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Immunization of *Aotus* monkeys with *Plasmodium falciparum* blood-stage recombinant proteins

(malaria/vaccine/recombinant antigens)

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ABSTRACT **The current spread of multidrug-resistant malaria demands rapid vaccine development against the major pathogen *Plasmodium falciparum*. The high quantities of protein required for a worldwide vaccination campaign select recombinant DNA technology as a practical approach for large-scale antigen production. We describe the vaccination of *Aotus* monkeys with two recombinant blood-stage antigens (recombinant p41 and 190N) that were considered as vaccine candidates because parasite-derived antigen preparations could protect susceptible monkeys from an otherwise lethal malaria infection. In contrast to the natural antigen, recombinant p41 protein (*P. falciparum* aldolase) could not protect monkeys, although all animals seroconverted. 190N antigen, a recombinant protein containing conserved sequences of the major merozoite surface antigen p190, protected two of five monkeys from critical levels of infection with the highly virulent FVO isolate of *P. falciparum*. However, the B- and T-cell responses to 190N antigen were similar in protected and unprotected animals so that other unknown factors may contribute to protection. Higher purity or lack of protective epitopes or different structure of protective epitopes in the recombinant proteins might explain the better performance of parasite-derived antigens in vaccination trials. The partial protection obtained with 190N antigen suggests that this molecule could contribute to a vaccine mixture against *P. falciparum*.**

The unicellular protozoan *Plasmodium falciparum* is the major pathogen causing lethal malaria in man. The sporozoite stage of the parasite is transmitted by the bite of a female *Anopheles* mosquito, invades the liver of the host, and develops into a mature schizont. Subsequent release of merozoites into the blood stream starts the infectious cycle, which ends with the manifestation of clinical malaria symptoms. Insecticide spraying briefly controlled the spread of the disease until resistant mosquito vectors developed. More recently, the worldwide spread of drug-resistant malaria has eliminated the hope of eradicating malaria by chemotherapy. The partial failure to control malaria by drugs explains the urgent necessity of additional means for malaria control, such as vaccines.

Successful experimental immunization trials with irradiated sporozoites (1), blood-stage parasites (2), and later with purified parasite antigens (3–5) demonstrated that *P. falciparum* is vulnerable to immune attack in experimental infections. The major coat protein of merozoites referred to as p190, gp195, or PSA (polymorphic schizont antigen) is a polymorphic glycoprotein with M_r between 190,000 and 205,000. Parasite-derived p190 and its processed products purified from parasite cultures could protect *Aotus* monkeys completely from malaria, and therefore, recommends it as a potential vaccine candidate (5). Because of the problem of antigen polymorphism (6, 7), two conserved regions of the p190 molecule (amino acid residues 146–312 and 1059–1196; ref. 8) were expressed in *Escherichia coli* as a single fusion protein termed 190N (9). The use of strongly conserved regions of p190 reduces the risk of antigenic variation triggered by immunization. 190N antigen is recognized by sera of humans with acquired semiimmunity to malaria (9) and contains B- and T-cell epitopes (10). These properties made it an attractive subunit vaccine candidate based on the protective natural p190 molecule (9, 10).

The other recombinant protein included in this study is a protein of M_r 41,000 (termed p41), which was identified as the aldolase of the parasite (4, 11). The parasite-derived antigen preparation gave a marked degree of protection against malaria in immunized *Saimiri* monkeys (4). Probably for functional reasons, the entire polypeptide sequence is highly conserved in all *P. falciparum* isolates analyzed (11). The central function of aldolase for parasite metabolism makes it unlikely that mutants due to immunoselection will appear after immunization. Successful expression of the entire polypeptide in *E. coli* allowed us to vaccinate monkeys with the entire molecule (12). The sequence of the recombinant antigen lacks only five amino acid residues present in the natural antigen. This lack does not affect the enzymatic or immunological features of the recombinant antigen, which are virtually identical to the natural protein (12).

