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Immunology

# Protective immunization of the squirrel monkey against asexual blood stages of *Plasmodium falciparum* by use of parasite protein fractions

**(malaria/vaccination/induced protective immunity)**

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**ABSTRACT** **We had previously shown that two polypeptides of *Plasmodium falciparum* are preferentially recognized by antibodies of resistant squirrel monkeys *Saimiri sciureus*. Free parasites were isolated from synchronized cultures on human erythrocytes initially inoculated with infected *Saimiri* erythrocytes. Crude extracts were prepared from mature schizont stages and electrophoresed on preparative $NaDodSO_4$/polyacrylamide gels. Two groups of five monkeys were immunized with protein fractions eluted from the 75- and 100-kilodalton regions of the gels. Strong protection against challenge by the homologous strain of *P. falciparum* was induced in both groups. Analysis of specific anti-malarial antibodies revealed a homogeneous response of all the animals against a few polypeptides of the mature parasite.**

The course of infection with *Plasmodium falciparum* can be modified by immunization with erythrocytic forms of the parasite. The previously reported protection of *Aotus* monkeys against blood-stage infection by *P. falciparum* was achieved using crude extracts of merozoïtes and schizonts injected in association with Freund's complete adjuvant (1–4) or with synthetic adjuvants (5–7). It has also been shown that immunization with purified antigens from the parasite *Plasmodium yoelii* can protect mice against murine malaria (8).

In the present report we describe the vaccination of squirrel monkeys with two partially purified protein fractions isolated from *P. falciparum* grown *in vitro*. We have previously reported that drug-controlled infection by *P. falciparum* (FUP strain) induces a strong protective immunity in squirrel monkeys (9, 10). Purified IgG from immune monkeys can cure the blood infection of naive recipient monkeys (10–12). Immunochemical analysis of IgG preparations that protect *in vivo* was used to define parasite protein antigens eliciting a protective immune response. Three polypeptides of 50, 76, and 100 kilodaltons (kDa) were strongly recognized by antibodies present in the protective IgG preparations (ref. 10; unpublished observations) and could thus be considered as possible protective antigens. Squirrel monkeys were immunized with two protein fractions eluted from the 75- and 100-kDa regions of preparative $NaDodSO_4$/polyacrylamide gels. Strong protection against challenge by the homologous strain of *P. falciparum* was induced in both groups.

## MATERIALS AND METHODS

**Monkeys.** The squirrel monkeys *Saimiri sciureus* of karyotype 14.7 captured in Guyana are maintained at the Pasteur Institute of Cayenne (French Guyana). Adult females weighing 620 ± 10 g were selected for the present work.

**Parasites.** The Uganda Palo Alto (FUP) strain, adapted to the squirrel monkey (9), has been maintained since 1978 by serial transfers in monkeys. Continuous subcultures in human A+ erythrocytes, initiated with infected monkey erythrocytes were carried out in RPMI 1640 medium (GIBCO)/35 mM Hepes/10% human serum A+, working with a 5% hematocrit. Tissue culture flasks were incubated at 37°C under a continuous flow of humidified 2% $O_2$/3% $CO_2$/95% $N_2$.

**Preparation of Fractions 1 and 2.** Cultures of *P. falciparum* (FUP strain) in A+ human erythrocytes (13) were synchronized by the sorbitol technique (14). Infected cultures were collected when parasitemia reached 5–10% and parasites were at the schizont stage. After two washes in RPMI 1640, the erythrocytes were lysed by saponin (0.025%, 10 min at 37°C), and the suspension was chilled and centrifuged at 4°C (10,000 × *g* for 10 min). The pellet was suspended in RPMI 1640, loaded on a preformed Percoll gradient (20%/40%) and centrifuged at 5,000 × *g* at 4°C for 30 min. Free parasites, collected at the 20%/40% interphase were washed twice in phosphate-buffered saline ($P_i$/NaCl) and frozen at −20°C in the presence of 1 mM *N*-α-tosyllysine chloromethyl ketone/1 mM phenylmethylsulfonyl fluoride. Total parasite extracts were prepared directly in 5 vol of sample buffer (62.5 mM Tris·HCl, pH 6.8/6% $NaDodSO_4$/5% 2-mercaptoethanol/20% glycerol), boiled for 10 min, and centrifuged at 15,000 × *g* for 10 min. The supernatant was applied to a 7.5% preparative $NaDodSO_4$/polyacrylamide gel in parallel with fluorescein isothiocyanate-labeled molecular mass markers (bovine serum albumin, rabbit IgG, and ovalbumin). The 70- to 85-kDa and 90- to 120-kDa regions were located relative to the fluorescein isothiocyanate-labeled markers, the appropriate slices were excised, and the proteins were electroeluted overnight in Tris·glycine, pH 8.6/0.1% $NaDodSO_4$. Protein content was measured by the Coomassie method (Bio-Rad).

