Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87*, Suppl. III, 423-428, 1992

EFFICIENCE OF HUMAN *PLASMODIUM FALCIPARUM* MALARIA VACCINE CANDIDATES IN *AOTUS LEMURINUS* MONKEYS

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The protective efficacy of several recombinant and a synthetic Plasmodium falciparum *protein was assessed in* Aotus *monkeys. The rp41 aldolase, the 190L fragment of the MSA-1 protein and fusion 190L-CS. T3 protein containing the CS.T3 helper "universal" epitope were emulsified in Freund's adjuvants and injected 3 times in groups of 4-5 monkeys each one. The synthetic polymer Spf (66)30 also emulsified in Freund's adjuvants was injected 6 times. Control groups for both experiments were immunized with saline solution in the same adjuvant following the same schedules. Serology for malaria specific antibodies showed seroconversion in monkeys immunized with the recombinant proteins but not in those immunized with the polymer nor in the controls. Challenge was performed with the 10^5 parasites from the* P. falciparum *FVO isolate. Neither rp41 nor SPf(66)30 induced protection, whereas 190L induced significant delay of parasitemia. The fusion of the CS.T3 epitope to 190L significantly increased its protective capacity.*

Key words: *Plasmodium falciparum* protein – *Aotus* – recombinant peptides – vaccine candidates

Under natural conditions the development of clinical immunity to malaria in a susceptible host is a lengthy process and depends on the repeated exposure of the immune system to the parasite. The continuous contact with the *Plasmodium* in the endemic areas induce the development of effective immunity through the recognition of a broad range of parasite antigens. In addition, the time required for this proccess seems to be determined by the prevalence and degree of antigenic polymorphism of the exposed surface antigens in the plasmodial species and strains in the local environment (McGregor, 1986).

Analysis of *P. falciparum* genes and antigens have revealed that immunogenic proteins contain both variable and non-variable but naturally immunogenic structures. These conserved epitopes would be good candidates for the development of malaria vaccines because they will induce a broad spectrum of protection.

Several native immunogenic proteins from the surface of the asexual blood forms of *P. falciparum* have been isolated and tested for their protective capacity in experimental animals (Perrin et al., 1984, 1985; Siddiqui et al., 1987). Partial protection reached with two proteins p190 and p41 encouraged the cloning and characterization of the genes encoding these antigens (Mackay et al., 1985, Gentz et al., 1988; Certa et al., 1988). The p190 protein of MSA-1 (Major Merozoite Surface Antigen) is a polymorphic protein containing a dimorphic region that identifies the *P. falciparum* K1 and MAD-20 prototypes (Tanabe et al., 1987; Certa et al., 1987) as well as highly variable and conserved domains (Hall et al., 1984). The p41 corresponds to the parasite aldolase, and is the most conserved polypeptide of the parasite known (Certa et al., 1988).

Recently, several peptides of different *P. falciparum* proteins were synthetized and polymerized to a high molecular weight protein, SPf(66)30 (Patarroyo et al., 1987). This pro-

This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and from Beneficencia del Valle, Cali, Colombia.

tein was tested both in humans and monkeys and although results are controversial, the vaccine design seems to be useful in the field (Patarroyo et al., 1988; Rodriguez et al., 1990; Ruebush et al., 1990).

We present here a series of studies aimed to test the protective capacity of two recombinant proteins comprising a conserved fragment of the MSA, the r190L and r190L-CS.T3 construct, as well as the full-length p41 expressed in *Escherichia coli*. We also tried to reproduce and expand the experiments carried out previously using the synthetic protein SPf(66)30.

MATERIALS AND METHODS

Monkeys — Aotus lemurinus griseimembra from the Northern region of Colombia and bred in the Primate Centre of Universidad del Valle (Cali, Colombia) were matched for weight, (800 g), size and age and randomly assigned to groups of 4-6 animals. Monkeys belonged to karyotypes II and III both of them highly susceptible to *P. falciparum* infection.

