per man hour.

Hourly collections of mosquitoes on human baits were analysed as per Fisher's 'F' test. High intensity of both *A. sundaicus* (F=5.6158) and *Cx. quinquefasciatus* (F=7.4315) was observed during mid part of the night (2100-0200 hrs). Collections of the early evening (1900-2000 hrs) and early morning hours (0300-0400 hrs) were significantly low (Table 2). Similar biting phenomenon was observed by Sundaraman *et al.* (1957) in case of *A. sundaicus* in Indonesia (nearest land mass to Nicobar group of islands). During a year long study, Das *et al.* (1987) also observed peak biting period of *Cx. quinquefasciatus* between 0030 hrs and 0230 hrs, however high density of the species was prevailing all through the night mainly from 1700-0500 hrs.

Table 2. Hourly collection of adult mosquitoes in Kamorta Island

Time (hrs)	*A. sundaicus*	*Cx. quinquefasciatus*
1900—2000	14*	54*
2100—2200	32	154
2300—2400	29	191
0100—0200	26	235
0300—0400	7*	95*
LSD	2.6933	20.5128
F-value	5.6158	7.4315

* Level of significance 0.05.

In Great Nicobar Island altogether 577 larvae, belonging to six species under two genera, were collected from various breeding habitats along the coastal road leading to Indira point (earlier known as Pygmalion point). Largest collection of *A. sundaicus* (94.1%) was mostly made from kutcha(unlined) wells, brackish water nallahs and roadside ditches, along the coastal margin of the island (Table 3). Mixed breeding of *A. sundaicus* with *A. vagus, Cx. quinquefasciatus, Cx. tritaeniorhynchus* and *Cx. bitaeniorhynchus* was observed in many habitats like kutcha wells, fresh water nallahs and roadside ditches. This may be due to adverse conditions prevailing during the survey which compelled the species to co-breed. Moreover, the versatile adaptability of *A. sundaicus* to different breeding habitats has also been reported earlier (Rao, 1984).

In Kamorta a total of 975 larvae belonging to 4 species under 3 genera were encountered from three different breeding habitats (Table 3). *A. sundaicus* was also found breeding in water pockets of a small hill stream in Ramjaw village.

Results of the larval susceptibility tests conducted in both the islands are summarised in Table 4. It is revealed from the present study that the larvae of *A. sundaicus* are highly susceptible to Temephos (Abate 50E); LC_{30}, LC_{50} and LC_{90} values being 0.0012 ppm, 0.0014 ppm, 0.0022 ppm in Great Nicobar and 0.0013 ppm, 0.0015 ppm, 0.0024 ppm in Kamorta Island, respectively. Kalra (1981) also found that *A. sundaicus* larvae were highly susceptible to DDT, the LC_{90} value being 0.005 ppm at South and Little Andaman. Insecticide resistance in *A. sundaicus* has not yet been reported from the Indian subcontinent. However, this species is resistant to DDT and Dieldrin in neighbouring Indonesia and to Dieldrin in Malaysia (WHO, 1976).

Table 3. Habitatwise larval collection in Kamorta and Great Nicobar Islands

Mosquito species	Great Nicobar					Kamorta			
	Brackish water nallahs pH 7.9-8.2	Fresh water nallahs pH 7.3-7.8	Road side ditches pH 7.7-8.2	Kut-cha wells pH 7.3	Total	Tanks with clear water pH 7.65	Brackish waters pH 7.65	Stream water pockets pH 8.13	Total
Anopheles sundaicus	267	1	28	247	543	—	955	5	960
A. barbirostris	—	4	—	—	4	—	—	—	—
A. vagus	—	4	—	—	4	—	—	—	—
Culex bitaeniorhynchus	—	1	—	—	1	—	—	—	—
Cx. tritaeniorhynchus	—	4	6	9	19	2	—	—	2
Cx. malayi	—	—	—	—	—	4	—	—	4
Cx. quinquefasciatus	—	—	—	6	6	—	—	—	—
Orthopodomyia anopheloides	—	—	—	—	—	9	—	—	9
Total	267	14	34	262	577	15	955	5	975