**Immunization and Challenge Procedures.** The monkeys were splenectomized 2–3 weeks before the beginning of the experiment. They were immunized on days 0, 21, and 41 with 100 μg of protein in $P_i$/NaCl/0.1% $NaDodSO_4$, emulsified in an equal volume of Freund's complete adjuvant for the first immunization and Freund's incomplete adjuvant for the others. Immunization was done by subcutaneous injection at five points in the back and at one point in the posterior footpad. Control animals received only $P_i$/NaCl/$NaDodSO_4$ with Freund's complete adjuvant and Freund's incomplete adjuvant in the same schedule. All monkeys were challenged on day 55 by intravenous injection of 50 × $10^6$ parasitized monkey erythrocytes. Random group organi-

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Abbreviations: kDa, kilodalton(s); $P_i$/NaCl, phosphate-buffered saline.

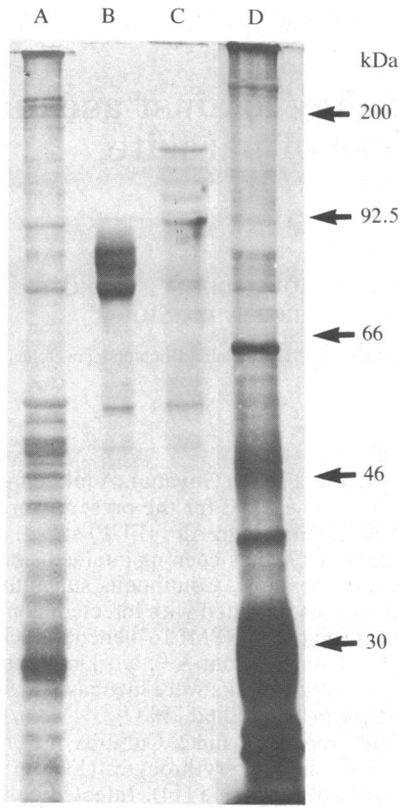


FIG. 1. A silver-stained 7.5% $NaDodSO_4$/polyacrylamide gel showing the major protein components of fractions 1 and 2. Lanes: A, total extract of free parasites; B, fraction 1; C, fraction 2; D, normal human erythrocyte extract.

zation was used to permit double-blind tests. The course of parasitemia in individual monkeys was scored daily on Giemsa-stained blood smears. Samples of serum of individual monkeys were collected throughout the immunization to check the appearance of a specific humoral response.

**Immunofluorescence Assay.** Immunofluorescence assay was carried out on acetone-fixed smears of a shizont-rich preparation of *P. falciparum* (FUP strain) obtained from infected monkeys. Specific antibodies contained in serial dilutions of serum were revealed with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin (Institut Pasteur Production).

**Immunoprecipitation.** Immunoprecipitation analysis of [$^{35}$S]methionine-labeled schizont-specific proteins by antiserum of vaccinated monkeys was done using parasite extracts as described in the legend to Fig. 3.

## RESULTS AND DISCUSSION

The presumptive antigens in the 75-kDa (fraction 1) and 100-kDa (fraction 2) regions were eluted from the preparative gel slices and gave on average 0.5% of the protein content of the crude extract. Pooled eluted fractions reanalyzed on 7.5% $NaDodSO_4$/polyacrylamide gels and stained with silver nitrate showed four major polypeptide bands at 72, 76, 85, and 90 kDa for fraction 1 (Fig. 1, lane B) and five bands of 90 (92), 96, 100, 105, and 120 kDa for fraction 2 (lane C). Some smaller components were also seen in both fractions; they could either be contaminating proteins such as the 72-kDa band seen in the fraction 2 or degradation products, as is probably the case for the minor bands in the 50-kDa region.

Immunization experiments were carried out on splenectomized squirrel monkeys. The experimental groups were composed of 5 animals that received three subcutaneous injections containing 100 $\mu$g of protein. As a control, 10 animals received only $P_i$/NaCl/$NaDodSO_4$ with Freund's complete adjuvant and Freund's incomplete adjuvant. Hematological, parasitological, microbiological, and serological tests were carried out periodically on blood samples collected before and during the vaccination period. All the animals tolerated vaccination well. No lesions were observed at the site of injection, and the monkeys seemed healthy and normally active. No anemia was detected in vaccinated or control animals. Parasitological tests done before the vaccination showed that most animals were infected with trypanosomes and filarial worms, but there was no correlation between the immune response and the presence of these parasites (data not shown).

