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Naturally Acquired Human Antibodies Which Recognize the First Epidermal Growth Factor-Like Module in the \textit{Plasmodium falciparum} Merozoite Surface Protein 1 Do Not Inhibit Parasite Growth In Vitro

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Merozoite surface protein 1, one of the major surface proteins of the invasive blood stage of the malaria parasite, is a prime candidate for the development of a vaccine against the human disease. Previously, monoclonal antibodies which both inhibited the growth of \textit{Plasmodium falciparum} in vitro and bound to the first of two epidermal growth factor-like modules located near the carboxy terminus of the protein had been identified. In this study, we have used affinity chromatography on a recombinant fusion protein corresponding to the first epidermal growth factor-like module in \textit{P. falciparum} merozoite surface protein 1 to prepare antibody induced by natural infection. The antibody was purified from the total immunoglobulin G fraction of adult West African donors, shown to passively confer immunity against falciparum malaria. Such affinity-purified antibodies were shown to recognize the native protein by a number of separate criteria and to block the binding of an inhibitory monoclonal antibody, but they failed to inhibit parasite invasion in an in vitro growth assay. These results indicate that antibody alone is not sufficient to interfere with erythrocyte invasion.

There is an urgent need to develop a more effective vaccine against malaria. Much work on vaccine development has been focused on merozoite surface protein 1 (MSP-1), a high-molecular-mass protein synthesized by the intracellular schizont of the asexual blood and liver stages and expressed on the surface of merozoites released from the ruptured schizont (18, 24). MSP-1 preparations purified from a number of malaria parasites have, by immunization, induced significant levels of protection against challenge with blood-stage parasites in both rodent (26) and simian (20, 21, 41, 46) experimental model systems. Investigators have used parts of MSP-1, either expressed in \textit{Escherichia coli} (17, 20, 23, 28, 33) or as synthetic peptides (13, 40), to induce complete or partial protection or to delay the progress of infection. A synthetic peptide polymer, which includes a sequence from the N terminus of \textit{Plasmodium falciparum} MSP-1, produced encouraging results in a recent clinical trial (49).

MSP-1 is modified by proteolysis leading to shedding of the molecule except for a small C-terminal fragment, which can be detected on the surface of the parasite in a newly infected erythrocyte. Prior to the release of merozoites from the mature schizont, \textit{P. falciparum} MSP-1 is processed by protease(s) to at least four major polypeptides, held together in a noncovalent complex on the merozoite surface (35). One of these, a 42-kDa membrane-bound fragment from the C terminus (designated MSP-1\(_{42}\)), undergoes a second proteolytic cleavage to produce MSP-1\(_{33}\) and MSP-1\(_{19}\) (1, 6). The membrane-bound MSP-1\(_{19}\) polypeptide, comprising two cysteine-rich epidermal growth factor (EGF)-like modules (4), is carried into the new erythrocyte on the surface of the invading merozoite (2).

The EGF-like modules are found in MSP-1 in all species of malaria parasite examined (16, 25), and there is evidence to suggest that they are an important target of protective immunity. In studies using the rodent malaria models \textit{Plasmodium yoelii} and \textit{Plasmodium chabaudi}, MSP-1-specific monoclonal antibodies (MAbs) passively protected mice against challenge infection with homologous blood-stage parasites (8, 32, 34). Two of these antibodies bind to discontinuous disulfide-constrained epitopes within the EGF-like modules (10, 37). Mice immunized with the cysteine-rich region of \textit{P. yoelii} MSP-1, expressed as a fusion protein in \textit{E. coli}, were partially or completely protected against challenge infection with \textit{P. yoelii} parasites (17, 33). A number of investigators have reported \textit{P. falciparum} MSP-1-specific MAbs which inhibit the in vitro growth of the parasite (2, 15, 42), and these antibodies react with MSP-1\(_{10}\). The EGF-like modules have been expressed in a correctly folded form in insect cells (11, 39), yeast cells (30), and \textit{E. coli} cells (9, 12). Serum from rabbits immunized with a correctly folded insect cell product representing MSP-1\(_{42}\) but not with an identical but incorrectly folded yeast cell product, completely inhibited \textit{P. falciparum} growth in vitro (11). We have reproduced the individual EGF-like modules of the \textit{P. falciparum} MSP-1 by expression as fusion proteins in \textit{E. coli} and have shown that a number of MSP-1-specific MAbs, including the invasion-inhibitory antibodies 5B1 (42) and 12.8 (2), bound to disulfide-constrained epitopes in the first of the EGF-like modules (MSP-1--EGF1) (12).

