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Plasmodium falciparum: Protein Antigens Identified by Analysis of Serum Samples from Vaccinated *Aotus* Monkeys

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Serum samples from *Aotus trivirgatus* subsp. *griseimembra* monkeys obtained at different stages of a vaccination experiment were analyzed for total antibody titer to *Plasmodium falciparum* and were used for identifying protective antigens of the human malaria parasite. Total malarial antibody titers were higher in serum samples from protected monkeys (vaccinated with antigen in an adjuvant) than in those from unprotected monkeys (vaccinated with either antigen or adjuvant only). Parasite proteins were labeled with [^{3}H]isoleucine, solubilized with nonionic detergent, and reacted with immune *Aotus* sera. Immunoprecipitates obtained were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Thirteen protein antigen bands in the molecular weight range 73,000 to 180,000 were resolved. Serum samples obtained from protected *Aotus* monkeys reacted more intensely with these proteins than samples from unprotected monkeys did. Evidence is presented that the protective antigen is not a single, normally nonimmunogenic, protein that is recognized only in protected monkeys. Rather, the present data indicate that a heightened immune response to multiple proteins correlated with in vivo protection to *P. falciparum* in *Aotus* monkeys. This finding may have a significant bearing on strategies for the development of a human *P. falciparum* vaccine.

Success in immunizing *Aotus trivirgatus* subsp. *griseimembra* monkeys against *Plasmodium falciparum* has laid the foundation for developing a human malaria vaccine (15, 19–21, 23). The vaccines used in such studies are composed of the whole parasite, isolated from asexual blood stages of *P. falciparum*, and an adjuvant. One of the potential problems in using such a preparation as a human vaccine is the difficulty of removing host erythrocytic material which may induce autoimmune responses. Thus, recent studies have concentrated on identifying protective antigens that may be purified and used as a vaccine for *P. falciparum*. The validity of this approach has been demonstrated in some work on nonhuman malaria parasites. Monoclonal antibodies directed toward surface antigens of sporozoites have been found to neutralize the infectivity of *Plasmodium berghei* and *Plasmodium knowlesi* sporozoites (6, 26). A cDNA clone which expresses one of these surface antigens in *P. kowlesi* has been isolated (8). This points to the possibility of producing a sporozoite vaccine by recombinant DNA technology. A protein antigen isolated from the late asexual erythrocytic stages of *Plasmodium yoelii*, when given in Freund complete adjuvant, has been found to induce protection against infection by the same rodent malaria parasite (9).

A number of workers have attempted to use human serum samples obtained from malaria-endemic areas for identifying protective antigen(s) of the asexual blood stages of *P. falciparum* (3, 4, 10, 12, 17, 18). The difficulties in using human serum samples for antigen identification are many. Very often, the clinical history of the patient is obscure, and the effect of drug treatment on the antibody response is hard to evaluate. Preimmune serum samples from the same patients as proper controls are usually not available. Attempts have been made to try to correlate the inhibition of in vitro growth of the parasite with antibody reactivity to possible protective antigens. However, the level of inhibitory activity as demonstrated in vitro does not necessarily reflect ability to inhibit infection of live parasites, as has been shown in studies with monkeys (5). Serum samples from *Aotus* monkeys that survived many challenges with *P. falciparum* have been used in one study, but preimmune serum samples from the same animals as controls were lacking (18).

In the study reported here, we have analyzed serum samples from *Aotus* monkeys obtained during a successful vaccination experiment (21). The serum samples from protected and unprotected monkeys were used to immunoprecipitate biosynthetically labeled protein antigens from *P. falciparum*. The unique features of the present work are the availability of preimmune serum samples as well as serum samples at different stages of vaccination and the fact that the in vivo protection of the monkeys against challenge with live parasites was documented.

MATERIALS AND METHODS

***Aotus* serum samples.** In a vaccination experiment reported earlier (21), *Aotus* monkeys were immunized 3 weeks apart with two doses of a vaccine prepared from schizont stage-enriched *P. falciparum*. Two monkeys, A299 and A305, were given only the parasite, and one monkey, A300, was given only CP 20,961 (Pfizer Inc., New York, N.Y.) adjuvant. Other monkeys were immunized with the parasite in conjunction with Pfizer adjuvant (A297, A320, and A322), stearoylmuramyl dipeptide (A310 and A321), or Freund complete adjuvant (A301). All animals given the vaccine with adjuvants (protected) survived the challenge with live parasites, whereas the other three monkeys (unprotected) died. Serum samples were obtained from each monkey 30 days before the first vaccination (preimmune serum samples), 14 days after the first vaccination (primary serum samples), and 14 days after the second vaccination (secondary serum samples). Postchallenge serum samples were obtained from protected monkeys 52 days after challenge. In those monkeys, the parasitemia became negative, and he-

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matocrit levels returned to normal, between 26 and 46 days after challenge.

