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REVIEW

PROTECTIVE ANTIGENS, IMMUNE MECHANISMS AND SYNTHETIC VACCINES IN MALARIA - 1994

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Abstract: An effective vaccine will greatly reduce mortality and morbidity against malaria and in combination with other control measures, can eradicate malaria from Sri Lanka. The possible types of synthetic malaria vaccines that may be directed against different target stages of the parasite are reviewed. The evidence for cellular and humoral mechanisms of immunity operating against sporozoites, liver stages, asexual blood stages and sexual stages are briefly discussed with reference to the major target antigens in each stage. A novel approach to blocking malaria transmission through immunization with mosquito antigens is briefly mentioned. Preliminary results of a clinical trial in Sri Lanka to determine the immunogenicity and safety of malaria peptides conjugated to diphtheria toxoid as candidate vaccines, are discussed.

Key words: Immunity, malaria, *Plasmodium falciparum, Plasmodium vivax,* surface antigens, vaccines

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 y.[1] These figures represent 4% and 25% of the total deaths for the respective age groups. Malaria continues to be prevalent in Sri Lanka with approximately 400,000 cases and 15 deaths being reported in 1991, excluding the North. Of these 76,500 infections were caused by *Plasmodium falciparum* and the rest by *Plasmodium vivax.*[2] Significant progress has recently been achieved in characterizing protective antigens and in understanding the immunology of malaria.[3-6] This has been particularly apparent in the case of *P. falciparum*, where the need for new methods of disease control is greatest, and is partly due to the ability to culture the parasite *in vitro.* Indeed humans trials are currently under way in several laboratories using vaccines based on synthetic peptides and recombinant proteins. The advantages of an immunological approach to the control of malaria are that a vaccine is easy to use, inexpensive, relatively long lasting and avoids the development of drug resistance in parasites.

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 and liver stages (exoerythrocytic forms or EEF) is designed to prevent infection and therefore the development of the

symptoms of the disease. Immunization with antigens based on the asexual blood stages, while not preventing infection, is primarily 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 block the transmission of disease to uninfected individuals in the population. An effective vaccine when used with other malaria control measures can be expected to completely eradicate malaria from Sri Lanka.

NATURE OF A VACCINE

Attenuated or killed pathogens have in the recent past provided some very successful vaccines. The smallpox and polio vaccines are two examples. Adequate quantities of malaria parasites however cannot be obtained from *in vitro* culture for this purpose. The use of blood derived material for vaccination also carries with it the risk of transmitting infectious viruses. It would also contain large amounts of immunologically irrelevant material from erythrocytes and parasites that can give rise to autoimmune reactions and other undesirable side effects. Vaccine based on defined molecules (molecular or subunit vaccines) that are capable of inducing the necessary protective immune response are being developed to overcome these drawbacks.[7]

Molecular vaccines can take a number of forms. Proteins can be produced in large amounts by recombinant DNA techniques in bacteria, yeast, eukaryotic cell lines or even genetically engineered plants and animals. The quantities of protein required for a mass vaccination campaign can theoretically be produced by these methods. A recombinant vaccine composed of the surface antigen of the Hepatitis B virus produced and purified from yeast has been commercially available for several years.

Peptide fragments from viral coat proteins can inhibit precipitation of the virus by an anti-serum and immunization with a short peptide fragment of the virus coupled to a carrier could induce the formation of virus neutralizing and precipitating antibodies. A synthetic peptide vaccine for contraception 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.[8] Because synthetic peptides are small molecules they are usually made immunogenic by coupling to larger protein molecules referred to as carriers. Tetanus and diphtheria toxoids are widely regarded as suitable carriers for human immunizations. The carrier molecule is necessary to stimulate thymus processed helper lymphocytes or T helper cells that cooperate with the B lymphocytes which differentiate to produce antibodies to the peptide epitope.

Recombinant vectors, (viral or bacterial) carrying the gene for the relevant antigen may also be used to deliver an immunizing dose of the antigen. A suitable viral vector for human use is Vaccinia, the virus that was the basis for immunization against small pox and therefore of which we have considerable experience. Other viral vectors that have been considered for immunization include Herpes, Adenovirus

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 circumsporozoite (CS) protein gene of *Plasmodium berghei*, a mouse malaria parasite, was cloned into an attenuated strain of *Salmonella typhimurium* and the recombinant *Salmonella* used to orally immunize mice against *P. berghei* infection, with some success. The use of a similar vaccine against *P. falciparum* sporozoites based on attenuated *S. typhi may* therefore be possible.