We describe the testing of both vaccine candidate recombinant proteins (rp41 and 190N) in *Aotus* monkeys. In contrast to the natural antigens, rp41 gave no protection in two independent experiments and 190N protected two of five animals from malaria after immunization and challenge with the highly virulent FVO isolate of *P. falciparum*. Probable explanations for the different results from experiments with recombinant and parasite-derived antigens are discussed.

MATERIALS AND METHODS

Monkeys. The monkeys (*Aotus trivirgatus griseimembra*) were of Colombian origin and bred in the animal facilities of the Universidad del Valle (Cali, Colombia). Each experimental group contained five animals matched for size and age. The monkeys had the karyotypes II and III, and each animal weighed ≈800 g. No previous exposure to *P. falciparum* was evident according to clinical history, antimalarial antibody, or peripheral blood mononuclear cell stimulation.

Antigens. The essentially full-length p41 protein expressed in *E. coli*, recombinant p41 (rp41), and 190N were purified as

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Abbreviations: rp41, recombinant p41; PRBC, parasitized erythrocytes (red blood cells); IFA, indirect immunofluorescence.

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described (8, 12). 190N antigen was further purified by an additional SDS/gel purification step followed by overnight dialysis and lyophilization. Both antigens and the control antigen (egg white lysozyme; Sigma) were dissolved in phosphate-buffered saline (PBS) at 700 μg/ml. The antigen preparation displayed a single band on SDS/polyacrylamide gels directly before immunization (data not shown).

Immunization. Antigen was injected at day-0, -21, and -42 of the experiment. At the same time the animals were bled for immunological assays. Each dose consisted of 350 μg of antigen dissolved in 500 μl of PBS. Immediately before immunization the antigen solution was mixed with 500 μl of complete Freund's adjuvant for the first dose or with 500 μl of incomplete Freund's adjuvant for the booster injections. The sample was divided in four 250-μl aliquots and injected s.c. into four different spots on the back of each animal.

Parasites. Three *P. falciparum* isolates, FUP Palo Alto (Uganda), FCB-1 (Colombia), and FVO (Vietnam) previously adapted to *Aotus* monkeys were used. Parasite liquid nitrogen stocks were thawed and used directly to infect the monkeys.

Parasite Challenge. Fifteen days after the third immunization, all animals were challenged with 5×10^6 parasites of the FVO isolate of *P. falciparum* injected i.v. Parasitized erythrocytes (PRBC) were obtained from a monkey donor with exponential parasite growth. After challenge, the parasitemia was monitored daily independently by us and the Cali Malaria Centre by both thin and thick smears stained with Giemsa dye. Animals with 10% parasitemia were considered unprotected and immediately treated with Fansidar (Hoffmann–LaRoche). In one case (animal A17) Fansidar was given at 6.5% parasitemia to save the animal, which showed severe malaria symptoms.

Determination of Protein Concentration. Protein concentrations (antigen, PBRC) were determined colorimetrically by using a commercial protein assay (Bio-Rad) and the protocol supplied by the manufacturer (Bio-Rad, D-8000).

Analysis of Immune Response. Immune response in sera was maintained at day-0, after the first, second, and third immunizations, and, in addition, 5 or 10 days after parasite challenge. Antibody levels and specificities were determined by indirect immunofluorescence (IFA), ELISA, and immunoblotting.

IFA. IFA was accomplished by using serial dilutions of antisera in PBS and analyzed on multispot slide preparations of FCB-1 parasites fixed with acetone (4).

ELISA Assay. ELISA was done essentially as described by Perrin *et al.* (4).

Immunoblot. Immunoblot analysis was performed as described (13). Each strip had one lane with a FCB-1 parasite lysate (30 μg of protein per lane) and two lanes with purified rp41 and 190N antigens (0.5 μg each). The monkey sera used were diluted 1/1000 in PBS. Antigen–antibody complexes were detected with ^{125}I-labeled protein G (Amersham) followed by autoradiography. Rabbit serum to rp41 or 190N antigens (9, 11) was included as a positive control.