Determination of total malaria antibody titers. Both the indirect immunofluorescent antibody (IFA) assay and the radioimmunoassay (RIA) were used. The IFA test was carried out as described by Voller and Bray (24) with acetone-fixed whole infected erythrocytes as the antigen. The titer was defined as the reciprocal of the highest dilution of serum showing 2+ fluorescence. The RIA was developed in this laboratory by using whole infected erythrocytes as the antigen. Briefly, 96-well microtiter plates were first coated with poly-L-lysine (molecular weight, 150,000 to 300,000; Sigma Chemical Co., St. Louis, Mo.). Then, 10^6 *P. falciparum*-infected erythrocytes enriched in the schizont stage by Percoll density gradient centrifugation (13) and suspended in 50 μl of phosphate-buffered saline (PBS) were added to each well of the microtiter plate and incubated overnight. Infected erythrocytes immobilized on the plates were fixed and rendered permeable by treatment at 4°C with 1% formaldehyde in PBS for 10 min, followed by a 10-min incubation with absolute methanol. Unreactive sites were blocked with a 1% bovine serum albumin solution. At least six fivefold dilutions of each serum (50 μl) were placed in the wells in duplicate or triplicate and were allowed to incubate for 1 h. Rabbit anti-monkey immunoglobin serum (Cappel Laboratories, Cochranville, Pa.) diluted 1:600 was used as the second antibody. Protein A (Pharmacia Fine Chemicals, Piscataway, N.J.) was iodinated with $Na^{125}I$ by using lactoperoxidase and glucose oxidase immobilized on hydrophilic beads (enzymobeads; Bio-Rad Laboratories, Richmond, Calif.) as recommended by the manufacturer (1). To each well, 12.5 nCi of ^{125}I-labeled protein A was added. After washing, bound radioactivity was counted in a Beckman Gamma 4000 with a DP-5000 data reduction system. The RIA titer was defined as the reciprocal of the serum dilution binding 20% of the input ^{125}I-labeled protein A. A detailed report of the sensitivity and reproducibility of this assay will be published separately.

In vitro culture of *P. falciparum* for metabolic labeling. *P. falciparum*, Uganda-Palo Alto strain, was grown in RPMI-1640 tissue culture medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% human type O^+ serum and 5% hematocrit of human erythrocytes as reported by Siddiqui and Palmer (22).

Metabolic labeling of *P. falciparum* cultures with [^{3}H]isoleucine. In vitro parasite cultures with parasitemias higher than 10% were used for labeling. Infected erythrocytes were washed with RPMI-1640 isoleucine-free medium, and then L-[4,5-^{3}H(N)]isoleucine, specific activity 75 to 105 Ci/mmol (New England Nuclear Corp., Boston, Mass.), was added to the isoleucine-free medium supplemented with 10% human serum. The ratio of added [^{3}H]isoleucine to parasite culture was 1 mCi/0.5 ml of packed erythrocytes, and the duration of labeling was 6 h. In the study of optimal duration of labeling, durations of 0.5, 2.0, 4.0, and 24.0 h were used.

Extraction of labeled parasite proteins and their immunoprecipitation by *Aotus* serum samples. At the end of the labeling period, infected erythrocytes were harvested and washed three times with PBS. After three freeze-thaw cycles, 7 volumes of lysing buffer was added, and the mixture was incubated for 1 h at room temperature. The composition of the lysing buffer was Tris-hydrochloride, 50 mM (pH 8.0); Nonidet P-40, 0.5%; EDTA, 5 mM; iodoacetamide, 5 mM; antipain, 10 ppm (μg/ml); and pepstatin A, 10 ppm (μg/ml). The lysate was clarified by centrifuging in a Beckman L2-65B ultracentrifuge at 80,000 × *g* for 1 h. All procedures were carried out aseptically, and the labeled lysates were stored at 4°C. *Aotus* serum (15 μl) was added to 4 μCi of lysate and incubated at room temperature with constant shaking for 1 h. To each sample, 0.1 ml of a 20% suspension of protein A-bearing *Staphylococcus aureus* cells (Enzyme Center Inc., Boston, Mass.) in PBS was added by

TABLE 1. IFA titers of serum samples from control and vaccinated *Aotus* monkeys[a]

