INFECTION AND IMMUNITY, June 1997, p. 2329–2338 Vol. 65, No. 6
0019-9567/97/$04.00+0

Assessment of the Humoral Immune Response against *Plasmodium falciparum* Rhoptry-Associated Proteins 1 and 2

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Received 15 November 1996/Returned for modification 28 January 1997/Accepted 24 March 1997

Naturally occurring antibody responses to *Plasmodium falciparum* rhoptry-associated proteins 1 and 2 (RAP-1 and RAP-2) were measured with recombinant and parasite-derived forms of the antigens. For comparative purposes, responses to multiple forms of three other malarial antigens were also examined. The sera of 100 Papua New Guineans were screened for antibodies. Eighty-six and 82% of individuals over 30 years of age had antibodies that recognized parasite-derived RAP-1 and RAP-2, respectively. Importantly, we found that recombinant and native antigens share linear epitopes seen by the human immune system; thus, the recombinant proteins may be adequate human immunogens. However, antibodies affinity purified on recombinant RAP-1 reacted with other antigens in addition to parasite-derived RAP-1. Thus, the antigenicity of RAP-1 may have been overestimated previously. The recognition of RAP-1 and RAP-2 correlated with age and with the recognition of recombinant forms of the ring-infected erythrocyte surface antigen, merozoite surface protein 1, and merozoite surface antigen 2 (MSA2) antigens. Antibodies to these antigens appear to be generated in response to the total exposure to malaria of the host. Antibodies to conserved regions of MSA2 had stronger correlations with both age and the recognition of other antigens than did the full-length recombinant MSA2 molecule. In contrast to results with the other antigens, there was no significant difference in the ages of individuals with a certain antibody titer to the full-length recombinant or parasite-derived MSA2 molecule, but antibodies to these two antigens did correlate with parasitemia. For all antigens tested, antibody levels after two infections can approach the peak levels of antibodies obtained in immune individuals.

Several proteins have been identified from the rhoptry organelles of *Plasmodium falciparum*. These include two protein complexes, a high-molecular-mass complex, RhopH (containing 140-, 130-, and 110-kDa proteins [4, 10]), and a low-molecular-mass complex, QF3 (5, 33). The second complex consists of two proteins, namely, rhoptry-associated proteins 1 and 2 (RAP-1 and RAP-2, respectively) (27).

RAP-1 and RAP-2 are considered candidate antigens for a subunit malaria vaccine. Monoclonal antibodies (MoAbs) directed against RAP-1 and RAP-2 can give substantial inhibition of parasite invasion in vitro (8, 15, 20, 26, 33). Three of four *Saimiri scureus* monkeys immunized with a mixture of RAP-1 and RAP-2 isolated from the K1 line of *P. falciparum* were protected from a subsequent challenge with the Uganda Palo Alto line (28), with substantially lower peak levels of parasitemia and lower initial rates of growth than those of the control animals. Protection from *P. falciparum* challenge was also obtained in *Saimiri* monkeys immunized with a mixture of parasite aldolase, serine protease, and QF3 (25).

The genes coding for RAP-1 and RAP-2 have been cloned (29, 31), revealing no homology to other known genes. Further, RAP-1 and RAP-2 show minimal sequence polymorphisms between *P. falciparum* isolates (17, 19, 29, 31, 38). Thus, the antigenic diversity that presents a major difficulty for other malaria vaccine candidates may not be important for RAP-1 and RAP-2.

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Recombinant forms of both proteins have been produced (rRAP-1 and rRAP-2) and found to be immunogenic in animal models (37, 38). Immunization of mice and rabbits with denatured rRAP-1 and rRAP-2 produced polyclonal antibodies which recognized linear epitopes in the recombinant proteins and gave partial inhibition of parasite invasion in vitro. To determine if human antibodies resulting from a natural infection with *P. falciparum* also recognize linear epitopes within the recombinant proteins, this study assessed the antigenicities of these recombinant forms of RAP-1 and -2.

First, we examined the humoral responses to rRAP-1 and rRAP-2 in the sera of a population from an area of Papua New Guinea (PNG) where malaria is endemic. These responses were then compared to responses against parasite-derived forms of RAP-1 and -2 (herein designated *P. falciparum* RAP-1 and -2). Studies have been performed with recombinant forms of RAP-1 (18, 22) but never with parasite-derived forms or with either type of RAP-2. The two above-described studies found that following natural infection with *P. falciparum*, 29 of 30 immune and semi-immune donors had rRAP-1-reactive immunoglobulin G (IgG) with higher reactivity to the N-terminal 294 amino acids than to the C-terminal 466 amino acids. This reactivity was not directed against the serine repeat region (18). At least one T-cell epitope in the N-terminal 294 amino acids of rRAP-1 was recognized by 14 of 15 malaria-immune human donors (22).

Second, we established how quickly an antirhoptry protein response arises by examining the responses to both the recombinant and parasite-derived RAP-1 and RAP-2 proteins in the sera of volunteers undergoing a single defined *P. falciparum*

infection. The boosting effect on titers of antibodies of a subsequent infection was also examined.

Third, we assessed the relative antigenicities of rRAP-1 and -2 compared with those of other malarial vaccine candidates by examining the responses of the same PNG sera to three other malarial proteins. These were recombinant and parasite-derived forms of merozoite surface antigen 2 (MSA2 [36]), recombinant forms of merozoite surface protein 1 (MSP1 [16]), and a recombinant form of ring-infected erythrocyte surface antigen (RESA [11]). These three antigens are all potential vaccine candidates, on the basis of in vitro inhibition of erythrocyte invasion by antibodies raised against the molecules and by protection seen in vivo in primate and mouse models (21). RESA, MSA2, and MSP1 have been more extensively studied than RAP-1 and -2, making them ideal for comparative purposes.

MATERIALS AND METHODS

Parasite culture. *P. falciparum* clone 3D7 from the Netherlands isolate NF54 (44) was cultured in vitro as described previously (43).

PNG serum collection. Sera were collected from 100 individuals; 21 were 0 to 5 years old, 19 were >5 to 10 years old, 19 were >10 to 15 years old, 19 were >15 to 30 years old, and 22 were >30 years old. The collection was from 12 villages from the Madang province of PNG. *P. falciparum* is endemic in Madang, with perennial transmission peaking in the wet season (November to March). Sera were collected as part of a series of epidemiology studies (6) from June to August 1982 and stored as a reference collection at −70°C. At the time of collection, thick blood films were prepared, stained with 4% Giemsa stain, and scored for malaria parasites: a score of 0 indicated a negative test, 1 indicated <80 parasites/mm^3, 2 indicated 81 to 200 parasites/mm^3, 3 indicated 201 to 600 parasites/mm^3, 4 indicated 601 to 1,400 parasites/mm^3, 5 indicated 1,401 to 3,400 parasites/mm^3, 6 indicated 3,401 to 8,000 parasites/mm^3, 7 indicated 8,001 to 19,960 parasites/mm^3, 8 indicated >19,960 parasites/mm^3, and 9 indicated missing data. Serum samples were kindly supplied by PNG Institute of Medical Research.

Defined *P. falciparum* infections. Two volunteers, one with no previous malarial exposure and the other with a prior *Plasmodium vivax* infection, were infected by *Anopheles stephensi* (Dutch) mosquitoes carrying sporozoites of *P. falciparum* clone 3D7 as part of a project to develop a challenge system for the testing of malaria vaccines (7). The course of infection was allowed to run for 14 days for the first volunteer and 13 days for the second volunteer (parasite densities were 200 and 18 parasites per μl of whole blood at treatment, respectively. One unit of whole blood was taken from each volunteer prior to treatment and cryopreserved. Blood samples were taken daily, and the plasma was used for this study. One year following the initial sporozoite infection, both volunteers were rechallenged with a blood stage inoculum of 3,000 parasites prepared from their previous infection, and the infection was allowed to run for 8 days.

