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HIGH PREVALENCE OF NATURAL ANTIBODIES AGAINST *PLASMODIUM FALCIPARUM* 83-KILODALTON APICAL MEMBRANE ANTIGEN (PF83/AMA-1) AS DETECTED BY CAPTURE–ENZYME-LINKED IMMUNOSORBENT ASSAY USING FULL-LENGTH BACULOVIRUS RECOMBINANT PF83/AMA-1

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Abstract. The 83-kilodalton (kD) apical membrane antigen of *Plasmodium falciparum* (PF83/AMA-1) is a potential asexual blood stage vaccine component. This antigen has been expressed as a full-length, nonfusion, recombinant baculovirus protein (PF83-7G8-1) using the authentic predicted signal peptide for appropriate postsynthetic routing. When purified by a novel high-performance, ion exchange chromatography (HPIEC) method, PF83-7G8-1 induced polyclonal antibodies in rats that immunoprecipitated both 83- and 66-kD forms of PF83/AMA-1 from ^{35}S-methionine metabolically labeled parasite extracts. Using HPIEC-purified PF83-7G8-1 in combination with a rat monoclonal antibody against the highly conserved carboxy-terminal (CT) region of PF83/AMA-1, we developed a CT-capture–enzyme-linked immunosorbent assay to measure naturally acquired responses against the entire PF83/AMA-1 molecule. Analysis of populations from villages in Guinea-Bissau and in an area of high malarial transmission in Senegal demonstrated a very high prevalence (94–100%) of naturally acquired serum IgG responses to PF83/AMA-1. Analysis of these natural responses showed that PF83/AMA-1 may be a well-recognized asexual parasite antigen. A statistically significant age-related change in antibody levels to PF83/AMA-1 was observed in Guinea-Bissau. No such correlation was observed in the Senegalese population, although an age-related antibody response was seen for total parasite antigen. No significant correlation was observed between PF83/AMA-1 responses and the parameters of parasite load and malaria-related fever.

Current efforts to develop a vaccine against *Plasmodium falciparum* target all vertebrate life cycle stages of the parasite for immunointervention. Within the erythrocytic cycle, which is responsible for clinical disease, several asexual blood stage proteins have been characterized and are putative components for inclusion in a malaria vaccine.[1–7] The *P. falciparum* merozoite 83-kilodalton (kD) apical membrane antigen (PF83/AMA-1) (Thomas AW, unpublished data)[1,2] is one of these molecules. A strong basis for the vaccine candidacy of PF83/AMA-1 is the result of work by Deans and others,[8] who identified a 66-kD *P. knowlesi* late-stage apical protein (subsequently called PK66), which was the target of inhibitory monoclonal antibodies (MAbs) in vitro. These antibodies retained their activity as Fab fragments and appeared to inhibit parasite development in a manner independent of schizont maturation,[9] suggesting a functional role for PK66 in erythrocyte invasion. Strong protective responses were induced in rhesus monkeys by immunization with PK66 in combination with exposure to *P. knowlesi*.[10] The PF83/AMA-1 antigen is an 83-kD analog of PK66 (Thomas AW, unpublished data).[1,2] Molecular analysis of PF83/AMA-1 and PK66/AMA-1 has shown that the two species are biologically conserved with regard to late-stage synthesis in mature schizonts and differential subcellular localization within schizonts and free merozoites.[11] In addition, initial work suggests that polymorphism in PF83/AMA-1 is limited.[12] Serum antibodies acquired during the course of naturally occurring infec-

tion have been reported for other malaria vaccine candidate antigens;[13–15] however, the prevalence of naturally acquired antibodies against PF83/AMA-1 has not been previously reported. Here, we describe the development and characterization of a eukaryotic baculovirus, full-length PF83/AMA-1 recombinant molecule (PF83-7G8-1), a carboxy-terminal-capture–enzyme-linked immunosorbent assay (CT-capture-ELISA) using recombinant PF83-7G8-1, and analysis of naturally acquired serum antibodies against PF83/AMA-1 in Guinea-Bissau, an area of moderate endemicity, and in Senegal, an area holoendemic for *P. falciparum*.

MATERIALS AND METHODS

Parasites. *Plasmodium falciparum* strain 7G8 (Brazilian isolate cloned at the Walter Reed Army Institute of Research, Washington, DC) and a cloned line of *P. falciparum* NF54 named CVD-1 were maintained as previously described.[16,17] The schizonts of CVD-1 were matured in culture in the presence of chymostatin and leupeptin to arrest merozoite release.[18] Pelleted schizonts were snap-frozen and stored in aliquots of 1×10^8 at −180°C. Schizont pellets were rapidly thawed and extracted by sonication in extraction buffer without detergent (1×10^8/ml),[19] microfuged, and filtered (Millex-GV 0.22 μM; Millipore, Bedford, MA). The parasite antigen preparation was maintained in aliquots at −80°C.