Table 4. Susceptibility of *Anopheles sundaicus* larvae to Temephos (Abate 50E)

Place	Regression equation	LC_{30} ppm	LC_{50} ppm	LC_{90} ppm
Great Nicobar Island	Y = 6.933 + 3.995	0.0012	0.0014	0.0022
Kamorta Island	Y = 6.5 x + 3.83	0.0013	0.0015	0.0024

Table 5. Susceptibility of adult anopheline and culicine mosquitoes against 4% DDT in Kamorta Island

Mosquito species	No. of mosquitoes treated	No. of mosquitoes died	Mortality %	Remarks
An. sundaicus	101	100	99.01	Susceptible
Cx. quinquefasciatus	525	163	31.05	Resistant
Cx. vishnui	39	39	100.0	Susceptible

No mortality observed in control. As per WHO criteria: 98-100% mortality—Susceptible; 80-97% mortality—Verification required; Below 80% mortality—Resistant.

Susceptibility status of adult *A. sundaicus, Cx. quinquefasciatus* and *Cx. vishnui* (the vectors of malaria, filariasis and Japanese Encephalitis respectively) was determined in Kamorta Island (Table 5). Results showed that *A. sundaicus* and *Cx. vishnui* were susceptible to 4% DDT (one hour exposure) while *Cx. quinquefasciatus* was resistant, as only 31% mortality was achieved. Kalra (1981) also reported the resistance of *Cx. quinquefasciatus* to 4% DDT from South and Little Andaman.

Live specimens of *A. sundaicus* collected on human bait were dissected for gut and gland infection with sporozoites while *Cx. quinquefasciatus* were dissected for infective stages of filarial worm. However, none was found positive for the malaria and filarial parasite respectively (Table 6). Parity status of *A. sundaicus* and *Cx. quinquefasciatus* was observed to be 40.42% and 8.16%, respectively. High parity rate of *A. sundaicus* provides a strong indication for its role in malaria transmission. In conjunction with high parasitic load in the community this may be a contributing cause of the high incidence of malaria in Great Nicobar and Kamorta Islands.

Table 6. Dissection results of vectors in Kamorta Island

Mosquito species	No. dissected	No. positive for sporozoite/ infective stages of filariasis	Parity %
Anopheles sundaicus	47	Nil	40.42
Culex quinquefasciatus	147	Nil	8.16

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SHORT NOTES

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Treehole Breeding of Mosquitoes in Nadiad, Kheda District (Gujarat)

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Earlier attempts to know the treehole breeding habit of anopheline mosquitoes in India were made by Christophers (1916), Iyengar (1929), Puri (1931), Rao (1984) and Nagpal and Sharma (1985). They reported *An. barianensis*, *An. culiciformis*, *An. asiaticus*, *An. annandalei*, *An. annandalei* var. *djajasanensis*, *An. sintoni* and *An. culicifacies* to be breeding in treeholes. In addition, Barraud (1934), Kalra and Wattal (1965), Shetty and Geevarghese (1977) and Panicker and Rajagopalan (1978) have observed treehole breeding habit of mosquitoes belonging to genus *Aedes, Armigeres, Culex, Ficalbia, Harpagomyia, Heizmannia, Orthopodomyia, Tripteroides, Uranotaenia* and *Toxorhynchites.* Among the above cited reports dealing with anophelines one by Nagpal and Sharma is notable for the observation that a vector species *An. culicifacies,* which is a profuse ground water breeding mosquito, is adapting itself to a new environment. The present study is undertaken with the aim to know the treehole breeding habit of mosquitoes during the monsoons in villages of Nadiad of Kheda district in Gujarat. During the course of larval survey (27th July to 5th September 1988) small (4.98 x 2.54 cm) to large (30.48 x 15.24 and 45.72 x 15.25 cm) treeholes were searched for mosquito breeding.

Larvae of all stages and pupae were collected with the help of a pasteur pipette. Sometimes the holes were flushed with water and this water alongwith immatures was collected. All immatures were taken to the laboratory and reared on larval food. Pupae were kept separately for eclosion. Adults that emerged were identified according to the key of Christophers (1933) and Barraud (1934). Results of the present survey are given in Table 1.

