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Roles of Conserved and Allelic Regions of the Major Merozoite Surface Protein (gp195) in Immunity against *Plasmodium falciparum*

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The *Plasmodium falciparum* major merozoite surface protein gp195 is a candidate antigen for a vaccine against human malaria. The significance of allelism and polymorphism in vaccine-induced immunity to gp195 was investigated in this study. Rabbits were immunized with each of two allelic forms of gp195 that were affinity purified from the FUP and FVO parasite isolates. gp195-specific antibodies raised against one allelic form of gp195 cross-reacted extensively with the gp195 of the heterologous allele in enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays. Competitive binding ELISAs with homologous and heterologous gp195s confirmed that a majority of the anti-gp195 antibodies produced against either allelic protein were cross-reactive. Moreover, the biological activities of the gp195 antibody responses were also highly cross-reactive, since anti-gp195 sera inhibited the in vitro growth of the homologous and heterologous parasites with equal efficiency. The degree of cross-reactivity with strain-specific and allele-specific determinants of gp195 in the ELISA was low. These results suggest that the immunological cross-reactivity between the two gp195 proteins is due to recognition of conserved determinants. They also suggest that a gp195-based vaccine may be effective against blood-stage infection with a diverse array of parasite isolates.

The *Plasmodium falciparum* major merozoite surface protein gp195 is a 195-kDa polypeptide that is synthesized at the schizont stage and then processed into a number of fragments found on the merozoite surface (12, 21). Vaccination of monkeys with parasite gp195 or recombinant and synthetic peptide fragments of gp195 conferred complete or partial protection against lethal blood-stage infections (8, 16, 20, 22, 42–44, 54). Thus, gp195 is a candidate human vaccine antigen against malaria. Genetic analyses of gp195 from a number of parasite isolates indicate that gp195 exists primarily in two allelic forms (56, 57). Allele-specific sequences occur as segments or blocks along the gp195 molecule and are flanked by conserved segments or blocks (56). A novel sequence within a region designated block 2 of gp195 (56) defines a third allelic form of gp195 (5, 46). Each parasite possesses one allelic form of gp195. Structural analyses also show that recombinations occur between gp195 alleles that give rise to hybrid gp195 molecules (30, 45, 46, 56). In addition to allele-specific and conserved sequences, a short segment of amino acids composed of tripeptide repeats is located at the N-terminal region of gp195. Both the sequence and length of the tripeptide repeats vary among different parasite isolates (56). Studies with gp195-specific monoclonal antibodies have also identified conserved, allele-specific, and strain-specific B-cell determinants (1, 10, 17, 24, 35, 39, 47). Epidemiological surveys show that parasites possessing different gp195 alleles or recombinants coexist in populations in which malaria is endemic; although one particular form of gp195 may dominate, multiple alleles can coinfect a single individual (30, 31, 51, 52). Despite all of these studies, the significance of conserved, allele-specific or strain-specific regions in gp195-induced immunity in humans remains uncertain. There is evidence that naturally acquired immunity to malaria is strain specific (29, 39), and in vitro studies have shown that human immune sera inhibit parasite growth in a strain-specific manner (18, 55, 62). However, it is difficult to correlate these results with gp195-specific immunity because of the potential contribution of other malarial antigens, many of which possess strain-specific determinants, in naturally acquired immunity. In addition, clinical immunity to blood-stage infections may be a concerted response to a number of protective antigens, and an isolated component of the immune response to a particular antigen (e.g., gp195) may not by itself be sufficient to confer protection. A number of vaccination studies with gp195 in *Aotus* or *Saimiri* monkeys have been performed; the degree of protection varies from partial to complete (16, 42, 44, 54). The best protection was observed in one study that utilized the homologous parasite strain as the challenge inoculum (54). However, variations in the antigen preparations, immunization protocols, monkey species, immunogenicity of gp195, challenge inoculum, and virulence of parasite isolates could account for the differences in the degree of protection among the vaccination studies. Recently, Etlinger et al. showed that immunization of *Saimiri* monkeys with native gp195 from the K1 isolate protected against heterologous challenge with the FUP isolate (11). However, sequence comparisons showed that the K1 and FUP gp195s share identical allelic sequences for the first 375 amino acids (25% of the whole protein), whereas the remainder of the FUP sequence is similar to the MAD20 allele (7). The protection afforded in this study could have been due to epitopes within conserved blocks or to the recognition of identical epitopes in the allelic block within the first 375 amino acids of both FUP and K1 gp195s. Therefore, the roles of allele and conserved regions of gp195 in protective immunity remain unclear. To begin to understand the significance of allelism in vaccine-induced immunity to gp195, we immunized rabbits with gp195s from two parasite isolates that possess different allelic gp195 sequences. The isolates used in this study are FUP (Uganda-Palo Alto) and FVO (Vietnam-Oaknoll), which represent the MAD20 and K1 alleles, respectively. These isolates were chosen for analysis because they have differences in gp195

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gene structure and have both been adapted to give consistent blood-stage infections in *Aotus* monkeys and therefore are of potential interest for evaluation in this animal model. We found that immunization with the gp195 of one allele induced antibodies that were highly cross-reactive with the gp195 of the heterologous allele. Moreover, these anti-gp195 antibodies were equally efficient in inhibiting the in vitro growth of parasites with either gp195 allele. Our studies suggest that allelic epitopes may not contribute significantly to vaccine-induced immunity to gp195.

MATERIALS AND METHODS

Parasites. The FUP and FVO isolates of *P. falciparum* were used. The histories of these two isolates were described previously (15, 53, 58). In vitro cultivation of these parasites was performed by established methods (26, 61). Synchronized parasite cultures were obtained by using sorbitol lysis (33).

Isolation of gp195. gp195s from the FUP and FVO isolates were purified from Nonidet P-40 detergent extracts of in vitro-cultured parasites by using monoclonal antibody affinity chromatography as previously described (54). The monoclonal antibody, MAb 5.2, used for purification was specific for the C-terminal, conserved region of gp195 (data not shown). The isolated proteins were visualized by silver staining of gels after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. All bands detected by silver staining were recognized by anti-gp195 monoclonal antibodies on immunoblots (data not shown).

Immunoblotting. FUP and FVO parasite cultures were synchronized by sorbitol lysis to obtain a majority of schizont and ring stages. Membrane debris was removed by Percoll gradient centrifugation (32). Parasite proteins were extracted with 1% SDS in borate-buffered saline (pH 8.0) as previously described (6). The extracted proteins were clarified by ultracentrifugation, separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, and electrophoretically transferred to nitrocellulose as described previously (60). Immunoblotting with rabbit anti-gp195 sera was performed as follows. Nitrocellulose strips were blocked with 4% nonfat powdered milk in borate-buffered saline. The strips were incubated with rabbit serum at a 1/500 dilution for 1 h. After the strips were washed with borate-buffered saline containing 0.5 M NaCl, they were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (heavy and light chain specific; Zymed Laboratories) at a 1/1,000 dilution for 1 h and washed with borate-buffered saline–0.5 M NaCl. Reactive protein bands were visualized by incubation with the enzyme substrate (nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate; Kirkegaard and Perry Laboratories).