Antigens – Four different *P. falciparum* proteins were tested: recombinant aldolase (rp41), 190L and 190L-CS.T3 and the synthetic polymer SPf(66)30. The aldolase was essentially in its full length and enzymatically active. 190L is a fragment from the amino terminus of the MSA-1 protein (residues 143-312) (Gentz et al., 1988) and 190L-CS.T3 was a construct including 190L and the universal CST.3 T-helper epitope described by Sinigaglia et al. (1988). These proteins were purified by metal chelate affinity chromatography followed by preparative SDS-polyacrylamide gel purification as described before (Döbeli et al., 1990). SPf(66)30 was synthetized by polymerization of three peptides from the 83kD, 55kD and 35kD molecular weight asexual blood stages and a single tetrapeptide NANP from the circumsporozoite (CS) protein (Patarroyo et al., 1988). The polymer ready to be used was kindly provided by Dr M.E. Patarroyo from the Immunology Institute of the Universidad Nacional de Colombia.

Immunization and challenge – Recombinant proteins were emulsified in complete (first dose) or in incomplete (booster injections) Freund's adjuvants. Each dose contained 350 µg/protein in a volume of 0,5 ml and was injected subcutaneously on the thorax and abdomen at days 0, 21, 42. The synthetic polymer was also emulsified in Freund's adjuvants as described above but 6 immunization doses, each containing 250 µg of peptide were injected at days 0, 30, 45, 60, 75 and 90. A control group was immunized with saline solution emulsified in Freund's adjuvants following the same schedules. Challenge was performed using 10^5 *P. falciparum* FVO parasites on ay 57 in the experiment using recombinant proteins and on day 100 in the one with the polymer. Serum was collected before each immunization and on days 5, 10, 15 after parasite challenge. Animals were drug cured whenever parasitemia reached a level of 10% or when appeared to be critically ill./

Analysis of the immune response – Antibody levels were determined by IFA (Indirect Immunofluorescence Assay) using as antigen *P. falciparum* FVO parasitized red blood cells (pRBC) directly obtained from highly parasitized donor monkey and by ELISA using as coating antigens the same proteins used for immunization as described before (Herrera et al., 1990).

RESULTS

Antibody response – Specific antimalarial antibody levels where determined by IFA before the immunization and all monkeys were negative. In animals immunized with the recombinant proteins antibody titres became first positive by the second immunization dose and reached levels between 1:80-1:5120. In most of the animals the parasite challenge boosted the antibody response and an increase in titers was evident 10 days after infection. There was no difference between the IFA titres neither before nor after challenge in these 3 groups. Antibodies against the recombinant proteins determined by ELISA showed high titers at prechallenge bleeding and were only slightly increased during the infection. (Table I).

In animals immunized with the synthetic polymer, no antiparasite antibodies were detected by IFA after the 6 immunization doses whereas they were evident by day 10 of the infection and increased on day 15. Sera were also studied by ELISA using as antigen the polymer and although the adsorbance in the test sygnificantly increased from the pre-immune to the pre and postchallenge bleedings there was no difference in this increase between the immunized and control groups (Table II).

TABLE I

Antibody response in *Aotus* immunized with recombinant proteins

	Pre-immune	Pre-challenge		Post-challenge	
Monkey	IFA/ELISA	IFA	ELISA	IFA	ELISA
rp41					
M39	0	320	128000	1280	256000
M 8	0	320	256000	2560	256000
M58	0	80	64000	640	128000
M36	0	640	128000	1280	128000
M10	0	320	128000	640	256000
190L					
C 9	0	5120	128000	5120	128000
M18	0	2560	16000	2560	32000
M45	0	320	16000	640	32000
M61	0	320	16000	640	64000
190L-CS.T3					
F41	0	5120	32000	5120	64000
F151	0	80	64000	1280	64000
M 3	0	320	8000	640	16000
M24	0	640	64000	1280	128000
Control					
M22	0	0	ND	320	ND
M38	0	0	ND	1260	ND
M55	0	0	ND	640	ND
M 4	0	0	ND	320	ND

IFA titers are expressed as the reciprocal of the final positive serum dilution. ELISA titers are expressed as the reciprocal of the dilution that yields an absortion of 1.0 unit at 405 nm. Preimmune, corresponds to day 0 of the experiment before immunization prechallenge to day 57 and postchallenge 10 days after infection.

TABLE II

Antibody response in *Aotus* immunized with the synthetic protein

	Pre-immune		Pre-challenge		Post-challenge	
Monkey	IFA	ELISA	IFA	ELISA	IFA	ELISA
M28	0	0052	0	0192	320	0560
M32	0	0006	0	0220	ND	ND
M67	0	0006	0	0258	1.280	0851
M70	0	0584	0	0411	320	0970
V02	0	0005	0	0427	320	0713
Control						
M10	0	0006	0	0.191	1.280	0226
M44	0	0008	0	0.184	320	0255
M59	0	0029	0	0.115	320	0241
M62	0	0002	0	0.169	1.280	0206

IFA titers are expressed as the reciprocal of the final positive serum dilution. For ELISA sera were diluted 1:100. Titers are expressed as the absorbance at 490 nm. Sera were considered positive when O.D. igual 1.0 at this wave lenght. Postchallenge bileding corresponds to day 15 of infection.

