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Vaccine 19 (2001) 204–212

Vaccine

www.elsevier.com/locate/vaccine

Identification of a major B-cell epitope of the *Plasmodium falciparum* glutamate-rich protein (GLURP), targeted by human antibodies mediating parasite killing

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Received 17 December 1999; received in revised form 17 April 2000; accepted 8 May 2000

Abstract

The antigenicity of the glutamate-rich protein (GLURP) of *Plasmodium falciparum* was comprehensively evaluated in epitope-mapping studies utilizing a phage display library, synthetic peptides and anti-GLURP IgG preparations previously shown to promote strong antibody-dependent cellular inhibition (ADCI) effects. We identified six major B-cell epitopes within the nonrepetitive region R0, corresponding to amino acid residues 173 to 187 (P1), 193 to 207 (P3), 216 to 229 (P4), 264 to 288 (P11), 343 to 357 (P10), and 407 to 434 (S3). Of these, four (P1, P3, P4, and S3) were frequently recognized by high-titered IgG antibodies in plasma samples from immune Liberian adults (prevalence: 29.1–45.0%). The three epitopes P1, P3, and P4 contained a common motif (seven out of nine positions are identical) and may thus constitute a family of structurally related epitopes. This leaves two distinct epitopes, one (P3) representing this new epitope family and S3 as targets for biologically active antibodies. Human IgG antibodies from single plasma samples were affinity-purified against these peptides. P3-specific IgG preparations were consistently more effective in ADCI than S3-specific IgG. Among the different GLURP epitopes, we therefore suggest that the P3 epitope is potentially the most important epitope in GLURP for the development of clinical immunity to malaria in man.

Keywords: *Plasmodium falciparum*; Glutamate-rich protein; B-cell epitope; Antibody-Dependent Cellular Inhibition; Phase-display

1. Introduction

Experiments with transfer of Immunoglobuline G (IgG) from clinically malaria immune individuals to malaria patients have consistently shown that antibodies are the main component in acquired immunity to asexual blood stages of *Plasmodium falciparum* [7,21,24]. However, the exact mode of action is still debated [10,11]. Druilhe and coworkers found that co-operation of antibodies with monocytes is critical for acquired protective immunity [4]. They further showed that cytophilic antibodies (IgG1 and IgG3) directed against merozoite surface antigens induces monocytes to secrete tumor necrosis factor α together with other soluble factors, which then block intra-erythrocytic parasite growth at the ring stage [6]. This mechanism is referred to as antibody-dependent cellular inhibition (ADCI) [4,15,19]. Among the cytophilic IgG subclasses, IgG1 seems to play a more important role than IgG3 in Kenyan adults since IgG preparations with the highest levels of IgG1 antibodies were associated with the highest ADCI activity [25]. The importance of cytophilic antibodies for malaria immunity is further supported by the predominance of cytophilic

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0264-410X/00/$ - see front matter
PII: S0264-410X(00)00181-X

IgG1 and IgG3 subclasses in immune Africans adults [5].

The 220 kDa glutamate-rich protein (GLURP) has been located in all the developmental stages of *P. falciparum* in humans, including on the surface of newly released merozoites [2]. Affinity-purified human IgG antibodies to GLURP are able to promote a strong ADCI effect in vitro [27] indicating that GLURP may be a target for protective immunity. This is supported by the observation that high levels of GLURP-specific antibodies were correlated to protection against high parasitemia [3,13] and to protection against disease [12]. It is therefore of interest to dissect the antibody response to GLURP in order to identify amino acid sequences which could be involved in acquired immunity to malaria. Since the ADCI assay may serve as an in vitro correlate of acquired immunity [4,9,16], we have chosen to focus on B-cell epitopes which are targeted by antibodies that are effective in this assay. In the present work, we report the identification of two epitopes in GLURP which are targets of ADCI-effective antibodies.

2. Materials and methods

2.1. DNA techniques

Restriction enzymes (New England Biolabs, Beverly) and Ready-To-Go T4 DNA Ligase (Pharmacia Biotech, Stockholm, Sweden) were used as recommended by the suppliers. PCR was carried out using standard PCR conditions and the primers SD15, (TTG TTA TTA CTC GCG GCC CAG CCG) and SD16, (GTT CTG CGG CCG CCC GTT TGA TCT C). Nucleotide sequencing was performed directly on the PCR products using SD16 as sequencing primer and the Taq DyeDeoxy termination cycle sequencing method.

