Perspectives for Malaria Vaccination [and Discussion]

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Perspectives for malaria vaccination

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The need for vaccines to relieve the current global resurgence of malaria is apparent. Immunity is specific for each species of human malaria and for each stage in the life cycle. Once protective immunogens have been identified for one species, the homologous molecules in other species may lead to protection. The usefulness of a particular immunogen will be determined, in part, by its antigenic diversity in the population and the potential for boosting during natural infection. Successful immunization with malarial antigens may require adjuvants to induce effective, long-lived immunity. If different vaccines become available against each stage in the life cycle, then the composition of a particular vaccine may be tailored for different objectives: protection for short periods (for example, during epidemics and for tourists), decrease in disease and death, and malaria eradication.

Introduction

Today, the control of malaria in tropical Africa has been all but abandoned; control in Asia and Latin America has deteriorated since the early 1960s. As the cost of insecticides increases, so will the cost of these programmes, and as insecticide resistance develops, this expensive method will become ineffective. Alternative methods for control are of limited value. Much of Africa, for example, depends on drug treatment of patients rather than an attempt to control transmission. Unfortunately, this may accelerate the appearance of drug-resistant parasites. The goal of the World Health Organization, 'health for all by the year 2000', will require major advances in techniques for disease control. One area where science may offer such hope in the near future is research towards a malaria vaccine.

There have been three major periods of scientific discovery in malaria: the first just before the turn of the century with the dual discoveries of the pathogen and the vector; the second in the 1930s and 1940s with the introduction of larvicides, synthetic antimalarial drugs and the discovery of DDT; and the third, taking place today, with the application of new techniques of immunochemistry and molecular biology for the development of malaria vaccines.

These first two periods of discovery raised great expectations. Ross, who discovered the mosquito vector, thought that malaria could be eliminated from Freetown, Sierre Leone, by a few people sweeping puddles; malaria remains untouched in Freetown to this day. The first successful application of the new knowledge was by Gorgas who controlled malaria and yellow fever during the building of the Panama Canal. The problem, however, was that these methods were too costly for most countries. The major advances of the 1930s and 1940s were towards more effective and less costly methods of attacking the mosquito to control malaria. Hackett of the Rockefeller Foundation and his Italian colleagues introduced the use of larvicide to kill

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the surface feeding Anopheline larvae, a concept used today for malaria control and the basis of attack on the blackfly, the vector for river blindness or onchocerciasis. Within less than a decade, the insecticide effect of DDT was to be discovered by Mueller in Switzerland and with this came the hope of malaria eradication, at least in areas outside Africa. By the early 1960s the maximum reduction of malaria cases was realized, but, within a few years, malaria was to come back with a vengeance because of DDT resistance. We now look to vaccines in this never-ending struggle with malaria and the mosquito. Although vaccines will contribute to malaria control, as have previous important discoveries, malaria will not be eliminated worldwide by vaccines alone. Novel ways to control malaria through the mosquito vector are needed. Unfortunately, vector research has not kept pace with the rapid advances in parasite biology, immunology and biochemistry.

Of the four species of *Plasmodium* in humans, *P. vivax* and *P. falciparum* are the most common. *P. falciparum* causes much of the morbidity and mortality associated with malaria, and therefore a vaccine against this infection has been given the first priority. The breakthrough in *in vitro* cultivation of *P. falciparum* by Trager & Jensen (1976) has greatly accelerated research on this organism.

Malaria infection in man is initiated with the injection by Anopheline mosquitoes of sporozoites that rapidly localize in liver cells. Here, each sporozoite can develop into 20 000 or more merozoites; each merozoite, after leaving the hepatocyte, is capable of infecting an erythrocyte. Inside the erythrocyte, the parasite reproduces asexually from rings to schizonts, the stage undergoing nuclear division. The mature schizont contains individual merozoites, each capable of invading other erythrocytes. This cycle of repeated rupture and invasion of erythrocytes by asexual parasites causes the clinical disease. Some of the rings, instead of continuing to proliferate as asexual parasites, differentiate into gametocytes, the form infectious to mosquitoes.

Vaccines are now being developed against the various malarias and all stages in life cycle because the protective antigens appear to be unique to each species and stage.

Immunity to sporozoites

The characteristics of sporozoite immunity are best illustrated by the vaccine trials with irradiated sporozoites in man (Clyde *et al.* 1973). Because of the irradiation, the volunteers did not become infected. Repeated inoculation did induce immunity, which extended to other strains of *P. falciparum* but not to challenge with *P. vivax* sporozoites. It was therefore established that immunity was species-specific but importantly covered all strains of *P. falciparum*. The individuals immunized with sporozoites were challenged with asexual erythrocytic parasites to determine if immunity would extend to this stage. They were found to be fully susceptible to challenge, and thus it was established that immunity to sporozoites was completely stage-specific as had been previously shown for the rodent model (Nussenzweig *et al.* 1969).

Effective immunity to sporozoites has the attractive feature that the infection is completely prevented. The immunized individual would be free of disease and would not be a reservoir for transmitting malaria to others. The cloning of the gene for the sporozoite surface antigen of *P. knowlesi* has led to the exciting discovery of the antigenic epitope that is the target of the protective monoclonal antibodies (Ellis *et al.* 1983). This exciting result obtained by the New York University group (Nussenzweig, this symposium; Godson, this symposium) has raised the

hope that a vaccine is indeed a real possibility and, more specifically, that this antigen will lead to protection. Sporozoite immunity and the molecular biology of the sporozoite suface antigen will be discussed elsewhere in the symposium. We wish to focus on aspects of sporozoite immunity that might be less familiar to the general scientific community.