Monkey	Serum titer[b]			
	Preimmune	Primary	Secondary	Postchallenge
A300	<32	<64	<64	NA[c]
A299	<32	<64	128	NA
A305	<32	<64	512	NA
A297	<32	512	4,096	NA
A320	<32	64	2,048	4,096
A322	<32	128	1,024	1,024
A310	<32	128	2,048	4,096
A321	<32	128	1,024	4,096
A301	<32	256	2,048	8,192

[a] Control (unprotected) monkeys were given antigen alone (A299 and A305) or CP 20,961 adjuvant alone (A300). Protected animals were given antigen with CP 20,961 adjuvant (A297, A320, and A322), antigen with stearoylmuramyl dipeptide adjuvant (A310 and A321), or antigen with Freund complete adjuvant (A301). All monkeys were challenged with live parasites. For details, see Siddiqui et al. (21).

[b] The titer was defined as the reciprocal of the highest dilution of serum showing 2+ fluorescence. Acetone-fixed, whole, infected erythrocytes were used as the antigen by the method of Voller and Bray (24). Serum samples were obtained before vaccination (preimmune), after the first vaccination (primary), after the second vaccination (secondary), and after challenge with live parasites (postchallenge).

[c] NA, Not available.

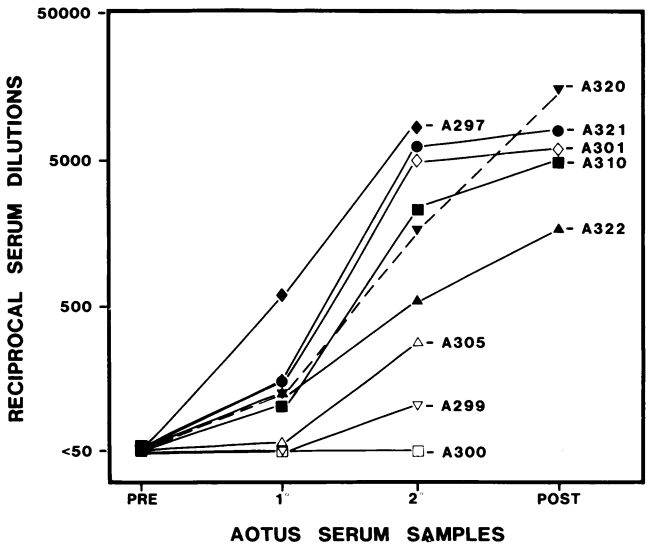

FIG. 1. Antibody response to intact *P. falciparum* in vaccinated *Aotus* monkeys as determined by RIA. The numbers beside each curve refer to the *Aotus* monkeys from which serum samples were obtained as described in Table 1, footnote *a*. The antibody titer given on the ordinate represents the reciprocal serum dilution that binds 20% of the input ^{125}I-labeled protein A as described in the text. The abscissa indicates the serum samples obtained at different times for each monkey. Abbreviations: PRE, preimmune; 1°, primary; 2°, secondary, POST, postchallenge.

the method of Kessler (11). After samples were washed three times with PBS containing 0.5% Nonidet P-40 and 0.1% bovine serum albumin, 200 μl of eluting buffer was added to each sample, and the samples were extracted at 100°C for 90 s by the method of Laemmli (14). Radioactivity was counted on a Packard Tri-Carb liquid scintillation spectrometer with a commercial scintillation cocktail, Biofluor (New England Nuclear Corp.).

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) was carried out in a 7.5% separating gel in a discontinuous buffer system as described by Laemmli (14). A vertical slab gel apparatus (Bio-Rad Laboratories) and a power supply (model ECPS 3000/150, equipped with a volt-hour integrator VH-1; Pharmacia Fine Chemicals) were employed. A constant current of 5 mA per slab was used, and each run was terminated when the cumulative value of volt-hours reached 900. Molecular weight standards used were ^{14}C-labeled myosin (200,000), phosphorylase b (97,000), ovalbumin (46,000), and carbonic anhydrase (30,000), supplied by New England Nuclear Corp.

The gel slabs were impregnated with 2,5-diphenyloxazole and dried in a vacuum gel dryer model SE 1125B (Bio-Rad Laboratories) for fluorography as described by Bonner and Laskey (2). Kodak X-O-Mat AR films and film casettes with calcium tungstate intensifying screens (Eastman Kodak Co., Rochester, N.Y.) were used. Each lane of the fluorograms was scanned on a Gilford spectrophotometer model 2400-S equipped with a linear transport (Gilford Instrument Lab., Oberlin, Ohio) at 500 nm to assess the distance of migration of each band more accurately. Molecular weights of the bands were estimated by the method of Weber and Osborn (25).