Lymphocyte Proliferation Assay. Peripheral blood mononuclear cells from each monkey were separated from heparinized blood obtained before the first and after the last immunization (day-42). Peripheral blood mononuclear cells were purified by Ficoll-Paque density gradient under the protocol of the supplier (Pharmacia). Cells were suspended at 5×10^6 cells per ml in RPMI 1640 medium (GIBCO) supplemented with 5% monkey B (−) serum, 2 mM L-glutamine, 25 mM Hepes, penicillin at 100 units/ml, and streptomycin at 100 μg/ml (GIBCO). Cells were cultured in triplicates of 200-μl aliquots in 96-well microtiter plates (Nunc) with either a FCB-1 *P. falciparum* crude-antigen preparation (10 μg of protein per ml), an erythrocyte lysate (5 μg/ml), lysozyme at 5 μg/ml, rp41 antigen at 5 μg/ml, or 190N antigen at 5 μg/ml. Control plates were stimulated with Con A at 10 μg/ml (Difco). Plates were incubated for 6 days at 37°C in 5% CO_2/95% air. Cells were pulse-labeled during the final 24 hr of culture with 1 μCi (1 Ci = 37 GBq) of tritiated methylthymidine (specific activity 2 Ci/mM, Amersham). Cultures were harvested onto glass-fiber filters by using an automated device, (miniMASH, Microbiological Associates) and processed for liquid scintillation counting.

Aldolase Inhibition Assay. Ten ng of rp41 aldolase was mixed with 1 μl of monkey serum, and inhibition of aldolase activity was determined colorimetrically by using a commercial kit (Sigma) according to the supplied protocol. Rabbit serum to rp41, that inhibited aldolase by 80% under the same conditions, served as a positive control, and preimmune serum of the same animal (G574) was used as a negative control.

Production of Rabbit Serum Against rp41 Antigen. A rabbit (G574) was immunized with one 200-μg dose of rp41 in complete Freund's adjuvant and two 200-μg doses of rp41 in incomplete Freund's adjuvant (Sigma).

RESULTS

Before *P. falciparum* challenge we had established the immune status of the experimental animals. In particular, we were interested whether the immune system stimulated with recombinant proteins would interact with the natural antigens presented by the parasite.

Humoral Immune Response. Table 1 summarizes the malaria-specific B-cell response of all immunized animals before parasite challenge. Animals injected with *P. falciparum*-derived recombinant proteins 190N and rp41 seroconverted. Chicken lysozyme was included as a nonrelated antigen in the control group. All sera of monkeys immunized with rp41 or 190N recognized the natural antigen on fixed parasites (IFA), in ELISA assays, and denatured in immunoblots (Table 1). The antibody titers against rp41 and 190N antigens are of the

Table 1. Prechallenge antibody titer in immunized monkeys

Monkey	IFA*	ELISA titer† PRBC	rp41	190N	Immunoblots‡
Antigen rp41					
A21	80	500	100.000	−	+
A17	80	1000	256.000	−	+
F6	320	1000	200.000	−	+
C3	320	1000	400.000	−	++
F27	1280	1000	256.000	−	++
Antigen 190N					
F71	320	1500	−	256.000	++
D73	80	500	−	100.000	+
F48	320	500	−	100.000	+
B12	20	500	−	128.000	++
B33	80	1000	−	16.000	+
Antigen LYS					
F104	−	−	−	−	−
C36	−	−	−	−	−
F56	−	−	−	−	−
F111	−	−	−	−	−
F67	−	−	−	−	−

LYS, Hen egg white lysozyme. −, Negative reaction in the assay.
*IFA results are expressed as reciprocal end-point dilutions.
†Results are expressed as the reciprocal of the dilution that yields an absorption of 1.0 unit at 405 nm. PRBC is a lysate of FCB-1 *P. falciparum* isolate; rp41 and 190N are recombinant proteins used for coating and immunization of monkeys.
‡Total proteins of the FUP Palo Alto lysate were electroblotted onto nitrocellulose membranes and incubated with a 1/1000 dilution of each monkey serum. +, Positive detection after overnight exposure; ++, strong reactivity under the same conditions.

same order of magnitude as in animals protected by immunization with the natural antigens (4, 5). Sera of semiimmune individuals from malaria endemic areas had similar ELISA values under the same assay conditions (PRBC: 1/10.000; rp41/1:1000; 190N 1/2400).