ELISA. Serum anti-gp195 antibodies from rabbits immunized with gp195 were measured by using an enzyme-linked immunosorbent assay (ELISA) with purified gp195s from FUP and FVO as the coating antigens (28). To measure the proportion of the anti-gp195 antibody response cross-reacting with the heterologous gp195, a competitive inhibition ELISA was performed with parasite-purified FUP and FVO gp195s. To measure the proportion of anti-FUP gp195 antibodies cross-reacting with FVO gp195, anti-FUP gp195 rabbit sera were diluted to a point on the descending portion of the ELISA titration curve. The diluted sera were mixed with various concentrations of FVO gp195, incubated for 1 h, and then allowed to react with FUP gp195-coated plates. As a control, diluted sera were similarly mixed with equal concentrations of FUP gp195 before reacting with FUP gp195-coated plates. Analogous inhibition ELISAs were performed to measure the cross-reactivity of anti-FVO gp195 sera with FUP gp195.

Indirect immunofluorescence antibody assay. Rabbit anti-gp195 sera were assayed for reactivity with gp195 on whole parasites by using acetone-fixed, schizont-infected erythrocytes as described previously (54).

In vitro parasite growth inhibition with anti-gp195 sera. Rabbit anti-gp195 sera were evaluated for their ability to inhibit the growth of homologous and heterologous parasites (FUP or FVO) by using established methods (28). Quarternary day 14 sera were used in inhibition assays. The corresponding preimmune sera collected before immunization were used as controls. Rabbit sera were heat inactivated at 58°C for 40 min and absorbed with fresh normal human erythrocytes before use. Parasite cultures were synchronized by sorbitol lysis to select for late trophozoite and schizont stages. Infected erythrocytes were adjusted to a parasitemia of approximately 0.5% and a hematocrit of 0.8%. Rabbit preimmune or immune sera were added to a final concentration of 15%, and 200-μl samples of the culture suspension were added in duplicate wells to a 96-well microtiter plate. Cultures were incubated for 72 h, and the parasitemia was determined by using microscopy. The degree of growth inhibition was calculated as follows: percent inhibition = $[(P - O) - (I - O)]/(P - O) \times 100\%$, where P is the parasitemia at 72 h of cultures incubated in preimmune sera; I is the parasitemia at 72 h of cultures incubated in immune sera, and O is the initial starting parasitemia at 0 h.

Rabbits and immunizations. Three rabbits (K41, K42, and K43) were hyperimmunized with purified FUP gp195, and three rabbits (K84, K85, and K86) were hyperimmunized with FVO gp195. Four immunizations were given intramuscularly at 21-day intervals. Each injection consisted of 50 μg of gp195 in complete Freund adjuvant (CFA). The amount of mycobacterium in CFA was successively halved for the secondary through the quarternary immunizations to minimize side reactions.

Characterization of gp195 genes from FUP and FVO isolates. Genomic DNA was obtained from in vitro-cultured FUP and FVO parasites by lysis with SDS and then phenol-chloroform extraction by standard methods (38). To characterize the allelic regions of the gp195 genes from these two isolates, synthetic oligonucleotide probes corresponding to published sequences of gp195 allelic regions of the K1 and FC27 (equivalent to MAD20 allele) isolates (36, 45) were synthesized by utilizing β-cyanoethyl phosphoramidite chemistry on an Applied Biosystems model 380B automated oligonucleotide synthesizer according to the recommendations of the manufacturer. Oligonucleotide probes were purified and desalted on Applied Biosystems oligonucleotide purification cartridges. Two allele-specific restriction enzyme fragments (based on the *Pst*I site at position 1445 of FC27 gp195 and the *Eco*RI site at position 4305 of FC27 gp195) were also probed with synthetic oligonucleotides and FUP gp195 genomic clones (7). Figure 1 summarizes the nucleotide sequences of the probes used. The probes were end labeled with [^{32}P]ATP by using T4 kinase under standard conditions (38). Briefly, 30 pmol of DNA was labeled with 10 U of T4 kinase and 150 μCi of [^{32}P]ATP (1,000 Ci/mmol; Amersham) in 0.05 M Tris–0.01 M $MgCl_2$–5 mM dithiothreitol–0.1 mM spermidine–0.1 mM EDTA at 37°C for 30 min. Reactions were terminated by the addition of 0.5 M EDTA. Labeled probes were purified by using Elutip-d columns (Schleicher and Schuell). Parasite DNA (10 μg) was digested

Probe 1	FC27	nucleotides 942-971	Allelic
Probe 2	K1	nucleotides 933-962	Allelic
Probe 3	FC27	nucleotides 1143-1172	Allelic
Probe 4	K1	nucleotides 1120-1149	Allelic
Probe 5	FC27	genomic clone/Pst1 site (1445)	Allelic
	K1	genomic clone/none	Allelic
Probe 6	FC27	nucleotides 3834-3863	Allelic
Probe 7	K1	nucleotides 3612-3641	Allelic
Probe 8	FC27	probe 7/EcoR1 site (4305)	Allelic
	K1	probe 8/none	Allelic

FIG. 1. Oligonucleotide probes used for the characterization of FUP and FVO gp195 allelic regions. The sequences of the probes are based on the dimorphic alleles represented by the K1 and FC27 isolates (36, 45). In some instances, allele-specific restriction sites (*Pst*I and *Eco*RI) were also analyzed.

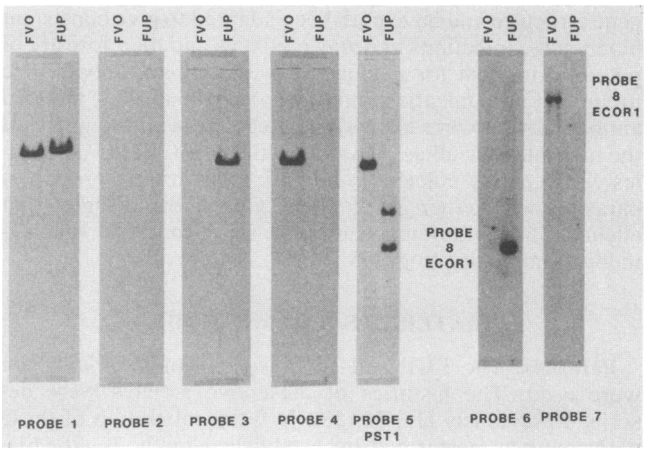

FIG. 2. Genomic blots of FVO and FUP DNA probed with the oligonucleotides listed in Fig. 1.

with *Eco*RI and separated on 0.7% agarose gels. Separated DNA fragments were denatured in 1.5 M NaCl–0.5 M NaOH for 1 h and neutralized with 1 M Tris–1.5 M NaCl (pH 8.0). They were transferred onto nitrocellulose filters in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and baked for 2 h at 80°C. Filters were probed with [^{32}P]ATP-labeled oligonucleotides in hybridization buffer (5× Denhardt solution, 5× SSC, 100 μg of sheared salmon sperm DNA per ml, 50 mM sodium phosphate buffer [pH 6.8], 1 mM sodium pyrophosphate, 100 μM ATP, 10% dextran sulfate, 20% formamide) at 37°C overnight. Filters were washed in 2× SSC–0.1% SDS and then in 1× SSC–0.1% SDS at 37°C. The hybridized fragments were visualized by autoradiography.