2.2. Construction of the phagemid library

One hundred μg of pGLURP [27] was fragmented with deoxyribonuclease I (Life Technologies, Roskilde, Denmark) as described [23] to yield fragments of approximately 50–300 baspairs in length. After phenol/chloroform extraction and ethanol precipitation, fragments were redissolved in 100 μl TE buffer (10 mM Tris–HCI. 1 mM EDTA, pH 7.5), and made blunt-ended with *Pfu* DNA polymerase (Stratagene La Jolla, CA). Fifty ng of fragmented DNA was ligated into 1 μg of the gene VIII-based phagemid cloning vector pG8H6 [14] (a kind gift from L. Frykberg), which had been digested with *Sma*I, and dephosphorylated with shrimp alkaline phosphatase (New England Biolabs). The ligation product was transformed into ultracompetent *E. coli* XL-2 cells (Stratagene) giving rise to 2×10^5 ampicillin-resistant transformants on Luria-Bertani (LB) agar plates containing 100 μg/ml of ampicillin, 1% glucose, and 10 μg/ml of tetracycline ($LB_{AmpGluTet}$). Transformants were pooled into $LB_{AmpGluTet}$ medium and kept in 15% glycerol at −80°C until use. Phages were produced by inoculating 100 μl of the pooled library with 10^{11} plaque forming units (pfu) of helper phage R408 (Stratagene). After incubation at room temperature for 20 min, the infected cells were mixed with 3 ml of 0.5% soft agar and poured onto LB plates containing 100 μg/ml ampicillin (LB_{Amp}). After incubation overnight at 37°C, the phage particles were eluted from the soft agar by shaking with 5 ml phosphate-buffered saline, pH 7.2, containing 0.1% of Tween 20 (PBST) and a cocktail of proteinase inhibitors (0.3 μM aprotinni, 0.1 μM leupeptin, 0.1 μM pepstatin A, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 0.2 mM *N*-a-p-Tosyl-L-lysine chloromethyl keone (TLCK)) at 4°C for 3 h. The suspension was sterile-filtered through a 0.2 μM filter (Sartorius AG, Göttingen, Germany) and kept at 4°C. The titer (colony form units) of the phage stocks were determined by infection of *E. coli* XL-2 cells, followed by plating on LB_{Amp} plates.

2.3. Panning

Plates (MaxiSorp™ Nunc, Roskild, Denmark) were coated with mouse anti-Human IgG (clone, HP-6017, Sigma, St. Louis, USA), diluted 1:3000 in 0.05 M Na_2CO_3, pH 9.6, for 3 h at room temperature, and blocked overnight with PBST containing 3% skimmed milk at 4°C. Human anti-GLURP IgG fractions [27] were added to the wells and incubated at room temperature for 1 h. After washing with PBST, phages (10^{10} colony forming units) were added to four wells and incubated for 3 h at room temperature. The wells were washed 10 times with PBST, and bound phages were eluted for 10 min at room temperature with 200 μl 0.1 M HCl–glycine, pH 2.2. The eluates were neutralized with 35 μl of 1M Tris–HCl, pH 9.1 and used to infect *E. coli* XL-2 cells, which were then grown overnight on $LB_{AmpGluTet}$ plates. The resulting colonies were pooled into 3 ml of LB medium, and 100 μl of the cells was infected with 10^9 pfu of R408 for production of phage stocks enriched for phages displaying B-cell epitopes of GLURP.

2.4. Computer analysis and synthetic peptides

The deduced amino acid sequence of the R0 region of GLURP was analyzed for potential B-cell epitopes using the method of Kolaskar and Tongaonkar [17]. Fifteen synthetic peptides were obtained from Schafer-N (Copenhagen, Denmark). All peptides were biotiny-

lated at the N-terminal end and blocked by an amide group at the C-terminal end.

2.5. Antibody preparations and plasma samples

Two polyclonal affinity-purified human anti-GLURP IgG fractions prepared previously [27] were investigated: one fraction, termed F2, reacted solely with the non-repetitive region of GLURP called R0, and another fraction, termed F3, reacted with epitopes shared between R0 and the repeat region R2 of GLURP [27]. The plasma samples were from blood donors clinically immune to malaria, living in an area of Liberia where malaria is holoendemic and transmission intense all year round. As controls served plasma from Danish donors never exposed to malaria.

