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Specificities of antibodies that inhibit merozoite dispersal from malaria-infected erythrocytes

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When malaria schizont-infected erythrocytes are cultured with immune serum, antibodies prevent dispersal of merozoites, resulting in the formation of immune clusters of merozoites (ICM) and inhibition of parasite growth. Antigens recognized by these antibodies were identified by probing two dimensional immunoblots of *Plasmodium falciparum* antigens with antibodies dissociated from immune complexes present at the surface of merozoites in ICM. Total immune serum recognized 88 of the 135 protein spots detected by colloidal gold staining, but antibodies dissociated from immune complexes recognized only 15 protein spots attributable to no more than eight distinct antigens. Antigens recognized by antibodies that inhibit merozoite dispersal include the precursor to the major merozoite surface antigens (gp195), a 126-kDa serine-repeat antigen (SERA), the 130-kDa protein that appears to bind to glycophorin (GBP130), and the approx. 45-kDa merozoite surface antigen. One other antigen (230/215-kDa doublet) was identified by using antibodies affinity purified from recombinant expression proteins. The identities of the other three antigens (150 kDa, 127 kDa and <30 kDa) were not determined. This approach provides a strategy for identifying epitopes accessible at the merozoite surface which may be important components of a multivalent vaccine against blood stages of *P. falciparum*.

Key words: Malaria; Surface antigen; Immune complex; *Plasmodium falciparum*; Merozoite; Vaccine

Introduction

It is likely that optimal malaria vaccines will contain multiple antigens from several of the various parasite developmental stages in order to reduce the number of parasites that successfully develop from one stage to the next. Probably, they will also contain multiple antigens from each stage in order to increase antibody avidity, and decrease the risk of selecting mutant parasites. A major obstacle to developing an effective vaccine against blood stage parasites is choosing protective antigens from among many possibilities [1,2]. In vitro, antibodies in malaria-immune sera inhibit dispersal of merozoites when schizont-infected erythrocytes rupture, resulting in formation of immune clusters of merozoites (ICM) and inhibition of parasite multiplication [3]. Several antigens are enriched in the immune complexes that form at the surface of merozoites in ICM [4]. In the present studies, antibodies were disso-

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Note: The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 86–23, 1985 edition.

Abbreviations: ICM, immune clusters of merozoites; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; TBS, Tris-buffered saline; IRBC, schizont-infected erythrocyte; mAb, monoclonal antibody; gp195, the 195-kDa precursor of the major merozoite surface antigens; SERA, the 126-kDa serine repeat antigen; GBP130, a 130-kDa glycophorin-binding protein; MSA, merozoite surface antigen; TX(i), Triton X-100-insoluble fraction of ICM; TX(s), Triton X-100-soluble fraction of ICM.

ciated from such immune complexes and used in immunoblotting experiments to identify their target antigens.

Materials and Methods

Immune serum. Immune serum was from *Aotus* monkey A076 immunized against the Camp strain of *Plasmodium falciparum* [5].

Parasite cultures for preparation of immune clusters of merozoites and antigens for immunoblotting. ICM were prepared as described previously [4] by culturing Metrizamide-enriched, Camp strain schizont-infected erythrocytes (IRBC) in 5% A076 immune serum for 4 h. The ICM were collected, after adding protease inhibitors, by centrifuging and washing three times with 25 pellet volumes of serum-free medium containing protease inhibitors (to remove unbound antibodies) [4]. Pellets were stored at −80°C. Antigens for immunoblotting were from IRBC cultured with 10 μg ml^{-1} leupeptin and chymostatin [6]. Parasites were collected as described for ICM and pellets were stored in liquid nitrogen.

Dissociation of antibodies from immune clusters of merozoites. Antibodies for immunoblotting were dissociated from the pH 8, Triton X-100-insoluble (TX(i)) and pH 8, Triton X-100 soluble (TX(s)) fractions of ICM by using acidic glycine buffer as shown in Fig. 1. Acid-dissociated antibody from 5×10^6 ICM was neutralized by dilution into 30 ml of 10 mM 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) buffer, pH 8.0, containing 150 mM NaCl, 1 mM EDTA (Tris-buffered saline; TBS) and 0.05% Tween 20.