The sporozoite is rapidly cleared from the circulation after it is inoculated into the vertebrate host. The liver selectively takes up sporozoites; the majority of sporozites are cleared on one passage through the liver (Danforth *et al.* 1982). Once within the hepatic parenchymal cells, the sporozoites are presumably protected from the effects of antibody. Therefore sporozoite immunity must block the parasite during a short period after the inoculation. A single sporozoite that escapes the immune response can produce an erythrocytic infection. The implications are twofold. First, partial immunity to this stage may not alter the course of the clinical disease. Even if only a fraction of the sporozoites survive to invade and develop in hepatic parenchymal cells, the ensuing asexual erythrocytic infection can cause a fatal infection. Second, blocking immunity against this stage must be fully effective at the time the sporozoites are first inoculated; there is no opportunity for the infection to boost the level of immunity against that inoculum.

It is possible that immunization with the repeating epitope could lead to effective, long-lived immunity. If this is not the case, however, alternative strategies must be considered in light of earlier studies on sporozoite immunity.

Sera from sporozoite-immunized animals (Spitalny & Nussenzweig 1973) and protective monoclonal antibodies (Yoshida *et al.* 1980) produced a circumsporozoite precipitation (c.s.p.). The monoclonal antibodies that produced the c.s.p. were directed against the repeating epitope of the sporozoite antigen. Most animals immunized with irradiated sporozoites developed c.s.p. antibodies and were protected (Spitalny & Nussenzweig 1973). C.s.p. antibodies, however, do not always correlate with protection. First, some mice developed c.s.p. antibodies after immunization and were not protected (Spitalny & Nussenzweig 1972). A similar observation was made in man where individuals who had c.s.p. antibodies were infected (Tapchaisri *et al.* 1983). Second, protection can exist in the absence of c.s.p. antibody. For example, B cell deficient (μ-suppressed) mice have been immunized with irradiated sporozoites (Chen *et al.* 1977). These mice were protected, although they had no detectable anti-sporozoite antibody.

While the target of immunity associated with c.s.p. antibody has been identified as the repeating epitope of the sporozoite surface protein, the mechanism of immunity and the target antigens are unknown in protected animals without c.s.p. antibodies. We do not know if the effector mechanism in these animals is antigen-specific (for example, cytotoxic T-cells) or non-specific (for example, activated macrophages). It is even possible that immune mechanisms induced by sporozoite antigens may not be limited to neutralizing circulating sporozoites. Sporozoite surface antigens may be deposited onto the surface of hepatocytes during invasion (Hollingdale *et al.* 1983).

Immunity to the hepatic schizonts

Research on possible malarial antigens exposed on the surface of hepatocytes has now been made possible by the development of techniques for culturing malaria parasites from the infectious sporozoite through the liver stage (Hollingdale *et al.* 1981).

Immunity to asexual erythrocytic parasites

Disease in malaria is caused by asexual erythrocytic parasites; no other stage including sexual erythrocytic parasites, the gametocytes, causes disease. Fever and the associated symptoms of headache, nausea and muscular pain occur at the time that schizont-infected erythrocytes rupture and presumably release pyrogens and other toxins responsible for these symptoms. It is ironic that, despite the accelerated research on molecular mechanisms in malaria, the cause of fever remains a mystery. High parasitaemia causes the complications of falciparum malaria: severe haemolytic anaemia, renal failure and cerebral malaria. Thus, to reduce moribidy and mortality, the primary objective of an asexual vaccine is the suppression of parasite proliferation.

There is no question that immunity develops in areas endemic for malaria and that this immunity suppresses asexual proliferation. Serious disease rarely occurs after the age of five in hyperendemic areas. Adults in these areas do become infected, however, and are important reservoirs for infecting mosquitoes, but the infection only produces a few days of fever. Thus, the goal of an asexual vaccine would be to convert a serious life-threatening disease to a minor illness, that is, to make an infection in a non-immune person like that of an immune adult.

Passive transfer of immunoglobulin from adult Gambians to *P. falciparum*-infected Gambian children controlled their infections (Cohen *et al.* 1961). The stage in the asexual erythrocytic cycle and the antigens against which this protective antibody was directed have not been identified. It is possible that all targets of protective immunity on the asexual parasite either undergo antigenic variation or are highly diverse within the parasite population. If this is so, it may be that only after repeated infection does one develop immunity to the repertoire of antigens. Such a situation would make vaccination difficult as is the case for influenza or Africa trypanosomiasis. It is known that immunity to malaria can be strain-specific. For example, during malaria treatment of patients with neurosyphilis, immunity that developed during the course of infection with one strain of *P. falciparum* did not protect against challenge with another strain (James *et al.* 1932). Furthermore, sera from adult Gambians, when passively transferred to splenectomized chimpanzees infected with *P. falciparum*, protected against an African strain but had no effect on an Asian strain (Sadun *et al.* 1966).