We show here that, in contrast to results obtained with MSP-1-specific MAbs, immunoglobulin G (IgG) affinity selected on MSP-1--EGF1 from the naturally acquired repertoire...
of adults with immunity to malaria did not prevent the invasion of erythrocytes by malarial merozoites in vitro. These antibodies did recognize the native protein and competed for antigen binding with a protective MAb.

MATERIALS AND METHODS

Expression construct and protein purification. The first EGF-like module in the Wellcome/T9-94 MSP-1 (4, 29), designated MSP-1-EGF1, was expressed as a fusion protein with glutathione S-transferase (GST) in E. coli. The production and characterization of the expression construct and the purification of the protein by chromatography on glutathione-agarose (47) have been described in detail elsewhere (12). The protein was dialyzed against 50 mM ammonium bicarbonate (pH 7.4) and quantified (the $A_{280}$ of 1 mg of protein solution ml⁻¹ was estimated at 1.9). Alternatively, the soluble EGF-like polypeptide was cleaved from the immobilized GST fusion partner by proteolysis with bovine factor Xa (Boehringer) (47).

Human IgG preparations. IgG derived from 178 adult inhabitants of Ivory Coast, West Africa (designated total-IgG), was used; the source and purification of this IgG and its clinical effect upon transfer to P. falciparum-infected recipients have been described previously in detail (43). Control IgG (designated control-IgG) was purified from heat-inactivated, pooled sera of healthy European A⁺ blood donors never exposed to malaria, by chromatography on protein A bound to glass beads (Prosep-A; Bioprocessing), according to the manufacturer’s instructions. IgG purity was estimated by analysis of Coomasie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and quantified on the assumption that 1 mg of IgG ml⁻¹ has an A280 of 1.4 (22). IgG preparations were concentrated to 5 to 10 mg ml⁻¹ by ultrafiltration (Amicon) and stored at 4°C, supplemented with 0.02% (wt/vol) sodium azide.

Affinity purification of MSP-1-specific IgG. IgG was affinity purified by chromatography on fusion protein representing MSP-1-EGF1 covalently linked to an insoluble matrix (22). Briefly, 35 mg of purified fusion protein (32,274 Da) was coupled to 7 ml (swollen gel volume) of cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer’s instructions. This affinity matrix was estimated to be of sufficient capacity to bind approximately 16 mg of IgG (on the assumption of only 1 IgG molecule bound per 10 fusion protein molecules). Nine hundred mg of total IgG was passed over the affinity matrix; IgG which did not bind (designated void-IgG) was collected. After being extensively washed with 100 mM and then 10 mM Tris- HCl (pH 8.0), bound IgG was eluted into 100 mM glycine-HCl (pH 2.5) and neutralized with 1/10 volume of 1 M Tris-HCl (pH 8.0). IgG reactive with the GST fusion partner or with native E. coli polypeptides was then removed by use of a bacterial acetone powder (22). Briefly, E. coli transformed with plasmid pGex-3X was induced to express GST and then lysed and clarified by centrifugation. Bacterial proteins in the supernatant were precipitated with acetone, harvested, and then dried. This powder was added at a ratio of 20:1 (wt/wt) over IgG, and then, after incubation at 4°C for 1 h, insoluble immunocomplexes were removed by centrifugation at 39,000 x g for 15 min. Affinity-purified IgG specific for MSP-1 (designated AP-IgG) was extracted from the supernatant by use of the immobilized fusion protein, concentrated by ultrafiltration, and stored as described earlier.