Synthetic peptides and recombinant proteins need adjuvants to enhance the immune response. Complete Freunds adjuvant is unsuitable for human use because of its severe side effects. Adjuvants presently available that are suitable for human use include muramyl dipeptide derivatives, aluminium hydroxide and more exotic reagents like the meningococcal outer membrane proteins.[9] One of the advantages of recombinant viral vectors for delivering a vaccine is that they do not require adjuvants.

IMMUNIZATION AGAINST SPOROZOITES

Evidence for immunity

Malaria infection in man is initiated by the inoculation 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 exoerythrocytic forms (EEF) that 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 inoculation of relatively small numbers of gamma ray irradiated sporozoites into rodents, monkeys and humans.[10] This has encouraged investigations into the development of a molecular vaccine against sporozoites.

The circumsporozoite (CS) protein as a target antigen

Sporozoites of all the malaria species are covered on the outside with a major coat protein referred to as the circumsporozoite or CS protein. The genes for the CS protein of *P. falciparum, P. vivax* and *P. malariae* have been cloned and their structure determined. 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.[11] In *P. falciparum* the repeat is composed of the sequence NANP (using the single letter code for amino acids: asparagine-alanine-asparagine-proline) with the variant sequence 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* sequenced so far, from different parts of the world contain the same immunodominant repeats, although the number of repeats varies between strains. In *P. vivax*, the predominant strain contains the repeat sequence

GDRAA/DGQPA while a variant contains the repeat ANGAGNQPG. More recently, a second surface protein of mol. wt. 42kDa present in both *P. falciparum* sporozoites and EEF, termed CSP-2, has been identified and shown to elicit protective antibodies.[12]

Humoral immunity against sporozoites

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. Parasites that have shed their coats are no longer infective. *In vitro* studies on sporozoite invasion of human hepatoma cells with monoclonal antibodies (mabs) to *P. berghei, P. vivax* and *P. falciparum* sporozoites suggest that the antibodies inhibit invasion.[13,14] However, high concentrations of antibodies are probably required for efficient inhibition.[15]

Role of cellular immunity against sporozoites

Immunization with irradiated sporozoites led to protection against *P. berghei* sporozoite challenge in B lymphocyte deficient but not in T lymphocyte deficient mice. This early observation on the role of T cells was not fully appreciated until more recent work confirmed the observation.[16] Other recent studies[17,18] have shown that gamma interferon produced by activated T cells is toxic to the EEF, and that cytotoxic T lymphocytes are involved in protective immunity in mice.[19]

The mechanism of action of gamma interferon and cytotoxic lymphocytes is not entirely clear. It is known that gamma interferon inhibits the growth of EEF in the liver.[17] Presumably T cells recognize 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. A class I antigen (HLA-Bw 53) and a class II haptotype (DRB1* 1302 - DQB1* 0501), that are common in West Africans but rare in other racial groups, have been associated with protection from severe malaria.[20] HLA-B53 restricted cytotoxic T lymphocytes against an antigen on EEF may provide one mechanism for this protection.[21]

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 recognized 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 recognizing an epitope based on $(NANP)_{40}$.[22] Using an algorithm for predicting regions of alpha helical amphipathicity in proteins a number of other T cell stimulating epitopes have been identified in non-repeat region of the *P. falciparum* CS protein.[23]

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.[23] Interestingly there was a correlation between these dominant T epitopes and the regions of the *P. falciparum* CS protein subject to variation between strains[24] suggesting that immune pressure at the level of T cells may be the driving force for such variation.