The p41 antigen had already been identified as the parasite aldolase, an essential glycolytic enzyme located in the cytosol (11). We asked whether inhibition of the enzymatic activity of p41 by antibodies contributes to protection. We therefore assayed aldolase activity in the presence of prechallenge sera of all experimental animals. As expected, only sera from the rp41 group could block the enzyme activity to the same degree as a rabbit control serum raised against parasite p41 (data not shown). A similar degree of aldolase inhibition was measured with sera from protected monkeys immunized with denatured or native parasite-derived aldolase (14).

We thus assumed the B-cell response determined in four independent assays to be sufficient for, at least, partial protection.

Cellular Immune Response. Lack of a correlation between protection and antibody titers in a previous and this trial suggested that other immune mechanisms, such as a cytotoxic T-cell response, could contribute to protection (4). We, therefore, measured T-cell stimulation in all experimental animals before immunization, after the second dose of antigen, and 10 days after parasite challenge (Fig. 1). The preimmune stimulation index with either pure antigen or PRBC as antigen had background levels in all animals tested. Prechallenge Con A-induced T-cell proliferation varied extensively depending on the animal. Both recombinant antigens induced antigen-specific T-cell proliferation with rp41, inducing significantly higher responses than 190N antigen (Fig. 1). PRBC antigen preparations stimulated T-cell proliferation in three of five rp41-immunized animals, and the rest had background levels (Fig. 1). This result is probably related to the low amounts of specific antigen available for stimulation in parasite lysates. The T-cell stimulation index 10 days after the parasite challenge dropped significantly, even with Con A as stimulator—perhaps due to parasite invasion.

Selection of the *P. falciparum* Isolate for Challenge. To avoid a primary immune response against the invading parasite, which could contribute to immunity, we wanted a *P. falciparum* isolate that rapidly replicates in Colombian *Aotus* monkeys. We thus infected three pairs of nonimmunized monkeys with the FUP Palo Alto (5×10^6 PRBC), FCB-1 (5×10^6 PRBC), or FVO (2×10^6 PRBC) isolates of *P. falciparum*. The course of infection was then followed by daily blood examination. The FVO isolate, for example, reached 10% parasitemia only 10 days after inoculation and required drug treatment, whereas the same parasitemia developed after 20 days with FCB-1 and after 15 days with the FUP Palo Alto isolate of *P. falciparum* (data not shown). In the same experiment, we measured B-cell response against the invading parasites during this test infection. None of the pairs responded 10 days after infection in ELISA assays with PBRC as antigen. Twenty days after infection, however, all monkeys seroconverted and developed significant titers of parasite antibodies (1/80 against FVO, 1/80 against FCB-1, and 1/20 against FUP Palo Alto; data not shown). We thus selected FVO for the challenge infection.

Course of Malaria Infection in Immunized *Aotus* Monkey. Fig. 2 shows the development of parasitemia in the three groups of animals immunized with either lysozyme (control) or the malaria vaccine candidates rp41 or 190N. As expected from the test infection, high parasitemia started to develop