Antigen preparation and control antibodies. *P. falciparum* RAP-1 and *P. falciparum* RAP-2 were affinity purified from Percoll-purified schizonts of in vitro-cultured *P. falciparum* clone 3D7 with two MoAbs (7H8/50 for *P. falciparum* RAP-1 and 3A9/48 for *P. falciparum* RAP-2 [5, 33]). Briefly, 6 liters of synchronous cultures of clone 3D7-parasitized erythrocytes was purified on colloidal silica gradients (32), giving 2 ml of 90% 3D7 schizonts. Schizont antigen was extracted three times with Triton X-100 (5) to give a 30-ml antigen preparation. *P. falciparum* RAP-1 and *P. falciparum* RAP-2 were affinity purified from this preparation by three passages through an immunoaffinity absorbent solution prepared by coupling both MoAbs (7H8/50 and 3A9/48) to cyanogen bromide-activated Sepharose 4B (5). *P. falciparum* RAP-1 and *P. falciparum* RAP-2 were eluted in 0.1 M glycine, pH 2.5, and neutralized by the addition of Tris to 0.3 M. A final purification step used isotachophoretic nonreducing sodium dodecyl sulfate (SDS)-electrophoresis to separate the two proteins in the native complex both from each other and from contaminants (39). After elution from affinity columns, SDS was added to 1% (wt/vol) and the eluate was dialyzed into 150 mM Tris–0.01% (wt/vol) SDS (pH 7.5). This material was loaded onto a 3- by 500-mm 4% polyacrylamide column prepared in 150 mM Tris (pH 7.5). The trailing buffer was 127 mM Tris–90 mM glycine–0.01% (wt/vol) SDS, and the current flux was kept to 0.03 mA/mm^2 (39). This ensured that although they were denatured, *P. falciparum* RAP-1 and *P. falciparum* RAP-2 were not reduced.

rRAP-1 and rRAP-2. Purified rRAP-1 and rRAP-2 were identical to material used in animal immunogenicity studies (37, 38). Both proteins are expressed from *Escherichia coli* expression systems. rRAP-2 codes for the entire mature sequence of the FCQ27/PNG allele of RAP-2, while rRAP-1 codes for a shortened form of the mature sequence of the K1 allele of RAP-1, amino acids 23 to 608.

MSP1. Recombinant antigen MSP1 (rMSP1) (or antigen 190L [14]) contains amino acids 147 to 321 of the K1 allele of MSP1, equivalent to blocks 3 (conserved) and 4 (variant) (41). The recombinant protein was expressed in *E. coli* with two additional histidines on the N terminus and four additional histidines on the C terminus and linked to a 17-residue sequence encoding the universal T-cell epitope from the CS protein to give a molecular mass of 25,209 Da (14). A second recombinant form of antigen MSA1, expressed from yeast, yMSP1$_{19}$ (23), was supplied by David Kaslow (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.) and codes for the C-terminal 19-kDa fragment of MSP1 from the K1 allele. This molecule is correctly folded to duplicate the epidermal growth factor-like domains in native MSP1 as judged by the binding of conformationally dependent MoAbs. yMSP1$_{19}$ was identified by the MSP1 conformationally dependent MoAb 4H9/19 (9).

MSA2. Recombinant antigen MSA2 (rMSA2) (or antigen 1624 or protein Ro 46-2924 [1, 40]) is an *E. coli*-expressed recombinant protein containing nearly the entire length of the mature version of the 3D7 allele of MSA2, with a molecular mass of 25,606 Da. Parasite-derived MSA2 (*P. falciparum* MSA2) was not affinity purified, but comparison of the binding of anti-rMSA2 sera to *P. falciparum* schizont extracts of the 3D7 clone as analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting clearly identified the location of *P. falciparum* MSA2 (hence of the 3D7 allele) as one of the dominant bands recognized by human antisera to malaria. Recombinant antigen NC (rNC) also corresponds to clone 3D7's MSA2 but encodes only the N- and C-terminal portions of MSA2 totally conserved across alleles (amino acids 21 to 43 and 168 to 224 of the 3D7 sequence [26a]).

RESA. Recombinant antigen RESA (rRESA) (or antigen 1505H) is an 89-kDa *E. coli* recombinant form of RESA and corresponds to the C-terminal 771 residues of the RESA polypeptide from the FCQ27/PNG strain of *P. falciparum*, with a molecular mass of 89,357 Da (2). Mouse sera raised against rMSP1, rMSA2, and rRESA were supplied by David Pye (CSL Ltd., Melbourne, Australia) and used as positive controls.

Schizont antigen. *P. falciparum* clone 3D7 refers to the recognition of total parasite schizont antigen from the 3D7 clone grown in vitro and extracted into Triton X-100 (5, 33).

Supplier. Antigens rMSA2, rRESA, and rMSP1 were all supplied by Saramane Pty Ltd. (Melbourne, Australia) and were identical to material used in several phase I human immunogenicity trials (30a, 40).

Immunochemical techniques. (i) SDS-PAGE. SDS-PAGE and immunoblotting procedures were as described previously (12), with PNG serum at a dilution of 1/400 in phosphate-buffered saline (PBS), anti-human IgG (gamma chain-specific) secondary antibodies conjugated to alkaline phosphatase, and nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) used in the development reaction. Immunoblot strips incubated with individual serum samples contained the following amounts of antigen: 6.3 ng of rRAP-1; 37.5 ng of rRAP-2; 25 ng of rMSP1, rRESA, and rMSA2; 50 ng of *P. falciparum* RAP-1, *P. falciparum* RAP-2, yMSP1$_{19}$, and rNC; and 0.75 μl of *P. falciparum* clone 3D7 extract. After development, immunoblots were scored by eye in the range of 0 to 6 for signal strength to give immunoblot antibody units.

(ii) ELISAs. Enzyme-linked immunosorbant assays (ELISAs) were performed with Nunc Immunoplates (Maxisorp) coated with 50 ng of rMSP1, rRESA, or rMSA2 per well; 100 ng of rRAP-2 per well; 25 ng of rRAP-1, *P. falciparum* RAP-1, or *P. falciparum* RAP-2 per well; and 50 μl of a 1/200 dilution of *P. falciparum* 3D7 extract in PBS per well. Primary antibodies in a 1/100 or 1/400 dilution were plated out in triplicate (with a control series for every plate). Anti-human IgG (gamma chain-specific) secondary antibody conjugated to horseradish peroxidase with *o*-phenylenediamine dihydrochloride as a substrate was used for detection, and absorbances at 450 nm were read. A regression line was fitted to the control series for each antigen, and the average of the triplicate readings of optical density at 450 nm (OD$_{450}$) for each serum sample was converted to antibody units based on the standard curve. When end point titers in sera were required, serial dilutions were used and end points were calculated as the inverse of the serum dilution which gave an OD$_{450}$ of 0.1 above background. Positive control mouse sera were used in both immunoblots and ELISAs. This was especially important in immunoblots, where control strips were run every third strip to identify the antigens being recognized (e.g., *P. falciparum* MSA2).

Controls. For both immunoblot analyses and ELISAs, background levels of antibody were estimated with the sera of 26 nonexposed individuals from Brisbane, Australia. Positive signals for the PNG sera for immunoblot analyses and ELISAs were then considered those greater than the mean plus 2 standard deviations of quantitations of signals in the Brisbane sera.

Affinity purification of antibody. Recombinant antigens rRESA and rRAP-1 were reacted separately with CNBr-Sepharose 4B (30 or 50 μg per ml of resin) for 16 h at 4°C in 0.2 M $NaHCO_3$–0.5 M NaCl, pH 8.8. Columns were blocked for 4 h at 4°C with 0.2 M glycine (pH 8.0) and then washed with 10 column volumes each of 0.1 M CH_3COOH–0.5 M NaCl (pH 4.0), binding buffer, and PBS. Sera from 18 individuals from PNG were diluted to 1/50 in PBS, and 2 ml of each batch was bound to 350 μl of affinity column for 16 h at 4°C. Individual resins were poured into columns at 4°C (nonbound fractions were retained) and washed with 5 ml each of PBS, PBS–0.5 M total NaCl, and PBS again. Elution was at room temperature with 0.1 M glycine (pH 2.5) or 3 M $MgCl_2$. Eluted antibody solutions were immediately neutralized with 2 M Tris, dialyzed at 4°C for 16 h in PBS, and concentrated in Centricon 10 microconcentration tubes (Amicon, Beverly, Mass.).

