Recent Advances in the Development of a Sporozoite Vaccine for Malaria*

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Much of the recent work on functional plasmodial antigens is based on the results of earlier investigations using intact, frequently attenuated parasites as immunogen. These studies had shown that immunisation with the extracellular "free" developmental stages of Plasmodia, namely, gametes,[1] merozoites[2,3] and sporozoites[4] resulted in the most effective protection.

The potential danger involved in vaccination with such intact parasite preparations, particularly the fear of undesirable side effects has, among other reasons, led to the search for alternative approaches, i.e., identification of the protective plasmodial antigens.

The use of hybridoma methodology[5] has been one of the main factors responsible for recent significant progress towards achieving this goal. The protective activity of a number of these antiplasmodial antibodies has been verified through the use of *in vitro* assays and the suppressive effects of passive transfer of these antibodies on malaria infection. In several instances, the identification of the corresponding plasmodial antigens has been followed by their purification and the assessment of their immunogenicity.

Researchers in several laboratories are presently combining the use of monoclonal antibodies with the cloning of the genes coding for the protective antigens. This should lead to mass production of these antigens, a requirement for the development and application of a malaria vaccine.

The development of synthetic peptide vaccines, an approach which, under experimental conditions, is being evaluated for its potential to prevent a number of viral infections,[6] might prove a viable alternative, and possibly a less costly future solution. For its applicability to malaria, however, this approach will require data on the amino acid composition of the protective epitopes of the malaria antigens. Initial results obtained by immunisation of animals with a synthetic dodecapeptide which contains the immuno-dominant epitope of the surface antigen of sporozoites of *P. knowlesi*, a simian malaria parasite, are described later in this manuscript.

In this report our discussion will focus only on advances in the development of a vaccine based on the use of sporozoites. If successful, such a vaccine could interrupt all parasite development and disease. Studies on vaccination against blood stages and sexual forms have been reviewed elsewhere.[12]

The blood stages of malarial parasites use many clever devices, including antigenic variation[7,8] to evade the host's immune response. Our studies deal with sporozoites, which in contrast to blood stages, may be a less resourceful stage of development of the malaria parasite.

Sporozoites mature in the salivary gland of the *Anopheles* mosquito, and are injected into the host during the bite. During their very short lifespan, sporozoites have to reach and penetrate hepatocytes where, in a few hours, they transfrom by schizogonic-like divisions into merozoites.

Inasmuch as sporozoites are "free" in the peripheral blood only for a few minutes, and because relatively few are injected by mosquito bite, many investigators previously believed that sporozoites were not immunogenic, and could not be destroyed by immunological attack. Both ideas are incorrect! Inoculation of small numbers of sporozoites attenuated by X-irradiation into rodents, monkeys and humans leads to the production of antibodies against the parasite surface and to protective immunity (reviewed 4, 9). Of the adults living in The Gambia, West Africa, a highly endemic area, 90 per cent have

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detectable levels of antisporozoite antibodies.[10]

Sporozoite-induced protective immunity is stage-specific and in most cases, species-specific,[11] but not strain-specific. Incubation of viable sporozoites with the sera of vaccinated and protected animals results in the appearance of a tail-like precipitate, designated the circumsporozoite or CSP reaction.[12,13] The CSP reactions are also stage-and species-specific. These findings suggest that protective immunity is at least in part antibody-mediated, and that the antigens involved in protective immunity and CSP reactions may be identical.

This was first demonstrated to be the case with *P. berghei*, a rodent malaria parasite. A monoclonal antibody (3D11) raised against the surface of *P. berghei* sporozoites displayed all the properties of polyclonal antisera obtained from animals vaccinated with whole X-irradiated sporozoites.[15] This antibody (3D11) not only identified a Mr 44,000 stage- and species-specific protein covering the surface membrane of sporozoites, but also neutralised their infectivity and mediated the CSP reaction.