Recombinant baculovirus protein expression and purification. Full-length PF83/AMA-1 (with putative signal peptide) of the 7G8 strain[12] was amplified by the polymerase chain reaction using oligonucleotides that incorporated extreme *Nhe* I sites. This product was cloned into the *Nhe* I site of the pJV (*Nhe* I) baculovirus expression vector[20] and sequenced. A single alteration from the published sequence was noted at basepair 856 from A to G, resulting in an amino acid change from asparagine (N) to aspartic acid (D). Transfection in *Spodoptera frugiperda* 9 (Sf9) cells and cloning by plaque assay were as described by Vialard and others.[20] Conditions for growth and maintenance of Sf9 cells and expression of PF83/AMA-1 protein by the recombinant virus were essentially according to Summers and Smith[21] in TC100 insect cell medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum. For analysis of glycosylation of PF83/AMA-1, tunicamycin (Sigma, St. Louis, MO), a specific inhibitor of asparagine-linked glycosylation,[22] was incorporated into the expression protocol at a concentration of 5 μg/ml 1 hr after infection.[23] Full-length recombinant PF83-7G8-1 was purified as described.[19] Essentially, the purification method used an approach to high-performance ion-exchange chromatography (HPIEC) in which elution conditions were not only defined by charge, but also by hydrophobicity. The HPIEC purification of whole recombinant PF83-7G8-1 protein from Sf9 cell extracts involved two successive elutions with sodium chloride gradients that differed only in the concentration of the nonionic detergent polyoxyethylenealkylether $C_{10}E_5$ (Kwant-Hoog Vacolie Recycling and Synthesis, Bedum, The Netherlands) present in the second gradient. This second elution provided a pooled fraction that was composed of 2–9% recombinant PF83-7G8-1 as determined by scanning laser densitometry. The CT-capture-ELISA and LOUVAIN/M (LOU/M) rat (Catholic University of Louvain, Louvain, Belgium) immunizations used HPIEC-purified recombinant PF83-7G8-1 protein.

Antibodies and human sera. The PF83/AMA-1 reactive rat MAb 28G2dc1 recognizes a highly conserved CT region, rat MAb 58F8dc1 recognizes an amino-terminal (NT) region, and rat MAb 19H8dc1 recognizes the merozoite surface protein-1 (MSP-1).[11] Antiserum was prepared against PF83-7G8-1 by multiple immunization of 8–12-week-old, male, LOU/M rats at two-week intervals subcutaneously with HPIEC-purified recombinant PF83-7G8-1 in Freund's complete and incomplete adjuvants.[24] Human sera were obtained from two different geographic areas: one of moderate *P. falciparum* endemicity, Cumura, Guinea-Bissau, and a holoendemic area/village, Dielmo, Senegal, in which transmission levels, when compared with other areas of westcentral Senegal, are unusually high as the result of the presence of a small permanent river.[25] The samples available from Guinea-Bissau collected during September 1991 were from 50 children 2–9 years of age. The samples from Senegal, which were collected during June and the beginning of July 1990 and 1991 (the beginning of the rainy season), were from 199 villagers between 2 and 86 years old; 51 villagers were 2–9 years of age.[25] Twenty-two percent (Guinea-Bissau) and 92% (Senegal) of the 2–9-

year old children and 65% (Senegal) of the overall population were positive for current *P. falciparum* infection as judged by thick blood films. Some individuals were also positive for *P. ovale* and/or *P. malariae.* The Dielmo population is the subject of an ongoing, long-term study initiated in 1990, for which the current data base enables the analysis of a wide range of malariometric parameters, and for which future comparisons will be even more informative as further data (such as human leukocyte antigen typing) becomes available.[25] The Guinea-Bissau study population is also part of an ongoing study that will permit us to address long-term issues more effectively in the future.

Immunoblotting. Recombinant expression of PF83-7G8-1 protein was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) essentially as previously described.[26] Gels were electroblotted onto nitrocellulose and probed with MAbs 28G2dc1, 58F8dc1, and 19H8dc1. The migration of prestained molecular mass markers (Amersham International, Hertogenbosch, The Netherlands) is noted alongside all figures. Blotted tracks contained the equivalent of 1×10^5 cells per track. Tracks were probed with monoclonal antibodies (5 μg/ml) for 1 hr at 20°C. All immunoblots were developed using alkaline phosphatase–conjugated reagents (Pierce, Oud Beijerland, The Netherlands).

Metabolic labeling of parasites. Synchronous schizont populations were prepared by double sorbitol treatment of asynchronous cultured parasites at time 0 and 32 hr later, thereby producing a ring population that had an age distribution from 0 to 4 hr. After parasite maturation, schizonts were purified[12] with Nycodenz (Nycomed, Oslo, Norway) and cultured at a concentration of 5×10^7 schizonts/ml. Schizonts were then metabolically pulse-labeled with 100 μCi/ml of ^{35}S-methionine (Amersham International) in methionine-free medium. After 2 hr, metabolically labeled schizonts were rapidly pelleted, washed, snap-frozen, and stored at −80°C.

Preparation and analysis of ^{35}S-methionine–labeled parasite material. Aliquots of 5×10^7 schizonts were solubilized,[27] precipitated with trichloroacetic acid (TCA), and counted by liquid scintillation. Samples containing equal TCA-precipitable counts per minute (cpm) were immunoabsorbed with human or pooled immune rat IgG coupled to protein G agarose (GammaBind Plus; Genex Corp., Gaithersburg, MD) or MAb coupled to cyanogen bromide–activated Q-Sepharose CL 4B[19] and washed as previously described.[27] Antigens were analyzed by SDS-PAGE and fluorography essentially as previously described.[26]

Enzyme-linked immunosorbent assay: CT-capture-ELISA and total parasite-ELISA. Sera from Guinea-Bissau were heat-inactivated at 56°C for 30 min. Samples from Senegal were not heat-inactivated because serum samples were limited. No difference in ELISA indices were observed between the two methods of serum preparation. High protein–binding, 96-well, flat-bottomed microtiter ELISA plates (Greiner, Labortechnik, Solingen, Germany) were coated with 1) 50 μl/well of MAb 28G2dc1 (16 μg/ml) in phosphate-buffered saline (PBS, pH 7.4) plus 0.02% NaN_3 for the CT-capture-ELISA, or 2) 50 μl/well of a 1:1,000 dilution of parasite sonicate in the same buffer, and incubated overnight at 4°C. Coated plates were washed five times with Tris-buffered saline, pH 7.4, plus 0.05% Tween 20 (TBS/Tween 20), then blocked with 200 μl/well of 3% bovine serum albumin (BSA) in PBS, pH 7.4, for 60 min at 37°C. The blocking buffer was discarded and the ELISA plates were flicked dry.

