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PROTECTIVE ANTIGENS FROM ERYTHROCYTIC STAGES: RECENT PROGRESS AND PERSPECTIVES FOR THE DEVELOPMENT OF A VACCINE

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The development of acquired immunity to malaria in man depends on the frequency and duration of exposure to the parasite. However, sterile immunity is never achieved in man and, even after years of exposure to malaria parasites, individuals living in holoendemic areas are still susceptible to infection by malaria parasites and low levels of parasitemia can be detected periodically in their blood. There is therefore a balance between the host immune response capable of recognising and controlling the infection (only low levels of parasitemia) and the capacity of the parasite to escape the immune response of the infected host.

These clinical and epidemiological observations clearly underline the difficulties encountered in the development of malaria vaccines which have to do as well as and hopefully "better than nature". Due to the stage specificity of the immune response towards plasmodia, several types of malaria vaccine may be envisaged. In this short review we will focus on approaches towards the development of vaccines based on asexual blood stages. Pioneer experiments by several groups in the late '70s demonstrated that immunization of monkeys with *P. falciparum* schizonts and merozoites may lead to a degree of protection similar to or possibly better than induced by natural infection (Siddiqui, 1977; Mitchell et al., 1978).

The development of the *in vitro* culture of asexual blood stages of *P. falciparum* (Trager & Jensen, 1976), advances in malaria antigen characterization using biochemical methods and monoclonal antibodies and the use of gene cloning technology have led to rapid progress in our understanding of the parasite biology. Perspectives for the development of malaria vaccines based on asexual blood stages now appear more complex but also possible using a systematic step-by-step approach.

Strategy for the development of a malaria vaccine based on asexual blood stages – The purification of schizonts and merozoites from plasmodia species infecting man (in particular *Plasmodium falciparum*) is not feasible on the large scale necessary for the production of vaccines. In addition, separation of the plasmodia from host components would be very difficult since parasites develop within the host's red blood cells. Another possible limitation linked to the use of whole parasites as immunogens is the number of parasite antigens which are irrelevant to the induction of protective responses. In this respect, immunization with killed or denatured parasites is likely to induce adverse immunopathological effects.

An alternative strategy has therefore been developed by several groups and can be summarized as follows:

1. Identification of parasite antigen(s) which are the target of protective immune responses.

2. Purification and biochemical characterization of these antigens.

3. Limited immunization trials in monkeys with defined and purified components.

4. Cloning and determination of the DNA sequence of genes corresponding to the protective antigens.

5. Immunization trials with fusion polypeptides obtained by DNA recombinant technology and with synthetic polypeptides resulting from information concerning the DNA sequence of the relevant genes.

6. Evaluation of the results in terms of extent of protection obtained and in relation to crossprotection for different *P. falciparum* isolates and other plasmodia species infecting man.

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7. Evaluation of potential vaccines in terms of production of antigens, safety tests, adjuvants, carriers (proteins, virus), etc.

We will concentrate here on immunization trials in monkeys with defined and purified components and on prospects for the use of fusion polypeptides or synthetic peptides as immunogens for the development of malaria vaccines based on asexual blood stages.

Immunization trials in Saimiri monkeys using defined *P. falciparum* antigens – The selection of the best candidates among the various malaria antigens is based on the assumption that these antigens should be expressed at the surface of merozoites and/or schizonts or involved in merozoites red blood cell interactions. A list of candidate antigens has been recently established at a WHO meeting (WHO, 1984). The availability of monoclonal and polyclonal antibodies directed against single components of *P. falciparum* allowed an evaluation of the extent and biological significance of the antigenic diversity (in particular for the 190-200Kd schizont- and merozoite-specific polypeptide (McBride, Walliker & Morgan, 1982)) and the purification of single *P. falciparum* components using affinity chromatography.