The variable tripeptide repeat sequence of the FVO gp195 gene was determined by amplification of the repeat region by the polymerase chain reaction (PCR) (49) with oligonucleotide primers corresponding to sequences flanking the repeat region of the gp195 gene of the K1 isolate. The sequences of the two primers were 5′-CCA ATT GGG AAT TCA CAA TGT GTA ACA CGT G-3′ and 5′-CCA ATT GGC TCG AGC TAA TTC AAG TGG ATG AG-3′. The amplified fragment was gel purified, subcloned into M13mp19, and sequenced by the dideoxynucleotide-chain termination method with a Sequenase version 1.0 kit (U.S. Biochemical) as recommended by the manufacturer.

Since FUP and FVO are not cloned parasites, potential contamination with the other gp195 allele was examined by PCR amplification of the 3′ dimorphic regions of gp195 with FUP and FVO genomic DNA. Four oligonucleotides were synthesized as primers for the PCR. Two primers corresponding to nucleotide positions 3586 through 3609 and 4510 through 4533 of K1 gp195 (36) and two primers corresponding to nucleotide positions 3877 through 3900 and 4888 through 4911 of FC27 gp195 (45) were used to amplify allelic blocks 14 through 16 to generate an expected PCR product of approximately 1 kb (56). Genomic DNA samples at concentrations of 5, 10, 15, and 20 μg/ml were amplified with each primer pair.

Recombinant gp195 polypeptides. Two recombinant polypeptides of the C-terminal region of gp195, p42 and p42A3, were expressed in the alcohol dehydrogenase 2–glyceraldehyde-3-phosphate dehydrogenase-regulated expression system of *Saccharomyces cerevisiae* with plasmid pBS24 (48). The p42 polypeptide is analogous to the natural cleavage product of gp195 (19) and corresponds to Ala-1333 through Ser-1705 of FUP gp195, and p42A3 (Ala-1333 through Leu-1587) corresponds to the N-terminal portion of p42 (7). The construction and expression of these recombinant polypeptides are described elsewhere (25).

Synthetic peptides. Two synthetic peptides (18-mers) corresponding to two sequence motifs of the FUP gp195 variable tripeptide repeat region [VR-1, $(SAQSGTSGT)_2$; VR-2, $SAQ(SGT)_5$] and a synthetic peptide (30-mer) corresponding to block 4 of the FUP allelic sequence, DKINEIKNPPPA NSGNTPNTLLDKNKKIEE (7), were produced by Merrifield solid-phase peptide synthesis (41) with t-Boc chemistry on an automated peptide synthesizer (Applied Biosystems).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to GenBank and EMBL and assigned the accession numbers M83091 and X63185, respectively.

RESULTS

Characterization of allelic regions of FUP and FVO gp195. Figure 2 shows the results of the Southern blots of FVO and FUP DNA probed with the oligonucleotides described in Fig. 1. With one exception, each allele-specific probe reacted exclusively with one of the two isolates. Allele-specific *Pst*I (probe 5) and *Eco*RI (probe 8) restriction enzyme sites were present in only one of the two isolates. Sequencing of the 5′ region of FVO gp195 (Fig. 3) also showed that FUP and FVO gp195 did not share allelic sequences at the 5′ block 2 region (Fig. 3B, amino acid positions 55 through 77). These results indicate that FUP gp195 and FVO gp195 differ in nearly all of the allelic regions. The only exception was noted with probes 1 and 2, which are specific for each of the two allelic sequences within the block 4 region of gp195 (56). Probe 1, which is specific for allelic block 4 of the FC27 isolate (45), hybridized with both FUP and FVO DNAs. Conversely, probe 2, which is specific for allelic block 4 of the K1 gp195 allele, hybridized to neither FUP nor FVO. This indicates that the gp195 sequences of the two isolates shared this allelic block, which encodes a sequence of 30 amino acids. The sharing of allelic sequences within block 4 of FVO and FUP gp195s is not unusual, since recombination between the gp195 alleles is very common in this region and, as a result, parasite clones that harbor both gp195 alleles and that share the same allelic sequences in block 4 have been found (45, 46, 57).

A.

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GAAAGTTATCAAGAACTTGTCAAAAAACTAGAAGCTTTAGAAGATGCAGTATTGACAGGTTATAGTTTATTTCAAAAG
  E  S  Y  Q  E  L  V  K  K  L  E  A  L  E  D  A  V  L  T  G  Y  S  L  F  Q  K
GAAAAAATGGTATTAAATGAAGGAACAAGTGGAACAGCTGTTACAACTAGTACACCTGGTTCAAAGGGTTCAGTTGCT
  E  K  M  V  L  N  E  G  T  S  G  T  A  V  T  T  S  T  P  G  S  K  G ▸S  V  A
TCAGGTGGTTCAGGTGGCTCAGTTGCTTCAGGTGGCTCAGTTGCTTCAGGTGGCTCAGTTGCTTCAGGTGGCTCAGTT
  S  G  G  S  G  G  S  V  A  S  G  G  S  V  A  S  G  G  S  G  A  S  G  G  S  V
GCTTCAGGTGGTTCAGGTAATTCAAGACGTACAA
  A  S  T  G  S  G  N ◂S  R  R  T
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B.

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                30          40          50          60          70          80
    Fvo  THESYQELVK KLEALEDAVL TGYSLFQKEK MVLNE..... .GTSGTAVTT STPGSKGSVA
  Mad 20 THESYQELVK KLEALEDAVL TGYSLFQKEK MVLNE..... .GTSGTAVTT STPGSSGSVT
Honduras THESYQELVK KLEALEDAVL TGYSLFQKEK MVLNE..... .GTSGTAVTT STPGSKGSVA
  Bandia       LVK KLEALEDAVL TGYSLFQKEK MVLNE..... .GTSGTAVTT STPGSKGSVA
     Fup  THESYQELVK KLEALEDAVL TGYGLFHKEK MILNEEEITT KGA..... .......SAQ
      K1  THESYQELVK KLEALEDAVL TGYSLFHKEK MILNEEEITT KGA..... .......SAQ
                                                                        ▸

                90         100         110         120         130         140
    Fvo  SGGSGGSVA. ..SGGSVASG GSGASGGSVA SGGSGN.... .......... .......SRR
  Mad 20 SGGSVASVA. .....SVASG GSGG...SVA SGGSGN.... .......... .......SRR
Honduras SGGSGGSVA. ..SGGSVASG GSGN...... .......... .......... .......SRR
  Bandia SGGSGGSVAS GGSGGSVASG GSVASGGSVA SGGSGN.... .......... .......SRR
     Fup SGTSGTSGTS GTSGTSGTSG TSAQSGTSGT SAQSGTSGTS AQSGTSGTSG TSGTSPSSRS
      K1 SGTSGTSGTS GPSGPSGT.. .......... .......... .......... ....SPSSRS
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FIG. 3. (A) Nucleotide sequence and the translated amino acids of the variable tripeptide region of FVO gp195. Arrowheads identify the variable tripeptide repeat region. (B) Comparison of the amino acid sequences of the variable tripeptide repeat regions of gp195s from the FVO, MAD20 (56), Honduras (23), Bandia (50), FUP (7), and K1 (36) parasite isolates. Sequences were aligned for maximal homology by using the Bionet ALP3/ALN3 alignment program. Arrowheads identify the variable tripeptide repeat regions. Spaces (.....) were inserted in sequences for maximal alignment.

