Molecular parasitology: Progress towards the development of vaccines for malaria, filariasis, and schistosomiasis

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Summary. Advances in molecular biology have allowed for the identification of potential vaccine candidates against several parasitic diseases. Antigens from various life stages of *Plasmodium* and *Schistosoma* species and filarial worms have been cloned, sequenced and tested as vaccines. Results to date in animal models have been promising. Modest levels of protection against experimental human malaria have been obtained using both sporozoite and blood-stage antigens. However, a greater understanding of the mechanisms which lead to immunity against parasites is required before effective vaccines can be developed.

Key words. Vaccines; immunity; cell-mediated immune response; humoral immune response; human challenge study.

Parasitic infections continue to cause a high rate of morbidity and mortality, predominantly in tropical and subtropical areas of the world (table). At present, no vaccine exists for the prevention of any parasitic disease in humans. However, the success of nematode (lungworm) vaccine for cattle demonstrates that effective immunity against parasites can be induced in a natural setting. Until recently, efforts to develop vaccines against parasites were frustrated by the inability to identify and produce protective antigens and to study the immunological basis of immunity at the molecular level. The use of monoclonal antibodies and recombinant DNA technology have allowed the identification, cloning, and sequencing of relevant parasite proteins. As a result of these advances, coupled with an increase in our understanding of the factors that mediate and govern the humoral and cellular immune responses, it is now possible to undertake a rational approach toward the development of parasitic vaccines.

Nowhere has the above been better demonstrated than for malaria. Therefore, a substantial portion of this article will be allotted to the discussion of the various approaches taken and difficulties faced in producing a malaria vaccine for widespread use.

Malaria

Several breakthroughs in the field of applied parasitology have allowed for the more efficient study of malaria vaccine development: a) the ability to culture the parasite in vitro; b) the development of a murine model for malaria; and c) the possibility of performing challenge studies in humans and primates to evaluate candidate vaccines.

Estimated worldwide morbidity and mortality attributed to selected parasitic diseases

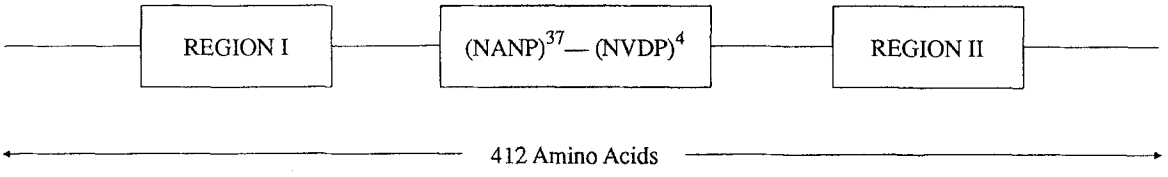
Disease	No. newly infected per annum	Deaths per annum
Malaria	200,000,000	2,000,000
Schistosomiasis	20,000,000	250,000
Filariasis	2,000,000	250,000*

*Deaths and blindness.

As with most parasites, the life cycle of the malaria parasite is complex and consists of many antigenically distinct stages. Infection is initiated when an infected feeding female anopheline mosquito releases sporozoites (from a few hundred to a few thousand) from its salivary glands into the bloodstream. The majority of sporozoites invade hepatocytes within minutes of entering the bloodstream. Each infected hepatocyte can yield up to 20,000 merozoites upon rupturing. These merozoites then invade erythrocytes and undergo asexual multiplication. The cycles of erythrocyte rupturing, release of merozoites, and the reinfection of erythrocytes are responsible for disease symptoms and death.

At first glance, a vaccine which would prevent infection, i.e., an anti-sporozoite vaccine, would be the most desirable. Such a vaccine would not only prevent disease in a susceptible individual, but also reduce transmission by lowering the number of infected reservoirs. The obvious drawback to such a vaccine is the possibility that a single sporozoite escaping the immune response would be sufficient to establish an infection. Given the short period of time in which the sporozoites remain in circulation and are accessible to neutralization by circulating antibody, this is a distinct possibility. The concept of anti-sporozoite immunity was given credence by the demonstration that animals and humans immunized with intact x-irradiated sporozoites were protected against parasitemia [10, 28].