2.6. Enzyme-linked immunosorbent assays (ELISA) with phage particles, recombinant GLURP, and synthetic peptides

ELISA with recombinant $GLURP_{94–489}$ (R0) and $GLURP_{705–1178}$ (R2) were performed as previously described [28]. In the initial screening of fractions F2 and F3 for anti-peptide reactivity, peptides were attached to strepavidine coated plates through an N-terminal biotin as previously described [20]. In all subsequent peptide-ELISA, microtiter plates (Maxisorb, Nunc) were coated overnight at 4°C with peptides either at 1 μg/ml (P1, P3, P4, and P11) or 2 μg/ml (S3 and S4) in 0.05 M Na_2CO_3, pH 9.6, and blocked with PBST containing 0.37 M NaCl and 3% (w/v) milk powder. Samples, diluted 1:50 into PBST containing 0.7 M NaCl and 1% (w/v) milk powder, were added to duplicate wells and incubated for 2 h at room temperature. Antibody binding was detected with peroxidase-conjugated rabbit anti-human immunoglobulin (code P-214, Dako). In all ELISA tests the cutoff levels were adjusted to a specificity of 95%, based on the examination of 100 Danish blood donors never exposed to malaria.

In the ELISA with fusion phages, microtiter plates were coated overnight at 4°C with mouse anti-human IgG (clone HP-6017, Sigma) diluted 1:3000 in 0.05 M Na_2CO_3, pH 9.6, blocked with PBST containing 3% (w/v) milk powder, and reacted for 2 h with either fraction F2 or F3 and diluted 1:100 in PBST containing 1.25% (w/v) milk powder. Phage particles were added at 10^{10} pfu/ml, and bound phages were revealed with horseradish peroxidase conjugated sheep anti-M13 antiserum (Pharmacia).

For isotype detection, monoclonal mouse anti-human IgG1-4 subclasses (clones NL16 (SkyBio, England), HP6002 (Sigma), Zg4 (Immunotech, Marseille, France) and RJ4 (Boringer Manheim, Germany)) were added to duplicate wells at 1, 1, 2, and 2 μg/ml, respectively, followed by incubation with peroxidase-conjugated rabbit anti-mouse Ig (code P0260, Dako) diluted 1000 fold. Plates were washed extensively with PBST between each incubation step.

2.7. Competition ELISA

Serial dilutions of synthetic peptides S3 and S4 were added to fraction F3, diluted 100 fold in 1.25% (w/v) milk powder in PBST. The mixtures were incubated overnight at 4°C and subsequently the effect on the binding to S3 and S4 coated ELISA plates was determined.

2.8. Affinity purification of human IgG using peptides

Peptide specific IgGs were purified by affinity chromatography from total IgG of each of four immune Liberian adults using as ligand, the synthetic peptides P3 or S3. Briefly, 4.7 mg of either P3 or S3 peptides were coupled to 4 mg of thiopropyl sepharose 6B (Pharmacia) in accordance with the manufacturer's instructions. Total IgG in column buffer (70 mM Tris, pH 8.6, 20 mM barbiturate, 0.5 M NaCl, 15 mM NaAzide) was first applied to a column containing bovine serum albumin (Sigma) coupled to thiopropyl sepharose 6B, and the mock elution from this column served as a negative control in subsequent biological experiments. The run-through was applied to the S3 column and the run-through from this column was applied to a column containing P3. The columns were washed extensively with column buffer. Bound IgG was eluted with 20 mM Tris, pH 8.6, 20 mM barbiturate, 3 M potassium thiocyanate, 15 mM sodium azide and subsequently dialyzed against the column buffer. All eluates were concentrated to 0.5 mg/ml in dialysis bags covered with polyethylene glycol (MW, 20.000, Sigma) and dialyzed extensively against PBS.

2.9. Direct growth inhibition assay, antibody-dependent cellular inhibition (ADCI) assay and indirect immunofluorescent assay (IFA)

Culture adapted *P. falciparum* NF 54 parasites at 0.5% parasitemia and 2% hacmatocrit were incubated with immunoglobulins at various concentrations between 5 and 135 mg/ml in 96-wells flat-bottom culture plates (TPP^R Switzerland) for 48 h. Mean final parasitemia (determined by ^{3}[H]-hypoxanthine uptake from triplicate wells) were compared with control wells containing control normal pool IgG and parasites.

ADCI assays were performed as described [28]. Briefly, IgG preparations were co-cultured with normal human monocytes and *P. falciparum* isolate NF 54 for 72 h in 96-wells flat-bottom culture plates. Each IgG preparation was used at various concentrations start-

ing from 5 mg/ml until a concentration was reached where the ADCI effect started to decline. This procedure was adopted because previous studies with anti-GLURP and anti-Msp3 antibodies had shown a dose related ADCI effect up to a certain IgG concentration followed by a decline in ADCI effect at increasing IgG concentrations. This observation may be explained as follows: in the ADCI assay, bridging of a monocyte and a merozoite by a specific antibody recognizing a surface structure on the merozoite leads to monocyte secretion of soluble factors that mediate parasite killing in vitro. At low IgG concentrations, the ADCI effect is weak due to the low number of activated monocytes. At increasing IgG concentration there will be an increasing ADCI-effect, up to an optimal ratio between antigen and antibody. At very high IgG concentrations, competition will occur between specific IgG antibodies bound to the Fcγ receptors on the monocytes and soluble specific IgG antibodies, for binding to surface epitopes on the merozoite; hence leading to a relatively lower number of activated monocytes. For clarity, we report here only the IgG concentration producing 100% ADCI activity. Parasitemia was estimated in thin smears from each well by microscopic examination of more than 40,000 erythrocytes. Monocyte-dependent parasite killing was expressed as described [27].