Clearly, our research towards an asexual vaccine is based on a more optimistic view that immunity against all isolates of a particular species of malaria can be induced by vaccination. For this to be the case, the vaccine must contain an antigen common to all parasites of a particular species. This antigen need not necessarily induce a protective immune response during natural infection. The parasite may cause immunosuppression or have immunodominant molecules that may interfere with induction of immunity to less dominant antigens. The latter may be more effective immunogens when purified and combined with an adjuvant. This has been shown to occur in transmission blocking immunity to gametes, the sexual forms; immunity does not develop despite repeated infection and the presence of these antigens in the circulating gametocytes (Gwadz & Green 1978). If, however, animals are immunized with gametes in adjuvant, subsequent infection boosts the level of antigamete immunity.

How does one sort through the numerous proteins of the asexual parasite to identify the candidates for a successful malarial vaccine? Simply stated, any antigen exposed to the immune system during invasion (for example, merozoite surface components) or any antigens exposed on the erythrocytes surface (for example, remnants of invasion or parasite molecules inserted

into the erythrocyte membrane by the intraerythrocytic parasite) are candidates for a vaccine. However, there is the problem of identifying all such molecules. In addition, other molecules, not exposed on a parasite surface, may be ideal for inducing non-specific immunity.

We will now discuss merozoite antigens, antigens in the erythrocytes membrane and nonspecific immunity.

Merozoite antigens

The merozoite is the only stage in the asexual cycle that is exposed to the immune system. This exposure is brief, since studies on *P. knowlesi* merozoites show that the invasion process takes 30 s (Dvorak *et al.* 1975). Antibody prevents the invasion of erythrocytes by agglutinating merozoites as they rupture from the infected erythrocyte. However, animals that have merozoite agglutinating antibody are not always protected *in vivo* (Miller *et al.* 1975 *b*). Antibody might also block invasion by binding to specific receptors on the merozoite that recognize the erythrocyte. In addition to blocking invasion, antibody attached to the merozoite surface could theoretically affect subsequent development of the intraerythrocytic parasite.

Multiple approaches have been taken to identify merozoite antigens for vaccine development, some of which will be discussed in papers to be presented at this symposium. We will review briefly these approaches.

Monoclonal antibodies derived from rodents immunized with whole parasites

With exceptions, most studies that produced monoclonal antibodies directed against merozoite antigens did not involve the use of purified, viable merozoites. Instead, the mice were immunized with schizont-infected erythrocytes or by natural infection in rodent malaria. The monoclonal antibodies were defined by indirect immunofluorescence pattern and by target antigen and were tested for their effects on invasion and development *in vitro* or for their effects on parasite multiplication *in vivo*. This approach has led to the identification of merozoite surface molecules (Deans, this symposium; Holder, this symposium), molecules associated with apical organelles (Holder, this symposium) and a soluble protein, the S-antigen, which is located in the vacuolar space surrounding the merozoites at the time of release from the infected erythrocyte (Kemp, this symposium). In the case of *P. yoelii*, the target antigen was purified and used in a vaccine trial (Holder & Freeman 1981).

One of the surface antigens of the merozoite has been identified in most studies of monoclonal antibodies to merozoites (Epstein *et al.* 1981; Freeman *et al.* 1981; Holder & Freeman 1982). This protein is synthesized throughout schizogony, is inserted into the schizont membrane (David *et al.* 1984), is processed at the time of merozoite release (David *et al.* 1984), is the major glycosylated protein synthesized during schizozony (Epstein *et al.* 1981) and is variant in the case of *P. falciparum* (McBride *et al.* 1982). The most unusual characteristic of this protein is that antibody directed against it is usually ineffective in blocking invasion. This includes not only monoclonal antibodies with some exceptions but also polyclonal sera from animals immunized with purified antigen. Holder & Freeman (1983) were able to protect animals against death from the lethal strain of *P. yoelii* by vaccination with this antigen, but sera did not passively protect non-immunized mice. Sera from rabbits immunized with a comparable molecular (M_r 230000 glycoprotein) purified from *P. knowlesi* immunoprecipitated the M_r 230000 glycoprotein and reacted with merozoites by indirect immunofluorescence, but the sera did not block invasion (P. H. David and L. H. Miller, unpublished data). It is possible that there are epitopes on the molecule that usually go unrecognized by the immune system which,

if isolated could induce blocking antibody. Such a possibility was raised by the finding that a monoclonal antibody inhibited *in vivo* proliferation of virulent *P. yoelii* (Majarian *et al.* 1984). This monoclonal antibody immunoprecipitated an M_r 230000 protein and gave rim immunofluorescence around merozoites, both characteristics similar to the surface molecule described by Holder & Freeman (1981). The assumption that both monoclonal antibodies are directed against the same protein on *P. yoelii* merozoites remains to be tested.

Perrin *et al.* (1981) identified monoclonal antibodies that reacted with schizonts and merozoites of *P. falciparum* (site of localization unknown). These antibodies caused intraerythrocytic death of the parasite without affecting invasion. This was unusual since immunoglobin from immune individuals has never been found to cause such effects in culture. A second unusual feature was that one of the monoclonal antibodies that caused intraerythrocytic death of *P. falciparum* was reduced by immunizing the mouse with *P. berghei*, a rodent malaria. This would mean that antibody-mediated immunity can cross species lines.