For the CT-capture-ELISA, 50 μl/well of pooled HPIEC-purified PF83-7G8-1 (75 ng/ml) in PBS, pH 8.0, plus 0.5% BSA was added to the plates and incubated for 90 min at 37°C. The plates were then washed five times with TBS/Tween 20. Sera diluted 1:200 in PBS, pH 8.0, plus 0.5% BSA were added in duplicate (50 μl/well) and incubated for 90 min at 37°C. The plates were then washed five times with TBS/Tween 20. A total of 50 μl/well of alkaline phosphatase–labeled goat anti-human IgG (H & L) (Pierce) diluted 1:2,500 in PBS, pH 8.0, plus 0.5% BSA was added and incubated for 60 min at 37°C. The plates were then washed five times with TBS/Tween 20 prior to the addition of 50 μl/well of *p*-nitrophenyl phosphate (1 mg/ml) in 100 mM diethanolamine (pH 9.8). The reaction times for the development of the CT-capture-ELISA and total parasite-ELISA were 25 and 60 min, respectively. The optical density at 405 nm (OD_{405}) was determined using a Titertek Multiscan PLUS MKII apparatus (ICN Biochemicals BV, Zoetermeer, The Netherlands). Bound IgG is reported as a ratio essentially as determined

by Fidock and others[28] and as follows: a panel of 40 randomly selected unexposed European sera from persons with no history of malaria exposure were used to determine a geometric mean for the CT-capture-ELISA background. Five nonimmune sera that represented the geometric mean were then selected and used as negative controls on each ELISA plate in the CT-capture- and total parasite-ELISAs. Sera from two individuals living in a holoendemic area of Cote d'Ivoire[29] were also included on each plate as positive controls. Individual ELISA index (EI) values were determined by dividing the arithmetic mean of experimental values by the geometric mean plus two times the standard deviation (SD) of the five negative control samples. Analysis of both populations using the geometric mean plus three times the SD of the five negative control samples did not change the prevalence of positive responses. Individuals with an EI less than one were considered nonresponders. Responders in the CT-capture-ELISA and total parasite-ELISA had OD_{405} readings at least 15% and 9.2%, respectively, above the geometric mean of the OD_{405} of the negative controls. The coefficient of variation (day to day experimental variation) of the nonimmune controls, as described by Venkatesan and Wakelin,[30] was 9.5% and 16% for the CT-capture- and total parasite-ELISAs, respectively.

RESULTS

The PF83-7G8-1 baculovirus recombinant protein. Full-length PF83/AMA-1 has been expressed as a recombinant baculovirus protein (PF83-7G8-1). The recombinant protein is initially expressed as a correctly oriented transmembrane protein in Sf9 cells because cell surface immunofluorescence with fresh, unfixed infected Sf9 cells is positive with the NT MAb (58F8dc1) but not with the CT MAb (28G2dc1). The same preparations after drying and methanol fixation are recognized by both NT and CT MAbs. Analysis of PF83-7G8-1 expression in Sf9, Sf21, and High Five™ (BTI-TN-5B1-4 derived from *Trichoplusia ni* egg cell homogenates; Invitrogen, San Diego, CA) cells demonstrated that PF83-7G8-1 is equally expressed in Sf9 and Sf21 cells; however, expression in High Five cells is reduced. Recombinant PF83-7G8-1 migrates under denaturation conditions as a 90-kD protein (Figure 1). To examine the possibility of N-linked glycosylation, PF83-7G8-1–infected Sf9 cells were cultured with and without tunicamycin. In the presence of tunicamycin, PF83-7G8-1 is expressed predominantly as an 83-kD protein similar to that of the native parasite protein (Figure 1). Maximum protein production for PF83-7G8-1 and the optimal time of harvest was between 72 and 96 hr postinoculation. Pooled sera from rats that were immunized with HPIEC-purified PF83-7G8-1 immunoprecipitated both 83- and 66-kD forms of native PF83/AMA-1 (Figure 2) and reacted with native PF83/AMA-1 by indirect immunofluorescent antibody assay, giving the expected punctate fluorescence pattern in mature schizonts.

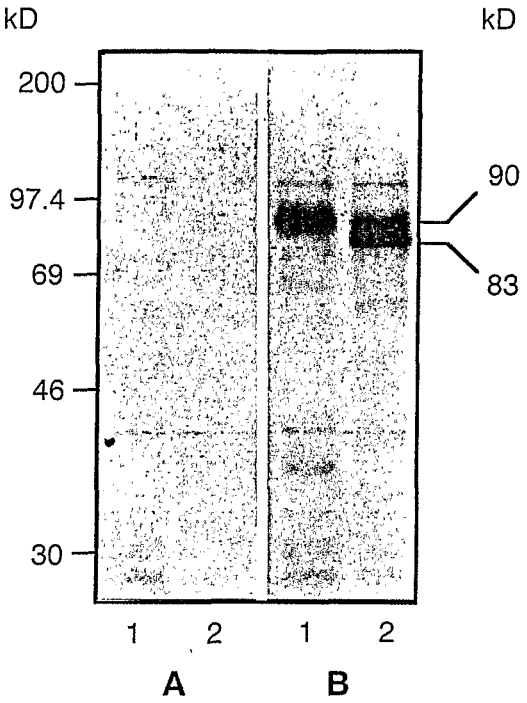

FIGURE 1. Expression and N-linked glycosylation of the 83-kilodalton (kD) apical membrane antigen of *Plasmodium falciparum* expressed as a full-length, nonfusion, recombinant baculovirus protein (PF83-7G8-1) The PF83-7G8-1–infected Sf9 cells were cultured for approximately 48 hr without (lanes 1) or with (lanes 2) tunicamycin, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotted with monoclonal antibody (MAb) 19H8dc1 (**A**) or pooled MAbs 28G2dc1 and 58F8dc1 (**B**).