Since both FUP and FVO are monkey-adapted isolates that have not been cloned, we investigated the possibility of contamination of these isolates with parasites bearing the second gp195 allele by using PCR techniques with oligonucleotide primer pairs specific for the 3′ allelic regions of the gp195 dimorphs and optimal (5 μg/ml) and excess (10, 15, and 20 μg/ml) concentrations of genomic DNA. Only one allelic sequence was detected with 5-μg/ml genomic DNA from FUP (Fig. 4, lanes 2 and 4) and FVO (lanes 3 and 5) isolates, thus indicating that the gp195 sequences of these isolates were homogeneous. Similar results were obtained with higher DNA concentrations (data not shown).

Sequencing of the variable tripeptide repeat region of FVO gp195. Figure 3A shows the nucleotide sequence and the translated amino acid sequence of the variable tripeptide region of FVO gp195. The repeat motif is composed of two tripeptide sequences, SVA and SGG. Figure 3B shows the comparison of the variable tripeptide repeat regions of gp195 genes of the FVO, MAD20, FUP, Honduras, K1, and Bandia isolates. These two tripeptide sequences are found in the MAD20, Bandia, and Honduras gp195 genes (23, 50, 56). However, differences were noted in the length and/or the arrangement of the repeats among these three isolates. The FVO gp195 repeat sequence is different from the FUP gp195 repeat motifs, which are composed of the tripeptide sequences SGT and SAQ (7).

Characterization of rabbit anti-gp195 antibodies. Hyperimmune sera from rabbits immunized with FUP and FVO gp195s were assayed by using the ELISA for antibodies recognizing FUP and FVO gp195s. Figure 5A shows the binding curves of rabbit anti-FUP gp195 (K41, K42, and K43) and anti-FVO gp195 (K84, K85, and K86) sera to FUP gp195. Anti-FUP gp195 and anti-FVO gp195 antibodies reacted equivalently with FUP gp195. The results were similar when FVO gp195 was used as the antigen in the ELISA (Fig. 5B). To characterize further the reactivity of these antibodies, rabbit sera were assayed for binding to native gp195 on acetone-fixed, schizont-infected erythrocytes. The reactivities of anti-FVO gp195 sera with acetone-fixed FUP and FVO parasites were very similar (Table 1). Similar observations were made with anti-FUP gp195 sera

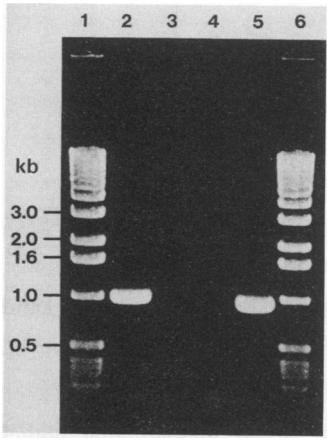

FIG. 4. Ethidium bromide staining of PCR products from FUP and FVO genomic DNA separated on a 1% agarose gel. Lanes 2 and 4 contain PCR products from FUP genomic DNA amplified with allele-specific oligonucleotide primers (see Materials and Methods) specific for FC27 (lane 2) and K1 (lane 4) gp195 alleles. Lanes 3 and 5 contain PCR products from FVO genomic DNA amplified with allele-specific oligonucleotide primers specific for FC27 (lane 3) and K1 (lane 5) gp195 alleles. Lanes 1 and 6 contain 1-kb DNA ladder molecular size markers (GIBCO/BRL).

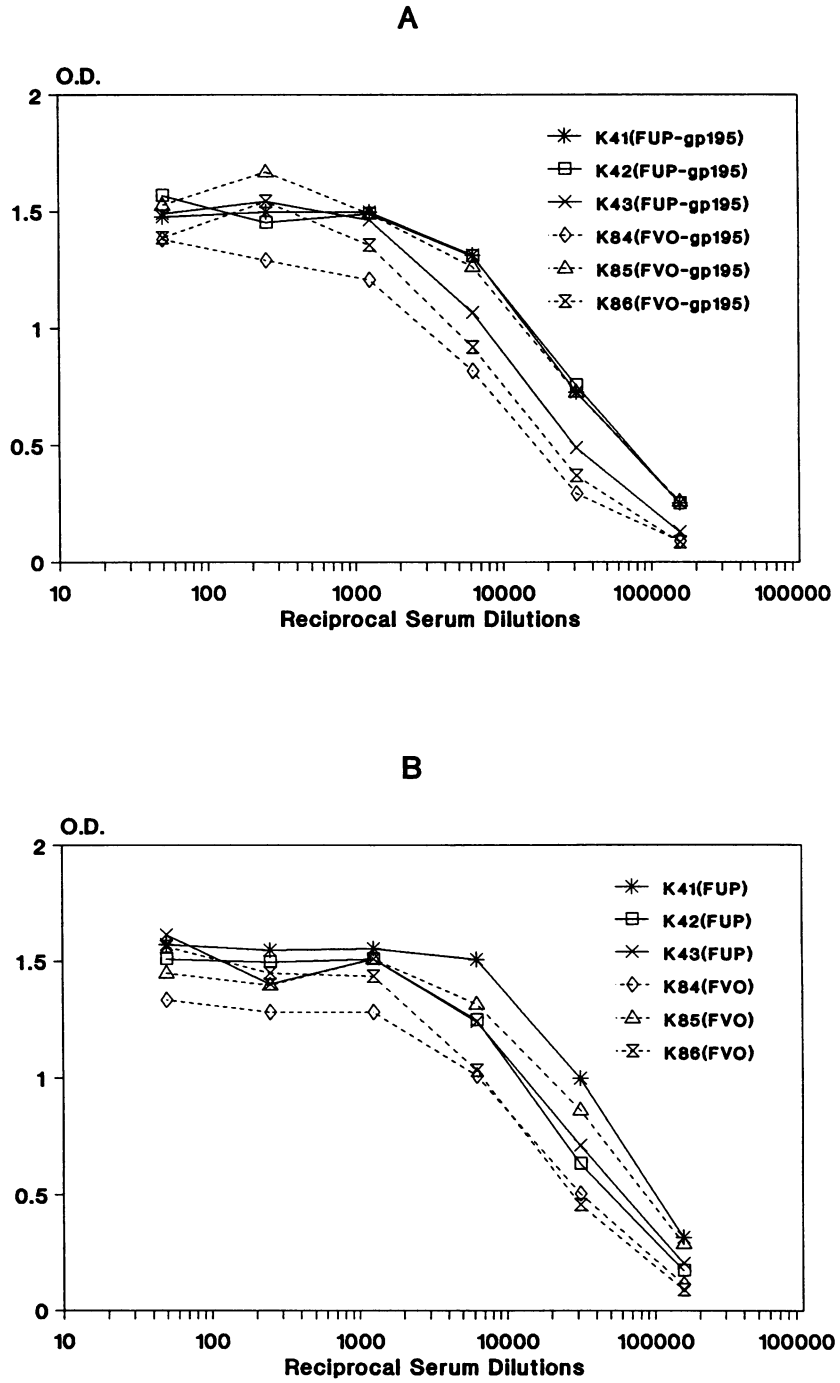

FIG. 5. ELISA titration curves of rabbit anti-FUP gp195 and anti-FVO gp195 sera against purified FUP gp195 (A) and FVO gp195 (B). Quarternary day 14 sera were used.