Culture and metabolic radiolabelling of P. falciparum. Clone T9/94 (48) was maintained in culture and synchronized when required, essentially as described previously (27); growth medium was supplemented with 0.5% (wt/vol) AlbuMAX (Life Technologies) instead of serum (34a). Synchronous cultures containing predominantly schizont-stage parasites with 2 to 4 nuclei were washed once in methionine-free RPMI 1640 and resuspended at a 10% hematocrit in the same medium supplemented with AlbuMAX plus 1.85 MBq of [35S]methionine (Amersham) ml⁻¹. After 3 to 4 h of growth, cells were pelleted, washed three times in RPMI 1640, and then frozen at -70°C until required.

Immunoprecipitation and Western blotting methods. IgG specificities were analyzed by immunoprecipitation followed by SDS-PAGE and fluorography of the radiolabelled parasite proteins, by previously described procedures (27).

For analysis by Western blotting (immunoblotting), identical samples of the MSP-1-EGF1 polypeptide without the GST fusion partner were subjected to nonreducing SDS-PAGE by a Tricine buffer system (45) and then electrophoretically transferred to nitrocellulose, essentially as described previously (6). Primary antibody binding was detected with appropriate anti-IgG alkaline phosphatase conjugates and a chromogenic substrate. In both immunoprecipitation and Western blotting procedures, the murine MAb 111.4 (29) was used as a positive control; this antibody binds to a disulfide-constrained epitope of MSP-1-EGF1 (12).

Molecular mass markers used were obtained from Gibco BRL (high molecular mass, prestained; 14.3 to 200 kDa) or BDH, Poole, United Kingdom (CNBr-cleaved horse heart myoglobin; 2,512 to 16,949 Da).

ELISA to measure anti-MSP-1 antibodies. The titer of IgG specific for MSP-1 was measured by enzyme-linked immunosorbent assay (ELISA), essentially as described previously (3). Briefly, ELISA plates were coated with S42AA (39), a recombinant insect cell product which includes the carboxy-terminal 271 amino acids (D-1433 to S-1723 [38]) of the Wellcome/T9-94 MSP-1 and includes both EGF-like modules. Plates were washed, and twofold serial dilutions of each IgG (2 x 10⁻⁴ to 4.9 x 10⁻⁸ g ml⁻¹) were applied to duplicate wells. Following incubation with enzyme-conjugated anti-human IgG, plates were developed with a chromogenic substrate and $A_{405}$ values were measured.

Competition ELISA. To determine whether the human antibodies could inhibit the binding of MAb s, the plates were first incubated with serial dilutions of each human IgG fraction. The plates were washed, and an optimal dilution of MAb determined by titration was added to the plate. The binding of MAb was measured by use of an enzyme-conjugated anti-mouse IgG and chromogenic substrate.

In vitro parasite invasion inhibition assay. Mature schizonts, purified from highly synchronous cultures by centrifugation onto a cushion of 63% isotonic Percoll (Pharmacia), were added to erythrocytes to give a parasitemia of 0.5 to 1%. Each test IgG was dialyzed extensively against RPMI 1640 and then added to four identical microcultures of P. falciparum, at a final concentration of 1 mg ml⁻¹ (the final hematocrit was 2%). Control cultures (six of each) containing either no added IgG or 5 mM EGTA [ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] were also included; EGTA at this concentration effectively inhibits merozoites’ invasion of erythrocytes (36, 50). After 24 h of growth at 37°C, culture blood smears were made from each well and then fixed with methanol and stained with Giemsa stain. Parasitemia was determined by counting of the number of young parasites within 8,500 or more erythrocytes. Multiple parasites within a single erythrocyte were recorded as one count.
RESULTS

Affinity purification of MSP-1-specific human IgG. IgG was purified from pooled IgG of West African adults (total-IgG) by affinity chromatography on MSP-1-EGF1 fusion protein representing the first EGF-like module in the P. falciparum Wellcome/T9-94 type MSP-1. A two-stage affinity selection protocol, utilizing elution by acid at pH 2.5, yielded 2.9 mg of IgG (AP-IgG), 0.3% of the 900 mg of total-IgG starting material. Analysis of each IgG fraction by SDS-PAGE under reducing conditions revealed essentially only two polypeptides, corresponding to the IgG heavy and light chains (data not shown). A significant quantity of IgG remained bound to the affinity matrix following sequential washes at pH 2.5 and subsequent washes with buffers at neutral pH containing 8 M urea or 1% SDS; such IgG was only eluted by boiling in SDS-PAGE sample buffer (data not shown), and further analysis of this IgG was not attempted.