Monoclonal antibodies derived from mice immunized with selected proteins

Merozoites of *P. knowlesi*, a monkey malaria, can be obtained in a purified, viable form that has permitted the biochemical characterization of the surface proteins. Certain proteins are selectively labelled on intact merozoites by lactoperoxidase-catalysed radioiodination. Among these proteins, we purified an M_r 140000 protein from SDS-polyacrylamide gels and incorporated it into liposomes to immunize mice for production of monoclonal antibodies (Hudson *et al.* 1983). Monoclonal antibodies to the M_r 140000 protein reacted with the merozoite surface and partly blocked invasion (Miller *et al.* 1983). Sera from a rabbit immunized with affinity purified M_r 140000 also blocked invasion (P. H. David, L. H. Miller & D. Hudson, unpublished data).

Merozoite receptors as immunogens

The first step in invasion of erythrocytes by merozoites is specific recognition between the two cells (Miller *et al.* 1977). The merozoite binds to the erythrocyte by any surface of the merozoite that randomly contacts the erythrocyte (Dvorak *et al.* 1975). The merozoite then reorients so that the apical portion is in contact with the erythrocyte and forms a junction between a cap region at the apical end of the merozoite and the erythrocyte (Aikawa *et al.* 1978). Although it is not known how the merozoite reorients, evidence exists that the initial recognition and the junction formation are mediated by specific receptors. The two steps in recognition, initial attachment and apical junction formation, are probably mediated by different receptors. This proposal is derived from the observation that *P. knowlesi* merozoites bind at a similar rate to Duffy blood group positive and negative erythrocytes, although they can not form a junction with Duffy negative erythrocytes (Miller *et al.* 1979). After junction formation, merozoites invade the erythrocyte by creating a vacuolar membrane continuous with the erythrocyte membrane and entering within the vacuole via a moving junction around the merozoite. The ligands on the erythrocyte involved in reception include determinants on the glycoprotein carrying the Duffy blood group antigen for *P. vivax* (Miller *et al.* 1976) and *P. knowlesi* (Miller *et al.* 1975), erythrocyte band 3 for *P. knowlesi*, and glycophorin for *P. falciparum* (Miller *et al.* 1977b; Perkins 1981; Pasvol *et al.* 1982). For the data on these erythrocyte receptors see Pasvol (this symposium).

From the viewpoint of vaccine development, it is the merozoite receptor that may be an

effective immunogen to produce atibody that will block reception. While antigenic diversity is a character of some malarial immunogens, it is presumed that the binding region on the merozoite receptor would be common to all strains of a particular species. If the binding region contains a conserved epitope that can be recognized by the immune system, it can induce receptor-blocking antibody to all strains of a particular species of malaria. The merozoite molecules involved in reception have yet to be identified.

The surface molecules of *P. knowlesi* merozoites is best characterized of all malarias. The merozoite surface proteins that may be involved in reception are as follows. An M_r 230000 glycoprotein is synthesized during schizogony (Epstein *et al.* 1981) and inserted into the membrane of the intraerythrocytic schizont. It is processed at the time of rupture of the infected erythrocyte and release of merozoites to a quartet of four proteins found on the merozoite surface: M_r 75000, 55000, 53000 and 44000 (David *et al.* 1984). The cleavage to the four components may form a receptor complex. A second surface protein of M_r 140000 is extremely trypsin-sensitive (Johnson *et al.* 1981). Treatment of merozoites with comparable, low levels of trypsin eliminates the ability of merozoites to bind erythrocytes. There is evidence for a role in receptor binding by a third component, a M_r 66000 protein (Deans *et al.* 1982; Deans, this symposium). It should be noted that monoclonal antibodies to all three components bind over the entire merozoite surface. None are localized over the cap-like junction region at the apical end of the merozoite.

Other candidate proteins for initial reception and junction formation include the following. The S-antigen of *P. falciparum* that was originally described by Wilson *et al.* (1969) is within the vacuolar space surrounding the merozoites at the time of their release from infected erythrocytes (Coppel *et al.* 1983). The contents of rhoptries, which are secreted from the apical end of the merozoite onto the erythrocyte during the invasion process, are now being identified by monoclonal antibodies. A monoclonal antibody to a M_r 235000 protein of *P. yoelii* has been shown to bind over the rhoptries by frozen section electron microscope (Oka *et al.* 1984). Monoclonal antibodies, that bind to the apical region of *P. falciparum* merozoites by indirect immunofluorescence, immunoprecipitate two sets of proteins: M_r 145000–135000 and M_r 40000–80000 (Campbell *et al.* 1984).

A more direct approach to identifying merozoite receptors would be the use of receptors from erythrocytes to bind soluble malarial proteins. Such an approach has led to the identification of certain molecules from *P. falciparum* schizonts (Jungery *et al.* 1983). Glycophorin and *N*-acetyl glucosamine are possible erythrocyte receptors for *P. falciparum*. Detergent-solubilized, metabolically labelled parasites were passed over an affinity column of glycophorin or *N*-acetyl glucosamine. Proteins of M_r 140000, 70000 and 35000 bound to the column, suggesting that they may be involved in reception. We have recently identified the glycoprotein carrying the Duffy blood group antigen (Hadley *et al.* 1984) which should make parallel studies in *P. knowlesi* and *P. vivax* possible. The Duffy blood group antigen is carried on a sialoglycoprotein which migrates in a SDS-7.5% polyacrylamide gel as a broad band between M_r 35000 and 43000 and a smear to M_r 65000, the width on a SDS-gel probably reflecting the variable glycosylation. The molecule has the peculiar character of aggregating on boiling in SDS with 2-mercaptoethanol.

