

Malaria vaccine: roadblocks and possible solutions

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Abstract

Malaria remains the most prevalent and devastating parasitic disease worldwide. Vaccination is considered to be an approach that will complement other strategies for prevention and control of the disease in the future. In the last 10 years, intense studies aimed at the development of a malaria vaccine have provided important knowledge of the nature of the host immunological mechanisms of protection and their respective target antigens. It became well established that protective immune responses can be generated against the distinct stages of *Plasmodium*. However, in general, protective immune responses are directed at stage-specific antigens. The elucidation of the primary structure of these antigens made possible the generation of synthetic and recombinant proteins that are being extensively used in experimental immunizations against the infection. Today, several epitopes of limited polymorphism have been described and protective immunity can be generated by immunization with them. These epitopes are being tested as primary candidates for a subunit vaccine against malaria. Here we critically review the major roadblocks for the development of a malaria vaccine and provide some insight on how these problems are being solved.

Key words

- Malaria
- *Plasmodium*
- Vaccine
- Immunity
- Infectious disease

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Introduction

Malaria remains the most prevalent and devastating parasitic disease worldwide, with a yearly estimate of more than 200 million cases due to *Plasmodium falciparum* and at least 40 million cases due to *P. vivax*. The population at risk is estimated at more than 2 billion people living in tropical regions of the world. The disease is most prevalent in Africa, where children under 5 years of age are the individual group at higher risk of death due to infection. Malaria transmission also occurs in large areas of South Asia, Latin

America, and Oceania. Although the pattern of disease transmission, infection and mortality in these endemic areas is quite diverse, malaria has never been a greater problem than it is today, and it is likely that this circumstance will not improve worldwide in the next five to ten years.

The main problem for controlling the disease is that only a few strategies are economically accessible, such as education, chemotherapy, vector control using insecticides or elimination of mosquito breeding sites, and more recently, insecticide-impregnated mosquito bed nets. It is certain that the best

way to control the disease on a short-term basis is investing more resources in currently available methods of control. It is well documented that even small investments in these areas are highly cost-effective. For example, a single program of insecticide-impregnated bed nets can reduce child mortality in an entire African country (1). Larger investments in malaria control programs would have an enormous impact on most endemic areas, reducing the number of cases and casualties.

Mass vaccination against malaria is not available, and it will not be feasible within this century. Research in this field was once considered as a priority that would lead to the most powerful method of control and prevention of the disease. However, in the past ten years this concept has changed. Malaria parasites display genetic mechanisms to generate drug-resistant strains and to avoid several immunological mechanisms of defense. Accordingly, in areas where drugs or eventually vaccines are used, parasites will have to be constantly monitored for the presence of resistant mutant strains. In spite of these limitations, mass vaccination is considered to be an approach that, in the future, may complement other strategies such as chemotherapy, vector control, etc., for prevention and control of the disease.

Why has a malaria vaccine not been developed?

Vaccines can prevent a number of infections by viruses and bacteria. The basis for vaccine research and mass vaccination against these microorganisms relies on certain facts. Viruses and bacteria can be easily cultivated *in vitro*, and they have a relatively simple life cycle. By generating large amounts of microorganisms *in vitro*, it is possible to obtain antigenic fractions that can be used for basic research, product development and mass vaccination. The fact that they have only a single form, a not so large

number of genes, and undergo limited morphological and biochemical alterations during their life cycle facilitates protective immune responses. However, many exceptions exist and a number of viral and bacterial infections cannot be prevented by vaccines.

These basic characteristics of viruses and bacteria are not shared by malaria parasites, and some of the concepts of vaccination generated by immunization against them cannot be applied to most parasitic diseases. For years, these limitations impaired the development of a malaria vaccine and, at some point, generated a very pessimistic view of the problem. From a different perspective, these roadblocks helped to create new concepts in vaccination that will be required for the development of an effective vaccine against malaria and perhaps other complex microbial infections.

Among the most critical factors that have delayed the advances in this field is the impossibility of generating large amounts of parasites in culture. Up to now, sufficient amounts of antigen can only be generated for basic research, but not for product development or mass vaccination. The second factor is the complexity of the malaria parasite life cycle. During their life cycle, malaria parasites undergo complex transformation generating several different forms that are morphologically and antigenically distinct. Therefore, individuals exposed to malaria have contact with multiple forms of the parasite expressing a variety of stage-specific proteins. Another problem imposed by its complex life cycle is the fact that distinct immunological effector mechanisms are responsible for eliminating different forms of the parasite. Taken together, these aspects led to the concept that an ideal malaria vaccine would be a complex mixture of antigens expressed at different stages of the parasite life cycle produced by chemical synthesis or recombinant DNA technology, or both. Due to the polymorphism frequently detected in several parasite antigens, a vaccine would

have to be elaborated using immunogenic regions of parasite antigens that are either invariant or display a limited polymorphism. Finally, an ideal vaccine will have to elicit distinct immunological effector mechanisms to attack the parasite at different stages of its life cycle.

