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Generalized immunological recognition of the major merozoite surface antigen (gp195) of *Plasmodium falciparum*

(malaria vaccine/asexual blood-stage antigen/immune-response gene control)

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ABSTRACT **The antibody response to the *Plasmodium falciparum* major merozoite surface antigen (gp195) of congenic mouse strains differing in *H-2* haplotype has been examined. All seven strains of mice were capable of producing gp195-specific antibodies. Generalized immune recognition of gp195 by mice of diverse *H-2* haplotypes distinguished gp195 from the *P. falciparum* circumsporozoite protein and the 230-kDa and 48/45-kDa gamete surface antigens. However, the *H-2* genetic locus appeared to influence the specificity of gp195-specific antibodies. Immunoblot patterns of mouse sera with parasite antigens revealed a complex pattern of reactivity with terminal and intermediate processing fragments of gp195. The majority of immunoblot bands observed were similar for all of the mouse strains; however, there were several strains that additionally recognized a few unique fragments or displayed more intense reactivities with specific processing fragments. These results suggest that while individuals of diverse major histocompatibility complex makeup are capable of recognizing the gp195 antigen, the recognition of specific gp195 B-cell and T-cell epitopes may be under control of the major histocompatibility complex.**

The global resurgence of malaria, which is due in part to the spread of drug-resistant *Plasmodium falciparum* and the reduced efficacy of vector control measures, has intensified efforts to develop a malaria vaccine (1, 2). The current subunit vaccine strategy for producing a malaria vaccine relies on the ability of single parasite antigens to confer protective immunity in animal models or to induce antibodies that inhibit parasite growth *in vitro* (3). Antigens expressed during several stages of the complex life cycle of *P. falciparum* are being investigated, and a number of parasite proteins have been identified as potential vaccine candidates (4). Research on these proteins has focused on the structural and immunological properties of native parasite proteins and their derivative synthetic peptides and recombinant polypeptides and on their ability to induce protective immunity to malaria in animal and human hosts. Recently, interest has developed in studying the immunogenicity of subunit malaria vaccine peptides and polypeptides in individuals of different genetic backgrounds, since an effective vaccine must be capable of eliciting an immune response in a genetically heterogeneous population. The first of these studies were performed on synthetic peptides bearing the major repetitive epitope, Asn-Ala-Asn-Pro (NANP), of the circumsporozoite protein of *P. falciparum* (5, 6). When congenic mice of different *H-2* haplotypes were immunized with either an $NP(NANP)_5NA$ 24-mer peptide (5) or an $(NANP)_{40}$ peptide (6), only mice of the $H\text{-}2^b$ haplotype were able to produce an anti-NANP antibody response. Lymph node proliferation studies of mice immunized with a recombinant polypeptide (R32tet32) containing the circumsporozoite protein repeat demonstrated that T cells of mice only of the $H\text{-}2^b$ and $H\text{-}2^d$ haplotypes were capable of being stimulated with $(NANP)_n$ constructs (5). Restriction of responsiveness to a limited number of *H-2* haplotypes also has been reported for a recombinant *Plasmodium vivax* circumsporozoite protein (7).

This analysis of immune responsiveness has been extended to other malaria antigens, including a 230-kDa gamete surface antigen, a 48/45-kDa gamete surface antigen, and a 25-kDa zygote/ookinete antigen (8). After immunization with purified *P. falciparum* zygotes, only two of six congenic mouse strains produced antibodies that immunoprecipitated the 230-kDa antigen, while another two strains produced antibodies specific for the 48/45-kDa antigen. In contrast, an antibody response to the 25-kDa antigen was observed for most mouse strains. These studies demonstrated that genetic nonresponsiveness is not limited to synthetic peptides or recombinant polypeptides based on malaria antigens but is also a feature of some native malaria protein antigens.

In toto, these results have profound implications for development of subunit malaria vaccines. In order to be effective, a vaccine must stimulate an immune response in individuals of diverse genetic makeup. If widespread, genetically controlled nonresponsiveness is a common characteristic of malaria antigens, the feasibility of the subunit vaccine strategy will be severely compromised.