Assessment of nonspecific trapping of antibodies during immune complex formation. Metrizamide-enriched IRBC were cultured for 4 h in 5% A076 immune serum or 5% A083 normal serum containing 10 μg (2.15×10^6 cpm μg^{-1}) ^{125}I-labeled A083 IgG. Triplicate samples were processed according to Fig. 1, the whole sample from each step was quantitated by gamma radiation counting, and sample standard deviations were calculated.

Antibodies. Monoclonal antibodies (mAb) 7B11, 7H10, 3H7 and 7F1, which react with the precursor to the major merozoite surface antigens (gp195), and mAb 5E3, which reacts with the serine repeat antigen (SERA), have been described previously [7,8]. Polyclonal monospecific antibodies against SERA were raised by immunizing female CAF1 mice with a peptide having the sequence FESNSGSLEKKKYVKLPSNG [9] covalently coupled to keyhole limpet hemocyanin by using water soluble carbodiimide or glutaraldehyde at a ratio of 1 mg peptide per mg KLH. Mice were primed with 100 μg of conjugate inoculated subcutaneously in 100 μl 50% Freund's complete adjuvant followed by intraperitoneal injections of 100 μg of conjugate in 100 μl normal saline 4 and 6 weeks later. Polyclonal monospecific antibodies against gp195, p230/215 (doublet) and MSA were affinity-purified with

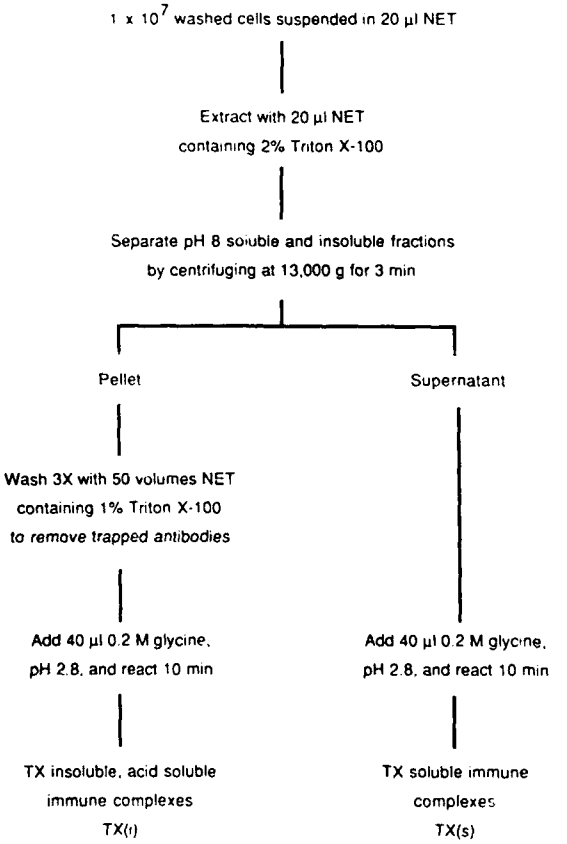


Fig. 1. Preparation of antibodies for immunoblotting from pH 8, Triton X-100-insoluble (TX(i)) and pH 8, Triton X-100-soluble (TX(s)) fractions of immune clustered merozoites.

antigen expressed by recombinant λgt11 clones a34, a126, and a137, respectively, as described previously [9,10]. Malaria DNA from clone a126 hybridizes with clone a33 (unpublished), and antibodies purified from the recombinant protein expressed by clone a33 react with a 230/215-kDa doublet and a 70-kDa antigen [9]. Antibodies purified from the recombinant protein expressed by clone a126 recognized only the 230/215-kDa doublet.

Two-dimensional polyacrylamide gel electrophoresis and immunoblotting. Antigens from 2.5 × 10^6 IRBC cultured with 10 μg ml^{-1} leupeptin and chymostatin were extracted with 20 μl of 1% sodium dodecyl sulfate (SDS), and were prepared for isoelectric focusing by adding 42 mg urea, 16 μl 20% Triton X-100, and 79 μl 2 × Ampholines buffer [11]. The first dimension was non-equilibrium pH gradient electrophoresis for 4 h [12] and the second dimension was SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% separating gel [13]. Electroblotting [14] was at 4°C in a tank system for 14 h at 85 V and blots were blocked with TBS containing 0.3% Tween 20. Immunoblotting reactions were as described [10]. After autoradiography, blots were stained with 25 ml of Aurodye colloidal gold (Janssen, Piscataway, NJ) according to the manufacturer's directions. Efficiency of electrophoretic transfer was evaluated by comparing Aurodye-stained blots with Gelcode silver-stained gels (Pierce, Rockford, IL).

