RECENT ADVANCES IN THE IMMUNOLOGY OF MALARIA

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Within the past decade after the successful development of the continuous *in vitro* culture technique of *Plasmodium falciparum* by Trager and Jensen (1976), the world has witnessed a rapid progress made in the study on immunology as well as biology of malaria so much so that even scientists working on malaria find it difficult to keep abreast with such a rapid development. This review is an extension to that reported previously (Tharavanij, 1981) with the objective of bringing to attention of our colleagues the advances made recently in the following areas of immunology of malaria including the host immune mechanisms, parasite evasion mechanisms and vaccine development.

IMMUNE MECHANISMS

Non-specific immunity

Innate resistance:

Factors related to red blood cells: (a)Receptor: A substantial body of information has been accumulated to show that only Duffy positive cells are susceptible to *P. vivax* and *P. knowlesi*. The nature of the receptor is not Duffy antigen *per se*, since Duffy negative red blood cells could be rendered susceptible to *P. knowlesi* infection by treatment with trypsin or neruaminidase even though they remain Duffy negative. In addition red blood cells from some monkeys are invaded by *P. knowlesi* though Duffy antigen is not detectable serologically on the cells. It is concluded that the actual receptor is not likely to be the Duffy antigen itself on the erythrocyte surface but very closely related to it (Mins, 1982; Wyler, 1983).

The nature of receptor for *P. falciparum* on the rbc surface is not clear. It has been shown that En(a-) as well as Tn and Wr negative red blood cells are resistant to *P. falciparum*. These cells are glycophorin A deficient suggesting that glycophorin A is a candidate receptor molecule. Furthermore, addition of glycophorin A into a malaria culture inhibited merozoite invasion into rbc. The observation of increased invasion of *P. falciparum* in young red cells as opposed to an older population is not related quantitatively to the presence of glycophorin A suggests that merozoite invasion is a complicated event involving glycophorin A which is known to connect to the red blood cell cytoskeleton (composed mainly of spectin and actin) through linkage with two other proteins: so called band 3 and ankyrin. Perhaps glycophorin A merely serves to trigger the membrane cytoskeleton, the change of which then allows merozoite invasion into the cells (a review by Wyler, 1983; Pasvol and Jungery, 1983).

(b) Haemoglobin: Only HbS has been convincingly demonstrated to confer protection against falciparum malaria. Parasites grown *in vitro* in HbS cells had retarded growth only when these cells are exposed to low oxygen tension (5%). Electron microscopic study showed that needle like aggregates of deoxyhaemoglobulin S disrupt the parasites in cells from sickle homozygotes and vacuolization in cells from sickle heterozygotes (Friedman, 1979a). Whether the parasites are killed by the sickling process or they are killed by other process

associated with sickling is not entirely clear. Friedman (1979b) suggested that parasite death was due to loss of erythrocyte potassium rather than the process of sickling, since maintenance of intracellular potassium reversed parasite damage but could not prevent sickling.

(c) The enzyme: The hypothesis that G6PD-deficient red blood cells confer protection against malaria remains controversial. It has been observed that G6PD-deficient cells are seen to have fewer parasites (Luzzatto *et al.*, 1969) and that the heterozygous females and not the homozygous or the hemizygous males are protected. This hypothesis was substantiated by Bienzle *et al.*, (1979) who showed that female heterozygotes (GdA-/GdB) have less parasite densities and less numbers of red cells infected than hemizygous boys. According to Luzzatto (1983), *P. falciparum* invaded red blood cells normally, but maturation of intracellular parasites is damaged or impaired. The parasites that do develop will then have a normal behaviour on their next round in G6PD deficient cells, suggesting that an adaptive changes has taken place (Luzzatto, 1983).

Acute phase reactants: Among the acute phase reactants produced during acute malaria, the alpha-1 acid glycoprotein (AGP) appears to have some protective effect against *P. falciparum*. Friedman (1983) showed that AGP concentration achieved during malaria is sufficient to inhibit parasite multiplication by 80 per cent. Its inhibitory activity depends on and is the function of its sialic acid component and its higher-order structure. AGP acts by blocking parasite-erythrocyte interaction during the invasion process.

Acquired resistance:

Non-specific immunity to malaria can be induced by BCG, live *Brucella abortus*, killed *Corynebacterium parvum* and endotoxin, the mechanisms of which have been speculated in the previous review (Tharavanij, 1981). One of the mechanisms is the release of factors including the tumour necrotic factors (TNFs) from activated macrophages which then kill the parasites (Clark *et al.*, 1981; Taverne *et al.*, 1981). TNFs have been shown to be equally effective in killing of *P. vinckei petteri*, *P. yoelii* and *P. berghei in vitro*, but they have different *in vivo* effects which have been shown to be related to virulence (Taverne *et al.*, 1982). The non-lethal *P. vinckei petteri* is most susceptible, *P. yoelii* is intermediate and *P berghei* is resistant to the effect of passively transferred TNF containing sera (Taverne *et al.*, 1982).

