Articles

Research Toward Malaria Vaccines

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Malaria exacts a toll of disease to people in the Tropics that seems incomprehensible to those only familiar with medicine and human health in the developed world. The methods of molecular biology, immunology, and cell biology are now being used to develop an antimalarial vaccine. The *Plasmodium* parasites that cause malaria have many stages in their life cycle. Each stage is antigenically distinct and potentially could be interrupted by different vaccines. However, achieving complete protection by vaccination may require a better understanding of the complexities of B- and T-cell priming in natural infections and the development of an appropriate adjuvant for use in humans.

MALARIA, ALTHOUGH INFREQUENT IN THE DEVELOPED world, occurs in hundreds of millions of people each year in the tropical countries and is a potential threat to hundreds of millions of others. It is therefore surprising that there has not been a major advance in malaria control since the 1940's, when insecticides and synthetic antimalarial drugs were introduced. Two events in the 1970's, however, changed the direction of research in malaria. One of these was the availability of culture forms of the major human malaria pathogen, *Plasmodium falciparum* (*1*), which allowed analysis of the molecular basis of parasite structure and function. The other was the demonstration that antigens involved in protective immunity could be identified by applying methods of modern biology to malaria research.

There are four species of human malaria: *P. falciparum*, a major human pathogen responsible for practically all malaria-related deaths, *P. vivax*, also widespread and causing considerable morbidity, and the generally less prevalent species *P. ovale* and *P. malariae*. Within each species there are numerous stages in the parasite life cycle (Fig. 1), each morphologically and antigenically distinct. For each stage there appear to be several antigenic targets on different molecules that could be suitable for immunization. Thus an antimalarial vaccine will probably be a multicomponent vaccine. In this article we describe recent progress toward this goal and highlight the problems that still must be overcome. Our discussion is oriented around each stage in the life cycle of the parasite as shown in Fig. 1.

Considerations in Vaccine Design

In contrast to most vaccines against viral and bacterial infections, malaria vaccines must be produced through recombinant DNA technology or peptide synthesis. This is because the asexual and sexual red cell stages of the parasite that would be required for antigen production can only be cultured in limited quantities, and such cultures are expensive in reagents (for example, human serum) and potentially contaminated by host red cell antigens and infective agents. Sporozoites can only be obtained in small numbers from the salivary glands of infected mosquitoes. The design of a subunit vaccine against malaria requires an appreciation of the relative roles of the antibody-dependent and -independent immune mechanisms that humans develop against each stage in the life cycle, as well as knowledge of the parasite antigens and of the most effective means of priming human B and T cells.

After protein targets of an antibody-mediated protective immunity have been defined, synthetic or genetically engineered peptides representing selected areas of these molecules are incorporated into a vaccine. Problems arise, however, if the binding sites, or epitopes, are not formed by a continuous amino acid sequence but rather are produced, as a result of protein folding, by juxtaposition of amino acids from different regions of the same polypeptide chain or from two or more chains. Another consideration is that peptides synthesized in bacteria may not form the correct disulfide bonds; synthesis in other cells may be required.

Helper T cells participate in T-cell–dependent antibody production against the sporozoite surface antigen (*2*), as they do against most protein antigens. Usually, the epitopes recognized by B cells and T cells on antigen molecules are distinct. During immunization, a T-cell epitope covalently linked to a B-cell epitope may be sufficient to prime for T-cell help. For example, injection of the repeating tetrameric epitope asparagine-alanine-asparagine-proline $(NANP)_n$ of the circumsporozoite (CS) protein of *P. falciparum* coupled to a peptide protein carrier, such as tetanus toxoid, elicits the production of antibody to the peptide that reacts with the CS protein on sporozoites. However, boosting of the immune reaction during a subsequent natural infection with sporozoites may not occur if the T cells of the immunized individuals have only been primed to respond to the carrier protein.

If T cells are needed for the induction of antibody-independent protective effects through lymphokines, then vaccines should be engineered to contain T-cell–inducing epitopes from malarial proteins themselves. Sensitized T cells that are a source of γ-interferon have a potent inhibitory effect on the liver stages of the malaria parasite (*3*). Vaccines that contain T-cell epitopes would offer a dual advantage during infection: antibody response would be amplified and T-cell–dependent killing mechanisms would be triggered. The ability of an individual to respond to a given T-cell epitope is determined by immune response (*Ir*) genes. *Ir* genes control

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immune responses in humans (*4*), but have been more thoroughly studied in the mouse (*5*). Recently, it was shown that the T cells from only one of seven congenic mouse strains bearing different allelic forms of an *Ir* gene recognized the tandemly repeated tetramer NANP of the CS protein of *P. falciparum* (*6*). Since T cells of some humans may also fail to recognize some epitopes in the CS protein, or other malarial antigens, the presence of multiple T-cell epitopes in vaccines could improve the chance that all individuals will respond. Furthermore, the incorporation of multiple T-cell epitopes might decrease the impact of possible polymorphic forms of parasite antigens not containing a particular T-cell epitope.