RESULTS

Antibody titers of *Aotus* serum samples obtained at different stages of a vaccination experiment. Total malaria antibody titers were determined by the IFA test (Table 1) and by the RIA (Fig. 1). Both tests used intact parasites as antigen. The results show that higher malaria antibody titers were elicited in primary and secondary serum samples from protected monkeys than in those from unprotected monkeys. Further-

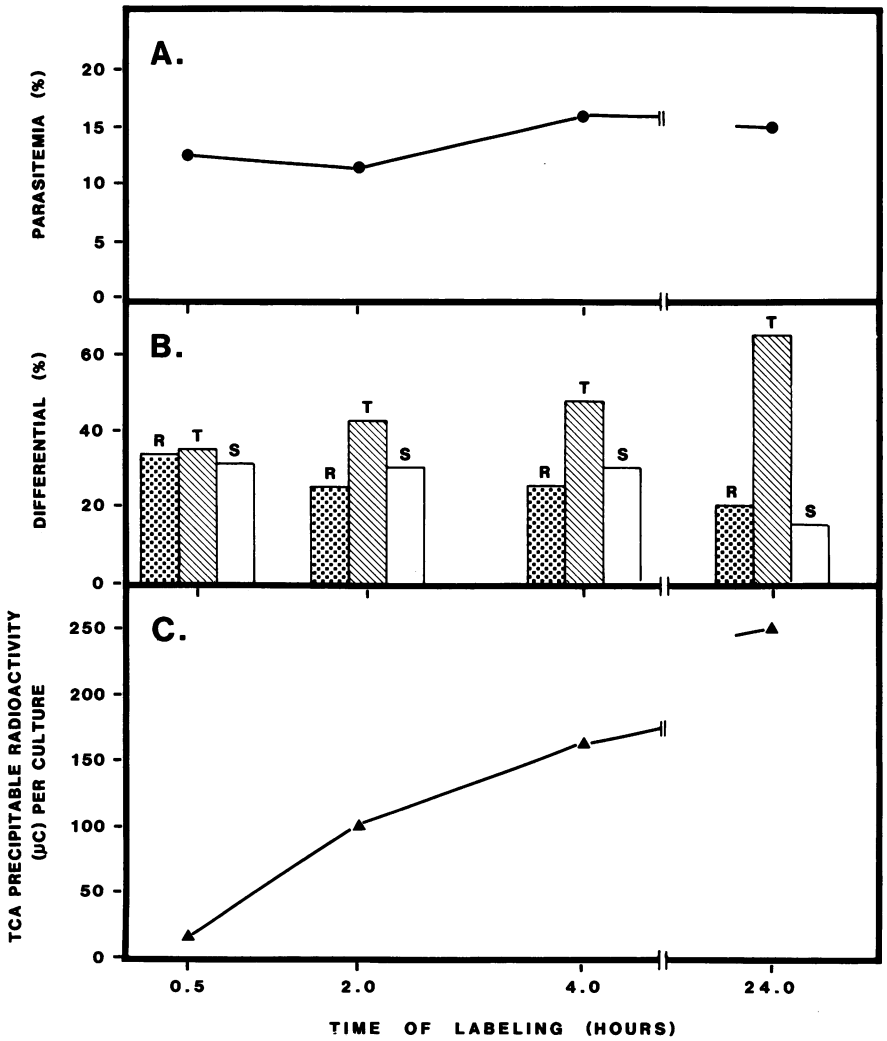

FIG. 2. Metabolic labeling of *P. falciparum* cultures with [^{3}H]isoleucine. (A) Changes in the percentage of parasitemia in the course of labeling. (B) Histograms indicating changes in differentials: R, Ring stage; T, trophozoite stage; S, schizont/segmenter stage. Parasitemias and differentials were determined microscopically by counting thin film slides of infected erythrocytes in Leishman stain. (C) Increase in incorporated trichloroacetic acid (TCA)-precipitable radioactivity with time of labeling. Curves in (A) and (C) are broken between 4 and 24 h.

TABLE 2. Immunoprecipitation of [^{3}H]isoleucine-labeled *P. falciparum* proteins by serum samples from *Aotus* monkeys

Monkey[b]	% of labeled proteins immunoprecipitated by serum sample[a]:			
	Preimmune	Primary	Secondary	Postchallenge
A300	1.5	1.3	1.3	NA[c]
A299	1.4	1.4	3.0	NA
A305	1.5	1.6	2.8	NA
A297	1.3	3.4	8.6	NA
A320	1.3	2.8	7.7	9.6
A322	2.5	2.6	4.8	8.1
A310	2.0	2.3	4.5	7.9
A321	1.8	2.0	3.9	5.9
A301	2.8	2.9	6.2	10.7

[a] The percentage of protein was calculated as [(counts per minute eluted from *S. aureus*)/(counts per minute precipitable by trichloroacetic acid)] × 100. For description of serum samples, see Table 1, footnote *b*.