The non-specific resistance induced by different stimulants is mediated through different mechanisms. Nude mice can be protected against *P. vinckei* and *P. chabaudi* infections by prior injection of *C. parvum* but not of BCG (Eugui *et al.*, 1982) suggesting that the effector phase of immunity activated by *C. parvum* by passes a requirement for T lymphocytes.

Specific acquired resistance

Antibody-mediated immunity

Against sporozoites: The fact that sporozoites injected by mosquito bites are few in number, and they are free in the peripheral blood only for a few minutes, led many investigators to believe in the past that sporozoites were non-immunogenic and could not be destroyed by the host immune mechanisms. Ample evidence is now available to show that inoculation of small number of sporozoites from X-irradiated mosquitoes into man or animals leads to the production of antibodies against the parasite surface antigen known as circumsporozoite (CS) protein, and protective immunity (Vanderberg *et al.*, 1969; Clyde *et al.*, 1975; Nussenzweig, 1977). Detectable levels of antibody

against CS protein has been demonstrated in 90% of adults living in the Gambia, Africa (Nardin *et al.*, 1979) and in 31.7% of people living in an endemic area at Kanchanaburi, Thailand (Tapchaisri *et al.*, 1983). Sporozoite-induced immunity is stage and species specific but not strain specific. The protective role of antibody against CS protein has been demonstrated against both animal and human malaria. Transfer of immune sera into mice reduced considerably the number of exoerythrocytic forms. The protection was not absolute, since animals eventually developed patent infection (Nussenzweig *et al.*, 1972). Failure to confer complete protection could be due to insufficient quantity of the antibody. Hence passive transfer of as small as 10 μg of MAB against CS protein of *P. berghei* abolished or profoundly diminished the infectivity of 10^3 sporozoites. Furthermore, Fab fragments of this MAB were as effective as the intact antibodies in mediating protection (Potocnjak *et al.*, 1980) indicating that this MAB functions by blocking the attachment of sporozoites to host receptor cells presumably hepatocytes. Species-specific MAB against the CS protein has been shown to neutralize infectivities of *P. falciparum* or *P. vivax* in chimpanzees (Nardin *et al.*, 1982). MABs against the CS proteins of *P. berghei* or *P. falciparum* have been shown to neutralize the infectivities of homologous plasmodia in hepatoma cell culture (Hollingdale *et al.*, 1982; 1984).

Against the blood stages: The protective role of antibody against the blood stage parasites has been well documented. In *P. falciparum* infections, large amounts of IgG from immune adults (doses equivalent to 10-20% of the recipient's own IgG) significantly reduced parasitaemia in heavily infected children, but this protection was of short duration (Cohen *et al.*, 1961). Protection appears to be most effective against strains prevalent in the same geographical location since passive transfer in chimpanzees of pooled gamma globulin from immune West Africans could protect only against challenge with the West African strain of *P. falciparum* but not against the Southeast Asian strains (Sadun *et al.*, 1966). The transferred antibody is less effective when it is adminstered to T cell depleted (Jayawardena *et al.*, 1977) or splenectomized recipients (Brown *et al.*, 1974). The protective function of humoral antibody coud also be demonstrated in B cell deficient animal such as mice treated with anti-μ serum or bursectomized birds. Anti-μ treatment rendered mice susceptible to infection with other wise avirulent *P. berghei yoelii* (Roberts *et al.*, 1977). The mechanisms whereby antibody confer protection has been a subject of intense investigations in the past decade. There are at least 4 mechanisms by which the antibodies can act:- interaction with extra-cellular merozoite intra-erythrocytic death of the parasites, reversal of cytoadherence and the combined action of antibody and effector cells in the form of opsonization or antibody-dependent cell-mediated cytotoxicity (ADCC).

(a) Interaction with extra-cellular merozoites: The antibody can act by inhibition of merozoite invasion (Cohen *et al.*, 1969; Phillips *et al.*, 1972), merozoite clustering (Chulay *et al.*, 1981), and inhibition of merozoite dispersal (Green *et al.*, 1981). Merozoite invasion inhibition has been shown to species-specific, not complement-dependent, and is mediated by IgM and IgG and its $(Fab')_2$ fragments but not its monovalent Fab (Cohen and Butcher, 1970). It is most active against homologous strain than against the heterologous strain (Wilson and Phillips, 1976). Merozoite inhibitory activity correlates with clinical immunity in 70-80% of human or monkey sera studies (Cohen, 1979).