(Table 2). Our results indicate that polyclonal anti-gp195 antibodies cross-reacted extensively with gp195 proteins of both dimorphic alleles. To determine the degree of immunological cross-reactivity of the rabbit anti-gp195 sera, a competitive binding ELISA was performed with either FUP or FVO gp195 as the competing antigen with FUP and FVO gp195-coated plates. For the anti-FUP gp195 antibodies, maximum inhibition (90 to 95%) of binding was achieved by using approximately 400 μg of either the homologous (FUP) gp195 or heterologous (FVO) gp195 per ml (Fig. 6). The average 50% inhibition concentrations were 100 μg/ml for the homologous gp195 and 55 μg/ml for the heterologous gp195. Figure 7 shows the competitive binding of anti-FVO gp195 sera (K84, K85, and K86) to FVO-gp195 coated plates by FVO and FUP gp195s. The results were similar to that of the anti-FUP gp195 rabbit sera. The average 50% inhibition concentrations were 105 μg/ml for homologous (FVO) gp195 and 200 μg/ml for heterologous (FUP) gp195. Since compe-

TABLE 1. Indirect immunofluorescence antibody reactivity of rabbit anti-FVO gp195 antibodies with FUP and FVO parasites[a]

Source of anti-FVO gp195	Parasite	Reactivity of the following reciprocal serum dilutions:					
		50	250	1,250	6,250	31,250	156,250
K84	FUP	++++	++	+	−	−	−
K85	FUP	++++	+++	++	−	−	−
K86	FUP	+++	++	+	−	−	−
K84	FVO	++++	+++	++	+	−	−
K85	FVO	++++	+++	++	+	−	−
K86	FVO	+++	++	++	−	−	−

[a] ++++, strongest reactivity; +, weakest reactivity; −, no reactivity.

tition with the heterologous gp195 antigen was similar in efficiency to that with the homologous protein, it appears that a major proportion of the polyclonal anti-gp195 antibody response to both allelic proteins was cross-reactive.

In addition to analysis with the ELISA and the indirect immunofluorescence assay, the specificities of anti-FUP and anti-FVO gp195 antibodies were analyzed by immunoblotting with whole-parasite extracts from the FUP and FVO isolates. The patterns of immunoblot reactivity of anti-FUP gp195 and anti-FVO gp195 sera with various processing fragments of FUP gp195 were similar (Fig. 8A). The same results were observed for the immunoblot patterns with FVO parasites as the antigen (Fig. 8B). These data provide additional evidence that the recognition of gp195 and its processing fragments from FUP and FVO parasites may be similar for the anti-FUP gp195 and anti-FVO gp195 polyclonal antibody responses. The recognition of multiple bands, corresponding to different intermediate and terminal gp195 processing products, on immunoblots by rabbit anti-gp195 sera was not surprising; mouse monoclonal and polyclonal anti-gp195 antibodies (6) and rabbit sera raised against recombinant gp195 polypeptides (data not shown) also reacted with similar sets of protein bands.

Since it appeared that most of the anti-gp195 response was induced against conserved epitopes, we were interested in determining whether the rabbit anti-gp195 sera contained antibodies that recognized allelic regions. To address this question, antisera were evaluated for reactivity with an *S. cerevisiae* recombinant polypeptide, P42A3, representing an allelic region of FUP gp195. P42A3 is a 30-kDa protein that corresponds to the first 254 amino acids of the N-terminal region of the C-terminal 42-kDa processing fragment of FUP gp195 (7). Anti-FUP gp195 antibodies from rabbits K41,

TABLE 2. Indirect immunofluorescence antibody reactivity of rabbit anti-FUP gp195 antibodies with FUP and FVO parasites[a]

Source of anti-FUP gp195	Parasite	Reactivity of the following reciprocal serum dilutions:					
		50	250	1,250	6,250	31,250	156,250
K41	FUP	++++	++++	+++	++	−	−
K42	FUP	++++	+++	++	+	−	−
K43	FUP	++++	+++	++	+	−	−
K41	FVO	++++	++++	+++	+	−	−
K42	FVO	++++	+++	+++	+	−	−
K43	FVO	++++	+++	++	−	−	−

[a] ++++, strongest reactivity; +, weakest reactivity; −, no reactivity.

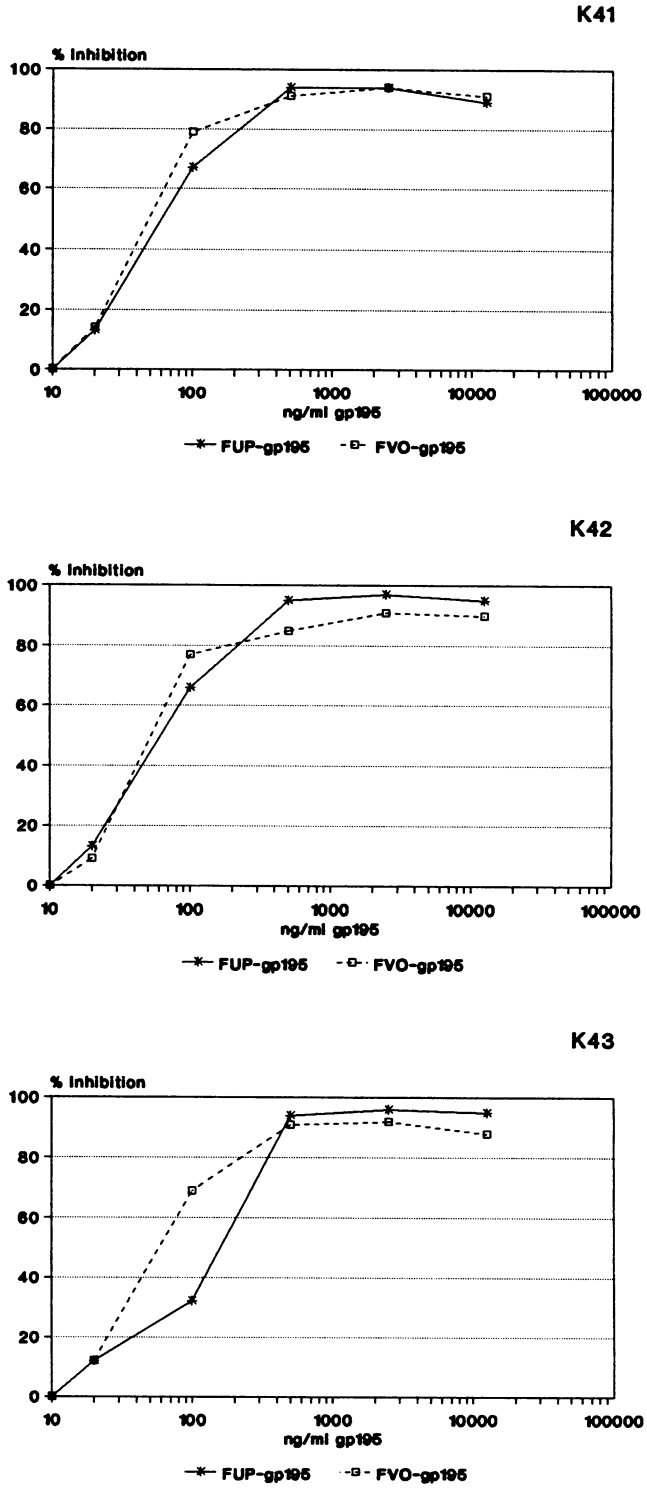

FIG. 6. Competitive binding ELISAs: inhibition of binding of rabbit (K41, K42, and K43) anti-FUP gp195 sera to FUP gp195-coated plates with FUP gp195 and FVO gp195 as competitors. Quarternary day 14 sera were used.