As shown in Table III parasitemia developed after a prepatent period of about 4 days in most monkeys. The group immunized with the aldolase rapidly increased the parasitemia and all monkeys reached levels above 10% between days 10 and 13 after challenge similar

TABLE III

Parasitemia development in animals immunized with recombinant proteins

	Day after challenge																	
Monkey	4	5	6	7	8	9	10	11	12	13	14	15	16	17	17	19	20	25
Control																		
M22	.1	.7	1.5	2.5	5.0	7.0	12.0[T]											
M38	.05	.5	1.0	2.5	4.7	8.0	10.5[T]											
M55	.05	.4	.7	1.5	3.0	6.2	9.5	12.8[T]										
M 4	.05	.1	.5	1.0	3.0	4.0	13.5[T]											
rp41																		
M39	.1	.7	1.0	1.5	3.0	4.5	14.0[T]											
M 8	.1	.1	2.5	2.5	4.0	4.3	16.0[T]											
M10	.08	.09	1.2	1.6	3.5	6.0	15.0[T]											
M36	.04	.04	.1	.5	1.5	2.5	3.9	16.0[T]										
M58	.05	.06	.1	.9	1.6	2.8	3.5	4.5	13.0[T]									
190L																		
M18	.05	.07	.2	.6	1.0	1.8	3.0	4.5	7.0	9.0	17.0[T]							
C 9	.04	.04	.4	.8	.8	1.5	2.5	4.0	5.0	6.0	6.5	7.0	9.5	11.5[T]				
M45	.05	.05	.6	1.0	1.2	1.8	2.8	4.2	5.3	6.2	6.5	6.8	9.0	10.5[T]				
M61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
190L-CS.T3																		
F151	.02	.02	.4	.6	1.0	2.3	2.5	5.0	9.5	12.0[T]								
M 3	.03	.02	.3	.6	1.1	2.2	2.6	2.56	1.4	2.6	2.8	2.9	3.0	3.0	3.0	3.0	3.2	0
M24	.03	.03	.4	.5	1.2	2.3	2.7	4.8	5.2	4.5	4.0	3.2	2.8	2.0	.8	.1	0	0
F41	0	0	0	0	0	0	0	0.5	1.5	0.2	0	0	0	0	0	0	0	0

Post challenge parasitemia is expressed as the percentage of infected red blood cells, determined on GIEMSA stained thin films. [T] = indicates the day and parasitemia at which animals were treated with antimalarials.

TABLE IV

Parasitemia development in *Aotus* immunized with SPf66-30

	Day after challenge																
Monkey	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	25
SPf(66)30																	
M28	+	0.1	0.1	0.2	1.1	1.6	3.7	12.2[T]									
M32	+	0.1	0.1	0.1	0.4	0.6	1.1	1.4	3.2	2.9	3.1	3.8	8.7	10.9[T]			
M67	–	0.1	01.	02.	08	1.1	2.0	3.1	3.7	4.1	3.0	1.8	1.9	1.7	1.2	0.6	–
M70	+	0.1	01.	01.	0.1	0.3	0.7	2.8	1.2	4.7	5.5	17.9[T]					
V02	–	–	–	0.1	01.	0.4	0.6	0.7	0.8	3.0	3.5	11.0[T]					
Control																	
M10	+	0.1	0.1	0.1	0.4	0.6	0.5	1.9	1.4	2.6	2.8	4.1	7.6	10.2[T]			
M44	–	0.1	0.1	0.1	0.3	1.2	5.3	15.1[T]									
M59	–	0.1	0.2	0.2	0.5	1.2	2.7	2.5	2.5	3.2	1.9	2.8	4.2	3.5	2.1	2.7	–
M62	–	0.1	02.	0.2	1.1	1.7	6.6	17.3[T]									

Post challenge parasitemia is expressed as the percentage of infected red blood cells, determined on GIEMSA stained thin films. [T] = indicates the day and parasitemia at which animals were treated with antimalarials.

to the control group whereas 3 of the 4 monkeys from the group immunized with the r190L delayed the development of the peak of parasitemia (days 14-17) and one was fully protected. In the group immunized with the 190L-CS.T3 construct, only 1 animal developed high parasitemia and had to be treated on day 13 whereas the other 3 developed lower

peaks and controlled the infection.