(b) Intra-erythrocytic death of malaria parasites: Addition of human immune sera from Sudan to the culture of *P. falciparum* induced 'crisis' forms (Jensen *et al.*, 1982). This parasitocidal effect of immune sera appears to be confined only to certain geographical locations, since immune sera from Flores, Indonesia could not cause intra-erythrocytic death even though they possessed merozoite invasion inhibitory activity (Jensen *et al.*, 1984).

(c) Reversal of cytoadherence: Erythrocytes infected with trophozoites or schizonts of *P. falciparum* are not normally present in the peripheral blood but sequestered along capillaries and vascular endothelial cells (Miller, 1969). It has been shown recently that 96-100% of amelanotic melanoma cells bound infected erythrocytes much better than endothelial cells of which only 4-59% were bound (Schmidt *et al.*, 1982). Immune sera revert cytoadherence (David *et al.*, 1983) and this reaction was strain specific (Udeinya *et al.*, 1983).

(d) Combined action of antibody and cells: (1) Opsonization: The value of opsonization in protection against malaria has been of some doubt. Passive transfer of immune serum in rodent malaria failed to enhance the *in vivo* clearance rate of ^{51}Cr-labelled parasitized cells (Wyler, 1982). Administration of silica which destroys phagocytic cells *in vitro* does not interfere with the recovery from malaria of vaccinated mice (Playfair and De Souza, 1979). Moreover, recovery from non-lethal *P. yoelii* infections coincided with decreased anti-bacterial activity of spleen, liver and peritoneal macrophages (Murphy and Lefford, 1979).

In human malaria, it has been recently demonstrated that polymorphonuclear leukocytes (PMN) from normal blood donors phagocytosed *P. falciparum* infected rbc *in vitro* to a greater extent than normal rbc (Celada *et al.*, 1983). The phagocytic activity was greatly increased by immune sera but not by sera from individuals recovering from a first acute *P. falciparum* infection. The phagocytosis enhancement was mediated by IgG (Celada *et al.*, 1983) and was independent of complement (Celada *et al.*, 1984).

(2) ADCC: The observation that K cell activity towards chicken erythrocytes is increased in malaria infection is taken as an indication that ADCC may be important in immunity to malaria (Greenwood *et al.*, 1977). Lymphocytes from West African infected children and immune adults were found to kill *P. falciparum* in the presence of immune serum (Brown and Smalley, 1980). Immune sera have been shown to arm peripheral blood monocytes from normal unsensitized individuals to phagocytose free merozoites but not intact schizonts. A marked difference in the level of merozoite phagocytosis was observed depending on immune status of individuals whose sera were tested, but not on the antibody levels measured by fluorescence or by precipitation tests. The activity was mediated by IgG and merozoite recognition by armed macrophages was not strain specific (Khusmith *et al.*, 1983).

Cell-mediated resistance

Against sporozoites: Acquired resistance to sporozoite infection is thymus dependent requiring T cells which function either by their helper role in the antibody production or by other mechanisms unrelated to their helper function. It has been shown that thymectomized, X-irradiated and bone marrow reconstituted mice (ATX-BM) or nude mice do not develop sporozoite neutralizing antibody or clinical immunity after vaccination with irradiated sporozoites. Reconstitution with thymus cells restores the capacity of such animals to synthesize antibody and become immunized (Spitalny *et al.*, 1977). The spleen does not have an essential inductive or

effector role in sporozoite immunity (Spitalny *et al.*, 1976).

The cell-mediated effector mechanisms in the absence of antibody is suggested by Chen *et al.*, (1977). Mice treated with anti-μ chain serum which suppresses humoral immunity are still clinically immune after vaccination with sporozoites. Nevertheless, attempts to adoptively transfer sporozoite resistance with sensitized cells have so far been unsuccessful (Spitalny *et al.*, 1976).