During the survey tree holes of 12 species of tree viz., *Acacia nilotica* (Acacia), *Ficus benghalensis* (Banyan), *Ficus racemosa* (Umetha), *Azadirachta indica* (Neem), *Delonix regia* (Flamboyant flame), *Dalbergia sisso* (Sisam), *Madhuca latifolia* (Mahuva), *Tamarindus indica* (Tamarind), *Acacia chundra* (Kaitha), *Acacia senagal* (Samdi), *Zizyphus oenoplia* (Jackal jujuba) and *Mangifera indica* (Mango) were found positive for mosquito breeding in 25 villages. All positive treeholes were at a distance of 20 to 100 metres from human dwellings. The height range of treeholes was 91.44 cm to 7.6 metres. Treeholes of *Amblica offacinalis* (Amla) and *Manikara hexandra* (Rayan) were negative. A total 241 treeholes of different trees were searched. Results revealed that treeholes were positive for

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Table 1. Mosquito breeding in tree-

S. No.	Name of tree	Tree-holes searched	No. of treeholes positive for					No. of immatures	
			An.	*Cx.*	*Ae.*	Mx.	Total	*An.*	*Cx.*
1.	*Acacia nilotica* (Acacia)	47			4		4		
2.	*Ficus benghalensis* (Banyan)	81			21	2	23		13
3.	*Mangifera indica (Mango)*	18	2		1	1	4	174	15
4.	*Delonix regia* (Flamboyant flame)	5			1		1		
5.	*Azadirachta indica* (Neem)	46			7	2	9		12
6.	*Ficus racemosa* (Umetha)	4			2		2		
7.	*Amblica offacinalis* (Amla)	2							
8.	*Manikara hexandra* (Rayan)	3							
9.	*Dalbergia sisso* (Sisam)	4			3		3		
10.	*Madhuca latifolia* (Mahuva)	3			1		1		
11.	*Tamarindus indica* (Tamarind)	7			2		2		
12.	*Acacia chundra* (Kaitha)	7			2		2		
13.	*Acacia senagal* (Samdi)	4		1	2		3		31
14.	*Zizyphus oenoplia* (Jackal Jujuba)	10			3		3		
	Total	241	2	1	49	5	57	174	71

Note : *An.* = *Anopheles; Cx.* = *Culex; Ae.* = *Aedes;* Mx. = Mixed ; Figures in parentheses are the percentages.

holes in Nadiad, Kheda (Gujarat)

collected		No. of adults emerged							
Ae.	Total	*Ae. aegypti*	*Ae. vittatus*	*Ae. unili-neatus*	*Ae. albo-pictus*	*Ae. thom-soni*	*An. subpi-ctus*	*Cx. quinque-fasciatus*	Total
158	158		39 (28.05)		100 (71.94)				139
210	223	40 (26.49)	18 (11.92)		80 (52.98)			13 (8.60)	151
28	217			14 (13.46)	12 (11.53)		70 (67.30)	8 (7.60)	104
6	6				6 (100.00)				6
54	66		11 (18.64)	6 (10.16)	29 (49.15)	2 (3.38)		11 (18.64)	59
10	10		6 (100.00)						6
85	85	18 (33.96)		35 (66.03)					53
5	5			5 (100.00)					5
17	17			13 (100.00)					13
14	14			3 (23.07)	10 (76.92)				13
23	54		12 (26.66)			6 (13.33)		27 (60.00)	45
23	23	3 (18.75)		2 (12.50)	8 (50.00)	3 (18.75)			16
633	878	61 (10.00)	86 (14.09)	78 (12.78)	245 (40.16)	11 (1.80)	70 (11.47)	59 (9.67)	610

Anopheles (2), *Aedes* (49), *Culex* (1) and mixed *(Culex + Aedes)* 5.