Prospects for a sporozoite vaccine

Two teams of reasearchers have already performed human trials with immunogens based on the CS protein of *P. falciparum*. In one trial a 12 amino acid synthetic peptide $(NANP)_3$ conjugated to tetanus toxoid with aluminium hydroxide as an adjuvant was administered.[25] Antibody production occurred in only 53% and 71% of the recipients of 100 μg and 160 μg of vaccine respectively (total of 29 volunteers) as judged in an ELISA assay. Three vaccinees with the highest antibody titres (1:200 and 1:400 by ELISA) 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 this may be due to epitope suppression resulting from previous exposure to tetanus toxoid or an 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 gene for tetracycline resistance read out of frame) was administered in alum as an adjuvant.[26] In general, the antibody response was poor with significant boosting in only 1/15 individuals and that also in the person receiving the highest dose of immunogen (800 μg). This individual had an ELISA titre of 1:800. Only this volunteer was protected against a 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 possible that the poor antibody response to the $R32tet_{32}$ in man is due to the genetic restriction in the recognition of $(NANP)_n$ by T cells. Therefore greater emphasis has to be placed on stimulating T cell immunity to sporozoites for efficient protection. 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. It is also clear that the epitopes recognized by cytotoxic T lymphocytes (CTL) may be an important constituent of a vaccine against sporozoites. An epitope recognized by CTL on the *P. falciparum* CS protein has been recently determined.[27] Interestingly this region is also subject to variation in different *P. falciparum* strains.[24]

Antigens of the EEF

Advances in *in vitro* culture of the EEF of *P. vivax* and *P. falciparum*[28] have permitted a greater exploration of the antigens of these stages which may be of importance for immunization 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.[29] Clearly the identification of more antigens of the EEF and their determination of their ability to be recognized by cytotoxic T cells is an important area for future investigation.

IMMUNIZATION AGAINST 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.[30] 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 hybridization with the insert, rapidly leads to a determination of the amino acid sequence of the antigen. 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 repetetive sequences in many parasite antigens and their extensive cross-reactivity is directed towards producing low affinity antibodies against antigens irrelevant to protection.[31] i.e. the malaria parasite may be cleverly producing an immunological *smoke screen.*

A second major approach has been to identify potentially protective antigens by the use of a combination of mabs, localization of the antigen within the parasite by immunofluorescence and immuno-electron microscopy and drawing analogies with protective antigens of murine malaria.

A third type of approach has utilized 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 against merozoite surface and rhoptry antigens, antigens in the parasitophorous vacuole as well as other novel parasite antigens.[32,33]

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 immunization with such parasite material.[34] The sera from immune owl monkeys inhibit parasite growth *in vitro.* 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.[33] Antibodies may bind to surface molecules on the merozoite and interfere with invasion. Since merozoite release and subsequent reinvasion is a very rapid process it is possible that the rate of binding of antibody molecules to merozoite components is a limiting factor

in immunity. High concentrations of anti-merozoite antibodies might therefore be required to inhibit invasion.

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.[35,36] Mouse studies have implicated tumour necrosis factor or TNF and hydrogen peroxide produced by activated macrophages in toxicity to intra-erythrocytic parasites.[37] Studies on human malaria suggest that T cells from immune individuals in endemic areas produce high levels of gamma interferon and the interleukin IL 2, in response to stimulation with parasite antigen.[38] 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,[39] 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 activated monocytes macrophages. Monocytes and macrophages may be activated by cytokines from antigen activated T cells. However, parasite inhibitory antibody was also found in the sera of children recovering from malaria, reaching a peak in convalescent children.[40]

Recent studies have implicated antibody-dependent phagocytosis by monocyte/macrophages[41,42] and neutrophils[43] in immunity to asexual blood stages. A balance between protective opsonising antibodies of the IgG1 and IgG3 isotypes and blocking antibodies of the IgG2 and IgG4 isotypes may influence the immune status of individuals.[41,42]

It is relevant to consider the various antigens that are possible candidates for inclusion in a vaccine against asexual blood stages. They are categorized 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. Antigens on the surface of parasitized red cells are particularly suitable as targets since they are readily accessible to host antibodies over a significant time period.[5] Recent work suggests that normal erythrocyte surface components may be modified in mature parasites.[44-47] Caution is therefore needed in interpreting reports on the presence of parasite-derived antigens exposed on the outside surface of the host erythrocyte. However a high mol. wt. (Mr >200 kDa) *P. falciparum* erythrocytic membrane protein or PfEMP1 has been reported to undergo antigenic variation, mediate cytoadherence to endothelial cells and to influence gametocytogenesis.[48,49]