Against the blood stages: A substantial body of information has been available for the protective role of T cells in immunity against the blood stages. Brown *et al.*, (1968) showed that *P. berghei* infection in normal rats was self-limited whereas the infection in neonatal thymectomized animals resulted in higher and more prolonged parasitaemia and increased mortality rate. Infection in nude mice with *P. yoelii* produced 70% parasitaemia and death and only mild disease in normal mice (Clark and Allison, 1974). A similar observation was made in *P. chabaudi* infected CBA nude mice (Eugui and Allison, 1980). Chicken were first rendered agammaglobulinaemic (by treatment with cyclophosphamide and testosterone), infected with *P. gallinaceum*, and then cured with chloroquine. The animals were again challenged but the animals survived, indicating the protective effect of B cell independent mechanisms (Rank *et al.*, 1976). Similar result was obtained in μ-suppressed mice infected with *P. yoelii* followed by clindamycin treatment (Roberts *et al.*, 1978, 1979). The T cell functions in protective immunity could be due to 2 different mechanisms comprisingt heir helper function for production of antibody by B cells, and the antibody independent mechanisms which mediated through macrophages by releasing ofreactive oxygen intermediates (Allison and Eugui, 1983) which have been shown to cause intra-erythrocytic death of malarial parasites (Clark *et al.*, 1983).

PARASITE EVASION MECHANISMS

Despite being exposed to several immune mechanisms of the hosts, some malaria parasites can survive in the host hostile environment sometimes causing recrudescence or relapses which are frequently found in clinical practice. Factors involved in the evasion mechanisms include intracellular location of parasites, antigenic variations and parasite modification of the host immune responses.

Intra-cellular location of plasmodia

Extra-erythrocytic development within hepatic parenchyma causes no cellular reaction indicating that infected liver cells does not express plasmodial antigen, and the parasite is protected from the immune attack by its intra-cellular location. The EE form (hypnozoite) has been described in the liver of primates infected with *P. cynomolgi* (Krotoski *et al.*, 1982a) and *P. vivax* (Krotoski *et al.*, 1982b).

The intra-cellular localization of plasmodia in rbc make them invulnerable to the attack by cytotoxic T cells despite the presence of malaria antigen on the surface of infected rbc. It is known that the action of cytotoxic T cells requires the additional presence of compatible MHC antigen on the surface of target cells, and rbc are notorious for the paucity of MHC gene products on their surfaces. It is not therefore surprising that attempts to demonstrate specific T cell cytotoxicity against parasitized rbc have been unsuccessful (Phillips *et al.*, 1970; Cohen and Butcher, 1971).

Antigenic variation

There are substantial evidence within several plasmodial species for the occurrence of many antigenically different strains. The difference in antigenicity and virulence between parent and relapse strains of *P. berghei* was first demonstrated by Cox (1959). Brown *et al.*, (1965) showed by means of schizont

infected cell agglutination test that *P. knowlesi* obtained during recrudescence along a course of chronic infection in rhesus monkeys contained agglutinin reacting to previously patent variants but not for those yet to appear. The presence of antigenic diversity of *P. falciparum* was demonstrated using an indirect fluorescent antibody assay and a panel of MABs (McBride *et al.*, 1982). Hommel *et al.*, (1983) demonstrated by a surface immunofluorescent technique that clones of *P. falciparum* from splenectomized squirrel monkey (S–) acquired new antigen (S+) after passive transfer into the spleen intact monkey. The switchover from S– to S+ phenotypes occurred as early as 14 days after transfer and almost complete by day 20.

The parasites undergoing antigenic variation are not recognized by the antibodies reactive against the preceding variants, and thus can evade from otherwise the hostile host immune mechanisms.

Modification of immune responsiveness

During acute malaria, there are changes insub-population of lymphocytes characterising by a reduction in the absolute number of circulating T cells and the relative increase in the percentage of B cells and null cells (Wyler, 1976; Greenwood *et al.*, 1977; Wells *et al.*, 1979). Changes in lymphocyte sub-populations are associated with modified immune responsiveness comprising immunosuppression, lymphocyte polyclonal activation, lymphocytotoxic antibodies and soluble circulating antigen leading as a consequence to the survival of the parasites.

(1) Immunosuppression : Immunosuppression caused by *P. falciparum* has been studied by Greenwood *et al.* (1972). It was shown that patients with falciparum, the humoral immune responses to *S. typhi* 'O' antigen and tetanus toxoid were suppressed whereas the response to *S. typhi* 'H' antigen was unaltered. The duration of suppression varies with the antigen used being less than 7 days for typhoid vaccine and more than 30 days for meningococcal vaccine (Williamson *et al.*, 1978). Suppression of the humoral immune response is mediated through adherent cells especially macrophages and T cells. Spleen cells from mice infected with *P. yoelii* do not exhibit a primary *in vitro* response to sheep rbc, but this defect can be restored by replacing the adherent cells with those from normal animals (Warren and Weidanz, 1976; Brown *et al.*, 1977). Antibody response to thymus independent antigen, the pneumopolysaccharide, in *P. yoelii* infected mice, was shown to be depressed, but such depression did not occur in nude mice or mice pre-treated with anti-thymocyte sera (Weidanz *et al.*, 1982). The immunosuppression by adherent cells is related to the duration of infection, and in *P. berghei* infected mice, the suppression was observed after the third day of infection (Wyler, 1978).