In the experiments carried out using the SPf(66)30 polymer, both the immunized and control groups delayed the development of parasitemia. Most of the animals showed patent parasitemia only on day 6 and a slow increase thereafter. In the immunized group 4 of the 5 animals developed high parasitemia between days 12-18 whereas 1 monkey (M67) reached 4.1% parasitemia by day 14 and spontaneously cured by day 21. In this control group, 3 of the animals had to be drug cured and 1 (M59) cleared the parasitemia by day 22 (Table IV).

DISCUSSION

Native p41 and p190 proteins isolated from *P. falciparum* by affinity chromatography had shown protective properties in *Saimiry* or *Aotus* monkeys (Perrin et al., 1984, 1985; Siddiqui, 1987).

The cloning and characterization of the p41 gene allowed the identification of its aldolase activity and its high degree of conservation (Certa et al, 1988). These characteristics together with the suggested location of the protein on the apical region of the merozoite surface conferred to this antigen a great potential as malaria vaccine candidate. In a series of studies we have tested several times the protective capacity of the recombinant aldolase and have found that although it can induce a good antibody response and lymphocyte proliferation *in vitro* it does not protect *Aotus lemurinus* (Herrera et al., 1990). The comparison of sera obtained after challenge from monkeys immunized with the native protein (*Saimiri*) and with the rp41 (*Aotus*) showed a number of additional antigens recognized by the *Saimiri*. Antigens isolated by affinity chromatography are potentially contaminated with other parasite antigens. In fact, the gene corresponding to a 29 Kd protein together with another coding for a fragment of the aldolase were cloned by using a polyclonal anti-native p41 antiserum suggesting the possibility of cross-reactive epitopes in the 2 proteins (Knapp et al., 1988). Moreover, recently it has been found that anti-aldolase antibodies locate the protein in the cytoplasm rather than in the roptry organelles of the merozoite surface (Howard, 1990).

The protection obtained with the 190L antigen is similar to that reached with a construct used previously in *Aotus* monkeys, which contained an additional conserved fragment 190M (190N) (Herrera et al., 1990). Fusion of 190L with the universal epitope CS.T3, did not increase the titres of antibodies. It was however, probably responsible for the increased production of $^{\tau}$-IFN which was correlated with protection in the immunized monkeys (Herrera et al., 1991). Although there was not a clear correlation between antibody titres against 190L and protection in this group a synergistic role of antibodies and and $^{\tau}$-IFN is likely to be required for good protection. $^{\tau}$-IFN plays an important role in the stimulation of both macrophages and neutrophils for the release of oxygen radicals (Kumaratilake et al., 1991).

Interpretation of the negative results obtained with the SPf(66)30 synthetic protein is complicated. It was originally reported as protective in humans (Patarroyo et al., 1988) but more recently in two studies carried out in *Aotus nancymai* monkeys (Rodriguez et al., 1990; Ruebush et al., 1990), basically no protection was achieved. In the experiment reported here one monkey was protected in each group, experimental and control. There was a significant delay in the development of parasitemia even in the control group which may have been induced by a non-specific immune response due to the number of immunization doses using Freund's adjuvants. In the experiments reported using these synthetic peptides (Patarroyo et al., 1988; Rodriguez et al., 1990; Ruebush et al., 1990), the antibody response to both parasite and peptides have been either poor or negative. In our experiments, the peptides failed to induce antibodies against the immunogen or the parasite. We do not have any obvious explanation for this lack of immunogenicity although genetic differences may exist between the 2 subspecies of *Aotus* used. However, the differences between previous experiments carried out with *A. nancymai* are against this hypothesis.

Finally, our results suggest a great potential for the use of the conserved 190L fragment fused with CS.T3. It induces high anti-parasite antibody titres and stimulates T-cells to produce t-IFN which might be either directly or indirectly involved in the parasite control. The biological mechanism, how SPf(66)30 protects humans remains obscure and can not be elucidated in the *Aotus* monkey model for malaria.

ACKNOWLEDGEMENTS

To Franco Rosero, Blanca Liliana Perlaza and Consuelo Clavijo for the excellent technical assistance and to Dr M.E. Patarroyo for providing us with the SPf(66)30 polymer.

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