A total of 878 larvae of genus *Aedes, Culex* and *Anopheles* were collected from 57 positive tree-holes of different tree species, and 610 adults (5 species of *Aedes,* 1 species of *Culex* and 1 species of *Anopheles)* emerged from the collected immatures. Of genus *Aedes* (633 immatures) four species belonged to sub-genus *Stegomyia* and one species of sub-genus *Christophersiomyia* adults emerged. Table 1 reveals that *Aedes albopictus* (40.16%) has a greater preference for treehole breeding followed by *Aedes vittatus* (14.09%), *Aedes unilineatus* (12.78%), *Aedes aegypti* (10.00%) and *Aedes thomsoni* (1.80%), respectively.

Three species of sub-genus *Stegomyia—Ae. aegypti, Ae. albopictus* and *Ae. vittatus* are known for transmission of Dengue and Chikungunya viruses in India but *Ae. aegypti* plays the main role. Hence, increase in density of these species during monsoon may contribute towards the transmission of these viral diseases.

Table 1 shows that maximum number of mosquito species were breeding in treeholes of *Azadirachta indica.* The dominating species was *Ae. albopictus* (49.15%) followed by *Ae. vittatus* (18.64%), *Cx. quinquefasciatus* (18.64%), *Ae. unilineatus* (10.16%) and *Ae. thomsoni* (3.38%). Further, *Ae. albopictus* was found breeding in treeholes of six other tree species, *Acacia nilotica, Ficus benghalensis, Mangifera indica, Delonix regia, Acacia chundra* and *Zizyphus oenoplia*. Similarly, *Ae. unilineatus* preferred to breed in treeholes of seven different tree species, *Mangifera indica, Azadirachta indica, Dalbergia sisso, Madhuca latifolia, Tamarindus indica, Acacia chundra* and *Zizyphus oenoplia*. Treeholes of *Mangifera indica, Azadirachta indica, Acacia chundra* and *Zizyphus oenoplia* were found with breeding of *Ae. albopictus* and *Ae. unilineatus.* The second most prevalent species among *Aedes* was *Ae. vittatus* which was observed breeding in treeholes of five different trees, *Acacia nilotica, Ficus benghalensis, Azadirachta indica, Ficus racemosa* and *Acacia senagal. Acacia nilotica* was found with breeding of two species, *Ae. vittatus* (28.05%) and *Ae. albopictus* (71.94%).

Ficus benghalensis treeholes had breeding of four species, *Ae. albopictus* (52.98%), *Ae. aegypti* (26.49%), *Ae. vittatus* (11.92%) and *Cx. quinquefasciatus* (8.60%). In treeholes of *Ficus racemosa* only *Ae. vittatus* was found breeding. *Cx. quinquefasciatus* (60.00%), *Ae. vittatus* (26.66%) and *Ae. thomsoni* (13.33%) were observed breeding in treeholes of *Acacia senagal.* From treeholes of *Zizyphus oenoplia,* breeding of *Ae. albopictus* (50.00%), *Ae. aegypti*(18.75%), *Ae. thomsoni* (18.75%) and *Ae. unilineatus* (12.50%) were obtained. In tree-holes of *Dalbergia sisso, Ae. aegypti* (33.90%) and *Ae. unilineatus* (66.03%) were observed. Similarly breeding of *Ae. albopictus* (76.92%) and *Ae. unilineatus* was seen in treeholes of *Acacia chundra.* Treeholes of *Delonix regia* had breeding of *Ae. albopictus.* Treeholes of *Madhuca latifolia* and *Tamarindus indica* supported only *Ae. unilineatus* breeding.

Larvae (71) of genus *Culex* were collected from 6 tree holes (1 *Culex* + 5 mixed for *Aedes* and *Culex*) of four different trees, *Ficus benghalensis, Mangifera indica, Azadirachta indica* and *Acacia senagal.* Only *Cx. quinquefasciatus* emerged from the immatures collected from treeholes.

During this study only two treeholes of *Mangifera indica* from villages Mahisha and Alina were positive for genus *Anopheles.* Immatures (174) of *An. subpictus* were collected from cavities formed among branches. *An. subpictus* breeding in treeholes is reported for the first time in India. Although it has not been incriminated as vector of malaria from Gujarat but it was found to harbour sporozoites in Pondicherry (Panicker *et al.*, 1981) and in Jagdalpur, MP (Kulkarni, 1983). Further

observations on the role of *An. subpictus* in malaria transmission in Gujarat would be of interest in relation to malaria dynamics and its control.