Immunosuppression of CMIR in man has been demonstrated mainly by the reduction of delayed cutaneous responses to lectins and soluble antigens especially candidin (Druilhe *et al.*, 1983), but the lymphocyte proliferative response to lectins remain intact (Greenwood *et al.*, 1972; MacDermott *et al.*, 1980; Ballet *et al.*, 1980). Specific malaria antigen induced lymphocyte proliferation has also been reported (Wyler *et al.*, 1977) Further study in *Aortus* monkeys showed that suppression of lectin induced blastogenic response can occur provided that parasitaemia exceeds 25% (Taylor and Siddiqui, 1978). Brasseur *et al.*, (1983) showed that patients with cerebral malaria as well as some patients with high parasitaemia even without cerebral involvement had diminished or negative cutaneous response to candidin during the acute phase. Blastogenic response to lectin was in general unimpaired except in a few cerebral cases, whereas proliferative responses to candidin were suppressed in parallel with the delayed cutaneous response to the same antigen.

In animal malaria, results for suppression of CMIR are conflicting. Mice with *P. berghei* malaria showed normal skin graft rejection or contact hypersensitivity to picryl chloride or oxazolone (Greenwood *et al.*, 1971), the responses to sheep rbc, chicken gamma globulin and dinitrofluorobenzene were shown to be depressed (Liew *et al.*, 1979).

What is the significance of immunosuppression in malaria in relation to parasite survival or the future vaccine development? Evidence in favour of parasite survival includes (a) The observation that infection to the blood stage parasites suppressed the antibody response to subsequent sporozoite vaccination. The response to sporozoite surface antigen was short-lived, the response to secondary challenge was undetectable and vaccination did not induce protection (Orjih and Nussenzweig, 1979); (b) Splenectomy of rhesus monkey with chronic *P. inui* infections may lead to self-cure suggesting that malarial resistance is reduced by the action of splenic cells probably having immunosuppressive activity (Wyler *et al.*, 1977). In relation to responses to the vaccine, malaria induced immunosuppression will obviously reduce the efficacy of the vaccines against other human pathogens.

(2) Polyclonal lymphocyte activation: Malarial parasites contain mitogen capable of non-specific stimulation of lymphocytes to proliferate thus reducing the number of immunologically committed lymphocytes reacting specifically to the malarial antigens. In man polyclonal lymphocyte activation has long been suspected by the observation that synthesis of gamma globulins in immune Africans is about 7 times greater than that of normal Europeans, whereas the rate of albumin synthesis is not different (Cohen *et al.*, 1961). Direct evidence for the stimulating effect of malaria antigen comes from the demonstration that addition of the supernatant from the culture of *P. falciparum* or sera from patients with falciparum malaria stimulated transformation of human peripheral blood lymphocytes (Wyler and Oppenheim, 1974; Strickland 1978). Only T cells and not B cells are stimulated by *P. falciparum* extracts (Wyler, 1978).

(3) Lymphocytotoxic antibodies: Antibodies cytotoxic for autologous lymphocytes optimally reactive at 15°C have been demonstrated in the sera of patients with falciparum or vivax malaria (Wells *et al.*, 1980). In a subsequent study, it was shown that antibodies belonging to IgM class were reactive against both T and B cells. Those directed against T cells were reactive only at 15°C but those against B cells were reactive at both 15°C and 37°C (Gilbreath *et al.*, 1983).

(4) Soluble circulating antigen: Soluble malaria antigen has been demonstrated in the sera from patients with malaria (Wilson *et al.*, 1975; Perrin *et al.*, 1979). Some of the soluble antigens might have formed immune complexes. The complexes have been shown to appear soon after the clearance of parasitized rbc and reach maximum value after 5-9 days. The peak of circulating immune complexes coincides with the decreased level of C4 and C3 with the appearance of C3d in the plasma (Perrin *et al.*, 1979). The immune complexes may play role in suppressing the. host resistance mechanisms against malaria. Culturing of lymphocytes in autologous plasma results in the reduced lympho-proliferative responses to lectin (Moore *et al.*, 1977) and this suppression could be due to immune complexes in the plasma. Immune complexes could interact with Fc receptors on the surface of macrophages thus interfering with the macrophage-mediated ADCC against malaria.