Characterization of affinity-purified human antibodies. ELISA, immunoprecipitation, and Western blotting experiments were carried out to demonstrate the specificity and to quantify the amount of MSP-1-specific antibody in AP-IgG in comparison with other Ig preparations.

The titers of MSP1-specific antibodies in the four Ig samples were compared by use of an ELISA based on the insect cell product S42ΔA, as shown in Fig. 1. Differences in anti-S42ΔA antibody titers between samples were estimated by comparison of IgG concentrations at fixed absorbance values; such approximations are valid in regions where plotted data lines remain parallel (31). By this method, the AP-IgG preparation was estimated to contain 100-fold more S42ΔA-specific Ig than the void-IgG. Note that the level of S42ΔA-specific Ig present in the void-IgG sample was reduced from that present in the total-IgG to approximately that in control-IgG, which was assumed to represent the background level. This result suggested that a substantial proportion of the S42ΔA-specific Ig originally present in the total-IgG preparation had been removed by the affinity purification procedure.

Immunoprecipitation experiments were conducted to demonstrate that the affinity purification procedure selected IgG which could recognize and bind to the native MSP-1. Figure 2 shows that the AP-IgG preparation (lane 3) and the control MAb 111.4 (lane 2) both immunoprecipitated a 190-kDa species from radiolabelled parasite material, corresponding in size to the intact MSP-1. No equivalent species was observed in immunoprecipitation reactions with either the total-IgG (Fig. 2, lane 4), void-IgG (lane 5), or control-IgG (lane 6) preparation or with protein A-Sepharose alone (lane 1). Other faster-migrating species were immunoprecipitated by all three polyclonal IgG preparations, and most of these were common among the samples (Fig. 2, compare lanes 3, 4, and 5); such species were not characterized further.

Western blotting was used to demonstrate that the AP-IgG preparation had enhanced levels of antibodies against the first MSP-1 EGF-like module compared with the other antibody preparations. Digestion of the GST fusion protein with factor Xa produced a 52-amino-acid polypeptide comprising MSP-1-EGF1 (residues 1631 to 1678 of MSP-1) preceded by four additional residues (GIQM), with a calculated molecular mass of 6,022 Da. Identical samples of this polypeptide were subjected to Tricine-SDS-PAGE under nonreducing conditions and transferred to nitrocellulose. Figure 3 shows that both MAb 111.4 (lane 1) and the AP-IgG preparation (lane 3) clearly detected the MSP-1-EGF1 polypeptide; this species was not detectable in the conditions of this assay with the total-IgG (lane 2), and neither the void-IgG nor the control-IgG preparation reacted with it (data not shown).
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FIG. 3. The titers of IgG specific for MSP-1-EGF1 within two polyclonal antibody preparations were compared by Western blotting. Identical amounts of the 52-residue (6,022 Da) polypeptide representing MSP-1-EGF1, derived from an E. coli-expressed fusion protein by site-specific proteolysis, were subjected to Tricine-SDS-PAGE and then transferred to nitrocellulose. Blots were probed with either MAb 111.4 (lane 1), total-IgG (lane 2), or AP-IgG (lane 3). Color development reactions were conducted in parallel and stopped at the same time. Protein molecular mass markers indicated were derived from CNBr-cleaved horse heart myoglobin, at 16,949 Da, 14,404 Da, 8,159 Da, 6,214 Da, and 2,512 Da.