Summary: merozoite antigens for vaccine development

The merozoite is vulnerable to attack by antibody mediated mechanisms. Immune sera block merozoite invasion of erythrocytes by one of two mechanisms: (i) agglutinating merozoites as they burst from the infected erythrocyte or (ii) blocking reception between merozoites and erythrocytes. It was originally shown that IgG and the $F(ab)_2$ from immune animals block invasion; $F(ab)_1$ had no effect on invasion (Cohen & Butcher 1970). This observation was corroborated by the finding that blocking of invasion by sera correlated with the ability of the sera to agglutinate merozoites (Miller *et al.* 1975 *b*). Which method of blocking and which antigen will be induce protection by blocking merozoite invasion is unpredictable. Fortunately, varied approaches are being undertaken which we hope will lead to the identification of a protective immunogen.

Parasite antigens in the erythrocyte membrane

Antibody-mediated mechanisms directed against antigens on the erythrocyte surface may kill the intraerythrocytic parasite as follows. First, antibody-coated erythrocytes bind to macrophages, leading to extracellular killing (see section on non-antibody mediated mechanisms of protection) or phagocytosis. Second, antibody blocks binding of infected erythrocytes to endothelial cells *in vitro* (Udeinya *et al.* 1983). *P. falciparum*-infected erythrocytes bind to venular endothelium via knob protrusions on the infected erythrocyte and are thus protected from destruction in the spleen. Antibody, by blocking sequestration, forces the parasite from this protected site to pass through the spleen (David *et al.* 1983). Third, the membrane of infected erythrocytes are permeable to anionic and neutral compounds, not taken up by normal erythrocytes (Kutner *et al.* 1983). If facilitated uptake of metabolites results from parasite proteins within the erythrocyte membrane, then antibody directed against these proteins may block such uptake.

Because of technical problems, progress in identifying parasite proteins in the erythrocyte membrane has been slow, and some proteins claimed to be in the erythrocyte membrane may be located elsewhere. Labelling of intact, infected erythrocytes by the methods usually used for surface labelling normal erythrocytes may label internal proteins. Infected erythrocytes are permeable to anionic probes that do not enter the normal erythrocyte (Kutner *et al.* 1983). In addition, lysed erythrocytes in the preparation exposed parasite membranes to the label (Howard *et al.* 1983). Infected erythrocyte membranes have been difficult to isolate free from the parasite. A membrane preparation from *P. knowlesi*-infected erythrocytes was prepared by nitrogen cavitation (Schimidt-Ullrich *et al.* 1979), but the possible contamination of this preparation by parasite membranes has never been excluded. Antigen from this preparation isolated from the 75000 Da molecular mass region of SDS-polyacrylamide gels was used to immunize monkeys (Schmidt-Ullrich *et al.* 1983). Such monkeys were partly protected against challenge with *P. knowlesi*. In another method, infected erythrocytes bound to affigel beads were lysed so as to leave only the erythrocyte membranes attached to the beads (Gruenberg & Sherman 1983). Unfortunately, it appeared that soluble proteins from the malaria parasite bound to the beads during lysis. A third method combines hypotonic lysis and density separation in one step (Aley *et al.* 1984). These membranes from *P. knowlesi*-infected erythrocytes contained erythrocyte band 3 and the variant antigen known to be in the erythrocyte membrane and did not contain the major glycoprotein of the parasite membrane. In addition

to the variant antigen, the membrane preparation contained a set of 10 parasite proteins of M_r 277000 to 48000. Further studies will be required, however, to determine which of the proteins are in the interior of the erythrocyte and which are exposed on the external surface of the erythrocyte to the immune system.

Although monoclonal antibodies have identified sporozoite, merozoite and gamete antigens with ease, none have been identified that are directed against parasite antigens of the erythrocyte membrane. Mice immunized with heterologous erythrocytes may respond predominately to foreign host antigens. In addition, parasites in continuous culture may no longer express or transport their proteins to the erythrocyte surface as they do *in vivo*. It has been demonstrated, for example, that *P. falciparum*-infected erythrocytes, maintained in culture, rapidly lose the ability to bind to endothelial cells (Udeinya *et al.* 1983 a), a phenomenon observed *in vivo* and with parasitized erythrocytes freshly isolated from man or monkey. Despite the technical problems, the use of monoclonal antibodies to identify surface components on infected erythrocytes needs greater emphasis in the future.

Three approaches have successfully identified parasite proteins in or associated with the erythrocyte membrane. The first strategy was to compare parasite proteins of a variant of *P. falciparum* that lacked knobs (knobless, K−) with the parent line (knobby, K+). Knobs (focal protrusions, 100 nm in diameter) appear on erythrocytes containing trophozoites and schizonts of *P. falciparum* and are the mechanism by which infected erythrocytes adhere along endothelium of post-capillary venules (Luse & Miller 1971). Whereas all *P. falciparum*-infected erythrocytes have knobs when initially isolated from man, some lose knobs on continuous *in vitro* cultivation (Langreth *et al.* 1979). On comparing a K+ and K− variant, Kilejian (1980) has shown that a histidine-rich protein, present in K+ parasites, was completely missing from K− parasites. Knobs consist of an electron-dense submembrane cup pushing out the overlying membrane. Leech *et al.* (1984 a) have shown that the histidine-rich protein and the electron-dense cups remain with the cytoskeletal proteins after extraction of the membranes with Triton X-100 or deoxycholate. There is no evidence at present that any part of this molecule traverses the erythrocyte membrane to be exposed to the immune system.