VACCINE DEVELOPMENT

Against sporozoites

As discussed in the preceding section, the candidate antigen is the CS protein, the antibody against which has been demonstrated in immune persons. In addition, MABs against. CS proteins of all plasmodia tested e.g. *P. berghei*, *P. falciparum* and *P. vivax* have been shown to neuralize infectivities of sporozoites of the corresponding species either *in vivo* or *in vitro*. Immunity against sporozoites is stage and species specific but not strain specific. With this property, it is therefore possible that the vaccine prepared against a given species of plasmodia will be broadly reactive against sporozoites of this species anywhere in the world.

To develop sporozoite vaccine, three general approaches could be made:- preparation of a large quantity of sporozoites free from mosquito tissue contaminants, preparation of CS proteins through recombinant DNA technology, and synthesis of the antigenic peptide once the structure of the immunodominant epitope of the CS protein is known. The first approach is untenable because of the difficulties in producing a large quantity of sporozoites in the laboratory. The latter two approaches are more appropriate, through which rapid progresses have been made. Using a recombinant DNA technology, the gene encoding the CS protein of *P. knowlesi* has been cloned in *E. coli* using plasmid pBR 322 as a cloning vector (Ellis *et al.*, 1983), the coding sequence determined and the CS peptide synthesized (Ozaki *et al.*, 1983; Godson *et al.*, 1983). The gene coding for CS protein is unsplit and present in the genome in only one copy. The CS protein has an unusual structure with the central 40% of the polypeptide chain present as 12 tandemly repeated amino acids flanked by regions of highly charged amino acids (Ozaki *et al.*, 1983). These tandemly repeated amino acids occur 12 times within the gene and account for at least one-third of the amino acid sequence of the CS protein (Godson *et al.*, 1983). Cloning of the gene coding for CS protein of *P. falciparum* has recently been accomplished by 2 groups of investigators using different approaches. The New York University group used the same procedure as the knowlesi system by preparing first mRNA from sporozoites dissected from mosquitoes from which cDNA was prepared, cut with restriction enzymes and then hybridized with strands of DNA from pBR322 plasmid cut with the same restriction enzymes. The recombinants were then transformed in *E. coli* and the gene products screened against MAB specific for CS protein (Enea *et al.*, 1984). Another approach carried out by the NIH group was different from the NYU group by 3 aspects:- First DNA from the blood stage parasites instead of mRNA from sporozoites was used as starting materials. Second mung bean nuclease which cut only at the coding sequence of the gene (at the begining and at the end of the exon) was used. Third the bacteriophage was used in place of the plasmid. With this novel technique, the entire coding sequence of the CS protein gene was known (Dame *et al.*, 1984). Whatever method used for cloning, similar result was obtained showing that the immunodominant peptide consists of tandemly repeats of 4 amino acids:- Asn-Ala-Asn-Pro. Dame *et al.*, (1984) showed a more complete sequence of sporozoite genes encoding a protein of 412 amino acids which consist of a signal sequence, a charged region, a central region of 41 repeats of four amino acids, two other charged regions, a probable cysteine loop, and an anchor sequence. Thirty-seven of the repeats in the central region are identical (Asn-Ala-Asn-Pro), four others have alternative sequence (Asn-Val-Asp-Pro). In addition, two conserved regions (region I and II) before and after the repeats have been identified. There is a homology between region II of *P. falciparum* and *P. knowlesi*, and thus

this conserved regions could be considered as a target for a vaccine to be broadly reactive against sporozoites of many species of human as well as animal malaria (Dame *et al.*, 1984).

Against blood stages

Research towards the development of vaccine against the blood stages has not made a rapid progress as with the sporozoites for several reasons:- First, there appear to have several candidates molecules associated with protection and thus it is very difficult to decide as to which molecule the concentrated effort should be spent. Second, antigenic diversity and antigenic variation have been demonstrated in the blood stages (McBride *et al.*, 1982; Hommel *et al.*, 1983). The ideal vaccine should be directed against the invariant antigen and not the variant antigen in order to confer a good protection against all strains.

The general approach to identify candidate antigens is to study the reaction between the immune sera or MABs with known biological functions and the antigenic epitopes of the parasites. Using this approach, several candidate antigens have been identified. The next approach is to clone the gene encoding these protein and then synthesized, provided that the peptide is not too long, or to put the gene in acceptable vehicles such as vaccinia virus.

Identification of candidate antigens: Several candidate antigens have been identified by biologically active MABs and are summarised below (WHO, 1984):-

(1) Antigens on the surface membranes of schizonts and merozoites of *P. falciparum:*

Mr 195 K antigen: This is a high M.W. antigen on the surface membrane of schizont and merozoites. The significance of this molecule is derived from two sources:- First, the analogous antigen (Mr 250 K) of *P. chabaudi* is immunogenic and immunization of mice with 8 μg dose twice with Freund's complete adjuvant and once intravenously induced immunity against homologous challenge. Passive transfer in mice of MAB against Mr 230 K of the schizont surface protein of *P. yoelii* confers protection against 17X (non-lethal) and 17XL (lethal) parasites. Second, some MAB against Mr 195 K antigen of *P. falciparum* partially blocked merozoite invasion. The general features of Mr 195K antigen are as follows:-

(a) Glycoprotein in nature as shown by incorporation of ^{3}H-glucosamine.