While genetic control of immune responsiveness to certain malaria antigens of the sexual stages of the parasite life cycle has been demonstrated, the effect of major histocompatibility complex (MHC) genes on responsiveness to asexual blood-stage antigens has not been examined. A prime candidate antigen for the development of immunity to the asexual blood stages of *P. falciparum* is the major merozoite surface protein (gp195), whose processing fragments are found on the surface of the infective merozoite (9). Several laboratories including our own have shown that vaccination of monkeys with purified gp195 or its processing fragments protects these animals against a lethal challenge dose of *P. falciparum* (10–13). In addition, a limited clinical trial with synthetic peptides containing gp195-related amino acid sequences has produced promising results (14). Therefore, it is important to determine whether immune responsiveness to gp195 is restricted to certain MHC haplotypes. To address this issue, the antibody responses of a panel of congenic mice differing in *H-2* haplotype and immunized with purified gp195 were measured. We observed that the antibody response to gp195 was not restricted to mice of a few *H-2* haplotypes but rather that a significant gp195-specific antibody response occurred in all mouse strains examined.

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Abbreviation: MHC, major histocompatibility complex.

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MATERIALS AND METHODS

Antigens. gp195 was purified from the *in vitro* cultivated Uganda–Palo Alto (FUP) isolate of *P. falciparum* as described (12). Parasite cultures used for immunoblots were grown to <10% parasitemia and consisted of a majority of schizont and ring stages. Membrane debris was separated from intact erythrocytes in a continuous 55% Percoll gradient as modified by Kramer *et al.* (15). Infected erythrocytes were diluted in 20 volumes of 0.85% NaCl and a final concentration of 0.01% saponin was added to lyse the infected erythrocytes. The whole parasites were collected by centrifugation and the parasite proteins were extracted in an equal volume of lysis buffer (62.5 mM Tris/2% SDS/0.001% antipain/0.001% pepstatin A/10 mM iodoacetamide, pH 8.0). The extract was clarified by ultracentrifugation and stored at −70°C.

gp195-Specific Antisera and Monoclonal Antibodies. Rabbit anti-gp195 sera were produced by hyperimmunization of rabbits with purified gp195 emulsified in complete Freund's adjuvant (16). Mouse monoclonal anti-gp195 antibodies were produced by fusing spleen cells of BALB/c or Swiss mice hyperimmunized with whole parasite lysates or with purified gp195 (17). Monoclonal antibody 5.2 was described previously (17); other gp195-specific monoclonal antibodies were provided by C. Locher and S. Case (ref. 18; C. Locher, unpublished work).

Immunization of Mice. Congenic mice (8–12 weeks old) differing in *H-2* haplotype but sharing the C57BL/10 genetic background were obtained from The Jackson Laboratory. The complete strain designations of the mice used in this study are C57BL/10 SnJ, B10.A/SgSnJ, B10.D2/nSnJ, B10.M/SN, B10.WB (69NS), B10.BR/SgSnJ, and B10.PL. Inbred *H-2*-congenic mouse lines (5–7 mice per group) were immunized intraperitoneally three times at 2-week intervals with 5 μg of parasite-purified gp195 emulsified in complete Freund's adjuvant. Mice were bled from the tail vein before immunization, 7 and 10 days after the primary injection, and 5, 7, and 14 days after the secondary and tertiary injections.

Serum Antibody Assay. Serum antibodies specific for gp195 were assayed by an enzyme-linked immunosorbent assay (ELISA) (19). Ninety-six-well vinyl plates were incubated with gp195 (0.5 μg/ml, 50 μl per well) in borate-buffered saline (BBS: 167 mM borate/134 mM NaCl, pH 8.0) at room temperature for 2 hr. The plates were washed and blocked with 1% bovine serum albumin in BBS for 1 hr, washed in BBS containing 0.5 M NaCl (HSBBS), and stored at −70°C. Mouse sera were serially diluted in BBS with 1% albumin and added to antigen-coated wells. After a 1-hr incubation at room temperature, plates were washed seven times with HSBBS and a 1:2000 dilution of peroxidase-conjugated goat anti-mouse IgG (heavy and light chain-specific; Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. After 1 hr at room temperature, plates were washed seven times in HSBBS and once in BBS. One hundred microliters of peroxidase substrate solution [containing H_2O_2 and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)] was added to each well and the absorbance at 410 nm was determined with a Dynatech 605 ELISA reader.

Indirect Immunofluorescence Assay. Assays were performed on acetone-fixed thin blood smears as described (17).