Identical results were obtained when malaria parasites of monkey *(P. knowlesi)* and human *(P. vivax* and *P. falciparum)* were studied.[16,17] When the circumsporozoite (CS) proteins were compared, we found that they had similar physico-chemical and antigenic properties. We therefore concluded that they were structurally related.[18] One important observation was that sporozoites could be neutralised, not only by the native monoclonal antibodies to CS proteins, but also by monovalent Fab fragments.[16,19] This implied that the simple binding of antibody to the parasites' surface somehow interfered with their infectivity. In fact, Dr. M. Hollingdale recently found that in the presence of the specific Fab fragments, sporozoites of several malaria species did not attach to target cells *in vitro*. He concluded that CS proteins play a role in the penetration process of the parasite into the host cells.[20,21]

As the sporozoite neutralising activity of different monoclonal antibodies of the same isotype was not identical, we compared their specificities by a competitive binding assay. The assay's principle is that if two monoclonal antibodies recognise the same region of a molecule, they will inhibit each other's binding activity. Much to our surprise, we found that every monoclonal antibody against the CS protein of *P. knowlesi* interfered with the binding of all others to the antigen. Identical results were obtained when studying the properties of panels of monoclonal antibodies to *P. vivax, P. falciparum* and *P. berghei*. This observation strongly suggested that all CS proteins have single immunodominant regions.[22] This was further confirmed when studying the properties of polyclonal antisera of animals and humans vaccinated with X-irradiated sporozoites. Pre-incubation of crude extracts of *P. knowlesi* and *P. falciparum* sporozoites with single monoclonal antibodies to the corresponding CS protein strongly inhibited the subsequent binding of the polyclonal antibodies. Another unexpected observation was that in all systems, every monoclonal antibody tested revealed an unique repetitive epitope. This was demonstrated by the ability of monomeric CS proteins to simultaneously bind two or more monoclonal antibodies in a two-site immunoradiometric assay (IRMA).[22]

In short, these findings indicated that the CS proteins are the most immunogenic constituent of sporozoites; that only one area of the CS proteins was recognised by the polyclonal and monoclonal antibodies; and finally, that this immunodominant region contained repetitive epitopes.

Taken together, these findings indicate that CS proteins might be used as vaccines against malaria. However, the major practical impediment for the vaccine's development is that mature sporozoites can be obtained only from the salivary glands of mosquitoes. In order to overcome this difficulty and obtain large amounts of CS proteins, two possible approaches exist: genetic engineering, and/or synthesis of the portion of the molecule which contains the relevant epitopes. Both approaches may be feasible.

Prior to applying recombinant DNA technology, we studied the biosynthesis of CS proteins in sporozoites of several malarial species. These experiments have shown that, in mature salivary gland sporozoites, between 5 to 15 per cent of the labelled amino acids incorporated into protein were found in the CS proteins and their intracellular precursors. Therefore, the mRNA corresponding to the CS protein should be relatively abundant.[16,19] Using mRNA extracted from the thoraces of *P. knowlesi*-infected mosquitoes, our graduate student, Joan Ellis, prepared cDNA and introduced fragments into plasmids, and then these into bacteria. More recently, J. Ellis found that some of the bacterial colonies were expressing a portion of the CS protein which was recognised by monoclonal antibodies. Three positive cDNA clones were identified, one of which (PEG81) contained a piece of *Plasmodium* DNA of only 350 base pairs. These clones were shown to be sporozoite-specific by Southern and Northern blot analysis.[23]

The sequence of nucleotides of PEG81 contained several repeats of 36 base pairs. One of the possible reading frames coded for a hydrophilic polypeptide rich in alanine and devoid of lysine and arginine. We reasoned that if this was the correct reading frame, the epitope should be resistant to trypsin, but sensitive to elastase, an enzyme which cleaves peptide bands at alanine residues. This was confirmed experimentally by subjecting the

in vivo P. knowlesi-synthesised CS protein, as well as the *E. coli*-derived fusion protein, to enzymatic digestion, followed by an immunoassay to detect the epitope.

In order to prove that this reading frame was correct, the corresponding dodecapeptide was synthesised and tested for reactivity with the monoclonal antibodies. We found that, at concentrations of $10-12^{M}$, the synthetic dodecapeptide inhibited the interaction of all the monoclonal antibodies with the authentic *P. knowlesi* CS protein.[24]

Therefore, the basic question raised was whether this peptide was immunogenic, particularly if it induced the formation of antibodies which reacted with the parasite, which hopefully would lead to sporozoite neutralisation and protection. A dimer of the dodecapeptide was conjugated (through the use of carbodiimide) to bovine gamma globulin (BGG) and keyhole limpet haemocyanin (KLH). Mice, rabbits and squirrel monkeys were immunised by the subcutaneous injection of these antigens emulsified in Freund's Complete Adjuvant (FCA).