(b) The reactive epitope is distributed evenly over the parasite surface membrane as demonstrated by immunoelectromicroscopy.

(c) Exhibit marked antigenic diversity in natural isolates.

(d) Conversion to Mr 83 K protein during terminal schizont maturation and merozoite release. The Mr 83 K protein is not detected in ring stage parasite, but can be identified in culture supernatant.

Glycophorin binding protein: The significance of glycophorin binding proteins in inducing protective immunity is derived from following source of information:-

(a) A doublet of Mr 155 130 K localized to the merozoite surface have been shown to bind glycophorin *in vitro.*

(b) Glycophorin is a potential receptor for attachment of *P. falciparum* merozoites to rbc. ^{125}I-glycophorin binds to free merozoites and soluble glycophorin added to an invasion assay inhibits merozoite invasion. It has been estimated that each merozoite bears approximately 5,000 binding sites for glycophorin.

Glycophorin binding protein could be found in the culture supernatant after release of merozoite from infected cells, and by washing of free merozoites. The protein is heat stable,

capable of withstanding heating at 100°C for 10 minutes, a property shared by S antigen. Incorporation of amino acid into this protein is found when the parasites are grown in the presence of ^{3}H-glycine but little uptake of ^{35}S-methionine.

Glycophorin binding protein shares many properties with the ring infected erythrocyte surface antigen (RESA), and human MAB directed against RESA has been shown to block merozoite invasion.

(2) Antigens in apical paired organelles of merozoites: Apical paired organelles have been shown to be associated with membrane junction formation and interiorization during merozoite invasion. Furthermore, an MAB reacting with these organelles can block merozoite invasion. Several antigens associated with the paired organelles have been identified using MABs which could be sub-divided into 2 classes:-

(a) Mr 40-90 K rhoptry proteins: There is an array of proteins in this group with different molecular weights all of which are reactive against the rhoptry protein. The molecules identified varied from one laboratory to the other being Mr of 82, 70, 67, 39, and 37 K in one laboratory 78, 63, 42 and 40 K in another laboratory, and 85, 73, 68 and 42 K in the third laboratory. Amongst these molecules, the Mr 68 and 42 K appear most promising as candidate antigens, since one MAB reacting with these two molecules have merozoite invasion inhibitory activity and is broadly reactive against all isolates tested

(b) Mr 100-150 K rhoptry proteins: The significance of these rhoptry proteins has not been clearly demonstrated. It has been shown that three MABs directed against Mr 145 135 and 104 were reactive against all 21 strains, of *P. falciparum* tested but not against nonhuman primate or rodent malaria. In another laboratory, the molecules identified were of Mr 150 and 140 K.

(3) Other schizont-merozoite proteins associated with erythrocyte invasion: There are several parasite proteins interacting with N-acetyl-D-glucosamine. The importance of this aminosugar is evident by the demonstration of merozoite invasion inhibition by adding it to the malaria culture. These parasite proteins are retained in the N-acetyl-D-glucosamine column when ^{35}S-methinine labelled *P. falciparum* is passed through. The molecular weights of these proteins were Mr 140, 70 and 35 K. They can be distinguished from glycophorin binding proteins by virtue of their ability to be labelled with ^{35}S-methionine which is poorly labelled in the glycophorin binding protein. The aminosugar reactive proteins might be part of the merozoite membrane or the rhoptry organelles and that they could participate in merozoite attachment by dint of their specific binding properties.

(4) Other schizont-merozoite antigen related to intra-erythrocytic death: MABs reacting with Mr 140, 82 and 41 K antigens have been shown to block parasite growth apparently by inhibiting terminal intra-erythrocytic development. The 140 and 41 K parasite antigen purified by affinity chromatography were used to immunize saimiri monkeys with 50-75 μg of protein per dose in the presence of Freund's complete adjuvant initially and incomplete adjuvant subsequently. The monkeys were protected when the antigens eluted directly from the column were used. When these antigens were further purified by SDS gel electroelectrophoresis, only purified 140 K antigen was protective but not the purified 41 K antigen.