Proteins of rhoptries and micronemes

The contents of the paired apical rhoptry organelles and associated micronemes of merozoites are extruded during invasion and have been postulated to give rise to the parasitophorous vacuole. Several proteins and protein complexes from rhoptries have been identified and cloned.[50-53] Antibodies against many rhoptry proteins have been shown to inhibit *P. falciparum* growth *in vitro*. The mechanism by which antibodies to rhoptry proteins exert their inhibitory effect is not known with certainity, but this could occur by their binding to proteins secreted during invasion and thereby interfering with the invasion process. One of the proteins synthesized in the apical complex as an 83 kDa precursor protein is reported to be processed to a 60 kDa molecule that is translocated to the merozoite surface.[53] This protein, termed the apical membrane antigen 1 or AMA - 1 gives rise to invasion inhibiting monoclonal antibodies.[53] It is currently undergoing human trials as an asexual blood stage vaccine.

Merozoite surface proteins

A 185-200kDa precursor glycoprotein (abbreviated to PMMSA and alternatively termed MSA-1 or MSP-1) that is processed to smaller proteins that are found on the merozoite surface was characterized in *P. falciparum* by gene cloning.[54,55] The oligosaccharide side chains of this antigen modulate the antigenicity of the native protein.[56,57] Analysis of the structure of the protein from several isolates suggests that the protein is coded for by dimorphic alleles, that can undergo limited genetic exchange within the molecule.[58] Immunization with PMMSA or synthetic peptides derived from the sequence protect both owl and squirrel monkeys against malaria.[59-62] Non-polymorphic T lymphocyte epitopes in PMMSA that are recognized by persons living in malaria endemic areas have been identified.[63] Proteolysis of PMMSA to yield several polypeptide fragments takes place on the merozoite surface.[64] An 83kDa fragment containing the amino terminus of the mature protein is reported to contain a binding site for the red cell cytoskeletal protein, spectrin.[65] However after shedding the rest of the PMMSA fragments, only the 19kDa polypeptide is carried into cells by invading merozoites.[66] The 19kDa fragment contains two epidermal growth factor like, cysteine-rich domains and may therefore function in binding to red cells or cell surface signalling.[66]

A 45kDa glycosylated and myristilated smaller surface antigen or GYMSSA (alternatively termed MSA-2 or MSP-2) that is unrelated to PMMSA has been identified.[33] Mabs against this antigen inhibit parasite growth *in vitro* and epitopes recognized by the mabs have been identified. The complete amino acid sequence of the antigen has been determined.[67] While GYMSSA shows some antigenic variation, conserved regions are clearly of value for vaccination. T epitopes on the molecule recognized by man were successfully identified.[63]

A 60 kDa merozoite surface protein that is reportedly involved in binding to erythrocytes during invasion is also a vaccine candidate.[68]

Proteins of the parasitophorous vacuole

Antibodies against a 110 kDa serine rich antigen of the parasitophorous vacuole have been shown to inhibit parasite growth *in vitro*. Immunization of owl monkeys with the recombinant antigen was also found to protect against falciparum malaria.[69] 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, but requires further investigation for confirmation.

Other target proteins

At least two other antigens, which may have at least a transient location in the parasitophorous vacuole, have been reported to be recognized by inhibitory mabs. These are the heat stable S antigen and a 26kDa antigen for which partial amino acid sequence has been obtained.[32] 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. 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 sequenced and antibodies against the molecule shown to inhibit merozoite invasion of red cells *in vitro*.[70,71]

Prospect for an asexual blood stage vaccine

Several examples have been given above where purified antigens have been used to immunize monkeys against falciparum malaria with considerable success. Three out of four squirrel monkeys were protected against fulminant infection when immunized with a 42 residue peptide derived from the N-terminal region of PMMSA.[61] Using sequence data obtained by N-terminal amino acid sequencing 55kDa and 35kDa *P. falciparum* proteins to synthesize small peptides and a peptide from the N-terminus of PMMSA, Pattaroyo *et al*[72] were able to obtain significant protection in owl monkeys after immunization with peptides coupled to bovine serum albumin in Freunds adjuvant. Immunization with a combination of the three peptides led to sterilizing 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 immunize volunteers with alum as an adjuvant. Four out of five individuals were protected by the immunization.[73] However, the original results of Pattaroyo in protecting owl monkeys with the SPf66 vaccine were