The monkeys were challenged with $50 \times 10^6$ parasites (FUP strain) on day 55. After the challenge, parasitemia was scored daily in Giemsa-stained smears. Nine out of the 10 control animals showed the typical acute course of parasitemia, which requires quinine chemotherapy within 8 days to avoid death (Fig. 2*A*). One animal immunized against fraction 1 responded like the controls. The remaining 4 showed high resistance to the challenge: a weak parasitemia (5%) was observed in 2 animals and disappeared within 3 weeks; only marginal parasitemia occurred in the others (Fig. 2*B*). All 5 animals immunized with fraction 2 showed good resistance to the challenge and the parasitemia became negative

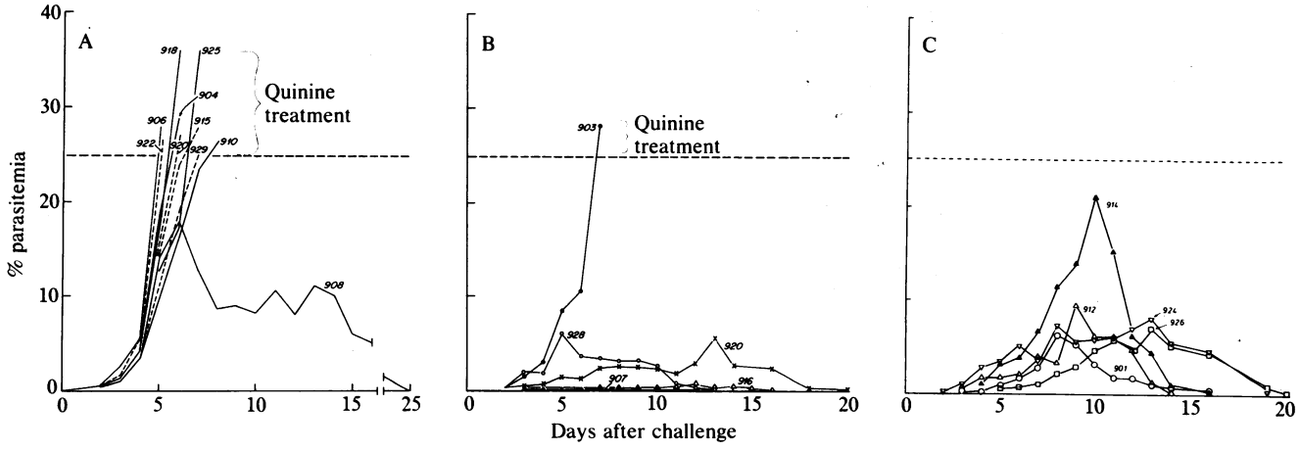


FIG. 2. Response of squirrel monkeys to challenge with $50 \times 10^6$ parasites of *P. falciparum*. (*A*) Controls. (*B*) Immunized against fraction 1. (*C*) Immunized against fraction 2. The course of parasitemia in individual monkeys (identified by numbers) was scored daily on Giemsa-stained blood smears. All counts were done twice using a double-blind label.

Table 1. Appearance of malarial antibodies in antiserum of squirrel monkeys during immunization

| Group | Animal no. | Antibody titer on day −2 | 9 | 29 | 50 |
|---|---|---|---|---|---|
| Control | 906 | Neg | ND | ND | Neg |
| | 910 | Neg | ND | ND | Neg |
| | 918 | Neg | ND | ND | Neg |
| | 922 | Neg | ND | ND | Neg |
| | 929 | Neg | ND | ND | Neg |
| Fraction 1-immunized | 903 | Neg | Neg | 1:20 | 1:40 |
| | 907 | Neg | Neg | Neg | 1:20 |
| | 916 | Neg | Neg | Neg | Neg |
| | 923 | Neg | Neg | 1:40 | 1:160 |
| | 928 | Neg | Neg | 1:80 | 1:320 |
| Fraction 2-immunized | 901 | Neg | Neg | 1:80 | 1:320 |
| | 912 | Neg | Neg | Neg | 1:20 |
| | 914 | Neg | Neg | 1:40 | 1:80 |
| | 924 | Neg | Neg | 1:20 | 1:80 |
| | 926 | Neg | Neg | 1:40 | 1:160 |

Samples of antiserum of individual monkeys were collected as follows: 2 days before the first immunizing injection (day −2), 9 days after the first injection (day 9), 8 days after the second injection (day 29), and 9 days after the third injection (day 50). Neg, negative; ND, not done.

within 3 weeks, even in the 1 case where it was >10% at day 10 (Fig. 2*C*). Biweekly parasitemia controls tested for 2 months after the challenge gave negative results in all the vaccinated animals; in 5 of the 9 quinine-treated controls recrudescence was detected, requiring a second cycle of chemotherapy.

