INFECTION AND IMMUNITY, Oct. 1985, p. 102–106
0019-9567/85/100102-05$02.00/0

Vol. 50, No. 1

Two Apparently Nonrepeated Epitopes on Gametes of *Plasmodium falciparum* Are Targets of Transmission-Blocking Antibodies

RICHARD CARTER,[1,2]* GILLIAN BUSHELL,[2] ALLAN SAUL,[2] PATRICIA M. GRAVES,[3] AND CHEV KIDSON[2]

Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205,[1] Queensland Institute of Medical Research, Herston, Brisbane, Australia 4006,[2] and Papua New Guinea Institute of Medical Research, Madang, Papua, New Guinea[3]

Received 18 March 1985/Accepted 20 June 1985

One-site and two-site immunoradiometric assays have been developed against an antigen on gametocytes of *Plasmodium falciparum*, using monoclonal antibodies (Mabs) which block transmission of the parasites to mosquitoes. Three such Mabs have been studied, each of which immunoprecipitates a complex of three gamete surface proteins of apparent M_r 260,000, 59,000, and 53,000 from Triton X-100 extracts of the parasites. The assays showed that the Mabs recognized one or the other of two distinct, nonrepeated epitopes on the target antigen(s). In the one-site assay certain combinations of two Mabs interacted at appropriate concentrations to enhance binding of the Mabs to the antigen. The same combinations of Mabs synergize to suppress infectivity of gametocytes to mosquitoes.

It was previously shown that certain monoclonal antibodies (Mabs) which react with a gametocyte and gamete surface stage-specific antigen suppressed infectivity of gametocytes of *Plasmodium falciparum* to *Anopheles freeborni* mosquitoes (8). In the absence of complement two such Mabs, IIC5-B10 and IA3-B8 (both isotype $\gamma 2_a$), were necessary for suppression in what was clearly a synergistic reaction. Although the Mabs were obviously not functionally equivalent, they each immunoprecipitated the same set of three gamete surface proteins of M_r 260,000, 59,000, and 53,000 from Triton X-100 extracts of surface-radioiodinated gametes of *P. falciparum* (8).

In the present study we have developed one- and two-site immunoradiometric assays (IRMA) to further investigate the properties of these Mabs and their target epitopes. The two-site IRMA is similar to that which has been used in epitope analysis of Mabs against the sporozoite CS protein in various species of *Plasmodium* (10).

MATERIALS AND METHODS

Parasites. Lines of *P. falciparum* were grown in culture as previously described for production of gametocytes (7) and for asexual parasites (9). The origins of *P. falciparum* lines 7G8 and FCQ-27 are given elsewhere (references 1 and 3, respectively).

Twelve to 14 days after expansion of a culture with fresh erythrocytes (RBCs), when most gametocytes were at stage III to IV of development (2), gametocytes were harvested and enriched on a Percoll gradient (8). Fractions recovered at interfaces between 40 and 60% Percoll in medium were combined and washed in RPMI 1640 and contained 20 to 50% gametocyte-infected cells; asexual stage-infected cells were a minority of all parasitized cells.

Asexually parasitized cells at 5 to 10% parasitemia were harvested from cultures 3 to 4 days after expansion with fresh RBCs.

Preparation of Mabs. Mabs IIC5-B10 ($\gamma 2_a$ isotype), IA3-B8 ($\gamma 2_a$), and IID2-A10 ($\gamma 1$) were produced as previously described (8). Preparations were made of ammonium sulfate-precipitated proteins from ascites fluid representing each of the three Mabs. These were dialyzed against phosphate-buffered saline and kept at 4°C as solutions of 1 mg of protein per ml in PBS with 0.01% sodium azide as preservative. Protein A-purified fractions of IA3-B8 and IIC5-B10 were made by passing the ammonium sulfate-precipitated proteins in PBS over a protein A-Sepharose column equilibrated with 90 mM sodium phosphate (pH 8.1) buffer. The bound antibody was eluted with 100 mM sodium citrate (pH 3.3) buffer, neutralized with 2 M Tris solution, and dialyzed against two changes of PBS.

Protein A-purified IIC5-B10 and IA3-B8 were radioiodinated by the Iodogen method (5); 200 to 500 μCi of ^{125}I was bound per 100 μg of protein A-purified Mab and made up to a stock solution of 30 μg of protein per ml in 10% bovine serum in PBS with 0.01% sodium azide.