The second erythrocyte membrane protein to be identified is the variant antigen of *P. knowlesi* (Howard *et al.* 1983). Howard (this symposium) has described this work in detail but the strategy for identifying this protein is worth emphasis. Cloned parasites were induced to undergo antigenic variation and the variants recloned. The two cloned variants differed in a surface antigen immunoprecipitated by variant specific antisera. The assumption that this is the only variant molecule between the two clones is reasonable. A similar approach identified a strain variant protein in the erythrocyte membrane of *P. falciparum* believed to be responsible for cytoadherence, the attachment between knob protrusions in infected erythrocytes and endothelium (Leech *et al.* 1984 b). Even if a region of the molecule involved in cytoadherence is variant, the portion involved in binding to endothelium must be constant and may contain epitopes common to all strains that can be recognized by the immune system.

Perlmann *et al.* (1984) have opened a new approach to the identification of parasite antigens in the erythrocyte membrane. They found that sera from adult Africans immune to malaria bound to the membrane of erythrocytes containing rings and trophozoites of *P. falciparum* if the infected cells were fixed briefly with glutaraldehyde and then air-dried. Unfixed cells did not bind antibody. The intensity of surface immunofluorescence decreased as the intraerythrocytic parasite matured. It is possible that merozoite molecules involved in invasion may be left

in the membrane surface of infected erythrocytes. Antibodies eluted from such fixed and air dried erythrocyte immunoprecipitated an M_r 155000 protein from metabolically labelled parasites and inhibited invasion *in vitro*.

Non-antibody mediated mechanisms of protection

In *P. chabaudi* infections in mice, the parasitaemia initially rises and then falls rapidly. B cell deficient mice, injected with anti-μ serum, can control *P. chabaudi* infections as effectively as normal mice (Grun & Weidanz 1981). In contrast, infection is lethal in nude mice. This indicates that T cells can have a protective role other than through helper function in antibody formation.

Taliaferro & Taliaferro (1944) were the first to observe abnormal intraerythrocytic parasites, which they called crisis forms, at the time of the sudden drop in parasitaemia. The role of the spleen in the induction of crisis was studied in the *P. berghei*-infected rat. During rising parasitaemia before crisis, the rate of clearance of abnormal cells through the spleen is markedly reduced below the rate seen in normal rats (Quinn & Wyler 1979). At crisis, the spleen is again able to clear abnormal cells and, for the first time, to clear parasitized erythrocytes. The blood flow through the spleen switches from an open to a closed circulation during rising parasitaemia. At crisis, the circulation through the spleen again becomes open, forcing the parasitized erythrocytes to filter through the cords where they come into close contact with macrophages (Wyler *et al.* 1981). Removal of the spleen during crisis leads to immediate recrudescence of the infection (Quinn & Wyler 1980). *P. falciparum* might be partly protected from splenic killing mechanisms, since erythrocytes containing the mature parasites are sequestered along venules and are spared from passing through the spleen.

Drawing on a diverse literature, Allison & Eugui (1983) have developed a theoretical model whereby an antibody-independent mechanism can lead to intraerythrocytic death of malaria parasites. The centrepiece of the model is induction of oxidant stress on contact with mononuclear cells in the spleen or in the peripheral circulation, a model independently proposed by Clark & Hunt (1983). Oxidant stress leads to death of the intraerythrocytic parasite. Such a mechanism would be enhanced by antibody directed against parasite antigens on the erythrocyte surface which would bind the infected erythrocyte to the macrophage or a K cell (antibody dependent cell cytotoxicity). The parasite, in turn, could evade such a mechanism by varying the antigens on the erythrocyte surface.

Recently, Jensen *et al.* (1983) discovered a circulating factor that led to crisis forms in culture. This factor was present in the sera of Sudanese adults who had no history of clinical malaria, despite living in an area endemic for malaria. They had no splenomegaly and low titres of antimalarial antibodies. The factor was not precipitated by 40 % ammonium sulphate and did not bind to protein-A Sepharose, suggesting that the factor was not immunoglubin as was thought at first (Jensen *et al.* 1982). The factor affected the developing parasite and did not block invasion, a step where antibody is known to work. Soluble factors (for example, tumour necrosis factor) have been previously proposed in non-specific immunity (Taverne *et al.* 1982), but the evidence for their role in malaria has been more indirect.

Whatever the mechanisms of non-antibody-mediated immunity (cellular or soluble factors), the primary question that we face for vaccine development is: can vaccination induce this and are some antigens more effective than others? At this time it is difficult to screen for those antigens that best induce this form of immunity. It is for this reason that the efficacy of a particular antigen for inducing non-specific immunity must be studied *in vivo*.