Most of the immunised animals produced relatively high titres of anti-peptide antibodies, the titres being lowest and most variable in the squirrel monkeys. The sera of these animals also reacted with intact sporozoites of *P. knowlesi*, both by the IFA and CSP reactions. The pooled sera of the peptide immunised rabbits also neutralised the sporozoites, i.e., abolished their infectivity (Gysin *et al*, unpublished results). In a preliminary experiment three squirrel monkeys immunised with the peptide conjugated to BGG, and two others immunised with peptide KLH, did not resist sporozoite challenge. Alternative methods of immunisation are presently being studied in order to identify preparations which would induce a maximal protective host response.

The genetic information shows that the *P. knowlesi* CS protein contains 12 tandemly repeated subunits of 12 amino acids. This explains the fact that monomeric CS proteins can simultaneously bind several molecules of monoclonal antibodies, and may also account for the extraordinary immunogenicity of this epitope. In fact, the CS protein covers the entire membrane of sporozoites, and nearly one half of each CS molecule consists of epitope repeats. Although there is no information regarding the secondary and tertiary structural features of CS proteins, nor any information on the manner in which they assemble on the sporozoites' membrane, it seems likely that the repeats form a regular and relatively compact array on the parasite surface. This quasi-polymeric structure, which may be important for some parasite function in the early stages of its interaction with the host, should at the same time facilitate recognition by specific receptors of cells from the immune system, and thereby enhance immunogenicity.

These findings are of obvious relevance to the practical development of a malaria vaccine. It is conceivable that, in the near future, genetically engineered or chemically synthesised portions of the CS protein of human malaria parasites will be available. Would such vaccines be effective? It has been frequently argued that, since immunity to sporozoites cannot protect against blood stages, vaccination with sporozoites has to lead to sterile immunity in order to be useful. In our view, this may be incorrect. There is suggestive evidence that the severity of the disease is related to the number of sporozoites injected during the mosquito bite. If this is correct, a vaccine which contributes to substantial reduction of the inoculum would still be useful. Moreover, for adults living in endemic areas with circulating antisporozoite antibodies, such a vaccine would enhance their naturally-acquired immunity.

Also encouraging is the observation that sterile immunity was achieved in experimental models and in humans vaccinated with X-irradiated sporozoites. The effectiveness of protection is dependent upon the dose of sporozoites used for challenge. The higher the dose, the less effective the protection. What is the size of the inoculum under natural conditions? It seems to vary according to the geographic area and the vector. But in most cases, the number of sporozoites injected by each mosquito, as well as the mosquito infection rate, is low. It is therefore conceivable that a vaccine containing only sporozoite antigens would completely protect a portion of the exposed population, and that, in those cases where a few sporozoites escape, the course of the infection should be less rapid and more benign. Ideally a malaria vaccine would also contain antigen from other stages of development.

In conclusion, we have described some unique structural and immunological properties of a protein from the membrane of sporozoites. We have further shown evidence that this protein is a logical target for the development of vaccines. Further studies are required to determine its function during the life cycle of the malaria parasites: the genetic mechanisms involved in its stage-specific expression, and the role of the intramolecular repetitive subunits.

Summary

Protective immunity against malaria has been achieved in hosts ranging from birds to man by repeated inoculation of irradiated sporozoites. The main antigens involved in protective immunity to sporozoites are the CS proteins, which are part of a family of proteins covering the whole surface membrane of the parasite and which have similar physico-chemical and antigenic properties. Monovalent fragments of monoclonal an-

tibodies to CS proteins neutralise sporozoite infectivity *in vitro* and *in vivo*. All monoclonal antibodies recognise a single immunodominant region within the various CS proteins, and this region contains repetitive epitopes. The recurrent immunodominant epitope of the CS protein of *P. knowlesi* has been identified, and shown to consist of 12 tandemly repeated subunits of 12 amino acids. The dimer of the dodecapeptide was coupled to protein carriers, emulsified in Freund's complete adjuvant, and injected into rodents and monkeys. All animals made antipeptide antibodies, and most of the antisera reacted with *P. knowlesi* CS protein. The neutralising ability of such antisera as well as the effectiveness of a peptide vaccine are now under evaluation.