In contrast to many B-cell epitopes, T-cell epitopes are usually defined by a continuous sequence of 10 to 20 amino acids (*7*). From the analysis of a few globular proteins, it appears that the number of T-cell epitopes is small (*7*). A method has recently been proposed for predicting which segments of a protein, based on amino acid sequence only, may contain the epitopes recognized by T-helper cells (*8*). The prediction is based on the observation that several defined T-cell epitopes can form amphipathic α helices, even if they do not form this structure in the native protein. Perhaps this method could be used to engineer more effective malaria vaccines through the prediction of T-cell epitopes that could be evaluated in animals and humans.

In addition to understanding the complexities of T-cell recognition of antigen, finding an appropriate adjuvant may be critical to the development of a human malaria vaccine. In monkeys, Freund's complete adjuvant (FCA) is ideal for vaccination with merozoite antigen, whereas a derivative of muramyl dipeptide (MDP) is a poor adjuvant (*9*). FCA, however, is unsuitable for human use. FCA has been shown in viral studies to be required for the generation of antibodies to weakly immunogenic but necessary epitopes (*10*). The identification of natural mediators of growth and differentiation of the immune system may lead to a new class of adjuvants. For example, interleukin-2 combined with an immunogen will convert a poor immune response into a strong immune response (*11*). Thus, although the components of a malaria vaccine will be produced by recombinant DNA technology or peptide synthesis, the design will depend on understanding the molecular basis of immunogenicity.

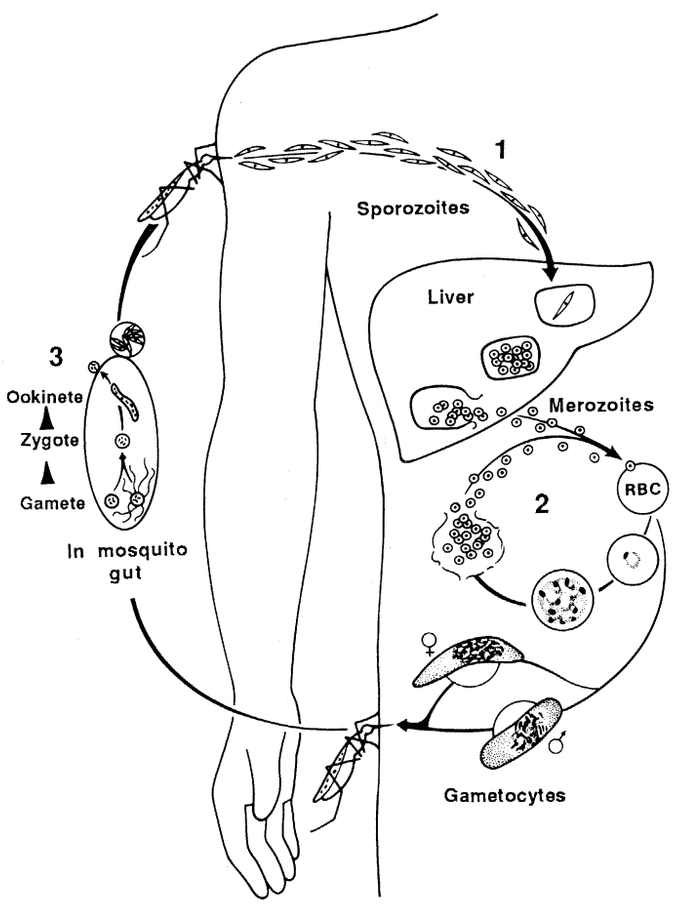

Fig. 1. Life cycle of the malaria parasite. 1. Sporozoites: Female anopheline mosquitoes, while they ingest a blood meal, inoculate sporozoites into the bloodstream that rapidly invade hepatic cells. Hepatic schizonts: One sporozoite develops into 20,000 merozoites within a hepatic cell. Merozoites rupture from hepatic cells and pour into the bloodstream to invade erythrocytes (RBC). 2. Asexual erythrocytic cycle: Asexual parasites mature within erythrocytes from rings to schizonts in 48 to 72 hours, the time varying with the malaria species. Merozoites are released to invade other erythrocytes. Disease and death in malaria is caused by this stage of the life cycle. 3. Cycle in mosquito: Some red cell parasites differentiate to gametocytes, which infect mosquitoes. Fertilization in the mosquito midgut occurs rapidly. Within 24 hours zygotes transform into ookinetes which penetrate the midgut to form oocysts and, later, sporozoites.

Sporozoite Vaccines

Although sporozoites remain in the circulation only for a brief period of time before entering hepatocytes, vaccination of rodents, monkeys, and humans with attenuated, x-irradiated sporozoites can lead to complete protection against malaria. The protective immunity, which is species- and stage-specific, but not generally strain-specific, is at least in part mediated by antibodies. When sporozoites are incubated with serum from the protected animals, their infectivity is neutralized and prominent deposits of antibody appear over the surface of the parasite and in the form of a tail-like precipitate; this is designated the circumsporozoite (CSP) reaction (*12*). Newborn rodents suckled by sporozoite-immunized foster mothers were totally resistant to sporozoite-induced malaria (*13*). Although the protective immunity to irradiated sporozoites is not long lasting, it can be boosted and maintained by the recurrent bites of infected mosquitoes. The frequent inoculation of malaria sporozoites into humans living in endemic areas may have a similar effect.