K42, and K43 reacted well with P42A3 (Fig. 9). However, the binding of anti-FVO gp195 antibodies (K84, K85, and K86) with the FUP-derived P42A3 was poor. In contrast, both anti-FUP gp195 and anti-FVO gp195 antibodies reacted

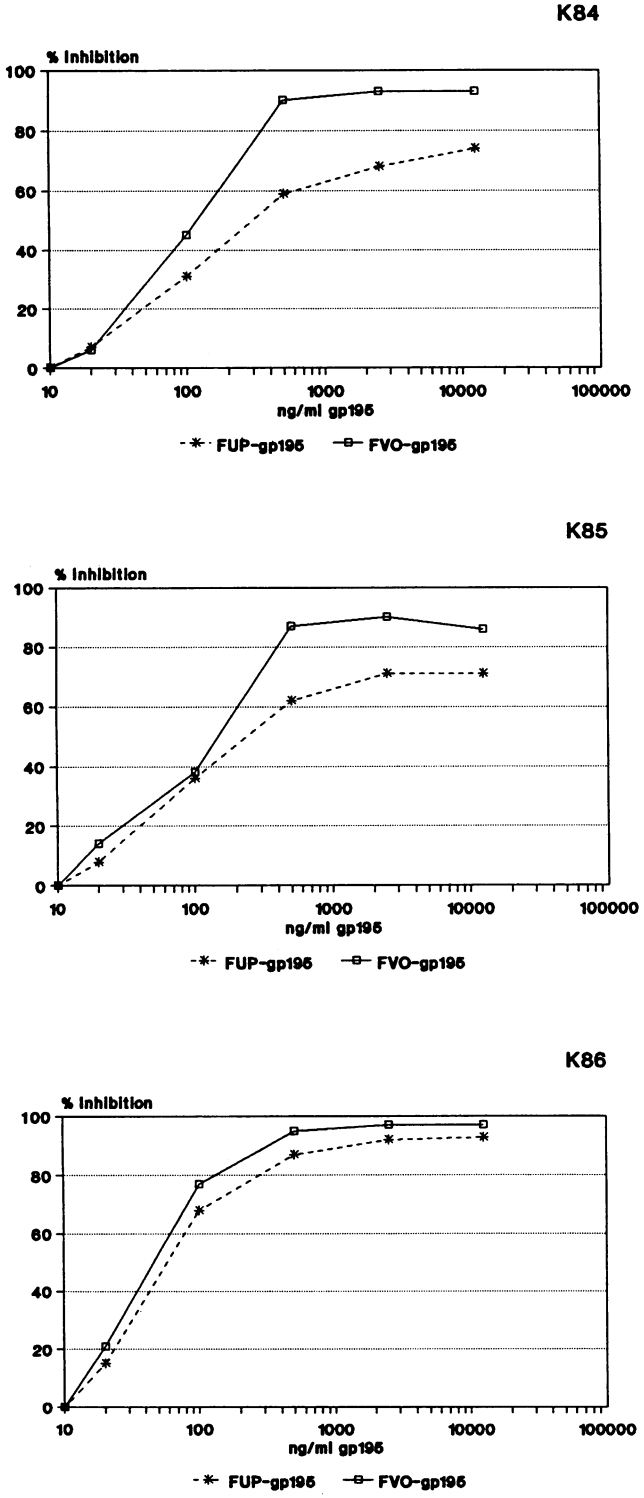

FIG. 7. Competitive binding ELISAs: inhibition of binding of rabbit (K84, K85, and K86) anti-FVO gp195 sera to FVO gp195-coated plates with FUP gp195 and FVO gp195 as competitors. Quarternary day 14 sera were used.

well with the full-length P42 recombinant polypeptide, which contains additional conserved sequences (data not shown). These results indicate that anti-gp195 sera contain antibodies that are allele specific, although these may not be a major component of the anti-gp195 response because of the high level of cross-reactivity of the anti-FUP gp195 and anti-FVO gp195 antisera. Furthermore, these results suggest that gp195 allelic sequences are poorly cross-reactive and that most of the binding observed between anti-FUP gp195 and anti-FVO gp195 antibodies and the heterologous gp195 molecules is due to conserved gp195 determinants.

To determine the role of the variable tripeptide repeat region in the cross-reactivity of anti-gp195 sera, the ELISA reactivity of anti-FUP gp195 and anti-FVO gp195 sera with two synthetic peptides, VR-1 and VR-2, corresponding to the tripeptide repeat motifs of the FUP gp195, was measured. Figure 10 shows the ELISA titers specific for VR-1 and VR-2. Anti-FUP gp195 sera from rabbits K41, K42, and K43 reacted well with both peptides. Titers ranged from 1/1,200 to 1/8,000 for VR-1 and from 1/4,200 to 1/20,000 for VR-2. For the anti-FVO gp195 sera from rabbits K83, K84, and K86, the ELISA reactivities with the peptides were much lower. Rabbit K85 had very low ELISA titers (<1/50) to both peptides. The only exception was the serum from rabbit K86, which showed a reactivity to VR-1 (titer, 1/1,000) that was comparable to those of some of the rabbit anti-FUP gp195 sera. These data indicate that, although the tripeptide repeat sequence of FVO gp195 differed from that of FUP gp195, a low level of antibody that was cross-reactive with FUP tripeptide repeat motifs was detected in most animals.

In vitro growth inhibition of FUP and FVO parasites by anti-FUP gp195 and anti-FVO gp195 rabbit sera. We previously showed that *Aotus* monkeys immunized with gp195-CFA are protected from lethal infections of *P. falciparum* (54). In addition, prechallenge sera from these protected monkeys strongly inhibited (>90%) parasite growth in vitro, whereas sera from unprotected monkeys did not (27). Thus, the ability of anti-gp195 sera to inhibit in vitro parasite growth may be indicative of a protective gp195-specific antibody response. Sera and purified immunoglobulins from rabbits immunized with gp195-CFA also efficiently inhibited parasite growth (27, 28). Therefore, the ability of the rabbit anti-gp195 sera to inhibit homologous and heterologous parasites was investigated by using this in vitro inhibition assay. The levels of inhibition of FUP and FVO parasites with anti-FUP gp195 sera were similar for the homologous and heterologous isolates (Table 3). In addition, anti-FVO gp195 sera inhibited the two isolates equally (Table 4). CFA immunization did not produce sera that inhibited the isolates; sera from rabbits previously immunized five times with *P. falciparum* rhoptry proteins in CFA (27) and sera from rabbits similarly hyperimmunized with each of two recombinant fragments of the gp195 polypeptide expressed in *S. cerevisiae* did not inhibit parasite growth (data not shown). These data indicate that the biological activity of the anti-gp195 antibodies is also highly cross-reactive.