Immunoblots of Parasite Antigens. Extracts of saponin-lysed parasites were mixed with an equal volume of Laemmli's sample buffer with (reduced) or without (unreduced) 10% 2-mercaptoethanol and electrophoresed in 10% polyacrylamide gels containing 0.1% SDS (20). The molecular size standards used were myosin (heavy chain, 200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa) (Amersham). Proteins were electrophoretically blotted onto nitrocellulose according to Towbin *et al.* (21). Protein-containing strips were blocked with 5% nonfat powdered milk in HSBBS at room temperature for 1 hr and incubated overnight with mouse antisera diluted 1:200 in HSBBS containing 1% nonfat powdered milk. The strips were rinsed with HSBBS and incubated with the secondary antibody [rabbit anti-mouse IgG (heavy and light chain-specific; Zymed Laboratories)] for 1 hr at room temperature. After rinsing as described above the protein-containing strips were incubated for 1 hr at room temperature with goat anti-rabbit IgG (heavy and light chain-specific) antiserum conjugated to alkaline phosphatase, rinsed, and developed with the enzyme substrate (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) (Kirkegaard and Perry Laboratories).

RESULTS

Seven strains of congenic mice differing in *H-2* haplotype were immunized three times with purified gp195 emulsified in complete Freund's adjuvant. While low responses [geometric mean titers (g.m.t.) ≤ 1:100] were observed for a few individuals of certain mouse strains (C57BL/10, B10.A, B10.BR) after two immunizations with gp195, after the third immunization all mice produced IgG titers >1:100 (Fig. 1). Mice of certain strains produced higher tertiary responses [e.g., B10.WB (g.m.t. = 1:7833), B10.A (g.m.t. = 1:6600)] than others [e.g., C57BL/10 (g.m.t. = 1:1900), B10.D2 (g.m.t. = 1:2870)].

To determine whether the different congenic mouse strains immunized with purified gp195 produced antibodies crossreactive with gp195 on the merozoite surface, an indirect immunofluorescence assay of the mouse sera was performed (Table 1). Consistent with the ELISA results, the sera of all mouse strains studied contained antibodies reactive by immunofluorescence with gp195. The staining pattern was the typical merozoite surface immunofluorescence obtained with other anti-gp195 antibodies (17). The highest reactivities were again observed for the B10.WB and B10.A strains.

These results established that mice of different *H-2* haplotypes are capable of producing an antibody response against purified gp195. In order to begin to study the specificity of the antibody response of the congenic mice to gp195, the reactivity of the antisera with gp195 and its processing fragments was examined on immunoblots (Fig. 2). Preimmune mouse sera showed low background reactivity with the parasite extract (Fig. 2*A*). Immune sera of each strain reacted strongly with an ≈200-kDa polypeptide corresponding in size to the high molecular weight gp195 precursor molecule (Fig. 2*B*). In addition, all strains produced antibodies recognizing a wide array of smaller proteins thought to correspond to terminal processing fragments and processing intermediates of gp195 (12). The overall immunoblot patterns were similar for the various congenic mouse strains. However, a few striking differences were observed for certain congenic mouse strains. B10.D2 (lanes 4) and B10.WB (lanes 6) antibodies reacted very strongly with a 50-kDa fragment that was recognized more weakly by other congenic strains. B10.D2 and B10.WB antibodies additionally recognized a 52-kDa fragment that was not recognized by other congenic strains, and B10.WB antibodies reacted with a unique 68-kDa fragment. B10.PL (lanes 7) antibodies produced a more intense 70-kDa band than antibodies of the other strains and also recognized a unique 65-kDa fragment. It should be noted that these immunoblots were performed using pooled sera of 5–7 mice per strain to minimize the effects of individual mouse variations.

The complex pattern of reactivity with gp195 processing fragments suggests that the mice of different congenic strains produced antibodies specific either for a variety of epitopes distributed throughout the gp195 molecule or for a few