Carboxyl-terminal-capture-ELISA. A capture-ELISA was developed to measure antibody responses against PF83/AMA-1 using the CT

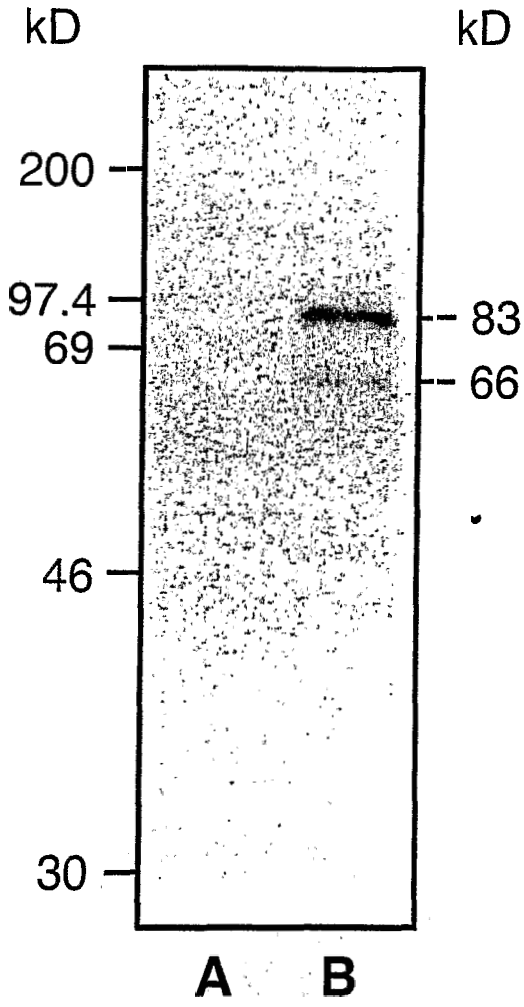

FIGURE 2. Immunoabsorption of ^{35}S-methionine metabolically labeled *Plasmodium falciparum* mature schizonts by pooled immune rat sera against high-performance, ion-exchange chromatography–enhanced β-galactosidase (**A**) and the 83-kilodalton (kD) apical membrane antigen of *P. falciparum* expressed as a full-length, nonfusion, recombinant baculovirus protein *Spodoptera frugiperda* 9 cell extract (**B**). Samples containing 5×10^5 counts per minute were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography.

MAb (28G2dc1) to capture HPIEC-purified PF83-7G8-1. The ELISA conditions were extensively tested to ensure that the measured human antibody responses were specific for PF83-7G8-1. This included comparison with a CT-capture-ELISA using the analogous fraction from HPIEC-fractionated protein extracts of Sf9 cells that had been infected with the wild-type baculovirus. In this assay, a randomly selected range of normal and hyperendemic sera gave OD_{405} values that were equivalent to those of the normal serum samples when tested against PF83-7G8-1. Responses to insect cell contaminants or rat MAbs did not therefore interfere with the readout of the assay.

Serum antibodies to PF83/AMA-1. Serum antibody levels against *P. falciparum* PF83/AMA-1 were measured by CT-capture-ELISA in 50 children, ages 2–9 years, from Cumura, Guinea-Bissau, an area of moderate endemicity, and in 199 villagers, ages 2–86 years, from Dielmo, Senegal, a holoendemic area. An initial analysis within the Dielmo population was undertaken to assess the relationship between endpoint titration and single dilution OD_{405} measurements in this ELISA. A close agreement between the two approaches was observed (Figure 3), and therefore all subsequent analyses used the simpler, single dilution measurement. All individuals in the Guinea-Bissau study and 94% of the Dielmo villagers (with a distribution independent of age) had detectable naturally acquired antibodies (IgG) to PF83/AMA-1. A statistically significant correlation between serum IgG levels specific for PF83/AMA-1 and age was observed in the 2–9-year-old population from Guinea-Bissau ($P < 0.005$, Spearman rank-order correlation coefficient = 0.43) Furthermore, a statistically significant correlation was observed between serum IgG levels and age when the children were put into groups 2–4 and 5–9 years of age ($P < 0.001$, by Wilcoxon sum rank test) (Figure 4). No similar correlation was observed within the age period examined for responses to total parasite antigen (Figure 4). No age-related response to PF83/AMA-1 was evident in the Dielmo population, although in contrast, within the same population, age-related responses to total parasite material were observed ($P < 0.001$, Spearman rank-order correlation coefficient = 0.38) (Figure 5). No statistically significant differences by sex in terms of humoral immune responses were observed in either population. No correlation could be determined between individual EIs and 1) PF83/AMA-1 immune responses and the development of fever or clinical malaria within Dielmo villagers from June to September 1990, 2) the presence of mixed infections within the Dielmo villagers, or 3) the parasite load evident on Giemsa-stained

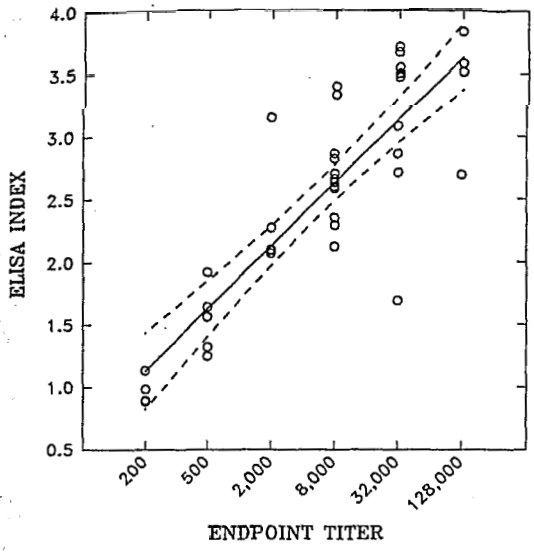

FIGURE 3. Correlation between enzyme-linked immunosorbent assay (ELISA) indices and endpoint titers from 40 random sera samples from Dielmo, Senegal. The solid line represents linear regression through the data; the dashed lines represent 95% confidence intervals.