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A Preliminary Malaria Survey in Bisra PHC, District Sundergarh, Orissa

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Bisra PHC is located in Sundergarh district of Orissa between 22°10' and 22°20' North latitude and 84°50' and 85°5' East longitude. It is surrounded by Singhbhum hill range in the North and in the East, Rourkela town in the West and villages under Birkera PHC in the South. Villages in Singhbhum hill range were highly endemic for malaria (Watts, 1924; Christophers, 1925; Senior White and Das, 1938). Malaria has returned with vengeance in this area. The spraying is poor and surveillance is inadequate. During 1986 and 1987 the SPR and SfR of Bisra PHC varied from 9 to 12 and 8 to 12 (Source: DMO). Health Department, Orissa Govt. suggested that Malaria Research Centre should open its field station to study the feasibility of the bioenvironmental control of malaria in Bisra PHC, because of persistent malaria transmission and the fact that spraying was not acceptable to the community. At the time of opening of field station a preliminary survey of malaria situation was carried out in Bisra PHC. Results of this study are reported in this paper.

Bisra PHC HQs is located 15 kms from Rourkela town. The PHC (rural) has a total area of 147 sq kms with 71 villages having a population of 46,072 (1981 census). Each village is further split into small hamlets locally known as 'tolas' which are often far from each other.

The land is mostly undulating interspersed with low hillocks having sparse forests. River 'Koel' runs along the northern limits of the PHC and four streams running through the PHC area feed the river. The area receives an annual normal rainfall of 165 cms, mostly during the monsoon months. During the non-monsoon months the land, however, remains dry and thus a large number of bed pools are formed in the streams and river which support heavy *Anopheles culicifacies* breeding.

Most of the inhabitants are aboriginals belonging to Oram, Munda, Kharia, Kisan, Bhuyia, Binjhia, Gonds, Khonds, Santhal, Lohar and Baraik tribes in order of prevalence. Some migrants particularly from Bihar, Rajasthan and Uttar Pradesh have settled in and around Bisra town. The main occupation is agriculture which largely depends on monsoons and during the rest of the year the people are engaged in labour

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work in industries or railways. Thus there is a daily flux of population between Bisra PHC villages and Rourkela town.

Although the establishment of Rourkela Steel Plant has transformed this area both economically and ecologically, malaria continues to persist as a major health problem.

A preliminary survey was conducted in the first fortnight of April 1988 in order to determine the present status of malaria in Bisra PHC. Active fever survey was carried out in nine villages within a radius of 15 kms from the PHC HQs. These villages were Dareikela, Kapranda, Tulsikani, Barsuan, Bhangurkela, Bijadihi Jabaghat, Sarubahal and Dhadari.

Blood smears were collected, stained with JSB stain and examined for the presence of malaria parasites. Adult patients with malaria were treated with chloroquine (900 mg for *P. vivax* and *P. malariae* and 1500 mg for *P. falciparum*). The children received proportionately low dosages. Spleens of primary school children (age below 9 yrs) were also examined for enlargement. Adult anopheline mosquitoes were collected in eight riverine and four non-riverine villages with the help of suction tubes both in the morning (0600 hrs to 0800 hrs) and in the evening (1800 hrs to 2000 hrs). The mosquitoes were indentified and dissected for the presence of sporozoites.

Table 1 gives the results of parasitological surveys. Fever surveys were carried out in nine villages and a total of 95 blood smears were collected. Of these, 26 (SPR 26.3%) were positive for malaria showing a Parasite Index (PI) of 3.61. Among the positives, 9 were *P. vivax*, 15 *P. falciparum* and 1 *P. malariae*. This is the first report of *P. malariae* from this area. Table 1 shows that SPR, SfR and PI recorded in this survey were much higher than that of the NMEP surveillance conducted from March to May in the corresponding and previous years. This may be the result of better case detection