Affinity purification was evaluated by performing standard and low-affinity

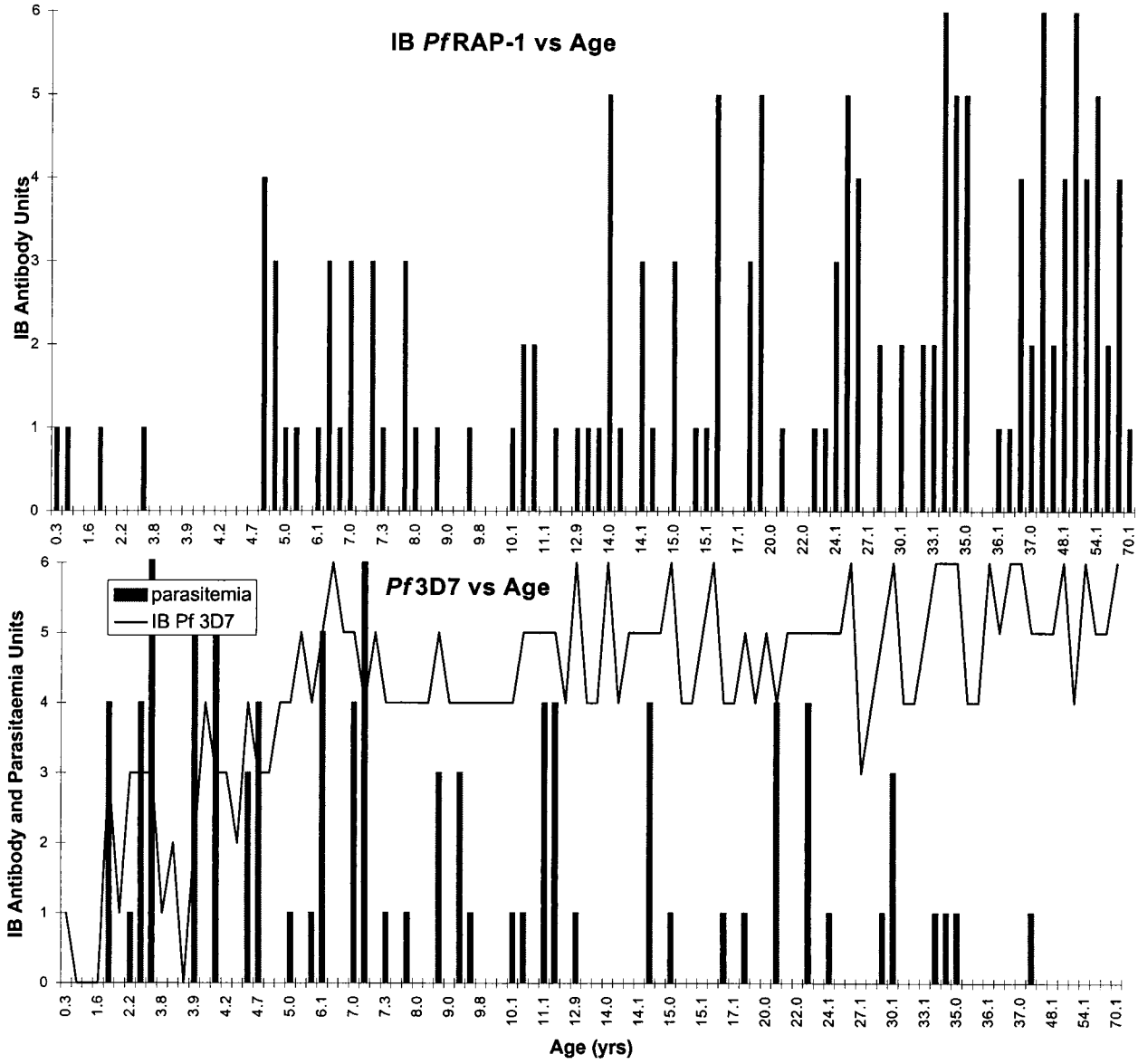

FIG. 1. Antibody responses of the sera of 100 individuals from PNG. Shown are antibody responses to parasite-derived RAP-1 compared to total amounts of schizont antigen, as measured by immunoblot analysis. The derivation of antibody units for the immunoblots (IB) and parasitemia are described in the text. *Pf*3D7, *P. falciparum* clone 3D7; *Pf*RAP-1, *P. falciparum* RAP-1.

Western blot experiments on *P. falciparum* clone 3D7 antigen extracts and on the recombinant protein used for purification. The eluted antibody was used either at an appropriate dilution without additions or after preincubation for 2 h at 4°C with either 2 μg of the recombinant protein used for purification or CNBr-Sepharose 4B preblocked with glycine. The conditions for low-affinity immunoblotting are described in reference 38.

Data analysis. Statistical analysis was performed with Epi Info version 6 to test by analysis of variance if there existed a significant difference in the ages of individuals with a certain discrete antibody level. The same test was used to analyze the ages of individuals when parasitemia was categorized. If age distribution was nonnormal, significance was tested by Kruskal-Wallis one-way analysis of variance. For these two tests, the resultant *P* value was multiplied by the number of tests performed, an application of the Bonferroni correction to avoid spurious values of *P*. The Wilcoxon rank sum two-sample test was used on contiguous sets of mean antibody levels. Spearman nonlinear correlation analyses were performed on sets of continuous data on antibody response to an antigen versus that to a second antigen or on age and level of parasitemia of the donor.

RESULTS

Antibody correlations with age. Immunoblotting and ELISAs were performed with the sera of 100 individuals from PNG against 11 recombinant and parasite-derived antigens. As an example, Fig. 1 summarizes the antibody responses of the 100 PNG sera to parasite-derived RAP-1 by immunoblotting. Also shown are the responses to total *P. falciparum* clone 3D7 schizont antigen and parasitemia. For both *P. falciparum* RAP-1 (Fig. 1) and *P. falciparum* RAP-2, the proportion of individuals with antibody to an antigen increased with age. In the age group of individuals over 30 years old, 19 of 22 individuals had antibodies to *P. falciparum* RAP-1 by immunoblot analysis and 18 of 22 had antibodies to *P. falciparum* RAP-2. Two of the three individuals in this age group who had

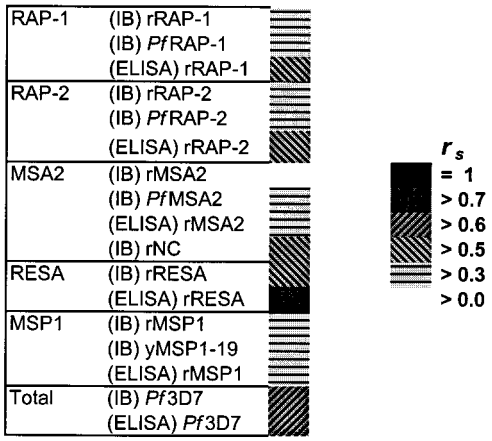

FIG. 2. Correlation of age with antibody response for 100 sera of individuals from PNG. Spearman correlation coefficients of donor age and antibody response to an antigen are shown. Antibody was measured by immunoblot analysis (IB) or ELISA. All correlation coefficients shown are significant ($P < 0.002$ for all antigens except rMSA2, where $P < 0.02$). The greater the r_s value, the higher the correlation between age and reactivity. *Pf*RAP-1 and -2, *P. falciparum* RAP-1 and -2, respectively; *Pf*MSA2, *P. falciparum* MSA2; *Pf*3D7, *P. falciparum* clone 3D7.

no detectable antibodies to *P. falciparum* RAP-1 also had no detectable antibodies to *P. falciparum* RAP-2, and the third individual had a weak reaction to *P. falciparum* RAP-2.

Establishment of the positive cutoff point as the mean plus 2 standard deviations of the scores of responses from the sera from 26 nonexposed individuals from Brisbane was not possible with the rRAP-1 immunoblots. Twenty-four of 26 of the Brisbane individuals were positive to rRAP-1 at a dilution of 1/400, with a mean scored signal strength of 2.2, giving a background signal cutoff of 5.3. This was 8.5-fold the background level of any other antigen. Consequently, immunoblot data for rRAP-1 was analyzed without the background signal being removed.

The proportion of individuals with antibody to an antigen increased with age for all antigens. To determine if this relation between age and antibody was significant, Spearman correlation analyses were performed on the sets of continuous data on antibody response to an antigen versus the age of the donor. Spearman correlation analyses were used, as the data distribution was nonnormal for both age and antibody responses (e.g., the mean age was 17.9 years, the median age was 13.0 years, and for antibodies to antigens rRAP-1 and rRAP-2 by ELISA, mean antibody units were 573.6 and 160.3, respectively, compared to median antibody units of 35.3 and 0). For Spearman correlation analyses, the greater the correlation coefficient (r_s), the higher the correlation between age and antibody reactivity. Antibody levels to all antigens positively correlated with age for both ELISA and immunoblot data (all P values were <0.002, except with antigen rMSA2, where P values were <0.02) (Fig. 2).

The strength of an antibody response to antigen rMSA2 was very high even in the youngest age group. Hence, the response had the lowest correlation coefficient ($r_s = 0.25$) and the lowest P value, and so its correlation with age was further analyzed by testing if there existed a significant difference in the ages of individuals with certain discrete antibody levels, that is, to see if older individuals had statistically higher antibody titers to rMSA2. The median ages of individuals with immunoblot signal scores of 0 to 6 against rMSA2 were not significantly different ($P = 1.0$ by analysis of variance). Nor were the median ages of individuals with immunoblot signal scores of 0 to 6 significantly different for antigens *P. falciparum* MSA2 ($P = 0.28$) and rMSP1 ($P = 0.56$). However, a similar analysis of immunoblot data for all other antigens did find a significant difference between the median ages of individuals with a particular immunoblot antibody response score of 0 to 6 (all P values were <0.05).