Thin layers of air-dried, acetone fixed, schizont-infected red blood cells were used as antigen for IFA. The IFA endpoint titre was defined as the highest positive dilution by immunofluorescence.

3. Results

3.1. Construction of a glurp gene-specific epitope library

An epitope library displaying peptides derived from the entire GLURP molecule was constructed by insertion of Dnase generated random pGLURP fragments into the phagemid vector pG8H6 [14]. The number of primary clones in the library was 2×10^5 transformants, of which 90% contained an insert. Nucleotide sequencing of 10 randomly selected clones revealed inserts derived from the vector and from the *glurp* gene itself.

3.2. Mapping of epitopes recognized by human anti-GLURP antibodies using phage display

Human IgG from immune adult Liberians affinity-purified on R0 produced two fractions. One, termed F2 reacted with only R0, while another termed F3, reacted with both R0 and R2 [27]. To identify epitopes within R0, the GLURP phage display library was first subjected to affinity selection (panning) on fraction F2. After the second round of panning, bound phage particles were eluted and used to infect *E. coli* XL-2 cells. DNA inserts from 109 transformants were amplified by PCR, revealing 8 different groups as judged by agarose gel electrophoresis. Fifty-seven percent (62/109) of the amplicons were the same size, and were probably derived from identical clones, since nucleotide sequencing of 16 amplicons showed the same $GLURP_{196–274}$ insert (Fig. 1A). Nucleotide sequencing of additional 34 PCR fragments revealed inserts derived from the $GLURP_{121–480}$ region in 23 clones (Fig. 1A) and from R2 or from pUC19 in 11 clones. One fusion phage was purified from each of the aforementioned eight groups identified within R0, all of which bound F2 antibodies by ELISA (Fig. 1A).

Epitopes recognized by the cross-reactive antibody specificities in IgG fraction F3 were also identified by panning. After the first round of panning, 19 clones were examined by nucleotide sequencing (summarized in Fig. 1B). Three clones contained inserts from the R0 region and 13 contained inserts from R2. The remaining three clones contained fragments from R1 ($n = 1$) and R0 ($n = 2$) inserted out of frame. The inserts from the R2 region all contained two or more

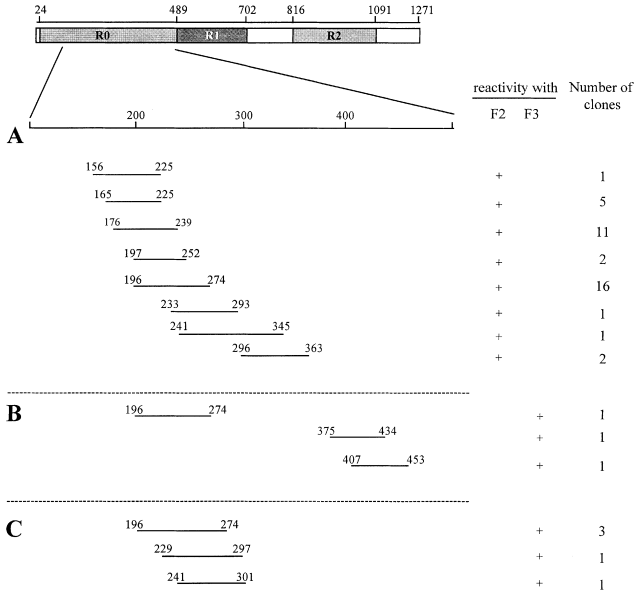

Fig. 1. Structure and properties of clones recognized by affinity-purified human anti-GLURP IgG preparations. The open box at the top indicates the amino acid sequence of GLURP. R0 indicates the non-repeat region, R1 indicates the central repeat region, and R2 indicates the carboxyl-terminal repeat region. Numbers above the box indicate amino acid residues relative to the Met start codon. The lines below indicate the positions of *glurp* inserts within R0 of different clones obtained after (A) two rounds of panning on fraction F2 (see text for an explanation), (B) one round of panning, and (C) two rounds of panning on fraction F3 (see text). To the right is indicated the reactivity of fusion phages with F2 and F3 by ELISA, and the number of clones that were sequenced.

copies of the repeat unit, DKNEKGQHEIVEVEEILPE, cloned in frame with gene VIII. A second round of panning on IgG fraction F3 resulted in the same distribution of fragments from GLURP (Fig. 1C). The reactivity of selected fusion phages with fraction F3 is illustrated in Fig. 1. Collectively, the data points to aa sequences between residues 156 and 453 as targets for human anti-GLURP IgG antibodies effective in ADCI experiments.