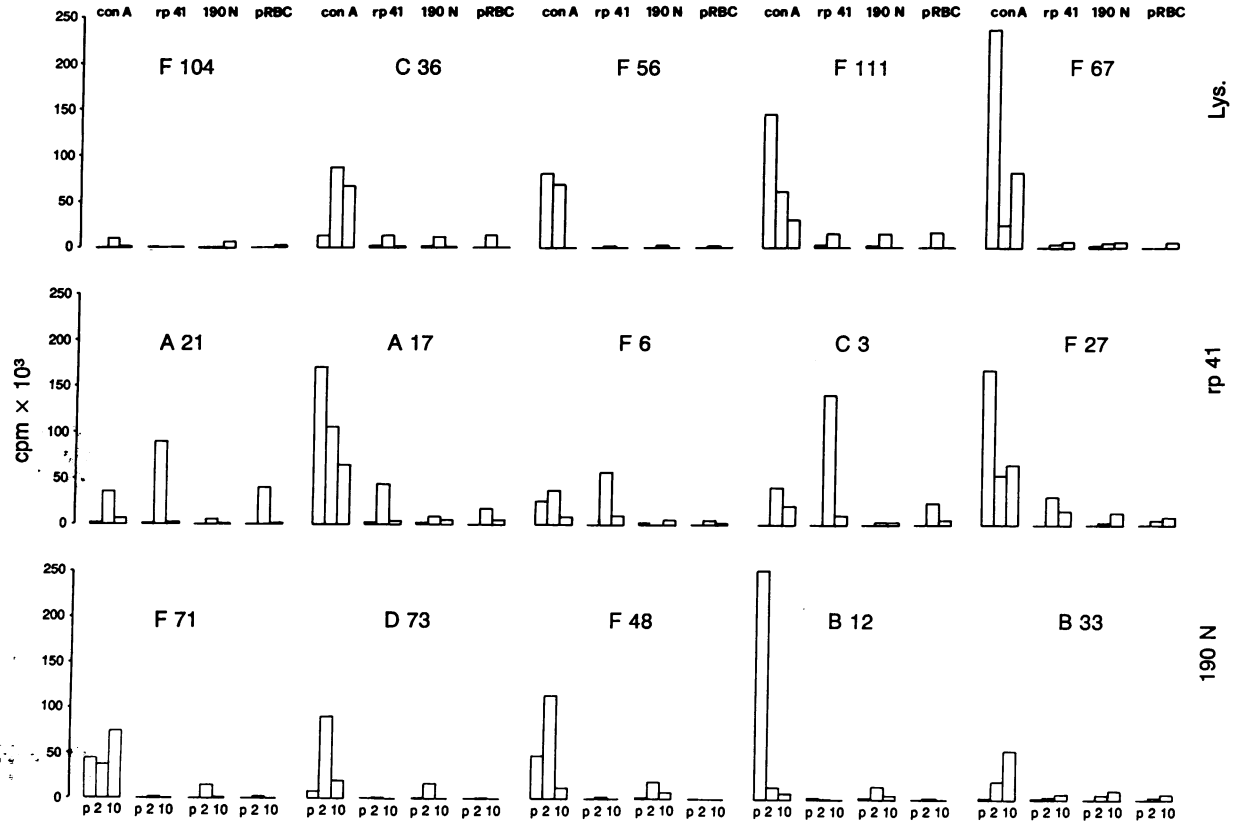

FIG. 1. Development of T-cell response after immunization with lysozyme (Lys., *Top*), rp41 antigen (*Middle*), and 190N antigen (*Bottom*). T cells of each animal were stimulated before immunization (p), after the second immunization (no. 2), and 10 days (no. 10) after challenge with either Con A (control, data set 1), rp41 (data set 2), 190N (data set 3), or PRBC (pRBC) (data set 4). The type of antigen for stimulation appears at the top of each data set, and the animal code appears below. ^3H counts incorporated are plotted on the *y* axis. Note that the response to control stimulator Con A varies extensively depending on the animal. Values given are the average of triplicate measurements.