Subsequent efforts at developing an anti-sporozoite vaccine focused upon identifying the protective epitope(s) expressed by sporozoites. The entire surface of the sporozoite is covered by a single protein termed the circumsporozoite (CS) protein. Monoclonal antibodies against the CS protein were found to afford protection in animal challenge studies [48]. Shortly thereafter, the gene coding for the CS protein of *Plasmodium falciparum* was cloned and sequenced [11]. The CS protein has a unique structure characterized by a central domain composed of 41 tetrapeptide units (fig.). There are 37 repeats consisting of asparagine-alanine-asparagine-proline (NANP) inter-



Schematic representation of the circumsporozoite protein of *P. falciparum*.

spersed with 4 repeats of asparagine-valine-aspartic acid-proline (NVDP). The immunodominant region of the CS protein is the NANP repeat. Antibody to this region neutralizes sporozoite infectivity [50]. Of equal importance is the fact that the NANP repeat sequence is highly conserved among *P. falciparum* isolates from different geographical areas [49]. Interestingly, the CS protein from other *Plasmodium* species has a similar repeat structure. However, the amino acid sequence of the immunodominant repeat varies considerably among species. There are two 15-amino acid sequences flanking the immunodominant repeat which show minimal interspecies variability. Studies with synthetic peptides corresponding to these conserved and variable regions have shown that only antibody to the species variable region can prevent penetration of human hepatocytes by sporozoites [3].

Based upon the above findings, several candidate vaccines have been produced and evaluated in humans. A highly purified recombinant protein termed $R32_{tet32}$ was produced in *E. coli* [2]. $R32_{tet32}$ has the following one letter amino acid code, MDP(NANP)^{15}NVDP(NANP)^{15}-NVDP, fused to the first 32 amino acids of a gene encoding resistance to tetracycline. This vaccine was found to be safe when administered to humans [2]. Unfortunately, only repeated high doses (800 μg) of vaccine engendered a good anti-NANP antibody response. Challenge studies showed that one volunteer, who possessed the highest antibody titer, was protected against parasitemia. Two others with lower titers were not protected. A synthetic peptide-based vaccine synthesized by chemically coupling $(NANP)^3$ to tetanus toxoid has also undergone clinical evaluation in humans [20]. This vaccine was also moderately immunogenic. One of six volunteers challenged with sporozoites was protected. Again, protection correlated with anti-NANP antibody levels.

Although somewhat disappointing, these two studies proved conclusively that high levels of anti-NANP antibody can afford complete protection against clinical disease. A critical issue raised by these studies was the feasibility of routinely engendering such a protective immune response in humans using defined vaccines. In other words, why were these two vaccines so poorly immunogenic? There are several possible reasons including a) the relatively small molecular weight of the $R32_{tet32}$ molecule; b) the method of conjugate synthesis; c) the selection of carrier protein; d) innate nonimmunogenicity of the NANP repeat; and e) lack of a sporozoite T-cell epitope.

In an attempt to address some of these possibilities, numerous conjugates have been constructed using various carrier proteins coupled to either synthetic NANP-bearing peptides or to a recombinant protein termed R32LR which has the following amino acid sequence, MDP-$[(NANP)^{15}NVDP]_2$LR. Preliminary studies in our laboratory demonstrated that conjugates produced with R32LR were consistently more immunogenic than those made with either $(NANP)^3$ or $(NANP)^6$ peptides (author's unpublished observations). To study the effect of carrier protein selection on the immune response to the NANP repeat, R32LR was covalently coupled to tetanus toxoid (Ttxd), cholera toxin (CT), choleragenoid (CG, the nontoxic binding domain of CT), and *Pseudomonas aeruginosa* toxin A (ToxA) [34]. When tested in rabbits, the anti-NANP antibody response was significantly higher for conjugates constructed with CT or ToxA than those made with Ttxd or CG. Surprisingly, the magnitude of the anti-NANP antibody response was not governed by the levels of anti-carrier antibody attained. However, CT, CG, and ToxA conjugates were found to be equally immunogenic in monkeys (P. Hauser, personal communication). The CT-R32LR and the ToxA-R32LR have been evaluated for safety and immunogenicity in humans (J. C. Sadoff, S. J. Cryz Jr, E. Fürer, D. Gordon, W. R. Ballou, and J. U. Que, manuscript in preparation). The ToxA conjugate gave a consistently higher immune response than the CT conjugate. Studies to determine the efficacy of the ToxA-R32LR vaccine are now underway.