These experiments indicate that compared with the void-IgG sample, a significantly greater proportion (approximately 100-fold by ELISA) of the antibodies within the AP-IgG preparation were directed against determinants present in the first EGF-like module of MSP-1.

Affinity-purified antibodies compete with a protective MAb for antigen binding. The different IgG preparations were assayed for their abilities to block the binding of two inhibitory MAbs to S42AA (Fig. 4). The AP-IgG fraction was able to compete with MAb 12.8 at a concentration of 2 μg ml⁻¹, but none of the other IgG was effective in this range. None of the antibodies inhibited the binding of MAb 12.10 at 10 μg ml⁻¹ (data not shown).

Parasite in vitro invasion assay. Highly synchronous microcultures of P. falciparum containing predominantly schizonts were incubated in the presence of various IgG preparations. After 24 h, encompassing schizont rupture-merozoite release and reinvasion of new erythrocytes, parasitemias were determined. The final parasitemia in cultures supplemented with African IgG preparations did not differ significantly from that of the control (Fig. 5), although EGTA was very effective at blocking invasion. The morphology of the parasites was normal on Giemsa-stained smears for all cultures, incubated with or without antibodies, and no agglutinated clusters of merozoites were observed in any sample.

DISCUSSION

We have investigated whether antibodies to the first EGF-like module of MSP-1 induced by natural infection are inhibitory to parasite growth in vitro. This biological property is manifested by some but not all MAbs specific for the C-terminal cysteine-rich region of MSP-1. Four murine MAbs which are specific for MSP-1 and which inhibit the growth of P. falciparum in vitro (2, 15, 42) have been described previously. Two of these antibodies, 12.8 (2) and 5B1 (42), bind to the first of two EGF-like modules in MSP-1 (12); another, 12.10, binds only if the two EGF-like modules are expressed together (9, 12). The present study was aimed at investigating whether or not naturally occurring antibodies with similar specificities

FIG. 4. Affinity-purified antibodies block the binding of an inhibitory MAb. ELISA plates coated in S42AA were preincubated with various concentrations of human IgG. Serial dilutions of AP-IgG (△), total-IgG (■), void-IgG (●), and control-IgG (▼) were used in triplicate. An optimal concentration of MAb 12.8 was then added, and the amount bound was determined. The ordinate represents A₄₉₂ as a measure of bound MAb, and the abscissa represents the IgG concentration.

FIG. 5. Invasion inhibition assay. Various IgG preparations were added to individual microcultures of P. falciparum to test for their abilities to inhibit parasite invasion of erythrocytes. Growth was also monitored in cultures containing either no added IgG or 5 mM EGTA, previously shown to effectively inhibit merozoite entry into erythrocytes. Bars on the chart represent mean final percentage parasitemias; error bars indicate standard distribution within each sample.
could manifest the same biological activity and therefore be responsible (at least in part) for the protection observed in immune individuals and in nonimmune individuals protected by passive transfer.

By affinity chromatography on the first EGF-like module of MSP-1 expressed as a fusion protein in E. coli, we selected a subset of IgG from a polyspecific IgG pool derived from West African adults and shown to passively confer immunity to malaria. We have previously concluded that MSP-1–EGF1 expressed in bacteria adopts the correct disulfide structure; it is reactive with MSP-1-specific MAb s, and this binding is diminished or abolished by prior treatment with thiol reagents (12). From 900 mg of total-IgG, 2.9 mg of IgG was selected by the affinity chromatography procedure. On the basis of these figures, and on the assumption that the AP-IgG is wholly specific for MSP-1, approximately 0.3% of the total-IgG population was recovered. Taking into consideration the fact that higher-affinity antibodies remained bound to the column during the elution process, the results suggest that large amounts of IgG are directed against the first EGF-like structure in MSP-1. No similar quantitative estimates for the proportion of MSP-1-specific antibodies in human serum have been previously reported.

The AP-IgG preparation was shown by ELISA to contain approximately 100-fold-enhanced levels of S42ΔA-specific antibodies compared with the void-IgG preparation. S42ΔA includes both EGF-like modules and parts of MSP-1 N terminal to this region; antibodies to these regions will increase absorbance values measured in the ELISA for the nonslected samples.