All the challenged animals (vaccinated and controls) showed pronounced anemia, with an average drop of 30% in erythrocyte count notwithstanding the considerably lower levels of parasitemia observed in vaccinated animals. This confirms previous observations that the extent of malarial anemia is incommensurate with the level of parasitemia and that some factor(s) other than plasmodial segmentation must therefore be destroying erythrocytes (15). However, in the vaccinated animals the development of anemia was delayed. All animals regained normal hematocrit values within 4 weeks after the challenge. Weight control tests carried out before the vaccination and 1 month after the challenge showed a variation of <2%.

The humoral response of the monkeys to the different immunizations was checked using two different techniques. In the first, anti-malarial antibodies were measured by indirect immunofluorescence assay and began to be detected after the second injection (Table 1), but they did not reach the levels observed in drug-controlled infections (9) (usually 1:320 to 1:1,280). With the second technique, immunoprecipitation of Triton X-100 extracts of [$^{35}$S]methionine-labeled parasites were done with serum samples collected 5 days before challenge. The serum of control animals did not show specific precipitation (Fig. 3*A*). The five animals immunized with fraction 1 gave a homogeneous response: two major bands of 72 and 90 kDa and minor bands of 96 and 100 kDa are seen in the immunoprecipitates (Fig. 3*B*). Surprisingly, no humoral response was detected against the 76- and 85-kDa polypeptides present in the total labeled extracts, even though these peptides were major components of fraction 1.