The acquisition of resistance to malaria with increasing age under natural conditions is associated with higher frequencies and higher titers of serum antibodies to sporozoites (*14*), which neutralize the infectivity of sporozoites in vitro (*15*). In children from a rural area of Tanzania, Del Giudice *et al.* observed a significant negative correlation between levels of antibody to the CS protein of *P. falciparum* and both parasitemias and splenic enlargement (*16*). However, it is difficult to evaluate the importance of antibodies to sporozoites in resistance to malaria because the serum of most adults in endemic areas also contains antibodies to blood forms of the parasite.

Interferons, particularly immune or γ-interferon, may also play a role in the protective immunity induced by x-irradiated sporozoites. The targets of interferon activity are not sporozoites but the next developmental stage of the parasite, that is, the exoerythrocytic, intrahepatic forms (EEF) (*3*). It is conceivable that the viable sporozoites injected during challenge of the vaccinated mice trigger the sensitized T cells to release γ-interferon, which in turn inhibits the development of EEF. In support of this idea is the observation that spleen cells of mice vaccinated with x-irradiated sporozoites release high levels of γ-interferon when challenged in vitro with parasite extracts (*17*). Also, some mice whose antibody production was suppressed at birth with goat antibody to the μ chain of

immunoglobulin and then vaccinated with x-irradiated sporozoites were partially protected against challenge with viable sporozoites (*18*).

The protective antigen (CS protein) of sporozoites has been identified in several malaria species by means of monoclonal antibodies (*19*). The CS protein covers the whole surface membrane of mature salivary gland sporozoites, and is shed when cross-linked by antibodies (CSP reaction). Passive transfer of 10 μg of monoclonal antibody to a mouse induces protection against sporozoite challenge. Neutralization of sporozoite infectivity has also been achieved with monovalent Fab fragments, implying that the binding of antibodies to the parasite surface interferes with their infectivity (*20*).

Indirect evidence suggests that the CS protein is essential for the parasite's survival in the mammalian host and is probably involved in the process of its penetration into hepatocytes. Indeed, CS antigens, which are one of the main proteins synthesized by mature salivary gland sporozoites, are absent, or present in small amounts, on the noninfective sporozoites from oocysts developing in the mosquito midgut (*21*). Fab fragments of monoclonal antibodies to CS proteins prevent the attachment of sporozoites to target cells in vitro (*22*). Recent evidence suggests that antibodies to the CS protein may also hinder the development of the parasite after it penetrates the target cells (*23*).

Circumsporozoite proteins display unusual immunological properties. They have an immunodominant epitope, repetitively represented in a single molecule. The remarkable immunogenicity of this epitope is highlighted by the finding that antibodies from humans living in endemic areas, or from two volunteers vaccinated with x-irradiated sporozoites of *P. falciparum* or *P. vivax* and protected against malarial infection, are mostly or exclusively directed against the repetitive epitope (*24*).

The structure of this epitope was clarified when the CS genes from various malaria parasites were cloned and the corresponding amino acid sequences determined (*25*). The central area of the CS polypeptides is formed by tandem repeats of amino acids, which vary in sequence among different species of malaria parasites. Although the repeats are remarkably conserved within each molecule, focal changes do occur. In the case of the human malaria parasites, *P. falciparum* and *P. vivax*, monoclonal antibodies to the respective CS proteins recognize the repeats in all isolates examined from different areas of the world (*26*). The amino acid sequence of the repeats from two strains of *P. falciparum* (*25*) and two strains of *P. vivax*, from areas widely apart, are almost identical (*27*). Other studies showed that a DNA probe corresponding to the NANP repeats hybridized with DNA from 23 isolates of *P. falciparum* (*28*).

The CS protein covers the whole surface of the parasite, and each CS molecule contains multiple copies of a single epitope. We calculate that the dominant, species-specific epitope of *P. falciparum* CS protein is represented 10^8 times on the sporozoite surface. The parasite should therefore be particularly vulnerable to attack by antibodies to repeats. Since antibodies to the epitopes formed by these repeats neutralize the infectivity of sporozoites, it seems logical to use the repeats as the basis for vaccine development. Antibodies against a few other areas of the CS molecule, which are more conserved among various species of malaria parasites, have little or no effect on the infectivity of sporozoites (*29, 30*).

To date, two candidate vaccines based on the NANP repeats have been developed. One of these, made by recombinant DNA technology, consists of fusion proteins made in *Escherichia coli* and contains NANP repeated 32 times; similar proteins with 16 and 48 repeats have also been made. When injected into mice, these products induce high titers of antibodies, which react with the authentic CS protein and block sporozoite invasion of human hepatoma cells in vitro (*31*). The other vaccine is a conjugate of a protein carrier with the synthetic repeat of NANP, which represents the epitope recognized by antibodies in the sera of humans living in endemic areas and of animals injected with sporozoites of *P. falciparum*. Rabbits and mice injected with the NANP-conjugate make high titers of antipeptide antibodies most of which also recognize the CS protein and neutralize parasite infectivity (*30, 32*).

These two new types of sporozoite vaccine are currently being tested in human volunteers to evaluate their safety, immunogenicity, and effectiveness. Available experimental evidence suggests that circulating antibodies reduce substantially the number of parasites developing in the liver. It has been argued that if one sporozoite escapes destruction a severe infection will result. A more optimistic outlook is that, in individuals where full protection is not achieved, the severity of the infection will be reduced and that they will have additional time to develop an immune response against the blood stage of the parasite.