We found that only the AP-IgG preparation contained enough antibodies of the correct specificity to generate an MSP-1-specific signal in Western blotting and immunoprecipitation experiments. Successful immunoprecipitation of radio labelled MSP-1 demonstrates that AP-IgG can bind to the native protein. These data support the ELISA results and suggest that a substantial proportion of antibodies within the AP-IgG preparation are specific for MSP-1–EGF1. In addition, the selected antibodies inhibited the binding of MAb 12.8, which binds specifically to the first domain, but had no effect on the binding of MAb 12.10, which is known to be directed to a conformational epitope distinct from that of 12.8; when S42ΔA was used as the antigen.

Immunity against malaria, which may be acquired by long-term residents in regions where malaria is endemic, can be passively transferred to young children who have not developed an effective immunity by the administration of IgG from immune adults (14, 19, 43). The mechanism for this protection remains unclear. Our findings that a concentration of 1 mg of total-IgG ml⁻¹ did not significantly affect parasite multiplication were not surprising. In previous experiments, identical IgGs had no inhibitory effect on either parasite growth or invasion in vitro at concentrations of up to 5 mg ml⁻¹; indeed sometimes stimulating parasite growth, at least of some strains (7). Nevertheless, this antibody preparation exerted a profound reduction in asexual parasitemia when administered to human malaria patients by intravenous inoculation (43), and it is perhaps significant that it was shown to inhibit parasite growth in vitro in cooperation with monocites (7). Although the invasion inhibition assay used in the present study was similar to that used previously (2), in which merozoite invasion was significantly inhibited by MAb at a concentration of only 100 μg ml⁻¹, a 10-fold-higher concentration of the polyspecific AP-IgG preparation of human origin did not hinder merozoite entry into erythrocytes. This discrepancy suggests that minor differences in the target epitopes may be critical in the function of the corresponding antibodies.

It has been demonstrated that MSP-1₉ displays multiple distinct Ig-binding sites, and competition assays suggest that these are clustered or overlapping (12, 51). Fine-structure mapping of these epitopes will be difficult to achieve, in view of their conformational nature. Expression of the constituent EGF-like domains individually does not reproduce all antigenic determinants formed when they are expressed together, such as the binding site for the invasion-inhibitory MAb 12.10 (12). Idiotypes of the 12.8-like specificity should be present in the AP-IgG, and this is confirmed by our results, but these antibodies do not have inhibitory activity. The method of affinity purification used here would not be expected to select antibodies with 12.10-like specificities. The antibodies which bound to the affinity matrix and could only be eluted after denaturation may have had a very high affinity for MSP-1–EGF1; although it was not possible to assay them, this subset of affinity-selected IgG may have possessed invasion-inhibiting activity.

Natural infection or immunization with MSP-1–EGF1 may stimulate the production of several different anti-MSP-1–EGF1 idiotypes of which only a subset exert the required biological effect(s). Hence, it may be difficult to achieve the requirement for relatively high concentrations of inhibitory Ig suggested both by a kinetic model of merozoite invasion (44) and by experiments using MAb s (2, 15, 42). In addition, the binding of inhibitory MAb s such as 12.8 may be blocked by the binding of other antibodies which are themselves not inhibitory in vitro (5, 51). It is encouraging that polyclonal Ig in serum from rabbits immunized with an insect cell product representing the carboxyl-terminal processing product MSP1₄₂ completely inhibited the multiplication of the malaria parasite in vitro (11). Further experiments in primates and human volunteers will be critical to determine whether or not the artificial antigen can induce blocking antibodies whereas natural infection does not, or alternatively whether or not it is the host species which determines the type of antibody produced. At least in a mouse malaria, immunization with the two EGF-like modules of P. yoelii has provided very significant protection against challenge infection (17, 31). In these experiments, it is possible that epitopes in the second EGF module or formed from the interaction of the two domains are most important as targets of inhibitory antibodies.

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