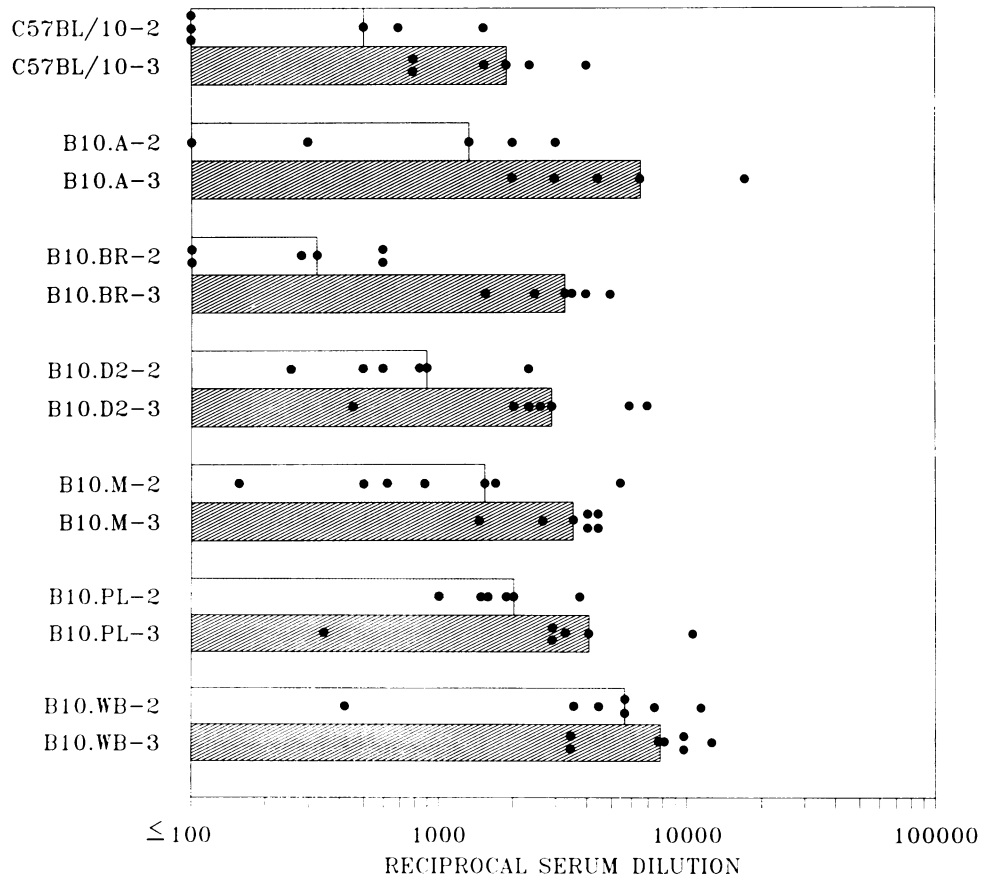

FIG. 1. Antibody responses of congenic mice immunized with purified gp195. Bars represent the geometric mean IgG ELISA titers of congenic mouse strains after two (stippled bars) and three (hatched bars) immunizations with purified gp195. Data points for individual mice are also shown (●). The results are expressed as the reciprocal of the serum dilution giving an absorbance reading of 0.1.

epitopes distributed on several intermediate and terminal gp195 processing fragments. The results of immunoblots of *P. falciparum* antigens with a set of gp195-specific mouse monoclonal antibodies (Fig. 2*C*) provide an indication of the heterogeneity of the murine anti-gp195 response. Each of the monoclonal antibodies reacts with the 200-kDa precursor molecule and a subset of fragments derived from this precursor. Most of the major bands recognized by the congenic mouse sera can be aligned with bands identified by the gp195-specific monoclonal antibodies. Many of the bands recognized by the congenic mouse sera appear to correspond to fragments reactive with the monoclonal antibody 5.2 (lane 8); however, additional bands observed with the congenic mouse serum antibodies correspond in size to fragments recognized by other gp195-specific monoclonal antibodies [24A1.7 (lane 9), 70 kDa; CE2.1 (lane 10) and S1.3 (lane 12), 65 kDa] as well as a few bands that may not be recognized by our panel of monoclonal antibodies (50 and 52 kDa). Thus, the antibody response to purified gp195 appears to be directed against several epitopes located in different regions of the molecule and does not appear to be focused on a single epitope as observed for the antibody response to the circumsporozoite protein of *P. falciparum* (22).

Table 1. Indirect immunofluorescence analysis of sera of congenic mice immunized with purified gp195

Mouse strain	*H-2* haplotype	Merozoite surface staining*				
		50	250	1250	6250	31,250
C57BL/10	*b*	4+	3+	2+	±	−
B10.A	*a*	4+	4+	3+	2+	+
B10.BR	*k*	4+	3+	3+	+	−
B10.D2	*d*	4+	4+	2+	+	−
B10.M	*f*	4+	3+	2+	+	±
B10.PL	*u*	4+	4+	3+	2+	±
B10.WB	*ja*	4+	4+	3+	2+	2+

*Sera were assayed at five dilutions (reciprocal of dilution is given above each column). Staining intensity scale: 4+, highest; ±, lowest detectable; −, not detectable.