The correlations described above between the titer of antibody to an antigen and age may reflect an increase in the number of individuals with antibodies to that antigen with age or increases in the level of antibody response to that antigen with age. To distinguish between these two possibilities, median titers of antibodies to antigens for one age group (e.g., 0 to 5 years) were compared with those of a second age group (e.g., >5 to 10 years). This was done both for all individuals within an age group (where a statistical increase in a median titers of antibody might reflect an increase in numbers of individuals with antibodies and/or an increase in the level of antibody to that antigen) and for only those individuals within the age group with detectable antibodies to the antigen (where a statistical increase in the median titer of antibody to an antigen reflected a real increase in the titer of antibody to that antigen with age). Table 1 shows the results of Wilcoxon rank sum pairwise tests comparing the median antibody responses within age groups for all individuals with those for responders alone (here the term responders refers to individuals with antibodies at levels greater than the background for that antigen). The primary difference in the responses to most antigens in Table 1 is between the age groups 0 to 5 and >5 to 10 years. This is due to an increase in the number of responders, as there is no significant difference in the median levels of antibodies among responders alone. Exceptions are the responses to total *P. falciparum* clone 3D7 and antigen rMSP1, which show both an increase in the number of responders and an increase in the level of response. Conversely, the differences between the median responses in the older age groups reflect real increases in the levels of antibodies to the antigens.

Median titers of antibodies to most antigens thus show a steadily increasing trend among age groups, with a first significant increase between ages 0 to 5 and >5 to 10 years, due to an increase in the number of responders, followed by a second significant increase after 15 or 30 years of age, due to an increase in the level of antibody per responder (summarized in Fig. 3 for the immunoblot results for rRESA and *P. falciparum* RAP-2).

Antibody correlations with parasitemia. The percentage of parasitemic individuals decreased with increasing age, as did the level of parasitemia (Fig. 1). When parasitemia was categorized into three groups (above the mean level of parasitemia, above no parasitemia, and no parasitemia), the median age of individuals within each group increased significantly as parasitemia decreased ($P = 0.05$ by Kruskal-Wallis one-way analysis of variance).

As this was not a longitudinal survey, the effect of antibody in the sera cannot be assessed for its potential effect on future parasite growth. However, for both immunoblot and ELISA data, antibodies to antigens rMSA2 and *P. falciparum* MSA2 were significantly higher in parasitemic individuals in the age group 0 to 5 years than in nonparasitemic individuals (all P values were <0.02). This was also true for antigen rRESA in the >10- to 15-year olds ($P < 0.05$). Spearman correlation analyses also showed antigens rMSA2 (immunoblotting and ELISA), *P. falciparum* MSA2 (immunoblotting), rRAP-2 (immunoblotting), and $yMSP1_{19}$ (immunoblotting) to correlate positively with parasitemia (all P values were <0.04).

TABLE 1. Significant Wilcoxon rank sum pairwise test *P* values on median titers of antibodies in the sera of donors grouped by age[a]

Test	Antigen	No. of responders to indicated antigen/total no. of individuals in age group (yr):		P_n	P_m	No. of responders to indicated antigen/total no. of individuals in age group (yr):		P_n	P_m	No. of responders to indicated antigen/total no. of individuals in age group (yr):		P_n	P_m
		0–5	>5–10			>10–15	>15–30			>15–30	>30		
Immunoblot analysis	rRAP-1	—	—	—	—	19/19	19/19	0.04	0.04	—	—	—	—
	rRAP-2	—	—	—	—	—	—	—	—	7/19	15/22	0.10	1.0
	P. falciparum RAP-2	5/21	11/19	0.04	0.10	—	—	—	—	11/19	18/22	0.10	1.0
	rMSA2	16/21	19/19	0.02	0.20	—	—	—	—	—	—	—	—
	P. falciparum MSA2	14/21	18/19	0.01	0.10	—	—	—	—	—	—	—	—
	P. falciparum clone 3D7	17/21	19/19	0.0002	0.0002	—	—	—	—	—	—	—	—
	rRESA	10/21	16/19	0.02	0.10	—	—	—	—	15/19	22/22	0.0002	0.02
	rMSP1	9/21	10/19	0.20	0.02	—	—	—	—	11/19	16/22	0.04	0.02
ELISA	rRAP-1	—	—	—	—	16/19	18/19	0.02	0.04	18/19	19/22	0.10	0.01
	rRAP-2	—	—	—	—	—	—	—	—	13/19	17/22	0.10	0.02
	rMSA2	16/21	19/19	0.10	1.0	18/19	19/19	0.02	0.02	—	—	—	—
	rRESA	18/21	19/19	0.20	1.0	—	—	—	—	19/19	22/22	0.001	0.001
	rMSP1	14/21	17/19	0.10	1.0	—	—	—	—	17/19	22/22	0.04	0.10
	P. falciparum clone 3D7	20/21	19/19	0.04	0.10	19/19	19/19	0.04	0.04	—	—	—	—

[a] Shown are the results of comparing the median titer of antibody to an antigen from an age group (e.g., 0 to 5 years) with that of the next oldest age group (e.g., >5 to 10 years) to determine if there is a significant difference between the two by the Wilcoxon rank sum pairwise test. Probabilities are shown both for comparisons of the median titers of antibodies in sera from all individuals within an age group (P_n) and for comparisons of the median titers of antibodies in sera from only those individuals with a titer above background (P_m) (*m* signifies the number of responders to an antigen in an age group). —, no significance exists for this comparison (some nonsignificant results are shown when the results from tests performed with ELISA data disagree with those performed with the immunoblot data).

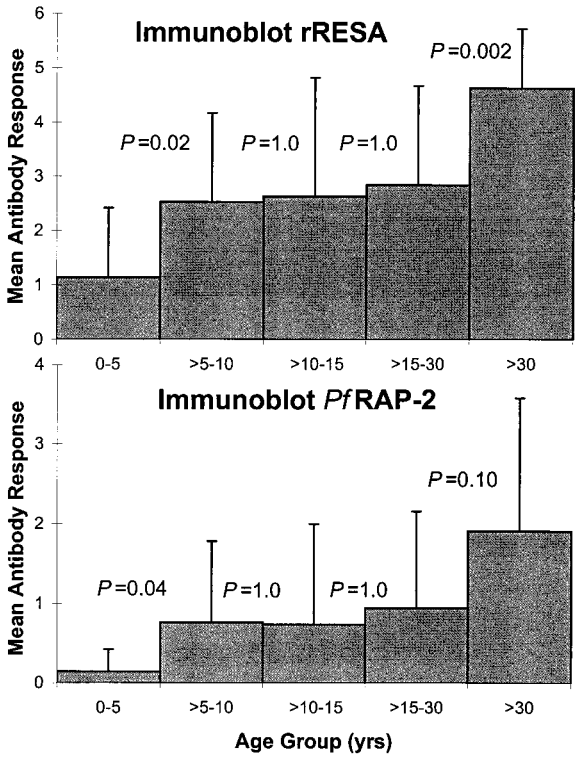

FIG. 3. Median titers of antibodies within an age group to the antigens rRESA and *P. falciparum* RAP-2 (*Pf*RAP-2) determined by immunoblot analysis. Probability values from Wilcoxon rank sum pairwise tests between median titers of adjacent age groups (e.g., 0 to 5 years with >5 to 10 years) are indicated.

Antibody correlations between antigens. The associations between antibody responses to one antigen and to the other antigens were also analyzed by Spearman correlation analyses (Fig. 4). The possible confounding effect that age may have had on correlations was controlled for by repeating the correlations within age groups (0 to 5, >5 to 10, >10 to 15, >15 to 30, and >30 years). Those antigens with the highest r_s value for the full correlation are also those antigens which gave significant correlation coefficients between antibody responses to different antigens in each of the five age groups.

For the recombinant and total *P. falciparum* clone 3D7 antigens, data from the two-assay system used (immunoblotting and ELISA) correlate well. This is important in allowing comparisons between the results of the two assays, as when *P. falciparum* RAP-1 and *P. falciparum* RAP-2 were used, since there was sufficient antigen for immunoblotting only. Some evidence of a correlation between the responses to RAP-1 and RAP-2 was observed. However, the strongest correlations were between recombinant and parasite-derived forms of the same antigen, e.g., between rRAP-2 and *P. falciparum* RAP-2, rMSA2 and *P. falciparum* MSA2, and rRAP-1 and *P. falciparum* RAP-1. The MSA2 antigens (rMSA2 and *P. falciparum* MSA2) had the smallest r_s values when they were compared with other antigens, although they were still significant (Fig. 4).