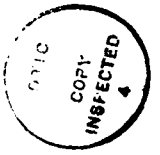
Results

Antigen spots recognized by immune serum. In autoradiographs of two-dimensional immunoblots prepared from IRBC cultured in the presence of leupeptin and chymostatin (Fig. 2A), antibodies in serum from an *Aotus* monkey (A076) that is immune to challenge with blood-stage Camp strain *P. falciparum* malaria parasites [5] reacted with 88 discrete spots. The location of each spot was determined by staining the blot for protein with colloidal gold (Fig. 2B) and aligning with the autoradiograph.

Antigen spots recognized by antibodies dissociated from the Triton X-100-insoluble or Triton X-100-soluble fraction of ICM. Only 15 of the 88 spots recognized by antibodies in immune serum were recognized by antibodies dissociated from immune complexes in the pH 8 Triton X-100 insoluble (Fig. 2C) or soluble (Fig. 2D) fractions of ICM. Spots labeled a–m were recognized by antibodies from the Triton X-100-insoluble fraction of ICM. Spots n and o were recognized exclusively by antibodies from the Triton X-100-soluble fraction.

Identification of specific antigens recognized by antibodies from immune clusters of merozoites. Most of the 15 spots recognized by antibodies dissociated from immune complexes were identified by comparison with the locations of antigens recognized by monoclonal or monospecific antibodies. Six of the spots (c–h) are associated with gp195. Spots c–g correspond to gp195 and its processed products p83, p73, p67 and p45, respectively, and these spots were recognized by antibodies affinity-purified with the recombinant protein expressed by clone a34 (Fig. 3). This clone contains DNA encoding the C-terminal half of the p83 domain and the N-terminal half of the p45 domain of Camp strain gp195 [10]. The specific assignments of antigen spots c–f were also confirmed by probing blots with mAb 7B11, which reacts with an epitope shared by gp195, p83, p73, and p67 [7]. MAb 7B11 was also included in experiments with monospecific antibodies against other antigens, as an internal control to provide reference markers for alignment.

Other antigens recognized by antibodies dissociated from ICM include the following: a and b, a 230/215 antigen (doublet) recognized by antibodies affinity-purified from the recombinant protein expressed by clone a126; h, gp45, recognized by mAbs against a processed product derived from the C-terminus of gp195 [7,10]; i, a 150-kDa antigen which was not identified with defined antibodies; j, SERA, the precursor to a 50-kDa exoantigen [15] recognized by mAb 5E3 [8] and by an immune serum raised against the peptide FESNSGSLEKKKYVKLPSNG synthesized based on the DNA sequence for SERA [9]; k, a 127-kDa antigen which was not identified with