Asexual Erythrocytic Parasites

The asexual erythrocytic parasite causes all the symptoms of malaria. The level of parasitemia is correlated with the severity of disease (*33*). Whether differences in parasite virulence or host factors modulate the frequency of complications, such as cerebral malaria, is unknown. Adults in areas of heavy transmission of *P. falciparum* are susceptible to repeated infections. However, they are usually asymptomatic because their immune system suppresses parasite proliferation. Many young children are unable to inhibit parasite replication and suffer mortality and morbidity from their infections. One goal of vaccination may be to transform the immune system of a nonimmune child to that of an immune adult, without requiring absolute resistance to infection.

What immune mechanisms are most effective? Antibody may specifically bind to antigens on the merozoite surface, blocking invasion, or to antigens on the surface of infected erythrocytes, leading to death of the intraerythrocytic parasite or destruction of the erythrocyte by phagocytes or killer cells. In addition, immunity may function through several other nonantibody-mediated mechanisms. In *P. chabaudi* infections in mice, for example, the parasitemia initially rises and then falls rapidly. T cells are required for the development of immunity (*34*). However, since mice that are made B cell–deficient by injection with antiserum to μ chain can control *P. chabaudi* infections as effectively as normal mice (*34*), the protective role of T cells is not that of helper for antibody formation. Other possible defense mechanisms may be through the release of lymphokines, activation of mononuclear cells in the spleen, and induction of oxidant stress on contact with parasitized erythrocytes (*35*). In addition, Jensen *et al.* (*36*) detected a nonimmunoglobulin serum factor in immune Sudanese adults that kills intraerythrocytic parasites in culture.

Immunity to a particular protein of the malaria parasite may function through both specific (antibody) and nonspecific effector mechanisms. For example, passive transfer of monoclonal antibody to a merozoite surface protein of *P. yoelii* rapidly decreased the levels of parasitemia in mice (*37*). Immunization of mice with the protein of *P. yoelii* induced protection (*38*), but sera from immunized mice did not transfer protection (*39*), suggesting the immunity may also be antibody-independent (*38*). It appears that immunization with the protein did not induce high titers of antibodies to the epitope recognized by the monoclonal antibody, because monoclonal antibodies protected against two variants of *P. yoelii*, whereas immunization protected against only one variant. What, then, is the mechanism of protection after immunization with this merozoite surface

antigen? The fact that intraerythrocytic forms were damaged at the time of rapid drop in parasitemia led Freeman and Holder to speculate that the effector mechanism is nonspecific (cellular) (*39*). This type of immunity in an immunized animal requires T cells and an intact spleen (*40*). While some idiotypes may protect (*37*), cell-mediated mechanisms more readily facilitate protection after immunization with the protein.

Vaccination with a number of purified proteins from asexual *P. falciparum* parasites protected New World monkeys from a fatal *P. falciparum* infection (*41*), but all the proteins were administered in FCA. Similarly, immunization of monkeys with *P. knowlesi* parasites required FCA (*42*). Other adjuvants were ineffective in *P. knowlesi*. An adjuvant equivalent to FCA that acts on T cells may be required for vaccination in humans. No such adjuvant is known today. It could be argued that one of the greatest advances in immunization against the asexual erythrocytic parasite will come from the development of a safe, effective adjuvant and that this may be as important as the choice of antigen.

Antibody-mediated protective mechanisms include neutralizing antibody that blocks merozoite invasion of erythrocytes and antibody that interacts with the surface of infected erythrocytes. Several strategies are being pursued in different laboratories to determine which of the many malarial proteins (and the correspondingly larger number of individual epitopes) might be useful vaccine targets. In one approach, monoclonal antibodies are produced against merozoites and screened for blockade of merozoite invasion of erythrocytes in vitro. The same assay can be used to screen polyclonal antisera against a purified antigen. Monoclonal antibodies can also be used to purify malarial antigens that are on the merozoite surface, even if the particular monoclonal antibody is non-inhibitory, because antibodies to other epitopes may block invasion.

A second approach in the search for vaccine targets involves the construction of genomic or complementary DNA (cDNA) expression libraries from the malaria parasite, the screening of the libraries with sera from malaria-immune persons, and preparation of a panel of antisera against the cloned expressed products (*43*). Thus, a battery of rabbit antisera has been produced in one laboratory against fusion proteins, each of which represents the expression of part of a malarial gene sequence. Such sera serve as probes for identifying the malarial protein and for studying the subcellular location of the protein within the malaria parasite or infected cell. This approach has led to the identification and sequencing of *P. falciparum* DNA encoding several genes of biological interest (for example, the highly variant S, or soluble, antigens) (*44*) including one that is localized on the erythrocyte membrane after invasion (*45*). Still unknown, nevertheless, is which among the many proteins identified are vaccine candidates and which, if any, of the expressed recombinant clones induce antibodies that block invasion or react with the erythrocyte surface. A further complexity is that antisera to repeating epitopes in the recombinant peptides sometimes cross-react with epitopes in other malarial proteins (*46*).

A third approach takes into consideration the biology of the parasite in vivo and attempts to identify functional molecules that may be exposed to the immune system, such as receptors for merozoite invasion of erythrocytes and for cytoadherence between *P. falciparum*–infected erythrocytes and endothelium. Parasite molecules that have important functional properties may contain conserved domains that could be used for vaccination.