Antibody responses to RAP-1 and RAP-2 in defined-infection sera. Antisera from two naive individuals infected with *P. falciparum* sporozoites of the 3D7 clone were screened for anti-rRAP-1 and -2 antibodies by ELISA and immunoblot analysis. Figure 5 shows the immunoblots with sera at a 1/200 dilution. Volunteer 1 had IgM antibodies that recognized rRAP-1 18 days after inoculation with sporozoites, coinciding with the first IgM response to *P. falciparum* clone 3D7 antigen extracts. Volunteer 2 (who had a lower parasitemia) showed the same pattern in the next serum sample (21 days postinoculation). In contrast to the IgM response, IgG responses to

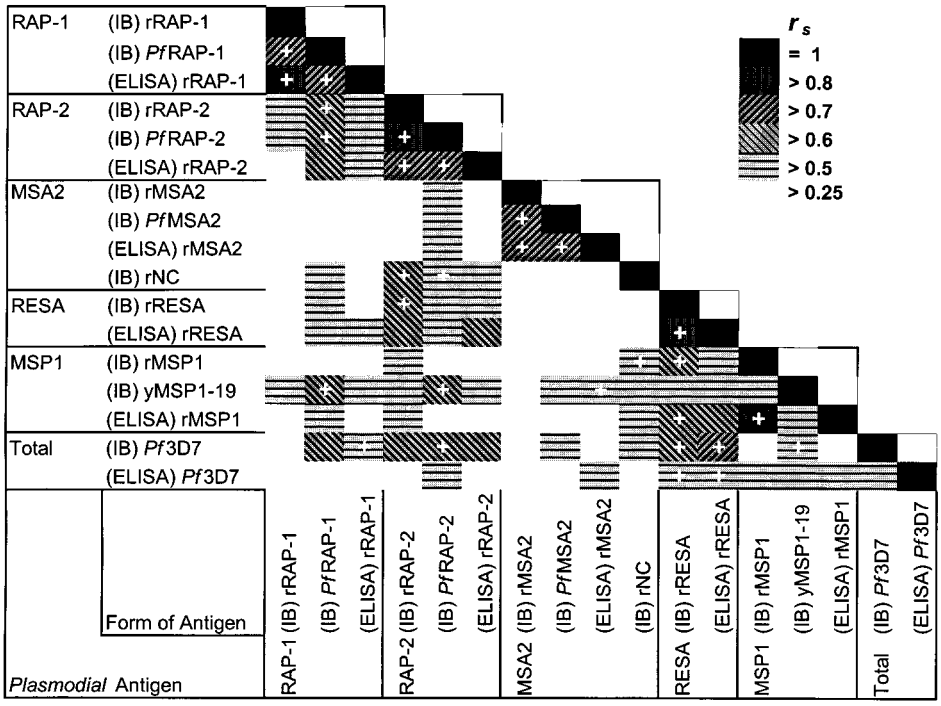

FIG. 4. Comparison of antibody responses to different antigens in the sera of 100 individuals from PNG. Spearman correlation coefficients of an antibody response to one antigen with that to another are shown. All correlation coefficients shown are significant ($P < 0.0002$ for r_s values of ≥ 0.5; $P < 0.01$ for r_s values of ≥ 0.25). A white + indicates that the correlation always remains significant when the regression is performed separately within age groups (0 to 5, >5 to 10, >10 to 15, >15 to 30, and >30 years), i.e., there are five significant values of r_s. IB, immunoblot analysis; *Pf*RAP-1 and -2, *P. falciparum* RAP-1 and -2, respectively; *Pf*MSA2, *P. falciparum* MSA2; *Pf*3D7, *P. falciparum* clone 3D7.

rRAP-1 preexisted in both samples. Both sera showed only poor activity against rRAP-2 (IgM or IgG) at this dilution.

The anti-rRAP-1 IgG response on day 52 was titrated by ELISA and compared to responses in positive (immune sera from PNG) and negative (sera from Brisbane) pools. The volunteers' sera had anti-rRAP-1 IgG titers of 1/2,056 and 1/855 by ELISA, compared to titers of 1/2,529 for the positive pool from immune donors from PNG and 1/40 for the negative pool.

These two volunteers were subjected to a repeated asexual blood stage infection with the same parasite line after a year. Figure 6 summarizes the immunoblot and ELISA analyses of the trial sera from volunteer 1 after subjection to several antigens at a 1/400 dilution (i.e., a dilution equivalent to that used in the analyses of PNG sera). The results with sera from volunteer 2 follow similar trends (data not shown). The second infection strongly boosted the antibody response, such that by immunoblotting, the peak titers reached after two controlled infections were equal to or in excess of the mean levels of antibodies in sera of individuals over 30 years old from PNG after exposure to rRAP-1 and *P. falciparum* RAP-1, rRAP-2 and *P. falciparum* RAP-2, rMSA2 and *P. falciparum* MSA2, and rMSP1 and $yMSP1_{19}$.

Antibody specificity. Sera showing various types of anti-rRAP-1 and -*P. falciparum* RAP-1 responses were purified on rRAP-1 affinity columns. As a control, the same procedure was followed for affinity purification of sera on rRESA columns. Examples are shown in Fig. 7.

From five sera positive for rRESA, antibodies affinity purified on rRESA columns gave a *P. falciparum* recognition pattern identical to that of the rRESA control sera and recognized no other bands and the recognition was blocked by preincubating the sera with soluble rRESA antigen. Three negative control sera (PNG sera negative for rRESA but positive for other *P. falciparum* antigens) purified the same way did not recognize RESA or rRESA.

In contrast to the rRESA results, sera purified on rRAP-1 columns had antibodies with diverse reactivities to *P. falciparum* antigens. This was the case whether the immunoblotting was performed under the low-affinity or more stringent standard immunoblot conditions. In only 4 of 10 cases where the purified sera gave multiple antigen recognition could any reactivity to *P. falciparum* RAP-1 be detected (confirmed by MoAbs to *P. falciparum* RAP-1). The relative strengths of the immunoblot signals to rRAP-1 and *P. falciparum* RAP-1 in the prepurification sera did not seem to have any influence over the reactivity pattern of the purified antibodies. Preincubation of the affinity-purified antibodies with soluble rRAP-1 removed all reactivity to rRAP-1 and *Pf*RAP-1 by immunoblot analysis but not to the other multiple *P. falciparum* antigens.

DISCUSSION

Antibody reactivities to rRAP-1 and rRAP-2 were markedly distinct. rRAP-1 was strongly recognized, but rRAP-2 was poorly recognized. This was not due to the inappropriateness of the rRAP-2 molecule, as the same pattern was obtained with the native forms of these molecules. However, the total number of responders among the 100 PNG sera was high, reaching 86% (RAP-1) and 82% (RAP-2) by immunoblot analysis in the age group of individuals over 30 years. Some evidence for correlation between the responses to these two molecules was observed (Fig. 4) and may reflect the ability of antibodies

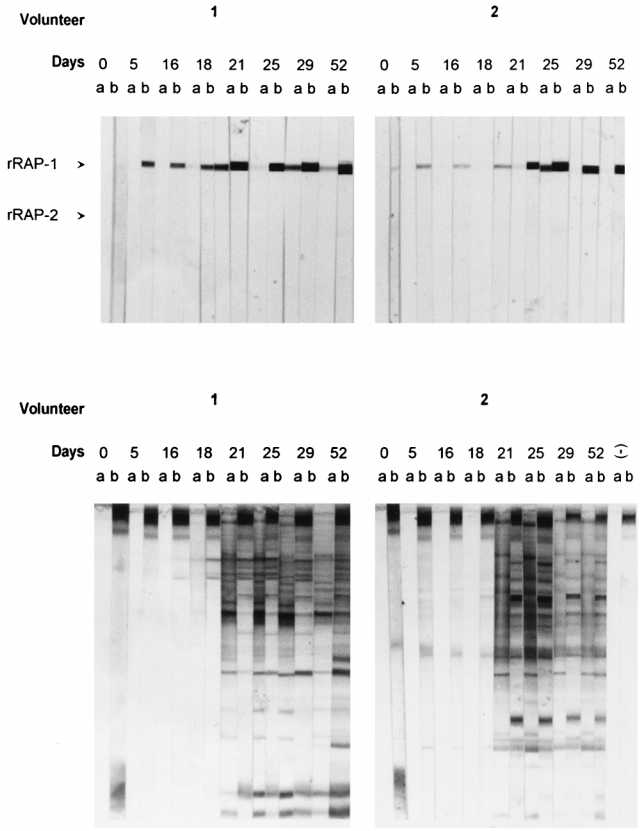

FIG. 5. First infection antibody responses to *P. falciparum*. Shown are IgG and IgM responses to rRAP-1, rRAP-2, and total *P. falciparum* clone 3D7 schizont antigen in an experimental sporozoite infection of two naive volunteers infected with *P. falciparum*. Lanes a show IgM responses, and lanes b show IgG responses for the samples at the indicated days postinoculation. Lanes (−) a and b show the reactivity of the secondary antibody alone.

raised against one of these antigens to cross-react with the second (37, 38).

Figure 4 also shows important correlations between parasite-derived and recombinant antigens (rRAP-1 and *P. falciparum* RAP-1, rRAP-2 and *P. falciparum* RAP-2, and rMSA2 and *P. falciparum* MSA2). As both the recombinant and parasite-derived molecules were denatured, a subset of conformationally dependent antibodies may not recognize either. However, the correlation between antibody recognition of parasite-derived and recombinant material is encouraging for vaccine development, as linear epitopes are shared between the denatured recombinant and parasite-derived proteins. This is an important finding for RAP-1 and RAP-2, for while immunization with SDS-denatured *P. falciparum* RAP-1 and -2 gave protection to monkeys from *P. falciparum* (28), no other comparable data exist on the possibilities of human immune responses to the recombinant proteins as immunogens.