Since Southern blot analysis indicated that the gp195 genes of FUP and FVO share one allelic region (block 4), the observed immunological and biological cross-reactivity may be due to reactivity with epitopes located within this region. Therefore, a synthetic peptide (30-mer) encompassing the entire block 4 region was synthesized, and the antibody response to this peptide was measured in an ELISA (Fig. 11). Antibody titers to the region 4 peptide were low (<1/1,000) compared with titers against native gp195 (>1/50,000). Two rabbits, K43 (anti-FUP gp195) and K85 (anti-FVO gp195), had no detectable peptide-specific antibodies. These data suggest that epitopes within allelic region 4 are not likely to be responsible for the observed immunological and

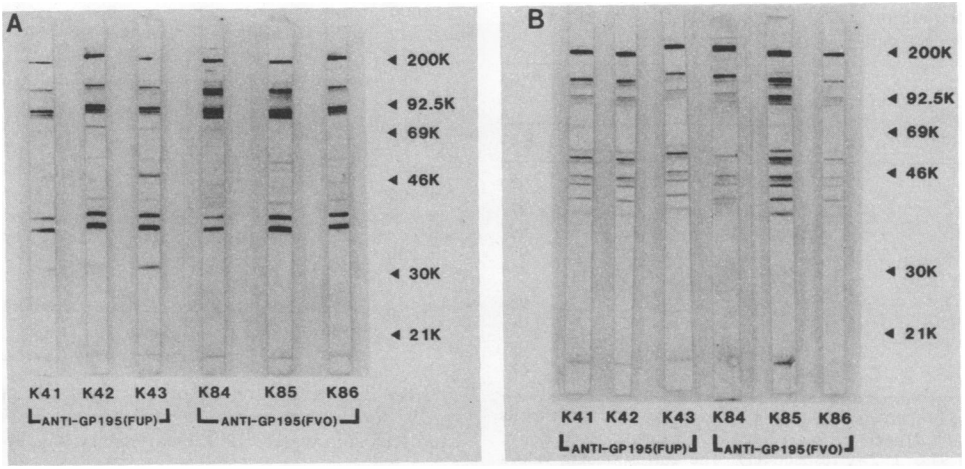

FIG. 8. Immunoblots of FUP (A) and FVO (B) gp195s and processing fragments from FUP and FVO parasite extracts with anti-FUP gp195 (K41, K42, and K43) sera and anti-FVO gp195 (K84, K85, and K86) sera. Arrows indicate the relative positions of molecular size markers.

biological cross-reactivity of the rabbit antisera produced against the FUP and FVO gp195 antigens.

DISCUSSION

We have shown that the gp195 molecules of the FUP and FVO parasites possess different allelic and variable tripeptide repeat sequences. Immunogenicity studies in rabbits showed that these proteins induced highly cross-reactive antibodies as measured by direct or competitive binding ELISAs with FVO and FUP gp195s. The same degree of cross-reactivity was observed in indirect immunofluorescence assays, indicating that similar cross-reactive determinants are present on native gp195s of FVO and FUP whole schizonts and merozoites. In addition, anti-FUP gp195 and anti-FVO gp195 antibodies shared considerable specificities; their binding patterns to various gp195 processing fragments on immunoblots of whole parasite antigens were similar. Cross-reactivity was not due to contamination of either parasite isolate with a minor population of parasites bearing the other gp195 allele, since no contaminating DNA was detected by using genomic blotting or by PCR amplification. These observations were not limited to a single animal species, since both congenic and outbred mice immunized with FUP gp195 produce antibodies with a similar degree of cross-reactivity with FVO gp195 (data not shown). Our results are not in disagreement with previous studies that have unequivocally established the presence of polymorphic epitopes on gp195 (17, 35, 39). Although the previous studies were based on monoclonal antibodies that identified individual allele- or strain-specific epitopes, our results are based on the polyclonal antibody response to the complete gp195 protein, in which the cross-reactive response appears to dominate.

Despite the extensive antibody cross-reactivity between the two gp195 proteins, allele-specific antibodies were detected in our studies. This is evident by the binding of anti-FUP gp195 antibodies with P42A3, a recombinant polypeptide composed of allelic amino acid sequences from the C-terminal region of FUP gp195. Anti-gp195 antibodies also reacted with synthetic peptides corresponding to the variable tripeptide repeats of FUP gp195. This indicates that antibodies to the variable repeat epitopes were also induced during immunization. An interesting finding was the detection of low levels of reactivity with the repeat peptides of anti-FVO gp195 sera despite the overall difference in the

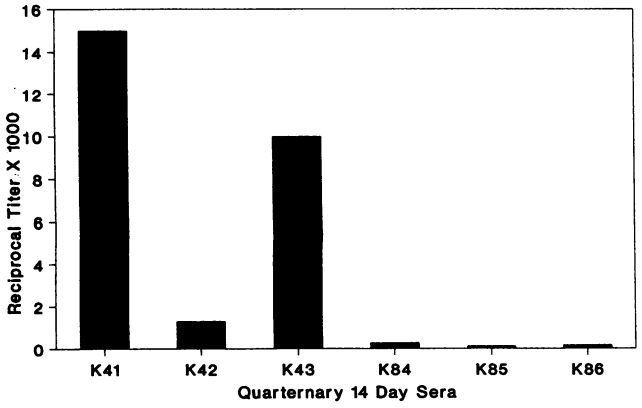

FIG. 9. Reactivity of rabbit anti-FUP gp195 and anti-FVO gp195 sera with *S. cerevisiae* recombinant polypeptide P42A3. Quarternary 14-day sera were used.

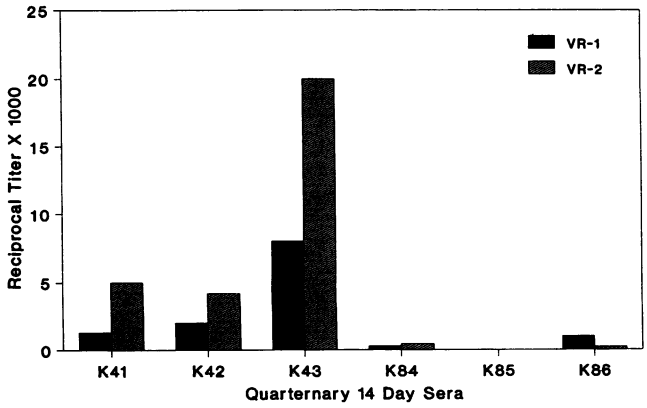

FIG. 10. Reactivity of rabbit anti-FUP gp195 and anti-FVO gp195 sera with synthetic peptides VR-1 and VR-2, corresponding to two sequence motifs of the variable tripeptide region of FUP gp195. The titers of the K85 sera were <1/50. Quarternary day 14 sera were used.