As an alternative to chemical coupling, fusion proteins containing NANP repeats have been synthesized and evaluated (P. Hauser, personal communication). $(NANP)^{32}$ has been fused to the first 81 amino acids of the gene encoding the NS1 protein of the influenza A virus and produced in *E. coli*. A second fusion protein consisting of $(NANP)^{15}$-NVDP fused to the amino-terminus of the pre-S2 gene of hepatitis B virus was produced in yeast. While both fusion proteins produced more anti-NANP antibody than $R32_{tet32}$ in monkeys, the hepatitis B construct was far more immunogenic than the influenza A construct.

It has been known for some time that immunization with sporozoites can engender antibody-independent immunity [33]. Recently, it was shown that a recombinant, attenu-

ated *Salmonella typhimurium* strain expressing gene coding for NANP could confer protection against experimental murine malaria in the absence of antibody production, presumably by cell-mediated immunity (CMI)[36]. $CD8^+$ T-cells play a critical role in the protective cellular immune response[44]. Theoretically, such cells could act directly to destroy sporozoite-infected hepatocytes or by the release of γ-interferon[37,44]. It is important to remember that the parasite multiplication occurs within a vacuole, limiting the expression of sporozoite antigens (specifically the CS protein) on the hepatocyte surface, a pre-requisite for T-cell recognition. However, as discussed by Good et al.[13], the CS protein may be shed during the invasion process and become associated with the hepatocyte surface.

The question then becomes two-fold: first, what are the T-cell factors which regulate the immune response to the NANP repeat; and secondly, can an effective anti-sporozoite cell-mediated immune response be induced in humans? The antibody response to the NANP repeat is governed by the class II major histocompatibility complex antigens, i.e., $CD4^+$ helper T-cells[14]. The recognition of a major T-cell epitope expressed within region II of the CS protein, termed Th2R, is extremely limited in mice[14]. The practical aspects of this finding are shown by the fact that peripheral blood lymphocytes from a full 40% of adults living in a malaria-endemic area did not undergo proliferation when exposed to T-cell epitopes[15]. These results can be interpreted to indicate that a substantial proportion of a given population may not be able to recognize critical epitopes expressed by the CS protein. Of equal concern is the fact that these T-cell epitopes cluster in a variable region near the carboxyl-terminus of the CS protein. Therefore, an individual who mounts an immune response following vaccination or natural exposure may not be capable of mounting an anamnestic response upon re-exposure to a sporozoite with a variant T-cell epitope. However, it remains to be seen whether highly immunogenic $(NANP)^x$-NVDP-containing vaccines can evoke a boosting response in humans.

The phenomena of nonresponsiveness to the NANP repeat and variability within the T-cell sites pose serious problems for sporozoite vaccine development. Numerous approaches are now being taken to overcome these parasite defenses. One critical question still to be answered is how great the absolute diversity is among T-cell epitopes of various sporozoite isolates. If it is limited, a 'cocktail' vaccine containing the relevant antigens could be formulated. Even if variation is widespread, it may be possible to identify cross-reactive amino acid sequences common to many isolates. Recent studies described above indicate that NANP-containing conjugate or fusion protein vaccines may offer a way to evoke high levels of antibody. Preliminary studies with the R32LR-ToxA conjugate indicate that after 2–3 immunizations, most vaccinees developed titers which, in previous challenge trials, afforded protection.

The problems of malaria vaccine development based upon sporozoite immunity must also be viewed with an eye to a specific user population. Travellers entering an endemic area for a short period of time may prove to be the simplest to protect. In such instances, repeated vaccinations (if needed) could be performed at the appropriate interval(s) before travel. Furthermore, in such cases, protective levels of antibody only need to be maintained for a short period of time.

The task of vaccinating those living in an endemic area is more complex. Of prime importance is that the vaccine be effective in children since this is the age group with the greatest morbidity and mortality attributed to malaria. Ideally, a malaria vaccine would be a component that could be added to existing childhood vaccine programs, especially that for diphtheria, tetanus, and pertussis. In this scenario, it would be essential for the vaccine to behave as a T-dependent antigen, since children $\leq 18-24$ months of age usually do not respond well to T-independent antigens. If, for some reason, the vaccine were not compatible with existing programs, it would be essential that one dose of vaccine could induce a protective immune response since the logistics and costs of administering multiple doses of vaccine at definite intervals in children $\geq 2-4$ years of age are insurmountable in most malaria endemic areas.