Antigen identification through recombinant DNA

In the usual sequence of events, monoclonal antibodies or polyclonal antibodies to a purified target antigen are used to identify the DNA coding for that particular protein (Ellis *et al.* 1983). Coppel *et al.* (1983) have reversed the process. A cDNA library of *P. falciparum* schizonts was expressed in λ phase gt11 as a fusion protein with β galactosidase, and the expressed protein identified with sera from hyperimmune individuals. Animals immunized with the fusion protein made antibody that could identify the parasite protein, its location in the cell and its effect on parasite multiplication in culture. The gene is already partly cloned and the reading frame identified. Most important, this approach may identify parasite proteins not recognized by other approaches described above. The one limitation of this approach is that antigens produced from expression of cDNA may not lead to protection if the important epitopes are at the amino-terminal end of a large protein.

Transmission blocking immunity

Malaria control and eradication programs use residual insecticides to shorten survival of mosquitoes that land on sprayed surfaces after taking a blood meal. This technique selectively affects those mosquitoes that may have become infected. Vaccines that block transmission by the mosquito would in effect supplement or replace these and other control measures. The targets could be the intraerythrocytic gametocytes or the extracellular forms in the mosquito midgut: gametes, zygotes and ookinetes. Antibodies taken with the blood meal into the mosquito midgut can block fertilization in the absence of complement, lyse gametes and zygotes in the presence of complement and block the development of zygotes. For discussion of these mechanisms and the target antigens on gametes, zygotes and ookinetes, see Carter (this symposium).

In contrast to the extensive work on the mosquito stages, no research has been performed on potential antigenic targets on gametocyte-infected erythrocytes. *P. falciparum* gametocytes require 10 days to develop from ring forms to mature gametocytes and then can remain for weeks in the circulation, infectious to mosquitoes. Erythrocytes that contain the immature gametocyte are not seen in the peripheral circulation, but, unlike asexual erythrocytic stages which adhere to endothelium via knobs, gametocytes do not induce knob protrusions on the erythrocyte membrane. Thus the mechanism of sequestration for immature gametocytes differs from that for asexual parasites. Similarly, the antigens on erythrocytes containing gametocytes may differ from those containing asexual parasites.

Transmission blocking vaccines can be used in malaria eradication programs and in combination with vaccines to other stages to slow the appearance of mutants. The effectiveness of transmission blocking vaccines in controlling or eradicating malaria from a region would depend on the duration of immunity after vaccination, the proportion of the population immunized and the intensity of transmission. In parts of Africa where, because of high levels of transmission, one individual can infect more than 500 others, a gamete vaccine would have little effect on transmission. In India or Sri Lanka where the transmission is less intense, this vaccine, possibly with other control measures might eliminate malaria.

Vaccine against all stages may select for mutants that will eventually become the predominant type in an immunized population. Such a course of events may occur after vaccination against the asexual erythrocytic parasite, the stage with the most antigenic diversity. The combination

of a transmission blocking vaccine with an asexual vaccine would block transmission of asexual erythrocytic infections resistant to the vaccine. The parasite population would thus remain unchanged except for selection of mutant populations resistant to both vaccines. Unfortunately, antigenic diversity has also been identified for gamete antigens of *P. falciparum*. Whether the level of diversity in gametes will present problems for vaccination must await experimental immunization trials with the antigen.

GENERAL CONSIDERATIONS

Immunogenicity and requirement for adjuvants

The ideal parasite antigen would be of sufficient immunogenicity not to require an adjuvant. Such will probably not be the case for most malarial antigens (Desowitz & Miller 1980), although the sporozoite antigen appears to be highly immunogenic and may not require an adjuvant (see Nussenzweig, this symposium). The slow development of the anti-sporozoite immunity, despite repeated infections in endemic areas, probably relates in part to the small number of sporozoites inoculated. Sporozoite protective immunity, if it can be induced with the sporozoite antigen, may be comparable to tetanus where no immunity to the toxin may exist after recovery from the disease; immunization with toxoid leads to protection. Adjuvant have been required for immunization of primates against asexual erythrocytic parasites (Desowitz & Miller 1980) and gametes (Gwadz & Green 1978).

It should be noted that epitopes important for protective immunity may go unrecognized during vaccination trials with whole parasite antigens. This may lead us to discard an antigen that may be an effective immunogen if presented to the host in a special way.

Antigenic diversity and antigenic variation

The discussion will be limited to variation within a species, since immunity appears to be species-specific. The repeating epitope, the major immunogen of the sporozoite antigen, appears to be highly conserved for *P. falciparum*. Thus, monoclonal antibodies to *P. falciparum* sporozoites completely block the binding of sera from human volunteers immune to sporozoites. A number of antigens of the asexual parasites have been found to be variant. For *P. falciparum*, these include the major schizont glycoprotein of M_r 195000 (McBride *et al.* 1982), the S-antigen (Wilson *et al.* 1969) and the antigen of the erythrocyte surface protein of *P. falciparum* (Hommel *et al.* 1983; Udeinya *et al.* 1983). There is evidence that a surface protein of *P. falciparum* undergoes antigenic variation (Hommel *et al.* 1983). Antigenic diversity and variation has been discussed in detail by Howard (this symposium). The range of antigenic diversity must be analyzed for each candidate immunogen. It is hoped that some antigenic regions will be constant because of their functional importance to the parasite (for example, receptors for erythrocyte invasion or cytoadherence to endothelial cells).