It was noted that while complex patterns of reactivity with gp195 and its processing fragments also were observed with sera of rabbits (Fig. 2*C*, lane 13) immunized with purified gp195, the number of bands seen in the present study was greater than observed previously (12, 17). A number of precautions were taken during parasite antigen extraction to minimize the presence of degradation fragments, including the separation of membrane debris from intact infected erythrocytes in a Percoll gradient and the inclusion of protease inhibitors in the lysis buffer. It is likely that the immunoblot protocol used in this study is more sensitive in detecting antibodies to certain intermediate and terminal gp195 processing fragments than previous protocols. These fragments may not have adsorbed efficiently to immunoabsorbents or may not have immunoprecipitated as stable antigen–antibody complexes with formalin-treated *Staphylococcus aureus* (17). Alternatively, many fragments may not be adequately labeled by radioactive amino acids for detection by fluorography. While the majority of processing fragments were not observed with parasite proteins electrophoresed under reducing conditions, a band corresponding to the gp195 precursor was detected by antibodies of all mouse strains, and strain-specific recognition of certain processing fragments was still observed (data not shown).

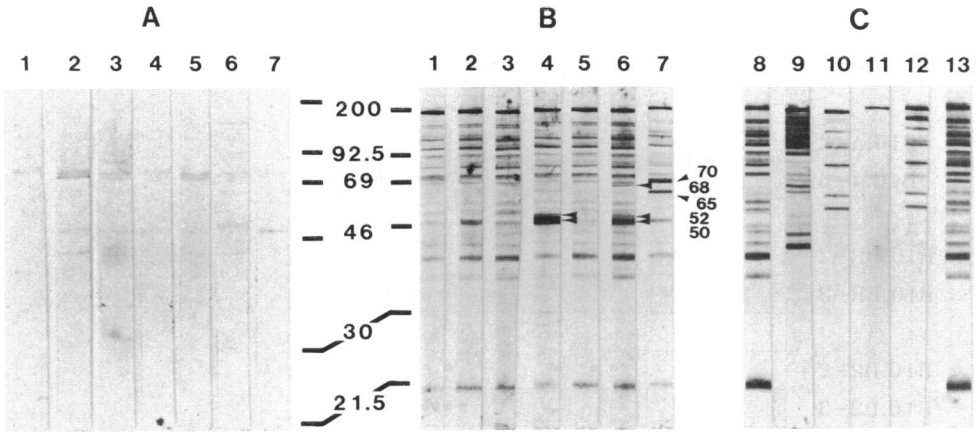

FIG. 2. Immunoblots of *P. falciparum* antigens separated under nonreducing conditions in SDS/10% polyacrylamide gels and reacted with pooled sera of congenic mice immunized with purified gp195. Molecular size markers are indicated in kilodaltons. (*A*) *P. falciparum* antigens were immunoblotted with pooled preimmune serum of congenic mice. Lanes: 1, B10; 2, B10.A; 3, B10.M; 4, B10.D2; 5, B10.BR; 6, B10.WB; 7, B10.PL. (*B*) *P. falciparum* antigens were immunoblotted with pooled tertiary sera of congenic mice immunized with purified gp195; lanes are in the order described above. (*C*) *P. falciparum* antigens were immunoblotted with anti-gp195 monoclonal antibodies 5.2 (lane 8), 24A1.7 (lane 9), CE2.1 (lane 10), DB8.2 (lane 11), and S1.3 (lane 12) and with a pool of rabbit anti-gp195 antisera (lane 13). Arrowheads indicate quantitative or qualitative differences in recognition of parasite antigens by the congenic mouse strains.

DISCUSSION

The ability of congenic mouse strains possessing seven different *H-2* haplotypes to produce an antibody response against gp195 distinguishes this blood-stage antigen of *P. falciparum* from several other malaria antigens including the circumsporozoite protein and the 230-kDa and 48/45-kDa gamete surface antigens (5–8). Generalized immune responsiveness to purified gp195 among the congenic mice indicates that this antigen possesses T-cell and B-cell epitopes capable of eliciting an antibody response in individuals of diverse MHC makeup. Antibodies produced by these congenic mouse strains displayed extensive crossreactivity with gp195 on the merozoite surface by indirect immunofluorescence. The typical merozoite surface staining pattern obtained with the mouse antisera suggests that the antibodies reacted with epitopes expressed by processed gp195 molecules on the mature merozoite surface.