The data presented here on multiple recombinant and parasite-derived forms of five asexual malarial antigens (and total schizont antigen) are consistent with observations made in previous studies on particular antigens (1–3, 13, 24, 30, 34, 35, 42). In those studies, adults were found to have significantly higher levels of antibody to MSP1 than children and infected children were found to have significantly more antibodies than noninfected children (13, 42). Antibody response to MSP1 is short-lived, with children losing antibodies faster than do adults (24, 35). Similar distributions of antibody and parasitemia among age groups hold for RESA and MSA2, with a higher antibody response at the beginning of the transmission season (more likely in adults) being predictive of a decrease in the incidence of clinical malaria (1, 2, 30). A similar protective correlation of antibodies against regions of MSP1 was also found in children (3).

We found, as have others (1, 2), that peak levels of antibodies do not arise until an individual is over 30 years old. However, antibody levels after one infection in healthy adults can approach these peak levels for antigen rRAP-1, but this antibody appears to be short-lived. A second infection strongly boosts this response, such that in experimental infections of two volunteers with *P. falciparum*, the peak titers reached after two controlled infections were equal to or in excess of the mean levels of antibodies in the age group of individuals over 30 years from PNG for rRAP-1 and *P. falciparum* RAP-1, rRAP-2 and *P. falciparum* RAP-2, rMSA2 and *P. falciparum* MSA2, and rMSP1 and yMSP1$_{19}$, and clear evidence of boosting of antibody response is evident for all antigens (Fig. 6). This pattern is apparently reflected in the boosting effect on antibody levels of a concurrent parasitemia which is seen in the age group 0 to 5 years in the PNG sera (13). Since this boosting is not visible in older age groups, repeated exposure to malaria may lead to a persistence of antibody rather than simply raising levels of antibodies. An enhancement of antibody persistence to multiple antigens which lasts until the next infection (in areas of perennial transmission) or until the start of the next transmission season may allow better control of infection and hence lower morbidity (1).

Antibody responses to the full-length MSA2 antigens (rMSA2 and *P. falciparum* MSA2) had the lowest r_s values when they were compared with antibody responses to other antigens, although they were still significant. Antigens rMSA2 and *P. falciparum* MSA2 elicited the highest antibody responses in individuals from 0 to 30 years of age (after responses to total *P. falciparum* clone 3D7 schizont antigen), while rNC elicited the lowest. The response to antigen rMSA2 showed only a weak correlation with age, and the mean titers of antibodies to antigens rMSA2 and *P. falciparum* MSA2 were not significantly different between age groups. In addition, antigens rMSA2 (by immunoblotting and ELISA) and *P. falciparum* MSA2 (by immunoblotting) correlated positively with parasitemia. Thus, rather than reflecting total malarial exposure (via age), high levels of antibodies to full-length MSA2 are equally likely in all age groups and may reflect exposure to the 3D7 allele. Conversely, although the number of responders was small (13 of 100), the response to rNC (i.e., the conserved regions of MSA2) had higher correlations with responses to the other antigens and reflects total malarial exposure.

The response to rRAP-1 had three unusual aspects: a high reactivity was found in nonexposed individuals, the strengths of signals to rRAP-1 correlated with those to *P. falciparum* RAP-1 but were in general stronger, and an increase in the level of IgG to rRAP-1 was visible in naive volunteers 5 days following inoculation with sporozoites. To investigate these aspects of the response to rRAP-1, antibodies from PNG sera were purified on rRAP-1 or rRESA affinity columns.

Antibody purified on rRESA columns reacts specifically with *P. falciparum* extracts in a pattern characteristic of the control antibody against this molecule. Preincubation of the extracted antibodies with rRESA in a soluble form removes this reactivity to *P. falciparum*.

However, for antibodies affinity purified on rRAP-1, we found strong evidence that the purified antibodies recognize specific antigens other than *P. falciparum* RAP-1, i.e., preincubation of the extracted antibodies with soluble rRAP-1 removed reactivity to *P. falciparum* RAP-1 but not to other *P.*

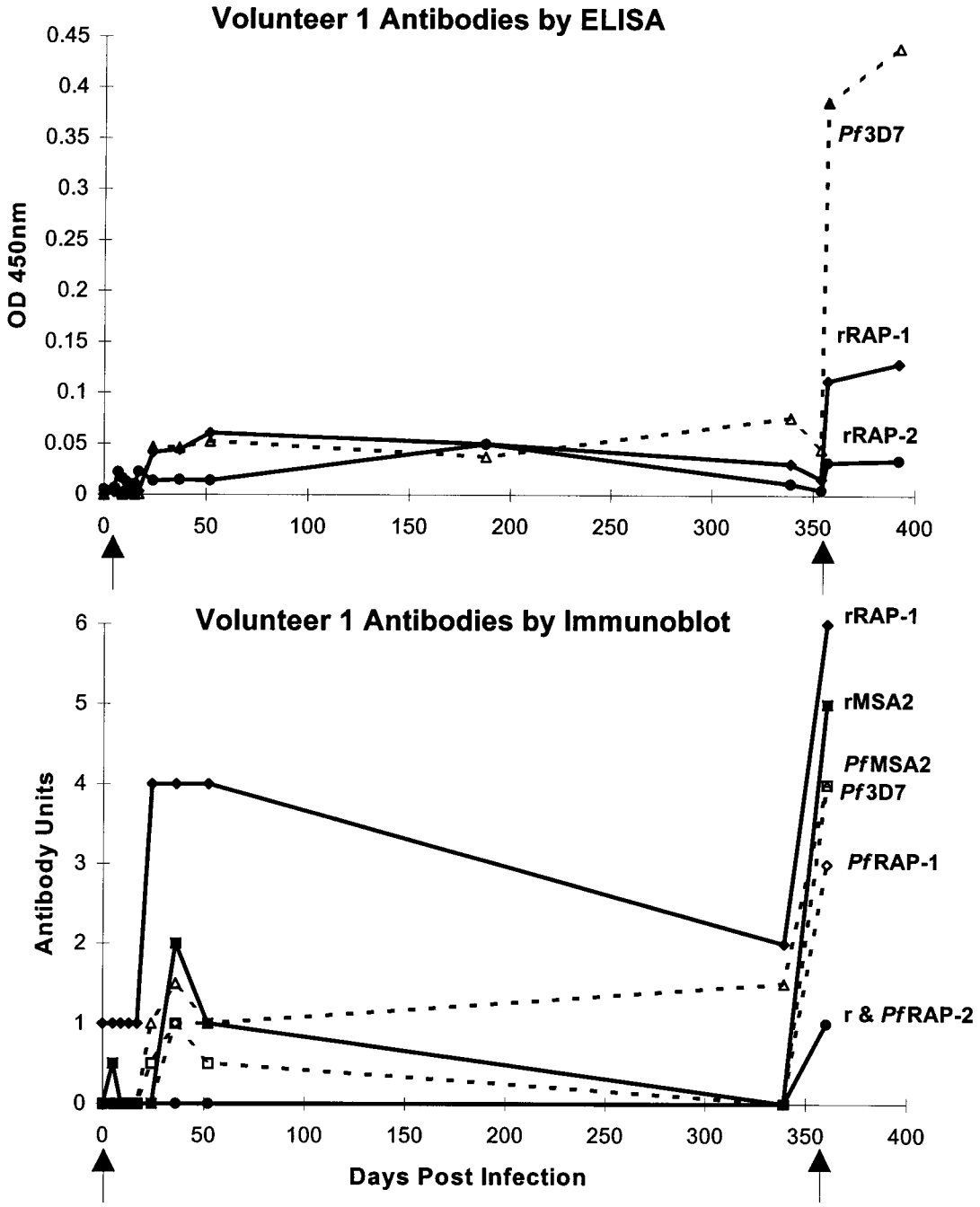

FIG. 6. IgG responses to multiple antigens in one volunteer over the course of two infections with *P. falciparum*. Antibodies were measured by immunoblot analysis and ELISA. Arrows indicate infection times. For RAP-1, RAP-2, MSA2, and total *P. falciparum* clone 3D7 (*Pf*3D7), shaded symbols and dashed lines refer to parasite-derived forms and filled symbols and lines refer to recombinant (r) forms. *Pf*MSA2, *P. falciparum* MSA2; *Pf*RAP-1 and -2, *P. falciparum* RAP-1 and -2, respectively.

falciparum antigens recognized. Such cross-reactivity can explain the high reactivity seen in unexposed Brisbane sera in this study and the high background seen in two previous studies (18, 22). It also helps to explain the early boosting of IgG reactivity to rRAP-1 observed in the day-5-postinoculation sera of the two volunteers. The timing of the boosting is probably too coincidental to be due to unrelated causes and may have arisen due to antigens on circulating sporozoites. Thus, the antigenicity of *P. falciparum* RAP-1 during natural infection with *P. falciparum* may have been overestimated in previous studies (18, 22) when only forms of rRAP-1 were used to estimate anti-*P. falciparum* RAP-1 antibodies.