Based on these concepts, in the past 15 years, great advances have been made in characterizing parasite antigens and the immunological effector mechanisms. The number of genes coding for antigenically relevant proteins that have been cloned and sequenced cannot find a parallel in any other parasitic disease. This work can serve as the basis for the development of a malaria vaccine. Nevertheless, it is important to highlight that studies on the characterization of antigens should continue since new protein families have been only recently described (2-4). Also, very few genes encoding *P. vivax* antigens have been identified, and it would be important to pursue studies in this direction since this parasite co-exists with *P. falciparum* on most continents where malaria transmission occurs.

The advances in the description of the immunological effector mechanisms which lead to parasite elimination also provided important insights on how an appropriate anti-plasmodial immune response can be elicited. Again, there is still more to understand about the immunological effector mechanisms in experimental models and humans. Most relevant was the fact that multiple antigens, expressed at distinct stages of the parasite, were described as a target for the host protective immunity, and therefore candidates to be part of a subunit vaccine against malaria (reviewed in Ref. 5).

Malaria parasite life cycle and stage-specific immunological mechanisms of parasite destruction

During a mosquito bite, sporozoites are inoculated into the bloodstream where they

remain for only few minutes, being targets for antibodies that react with a stage-specific surface antigen, the circumsporozoite (CS) protein (6). Sporozoites then invade hepatocytes and are no longer a target for anti-CS antibodies. Inside hepatocytes, sporozoites develop to schizonts and after a few days they are released into the bloodstream as merozoites. Liver schizonts express stage-specific antigens as well as antigens that are common to sporozoites and blood stages. Because liver schizonts are intracellular, the immunological effector mechanisms are only mediated by T lymphocytes. CD8⁺ T lymphocytes specific for several antigens have been described as the most powerful immunological mechanism that inhibits the development of liver stages of malaria. CD4⁺ lymphocytes also exert a certain inhibitory effect on the development of this form of the parasite (reviewed in Ref. 7).

When the tissue merozoites are released into the circulation, they also become a target for antibody-mediated immunity. A variety of surface antigens have been described as targets for antibodies that can inhibit erythrocyte invasion *in vitro* (reviewed in Ref. 8). Human protective antibodies also mediate opsonization by blood monocytes (9). The targets for antibodies that mediate opsonization are not precisely known, and it is likely that a number of them exist. Once merozoites invade red blood cells they transform into trophozoites, subsequently to schizonts, and finally are released as merozoites. It is certain that the intra-erythrocytic forms of malaria are targets for the CD4⁺ T cell-mediated immunity (10) and antibodies since some parasitic antigens occur on the surface of infected erythrocytes (2-4).

A small part of trophozoites transform into gametocytes that undergo development when ingested by susceptible mosquitoes. Gametocytes express on their surface stage-specific antigens which have been shown to be targets for antibodies that impair parasite development inside the mosquito, therefore

blocking the cycle and transmission (11,12). This type of immunity does not protect the host, but by reducing the number of infected mosquitoes and their parasitic load, it may diminish malaria transmission. Finally, stage-specific antigens expressed on the surface of sexual forms of the parasite (zygotes and ookinetes) can also be targets for transmission-blocking antibodies (13,14).

A schematic view of the parasite life

Figure 1 - Schematic representation of the life cycle of malaria parasites and description of host immunological mechanisms of protection.

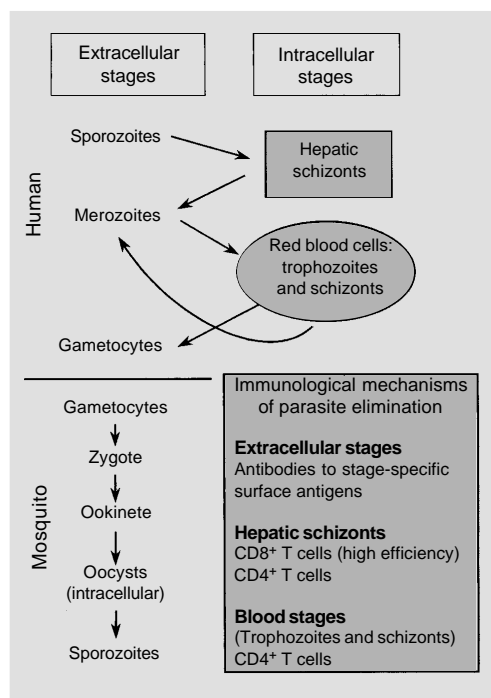
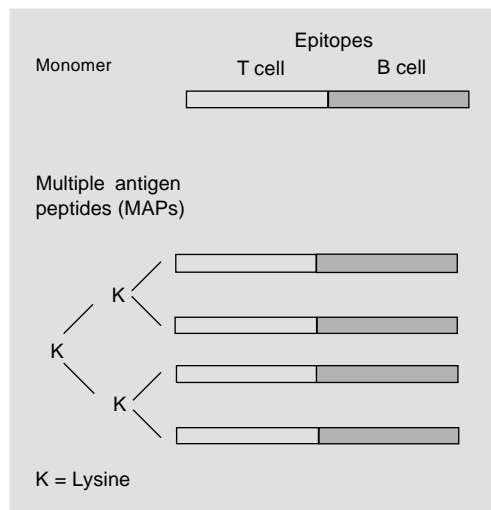


Figure 2 - Schematic representation of multiple antigen peptide polymers.



cycle and the different immunological mechanisms that are capable of eliminating each one of these forms is presented in Figure 1.