[b] Monkeys were treated as described in Table 1, footnote *a*.

[c] NA, Not available.

more, antibody titers in secondary serum samples from all of the monkeys given the parasite antigen were considerably higher than those of primary serum samples. Antibody titers of postchallenge serum samples were, in general, higher than those of the secondary serum samples from the same protected monkeys.

Biosynthetic labeling of *P. falciparum* by [^{3}H]isoleucine. To determine the optimal labeling conditions for in vitro-cultured *P. falciparum*, parasitized erythrocytes were cultured in the presence of [^{3}H]isoleucine for 0.5 to 24.0 h. Throughout this time period, the parasitemia (12 to 16%) remained essentially unchanged (Fig. 2A). The differential percentages of the various blood stages remained similar up to 4 h, but the number of parasites in the trophozoite stage increased from 35 to 65% at the end of 24 h (Fig. 2B). The uptake of [^{3}H]isoleucine increased rapidly for the first 4 h (Fig. 2C), but further incubation of the parasites to 24 h led to only a slight additional incorporation of radioactivity. Thus, a labeling time of 4 to 6 h was selected for optimal incorporation.

Immunoprecipitation of [^{3}H]isoleucine-labeled proteins. Protein antigens recognized by *Aotus* serum samples were immunoprecipitated from [^{3}H]isoleucine-labeled, Nonidet P-40-solubilized parasite proteins. All of the immunoprecipitations were performed under conditions in which the antibody was limiting. Earlier experiments established that linearity of the precipitated radioactivity occurred when up to three times the amount of *Aotus* antiserum was used. No major differences were noted when rabbit anti-monkey serum was incorporated as a second antibody, except that the protein concentration in the immunoprecipitate was greatly increased, causing distortions in SDS-PAGE patterns. Thus, the second antibody was not used routinely when the immunoprecipitates were subjected to SDS-PAGE. The percentage of labeled proteins immunoprecipitated with each of the serum samples (Table 2) gave a pattern similar to that expected from the IFA and RIA titers. More proteins were precipitated with secondary serum samples than with primary samples, and serum samples from protected monkeys precipitated more protein than samples from unprotected monkeys did. Quantitatively, postchallenge serum samples precipitated greater amounts of protein than the corresponding secondary serum samples.

SDS-PAGE patterns of immunoprecipitates. The protein antigens recognized by each of the serum samples were separated by SDS-PAGE according to relative molecular weights. Each lane of the fluorograms (Fig. 3) was scanned, and representative lanes are shown in Fig. 4. A comparison of the protein pattern of the lysate with that of immunoprecipitates of postchallenge serum samples from protected monkeys (top row of Fig. 4) showed that the immunogenic proteins that migrated in the molecular weight regions labeled a, b, c, e, g, i, and j were present in relatively low amounts in the lysate. Proteins that migrated in region d were invariably recognized by all immune serum samples and were even detected by primary serum samples from unprotected monkeys given a single dose of antigen (A299 and A305). Band l was prominent only in immunoprecipitates of monkey A301 and was probably obscured by bands k and m in the lysate. Other proteins that migrated in the lower-molecular-weight regions n to u were not schizont stage specific (unpublished data).

Immunoprecipitates from preimmune serum samples were used to indicate proteins that were bound nonspecifically under experimental conditions. Nonspecific binding occurred mainly in regions d, h, k, and m, which coincided with prominent bands in the total lysate. The recorder scans of individual lanes gave a quantitative assessment of the antibody response to different protein antigens by analysis of peak areas. Low reactivity was found in primary serum samples. Higher reactivity was observed with the corresponding secondary serum samples. Likewise, the postchallenge serum samples reacted more intensely with each of the parasite proteins than the secondary samples did. Comparison of protected and unprotected monkeys revealed higher