(5) Malarial antigens on infected erythrocytes: (a) Antigens on membranes of ring infected erythrocytes (RESA): These antigens were first demonstrated by Perlmann *et al.*, (1984) using an indirect immunofluorescent test on 1% glutaraldehyde fixed, ring-infected erythrocytes. Elution of the antibody from

ring-infected rbc showed their reactivities in the immunoblot technique against the proteins of Mr 155, 135 and 120 K. Evidence of these antigens as candidate proteins are:- First, a good correlation between the serum activity against RESA antigen and its capacity to inhibit invasion. Second, anti-RESA antibody eluted from ring infected erythrocytes showed an approximately 100-fold increase in inhibitory activity than that of the corresponding serum. Third, a human MAB specific for Mr 155 K protein blocks merozoite invasion *in vitro*.

(b) Antigens on membrans of mature trophozoite and schizont of *P. falciparum* infected erythrocytes: Knob protein: The knob protein highly rich in histidine is synthesized by K+ (knob positive) but not by K– (knob negative) strains. Part of the knob structure is derived from the altered host cell since the knob protein remains associated with the insoluble residue together with erythrocyte cytoskeleton following treatment of plasmodium-infected cells by some detergents. Earlier in its discovery, it was postulated that the knob proteins played role in cytoadherence during sequestration. This hypothesis is later refuted by the finding that not all K+ parasites have cytoadherent properties.

Cytoadherent proteins: Adherence of *P. falciparum* infected red blood cells is believed to be due to the presence of cytoadherent protein different from the knob protein. The protein is sensitive to tryptic cleavage. Different isolates appear to have cytoadherent proteins of different Mr within the range of 200-280 K, and different antigenicity. Thus a given serum can reverse cytoadherence of the homologous but not the heterologous isolates.

(6) Other protein antigen: There are other proteins of plasmodia capable of inducing protective immunity eventhough the sites of their actions remain obscure. According to Fandeur *et al.*, (1984), Saimiri monkeys are protected against *P. falciparum* challenge by a passive transfer of immune IgG reacting with proteins of Mr 90-100, 76 and 41 K. There is no correlation between parasite growth inhibitory activity of these IgG and protection. Monkeys immunized with purified Mr 90-100 K protein as well as Mr 76 K protein are protected against challenge with *P. falciparum*.

Cloning of genes encoded for 'candidate antigen': Scientists at the Walter and Eliza Hall Institute of Medical Research, Melbourne first succeeded in the cloning of genes encoded for S antigen of *P. falciparum* using phage λ gt11-Amp as a cloning vector (Kemp *et al.*, 1983). A substantial part of this protein consists of tandemly repeats of 11 amino acids (Pro-Ala-Lys-Ala-Ser-Gln-Gly-Gly-Leu-Glu -Asp) apparently strictly conserved within the protein (Coppel *et al.*, 1983). More recently, a gene encoded for 'RESA' antigen of *P. falciparum* was successfully cloned using again the phage λ gt11-Amp3 (Coppel *et al.*, 1984). The 'RESA' protein is composed of repeating sub-units of 8, 4 and 3 amino acids.

The cDNA for Mr 195 K protein of *P. falciparum* has been cloned in another laboratory and the bacterial lysate from this cDNA clone has been used to immunize mice to produce antibody specifically immunoprecipitated with Mr 195 K antigen of schizonts of *P. falciparum*.

Against the gametes

Vaccination with the gamete antigens is aimed at blocking transmission of malaria from man mosquitoes thus reducing chances for the sporozoites to re-infect man. This type of vaccine has no effect whatsoever in protecting the vacinees from malaria when they are bitten by infected mosquitoes or when they receive blood for transfusion. Transmission blocking immunity has been demonstrated against avian (Gwadz *et al.*, 1979), rodent (Mendis and Targett, 1979, 1981) and simian

(Gwadz and Green, 1978) malaria infections by vaccination with gametes. Antibodies against gametes can act at various levels e.g. agglutination of microgametes, prevention of fertilization or inhibition of zygote developments. Using MAB against gametes of *P. falciparum*, target antigens of this parasite has been identified to be molecules of Mr 255, 59 and 53 K.

CONCLUSION

This review has focussed in 3 areas of rapidly developing fields of malaria immunology comprising specific and non-specific immune mechanisms, parasite evasion mechanisms and vaccine development. Clear understanding of the host immune mechanisms as well as the parasite evasion mechanisms will help in planning a strategy for the future control of malaria. Rapid progress has been made in the study on host-parasite relationship and identification of 'candidate' antigens for use as the vaccine. Through recombinant DNA technology and synthesis of immunodominant 'protective' peptides, there is hope for malaria vaccine in the near future. The ideal vaccine should be multivalent directed against sporozoites, blood stages and gametes.

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