Table 1. Comparison of MRC and NMEP surveillance in 9 villages of Bisra PHC

	MRC survey April 1988	NMEP surveillance*					
		1987			1988		
		Mar	Apr	May	Mar	Apr	May
BSC/BSE	95	114	105	113	86	105	85
Positive	25	4	2	8	7	8	6
Pv	9	2	0	3	3	4	1
Pf	15	2	2	5	4	4	5
Pm	1	0	0	0	0	0	0
SPR	26.3	3.5	1.9	7.0	8.1	7.6	7.0
SFR	15.8	1.7	1.9	4.4	4.6	3.8	5.8
PI	3.61	0.57	0.28	1.15	1.01	1.15	0.86

*Source Bisra PHC.
Population of 9 villages 6918.

Table 2. Results of anopheline collection from the villages of Bisra PHC

April 1988 villages	*An. culicifacies*		*An.annularis*		*An. subpictus*		*An. vagus*		*An. splendidus*	
	No. collected	MHD	No. Collected	MHD	No. collected	MHD	No. collected	MHD	No. collected	MHD
Riverine, 8 villages	1870	63.1	98	3.15	55	2.02	31	1.07	1	0.03
Adjacent to forest and hills, 4 villages	103	6.9	9	0.67	24	1.55	19	1.2	0	0

during the survey. Out of 42 school children examined only 3 (7.1%) showed enlarged spleens.

A case of cerebral malaria was also detected in a boy of 5 years. At the time of blood smear collection, the patient had high fever associated with rigor and convulsions. The boy was under antibiotic therapy (ampicillin) by a local practitioner. On examination, the blood smear showed *P. falciparum* rings. The boy was treated with chloroquine and recovered. It may be noted that there was the death of a 22 year old woman in the neighbourhood, apparently due to malignant tertian malaria, a week before the detection of this case.

Table 2 gives the results of entomological studies. A total of 2210 mosquitoes belonging to five species viz., *An. culicifacies, An. annularis, An. subpictus, An. vagus* and *An. splendidus* were collected during the survey. Of all the anophelines, *An. culicifacies* was the most abundant species (89.2%). It was observed that villages adjacent to the river Koel had an average 63.1 man hour density (MHD) of *An. culicifacies* as compared to 6.9 in the non-riverine villages and the same was also true in the case of other anopheline species. A single specimen of *An. splendidus* was also collected from a riverine village. None of the 1973 *An. culicifacies* dissected showed gut or gland infection. This might be due to very low humidity (RH 30%) in April in this area resulting in reduced longevity.

Senior White and Das (1938) incriminated *An. fluviatilis, An. minimus, An. varuna* and *An. culicifacies* from Singhbhum hills and concluded that the first three species were mostly responsible for malaria transmission in this area and the role of *An. culicifacies* was of very little significance. During the present study, however, *An. fluviatilis, An. minimus* and *An. varuna* were not encountered. The absence of these erstwhile vectors and the prevailing malaria endemicity suggests that studies are required on the rôle of *An. culicifacies* in the malaria transmission in this region. It may be mentioned that recently Nagpal and Sharma (1986) have incriminated *An. culicifacies* in the transmission of malaria in six districts of Orissa. Studies on the susceptibility status of *P. falciparum* to chloroquine should also be taken up.

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Letter to the Editor

Malaria Among Bednet Users and Non-users

Sir—The use of a bednet is an effective personal protection measure against all mosquito-borne diseases. After the advent of DDT and other residual insecticides, this type of personal protection measure is often overlooked. In the literature of mosquito-borne diseases, much less attention has been given to the behaviour of the human host than to that of the parasite or the mosquito vectors (Gillet, 1975). Due to the development of resistance by mosquitoes to different insecticides and due to other environmental problems, such prophylactic measures are, however, gaining new importance in the present concept of Integrated Disease Vector Control (WHO/SEARO, 1983; Bang and Pattanayak, 1986).

The present study was carried out in a malaria endemic locality under Tengakhat Primary Health Centre (PHC) of Dibrugarh district in Assam, in order to know the malaria incidence pattern among bednet users and non-users. The locality comprises two tea gardens (Tarajan and Dhekiajuli Tea Estate) with a few villages adjoining a large forest range. A house to house survey was carried out by the surveillance workers to collect information in a predesigned proforma; such as, number of family members, their habit of the use of bednet, economic status etc. A proper surveillance both by active and passive methods was made during the malaria transmission season. The cases found positive were treated according to the species of parasite detected.