3.3. Epitope mapping using synthetic peptides

The epitope domains recognized by human anti-GLURP IgG antibodies were further analyzed by screening 14 synthetic peptides which mimic the most hydrophilic and antigenic domains between aa residues 156 and 453 in the R0 region (Fig. 2) as determined by computer analysis. Five peptides (P1, P3, P4, P10, and P11) bound antibodies in fraction F2, and two peptides S3 (aa residues 407–434) and S4 (R2 repeat unit) bound antibodies in the cross-reactive F3 fraction by ELISA.

To examine whether the peptides S3 and S4 share B-cell epitopes, they were tested for their ability to inhibit the binding of antibodies in fraction F3 to the homologous as well as the heterologous peptide. As shown in Fig. 3, the binding of antibodies in fraction F3 to peptide S4 was strongly inhibited by prior incubation with either S3 or S4. In contrast, peptide P3 did not modify this interaction. These results demonstrate that antibodies in F3 recognize a common motif present on the peptides S3 and S4.

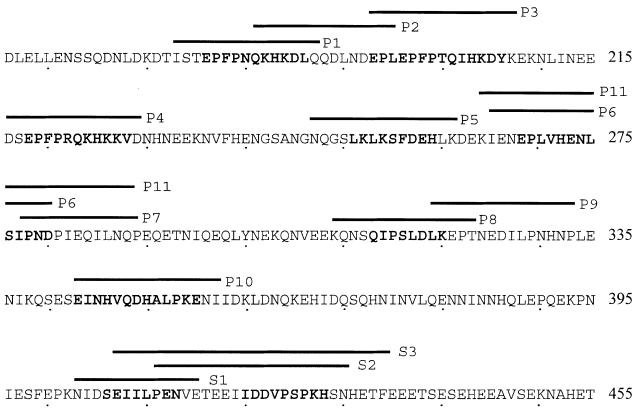

Fig. 2. Identification of B-cell epitope domains within the non-repetitive region of GLURP. Shown is the composite amino acid sequence (in single letter code) of the R0 sequences displayed on the surface of fusion phages. The lines above the aa sequence reflect the location of 14 synthetic peptides and represent the most hydrophilic and antigenic regions (in bold) as predicted by computer analysis [17]. Numbers to the right indicate positions relative to the Met start codon of GLURP.

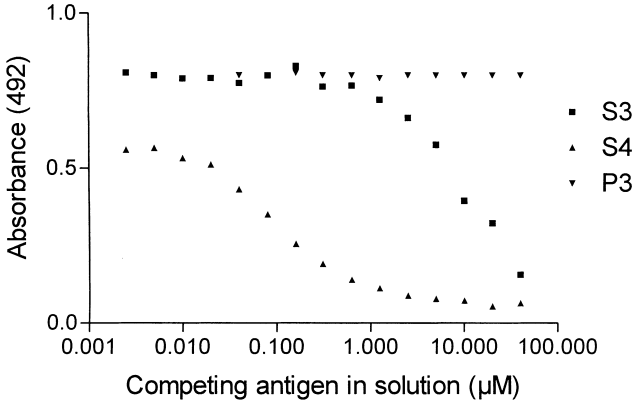

Fig. 3. Peptides S3 and S4 bear a common epitope. Affinity-purified human anti-GLURP IgG antibodies (fraction F3) were diluted 100-fold and preincubated with the peptides S3, S4, and P3 at the indicated concentrations before addition to wells coated with S4.

3.4. Antigenicity of GLURP recombinant and peptide antigens

The antigenicity of R0 and six peptides derived thereof was examined by ELISA using plasma samples from 79 clinically immune Liberian adults. Twenty eight (35.4%) showed IgG reactivity to peptide P1, 44 (55.7%) to peptide P3, 38 (48.1%) to P4, 2 (2.5%) to P10, 23 (29.1%) to P11, and 23 (29.1%) showed IgG reactivity to peptide S3 (Fig. 4). Seventy samples (88.6%) displayed IgG reactivity against the recombinant R0 protein and only 11 of these were non-reactive to the captioned peptides. Hence, the major epitopes in R0 have been identified.