Since all of the congenic mice were capable of producing anti-gp195 antibodies, we were interested in comparing the antibody specificities produced by mice of different *H-2* haplotypes. Effects of *H-2* haplotype on antibody specificity have been reported for several model protein antigens (23–25) and for the hepatitis B surface antigen (26). Immunoblot analysis of *P. falciparum* lysates electrophoresed under nonreducing conditions revealed a complex pattern of reactivity with the gp195 precursor and a collection of smaller fragments thought to represent distinct regions of the complete molecule (27–31). It was of interest that the immunoblot complexity of all mouse strains was decreased when parasite lysates were electrophoresed under reducing conditions. However, the numerous processing fragment bands were not resolved into a limited number of bands as would be expected if the formation of intermolecular, mixed disulfides were responsible for multiple bands detected under nonreducing electrophoresis conditions. It is possible that a majority of epitopes detected under nonreducing conditions are disulfide-dependent. However, the unique patterns of reactivity with gp195-derived fragments observed for some congenic strains under both reducing and nonreducing conditions suggest that while mice of diverse *H-2* haplotypes are capable of producing anti-gp195 antibodies, the production of antibody populations recognizing certain epitopes may be distinct for MHC-disparate individuals.

A few epitopes recognized by human T cells and B cells have been localized to conserved regions toward the amino terminus of gp195 (32, 33). Additional B-cell epitopes distributed throughout the molecule have been demonstrated by using mouse monoclonal antibodies, although these epitopes have not been mapped to defined amino acid sequences (31). The immunoblot patterns of the congenic mice corresponded to the combined patterns of several gp195-specific monoclonal antibodies. Epitope mapping studies will be needed to localize the epitopes recognized by the congenic mouse antibodies. Since not all antibodies against gp195 and its analogs in other *Plasmodium* species appear to affect parasite growth *in vitro* (34–38) and *in vivo* (39, 40), it is important to define the specificity of antibody subpopulations that possess biological activity and to measure the expression of these antibodies by mice of different *H-2* haplotypes.

Studies of immune responsiveness in a cohort of adults and children from the Gambia, a region of West Africa where *P. falciparum* malaria is holoendemic, indicate that genetic nonresponsiveness to certain malaria antigens in congenic mice may be paralleled by similar results in humans. The frequency of antibodies to the circumsporozoite protein epitope (NANP) was 63% and 62% in Gambian adults and children, respectively; ≈40% of adult sera immunoprecipitated the 230-kDa gamete surface antigen whereas only 6% reacted with the 48/45-kDa antigen (M. F. Good, personal communication). Variability in human immune responses to circumsporozoite protein or to gamete antigens were also reported for studies conducted in Papua New Guinea (41, 42), Tanzania (43), and Gabon (44).

Gabra *et al.* (45) conducted a study on the the serological reactivity of adults and children from the Gambia with several parasite antigens including a 23-kDa recombinant fusion polypeptide fragment of gp195 (45). Antibodies reactive with the gp195-based recombinant polypeptide were detected in the majority of children (≥93%) and adults (≥99%). However, the authors noted that relatively low levels of these antibodies were found in some individuals and there were great variations in the ELISA values among the samples studied. Production of antibodies to the same fusion polypeptide has also been measured in the Kassa district of Gabon, a mesoendemic malaria region (44). In this study, only 70% of adults had antibodies reactive with the recombinant fusion polypeptide; however, production of these antibodies appeared to be correlated with the control of parasitemia. The difference in frequencies of antigen-specific antibody production observed in these two studies may be

related to the different malaria-transmission rates and consequent differences in exposure to malaria antigens of the two regions. Prevalent antibody responses to purified gp195 (P190) also have been observed for sera of pregnant Nigerian women living in malaria-endemic regions (28).

The immunogenicity of purified gp195 in mice differing in *H-2* makeup supports the current subunit approach for development of a gp195-based malaria vaccine. In constructing a gp195-based subunit vaccine, it would be ideal to produce the complete precursor molecule as a single recombinant polypeptide. However, for practical reasons, it may be necessary to produce gp195 as several recombinant polypeptide fragments rather than a single, complete protein. It will be essential to evaluate the immune responsiveness of MHC-disparate individuals to these recombinant antigens before clinical trials are contemplated.

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