The receptors on parasites for erythrocyte invasion are obvious targets for antibody-mediated blockade of this obligatory step in the asexual cycle. Asexual malaria parasites invade new host erythrocytes through a specialized form called a merozoite (*47*). Initial attachment between merozoites and erythrocytes can occur between any point on the merozoite surface and the erythrocyte (*48*). The merozoite then reorients so that its apical end, which contains paired organelles (rhoptries and micronemes), comes in contact with the erythrocyte. A junction forms between the apical end of the merozoite and the erythrocyte (*49*). The apical organelles release their contents into the erythrocyte, causing a wave of deformation, with a vacuole forming in the erythrocyte opposite the apical end; the merozoite then enters the vacuole by movement of the junction around the merozoite.

Although antibodies to many parasite proteins on the merozoite surface have blocked invasion (*47*), it has been difficult to differentiate between merozoite agglutination and receptor blockade. An exception has been antibodies to the 66/44/42 kilodalton protein complex of *P. knowlesi* (*50*). Fab or $F(ab')_2$ fragments of antibodies blocked invasion and, surprisingly, were more effective than immunoglobulin G (*51*). The Fc fragment of immunoglobulin G appeared to interfere with its full activity.

Evidence suggests that merozoites recognize specific ligands on the erythrocyte surface: sialic acid on glycophorin A and a trypsin-sensitive, sialic acid–independent molecule for *P. falciparum* (*47, 52*), and the Duffy blood group antigen for *P. vivax* and *P. knowlesi*, a primate malaria (*47*). It appears that two ligands are involved in invasion: one for initial recognition and a second for apical junction formation. Merozoites of *P. knowlesi* can attach to human erythrocytes that are negative for the Duffy blood group antigen, although no junction forms and they cannot invade these cells. This attachment is specific since these merozoites do not attach to subprimate erythrocytes.

Since sialic acid on glycophorin A is a binding ligand for *P. falciparum*, glycophorin A coupled to Sepharose 4B (*53*) or aminoethyl-Bio-Gel (*54*) was used to bind parasite molecules that may be receptors. Human erythrocytes have also been used as ligands and bind four soluble proteins of *P. falciparum* (*55*). One of these (175 kD) binds with receptor-like specificity in that it does not bind to erythrocytes that have reduced susceptibility to invasion such as neuraminidase-treated human erythrocytes (*55*).

Rhoptries and micronemes release their contents onto the erythrocyte membrane during invasion as a vacuole forms. Perlmann *et al.* described an unusual pattern of immunofluorescence of the membrane of glutaraldehyde-fixed, air-dried parasitized erythrocytes treated with sera from an immune individual (*56*). Immunofluorescence was seen associated with the erythrocyte membrane of recently invaded, ring-containing erythrocytes, but disappeared as the parasite matured. Antibodies eluted from ring-infected erythrocytes react with a 155-kD protein. The gene coding for the 155-kD protein has been cloned and one of its domains contains negatively charged repeating sequences of eight amino acids (*45*). The protein in the merozoites appears to be localized in the microneme (*57*) and may be involved in membrane deformation and vacuole formation during invasion. Antibodies to the protein block invasion (*58*); vaccination of *Aotus* monkeys with a recombinant peptide from the protein by Collins *et al.* leads to partial protection (*41*).

Several proteins have been identified within rhoptries (*47*). A monoclonal antibody against a rhoptry protein of a rodent malaria parasite reduced the virulence of this infection (*59*). Immunization with a presumed rhoptry protein of *P. falciparum* (41 kD) in FCA by Perrin *et al.* gave partial protection to monkeys against a lethal infection (*41*).

In short, several merozoite proteins have been associated with erythrocyte invasion. Others may remain to be identified. The wide array of structures associated with merozoite entry increases the opportunity for identifying a malarial protein that will induce immunity.

Once inside an erythrocyte, the malaria parasite renders itself visible to the immune system by antigenic modifications of the

infected erythrocyte surface membrane (*60*). Since these antigens are accessible to immune recognition for a prolonged time (up to 36 hours) compared to the surface antigens on merozoites, which reinvade within minutes, they may be excellent targets for vaccination. The capacity of serum antibody from immune monkeys to transfer protection against *P. falciparum* to nonimmune animals was shown to be correlated with opsonization and phagocytosis of infected erythrocytes in vitro (*61*). In this series of experiments, antibodies which blocked merozoite invasion in vitro did not correlate with transfer of immunity.

Parasite molecules with functional roles at the host erythrocyte membrane may be useful targets for vaccination. *Plasmodium falciparum* requires exogenous iron as ferrotransferrin (*62*), and a malarial transferrin receptor has been identified at the surface of infected cells which internalizes and transports bound ferrotransferrin to the intracellular parasite (*63*). It is not yet known whether this malarial transferrin receptor is sufficiently different from the human receptor to serve as a useful vaccine target.