However, sera from an area of PNG where malaria is endemic demonstrate a widespread recognition of RAP-1 and RAP-2. Although one or two infections with malaria can generate a humoral response of similar magnitude in healthy adults, persistence of antibodies appears to be generated in response to the total exposure to malaria of the host.

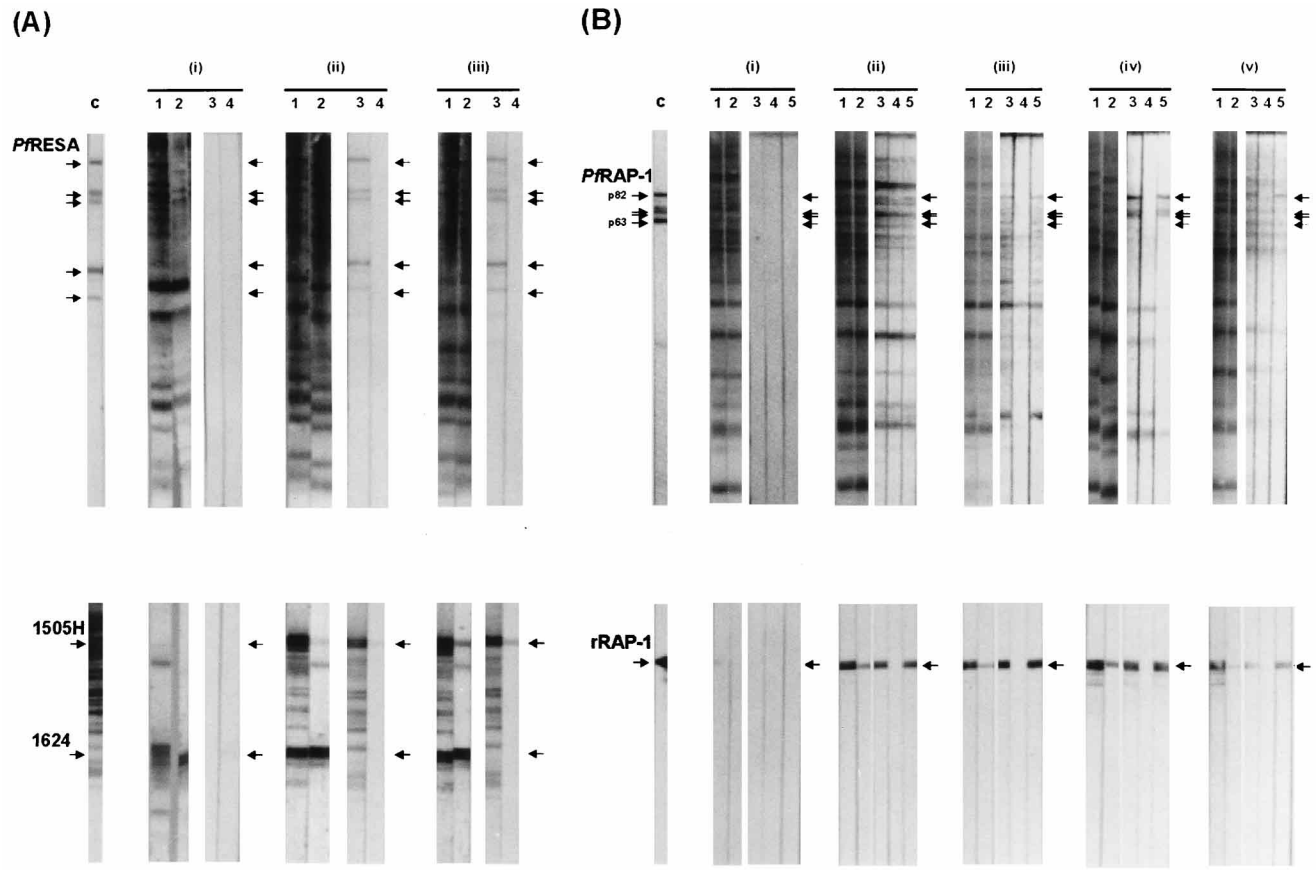

FIG. 7. Affinity purification of antibodies to RESA by antigen rRESA (A) and to *P. falciparum* RAP-1 (*Pf*RAP-1) by antigen rRAP-1 (B). The sera were immunoblotted against recombinant antigens in the bottom panels (rRESA [1505H], rMSA2 [1624], and rRAP-1) and Triton X-100 detergent extracts of 3D7 schizont antigen in the top panels, under nonreducing conditions. By affinity purification, the serum in gel i was negative to the purification antigen and sera in gels ii, iii, iv, and v were positive to the indicated antigens. Lanes: c, control antibody (7H8/50 for rRAP-1 and mouse anti-rRESA for rRESA); 1, total antibodies prior to purification; 2, antibodies that did not bind to the affinity column; 3, antibodies that eluted from the column; 4, sample in lane 3 preincubated with affinity purification recombinant protein; 5, sample in lane 3 preincubated with glycine-blocked CNBr-Sepharose 4B. *Pf*RESA, *P. falciparum* RESA.

ACKNOWLEDGMENTS

We express our gratitude to Patricia Graves for her assistance in locating the original PNG serum collection data files.

This study was funded by the National Health and Medical Research Council of Australia and the Australian Malaria Vaccine Joint Venture.

REFERENCES

1. **Al-Yaman, F., B. Genton, R. Anders, J. Teraika, M. Ginny, S. Mellor, and M. P. Alpers.** 1995. Assessment of the role of the humoral response to *Plasmodium falciparum* MSA2 compared to RESA and SPf66 in protecting Papua New Guinean children from clinical malaria. Parasite Immunol. **17:** 493–501.
2. **Al-Yaman, F., B. Genton, M. Falk, R. Anders, D. Lewis, J. Hii, H.-P. Beck, and M. P. Alpers.** 1995. Humoral response to *Plasmodium falciparum* ring-infected erythrocyte surface antigen in a highly endemic area of Papua New Guinea. Am. J. Trop. Med. Hyg. **52:**66–71.
3. **Al-Yaman, F., B. Genton, K. J. Kramer, S. P. Chang, G. S. Hui, M. Baisor, and M. P. Alpers.** 1996. Assessment of the role of naturally acquired antibody levels to *Plasmodium falciparum* merozoite surface protein-1 in protecting Papua New Guinean children from malaria morbidity. Am. J. Trop. Med. Hyg. **54:**443–448.
4. **Brown, H. J., and R. L. Coppel.** 1991. Primary structure of a *Plasmodium falciparum* rhoptry antigen. Mol. Biochem. Parasitol. **49:**99–110.
5. **Bushell, G. R., L. T. Ingram, C. A. Fardoulys, and J. A. Cooper.** 1988. An antigenic complex in the rhoptries of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **28:**105–112.
6. **Cattani, J. A., J. L. Tulloch, H. Vrbova, D. Jolley, F. D. Gibson, J. S. Moir, P. F. Heywood, M. P. Alpers, A. Stevenson, and R. Clancy.** 1986. The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. Am. J. Trop. Med. Hyg. **35:**3–15.
7. **Cheng, Q., G. Lawrence, C. Reed, A. Stowers, L. Ranford-Cartwright, A. Creasey, R. Carter, and A. Saul.** Measurement of *Plasmodium falciparum* growth rate *in vivo*: a test of malaria vaccines. Am. J. Trop. Med. Hyg., in press.
8. **Clark, J. T., R. Anand, T. Akoglu, and J. S. McBride.** 1987. Identification and characterisation of proteins associated with the rhoptry organelles of *Plasmodium falciparum* merozoites. Parasitology **18:**183–195.
9. **Cooper, J. A., L. T. Cooper, and A. J. Saul.** 1992. Mapping of the region predominantly recognised by antibodies to the *Plasmodium falciparum* merozoite surface antigen MSA 1. Mol. Biochem. Parasitol. **51:**301–312.
10. **Cooper, J. A., L. T. Ingram, G. R. Bushell, C. A. Fardoulys, D. Stenzel, L. Schofield, and A. Saul.** 1988. The 140/130/105 kilodalton protein complex in the rhoptries of *Plasmodium falciparum* consists of discrete polypeptides. Mol. Biochem. Parasitol. **29:**251–260.
11. **Coppel, R. L., A. F. Cowman, R. F. Anders, A. C. Bianco, R. B. Saint, K. R. Lingelbach, D. J. Kemp, and G. V. Brown.** 1984. Immune sera recognise an erythrocyte *Plasmodium falciparum* antigen composed of repeated amino acid sequences. Nature **310:**789–792.
12. **Epping, R. J., S. D. Goldstone, L. T. Ingram, J. A. Upcroft, R. Ramasamy, J. A. Cooper, G. R. Bushell, and H. M. Geysen.** 1988. An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in *Plasmodium falciparum*. Mol. Biochem. Parasitol. **28:** 1–10.
13. **Früh, K., O. Doumbo, H. M. Muller, O. Koita, J. McBride, A. Crisanti, Y. Touré, and H. Bujard.** 1991. Human antibody response to the major merozoite surface antigen of *Plasmodium falciparum* is strain specific and short-lived. Infect. Immun. **59:**1319–1324.
14. **Gentz, R., U. Certa, B. Takacs, H. Matile, H. Dobeli, J. R. L. Pink, M. MacKay, N. Bone, and J. G. Scaife.** 1988. Major surface antigen p190 of *Plasmodium falciparum*: detection of non-variable epitopes present in a variety of plasmodia isolates and their high yield expression in *E. coli*. EMBO J. **7:**225–230.