Synthetic peptides as candidates for a malaria vaccine

Synthetic peptides are extremely attractive candidates for vaccines since they can be produced on a large scale under extremely well-controlled conditions. In fact, a synthetic peptide coupled to a carrier antigen was one of the first immunogens to be tested in human trials against malaria (15). In the past ten years, only a single major advance has been made in this field that may have some impact on the development of a malaria vaccine. Branched peptides denominated multiple antigen peptides, or simply MAPs, were developed (Figure 2), and proved to be more immunogenic than linear peptides (16). Mice immunized with a MAP containing a B cell epitope of the CS protein coupled to a CD4⁺ T cell epitope produced very high antibody titers that mediate protective immunity against challenge with sporozoites of two distinct rodent malarial (16, 17). Details on the properties and advantages of these MAPs have been recently reviewed in reference 18. These optimistic results have justified human trials scheduled to start soon.

The “vaccine” developed by Dr. Manuel Patarroyo, a synthetic peptide denominated SPf66, experienced great success after initial immunization trials in monkeys and humans and was named “the first malaria vaccine” (19). This success sold a lot of hope to certain international organizations and newspapers. However, these results could not be duplicated in other trials on monkeys (20) or humans performed later in different parts of the world (21-23). The immunological basis for protection observed during the first group of trials was never elucidated. It is unknown whether SPf66 elicits a certain degree of protective immunity mediated by antibodies

or T cells, or both, as discussed in reference 24.

The concept of using synthetic peptides as an ideal vaccine against malaria has not advanced as expected in the past years. Other problems of using synthetic peptides are as follows. Peptides are relatively short. Recent advances in peptide synthesis have permitted the generation of linear peptides of more than 100 amino acids (25). This amount of antigenic information is still very limited if one considers that an ideal malaria vaccine would have to be the product of several genes. It seems unlikely that it can be obtained by chemical synthesis only. Also, correct peptide folding can be rarely achieved and most conformational epitopes may not be generated using synthetic peptides.

Recent advances in the generation of recombinant proteins and their impact on the development of a malaria vaccine

In contrast to synthetic peptides, protein production using recombinant DNA technology has advanced very fast in the past 12 years. In the mid 1980's, only very limited quantities of proteins could be generated in few prokaryotic expression systems. Today, a variety of expression systems are available to produce recombinant proteins, and their number continues to increase every year. The expression of foreign proteins can now be obtained in viruses, bacteria, and in lower and higher eukaryotic systems. They became a very important tool for basic research, product development and even mass production of vaccines such as hepatitis vaccine.

Different expression vectors can provide solutions for most of the initial problems of protein expression such as inappropriate protein folding, low protein yields and complex purification processes. By choosing the correct vector, it is now possible to select the microorganism that provides the best protein

folding, or produces the largest amount of protein or requires the easiest purification schedules. A second relevant point is the fact that these distinct vectors made possible the selection of the vehicle that elicits the type of immune response required for protection.

Recombinant proteins

CS protein

The CS protein is the most abundant protein that covers the entire sporozoite surface and is also expressed by malaria liver stages. Its biological function has recently been elucidated by deletion of the CS gene in the rodent malaria parasite *P. berghei*. "Knockout" of the CS gene generates a parasite strain which evolves normally as blood stages; however, sporozoites do not develop inside mosquito oocysts (26). This result strongly suggests that the CS protein is structurally required for generation of sporozoites. Other studies have also implicated this protein as a mediator of sporozoite binding and invasion of hepatocytes in mammalian hosts (27).

The structure of this protein has been thoroughly described (6). Briefly, the CS protein of all malaria species contains a central domain consisting of several amino acid repetitions. The amino acid sequence that composes each repetition is completely different among *Plasmodium* species. In *P. falciparum*, the only strain variability described so far is related to the number of the (NANP) X3 repetitions. In contrast, *P. vivax* isolates display an enormous variation in the sequence of these repetitions (28) and, therefore, *P. vivax* CS protein is not likely to be a candidate for a malaria vaccine.

The amino acid repetitions of the CS protein are known as targets for antibodies that can provide sterile immunity for experimental animals. Other regions of the CS protein (N- and C-terminal) contain epitopes that are recognized by CD4⁺ or CD8⁺ T cells

from several different MHC haplotypes of humans and rodents (reviewed in Ref. 7). It is also well established that T cells specific for epitopes of the CS protein confer protective immunity against liver stages of rodent malaria parasites. Therefore, the CS protein is a target for protective antibodies that block sporozoite invasion in hepatocytes and T cells that inhibit the development of liver stages of malaria.