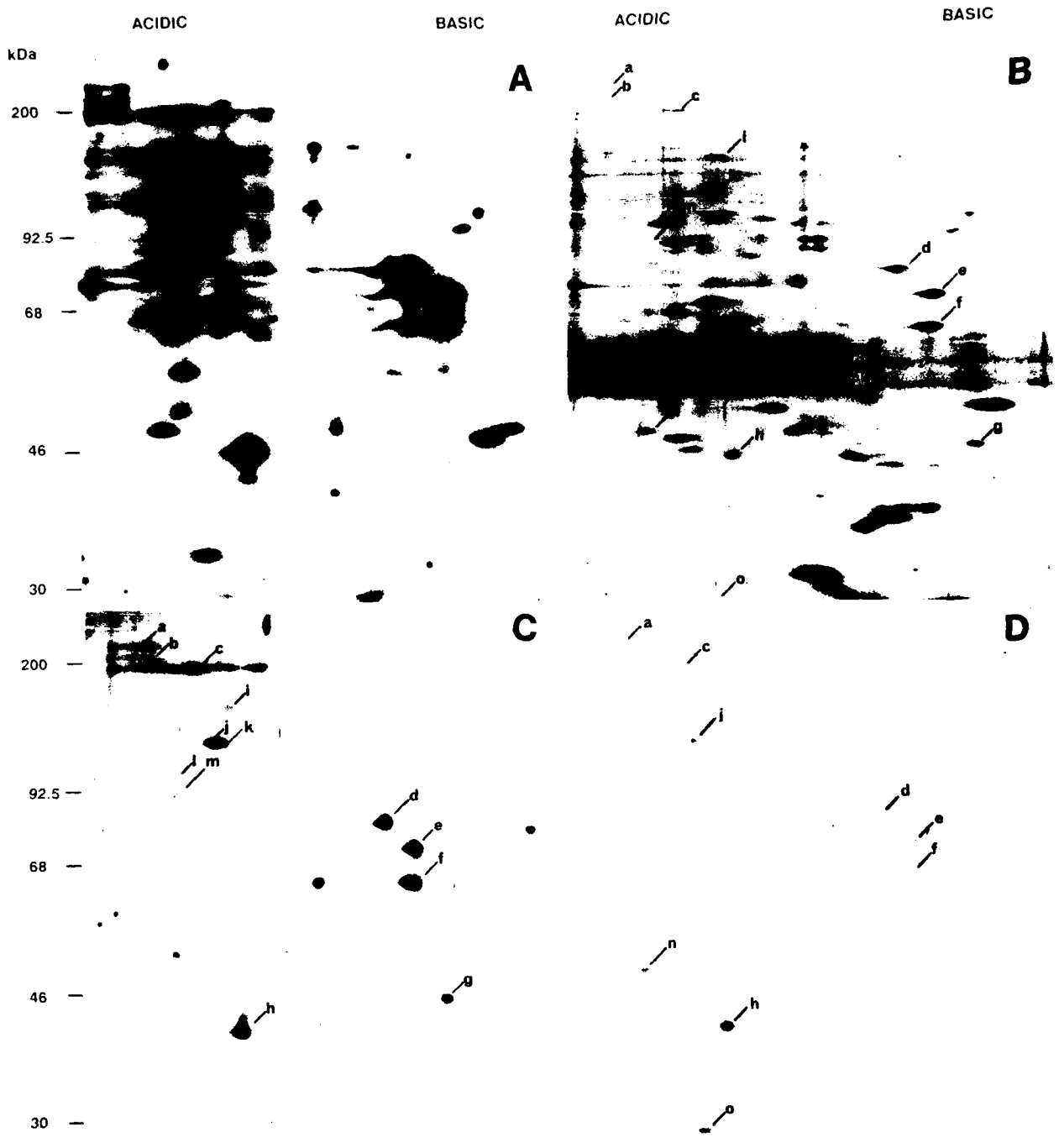


Fig. 2. *P. falciparum* antigens recognized by immune serum or by antibodies dissociated from immune complexes in immune clusters of merozoites (ICM). *P. falciparum* antigens were separated by two-dimensional electrophoresis, reacted with 25 μl of A076 immune serum, and subjected to autoradiography for 14 h (A) followed by staining with colloidal gold for 14 h (B). Alternatively, blots were reacted with antibodies dissociated from the TX(i) (C) or TX(s) (D) fraction of 5×10^6 ICM. Antigen spots recognized by antibodies dissociated from the ICM are noted in lower case letters.

Fig. 3. Monospecific antibodies against gp195 react with antigen spots recognized by antibodies dissociated from immune complexes. Blots of Camp strain antigens were reacted with antibody affinity-purified from the recombinant expression clone a34 which contains part of the gene from the major merozoite surface antigen (gp195). Autoradiography was for 14 h (A) followed by staining with colloidal gold (B). Antigen spots recognized by these antibodies include: gp195, p83, p73, p67, and p45 (c–g, respectively).

defined antibodies; l and m, a 96/92-kDa antigen (doublet) which corresponds to GBP130, a putative glycophorin-binding protein [16] recognized by rabbit antisera [17] raised against the region of tandem repeated primary structure for GBP130 [18]; n, MSA [39], recognized by antibodies affinity-purified from the recombinant protein expressed by clone a137 (unpublished); and o, an antigen of less than 30 kDa which was not identified with defined antibodies. The identity of spot h was confirmed by two-dimensional PAGE and fluorography of immunoprecipitates prepared from gp45 metabolically labeled with [^{3}H]isoleucine [7,10] and precipitated with mAbs 3H7 and mAb 7H10 (not shown). The assignments of antigen spots a–n are summarized in Table I.