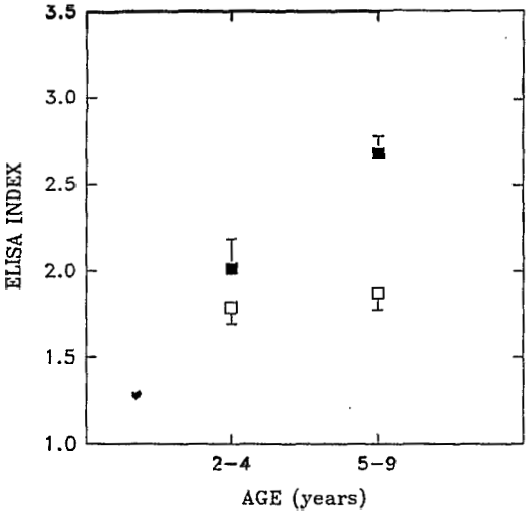

FIGURE 4. Results of carboxy-terminal (CT)-capture- and total parasite enzyme-linked immunosorbent assays (ELISAs) of serum samples from Guinea-Bissau. The CT-capture-ELISA results show a relationship between antibody levels (IgG) against the 83-kilodalton apical membrane antigen of *Plasmodium falciparum* (PF83/AMA-1) and age. ■ = arithmetic mean and SEM ELISA indices for responses to PF83/AMA-1; □ = arithmetic mean and SEM ELISA indices for total parasite antigen. Age group sizes (n) for those with PF83/AMA-1 and those with total parasite antigen, respectively, were 2–4 years of age, n = 16 and n = 15; 5–9 years of age, n = 34 and n = 34. Sera were diluted 1:200. One sample (from an individual in the 2–4-years-old age group) contained an insufficient quantity of serum to test against total parasite antigen. Excluding this sample, all individuals were seropositive for both total parasite antigen and PF83/AMA-1. The association between the ELISA index for PF83/AMA-1 and age are statistically significant ($P < 0.001$, by the Wilcoxon sum rank test).

blood films prepared at the time blood specimens were collected in either population. To compare responses with time, sera from approximately 10% of the Dielmo population were also assessed one year later (1991). Individual serum IgG levels specific for PF83/AMA-1 did not significantly differ between the 1990 samples and those taken during the equivalent period of 1991 ($P = 0.59$, by Wilcoxon matched-pairs signed ranks test). This comparison was made on a small number of individuals 2–4 (n = 4) and 5–9 (n = 2) years of age.

Relationship between the EI and the recognition of native PF83/AMA-1. Sera from two inhabitants from Guinea-Bissau, three and seven years of age (with EIs for PF83/AMA-1 of 1.30 and 2.28, respectively), were used to analyze human immune recognition of native PF83/AMA-1. As expected, a restricted range of parasite proteins was preferentially recognized by the sera (Figure 6). Although PF83/AMA-1 is a protein synthesized in such relatively small amounts that it is not, even during its period of maximal expression, discernible on fluorographs within the total synthetic activity, it was notable that immunoprecipitation with serum from the seven-year old with a high EI revealed bands of 83 and 66 kD. These bands were specifically removed by PF83/AMA-1 immunodepletion (Figure 6, C1 and C2). In contrast, immunoprecipitation with an equivalent amount of serum from the three-year old with a low EI showed a marked reduction in the recognition of total parasite antigens and immunoprecipitation of PF83/AMA-1 was undetectable. Similar results were obtained for another pair of serum samples with low and high EIs. This quantitatively minor parasite protein was therefore, in comparison with total parasite proteins, strongly recognized in the high EI sera, in which responses to both full-length (83 kD) and processed (66 kD) PF83/AMA-1 were evident.

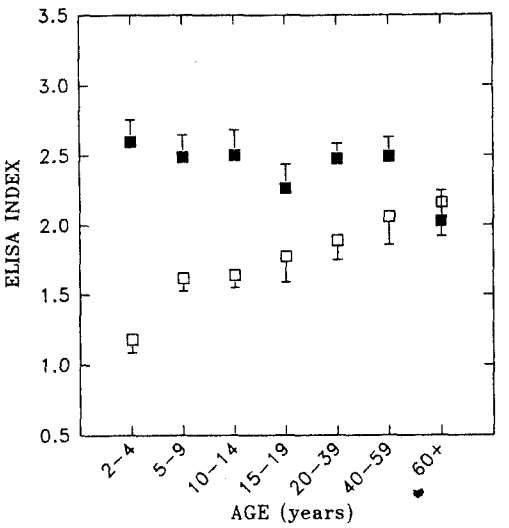

FIGURE 5. Results of carboxy-terminal-capture- and total parasite enzyme-linked immunosorbent assays (ELISAs) of serum samples from Dielmo, Senegal. The ELISA results show relationships between naturally acquired antibody levels (IgG) against the 83-kilodalton apical membrane antigen of *Plasmodium falciparum* (PF83/AMA-1) and total parasite antigen and age. ■ = arithmetic mean and SEM ELISA indices for responses to PF83/AMA-1; □ = arithmetic mean and SEM ELISA indices for total parasite antigen. Age groupings are as follows, in accordance with Trape and others.[25] Age group sizes (n) for those with PF83/AMA-1 and those with total parasite antigen, respectively, were 2–4 years of age, n = 20 and n = 5; 5–9 years of age, n = 32 and n = 9; 10–14 years of age, n = 24 and n = 14; 15–19 years of age, n = 22 and n = 11; 20–39 years of age, n = 56 and n = 12; 40–59 years of age, n = 28 and n = 14; 60 or more years of age, n = 17 and n = 14. Sera were diluted 1:200.