Recombinant proteins produced in *E. coli* or yeast have been widely used to study the induction of protective immunity using the CS protein. In fact, an *E. coli*-derived recombinant protein representing the repetitive domain of the *P. falciparum* CS protein was one of the first immunogens to be used in a human vaccination trial (29). This study demonstrated the safety and immunogenicity of this recombinant protein for human immunization. However, protection was relatively poor and only one out of six immunized volunteers was protected after exposure to infected mosquitoes. Although negative, these results were important since they suggested that, if improved, immunization with a recombinant CS protein could be of great use to elicit sterile protective immunity

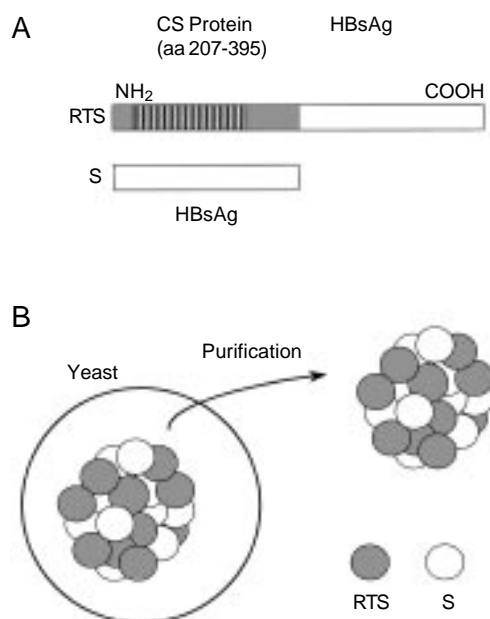
against malaria. Also very important was the fact that this and other bacterial recombinant proteins were extremely useful for testing new adjuvants for improvement of human antibody and cellular immune response (30). In spite of these efforts, in many subsequent studies *E. coli*-derived recombinant CS proteins could not improve sterile immunity beyond 2 of 11 volunteers challenged with *P. falciparum* sporozoites (31).

More recently, human trials were performed using a recombinant protein based on the sequence of the *P. falciparum* CS protein produced in yeast. This recombinant protein consisted of 189 amino acids (from 207 to 395) of the *P. falciparum* CS protein fused to hepatitis B surface antigen (S antigen). This fusion protein was denominated RTS and its gene was simultaneously expressed in *Saccharomyces cerevisiae* with the gene encoding the S antigen (32,33). The protein particle resulting from the expression of the RTS and S gene was denominated RTS,S (Figure 3). The S antigen is an integral membrane protein and the major antigen of hepatitis B virus. This protein is known to be highly antigenic for humans and it is currently used for mass vaccination in many countries.

Initial studies were performed by immunizing groups of volunteers with RTS,S emulsified in alum only, or alum plus 3-deacylated monophosphoryl lipid A (MPL) derived from *Salmonella minnesota* lipopolysaccharide. The second group of individuals had a consistently higher antibody titer to the repeat region of the CS protein and after challenge with sporozoites, 2 out of 8 volunteers were protected against malaria (32). In contrast, none of the individuals immunized with RTS,S in alum were protected. This study also demonstrated that an adjuvant formulation could be of great relevance for the induction of protective immunity against malaria in humans.

A recently published study was designed to evaluate three different adjuvant combi-

Figure 3 - Schematic representation of RTS,S particles. A, RTS and S proteins; B, RTS, S particles. HBsAg, Hepatitis B surface antigen (S antigen).



nations using as antigen the protein RTS,S. The volunteers were divided into three groups which were immunized with RTS,S in three distinct adjuvant preparations. The first group was immunized with RTS,S in alum plus MPL. The second group was injected with RTS,S in an oil-in-water emulsion. The third group was immunized with this emulsion plus MPL and QS21 (saponin). The antibody titers against sporozoites or the repeat region of the CS protein were significantly higher in individuals immunized with the last two preparations. After challenge with sporozoites, striking results were observed. While 6 of 7 individuals (85.7%) were completely protected against malaria in group 3, only 28.6% of the individuals were protected in group 2 and 12.5% in group 1 (33). The results imply that the correct adjuvant formulation is crucial to elicit a high degree of protective immunity. The precise mechanism of protection is unknown. Groups 2 and 3 had antibody titers that were not significantly different. Therefore, if protective immunity is dependent on antibodies, it is likely that the avidity or fine epitope specificity of these immunoglobulins is more important than their serum concentration. The basis of protective immunity will require detailed analysis of the humoral and cellular immune response to the CS protein. Based on this very promising result, field trials in endemic areas of malaria are expected soon.