not reproducible.[74] In their subsequent field trials with SPf66, protective efficacies of 82% against *P. falciparum* and 60% against *P. vivax* were reported.[75] The immunological basis for protection in *P. falciparum* and the cross-protection in *P. vivax* is not clear. In a more recent Phase III field trial with SPf66 in Colombia, no cross-protection with *P. vivax* together with an estimated 33.6% protection against a subsequent episode of *P. falciparum* malaria was reported.[76]

Apart from the effort to produce a vaccine that reduces or abolishes parasites in the blood (anti-parasite), the possibility vaccinating to reduce the pathology produced by blood stage infections (anti-disease) is being investigated. The existence of blood stage infections causing little illness in immune individuals suggests that an anti-disease vaccine may be possible. Glycophosphatidyl inositol anchors of merozoite surface antigens[33] are one of the targets for an anti-disease vaccine since such moieties are thought to be potent inducers of cytokines (e.g. TNF) that are responsible for many of the pathological effects of malaria infection.

IMMUNIZATION AGAINST SEXUAL STAGES AND MOSQUITOES (TRANSMISSION BLOCKING VACCINE)

Male and female gemetocytes ingested with blood undergo gametogenesis in the mid gut of the mosquito. Within ten minutes the gametes become extracellular and undergo fertilization. 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. Antibodies in the host to antigens on extracellular gametes can block the infectivity of *Plasmodium* gametocytes to mosquitoes.[77,78] This phenomenon termed transmission blocking immunity has also been shown to occur in *P. falciparum*[79] and *P. vivax*.[80] A complex of proteins recognized by transmission blocking mabs have been identified on the surfaces of both male and female gametes.[79] After fertilization new surface antigens appear in the zygote and the ookinete. Antibodies to a 25kDa surface antigen on zygotes and ookinetes also block transmission. The gene for the 25kDa antigen has been cloned and sequenced.[81] 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. Such regions may be the binding site to mosquito gut epithelial cells. The 230kDa protein present on the surface of macrogametes and zygotes and which is a target of transmission blocking antibodies, has been cloned and sequenced recently.[82] There are contiguous and cross-reactive epitopes shared between the 230kDa antigen and 48/45kDa glycoproteins also present on the gamete surface, that is also recognized by transmission blocking antibodies.[83] A 16kDa protein present on the surfaces of both sporozoites and macrogametes, and which is a potential candidate for a transmission blocking vaccine, has been recently identified.[84] However, a possible drawback with trans - mission blocking vaccines is the observation that depending on the concentration of antibodies used, enhancement of transmission can sometimes be observed.[85] It may not be easily possible to control immunization schedules in a population to ensure that blocking is favoured over enhancement.

Use of mosquito antigens for immunization

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.[86] Recently it has been shown that antibodies against mosquito components reduce the fecundity of mosquitoes.[87,88] The effect of anti-midgut antibodies on the infectivity of malaria parasites to *Anopheles* mosquitoes was also investigated and a significant reduction in oocyst numbers was observed in the presence of the antibodies in the blood meal.[89,90] The reduction in transmission may be due to antibodies to mosquito midgut receptors for 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.

TOWARDS CLINICAL TRIALS IN SRI LANKA WITH SYNTHETIC MALARIA VACCINES

Structural diversity in GYMSSA

It has been established that the 45kDa merozoite surface antigen, termed GYMSSA, is a candidate antigen for a malaria vaccine. The amino acid sequence of GYMSSA in several different laboratory isolates of *P. falciparum* have been determined. Protective regions of GYMSSA (epitopes) have been identified using monoclonal antibodies that inhibit parasite growth *in vitro* and these have largely been located in the immunodominant but variable repetitive region.[91] However, from protein biochemistry considerations it is possible to predict the existence of potential antibody binding sites (epitopes) in the regions of GYMSSA that are structurally conserved in different parasite isolates.[11] There is at present little data on antigenic diversity of *P. falciparum* in Sri Lanka and the relationship of the Sri Lankan strains of the parasite to the laboratory isolates studied elsewhere in the world.