TABLE 3. In vitro inhibition of *P. falciparum* FUP and FVO isolates by rabbit anti-FUP gp195 sera[a]

Parasite	Expt	% Inhibition by anti-FUP gp195 from rabbit:		
		K41	K42	K43
FUP	1	94	84	98
FUP	2	99	82	97
FUP	3	94	96	94
FUP	4	96	87	97
FVO	1	98	84	98
FVO	2	99	96	95
FVO	3	88	85	98
FVO	4	94	ND	96

[a] The average values for percent inhibition ± standard deviations of rabbits K41, K42, and K43 in experiments 1 to 4 against FUP parasites were 96% ± 2%, 87% ± 6%, and 97% ± 2%, respectively. The corresponding values against FVO parasites were 95% ± 5%, 88% ± 7%, and 97% ± 2%, respectively. ND, not determined.

tripeptide repeat sequences between the two gp195 genes. It is possible that similarities within the repeat motifs (e.g., SGTSG in FUP versus SGGSG in FVO) may trigger a cross-reactive response. Alternatively, conformational determinants generated from the tertiary folding of the tripeptide repeat motifs of the two gp195 proteins may themselves be cross-reactive.

The extensive immunological cross-reactivity of the polyclonal anti-gp195 response may be attributed to a preferential recognition of epitope(s) within the conserved blocks of the gp195 molecules or conserved sequences interspersed within the allelic blocks. Alternatively, allelic and variable repeat sequences themselves may be antigenically cross-reactive. To address these possibilities, anti-gp195 antibodies were examined for binding with P42A3 and with the variable repeat peptides. Only anti-FUP gp195 antibodies reacted with P42A3; anti-FVO gp195 antibodies did not. In addition, the reactivity of anti-FVO gp195 sera with variable repeat peptides was low. These results suggest that allelic and variable repeat epitopes are poorly cross-reactive and thus imply that conserved epitopes of gp195 are responsible for much of the observed cross-reactivity of the polyclonal anti-gp195 responses. Furthermore, since a major proportion

TABLE 4. In vitro inhibition of *P. falciparum* FUP and FVO isolates by rabbit anti-FVO gp195 sera[a]

Parasite	Expt	% Inhibition by rabbit anti-FVO gp195 from rabbit:		
		K84	K85	K86
FUP	1	72	77	97
FUP	2	82	95	90
FUP	3	75	84	93
FUP	4	75	92	84
FVO	1	93	81	85
FVO	2	95	88	82
FVO	3	93	100	78
FVO	4	91	97	78

[a] The average values for percent inhibition ± standard deviations of rabbits K84, K85, and K86 in experiments 1 to 4 against FUP parasites were 76% ± 4%, 87% ± 8%, and 91% ± 5%, respectively. The corresponding values against FVO parasites were 93% ± 2%, 92% ± 8%, and 81% ± 3%, respectively.

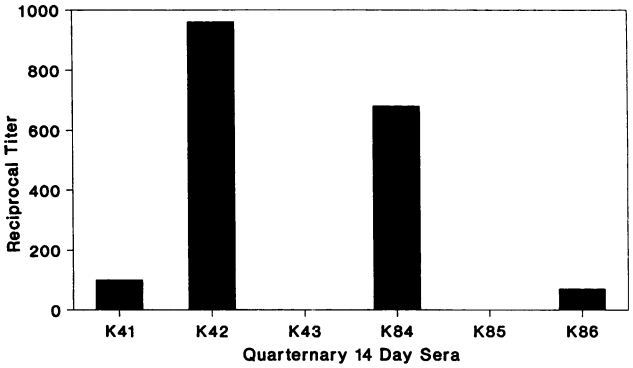

FIG. 11. Reactivity of rabbit anti-FUP gp195 and anti-FVO gp195 sera with the region 4 synthetic peptide. The titers of the K43 and K85 sera were <1/50. Quarternary day 14 sera were used.

of the antibody response was cross-reactive, our results suggest that the conserved region(s) of gp195 is immunodominant. This is in contrast to the characteristics of naturally acquired antibody responses to gp195 in humans living in areas where malaria is endemic (9, 13, 14). In these studies, antibodies from human immune sera were shown to preferentially react with allele-specific regions of gp195. One explanation for the difference in the results may lie with the different modes of presentation of the immunogen. Our studies utilize adjuvant-assisted immunization for the presentation of purified gp195 proteins, whereas humans from areas where malaria is endemic were exposed to gp195 in the context of whole parasites. Studies have shown that the specificities of immune responses in adjuvant-assisted immunization and exposure by natural infections can be quite different (59, 63). It is possible that, in the absence of a powerful adjuvant such as CFA, conserved regions of gp195 may be intrinsically less immunogenic than allelic epitopes, leading to a preferential production of allele-specific antibodies in malaria-infected individuals.

The immunological cross-reactivity of anti-gp195 antibodies is of biological significance. Rabbit gp195-specific antisera strongly inhibited parasite growth of the homologous and heterologous strains in vitro. Thus, the immunological cross-reactivity of these sera correlated with their biological activity against the blood-stage parasites. These results, together with the genetic and immunological studies of FUP and FVO gp195 proteins, suggest that allelic epitopes may not play a significant role in the inhibitory activity of the gp195-specific antibodies. We previously demonstrated that the ability of anti-gp195 antibodies to inhibit parasites in vitro correlated with protective immunity in *Aotus* monkeys (27). Our results provide indirect evidence that immunization with the FUP gp195 may confer immunity against FVO infections in vivo. Therefore, a logical extension of this study is to determine whether immune responses of similar characteristics can be induced in appropriate primate models and whether these responses can protect against heterologous parasite challenge in vivo.

The protective epitopes of *P. falciparum* gp195 remain to be determined. A monoclonal antibody, MAb 12.10, that recognizes the conserved region at the C-terminal 19-kDa processing fragment can inhibit parasites in vitro (1). Our present findings that conserved regions of gp195, rather than allelic epitopes, are responsible for immunological and biological cross-reactivity are in line with the results of these studies. However, genetic studies with gp195 show that,

within homologous or conserved regions, single amino acid changes take place among different isolates (7, 56). Although the significance of these amino acid changes remains to be determined for gp195, in the rodent malaria model, *Plasmodium chabaudi*, a protective monoclonal antibody specific for the merozoite surface protein 5C10/66 recognizes an epitope located in a region of microheterogeneity (34). An analogous region of microheterogeneity can be found in gp195 (34). In *Plasmodium yoelii*, a monoclonal antibody, MAb 302, specific for the analogous merozoite surface protein (Py230) can passively transfer protection against blood-stage infections in a strain-dependent manner (4, 37). Analyses have shown that the amino acid sequence recognized by MAb 302 is localized within the C-terminal, cysteine-rich region of Py230 (2, 3). A sequence from the conserved C-terminal region of *P. falciparum* gp195 can be aligned with the sequence recognized by MAb 302. This segment falls within a region of microheterogeneity. Therefore, although on one hand the ability of rabbit anti-gp195 sera to inhibit heterologous parasites may be due to recognition of conserved epitopes, on the other hand the observed cross-inhibition may be due to the presence of identical amino acid changes within a region(s) of microheterogeneity. Another possibility is that a vigorous polyclonal antibody response to different epitopes within the conserved regions may provide a concerted immune response that exerts a biological and/or protective effect despite the presence of amino acid changes within these regions. Additional genetic, immunological, and in vitro growth inhibition studies with isolates that have the same allelic regions as FVO and FUP but that exhibit microheterogeneity within conserved regions may help to resolve these possibilities.

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