Merozoite surface protein 1

Among the proteins of the blood stages of *Plasmodium*, the merozoite surface protein 1 (MSP1) has been the most intensively studied as a potential target for protective immunity. This protein is synthesized as a precursor of large molecular mass (180-230 kDa) during schizogony and is later processed to some of the major merozoite surface proteins (reviewed in Ref. 34). During the invasion process, proteolytic cleavage releases most of the molecule from the mero-

zoite surface and only a 19-kDa fragment of the C-terminal region (MSP1₁₉) is carried into the invaded red cells (35,36). The biological importance of MSP1 for parasite survival is unknown, but it is well established that antibodies that recognize its C-terminal region inhibit merozoite invasion *in vitro* (37,38), and confer passive immunity on naive mice (39). Initial studies of immunization with MSP1 purified from blood stages showed that rodents or monkeys could be successfully vaccinated against *P. yoelii* or *P. falciparum* infection, respectively (40,41).

The potential of this molecule for vaccine development motivated studies aimed at the generation of recombinant proteins containing portions of MSP1. Several recombinant proteins based on the MSP1 sequence of different *Plasmodium* species were used to immunize rodents and monkeys. A certain degree of protective immunity could be achieved in monkeys after immunization with recombinant proteins based on the sequence of the N-terminal region of MSP1 (denominated PL190) fused to a T cell epitope (42).

However, it was not until recently that remarkable protective immunity could be elicited in experimental models by immunization with a few such recombinant proteins. These studies used recombinant proteins based on the sequence of the C-terminal region (42 or 19 kDa) of MSP1 of distinct *Plasmodium* species (Figure 4). This region of the molecule, particularly the MSP1₁₉, is considered to be of limited polymorphism

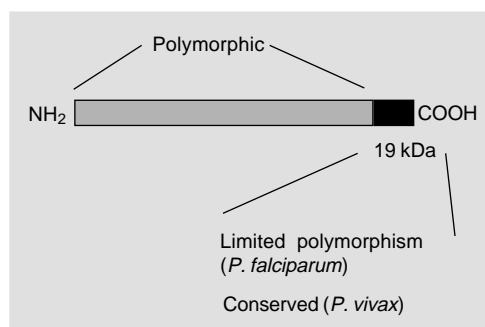


Figure 4 - Schematic representation of merozoite surface protein 1 of *Plasmodium* sp.

since only few amino acid substitutions are observed among different strains of *P. falciparum*. In the case of *P. vivax* MSP1₁₉, the variability is very restricted and only a single amino acid substitution has been reported (43). Also relevant is the fact that MSP1₁₉ contains epitopes that are recognized by antibodies and T cells of individuals naturally exposed to *P. falciparum* and *P. vivax* malaria (44,45).

Immunization of mice with an *E. coli*-derived recombinant protein containing MSP1₁₉ resulted in very effective protection upon lethal challenge with *P. yoelii* blood stages (46,47). The induction of protective immunity requires epidermal growth factor-like motifs present in MSP1₁₉ (48) and is mediated predominantly by antibodies (49). These results, therefore, suggest that epitopes in MSP1₁₉ are targets for the protective antibodies. It will be important to elucidate the mechanism used by specific antibodies to inhibit the development of blood stages since they may have an impact on the designing of a vaccine against malaria blood stages.

Using recombinant proteins based on the sequence of the *P. falciparum* MSP1 C-terminal region, three independent trials were performed using *Aotus* monkeys. A summary of these trials is presented in Table 1. In the first trial, animals were immunized with an *E. coli*-derived GST-MSP1₄₂ fusion protein or a yeast-derived recombinant MSP1₁₉ (50). The results showed that both

Aotus nancymai monkeys immunized with MSP1₁₉ in Freund's adjuvant recovered from blood stage infection without requiring treatment. In contrast, all three control animals required treatment by day 17 and only one out of three animals immunized with GST-MSP1₄₂ was partially protected. Two other monkeys (*Aotus vociferans*) were immunized with MSP1₁₉ in Freund's adjuvant and only one had a prolonged pre-patent period.

In the second trial, three *Aotus lemurinus griseinembra* monkeys were immunized with a baculovirus-derived recombinant protein containing a 42-kDa fragment of the C-terminal region of PfMSP1 emulsified in Freund's adjuvant (51). These animals developed very high titers of antibodies to the native protein as well as to the recombinant proteins representing MSP1₄₂ and MSP1₁₉. After challenge with blood stages, two of these monkeys never developed parasitemia and the third animal had a significant delay in the pre-patent period when compared to control animals immunized with adjuvant only.

The third trial was performed by immunizing *Aotus nancymai* monkeys with an *E. coli*-derived GST-MSP1₁₉ fusion protein. The adjuvant used was either alum or liposomes. The antibody titers were far lower than the titers observed in the other two immunization trials. Upon challenge with blood stages no protective immunity was observed in either group immunized with GST-MSP1₁₉ (52).