Partial immunity and the boosting of immunity during infection

At the time of immunization, the level of immunity will be at its maximum and will decrease with time thereafter. If initially the immunity is complete against a particular stage, a point in time will be reached when immunity is only partial. The effect of partial immunity on the infection and the boosting of immunity during infection will extend the life of a vaccine. Partial immunity to sporozoites would probably have little effect on the course of infection; any

sporozoites that survive may cause a fully virulent infection. Boosting would be unable to influence the course of infection. In contrast, the severity of disease from the asexual erythrocytic parasite is roughly proportional to the level of parasitaemia (Field & Niven 1937). Therefore, partial immunity that would reduce parasitaemia could lessen the severity of the disease. Furthermore, since the infection evolves over a week or more, boosting of immunity can occur. In the case of gamete immunity, boosting occurs during the blood infection (Gwadz & Green 1978); gametocytes in the blood synthesize the gamete antigens (Carter *et al.* 1984).

Analogous antigens

In general, antigens in one species have analogous antigens in other species. Analogy does not necessarily indicate that they have cross-reactive epitopes but that they serve a similar function in all species. A corollary would be that if the antigen leads to protection in one species, the analogous antigen may induce immunity in other species.

Examples of homologous antigens have been described from all stages in the life cycle. In all species, the sporozoite surface protein that is the target of protective monoclonal antibodies has intracellular higher molecular mass precursors that are processed as the molecule is transferred to the sporozoite membrane. Furthermore, the antigen has a repeating epitope in all species studied (Yoshida *et al.* 1981; Nardin *et al.* 1982; Zavala *et al.* 1983).

A major glycoprotein that appears on the merozoite surface is synthesized throughout the period of schizont development, inserted into the schizont membrane and processed to lower molecular mass proteins at the time of rupture of the schizont-infected erythrocyte and release of merozoites (Holder & Freeman 1981; Holder & Freeman 1983; David *et al.* 1984). [^{3}H]glucosamine is incorporated into this protein in all species studied except for a virulent strain of *P. yoelii*. Immunization with apparently analogous antigens in two species leads to partial protection (Holder & Freeman 1981; Schmidt-Ullrich *et al.* 1983).

A set of three proteins on the surface of *P. falciparum* and *P. gallinaceum* gametes are immunoprecipitated by monoclonal antibodies that block transmission to the mosquito (Carter *et al.* 1984). Proteins in both parasites consist of a high molecular mass protein (more than M_r 200000) and a lower molecular mass doublet (M_r ca. 50000). The proteins are synthesized simultaneously in the gametocyte before gametogenesis but do not appear to have a product–precursor relation.

Even molecules that have a function unique to a particular parasite may have analogous molecules in other parasites that lack the function. For example, cytoadherence to endothelium via knobs on the erythrocyte surface is characteristic of certain malarias (*P. falciparum*, *P. coatneyi* and *P. fragile*). A candidate for the binding molecule in *P. falciparum* has been identified (Leech *et al.* 1984) that has certain characteristics in common with the variant antigen of *P. knowlesi* (Howard *et al.* 1983). If this is indeed the case, one presumes that the variant antigen has evolved to take on an additional function in *P. falciparum*.

The importance of the concept of analogy is as follows. If a molecule that induces protection in one species is identified, analogous molecules should exist in other species. They need not have the same molecular mass or cross-reactive epitopes, but they would have the same structure, function and distribution within the parasitized cells. The presumption that the analogous molecule will lead to protection in all species must, of course, be tested in each case.

Concluding remarks

With the global resurgence of malaria, the rising costs of control and the failure of available techniques in areas such as Africa, the need for a malaria vaccine is apparent. The fact that vaccines against the various stages in the life cycle will be available in the future will permit tailoring of the vaccine for any given situation. The choice of vaccine (sporozoite, asexual or transmission blocking) may vary depending on the requirement: (i) short period of protection for non-immune people entering an endemic area (for example, tourists); (ii) curbing or preventing epidemics (for example, after a hurricane in Haiti); (iii) reducing mortality in an endemic area (for example, young children in Africa); and (iv) control or eradication of malaria. Because of the many unknowns about the immunogenicity and variability of antigens of various stages and the duration of protection, we cannot predict the ultimate form of the vaccine or combination of antigens that will be most useful in each situation.

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Discussion

R. R. FREEMAN (*Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.*). Dr Miller mentioned our experiments on immunization of mice against *P. yoelii* using a purified 230 000 molecular mass schizont antigen. We found that serum from the immunized mice contained a high titre of specific antibody, but did not confer protection on passive transfer. Dr Miller mentioned that another group of researchers has produced a monoclonal antibody, probably specific for the same *P. yoelii* antigen, which protects mice very effectively on passive transfer, and he suggested the possibility that our immunized mice may not have made an antibody response against a critical epitope on the antigen. I would like to point out that our immunized mice were effectively protected against challenge, presumably by virtue of a cell-mediated immune response. Is Dr Miller proposing that this antigen might be able to induce protective immunity via independent humoral and cell-mediated effector pathways simultaneously?

L. H. MILLER. Yes. Such an immunogen would be most useful in that it might induce antibody to block invasion and cell-mediated immunity to kill intraerythrocytic parasites.