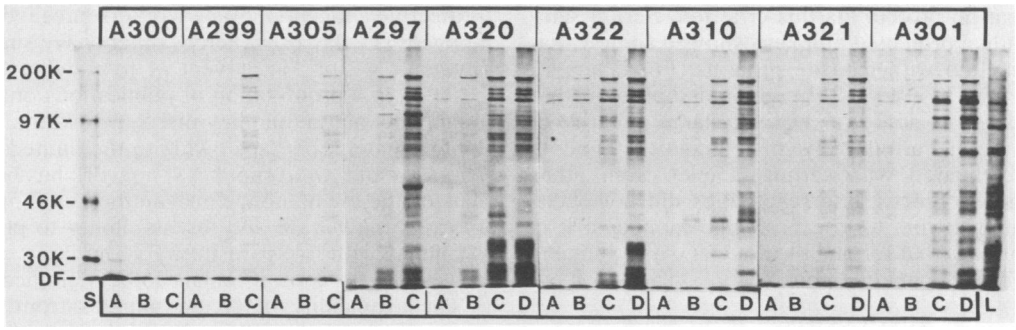

FIG. 3. SDS-PAGE of *P. falciparum* proteins. The proteins, labeled with [^{3}H]isoleucine, were immunoprecipitated with serum samples from unprotected (A300, A299, and A305) and protected (A297, A320, A310, A321, and A301) *Aotus* monkeys, analyzed by SDS-PAGE in 7.5% gel, and visualized by fluorography. Lanes show proteins immunoprecipitated with preimmune (A), primary (B), secondary (C), and postchallenge (D) serum samples. S, Molecular weight standards where K = 10^3; L, total parasite lysate; DF, dye front.