The survey covered a human population of 7743. Our observations reveal that 32.3 per cent of the population used bednets whereas the rest of them (67.6 per cent) were not in the habit of using bednets. The malaria positivity rate was considerably high among the bednet non-users. Among the bednet users only 1.8 per cent of the population suffered from malaria whereas among bednet non-users 5.9 per cent of the population had been affected by malaria.

Our study has shown that the use of bednet can markedly reduce the chances of man-mosquito contact, thereby, blocking the transmission in such a malaria endemic locality. It was also noticed in many cases that either the bednet does not have proper mesh or is torn and often it is not tucked under the mattress all the way round before sleeping. In such cases, the impregnation of bednet with repellent or insecticide may be effective (Curtis, 1983; Darriet *et al.,* 1984). In our entomological study *Anopheles dirus* Peyton & Harrison (*A. balabacensis*) had been incriminated as a vector species in this locality (Dutta *et al.,*1989). Being a highly anthropophilic species, it prefers to enter human dwellings at night for blood meals and generally avoids insecticide by not resting inside. In this situation, it can be assumed that proper use of bednets at night can reduce the chances of acquiring malaria.

Table 1. Distribution of malaria cases among bednet users and non-users

Variables	Number	Percentage
Total population	7743	—
Bednet users	2505	32.3
Bednet non-users	5238	67.6
Bednet users having malaria	46	12.8
Bednet non-users having malaria	312	87.2
Bednet users affected out of 2505	46	1.8
Bednet non-users affected out of 5238	312	5.9

88% *P. falciparum;* 10.6% *P. vivax* and 0.5% mix (*Pf* + *Pv*) out of total 358 cases.

As the people of this locality are of a lower socio-economic status and are less conscious about maintaining hygienic conditions, the application of personal protection measures through health education alongwith the spraying of residual insecticides may be effective in blocking transmission during the malaria season.

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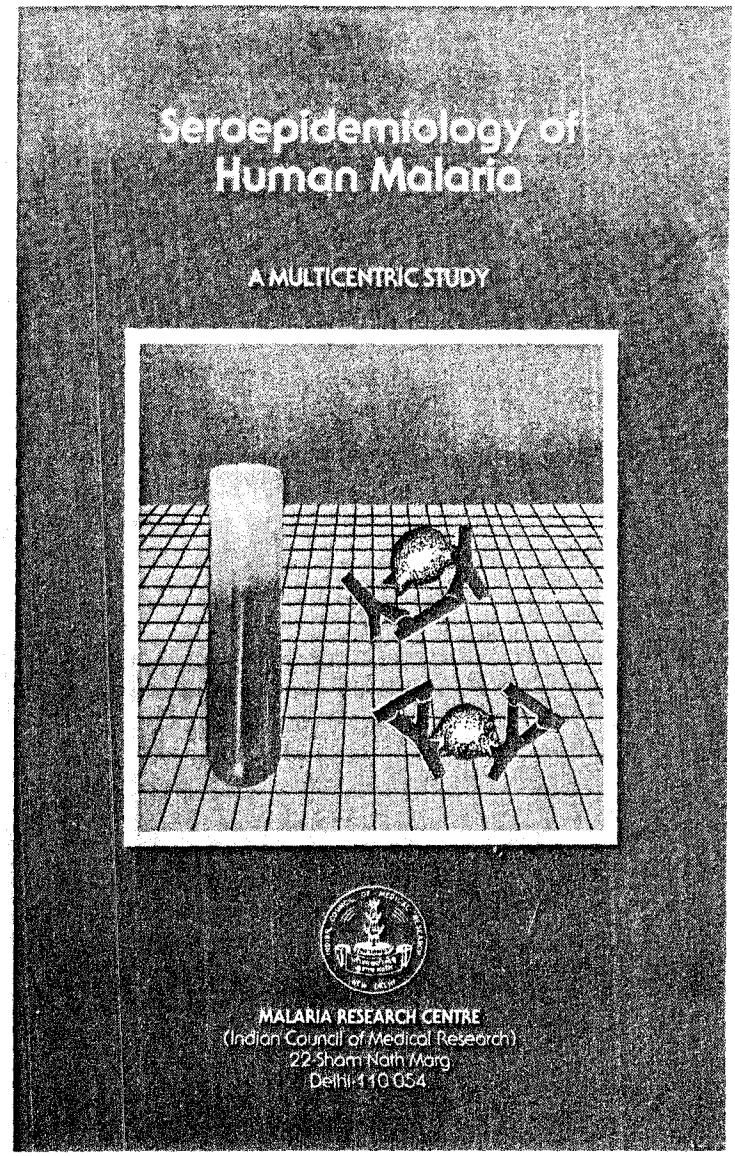