Specific accumulation of antibodies in immune complexes. Antibodies against these 15 spots accumulated specifically in the immune complexes that formed at the surface of merozoites in ICM and were not merely trapped during immune complex formation. Immunoblots probed with antibodies dissociated from 5×10^6 ICM gave an optimal autoradiograph in 8–14 h. The Triton X-100-insoluble and -soluble fractions from ICM obtained from a 0.5-ml culture prepared from 5×10^6 IRBC, 25 μl of immune serum, and 2.15×10^7 cpm of ^{125}I-labeled normal *Aotus* IgG contained only 0.016% (3440 ± 197 cpm) and 0.010% (2150 ± 161 cpm) of the total ^{125}I-labeled IgG, respectively. Although this was more than the amount of ^{125}I-labeled IgG recovered in the same fractions of parallel cultures containing normal serum (0.0002% (57 ± 36 cpm) and 0.004% (821 ± 73 cpm) respectively), no reactions were observed in 14 h exposures of autoradiographs of blots probed with 0.004 μl of immune serum (corresponding to 0.016% of 25 μl of immune serum). Furthermore, no antibodies were recovered from the cellular fractions of parallel control cultures containing uninfected erythrocytes (not shown).

TABLE I

Summary of antigens recognized by antibodies dissociated from immune clusters of merozoites and by selected monospecific antibodies

Spot	Size	pI	Identification	ICM antibody signal strength		Antigens reacting with monospecific antibodies	Reference
				Tx(i)	Tx(s)		
a	230 kDa	Acidic	p230 doublet upper band	++	+/−	a126[c]	
b	215 kDa	Acidic	p230 doublet lower band	+/−	−	a126[c]	
c	195 kDa	Acidic	gp195	++++	+	a34[c], mAb 7B11, mAb 7B2	[7,10]
d	83 kDa	Neutral	p83 from gp195	++++	++	a34[c], mAb 7B11, mAb 7B2	[7,10]
e	73 kDa	Neutral	p73 from gp195	++++	++	a34[c], mAb 7B11, mAb 7B2	[7,10]
f	67 kDa	Neutral	p67 from gp195	++++	+	a34[c], mAb 7B11, mAb 7B2	[7,10]
g	45 kDa	Neutral	p45 from gp195	+++	−	a34[c]	[10]
h	42 kDa	Acidic	gp45 from gp195	++++	+++	mAbs 7F1 and 3H7	[7]
i	150 kDa	Acidic	Not known	+	−	Not tested	
j	126 kDa	Acidic	SERA	++++	++	mAb 5E3, SERA antisera	[8]
k	127 kDa	Acidic	Not known	++	−	Not tested	
l	96 kDa	Acidic	GBP 130	+	−	serum 244-54	[17]
m	92 kDa	Acidic	GBP 130	+	+/−	serum 244-54	[17]
n	50 kDa	Acidic	MSA	−	++	a137[c]	
o	<30 kDa	Acidic	Not known	−	++	Not tested	

Electroblotting efficiency. Silver-staining two-dimensional gels that were not blotted revealed 143 spots, whereas colloidal gold-staining blots from companion gels revealed 135 spots (not shown). Proteins that were detected by silver-staining gels but not by colloidal gold-staining blots tended to be acidic and have sizes in the range of 20–35 kDa. For some proteins, transfer was incomplete because silver-staining electroblotted gels revealed low levels of residual large macromolecules. However, colloidal gold staining of the resultant blots revealed that a large proportion of each of these molecules was transferred to the nitrocellulose.

Discussion

Previous work, in which ICM antigens labeled with [^{3}H]isoleucine were analyzed by SDS-PAGE, indicated that 15 antigens were stabilized at the surface of merozoites by formation of immune complexes with antibodies from growth inhibitory immune serum [4]. Also in previous work [8], monoclonal antibodies were used to identify two of these ICM antigens as SERA [15,19,20] and ABRA [21,22]. The identities of the other ICM antigens were not known.

We now have used antibodies dissociated from ICM to probe two-dimensional immunoblots prepared from IRBC cultured with leupeptin and chymostatin. These antibodies recognized 15 polypeptides corresponding to eight antigens, whereas the whole immune serum recognized 88 polypeptides.