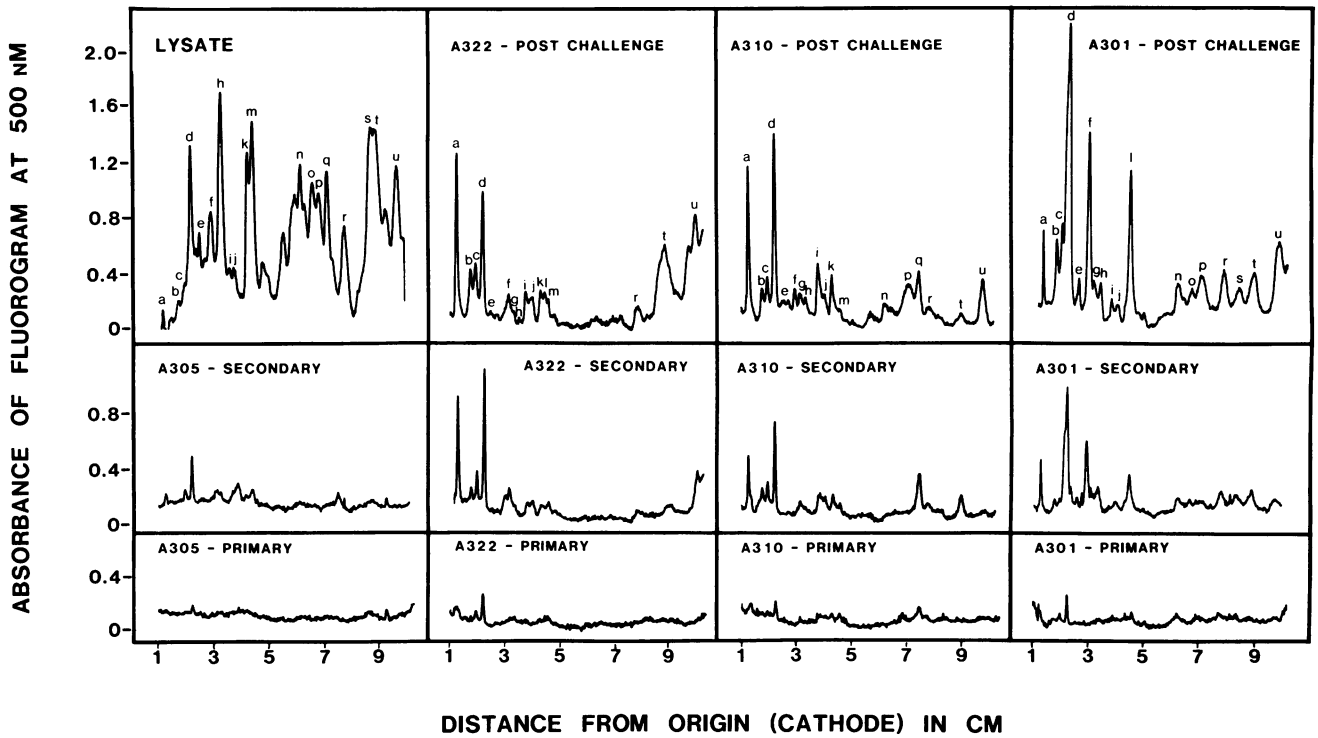

FIG. 4. Densitometric scans of representative fluorograms from Fig. 3. The [^{3}H]isoleucine-labeled lysate before immunoprecipitation and immunoprecipitates from an unprotected monkey (A305) and three protected monkeys (A322, A310, and A301) obtained from primary, secondary, and postchallenge serum samples are shown as indicated. Relative molecular weights ($\times 10^3$) of 13 parasite protein bands recognized by *Aotus* serum samples were: a, 180 ± 1.9; b, 152 ± 1.3; c, 143 ± 1.2; d, 132 ± 1.3; e, 121 ± 1.6; f, 107 ± 1.5; g, 102 ± 0.85; h, 95.9 ± 1.1; i, 86.4 ± 1.7; j, 82.7 ± 1.6; k, 77.4 ± 1.5; l, 75.0 ± 0.30; and m, 73.3 ± 1.5. The positions of these bands are also indicated in graphs for the lysate.

reactivity in serum samples from protected monkeys to each of the recognized protein antigens. Estimated molecular weights of bands are given in the legend to Fig. 4.

The next question we asked was whether there was a unique antigen recognized by all serum samples from protected monkeys but not by those from unprotected monkeys. To address this question directly, differences in antibody titers between serum samples from unprotected and protected monkeys were negated by applying the same number of immunoprecipitated counts from each of the secondary serum samples to SDS-PAGE (Fig. 5). If a unique, normally nonimmunogenic protein responsible for protection existed, it would be recognized by all serum samples from protected monkeys but not by those from unprotected monkeys. The results show that no protein fits this criterion. Serum samples from individual protected monkeys showed some variation in their reaction with parasite proteins. For example, serum samples from monkey A301 precipitated proteins that formed a doublet in region d, a relatively large quantity of proteins in band l, and a relatively small amount in bands k and m when compared with serum samples from other protected monkeys. Since these reactivities did not occur with serum samples from all the protected monkeys, it is unlikely that antigens that formed the doublet d and antigens in l were responsible for protection.

DISCUSSION

Development of a malaria vaccine by using a purified parasite antigen against *P. falciparum* will involve three initial stages: (i) identification and characterization of parasite antigens, (ii) purification of individual antigens, and (iii) testing the ability of purified antigens to protect against an in vivo challenge in experimental animals.

Thus far, a protein antigen isolated by affinity chromatography from the rodent malaria parasite *P. yoelii* has been found to protect mice against challenge with the same live parasite (9). In work on *P. falciparum*, progress has been relatively slow because of the technical difficulties in obtaining large amounts of parasites, the limited supply of *Aotus* monkeys, and the unavailability of proper hyperimmune serum samples for antigen identification and characterization. The use of human serum samples, although plagued with difficulties (see above), has led to some interesting findings leading toward the identification of *P. falciparum* protective antigen(s). Some workers have indicated multiple antigens (12, 17, 18), whereas others have suggested a single antigen (3, 10). The advent of hybridoma technology has resulted in a proliferation of publications on the production of monoclonal antibodies directed towards *P. falciparum*. Unfortunately, perhaps owing to the limited supply of both parasites and *Aotus* monkeys, no work has been reported to date on the use of monoclonal antibodies to purify an antigen from *P. falciparum* and test its ability to protect against a challenge with live parasites. By and large, protective antigens of *P. falciparum* remain to be identified.

The availability of serum samples from experimentally vaccinated *Aotus* monkeys has given us a unique opportunity to attempt to identify protective antigens of *P. falciparum*. Serum samples from protected and unprotected monkeys were analyzed by different immunoassays and by immuno-