One-site IRMA. Sheets of Gel Bond (Marine Colloids) were printed on their agarose-treated hydrophilic side with a grid of silicone rubber (Dow Corning Silicone Window and Glass Sealant) to leave an array of 2-mm-diameter spots surrounded by silicone rubber. The silicone rubber is sufficiently hydrophobic to prevent cross-contamination between the reagents on adjacent spots. Five microliters of a 20-μg/ml solution of poly-L-lysine (Sigma Chemical Co.) in distilled H_2O was applied to each spot and the sheets were incubated overnight in a moist chamber at 4°C. The sheets were rinsed with distilled H_2O and dried. A suspension of parasitized (prepared as above) or unparasitized RBCs at approximately 10^7 cells per ml in serum-free RPMI 1640 was applied at 10 μl per spot and allowed to settle overnight at 4°C in a moist chamber. This procedure allowed a uniform monolayer of cells to attach to the Gel Bond via the poly-L-lysine coating. The sheets were rinsed in PBS, excess fluid was shaken off, and the sheets were immediately dunked in acetone for 5 min at room temperature. The sheets were dried and stored desiccated at −20°C.

Assays were carried out as follows. To each acetone-fixed antigen-coated spot 5 μl of 10% bovine serum in PBS was applied as a blocking reagent and incubated at room temperature for 30 min. A 5-μl portion of a solution of unlabeled ammonium sulfate-precipitated or protein A-purified Mab in 10% bovine serum in PBS was then added as "competitor" prior to the final addition 30 min later of 5 μl of ^{125}I-labeled

* Corresponding author.

Mab in 10% bovine serum. The combined total (15 μl of solution) was incubated for 12 h at 4°C, and the sheet was subsequently washed once with PBS, soaked twice in 0.05% Tween 20 in PBS for 5 min per wash, washed once with distilled water, and dried; the sheet was then cut up into individual spots for counting on a gamma counter.

Two-site IRMA. Antigen from gametocyte-infected cells, harvested as described above, was prepared by extraction of the cells with NETT (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, pH 7.5) containing protease inhibitors (5 μg each of pepstatin, leupeptin, chymostatin, and antipain [Sigma] per ml) in a suspension of 5×10^7 to 20×10^7 RBCs (gametocyte infected or uninfected) per ml of extraction solution. After extraction for 5 min at room temperature with agitation, the suspension was centrifuged at 15,000 × *g* for 5 min and the supernatant was retained as antigen extract.

The two-site assay was conducted as follows. Round-bottomed detachable polystyrene wells (Dynatech, U.K. Immulon II, Removastrip U-wells) were treated with 30 μl per well of a solution of ammonium sulfate-precipitated or protein A-purified Mab in PBS and incubated for 2 h at room temperature. The wells were washed twice with 0.05% Tween 20 in PBS, and 100 μl of 10% bovine serum in PBS was added to each well as a nonspecific blocking reagent. After a 30-min incubation the blocking solution was removed and 30 μl of antigen extract (diluted as appropriate [see Fig. 4] in 10% bovine serum–0.05% Tween 20 in PBS) was added and incubated for 6 h at 4°C. The wells were washed twice with 0.05% Tween 20 in PBS and twice with 10% bovine serum in PBS, and 50 μl of a solution of ammonium sulfate- or protein A-purified Mab in 10% bovine serum in PBS was added and incubated overnight. This is the "competition" step in the two-site assay; if no competition step was involved the assay proceeded directly to the application of 30 μl of ^{125}I-labeled Mab (always preceded by two washes with 0.05% Tween 20 PBS and two washes with 10% bovine serum in PBS) diluted as appropriate in 10% bovine serum in PBS and incubated for 6 h or overnight at 4°C. The wells were then washed as usual followed by a rinsing of the entire plates in distilled water; individual wells were detached and counted in a gamma counter.

In all IRMAs each experimental point was determined in triplicate and results are given as the mean of the triplicate points.