Several of these eight antigens have been described previously. gp195 [23] (antigen spots c–h) and SERA [15,19,20] (antigen spot j) are polymorphic [24,25], are known to be associated with the merozoite surface [8,23,26,27], and can induce at least partial protection against blood stage malaria in monkeys [20,28,29]. Both gp195 and SERA also contain epitopes that are conserved among different parasite isolates, and some of these conserved epitopes are accessible at the surface of merozoites and participate in the formation of ICM (unpublished). GBP130 (antigen spots l and m) appears to bind to glycophorin [16] which has been implicated as an important ligand used by merozoites to invade erythrocytes [30–33]. Individually, gp195, SERA and GBP130 have

merit as vaccine candidates [17,28,29]. Co-administered as a multivalent vaccine, the antigens may elicit immune responses that act in concert to give increased protection [34,35].

In additional experiments, monoclonal antibodies that react with Pf EMP 2/MESA [36,37] or with a 240/225-kDa protein from rhoptries [38] reacted with antigen spots other than those corresponding to the 230/215-kDa doublet that correlates with spots a and b (unpublished). Several approx. 50-kDa malaria proteins have been described recently. Two of these are associated with the merozoite surface [39–41] and another is associated with rhoptries [39]. The 50-kDa antigen described in this present study is the same as the 45-kDa merozoite surface antigen known as MSA [39], as shown by comparing DNA sequences (manuscript in preparation), however its relationship to the protein described by Clark et al. [10] is not known. Spot i (150 kDa) may correspond to a low-molecular-weight form of the S antigen [42] or to PF155/RESA [43,44], and efforts to identify this antigen are in progress. Antigen spots k (127 kDa) and o (<30 kDa) are not related in an obvious way to any malaria antigens that have been described previously, other than having the same molecular weights as [^{3}H]isoleucine-labeled antigens identified by SDS-PAGE [4]. By analogy with gp195, SERA and GBP130, these other five antigens also are candidates for inclusion in a multivalent vaccine.

ABRA, which co-electrophoreses with antigen spot l (GBP130) during one-dimensional SDS-PAGE (unpublished), was identified previously as an ICM antigen [4,8], but it was not revealed as such in this present study. These results may be related in part to the fact that more antigen and higher concentrations of antibodies were used in the previous experiments [8]. In addition, ABRA is recognized as an ICM antigen when antibodies dissociated from ICM are used to probe two-dimensional immunoblots prepared with antigens from ICM, indicating that different molecular forms of ABRA are obtained from ICM vs. IRBC cultured with leupeptin and chymostatin (manuscript in preparation). Thus, ABRA is a ninth ICM antigen.

One other set of antigens, a 173-kDa triplet, was also stabilized at the surface of merozoites in ICM [4] but was not observed in this present study. Antibodies in A076 monkey serum do not precipitate the 173-kDa triplet [6], suggesting that these antigens are not recognized by antibodies during ICM formation but form strongly associated macromolecular complexes [7,45] with antigens that are targets for antibodies that inhibit merozoite dispersal.

Target antigens for antibodies that inhibit merozoite dispersal need not be integral membrane proteins. Although gp195 appears to be anchored to the merozoite membrane by diglyceride linkage to phosphatidyl inositol [46] and MSA partitions to the detergent phase after Triton X-114 extraction [39], other target antigens including SERA [19], ABRA [22] and GBP130 [47] do not have hydrophobic C-terminal sequences, and SERA, ABRA, and GBP130 do not appear to incorporate myristic acid or glucosamine (unpublished). SERA, ABRA, and GBP130 have been localized to the parasitophorous vacuole space where they appear to be associated with the merozoite surface. This association could result from the non-covalent interaction of these proteins with membrane anchored proteins such as gp195 or MSA.

A logical extension of these results is the use of antibodies dissociated from immune complexes to identify B-epitopes on antigen fragments produced by recombinant DNA or synthetic peptide techniques. Because immune complexes will form only at solvated, surface-accessible epitopes, it is likely that only a subset of all antibodies that react with a given antigen will be recovered, and these will be against surface epitopes. A similar analysis could be applied to any biological system for which functional antibodies are available. *P. falciparum* antigens that have merit as vaccine candidates are generally large proteins, can be difficult to express by current recombinant DNA technology, and may contain immunodominant or suppressor epitopes that interfere with the development of protective antibody responses. Application of these methods to B-epitope analysis may be especially important in developing a strategy for producing effective vaccines against organisms (such as malaria parasites) where synthetic or recombinant protein vaccines appear to offer the best prospects.

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