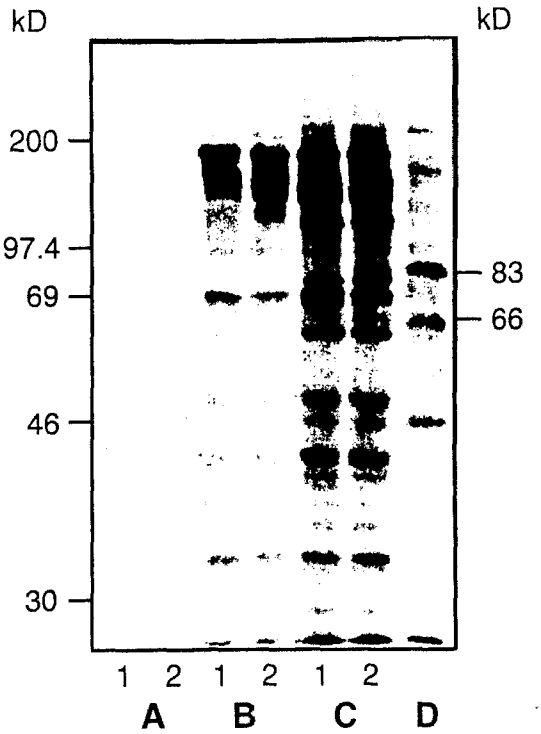

FIGURE 6. Analysis of the recognition of native 83-kilodalton (kD) apical membrane antigen of *Plasmodium falciparum* (PF83/AMA-1) by human sera from Guinea-Bissau. Extracts of ^{35}S-methionine metabolically labeled schizonts were immunoabsorbed with human IgG coupled to protein G/agarose. Serum samples were from a nonimmune adult male (**A**); a three-year-old girl, enzyme-linked immunosorbent assay (ELISA) index (EI) = 1.30 (**B**); a seven-year-old boy, EI = 2.28 (**C**); and a positive control (immunoabsorption of PF83/AMA-1 by anti–PF83/AMA-1 monoclonal antibodies (**D**). Lanes 1 and 2 represent parasite extracts immunodepleted of or containing PF83/AMA-1, respectively. Immunoprecipitates of metabolically labeled parasite extracts that contained 1.5×10^6 counts per minute (cpm) (**A–C**) and 6×10^6 cpm (**D**) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5% polyacrylamide gel) and fluorography.

DISCUSSION

We have used a new CT-capture-ELISA to show that PF83/AMA-1, a quantitatively minor, transiently expressed component of the mature schizont and merozoite, is highly recognized in humoral responses of all age groups in two different endemic areas of Africa. The recombinant PF83/AMA-1 used in this assay was a full-length molecule expressed in a baculovirus system in which the authentic predicted signal peptide was used to facilitate entry into an appropriate postsynthetic routing pathway. The transmembrane localization of the molecule after Sf9 expression suggests that this is indeed the case. Protection induced in vitro by PK66/AMA-1 appeared to be dependent upon a conformational epitope[27] and PF83/AMA-1 contains cysteine and proline residues that are almost all positionally conserved with PK66/AMA-1. Correct targeting of recombinant molecules has been shown to be necessary for the authentic expression of large fragments of other malarial proteins to form correct disulfide cross-links,[31] and this is expected to be critical in the production of authentic PF83/AMA-1. Under reducing conditions, recombinant PF83-7G8-1 has an apparent molecular mass that is 7 kD greater than

that of the native protein, which is attributable to aberrant (non-native) N-linked glycosylation. It is not yet clear whether N-linked glycosylation affects correct folding of this molecule or how it might alter potential recognition of immunological determinants.[32] Limited N-linked glycosylation of a baculovirus C-terminal product of MSP-1 did not interfere with its recognition by inhibitory MAbs that recognize conformational epitopes.[31]

The analysis of naturally acquired serum IgG responses by CT-capture-ELISA in areas of moderate and holoendemic *P. falciparum* demonstrated a high prevalence of serum IgG responses against PF83/AMA-1. Of particular interest, this included children 2–4 years of age. The limited reports to date regarding the prevalence of acquired serum IgG responses in children 1–4 years of age to other parasite antigens has shown a fairly large variation in serum positivity among exposed populations. For example, responses to the circumsporozoite protein CSP-1 could be measured in 10–53% of the children,[14, 15, 33] responses to MSP-1 in 70%,[13, 34] and responses to the ring-infected erythrocyte surface antigen (RESA) in 20–50% of the children in two different hyperendemic villages of western Kenya.[14] Although this variance may be attributable in part to differences in the constructs used (e.g., recombinant or synthetic peptide, fixed native material, etc.), and differences in the sensitivity of the methods for IgG detection used, it has been argued that the level of perennial transmission of *P. falciparum* within different hyperendemic areas is a factor.[14] It is evident that for PF83/AMA-1 there is a very high prevalence of naturally induced antibody responses to this antigen, even in the 2–4-year-old age groups of both the Dielmo and Cumura populations. Different levels of exposure to the parasite occur in the two populations (22% and 92% point prevalence of the 2–4-year-old children in Guinea-Bissau and Senegal, respectively). However, in combination with the relatively high sensitivity of the CT-capture-ELISA (which is measuring responses to the entire PF83/AMA-1 molecule in a conformation that is likely to mimic its native state), the parasite prevalence in both populations is sufficient to generate very high PF83/AMA-1–specific responses. The limited extent of the yearly variation observed in the level of PF83/AMA-1 responses in Dielmo is also a likely consequence of the relatively persistent exposure to infection.[14, 35] It is notable that in the above context, a recent study of the prevalence and level of responses to MSP-1 showed no significant differences in serologic responses between individuals living in areas of low and high seasonal malaria transmission.[35] Here, the seasonal exposure to *P. falciparum* observed in Guinea-Bissau was evidently sufficient to induce responses to PF83/AMA-1. In contrast to measures of prevalence, however, there is a clear age-related increase in the level of response to PF83/AMA-1 observed in children from Guinea-Bissau, and an age-related change in response to total parasite antigen in Dielmo in the absence of such a phenomenon for PF83/AMA-1. The changes observed in antibody levels to PF83/AMA-1 in Guinea-Bissau and total parasite antigen in Dielmo may be explained by an individual developing protective immunity with the occurrence of multiple parasite infections. The absence of this phenomenon for PF83/AMA-1 in the children from Dielmo suggests that prior exposure to *P. falciparum* infection, particularly in the light of the very strong response to PF83/AMA-1, has already been extensive enough to induce PF83/AMA-1 responses similar to those of adults.