Erythrocytes infected with *P. falciparum* also acquire the functional property of cytoadherence to endothelial cells as the parasite develops from the ring to trophozoite stage. Asexual *P. falciparum* parasites are only detected in peripheral blood smears as immature ring stages. Trophozoite- and schizont-infected erythrocytes are sequestered by specific attachment to endothelial cells lining the venules and capillaries (*64*). These mature parasitized cells may block blood flow and give rise to the classical neurological symptoms of cerebral malaria. This cytoadherence phenomenon, between the surfaces of *P. falciparum*–infected erythrocytes and endothelial cells, is mediated by knob protrusions of the erythrocyte membrane (*64, 65*) and underlying cytoskeleton (*66*). Laboratory-derived knobless variants (K^-) of *P. falciparum* do not attach to endothelial cells in vivo (that is, mature infected cells of K^- parasites appear in peripheral blood) (*67*). The K^- parasites also do not attach in vitro to endothelial cells or melanoma cells (*68*), which have been used as targets in a model of this cytoadherence process. Expression of knobs on the erythrocyte membrane can be linked directly to enhanced parasite survival, since K^- parasites are much less virulent in *Aotus* monkeys than knob-bearing (K^+) parasites from the same isolates (*67*). It has been suggested that through expression of knobs and cytoadherence, mature *P. falciparum* parasites avoid passage through the spleen, thereby avoiding exposure to localized specific and nonspecific mechanisms that would remove the altered erythrocytes (*69*).

A malarial protein of about 300 kD expressed on the surface of *P. falciparum* infected erythrocytes has been identified as the likely cytoadherence moiety on the basis of three properties. (i) The capacity of antisera to block or reverse cytoadherence in vitro matches their capacity to react with this protein at the surface of infected cells (*70*). (ii) This protein is exquisitely sensitive to tryptic cleavage at the cell surface in parallel with the loss of cytoadherence seen with trypsin-treated infected cells (*70*). (iii) Variants that are K^- and do not cytoadhere do not express this protein at the cell surface (*71*).

Since the ~300-kD protein is antigenically diverse with different isolates and appears to mediate cell attachment (*70*), it may bear two domains: a variant domain that facilitates immune evasion and a shared domain for cytoadherence. The fact that cytoadherence of different strains could be inhibited by antisera in a strain-specific manner (*72*) indicates that strain-specific epitopes are part of, or near, the cytoadherence molecule. In addition to variant epitopes (*70, 72, 73*), an antigenically invariant epitope was identified on the surface of infected erythrocytes isolated from Gambian patients (*73*). This conserved epitope, once identified, may be an important antigen for use in vaccines. The recent identification of host ligands for cytoadherence (*74*) may assist in the purification of the cytoadherence molecule and the particular domain involved in its function.

Transmission Blocking Immunity

The sexual phase of the life cycle of malaria parasites begins with the differentiation of erythrocytic parasites into male and female gametocytes (Fig. 1). After they are ingested by a mosquito along with the mammalian host's blood, the gametocytes undergo gametogenesis in the mosquito midgut. Within 10 to 20 minutes the gametes become extracellular and begin fertilization, still inside the mosquito gut. The zygotes so formed become mature ookinetes about 24 hours after fertilization (Fig. 1). The ookinetes are tissue invasive stages that penetrate the midgut wall, form oocysts, and ultimately become sporozoites.

The emergence of the gametes in the mosquito midgut exposes them to the antibodies ingested with the mosquito's blood meal. Antibodies developed in the mammalian host against gametes and zygotes of malaria parasites can block the infectivity of the parasites to mosquitoes (*75, 76*). Also, an apparently T-cell–mediated immunity can suppress the infectivity of gametocytes of a rodent malaria parasite to mosquitoes (*77*). In human populations antibodies develop during malarial infection that can block the transmission of the parasites to mosquitoes (*78*); malaria gamete-specific T-cell clones have been demonstrated in the peripheral blood of nonexposed donors (*79*). Because of the potential of these immune mechanisms to suppress the transmission of human malarial infections by mosquitoes, the general phenomenon is referred to as transmission blocking immunity.

The development of transmission blocking vaccines would create opportunities for the control and prevention of malaria distinct from those offered by protective vaccines against the sporozoites and asexual stages. Such vaccines, in addition to reducing the risk of malarial infection to the human population at large, could also be used to stop the spread of parasite mutants. They could act against parasites that may have become resistant to vaccines against sporozoite or asexual blood stages, unless genes for gamete proteins also underwent mutation. In a simian model, transmission blocking immunity produced by immunization with FCA was effective for many years and was readily boosted in response to malaria infection in the blood (*80*). This boosting was due to the presence of the target antigens in the gametocytes circulating in the bloodstream (*81*). This feature of the immunity helps ensure that antibodies are present when they are needed, that is, during an active blood infection. One potential risk of transmission blocking vaccines, however, is that transmission could be enhanced. Monoclonal antibodies and human sera that block transmission of *P. vivax* at high antibody concentrations have been found to enhance infection at low antibody concentrations (*82*).

Transmission-blocking antibodies are effective against two stages of parasite development: (i) the gametes, and thus they can prevent fertilization, and (ii) the zygotes and ookinetes, so they can prevent subsequent development in the mosquito (Fig. 1). Transmission-blocking monoclonal antibodies and their target antigens have been studied in various malarial species including the animal parasites *P. gallinaceum* (*83*) and *P. yoelii nigeriensis* (*84*) and the human parasites *P. falciparum* (*76, 85*) and *P. vivax* (*86*).