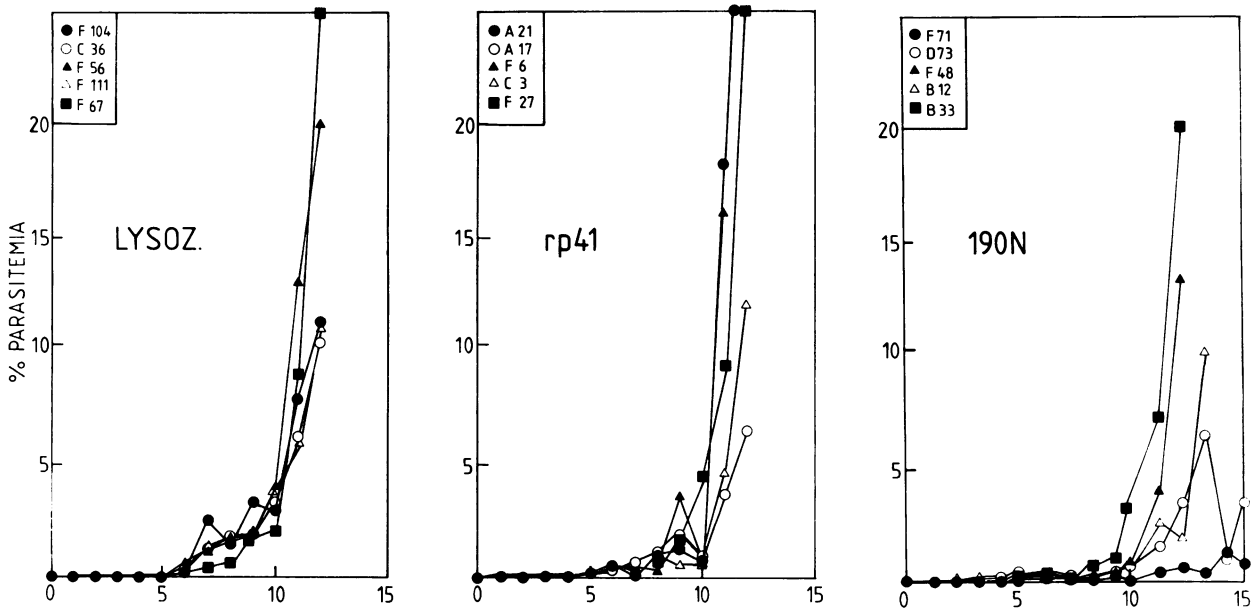

FIG. 2. Course of parasitemia in three groups of monkeys immunized with lysozyme (control group, *Left*), rp41 (*Middle*), or 190N (*Right*). Monkeys were challenged with the FVO isolate, and terminated curves indicate the point of chemotherapy in nonprotected animals. The two animals protected by 190N antigen (D73 and F71) had cleared the parasite at day-20 (data not shown). *x* axis shows days after challenge; animal code numbers appear in the insets.

after day-10 in nonprotected animals. Development of parasitemia in the control group and the animals immunized with rp41 was almost identical, although the mean parasitemia at day-9 was clearly lower than in the control group (Fig. 2). At day-10 rapid parasite replication occurred in all animals of the rp41 group, which were rescued by chemotherapy. Because the results of our vaccination trial deviate drastically from the result obtained with a natural p41 antigen preparation, we decided to repeat the experiment under the same conditions to exclude experimental errors. However, results were identical (data not shown).

Two of five animals in the group immunized with 190N antigen did not develop critical malaria symptoms; the peak parasitemia in these animals was 6% (D73) and 1.2% (F71). At day-20 these animals had completely cleared the parasites, and no blood-stage parasites were detected in the following 8 weeks. Another animal (B12) had a delayed onset of critical parasitemia. 190N antigen is thus significant as a p190-derived recombinant protein that partially protects *Aotus* monkeys from malaria.

DISCUSSION

We have tested two recombinant *P. falciparum* antigens (rp41 and 190N) as a malaria vaccine in the *Aotus* monkey animal model. The corresponding parasite-derived antigens protected previously immunized monkeys from malaria (3–5). After immunization with the affinity-purified recombinant proteins, all animals seroconverted and developed similar parasite-specific antibody titers. The antibodies recognized their natural equivalent in the native (IFA, ELISA) or denatured conformation (immunoblot). In addition, both antigens induced antigen-specific T-cell proliferation. Furthermore, rp41 antibodies inhibit the aldolase activity to the same degree as in animals immunized with the natural protein (14). Despite this parasite-specific humoral and cellular response, rp41 antigen did not protect monkeys, but 190N antigen induced partial malaria immunity.