Analysis of the 1990 samples from the Dielmo population showed no correlation to 1) parasite load at the time of sampling and 2) clinical malaria episodes between the time of sampling and September 1990[25] with individual EI against PF83/AMA-1. Thus, a protective component among the total naturally acquired PF83/AMA-1 antibodies as determined by the CT-capture ELISA could not be distinguished during the short-term follow-up period reported here. Although some protective trends within certain subpopulations were observed, they were not large enough to determine statistical significance, and a more extended follow-up will be required to draw definitive conclusions. We anticipate that, while the responses measured here against the full-length recombinant PF83/AMA-1 represent the overall recognition of the molecule within the population, protective responses will be determined by the specific recognition of defined regions or determinants, as has been shown for PK66/AMA-1[27] and the C-terminus of MSP-1,[36, 37] or may be restricted to particular cytophilic isotypes.[29]

Our initial analysis of the naturally acquired immune responses against PF83/AMA-1 has

shown that both the 83- and 66-kD forms may be well-recognized in comparison with most other parasite proteins of the maturing schizont and merozoite. Naturally acquired antibodies against the rhoptry-associated protein-1 (RAP-1) also appear to be strongly represented in the individual repertoire[7] and are highly prevalent.[38] Whether this shared characteristic may be a more general feature of rhoptry and apical proteins of the merozoite is as yet unclear.

The rapid response to PF83/AMA-1 requires more detailed investigation in younger populations of Guinea-Bissau and Senegal. Furthermore, the long-term analysis possible in both population groups will enable this study to be extended to a longitudinal investigation correlating PF83/AMA-1 responses to malariometric parameters. The CT-capture-ELISA reported here will also be of more general value in the analysis of immune responses due to specific immunization with various constructs of PF83/AMA-1.

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REFERENCES

1. Waters AP, Thomas AW, Deans JA, Mitchell GH, Hudson DE, Miller LH, McCutchan TF, Cohen S, 1990. A merozoite receptor protein from *Plasmodium knowlesi* is highly conserved and distributed throughout *Plasmodium. J Biol Chem 265:* 17974–17979.
2. Peterson MG, Marshall VM, Smythe JA, Crewther PE, Lew A, Silva A, Anders RF, Kemp DJ, 1989. Integral membrane protein located in the apical complex of *Plasmodium falciparum. Mol Cell Biol 9:* 3151–3154.
3. Siddiqui 'WA, Tam LQ, Kramer KJ, Hui GSN, Case SE, Yamage KM, Chang SP, Chan EBT, Kan SC, 1987. Merozoite surface coat precursor completely protects *Aotus* monkeys against *Plasmodium falciparum. Proc Natl Acad Sci USA 84:* 3014–3018.
4. Collins WE, Anders RF, Pappaioanou M, Campbell GH, Brown GV, Kemp DJ, Coppel RL, Skinner JC, Andrysiak PM, Favoloro JM, Corcoran LM, Broderson JR, Mitchell GF, Campbell CC, 1986. Immunization of *Aotus* monkeys with recombinant proteins of an erythrocyte surface antigen of *Plasmodium falciparum. Nature 323:* 259–262.
5. Aikawa M, Torii M, Sjolander A, Berzins K, Perlmann P, Miller LH, 1990. Pf155/RESA antigen localised in dense granules of *Plasmodium falciparum* merozoites. *Exp Parasitol 71:* 326–329.
6. Adams JH, Sim BKL, Dolan SA, Fang X, Kaslow DC, Miller LH, 1992. A family of erythrocyte binding proteins of malaria parasites. *Proc Natl Acad Sci USA 89:* 7085–7089.
7. Ridley RG, Takacs B, Etlinger H, Scaife JG, 1990. A rhoptry antigen from *Plasmodium falciparum* is protective in *Saimiri* monkeys. *Parasitology 101:* 187–192.
8. Deans JD, Alderson T, Thomas AW, Mitchell GH, Lennox ES, Cohen S, 1982. Rat monoclonal antibodies which inhibit the in vitro multiplication of *Plasmodium knowlesi. Clin Exp Immunol 49:* 297–309.
9. Thomas AW, Jeans JA, Mitchell GH, Alderson T, Cohen S, 1984. The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes. *Mol Biochem Parasitol 13:* 187–199.
10. Deans JA, Knight AM, Jean WC, Waters AP, Cohen S, Mitchell GH, 1988. Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66 kD merozoite antigen. *Parasite Immunol 10:* 535–552.
11. Narum DL, Thomas AW, 1994. Molecular characterization of PF83/AMA-1, a *Plasmodium falciparum* vaccine candidate merozoite antigen. *Mol Biochem Parasitol 67:* 59–68.