Some of these *P. falciparum* polypeptides purified from extracts obtained from *in vitro* cultures of asexual blood stages have been used for immunization experiments in Saimiri monkeys (Perrin et al., 1984, 1985; Hall et al., 1984; Dubois et al., 1984). Some of the results obtained using three polypeptides are reported here, the peptides being: (a) 200Kd schizont-specific; (b) 140Kd schizont- and merozoite-specific; (c) 41Kd associated with rhoptries. The latter peptide has been used either after solubilization in nonionic detergent ("native antigen") or following treatment with SDS ("denatured antigen") (Perrin et al., 1985). Groups of four *Saimiri sciureus* monkeys of the Guyana type were immunized at 2-3 week intervals with low amounts of purified polypeptides (less than 100 μg per injection) emulsified in Freund's complete adjuvant for the first immunization and Freund's incomplete adjuvant for the second and third immunizations. Control animals were injected with adjuvants according to the same schedule. The monkeys were challenged by an intravenous injection of 5×10^7 parasitized erythrocytes (Palo Alto FUP strain of *P. falciparum*). The course of parasitemia in individual monkeys was scored on Giemsa-stained blood smears. In all the immunized monkeys a delay in the appearance of patent parasitemia was observed but all the monkeys had at least a low parasitemia for a week. All the immunized monkeys but one recovered spontaneously without antimalarial therapy (Table I). In the experi-

TABLE I

Peak parasitemia observed in various groups of immunized monkeys*

Immunogens		Peak parasitemia (%)		Day
200 Kd	1	13	(intercurrent infection)	15
	2	6		13
	3	2		16
	4	2		16
140 Kd	1	4		8
	2	2		15
	3	2		16
	4	1		11
41 Kd "native"	1	1		12
	2	0.2.		34
	3	0.08		35
	4	0.05		28
41 Kd "denatured"	1	13		14
	2	11		16
	3	3		16
	4	2		14

*6 over 8 intact control monkeys had parasitemia higher than 20% by day 14, the two last monkeys had peak parasitemia of 11 and 12% before recovery.

TABLE II

Prechallenge antibody titres measured by indirect immunofluorescence

Immunogens	Antimalarial antibody titres Mean	Range	Specificity
200 Kd	1/256	1/64 – 1/1024	Surface schizont Schizont diffusely Merozoite surface
140 Kd	1/900	1/512 1/1024	Schizont diffusely Merozoite diffusely and spots
*41 Kd native	1/320	1/160 – 1/640	Schizont diffusely+ Merozoites:
*41 Kd SDS	1/320	1/160 1/640	2 spots/merozoites diffusely

*By using a solid phase ELISA assay with plastic wells coated with either native or denatured 41 Kd, it was found that both groups of monkeys have reciprocal Ab titres in the order of 1/5000 using wells coated with native 41 Kd. On wells coated with 41 Kd SDS the Ab titres increased upto 1/200'000 for monkeys immunized with the 41 Kd SDS (Perrin et al., 1985).

ments using the 41Kd polypeptides, it appeared that the nature of the immunogen ("native" or "denatured" by SDS) had a marked influence on the level of protection in the immunized monkeys (Perrin et al., 1985). In contrast to the results observed in immunized monkeys, six out of eight control monkeys showed a typical acute course of parasitemia and required antimalarial therapy. The humoral response of the control and immunized monkeys was tested on sera by indirect immunofluorescence and immunoprecipitation of ^{35}S-methionine-labelled *P. falciparum*. The antibody titers and the specificity of the antibodies on individual sera collected before challenge are reported in Table II. Relatively good antibody titers were raised by immunization and these antibodies reacted with the antigens used for immunization (data not shown). The partial protection observed following immunization was not strain-specific since different isolates of *P. falciparum* were used to prepare the antigens (SGE2) and for the challenge infection (Palo Alto). However, this observation is not fully conclusive since only two isolates were involved and since two of the polypeptides used (190-200Kd and 41Kd) have been shown to contain some epitopes involved in antigenic diversity (WHO, 1984; Schofield et al., 1986). These and other data reported by other investigators (Hall et al., 1984; Dubois et al., 1984) demonstrated that immunization with limited amounts of selected purified and possibly denatured *P. falciparum* polypeptides induces in monkeys a degree of resistance at least equal to that obtained in monkeys immunized with whole merozoites and schizonts (Siddiqui, 1977; Mitchell et al., 1978).