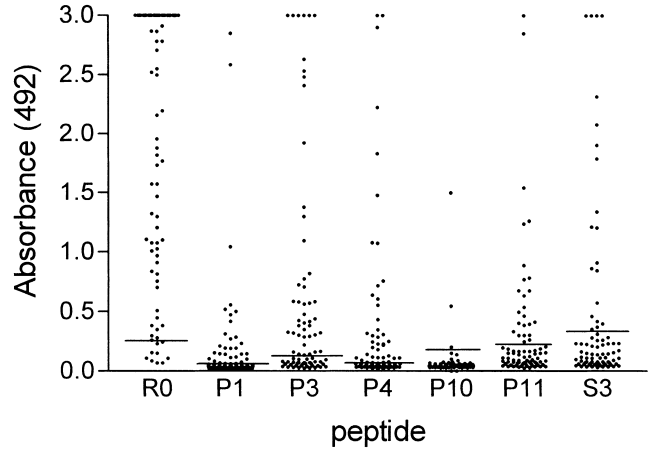

Fig. 4. IgG responses to R0, and synthetic peptides derived from R0. Each point represents one plasma sample. The horizontal lines mark the cutoff-levels for 95% specificity.

Table 1
ELISA titers in IgG preparations obtained by affinity chromatography on peptide-columns. The numerical extension on sample names indicates the plasma from which the IgG preparation was purified. For example, aS3-1 and aP3-1 denote IgG affinity-purified from Plasma 1

IgG affinity purified on		ELISA titer[b] to		
S3[a]	P3[a]	S3	S4	P3
aS3-1		6400	12,800	100
aS3-2		25,600	25,600	100
aS3-3		12,800	12,800	100
aS3-4		25,600	25,600	100
	aP3-1	100	100	3200
	aP3-2	100	100	6400
	aP3-3	100	100	12,800
	aP3-4	100	100	3200

[a] The preparation of the IgG is described in the text.
[b] ELISA titers are for total IgG in affinity purified anti-peptide preparations from four Liberian blood donors.

3.5. Affinity-purification of human antibodies against epitopes within R0

To study in detail the antibody response to single epitopes, peptide-specific IgG was affinity-purified from the plasma of four immune Liberian adults. Total IgG from each donor was applied sequentially to affinity columns containing S3 or P3. The run-through fraction from each peptide-column was completely depleted for antibody reactivity against the respective peptide. The combined use of the two peptide columns absorbed between 50% and 75% of the anti-R0 antibody reactivity in the four IgG samples. ELISA end point titers of the IgG preparations are listed in Table 1 and their IgG subclass content is listed in Table 2. The three anti-P3 IgG preparations purified from plasma samples 2, 3, and 4, displayed a great deal of cross-reactivity with the P1 and P4 peptides, while the IgG preparation obtained from plasma 1 was specific for the P3 and P4 peptides (data not shown). All IgG preparations had a predominance of cytophilic antibodies.

Table 2
Immunoglobulin concentrations in IgG preparations affinity purified on P3 or S3. The amount of each IgG subclass is expressed as the ELISA endpoint titer on plates coated with R0. The numerical extension on sample names indicates the plasma from which the IgG preparation was purified. For example aS3-1 and aP3-1 denote IgG affinity-purified from Plasma 1

IgG preparation	ELISA titer of			
	IgG1	IgG2	IgG3	IgG4
aP3-1	400	50	50	100
aP3-2	1600	3200	1600	200
aP3-3	3200	800	1600	200
aP3-4	400	400	400	50
aS3-1	12,800	6400	25,600	6400
aS3-2	12,800	1600	25,600	1600
aS3-3	25,600	12,800	51,200	3200
aS3-4	800	800	3200	800

3.6. IFA, direct growth inhibition and ADCI with affinity-purified anti-R0 and anti-peptide antibodies

To study the biological activity of affinity purified anti-GLURP antibody preparations, IFA, direct growth inhibition and ADCI assays were performed. All anti-GLURP antibodies preparations recognized the native antigen as demonstrated by IFA and all preparations had high IFA endpoint titres (Table 3). Total IgG from each of the four immune Liberian donors and pooled IgG from hyperimmune donors were used as positive controls. Mock-elution fraction from the BSA-column and total IgG from a pool of