Table 1 - Summary of vaccination experiments in monkeys with recombinant proteins derived from the C-terminal region of *Plasmodium falciparum* MSP1.

| Recombinant antigen | Source | Adjuvant | Aotus species | Number of animals | | | Ref. |
|------------------------|-------------|-----------|----------------------|-------------------|---------------------------|---------------------|------|
| | | | | Vaccinated | Delayed pre-patent period | Complete protection | |
| GST-MSP1 ₄₂ | Bacterial | Freund's | <i>A. nancymai</i> | 3 | 1 | None | 50 |
| MSP1 ₁₉ | Yeast | Freund's | <i>A. nancymai</i> | 2 | 2 | 2 | 50 |
| MSP1 ₁₉ | Yeast | Freund's | <i>A. vociferans</i> | 2 | 1 | 0 | 50 |
| MSP ₄₂ | Baculovirus | Freund's | <i>A. lem. gris.</i> | 3 | 3 | 2 | 51 |
| GST-MSP1 ₁₉ | Bacterial | Alum | <i>A. nancymai</i> | 3 | None | None | 52 |
| GST-MSP1 ₁₉ | Bacterial | Liposomes | <i>A. nancymai</i> | 3 | None | None | 52 |

The results of these trials strongly suggest that immunization with the C-terminal region of MSP1 can elicit protective immunity against malaria in non-human primates. Also, they suggest that the immune response to MSP1₁₉ of other species can be protective. In fact, immunization of *Saimiri* monkeys with a baculovirus-derived recombinant protein of PvMSP1₁₉ elicited strong protective immunity to *P. vivax* blood stages, significantly reducing the parasitemia in this experimental model (Dr. John Barnwell, personal communication). Unfortunately, so far, protective immunity could only be generated using a strong adjuvant such as Freund's, which is not allowed for human use. As was the case for the immunization with the CS protein, the adjuvant formulation seems to play a key role in the induction of protective immunity to malaria. This possibility is also supported by experiments in the mouse model showing that the adjuvant, the host genotype and the fine epitope specificity of antibodies significantly influence the protective immunity elicited by immunization with the C-terminal region of MSP1.

Serine repeat antigen

Serine repeat antigen (SERA) is a 110-kDa protein produced in large amounts by trophozoite-schizont blood stages of *P. falciparum* and *P. vivax* (53). The deduced amino acid sequence of SERA obtained from different isolates of *P. falciparum* suggests that most of the protein is highly conserved, except for the region flanking the serine repeats. Two recombinant proteins were generated in *S. cerevisiae*. SERA 1 and SERA N represented amino acids 24 to 285 and 24 to 506, respectively. Immunization of Panamanian *Aotus* monkeys with either recombinant protein emulsified in Freund's adjuvant elicited strong protective immunity capable of reducing blood stage parasitemia by at least 10-fold. Subsequent studies were carried out using only the recombinant SERA 1

with adjuvants suitable for human use. The overall results from these trials showed that 9 of 14 monkeys immunized with SERA 1 in the presence of Freund's adjuvant and 7 of 11 monkeys immunized with SERA 1 and MF75.2 adjuvant did not develop detectable parasitemia after challenge with blood stages of *P. falciparum*. However, several of the immunized and protected monkeys developed recurring low-grade infection after a prolonged period. The basis for the protective immunity observed in these animals has not been thoroughly studied, but sera from the immunized animals displayed antibodies that inhibited parasite growth *in vitro* (53).

Another successful attempt to use recombinant proteins was made by immunization of animals with yeast-derived recombinant proteins based on the sequence of the zygote and ookinete stage-specific antigens Pf25 and Pf28. The monkeys and mice immunized with these recombinant proteins developed antibodies that blocked development of oocysts, providing strong transmission-blocking immunity (13,14).

Several other antigens are being actively studied as potential candidates to be part of a malaria vaccine. These antigens have been produced by recombinant DNA technology. In most cases, few data are available to date on their ability to elicit protective immunity in experimental models.

Recombinant viruses and other live vectors

Recombinant live viruses expressing foreign antigens have the potential to be used as delivery systems designed to elicit protective immune responses against several microbial infections. Recombinant viruses can be easily produced on a large scale and, in many cases, the non-recombinant form of these viruses has been successfully used for mass vaccination of humans. As the first virus that could be genetically manipulated, vaccinia virus was used in most studies per-

formed so far. However, recent developments in the field of genetic manipulation of viral DNA and RNA made possible the utilization of a number of other viruses such as influenza, adenovirus, sinbis, and pseudorabies virus, among others.

The vast majority of studies were performed using different recombinant viruses expressing the CS protein. The fact that its B cell epitope (54) and CD8⁺ T cell epitope (55,56) have been mapped allowed the generation of recombinant viruses containing either or both epitopes. These studies were performed on mice and unequivocally showed that a number of recombinant viruses can be used to elicit protective immunity against sporozoites and liver stages of malaria.