Finally, careful consideration must be given to the final vaccine formulation. To date, only alum-adsorbed vaccines have been evaluated (largely due to the fact that only alum-based adjuvants are licensed for human use). However, several additional possibilities exist. The formulation of malaria antigens within liposomes has shown some promise in animal studies[27]. Potent new adjuvants based upon lipid A or muramyl dipeptide derivatives may also prove useful for malaria vaccines[7,35]. An intriguing possibility is to use live-attenuated viral or bacterial vectors to deliver relevant malarial antigens[36].

Filariasis

Filariasis is an arthropod-borne disease caused by several parasites. *Brugia malayi, Onchocerca volvulus,* and *Wucheria bancrofti* are the most important causes of disease in humans. A variety of disease syndromes can be caused by infection, the most frequent being cutaneous or lymphatic in nature. It is important to note that onchocerciasis is a leading cause of blindness worldwide. It is believed that the majority of disease symptoms manifested are mainly due to hyperresponsiveness of the host immune system. This is best illustrated by the hypersensitivity response seen during lymphatic filariasis in tropical eosinophilia[31]. Disease is restricted to those geographical areas where the mosquito vector can readily breed (South and Central America, Southern Pacific Basin, Southeast Asia, and Africa). Studies on the pathogenesis of filariasis have been limited by host-specificity and nu-

tritional requirements of the different parasite stages. In the absence of in vitro cultivation techniques, parasites are usually extracted from skin nodules or lymphatic tissue.

The human immune response to filarial antigens during infection has been studied in considerable detail[16]. A vigorous humoral immune response within the immunoglobulin G, A, M, and E (IgG, IgA, IgM, and IgE, respectively) classes to a variety of parasite antigens has been documented[1,16]. Infection also engenders a broad cross-reactive antibody response whose significance is unknown. There is some evidence to suggest that a higher proportion of IgE antibody than of IgG is specific for the infecting parasite. Interestingly, there is some degree of correlation between IgG subclass levels and clinical states. IgG_4 levels were found to be significantly higher in patients with nonsymptomatic microfilaremia than in individuals presenting with marked symptomology[21]. Such antibodies, however, were able to recognize a number of parasite antigens. A possible detrimental effect of such a pronounced humoral antibody response is the formation and deposition of immune complexes[40].

Evidence to indicate a protective role for antibody in filariasis is mounting, but far from conclusive. Patients infected with *W. bancrofti* who remain amicrofilaremic have elevated levels of IgG to a 25 kDa parasite antigen as compared to microfilaremic patients[22]. A murine monoclonal antibody which recognizes a 70 kDa antigen expressed by *B. malayi* can decrease the level of microfilaremia when passively transferred to experimentally infected animals[8]. A subpopulation of antibodies to either *O. volvulus* microfilaria or infective larvae has been shown to promote the uptake and subsequent killing by neutrophils and eosinophils[17]. Such functional antibodies deserve closer study to ascertain their involvement in protective immunity. Recently, Freedman and co-workers[12] have found a direct correlation between response to a 43 kDa larval stage antigen and freedom from bancroftian filariasis in individuals living in endemic areas.

There is some evidence to indicate the acquisition of natural immunity in individuals living in endemic areas. First, the incidence rates for microfilaremia increase until age 30–35, but after this time the infection rate remains constant or declines slightly. Perhaps of greater importance is the finding that a small proportion of the adults who are amicrofilaremic have no history of prior infection[47].

The cell-mediated immune response to filarial antigens may play a crucial role in immunity to disease. Individuals suffering from filariasis have a markedly suppressed cell-mediated immune response to nonspecific mitogens and to filarial antigens[32]. Infection seems to result in the generation of specific suppressor T-cells. Nonresponsiveness is more pronounced in individuals who have a high parasite burden. Unfortunately, little is currently known about which antigen(s) are involved in this process. Each life cycle stage can express more than 100 antigens, although there seems to be a high proportion of antigenic relatedness even between genera[26,38]. The identification of antigen(s), preferably shared, which can evoke either a protective cell-mediated or humoral immune response is essential in allowing a rational approach to vaccine development.