Thé mechanism of protection induced by immunization with whole merozoites and/or schizonts (Siddiqui, 1977; Mitchell et al., 1978) and with purified polypeptides is difficult to elucidate because in immunized monkeys low to substantial levels of parasitemia appeared after 24h, increased over the following days and persisted for at least a week, suggesting that active multiplication of the parasite occurred. At least three alternatives may explain this observation: first, antimalarial antibodies may play a key role in the control of parasite multiplication but complete elimination of the parasites may depend on naturally induced cellular immune responses, macrophage activation and/or cytokine production. These mechanisms may be necessary, particularly in the context of parasites which develop mainly within the cells of the host with only short exposure of merozoites to antibodies. Second, epitopes involved in antigenic diversity may be immunodominant and divert the development of truly protective antibodies which have to be directed against constant epitopes to achieve a protection across various isolates. Finally, the parasite itself modifies the host immune responses and this may account for the inability of the host to mount a proper immune response. In relation to an improvement in the induction of protective immune responses, it still remains to be explored whether or not the use of a cocktail of several of the putative protective antigens will improve the quality of the immunity induced by immunization.

Immunization trials using fusion and synthetic polypeptides – A number of genes coding for *P. falciparum* polypeptides have been identified in several laboratories (Coppel et al., 1983; Kochan, Perkins & Ravetch, 1986). Several of these genes code for polypeptides which are candi-

dates for vaccine development and others code for polypeptides whose potential has still to be evaluated. Determination of the DNA sequence of the cloned genes and the corresponding amino acids has revealed the presence of tandem repeats of three to 50 amino acids for every antigen for which extensive sequences were available. Some of those repeats probably play a key role in merozoite attachment to the red blood cell membrane and are therefore obvious candidates for immunization trials (Kochan, Perkins & Ravetch, 1986). Other repeats such as those found in the S antigen family (Coppel et al., 1983; Kemp et al., 1983) and within the 190-200Kd polypeptide (Cheung et al., 1985; Mackay et al., 1985) are likely to be involved in antigenic diversity. The crucial step in the use of antigens produced by gene cloning technology includes the insertion of the genes into good expression vectors under the control of adequate promoters, the purification of fusion polypeptides and the evaluation of the immunogenic and biological properties of the fusion polypeptides. At this point, it is evident that there are a number of publications on the cloning of malaria genes but very little on the testing of the products derived from such cloning.

As mentioned previously, monkeys immunized against the 190-200Kd polypeptides are resistant to a blood-induced challenge infection. Several groups have reported the partial or complete sequence of the gene coding for this antigen (Cheung et al., 1985; Mackay et al., 1985) and it has been shown that the 83Kd polypeptide which is one of the main merozoite surface components and is derived from the 190-200Kd polypeptide, corresponds to a N terminal region of the coding sequence (Cheung et al., 1985; Mackay et al., 1985). Sera from rabbits immunized with a fusion polypeptide expressing an N terminal peptide of 10Kd have been shown to react with the merozoite surface of several *P. falciparum* isolates (Cheung et al., 1985). Only two of the first 55 residues of the N terminal region have been shown to differ for three *P. falciparum* isolates (Mackay et al., 1985). This region of unique sequence is followed by a region containing tripeptide repeats which diverge significantly among various isolates (Mackay et al., 1985). A 43 amino acid peptide corresponding to the N terminal region (Table III) was selected, synthesized, coupled by glutaraldehyde to tetanus toxoid and used to immunize four Saimiri monkeys (3 x 1.5 mg/monkey of the conjugate containing 15% wt/wt of the peptide, in Freund's complete adjuvant the first time and in Freund's incomplete adjuvant the second and third times). Four control Saimiri were immunized according to the same schedule with glutaraldehyde-treated tetanus toxoid. All the control monkeys and one immunized monkey required antimalarial therapy following a rapidly progressing parasitemia and the peak parasitemia for the three monkeys who recovered spontaneously was 19%, 9.2% and 3.4%. The prechallenge sera of the immunized monkeys had only low antimalarial antibody titers as measured by indirect immunofluorescence (between 1/100 and 1/200) and relatively good antibody titers against the fusion polypeptide containing the N terminal of the 190-200Kd polypeptide, as measured by solid phase ELISA (1/5,000-1/10,000). The antibody detected by indirect fluorescence clearly delineated the surface of merozoite and reacted diffusely on immature schizonts (Cheung et al., PNAS, 1986).