Several important concepts emerged from these studies. First, protective immunity elicited by distinct recombinant viruses varies in magnitude. Several different recombinant

viruses containing the entire *P. yoelii* protein, or its B cell or CD8⁺ T cell epitope were compared in terms of their ability to elicit protective immunity against pre-erythrocytic stages of malaria. Individually, immunization of mice with a recombinant adenovirus expressing the entire CS protein of *P. yoelii* produced the highest degree of immunity to a challenge with sporozoites (Table 2; 57). On the other hand, in mice immunized with a recombinant influenza virus containing only the CD8⁺ T cell epitope of the CS protein, no inhibition of liver stage development was observed (58). Immunization with either virus induced an easily detectable CD8⁺ T cell-mediated immune response, and protective immunity mediated by recombinant adenovirus was mainly mediated by this lymphocyte subpopulation (57). To our knowledge, up to now, experimental malaria has been the model with the largest number of recombinant viruses compared for their ability to

Table 2 - Summary of experiments using recombinant viruses to elicit protective immunity against pre-erythrocytic stages of *Plasmodium yoelii*.

| Recombinant | Antigen expressed | Number of doses | % of liver stage inhibition | Complete protection | Mechanism of protection | Ref. |
|----------------|---|-----------------|-----------------------------|---------------------|--|-------|
| Adenovirus | Entire CS protein | 1 | ~94% | 40% | Mainly CD8 ⁺ T cells | 57 |
| Influenza + | CD8 ⁺ T cell epitope | 2 | >95% | 60% | CD8 ⁺ T cells | 58,59 |
| Vaccinia | Entire CS protein | | | | | |
| Influenza + | CD8 ⁺ T cell epitope + B cell epitope | 4 | >95% | 78-81% | Antibodies and CD8 ⁺ T cells | 59 |
| Vaccinia | Entire CS protein | | | | | |
| Influenza | CD8 ⁺ /B cell epitopes | 3 | ~85% | 50% | Antibodies and CD8 ⁺ T cells | 59 |
| Vaccinia | Entire CS protein | 2 | ~55% | None | CD8 ⁺ T cells | 58,59 |
| Vaccinia | CD8 ⁺ T cell epitope | 2 | ~75% | None | CD8 ⁺ T cells | 58,59 |
| Influenza | B cell epitope | 3 | ~85% | None | Antibodies | 59 |
| Influenza | CD8 ⁺ T cell epitope | 2 | <15% | None | None | 58 |
| Pseudorabies | Entire CS protein | 2 | Unknown | None | None | 59 |
| Vaccinia + | Entire CS protein | 2 | <15% | None | None | 58 |
| Influenza | CD8 ⁺ T cell epitope | | | | | |

elicit protective immunity. The results obtained with this system emphasize the importance of detailed comparative studies to determine the best recombinant virus capable of inducing the highest degree of protective immunity to a certain pathogen.

Distinct recombinant viruses generate protective immunity by different effector mechanisms reacting to different stages of the parasite. Immunization with recombinant vaccinia and adenovirus expressing the CS protein elicits protective immunity that is mainly mediated by CD8⁺ T cells against liver stages (57,58). In these two models depletion of CD8⁺ T cells, before challenge with parasites, completely reversed protective immunity. Immunization with a recombinant vaccinia virus expressing only the CD8⁺ T cell epitope was as efficient as immunization with a virus containing the full-length CS gene (59). In contrast, protective immunity induced by immunization with a recombinant influenza virus could only be obtained using a recombinant virus expressing the CS B cell epitope that is present on the surface of sporozoites (59).

A third concept emerging from these studies was that sequential immunization with two distinct live viruses expressing the same CD8⁺ T cell epitope has the potential to increase significantly protective immunity mediated by CD8⁺ T cells against liver stages (58,59). Initial results demonstrated that priming of mice with a recombinant influenza virus followed by a booster injection with a recombinant vaccinia virus, both expressing the CD8⁺ T cell epitope, elicited a high degree of protective immunity against a sporozoite challenge (59). Similar protective immunity could not be achieved with two doses of either influenza or vaccinia virus. This unexpected observation was explained by the fact that mice immunized with these two different carrier viruses have a frequency of malaria peptide-specific CD8⁺ T cells 20-30 times higher than that of animals immunized twice with recombinant vaccinia or influ-

enza virus (60). The frequency of malaria-specific CD8⁺ T cells depends on the order of immunization with these two viruses. In mice primed with recombinant vaccinia virus followed by a booster injection with recombinant influenza virus, the frequency of CD8⁺ T cells was 10 times lower (60). No protective immunity could be detected in this case (59). The reasons for these differences remain unknown, but it is clear that the immune response mediated by CD8⁺ T cells can be greatly increased by certain combinations of recombinant viruses that can be used to increase protective immunity against liver stages of malaria.

Immunization of experimental animals with a recombinant virus expressing the zygote stage-specific antigen Pf 25 also generated immunity against malaria. The sera from monkeys and mice immunized with this recombinant virus had antibodies that could significantly inhibit the development of oocysts, providing strong transmission-blocking immunity (61).

Several improvements can be made since DNA viruses, such as vaccinia or adenovirus, may carry in their genome significantly higher amounts of genetic information. This allows the expression of several relevant epitopes of different plasmodial proteins instead of a single one. In fact, a recombinant vaccinia virus has been recently developed expressing 7 genes of distinct stages of *Plasmodium falciparum* (62).