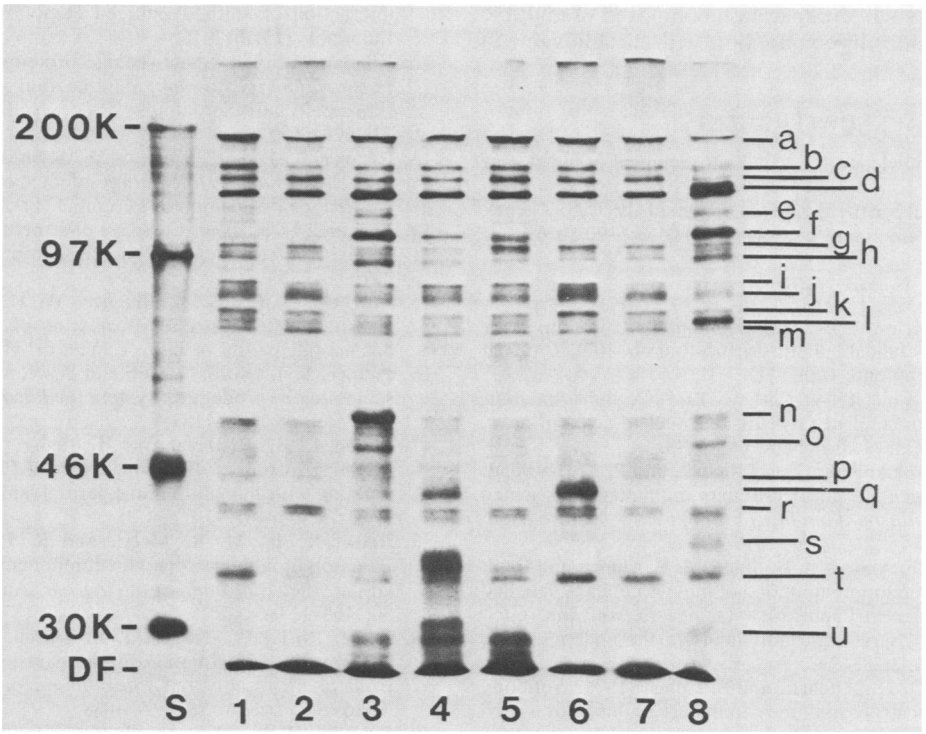

FIG. 5. [^{3}H]-labeled proteins of *P. falciparum* immunoprecipitated with secondary serum samples from vaccinated *Aotus* monkeys, analyzed by SDS-PAGE, and visualized by fluorography. Samples 1 through 8 were adjusted to consist of 54 nCi of immunoprecipitates obtained by using secondary serum samples from monkeys A299, A305, A297, A320, A322, A310, A321, and A301, respectively. S, ^{14}C-labeled protein standards; DF, dye front. Molecular weights of bands a through m are as given in the legend to Fig. 4.

precipitation of labeled parasite proteins. The fact that antibody titers were higher in secondary than in primary serum samples demonstrated that two doses of the vaccine were required to induce a strong antibody response. The fact that antibody titers were higher in primary and secondary serum samples from protected monkeys than in those from unprotected monkeys indicates that antibodies correlate with immunity to *P. falciparum*. The relative contributions of humoral and cell-mediated immune responses to malaria have not been fully evaluated, but our results substantiate earlier findings that the antibody response is important in protection (7). The antibody responses to individual parasite proteins of differing molecular weight in protected and unprotected monkeys were compared by SDS-PAGE. The use of preimmune serum samples from individual monkeys was a critical control in this analysis because the length of exposure of the fluorograms should minimize nonspecific reactivity but allow for the detection of minor, but potentially important, protein antigens. Densitometric tracings of SDS-PAGE patterns obtained from immunoprecipitates performed at antibody excess showed that the most striking difference between serum samples from protected and unprotected monkeys was that the former had a higher reactivity to all of the identified protein antigens. Surprisingly, when differences in antibody titer between protected and unprotected monkeys were negated by applying equal numbers of immunoprecipitated counts, there was no evidence of a unique antibody response to an individual parasite protein in protected monkeys. Obviously, protein with a very low isoleucine content, a very low turnover rate, or a very low solubility in Nonidet P-40 will escape detection. Polysaccharide or lypopolysaccharide antigens will not be detected either. However, neither the use of another labeled amino acid, [^{35}S]methionine, nor the use of a sugar stain, periodic acid-Schiff reagent, has shown a unique antigen in serum samples from protected monkeys (unpublished data).

It should be noted that there are differences between *Aotus* monkeys and humans in the degree and extent of acquired immunity to *P. falciparum*. In *Aotus* monkeys, the acquired immunity is long lasting, whereas in humans it is transient. For this reason the use of *Aotus* serum samples is preferable to the use of human serum samples for the identification of possible protected antigens. Serum samples from *Aotus* monkeys known to be persistantly protected are more likely to contain antibodies that react with protective antigens. Owing to the transient nature of immunity to *P. falciparum* in humans, it is uncertain whether hyperimmune human serum samples would contain such antibodies. Obviously, extrapolation of data from *Aotus* monkeys to human requires further confirmation in future clinical trials.

Our findings suggest that multiple antigens may be required to induce protective immunity. This has a significant bearing on the rationale for the development of a safe and effective human malaria vaccine. The only direct test to establish that a purified antigen preparation can induce protective immunity is to immunize and challenge experimental animals. With recent advances in the large-scale in vitro production of *P. falciparum* (16) sufficient amounts of a pool of antigens recognized by *Aotus* immune serum samples can be prepared by affinity chromatography. Thus, a prudent

approach is to test such a preparation composed of multiple antigens as a vaccine, in conjunction with an adjuvant, in *Aotus* monkeys.

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