190N antigen contains ≈20% of the natural p190 protein sequence in an unnatural array, and thus epitopes required for complete protection could be either missing or in an adverse configuration. This interpretation would help explain why only two of five *Aotus* monkeys were protected against malaria infection. One protected animal (F71) had the highest titers of p190 antibodies in all assays and the lowest peak parasitemia (1.2%). However, another animal (D73) had clearly lower antibody levels, although it was protected (6%). The T-cell response in the protected animals was similar to that of nonprotected monkeys (Fig. 1). This result may suggest that sterile immunity requires stimulation of a yet-unidentified subset of T cells by an epitope missing on 190N antigen but present on the natural p190 molecule. This explanation is consistent with the relatively poor stimulation obtained with PRBC as antigen.

Interpretation of the negative result obtained with rp41, which contrasts with the result obtained with natural antigen, is more complicated (4). The following differences could account for the different outcome of the two independent experiments:

(*i*) rp41 antigen lacks the first five amino acids of the natural antigen due to attachment of an affinity sequence required for purification; this pentapeptide could form an important epitope.

(*ii*) We cannot exclude that the p41 antigen preparation used in the previous trial (4) contained minor but highly immunogenic contaminants that contributed to protection. This explanation is unlikely because prechallenge sera of protected animals immunoprecipitated only one major band of M_r 41,000 from parasite extracts (4).

(*iii*) The FVO isolate of *P. falciparum* is more virulent than FUP Palo Alto, requiring early control of parasite attack. In Fig. 3 we compared postchallenge serum specificities of p41-protected and unprotected animals immunized with the recombinant antigen. It is evident that the animals immunized with natural p41 antigen and challenged with FUP Palo Alto detected several additional parasite antigens. This crossreactivity was missing in the sera of the monkeys immunized with rp41 antigen and challenged with FVO. We have shown above that a period of 20 days is sufficient for a primary immune response to the invading parasite. It is, therefore, difficult to determine whether the vaccine or the response against the other *P. falciparum* components protected against malaria in previous protection trials.

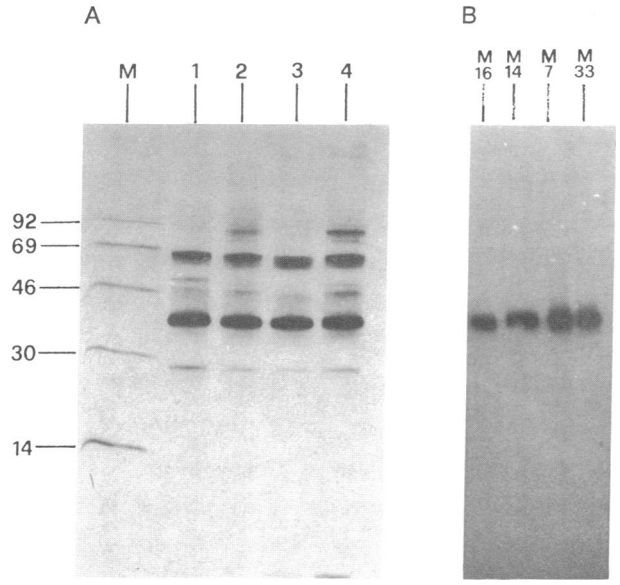

FIG. 3. Immunoblot analysis of postchallenge sera in protected and nonprotected monkeys. (*A*) Total parasite lysates were probed with postchallenge sera of monkeys protected by immunization with SDS/gel-purified natural p41 (4). Lane M, $M_r \times 10^{-3}$ markers. (*B*) Same analysis with postchallenge sera from monkeys immunized with rp41 antigen. Only p41 antigen is detected among *P. falciparum* proteins 20 days after challenge with the FVO isolate. Sera were used at 1/1000 dilution. Codes for animals appear at top of lanes. Codes in *A* were adapted from Perrin *et al.* (4); codes in *B* are for animals of the second rp41 protection trial.

Our results eliminate *P. falciparum* aldolase (p41) as a vaccine candidate for malaria. The partial inhibition obtained with 190N antigen, the ability to produce and purify large amounts of the recombinant protein, and the biological properties of this protein suggest that 190N antigen could become part of a malaria vaccine mixture that is yet to be completed.

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