A second area for potential advances in this field is the improvement of the immunogenicity of these recombinant viruses obtained by expressing immuno-stimulatory cytokines, or co-stimulatory receptors. In parallel to the increase in the immunogenicity of these viruses, their virulence can be reduced by the generation of attenuated viral strains, important for the use in children and pregnant women. A variety of attenuated strains of these viruses are being developed today mainly by genetic manipulation, and many of the viruses used in the studies described

above were either attenuated (vaccinia and influenza) or replication defective (adenovirus) strains.

A human trial has been scheduled using a recombinant vaccinia virus expressing 7 genes of distinct stages of *Plasmodium falciparum* (62). However, this trial should be viewed with caution because there is no evidence that immunization with this virus elicits protective immunity in non-human primates. A second major concern in the use of recombinant viruses for human trials is the fact that no evidence is available that a recombinant virus generates protective immunity against blood stages of malaria. As mentioned above, an ideal vaccine carrier should elicit protective immunity to different stages of malaria parasites. This problem could be solved eventually by a simultaneous immunization with a mixture of blood stage recombinant antigens in adjuvant.

A recombinant *Salmonella* vector expressing the CS protein was also proposed as a possible candidate vaccine against malaria. Initial studies in rodent malaria induced by *P. berghei* sporozoites suggested that this vehicle could elicit protective immunity against pre-erythrocytic stages of malaria (63). A subsequent study using a more virulent strain of rodent malaria (*P. yoelii*) was not successful. Based on the initial results obtained with *P. berghei*, a recombinant *Salmonella* vector expressing the *P. falciparum* CS protein was developed but its first human trial was disappointing, with most individuals failing to respond (64).

DNA vaccination

Plasmid DNA is the most recently developed delivery system designed to elicit an immune response to microorganisms. This type of immunization has the potential to revolutionize the field of vaccination since, for the first time, the antigen formulation does not contain any type of protein. It consists of an expression vector (plasmid DNA)

in which the gene of a microbial antigen is placed under the control of a strong promoter for expression in eukaryotic cells. Once injected into animals, the plasmid transfects cells and drives the expression of the foreign gene *in vivo*. In this system, the antigenic protein is produced *in situ* by the transfected cell for long periods of time. This approach is relatively simple when compared to other expression systems, as confirmed with a number of microbial genes including *Plasmodium* genes (reviewed in Ref. 65).

The initial study performed in malaria used an expression vector containing the entire CS gene of *P. yoelii*. Immunization with this plasmid DNA resulted in a strong immune response mediated by antibodies and CD8⁺ T cells, and provided a high degree of protective immunity in BALB/c mice against a challenge with sporozoites dependent on CD8⁺ T cells (66). This protective immunity was genetically restricted and only BALB/c mice were effectively protected. Immunization with a second plasmid carrying the gene of the Py17Hep antigen was also shown to generate a significant degree of protective immunity against a challenge with *P. yoelii* sporozoites. Similarly, this protective immunity was mediated by CD8⁺ T cells and was genetically restricted (67). In order to circumvent this strong genetic restriction, several mouse strains were immunized with both plasmids. This type of immunization led to a high level of protective immunity in 4 out of 5 mouse strains (68). This experiment highlights the importance of immunization with multiple malaria antigens to elicit a high degree of protective immunity in individuals with distinct genetic backgrounds. Based on these results, human vaccination trials are scheduled to be initiated as early as in 1997.

The immunogenicity of this type of vector can be further enhanced by co-immunization with plasmids containing genes for cytokines or co-stimulatory molecules of the immune system (69).

Conclusions

The study of malaria is a field that has evolved very fast over the last ten years. Based on the results obtained in studies directed towards the development of a malaria vaccine, we now know more about the parasite itself, malaria pathogenesis and immunity. These studies have also generated basic knowledge in a variety of fields such as parasitology, molecular genetics, biochemistry, and immunology. The fact that a product for mass vaccination has not been developed during this period is a consequence of numerous roadblocks. Some of them, such as antigenic polymorphism and antigenic variation, were predictable, based on studies with other microorganisms. Others, such as the stage specificity of antigen expression and immunological mechanisms of protection, were found during the course of these studies. Yet, in spite of them, a significant advance was made in this field. Today, several epitopes of limited polymorphism have been described and protective immunity can

be generated by active immunization. Immunization was often performed with a single antigen which generated stage-specific protection. Therefore, if several antigens can be assembled in the same vaccine, they can provide higher levels of protection by attacking the parasite at different points of the life cycle. This can be accomplished in the near future provided that it is demonstrated that immunity to one epitope does not interfere with immunity to another. Unfortunately, no one can predict if and when a product will be available for mass vaccination. During this time, it is a reasonable understanding that considerable investments should be made in programs for education, vector control, insecticide-impregnated bed nets and chemotherapy.

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