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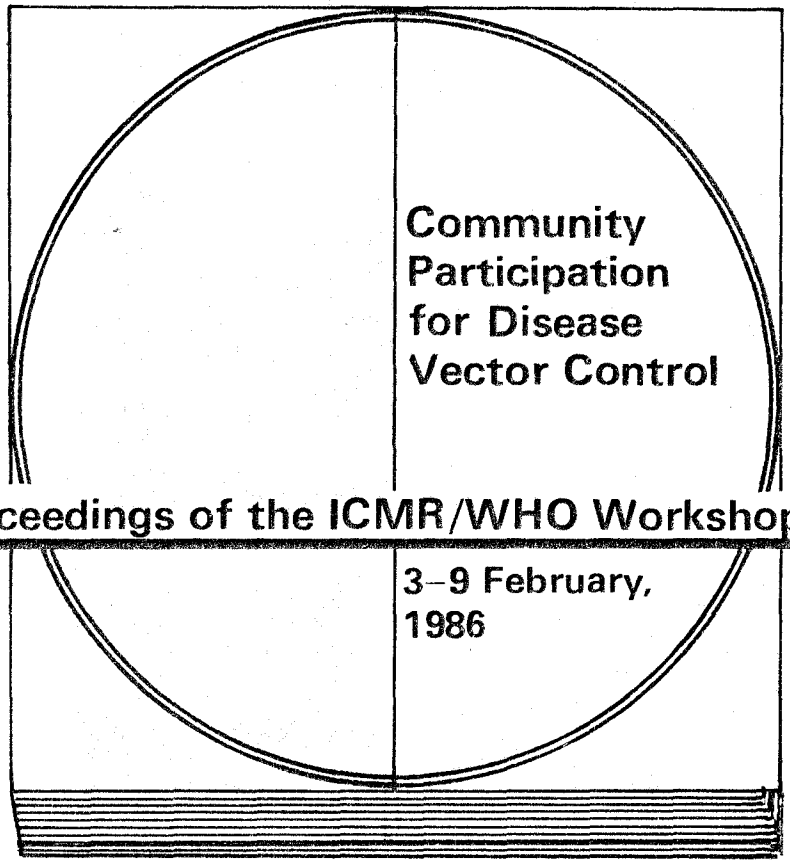

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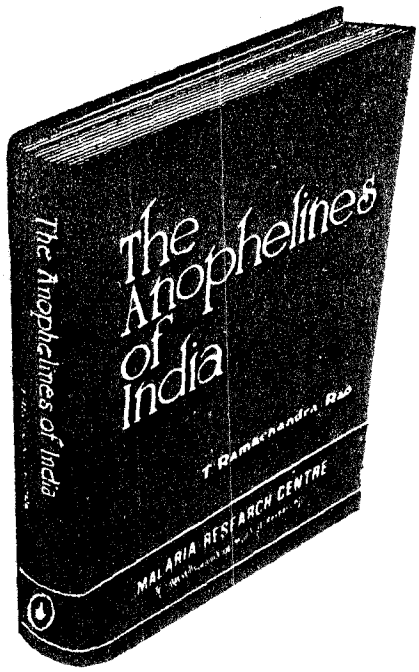

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