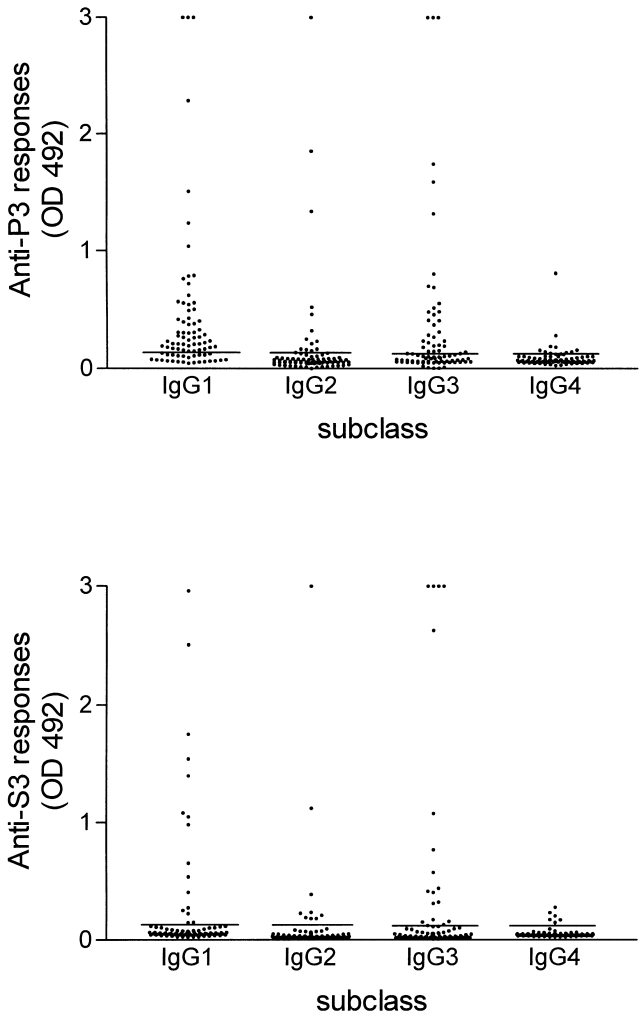

Fig. 5. IgG subclass responses to synthetic peptides P3, and S3. Each point represents one plasma sample. The horizontal lines mark the cutoff-levels for 95% specificity.

healthy European donors who had never been to malaria endemic areas were used as negative controls. Total IgG from the four immune donors, pool hyper-immune donors and all IgG preparations from P3- and S3-columns were effective in ADCI with anti-P3 being consistently more effective than anti-S3 IgG. None of the IgG preparations had a direct inhibitory effect on parasite growth in the absence of monocytes and the mock-elution fraction from the BSA-column did not promote either direct or indirect inhibition of *P. falciparum* growth. All anti-peptide antibody preparations had much stronger ADCI effects than unfractionated IgG. In general, anti-peptide IgG preparations were 10 times more efficient than total IgGs. There was some correlation between the IFA titre of a given IgG preparation and its ADCI effect. The two IgG preparations F2 and F3 were tested in the same experiments and found to promote 60% SGI at 135 μg/ml as previously reported [27]. Thus, purified anti-peptide IgG from human plasma are more efficient in ADCI assays than anti-recombinant IgG preparations.

3.7. Subclasses of IgG antibodies binding to P3, and S3 synthetic peptides

Plasma samples from 79 adult Liberian blood donors were tested in ELISA for the presence of specific antibodies of the IgG1-4 subclasses against peptides P3 and S3. The results obtained are presented in Fig. 5. Of the four IgG subclasses, cytophilic antibodies (IgG1 and IgG3) were the predominant subclasses against the two peptides.

4. Discussion

Passive transfer experiments have unequivocally demonstrated that IgG antibodies obtained from clinically immune Africans adults can abrogate or completely clear *P. falciparum* asexual parasites in malaria patients [7,21,24]. In an attempt to identify antibody specificities participating in this type of clinical immunity, we have investigated antibodies to the glutamate rich protein (GLURP). These antibodies may play a role in the development of clinical immunity because human IgG to GLURP mediates a strong monocyte-dependent inhibition of parasite growth in vitro [27]. When comparing the biological activity of different anti-GLURP IgG preparations, antibodies to the non-repetitive region, R0, were consistently more efficient in ADCI than antibodies against the repetitive region, R2 [27].

Using affinity-purified polyclonal human anti-GLURP IgG antibodies, we identified six epitopes between aa residues 173–187 (P1), 193–207 (P3), 216–229 (P4), 264–288 (P11), 343–357 (P10), and 407–434 (S3). The epitopes P1, P3, and P4 were adjacent and contained a common motif (seven out of nine positions are identical). Therefore, they most likely represent variants of the same epitope and thus constitute a new family of structurally related epitopes in GLURP. This is supported by the observation that affinity-purified anti-P3 IgG preparations showed cross-reactivity with the P1 and P4 epitopes, and that Liberian plasma samples frequently display reactivity against all three peptides. In addition, the presence of a new family of cross-reactive epitopes in this region is consistent with the observation that 57% of the fusion phages obtained by affinity selection on IgG fraction F2 contain the same fragment from residue 196–274.