Since regions of GYMSSA that are conserved in different laboratory isolates are likely to be used for vaccination in Sri Lanka, it becomes essential to determine whether these regions are also present in Sri Lankan isolates of *P. falciparum*. GYMSSA genes were therefore amplified from infected blood samples collected from different regions of Sri Lanka by the polymerase chain reaction (PCR). The amplified DNA was classifiable into one of two size classes. A sample of DNA from each size class was then DNA-sequenced and the results showed that at least two different allelic forms of GYMSSA are present in Sri Lanka.[92] However the regions of the molecule targetted for vaccine development were present in both alleles.

Natural antibody response to epitopes on merozoite surface antigens

Determining antibody levels and the prevalence of antibodies in persons living in malaria endemic areas to putative vaccine candidate antigens and epitopes provides preliminary information on the immunogenicity of the vaccine in man. With this in mind, antibodies against two epitopes of GYMSSA and two epitopes of PMMSA were measured by radioimmunoassay in villagers in the Matale and Polonnaruwa dis-

tricts. At both locations, a significant proportion of adults and children produced antibodies against peptides containing the epitopes.[93] At the Polonnaruwa site, where a higher rate of malaria transmission was observed, it was clearly seen that antibody levels to merozoite and sporozoite surface antigen epitopes declined markedly three to four months after the end of the transmission season.[93] Thus immunity to malaria may wane every year in the low transmission season due to the absence of antigenic stimulation.

Immunogenicity of merozoite surface antigen peptides in mice

Peptides corresponding to conserved GYMSSA and PMMSA epitopes were chemically linked to bovine serum albumin as a carrier protein. The conjugates were used to immunize Balb/c mice in saline and with the following adjuvants: Freund's adjuvant, two muramyl dipeptide derivatives (muramyl dipeptide is the minimal adjuvant determinant in mycobacterial cell walls) and aluminium hydroxide. Antibody levels against peptides were then measured by enzyme-linked immunosorbent assay. Freund's adjuvant produced the highest titre of antibodies (up to 10^{-6} after three injections). Antibody levels with alum-absorbed antigen reached titres of 10^{-3} and 10^{-4} while antigen in saline alone yielded a titre of 10^{-3}.[94,95] GYMSSA and PMMSA peptides that were chemically linked to diphtheria toxoid as a carrier, although possessing lower peptide: carrier ratios, were also immunogenic in mice when used with alum as an adjuvant (unpublished data). It would therefore appear that immunization with alum, an adjuvant that is suitable for use in man, may yield antibody levels that can be protective in man.

Phase I clinical trial on the safety and immunogenicity of peptide-diphtheria toxoid conjugates

Peptides derived from conserved regions of GYMSSA and PMMSA were synthesized by solid-phase chemical procedures and then covalently linked to diphtheria toxoid to form a stable complex. The peptide-carrier conjugates absorbed onto aluminium hydroxide were then used to immunize human volunteers originating from the Kandy and Nuwara Eliya districts with no known previous exposure to malaria. No adverse effects other than temporary mild fever, temporary pain at the injection site and the occasional incidence of aching muscles were observed in the vaccinees. The clinical chemistry and blood cell profiles of the vaccinees remained normal after three injections of the vaccine. Antibody levels were measured before and after immunization and preliminary results demonstrated a significant boosting effect from the second and third injections.[96] A single drawback appeared to be the development of type III hypersensitivity to the carrier diphtheria toxoid.[96] These results demonstrate the potential and limitations of synthetic peptide-based vaccines against malaria. Carriers other than toxoids, alternative methods of presenting peptides and the possible use of recombinant proteins need to be investigated in further studies.

CONCLUSIONS

There is considerable research effort currently devoted to the development of a malaria vaccine in many laboratories thoughout the world. The SPf66 Pattarayo

vaccine is currently being tested by the World Health Organization in Tanzania and the results of the trial are expected to become available before the end of 1994. Guidelines for ethical and other aspects of malaria vaccine trails have been drawn up by the World Health Organization.[97] With adequate financial, logistical, moral and technical support to the local scientific community, much headway in vaccine development and other ways of controlling malaria can also be made in this country.

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