15. **Harnyuttanakorn, P., J. S. McBride, S. Donachie, H.-G. Heidrich, and R. G. Ridley.** 1992. Inhibitory monoclonal antibodies recognise epitopes adjacent to a proteolytic cleavage site on the RAP-1 protein of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **55:**177–186.
16. **Holder, A. A., M. J. Lockyer, K. G. Odink, J. S. Sandhu, and V. Riveros-Moreno.** 1985. Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. Nature **317:**270–273.
17. **Howard, R.** 1992. The sequence of the p82 rhoptry protein is highly conserved between two *Plasmodium falciparum* isolates. Mol. Biochem. Parasitol. **51:**327–330.
18. **Howard, R. F., J. B. Jensen, and H. L. Franklin.** 1993. Reactivity profile of human anti-82-kilodalton rhoptry protein antibodies generated during natural infection with *Plasmodium falciparum*. Infect. Immun. **61:**2960–2965.
19. **Howard, R. F., and C. Peterson.** 1996. Limited RAP-1 sequence diversity in field isolates of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **77:**95–98.
20. **Howard, R. F., H. A. Stanley, G. H. Campbell, and R. T. Reese.** 1984. Proteins responsible for a punctate fluorescence pattern in *Plasmodium falciparum* merozoites. Am. J. Trop. Med. Hyg. **33:**1055–1059.
21. **Howard, R. J., and B. L. Pasloske.** 1993. Target antigens for asexual malaria vaccine development. Parasitol. Today **9:**369–372.
22. **Jakobsen, P. H., L. Hviid, T. G. Theander, E. A. Afare, R. G. Ridley, P. M. H. Heegaard, D. Stuber, K. Dalsgaard, and F. K. Nkrumah.** 1993. Specific T-cell recognition of the merozoite proteins rhoptry-associated protein 1 and erythrocyte-binding antigen 1 of *P. falciparum*. Infect. Immun. **61:**268–273.
23. **Kaslow, D. C., G. Hui, and S. Kumar.** 1994. Expression and antigenicity of Plasmodium falciparum major merozoite surface protein (MSP1(19)) variants secreted from Saccharomyces cerevisiae. Mol. Biochem. Parasitol. **63:**283–289.
24. **Müller, H. M., K. Früh, A. von-Brunn, F. Esposito, S. Lombardi, A. Crisanti, and H. Bujard.** 1989. Development of the human immune response against the major surface protein (gp190) of *Plasmodium falciparum*. Infect. Immun. **57:**3765–3769.
25. **Perrin, L. H., B. Merkli, M. S. Gabra, J. W. Stocker, C. Chizzolini, and R. Richie.** 1985. Immunization with a *Plasmodium falciparum* merozoite surface antigen induces a partial immunity in monkeys. J. Clin. Invest. **75:**1718–1721.
26. **Perrin, L. H., E. Ramirez, P. H. Lambert, and P. A. Miescher.** 1981. Inhibition of *P. falciparum* growth in human erythrocytes by monoclonal antibodies. Nature **289:**301–303.
26a. **Prescott, N.** Unpublished data.
27. **Ridley, R. G., H. Lahm, B. Takacs, and J. G. Scaife.** 1991. Genetic and structural relationships between components of a protective rhoptry antigen complex from *Plasmodium falciparum*. Mol. Biochem. Parasitol. **47:**245–246.
28. **Ridley, R. G., B. Takacs, H. Etlinger, and J. G. Scaife.** 1990. A rhoptry antigen of *Plasmodium falciparum* is protective in *Saimiri* monkeys. Parasitology **101:**187–192.
29. **Ridley, R. G., B. Takacs, H. Lahm, C. J. Delves, M. Goman, U. Certa, H. Matile, G. R. Woollett, and J. G. Scaife.** 1990. Characterisation and sequence of a protective rhoptry antigen from *Plasmodium falciparum*. Mol. Biochem. Parasitol. **41:**125–134.
30. **Rooth, I., H. Perlmann, and A. Bjorkman.** 1991. *Plasmodium falciparum* reinfection in children from a holoendemic area in relation to seroreactivities against oligopeptides from different malarial antigens. Am. J. Trop. Med. Hyg. **45:**309–318.
30a. **Saul, A., et al.** Unpublished data.
31. **Saul, A., J. Cooper, D. Hauquitz, D. Irving, Q. Cheng, A. Stowers, and T. Limpaiboon.** 1992. The 42-kilodalton rhoptry-associated protein of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **50:**139–150.
32. **Saul, A., P. Myler, T. Elliot, and C. Kidson.** 1982. Purification of mature schizonts of *Plasmodium falciparum* on colloidal silica gradients. Bull. W. H. O. **60:**755–759.
33. **Schofield, L., G. R. Bushell, J. A. Cooper, A. J. Saul, J. A. Upcroft, and C. Kidson.** 1986. A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognised by inhibitory monoclonal antibodies. Mol. Biochem. Parasitol. **18:**183–195.
34. **Shi, Y. P., U. Sayed, S. H. Qari, J. M. Roberts, V. Udhayakumar, A. J. Oloo, W. A. Hawley, D. C. Kaslow, B. L. Nahlen, and A. A. Lal.** 1996. Natural immune response to the C-terminal 19-kilodalton domain of *Plasmodium falciparum* merozoite surface protein 1. Infect. Immun. **64:**2716–2723.
35. **Sinigaglia, F., B. Takacs, H. Jacot, H. Matile, J. R. Pink, A. Crisanti, and H. Bujard.** 1988. Nonpolymorphic regions of p190, a protein of the Plasmodium falciparum erythrocytic stage, contain both T and B cell epitopes. J. Immunol. **140:**3568–3572.
36. **Smythe, J. A., R. L. Coppel, G. V. Brown, R. Ramasamy, D. J. Kemp, and R. F. Anders.** 1988. Identification of two integral membrane proteins of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **85:**5195–5199.
37. **Stowers, A., N. Prescott, J. Cooper, B. Takacs, D. Stueber, P. Kennedy, and A. Saul.** 1995. Immunogenicity of recombinant *Plasmodium falciparum* rhoptry associated proteins 1 and 2. Parasite Immunol. **17:**631–642.
38. **Stowers, A. W., J. A. Cooper, T. Erhhardt, and A. Saul.** 1996. A peptide derived from a B cell epitope of *Plasmodium falciparum* rhoptry associated protein 2 specifically raises antibodies to rhoptry associated protein 1. Mol. Biochem. Parasitol. **82:**167–180.
39. **Stowers, A. W., K. J. Spring, and A. Saul.** 1995. Preparative scale purification of recombinant proteins to clinical grade by isotachophoresis. Bio/Technology **13:**1498–1503.
40. **Stürchler, D., R. Berger, C. Rudin, M. Just, A. Saul, C. Rzepczyk, G. Brown, R. Anders, R. Coppel, G. Woodrow, D. Pye, F. Sorennson, D. Gillessen, H. Matile, and R. Reber-Liske.** 1995. Safety, immunogenicity and pilot efficacy of *Plasmodium falciparum* sporozoite and asexual blood-stage combination vaccine in Swiss adults. Am. J. Trop. Med. Hyg. **53:**423–432.
41. **Tanabe, K., M. MacKay, M. Gorman, and J. Scaife.** 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. **195:**273–278.
42. **Tolle, R., K. Früh, O. Doumbo, O. Koita, M. N'Diaye, A. Fischer, K. Dietz, and H. Bujard.** 1993. A prospective study of the association between the human humoral immune response to *Plasmodium falciparum* blood stage antigen gp190 and control of malarial infections. Infect. Immun. **61:**40–47.
43. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
44. **Walliker, D., I. A. Quakyi, and T. E. Wellems.** 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science **236:**1661–1666.

Editor: S. H. E. Kaufmann