The surfaces of gametes and zygotes of *P. gallinaceum* and *P. falciparum* bear a molecular complex consisting of a protein of 230 kD and two glycoproteins of 48 and 45 kD (*81, 87*). The components of the complex are synthesized simultaneously during growth of gametocytes and remain associated after extraction in the deter-

gent Triton X-100. Monoclonal antibodies react by immunoblot either with the 230-kD protein or with the 48/45-kD glycoproteins, indicating that the latter are structurally related to each other but not to the 230-kD protein (*76*). In *P. falciparum*, target epitopes of fertilization-blocking monoclonal antibodies occur on the 230-kD protein (*88*) and on the 48/45-kD molecules (*76, 85*). Several different epitopes have been defined on the 48/45-kD glycoproteins of *P. falciparum*; none of the epitopes appear to be repeated (*89*). Individual monoclonal antibodies to one of these epitopes are potent blockers of transmission (*76*). No variants of this epitope have been found among different isolates of *P. falciparum*, although one of the other epitopes on the 48/45-kD glycoproteins may vary in some isolates (*90*).

After fertilization and during the subsequent transformation of the zygote to an ookinete, many of the original gamete surface antigens are shed from the zygote surface and new antigens are expressed (*91*). These include a protein of 25 kD that appears on the surface of developing zygotes of *P. falciparum* and is a target of post-fertilization transmission-blocking monoclonal antibodies (*76*).

All the epitopes recognized by transmission-blocking monoclonal antibodies against gametes or zygotes are destroyed by reduction (*76*), indicating that these epitopes are dependent on tertiary structure maintained by disulfide bonds.

Antigenic Variation and Antigenic Diversity

An obligatory property of any putative vaccine molecule must be its expression by all natural isolates of the malaria parasite with only a limited number of alternative antigenic forms. This property may be fulfilled by the repeat sequence epitope on the CS proteins, which appear to be invariant in natural isolates (*26, 28*). However, in two species of simian malaria, *P. knowlesi* and *P. cynomolgi*, different isolates of each species (or subspecies) fail to cross-react with monoclonal antibodies against the sporozoite repeats (*92*), and the repeats in *P. knowlesi* have completely different deduced amino sequences (*93*).

Considerable evidence has accumulated for genetic diversity in natural isolates of *P. falciparum* [for a review, see (*94*)], including expression of different isozymes, different sensitivities to antimalarial drugs, and expression of alternative epitopes on particular malarial proteins. For example, a panel of monoclonal antibodies against a single glycoprotein on the surface of *P. falciparum* merozoites has differing patterns of reactivity among different clones of *P. falciparum*, including clones from a single human isolate (*95*). The molecular basis for this diversity has now been defined (*96*).

In addition to antigenic diversity in the natural parasite population, potential mechanisms have been described for the generation of new antigenic phenotypes. Malaria parasites may express new antigenic phenotypes through mutation, through recombination within a gene during meiosis, or through expression of alternative forms of a multigene family as occurs in African trypanosomes. For example, erythrocytes infected with mature asexual parasites of *P. knowlesi* express one of a set of malarial variant antigens (180 to 230 kD) on the erythrocyte surface (*97*). Cloned parasites undergo antigenic variation in vivo such that the variant populations express completely different antigenic forms of this malarial protein on the infected cell surface (*98*). Antigenic variation in malaria differs from that in the trypanosomes, because the malaria parasites are induced, by variant-specific antibody, to undergo antigenic variation and do not appear to be killed in the process. Through this process the asexual parasite evades parasiticidal variant-specific immune responses enabling it to establish chronic infections and repeatedly reinfect immunized animals. The molecular and genetic basis of these variations are unknown, although on the basis of serological studies it appears likely that there are significant structural differences among the alternative forms of this surface antigen. A similar finding was described for one isolate of *P. falciparum* during reinfection of squirrel monkeys (*99*).

The second example is provided by a 140-kD surface protein of *P. knowlesi* merozoites that was chosen for vaccination studies in rhesus monkeys because antibodies to it blocked invasion in vitro (*100*). This protein was invariant in parasites from chronically infected monkeys. However, within 3 weeks after challenge of immunized monkeys with cloned parasites, variants expressing proteins of markedly different molecular weight (180 to 65 kD) and of different reactivity with monoclonal antibodies to the 140-kD protein had replaced the original cloned population (*101*).

Variation in surface epitopes on gametes has also been described (*90*).

In summary, most antigenic diversity has been described for the asexual erythrocytic parasites. This may be in part because these forms are subjected to the intense pressure of the immune system and because they multiply rapidly in the blood and can give rise to mutants. With 1% of erythrocytes containing parasites in a 40-kg human, there are 10^{11} circulating parasites from which to select mutants. The asexual parasites may continue to proliferate for more than 1 month.

Tandemly Repeated Peptides

One of the most intriguing observations to stem from the application of molecular biological methods to malaria is the discovery of numerous malarial proteins with large tracts of repeated amino acid sequences. The extraordinary fidelity of some of these repeat sequences (especially at the amino acid level) suggests that the malaria parasite possesses some very unusual, perhaps unique, genetic mechanisms for their generation and maintenance. At present we can only guess at their significance. Here we describe some examples of these protein sequences.