Previously reported ELISA competition experiments have demonstrated that R0 and R2 share an epitope recognized by fraction F3 antibodies [27]. In the present study, two peptides S3 (aa residues 407–434) and S4 (the repeat unit of R2) both reacted with fraction F3 and were therefore expected to bear a common epitope. This was further supported by the finding that affinity-purified antibodies to S3 reacted strongly with S4 in ELISA. The two peptides both contained the motifs EXILPEX and EXVEXEEI (identical amino acids are underlined differences are indicated by X) which could represent the common antibody binding sequences. Alternatively, a common epitope might also

Table 3
IFA end point titres and specific growth inhibition index (SGI) of total IgG and affinity-purified anti-P3 and anti-S3 antibodies. The results are means of four independent assays

Plasma	IFA endpoint titres			IgG concentration producing 100% SGI (mg/ml)		
	Total			Total		
	IgG	aP3	aS3	IgG	aP3	aS3
Plasma 1	6400	800	6400	200	19	25
Plasma 2	6400	3200	6400	200	6	27
Plasma 3	> 6400	> 6400	3200	120	9	21
Plasma 4	6400	1600	> 6400	200	6	10

result from the combination of non-continuous amino acid residues, to create a conformational epitope of which S3 and S4 are mimics. This hyphothesis is supported by the observation that three of the four anti-S3 IgG preparations were non-reactive with peptides S1 and S2, each containing one of the motifs underlined above (Fig. 2). Further, if antibodies in the F3 fraction recognize a conformational epitope, this might explain why we were unable to more precisely delineate the cross-reactive epitope within the 241–274 region (Fig. 1).

To assess the relative importance of the antibody responses to GLURP B-cell epitopes for the development of protective immunity in man, we examined the ADCI-promoting activity of affinity-purified anti-peptide antibodies from four clinically malaria immune donors. We used antibodies prepared from single plasma samples rather than pooled samples, assuming that individual differences in response to different epitopes would result in distinct biological activities, thereby providing insight into potentially important features of protective responses to malaria in man. The P3 and S3 epitopes were chosen for these studies because they proved to be frequently recognized by high-titered antibodies in plasma from clinically immune Liberians and therefore constitute dominant B-cell epitopes. Further, they each represent two distinct families of cross-reactive epitopes. One family containing the P1, P3, and P4 epitopes, and the other containing the S3 and S4 epitopes. The affinity-purified anti-P3 IgG preparations were consistently more effective in ADCI than the anti-S3 IgG preparations notwithstanding that the latter also contained a relative excess of cytophilic antibodies. Thus, specific IgG antibodies from a single individual may be highly inhibitory when directed against the P3 B-cell epitope, while less inhibitory when directed against the S3 epitope. One of the anti-P3 IgG preparations (aP3-1) exerted less ADCI-effect than the other three, suggesting that other factors — apart from the epitope specificity — are important for the ADCI effect. The cytophilicy has been proposed to be an important factor [5] and in this respect, we note that this IgG preparation contains a relatively lower level of IgG3 compared to the three other anti-P3 IgG preparations. In general, IgG1 and IgG3 subclasses predominate in antibodies binding to the P3 and S3 epitopes, the total amounts of IgG3 being particularly high as this subclass is usually a minor part of total IgGs.

Synthetic peptide vaccines against malaria have attracted much attention (for a review see [18]) and a multicomponent (several antigens) vaccine is the ultimate goal for many malaria vaccine developers. The choice of components in such a vaccine is the crucial issue as best demonstrated by the variable results obtained with the vaccine developed by Patarroyo and colleagues [1,8,22,29]. We here offer a rationale, based on ADCI experiments and epidemiological data, for the inclusion in a malaria vaccine of well defined B-cell epitopes in the R0 region of the GLURP molecule. The suitability of these B-cell epitopes for vaccine development purposes is further illustrated by the fact that they are completely conserved in 29 field isolates of diverse geographical origin and 15 *P. falciparum* laboratory strains [26].

In conclusion, the dissection of the immune responses to the R0 domain of the GLURP molecule has led to the identification of at least two epitopes which trigger an IgG3 response capable of eliciting strong ADCI effects. If presented in a form and context that enables them to elicit a biologically active antibody response, they are therefore realistic candidates to be tested in an experimental malaria vaccine.

Acknowledgements

We thank I. Rosenstadt and M. Paulli Andersen for technical assistance and N. Axelsen and J. Vuust for their comments on the manuscript. This work was supported by the Scientific and Technological Cooperation with the Developing Countries program of the Commision of the European Communities contract CT 950021 and by the Research center for Medical Biotechnology under the Danish Biotechnology Research and Development program. Dr. Soe Soe was supported by a fellowship from the RSWG committee of the UND/WB/WHO-tdr.

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