12. Thomas AW, Waters AP, Carr D, 1990. Analysis of variation in PF83, an erythrocytic merozoite vaccine candidate antigen of *Plasmodium falciparum. Mol Biochem Parasitol 42:* 285–288.
13. Muller HM, Fruh K, von Brunn A, Esposito F, Lombardi S, Crisanti A, Bujard H, 1989. Development of the human immune response against the major surface protein (gp190) of *Plasmodium falciparum. Infect Immun 57:* 3765–3769.
14. Deloron P, Campbell GH, Brandling-Bennett D, Roberts JM, Schwartz IK, Odera JS, Lal AA, Osanga CO, de la Cruz V, McCutchan TM, 1989. Antibodies to *Plasmodium falciparum* ring-infected erythrocyte surface antigen and *P. falciparum* and *P. malariae* circumsporozoite proteins: seasonal prevalence in Kenyan villages. *Am J Trop Med Hyg 41:* 395–399.
15. Nardin EH, Nussenzweig RS, McGregor IA, Bryan JH, 1979. Antibodies to sporozoites-their frequent occurrence in individuals living in an area of hyperendemic malaria. *Science 206:* 597–599.
16. Trager W, Jensen JB, 1976. Human malaria parasites in continuous culture. *Science 193:* 673–675.
17. Davis JR, Cortese JF, Herrington DA, Murphy JR, Clyde DF, Thomas AW, Shahida B, Cochran MA, Thanassi J, Levine M, 1992. *Plasmodium falciparum:* in vitro characterization and human infectivity of a cloned line. *Exp Parasitol 74:* 159–168.
18. Lyon JA, Haynes JD, 1986. *Plasmodium falciparum* antigens synthesized by schizonts and stabilized at the merozoite surface when schizonts mature in the presence of protease inhibitors. *J Immunol 136:* 2245–2251.
19. Narum DL, Welling GW, Thomas AW, 1993. Ion-exchange/immuno-affinity purification of a recombinant baculovirus *Plasmodium falciparum* apical membrane antigen, PF83/AMA-1. *J Chromatogr 657:* 357–363.
20. Vialard J, Lalumiere M, Vernet T, Briedis D, Alkhatib G, Henning D, Levin D, Richardson C, 1990. Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the beta-galactosidase gene. *J Virol 64:* 37–50.
21. Summers MD, Smith GE, 1987. *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures.* College Station, TX: Texas Agricultural Experiment Station, Bulletin No. 1555.
22. Takatsuki A, Kohno K, Tamura G, 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. *Agric Biol Chem 39:* 2089–2091.
23. Smith GE, Summers MD, Fraser MJ, 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol Cell Biol 3:* 2156–2165.
24. Bazin H, ed., 1990. *Rat Hybridomas and Rat Monoclonal Antibodies.* Boca Raton, FL: CRC Press, Inc.
25. Trape JF, Rogier C, Konate L, Diagne N, Bouganali H, Canque B, Legros F, Badji A, Ndiaye P, Brahimi K, Faye O, Druilhe P, Pereira da Silva L, 1994. The Dielmo Project: a longitudinal study of natural malaria infection in a community living in a holoendemic area of Senegal. *Am J Trop Med Hyg 51:* 123–137.
26. Deans JA, Thomas AW, Cohen S, 1983. Stage-specific protein synthesis by asexual blood stage parasites of *Plasmodium knowlesi. Mol Biochem Parasitol 8:* 31–44.
27. Deans JA, Jeans WC, 1987. Structural studies on a putative protective *Plasmodium knowlesi* merozoite antigen. *Mol Biochem Parasitol 26:* 155–166.
28. Fidock D, Lepers JP, Gras-Masse H, Brarhimi K, Benmohamed L, Meis J, Langsley G, Raharimala L, Mellouk S, Guerin-Marchand C, Tartar A, Druilhe P, 1994. *Plasmodium falciparum* liver-stage specific antigen LSA-1 is well conserved and contains potent B- and T-determinants. *J Immunol 153:* 190–204.
29. Bouharoun-Tayoun H, Druilhe P, 1992. *Plasmodium falciparum* malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infect Immun 60:* 1473–1481.
30. Venkatesan P, Wakelin D, 1993. ELISAs for parasitologists: or lies, damned lies and ELISAs. *Parasitol Today 9:* 228–232.
31. Murphy VF, Rowan WC, Page MJ, Holder AA, 1990. Expression of hybrid malaria antigens in insect cells and their engineering for correct folding and secretion. *Parasitology 100:* 177–183.
32. Luckow VA, Summers MD, 1988. Trends in the development of baculovirus expression vectors. *Biotechnology 6:* 47–55.
33. Marsh K, Hayes RH, Carson DC, Otoo L, Shenton F, Byass P, Zavala F, Greenwood BM, 1988. Anti-sporozoite antibodies and immunity to malaria in a rural Gambian population. *Trans R Soc Trop Med Hyg 82:* 532–537.
34. Blackman MJ, Holder AA, 1993. Use of a recombinant baculovirus product to measure naturally-acquired human antibodies to disulfide-constrained epitopes on the *P. falciparum* merozoite surface protein-1 (MSP-1). *FEMS Immunol Med Microbiol 6:* 307–316.
35. Riley EM, Morris-Jones S, Blackman MJ, Greenwood BM, Holder AA, 1993. A longitudinal study of naturally acquired cellular and humoral immune responses to a merozoite surface protein (MSP1) of *Plasmodium falciparum* in an area of seasonal malaria transmission. *Parasite Immunol 15:* 513–524.
36. Hui GH, Hashiro C, Nikaido C, Case SE, Hashimoto A, Gibson H, Barr PJ, Chang SP, 1993. Immunological cross-reactivity of the C-terminal 42-kilodalton fragment of *Plasmodium falciparum* merozoite surface protein 1 expressed in baculovirus. *Infect Immun 61:* 3403–3411.
37. Blackman MJ, Heidrich HG, Donachie S, Mc-

Bride JS, Holder AA, 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J Exp Med 172:* 379–382.

38. Howard RF, Jensen JB, Franklin HL, 1993. Reactivity profile of human anti-82-kilodalton rhoptry protein antibodies generated during natural infection with *Plasmodium falciparum. Infect Immun 61:* 2960–2965.