1) The major surface protein on sporozoites consists of a very large tandemly repeated structure flanked by nonrepeat protein sequence (*24*). This repeat structure is different in different species of malaria parasite and in different strains (or subspecies) of some simian malarias (*102*). In all cases the repeat region makes up more than one-third of the protein.

2) The avian species *P. lophurae* synthesizes a protein that constitutes roughly 10% of its total protein in asexual blood stages, consists of 72% histidine (*103*), and includes extensive sequences containing five to nine contiguous histidine residues (*104*). Its function is unknown.

3) Four proteins of the *P. falciparum* merozoite that are expressed late in parasite development contain repeats. First, a 155-kD protein is distributed in the erythrocyte membrane of the newly invaded erythrocyte (*45*). Second, a 130-kD protein contains repeats of 50 amino acids (*105*) and is distributed over the merozoite surface. Third, the 195-kD glycoprotein on the surface of schizonts and merozoites has a short run of repeats that varies among strains (*96*). Fourth, a soluble extracellular, heat-stable antigen released from *P. falciparum* asexual parasites (S-antigen) has marked antigenic diversity in parasite isolates from different patients (*106*). In one isolate, the S-antigen contained a highly repeated sequence of 11 amino acids (*44*); in another, the S-antigen had an unrelated 8–amino acid repeat (*107*). Degeneracy of the repeats occurs at the carboxyl terminus of the repeats. These sequence data account for the observed antigenic diversity of the *P. falciparum* S-antigens; the size diversity results from different numbers of repeats. Although the

amino terminal sequence flanking the repeats is highly conserved (*108*), antibodies immunoprecipitate the protein in a strain-specific manner, suggesting that the repeat is immunodominant. A monoclonal antibody specific for a particular S-antigen partially blocked merozoite invasion of erythrocytes in vitro (*108*), suggesting that it may play some role in invasion.

Current speculations on the role of these repeat structures include the idea that they may divert the immune response away from other antigenic targets that are important for parasite survival. This does not seem plausible in the case of the sporozoite repeats, since antibodies to the repeats prevent the development of the parasite. It is also possible in some cases that while the primary sequences of repeats differ in different parasite isolates, the secondary or tertiary protein structures might be sufficiently similar to retain the protein's functional properties. The parasite may thus have a mechanism for deleting and replacing particular repeats with others of different antigenicity but conserving function in response to immune pressure.

Conclusion

The first clinical trials of sporozoite vaccines are now in progress. Various candidate antigens are being investigated for vaccination against parasites in other stages in the life cycle. Despite the enthusiasm and excitement that we and other malaria researchers feel, we would be wrong to predict the impact of vaccines—yet to be tested in humans—on malaria in the Tropics. Countries afflicted by malaria should maintain their efforts to contain the disease by mosquito control and chemotherapy. Basic research leading to the development of novel methods of vector control and toward novel drugs should be encouraged. It is our hope that vaccines will supplement and help to overcome the failures of mosquito control and chemotherapy, which have been the mainstay of antimalarial campaigns since the turn of the century.

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Randomly Exact Methods

J. D. Doll and D. L. Freeman

Important advances in the understanding of "random" processes have produced a variety of stochastic algorithms that offer unprecedented scope and utility in the study of physical systems. These algorithms represent a departure from the usual philosophy inherent in the study of many-body problems and have a number of significant features. Chief among these features are simplicity, weak dependence on dimensionality, and ease of transition between classical and quantum-mechanical descriptions. These methods are also readily adapted for use on massively parallel computer architectures. These new stochastic methods represent a valuable addition to the tools available for the analysis of both equilibrium and time-dependent many-body problems.

A curious feature of physical science is that there are few problems for which the fundamental equations of the underlying theory can be solved exactly and the resulting predictions compared directly with macroscopic observations. The cases for which this can be done are vital for testing the adequacy of basic theories, but mathematical difficulties prevent us from making such connections on a general basis. Our understanding of macroscopic physical phenomena, therefore, is of necessity based on intuition formed from these few important examples and from results of approximate calculations. Often these approximate calculations are inadequate and typically contain untested assumptions. In areas such as chemistry and condensed matter physics, this situation is especially frustrating since it is generally felt that the underlying microscopic theory is complete. Were we in a position to solve the basic equations exactly, meaningful predictions of the equilibrium and time-dependent properties of materials, both real and hypothetical, would be possible. Such predictions could be of significant assistance, for example, in the search for new substances with desirable physical, chemical, or biological properties. A new class of numerical methods based on the study of random processes (*1, 2*) offers the possibility of a general solution to this unsatisfactory situation. These "randomly exact" methods represent an unusual combination of simplicity, generality, and power.

Physical science and mathematics are largely concerned with the study of order. Thus the study of random processes is, at first glance, somewhat odd. Experience has shown, however, that many apparently random processes when more deeply studied exhibit regular behavior. This being the case, a common viewpoint concerning random phenomena could be termed "hostile tolerance," hostile because of the implicit and often untidy chaos, but tolerant since much of the chaos can ultimately be understood or eliminated. In this article we describe the evolution of this position of tolerance into one of advocacy.

The study of random phenomena has historically yielded a rich harvest of interesting mathematics. An important example is the distribution of random errors. Repeated measurements of a well-

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