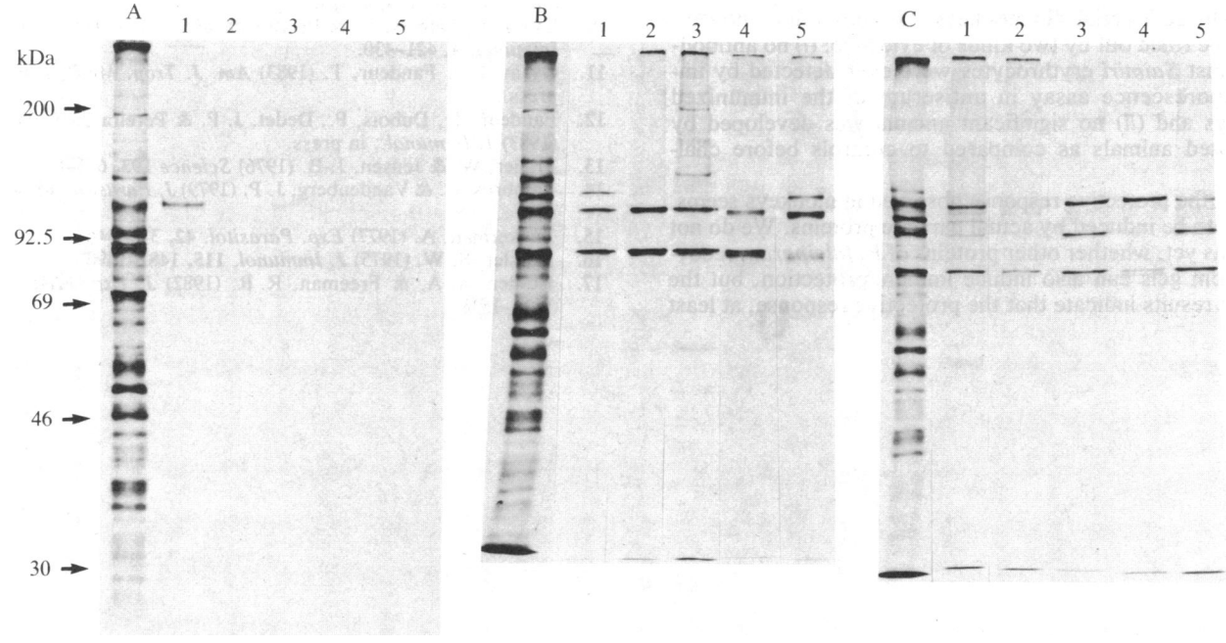


FIG. 3. Immunoprecipitation of [$^{35}$S]methionine-labeled parasite extract with antiserum of control animals (*A*), animals immunized with fraction 1 (*B*), or animals immunized with fraction 2 (*C*). For the preparation of the parasite extract, cultures of *P. falciparum* (FUP strain) in A+ human erythrocytes were synchronized with sorbitol (14) and the mature trophozoites were incubated with [$^{35}$S]methionine at 200 $\mu$Ci/ml (Amersham; 800 Ci/mmol; 1 Ci = 37 GBq) in methionine-free medium for 6 hr. They were collected at the mature schizont stage and washed three times in RPMI 1640. The parasite pellet was solubilized in 50 mM Tris·HCl, pH 8.0/1% Triton X-100/10 mM EDTA/400 mM NaCl/1 mM phenylmethylsulfonyl fluoride/1 mM *N*-$\alpha$-tosyllysine chloromethyl ketone/5 mM iodoacetamide. The supernatant was collected after centrifugation at 12,000 × *g* for 15 min at 4°C. Radiolabeled parasite extracts (400,000 cpm) were incubated overnight at 4°C with 5 $\mu$l of antiserum. Immunocomplexes were isolated in fixed *Staphylococcus aureus* Cowan strain (16) and, after six washes, were extracted with 40 $\mu$l of NaDodSO$_4$/polyacrylamide buffer. Identical volumes of immunocomplex extracts were electrophoresed on 3–10% NaDodSO$_4$ gel, and labeled compounds were revealed by fluorography. Pattern of total extract is shown in the first lane of each group. Individual antisera tested are as follows: (*A*) lanes 1–5, from monkey 906, 908, 910, 920, and 929, respectively; (*B*) lanes 1–5, from monkey 916, 923, 928, 907, and 903, respectively; (*C*) lanes 1–5, from monkey 926, 912, 914, 924, and 901, respectively.

These peptides are, however, strongly precipitated by antiserum of animals immunized by infection and drug treatment (10), suggesting that the antigenic determinants of these proteins were modified during the preparation of the purified fractions. The antiserum of animals immunized against fraction 2 immunoprecipitated the same proteins, with an additional band of 110 kDa (Fig. 3*C*). The similar humoral response of the two groups of monkeys suggests that there is a strong antigenic similarity between the two fractions. They could contain different products resulting from the specific processing of a parasite constituent, as already described for *P. falciparum* (17). In this sense, preliminary results in our laboratory show that some hybridomas, raised in BALB/C mice against the components of the same protein fraction used in the present vaccination experiments, secrete monoclonal antibodies that recognize the same protein family as that immunoprecipitated by antiserum of immune monkeys. In any case, the comparable resistance observed within the two groups could be directly related to the homogeneity of the humoral response. Complete and quantitative analysis of the serologic data collected before and during the challenge will be described elsewhere.

Our results clearly show that certain proteins of the human parasite *P. falciparum* recovered from $NaDodSO_4$/polyacrylamide gels can induce a protective immune response in monkeys against a challenge with blood-stage parasites. These peptides were eluted from gel areas corresponding to 75 and 100 kDa. Previous experiments have clearly shown, through metabolic labeling with [$^{35}$S]methionine, peptides of parasite origin of identical size. Nevertheless, a certain degree of contamination with proteins of the erythrocytes could be detected by immunoprecipitation in our parasite fractions. It could be argued that antibodies directed against these contaminants could also play a role in the protection elicited by gel fractions in monkeys. This possibility, however, can be ruled out by two kinds of evidence: (*i*) no antibodies against *Saimiri* erythrocytes were ever detected by immunofluorescence assay in antiserum of the immunized monkeys and (*ii*) no significant anemia was developed by vaccinated animals as compared to controls before challenge.

Thus, the protective response observed in monkeys seems, indeed, to be induced by actual parasite proteins. We do not know, as yet, whether other proteins of *P. falciparum* recovered from gels can also induce immunoprotection, but the present results indicate that the protective response, at least at the humoral level, seems to be associated only with a specific response to parasitic components. It is a promising first step in the development of a malarial vaccine based on protein antigens synthesized either chemically or biologically using recombinant DNA techniques. Further experiments, however, are necessary to identify the immunogen(s) precisely as well as to establish the vaccination potential of the different fractions against heterologous strains.

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