REFERENCES

1. Carter R, Gwadz RW. Infectiousness and gamete immunization. In: Kreier JP, ed, Malaria vol. 3. New York: Academic Press, 1980:263-97.
2. Cohen S. Progress in malarial vaccine development. Brit Med Bull 1982; 38:161-5.
3. Epstein N, Miller LH, Kaushel DC, et al. Monoclonal antibodies against a specific surface determinant on malarial *(Plasmodium knowlesi)* merozoites block erythrocytic invasion. J Immunol 1981; 127:212-7.
4. Cochrane AH, Nussenzweig RS, Nardin EH. Immunization against sporozoites. In: Kreier JP, ed, Malaria, vol. 3. New York: Academic Press, 1980:163-202.
5. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256:495-7.
6. Arnon R. Synthetic vaccines. Proc 5th Int Cong Immunol, Kyoto, Japan. Immunology 1983; 83:1327-41.
7. Brown KN, Brown IN. Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. Nature 1965; 208:1286-8.
8. Hommel M, David PH, Oligino LD. Surface alterations of erythrocytes in *Plasmodium falciparum* malaria. Antigenic variation, antigenic diversity, and the role of the spleen. J Exp Med 1983; 157: 1137-48.
9. Nussenzweig RS. Progress in malaria vaccine development: characterization of protective antigens. Scand J Infect Dis 1982; (Suppl.). 36:40-5.
10. Nardin EH, Nussenzweig RS, McGregor LA, Bryan JH. Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. Science 1979; 206:597-9.
11. Nussenzweig RS, Vanderberg JP, Most H. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*, III. Specificity of the protection. Nature 1969; 222:488-9.
12. Vanderberg JP, Nussenzweig RS, Sanabria Y, Nawrot R, Most H. Stage specificity of antisporozoite antibodies in rodent malaria and its relationship to protective immunity. Proc Helm Soc (Wash) 1972; 39: 514-25.
13. Cochrane AH, Aikawa M, Jeng M, Nussenzweig RS. Antibody induced ultrastructural changes of malarial sporozoites. J Immunol 1975; 116:859-67.
14. Vanderberg J, Nussenzweig R, Most H. Protective immunity produced by the injection of X-irradiated sporozoites of *P. berghei*, V. *In vitro* effects of immune serum on sporozoites. Mil Med 1969; (Suppl.) 134:1183-90.
15. Yoshida N, Potocnjak P, Aikawa M, Nussenzweig V, Nussenzweig RS. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasites. Science 1980; 207:71-3.
16. Cochrane AH, Santoro F, Nussenzweig V, Gwadz RW, Nussenzweig RS. Monoclonal antibodies identify the protective antigens of sporozoites of *Plasmodium knowlesi*. Proc Natl Acad Sci USA 1982; 79:5651-5.
17. Nardin EH, Nussenzweig V, Nussenzweig RS, et al. Circumsporozoite (CS) proteins of human malaria parasites *P. falciparum* and *P. vivax*. J Exp Med 1982; 156:20-30.
18. Santoro F, Cochrane AH, Nussenzweig V, et al. Structural similarities between the protective antigens of sporozoites from different species of malaria. J Biol Chem 1983; 258:3341-5.
19. Yoshida N, Potocnjak P, Nussenzweig V, Nussenzweig RS. Biosynthesis of Pb44, the protective antigen of sporozoites of *Plasmodium berghei*. J Exp Med 1981; 154:1225-36.
20. Hollingdale MR, Zavala F, Nussenzweig RS, Nussenzweig V. Antibodies to the protective antigen of *Plasmodium berghei* sporozoites prevent entry into cultured cells. J Immunol 1982; 128:1929-30.
21. Hollingdale MR, Nardin EH, Tharavanij S, Schwartz AL, Nussenzweig RS. Inhibition of entry of *Plasmodium falciparum* and *Plasmodium vivax* sporozoites into cultured cells: an *in vitro* assay of protective antibodies. J Immunol 1984; 132:909-13.
22. Zavala F, Cochrane AH, Nardin EH, Nussenzweig RS, Nussenzweig V. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. J Exp Med 1983; 157:1947-57.
23. Ellis J, Ozaki LS, Gwadz RW, et al. Cloning and expression of the *Plasmodium knowlesi* sporozoite surface antigen in *E. coli*. Nature 1983; 3021:536-8.