Several studies demonstrated that irradiated infective larvae, when used to immunize monkeys, cats, or mice, conferred a degree of protection against subsequent challenge with *B. malayi* or *B. pahangi*[19,30,46]. Kazura and Davis[24] have shown that immunization of mice with soluble *B. malayi* microfilarial antigens protected them against a subsequent microfilarial challenge. Protection was mediated by IgG antibody. Subsequently, it was shown that serum from animals immunized with irradiated larvae recognized 4 microfilarial antigens (M_r = 150 k, 75 k, 42 k, and 25 k)[23]. The antibody response was not specific for microfilariae, but also recognized other parasite stages, confirming previous reports of antigenic relatedness between stages. Immunization of mice with a 25 kDa and a 60 kDa filarial antigen has also been shown to enhance the clearance of microfilariae from the blood[19]. Whether the two 25 kDa antigens used in the studies described above are identical is unknown. The 60 kDa antigen has been cloned and sequenced[29]. Immunization of jirds with a vaccinia virus vector expressing this antigen resulted in a markedly decreased microfilaremia following challenge[29].

Schistosomiasis

Schistosomiasis is a chronic debilitating disease caused by *Schistosoma mansoni, S. haematobium*, or *S. japonicum*. Approximately 250 million people suffer from the disease worldwide. Humans are infected by coming into contact with water contaminated with cercariae which penetrate the skin. At present, disease control is centered on chemotherapy and on improving sanitation. A considerable body of evidence exists that indicates at least partial immunity to infection can develop in endemic areas. Patients with a primary infection are resistant to superinfection, which indicates the presence of a naturally-acquired immune state[45]. Numerous studies in hyperendemic areas have shown an increased resistance to reinfection once the parasite has been eradicated by chemotherapy[18,43]. There also appears to be an age-dependent acquisition of immunity in endemic areas, as measured by either prevalence rates or intensity of infection[5].

Resistance to infection appears to be mediated by an immune response to certain schistosomal antigens. A key factor appears to be the ability of lymphocytes to proliferate when exposed to schistosomal antigens[42]. Resistance to reinfection in cured patients was found to depend upon the degree to which lymphocyte proliferation occurred in response to certain cercarial, egg, and adult worm antigen(s)[5]. Patients with a poor response were

usually reinfected, whereas those with a good response remained parasite-free.

The effector cells which mediate immunity to schistosomiasis appear to be eosinophils. Sturrock et al.[41] found that eosinophilia in cured patients correlated with a subsequent lack of infection. This was of considerable interest since it has been known for some time that the schistosomula of *S. mansoni* can be destroyed by eosinophils in the presence of immune sera[6]. From studies involving Kenyan children, it appears that IgG antibodies which recognize an egg antigen which shows cross-reactivity with a schistosomula antigen are responsible for mediating the eosinophil-dependent killing of schistosomula[5]. Interestingly, reinfection in Kenyan children appeared to correlate with increased levels of anti-egg antibody. Butterworth and Hagan[5] have proposed that such cross-reactive antibodies serve to block the binding of effector antibody to schistosomula, thereby preventing eosinophil-dependent killing. Such a hypothesis is consistent with findings in a rat model of schistosomiasis[9]. Additional support for this hypothesis comes from the observation that IgM antibody, which recognized both egg and schistosomula antigens, could block antibody-dependent killing of schistosomula[25].

The key to developing an effective vaccine against schistosomiasis is dependent upon identifying an antigen present on the schistosomula which can induce an antibody response that supports the eosinophil-dependent killing of the schistosomula. A large number of antigens, either protein, carbohydrate, or glycoprotein, have been identified, isolated, and characterized from *S. mansoni*[39]. Passively transferred antibody to at least 11 of these antigens has been shown to afford protection against experimental infections[39]. Nine of the antigens have been purified to a variable degree and used to induce a protective immune response in animals following active immunization[39].

The most promising candidate vaccine is a 28 kDa protein, known to be a glutathione S-transferase, expressed on the surface of *S. mansoni, S. bovis, S. haematobium*, and *S. japonicum*[4]. The gene encoding this antigen has been cloned in *E. coli*. A recombinant fusion protein expressing the major protective epitopes of the 28 kDa protein was produced in *E. coli* and subsequently purified. Immunization of rats with this fusion protein induced antibody which mediated eosinophil-dependent killing of schistosomula. Rats and hamsters immunized with this protein were protected when challenged with *S. mansoni* cercariae as determined by the level of parasite burden. Immunization of baboons engendered an eosinophil-dependent cytotoxic response equal to or exceeding that observed with serum from naturally-infected baboons.

Studies are now underway to evaluate the potential of the 28 kDa protein as a human vaccine. Even a vaccine which could induce a partially-immune state in humans would be expected to have a considerable impact on disease severity and transmission, since schistosomes do not replicate in their human hosts.

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