TABLE III

Amino-acid sequence of the peptide expressed by the cDNA clone coding for part of the 200 Kd

Ser[1]	–	Tyr	–	Gln	–	Glu	–	Leu	–	Val	–	Lys[20]	–	Lys
Leu	–	Glu	–	Ala		Leu	–	Glu	–	Asp	–	Ala[15]	–	Val
–	Leu		Thr	–	Gly	–	Tyr	–	Ser	–	Leu	–	Phe	
Gln		Lys	–	Glu		Lys	–	Met		Val	–	Leu[30]		Asn
–	Glu	–	Gly		Thr	–	Ser		Gly		Thr	–	Ala	
Val		Thr		Thr	–	Ser	–	Thr[43]		Thr		Pro	–	Gly
–	Ser		Gly		Ser		Gly[46]	–	Gly		Ser		Val	
–	Thr		Ser		Gly	–	Gly		Ser	–	Gly	–	Gly	
Ser	–	Val	–	Ala		Ser	–	Val[60]	–	Ala		Ser	–	Gly
–	Gly		Gly		Ser	–	Gly	–	Gly		Ser	–	Gly[75]	–
Asn		Ser[79]		Arg	–	Arg	–	Thr		Asn	–	Pro	–	Ser
	Asp		Asn	–	Ser	–	Ser		Asp	–	Ser	–	Asp	Als
–	Lys	–	Ser	–										

The peptide used for immunization corresponds to the first 43 amino acids.

These results indicate that synthetic peptides, corresponding to the N terminal part of a major antigen of merozoite surface, can induce an immune response in monkeys which attenuated the severity of a blood induced infection in the immunized animals. The 43 amino acid peptide was selected on the basis of preliminary experiments conducted in rabbits with various peptides corresponding to different parts of the N terminal of the 190-200Kd polypeptide. In rabbits immunized according to the same schedule, 5 to 8 times higher antibody titres were measured.

This raises the question of proper coupling of the synthetic polypeptide, selection of the carrier, etc. There is still a wide margin for improvement in these respects.

The availability of malaria fusion peptides and synthetic polypeptides presents several advantages in terms of standardization and production, but the selection for vaccine development of the best products poses several problems: (a) Is the tertiary structure of the candidate antigens crucial? (Perrin et al., 1985). (b) Tandem amino acid repeats are probably important for the binding of the glycophorin binding protein to erythrocytes and, therefore, for penetration of merozoites. Other tandem amino acid repeats may have adverse effects by diverting the host to raise antibodies against adjacent epitopes. Consequently, it may be of interest to excise, from the cloned malaria genes, the portion of the gene coding for the repeats. (c) It may be of interest to construct a malaria gene complex coding for a fusion polypeptide expressing epitopes of several malaria antigens. This implies a molecular dissection of the various genes, the study of immune response in laboratory animals to various parts of each parasite antigen involved in protection, for example, using synthetic polypeptides. It is effectively possible that, for the development of an efficient vaccine, large fragments of several polypeptides have to be combined. In this respect, we have shown a greater range of antibody titres in populations living in endemic areas, when using a fusion polypeptide corresponding to the N terminal part of the 190-200Kd polypeptide, than when using whole *P. falciparum* extract (role of genetic background?) (Gabra et al., 1986).

In conclusion, a number of tools have now been developed, but we are still facing the same issues: Can we do better than nature in terms of immunization against malaria? Which antigen(s) should be selected? The possibility of answering these questions depends on a close collaboration between parasitologists, immunologists and molecular biologists.

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