RESULTS

One-site IRMA. The specificity of reaciton of the antigamete Mabs in the one-site IRMA was tested as follows. Preparations of cells attached to poly-L-lysine-treated Gel bond sheets, air dried and fixed with acetone, were made with (i) RBCs infected with gametocytes of *P. falciparum* line 7G8, (ii) RBCs infected with trophozoites and schizonts of line FCQ-27, and (iii) uninfected RBCs (Fig. 1). The method of preparation allows antibody to penetrate the cell membranes and react with the internal cell constituents. The purpose of the tests was to demonstrate that in the one-site IRMA the reaction of antibodies was antigen specific. The use of two different isolates as a source of gametocytes or asexual stages was a matter of convenience. FCQ-27 is a very poor gametocyte producer; therefore, use of this isolate for preparation of asexual parasites ensured virtual absence of sexual stages from the preparation. The two Mabs used in the study are known from previous work to react only with sexual stages of *P. falciparum* (8). ^{125}I-labeled IIC5-B10 bound only when 7G8 gametocytes were attached to the Gel

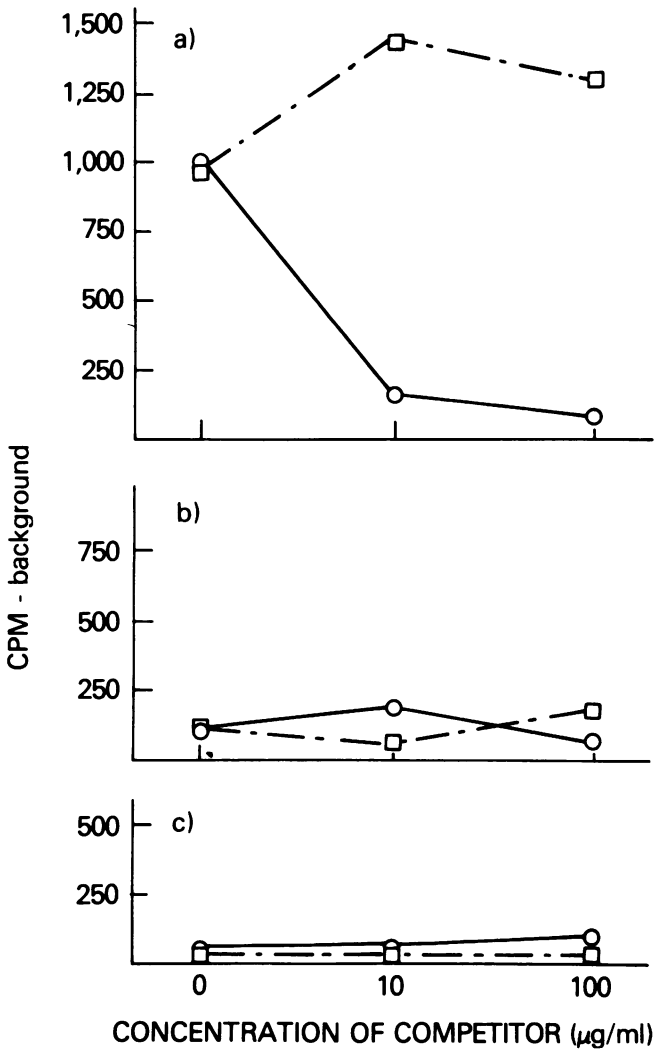

FIG. 1. Antigen specificity of the one-site IRMA, using ^{125}I-labeled IIC5-B10 to compare (a) 7G8 gametocyte-infected blood with (b) FCQ-27 trophozoite-infected blood with (c) uninfected RBCs as antigen. All preparations were made to give approximately 10^5 RBC per spot. ^{125}I-labeled IIC5-B10 was used at a concentration of 1.5 μg of protein A-purified Mab per ml of 10% bovine serum in PBS. Binding of the ^{125}I-labeled IIC5-B10 was competed with unlabeled protein A-purified IIC5-B10 (○) or IA3-B8 (□) as described in Materials and Methods. All values given are the mean of triplicate samples.

Bond (Fig. 1a) but not when FCQ-27 trophozoites (Fig. 1b) or uninfected RBCs (Fig. 1c) were used. Moreover, the gametocyte-bound ^{125}I-labeled IIC5-B10 was specifically competed by unlabeled IIC5-B10 but not by IA3-B8 at the same concentrations (Fig. 1a). Indeed, the presence of IA3-B8 appeared to enhance the binding of ^{125}I-labeled IIC5-B10.

Competition analysis with one-site IRMA. The effects of competition between homologous and heterologous Mabs in the one-site assay was further investigated. Mabs IIC5-B10, IA3-B8, and IID2-A10 at concentrations from 0.1 to 100 μg/ml were tested in competition with ^{125}I-labeled IIC5-B10 at 3 μg/ml with 7G8 gametocytes as antigen (Fig. 2A). Competition with the homologous Mab, IIC5-B10, was confirmed and was 50% effective at about 1 μg/ml, i.e. at a

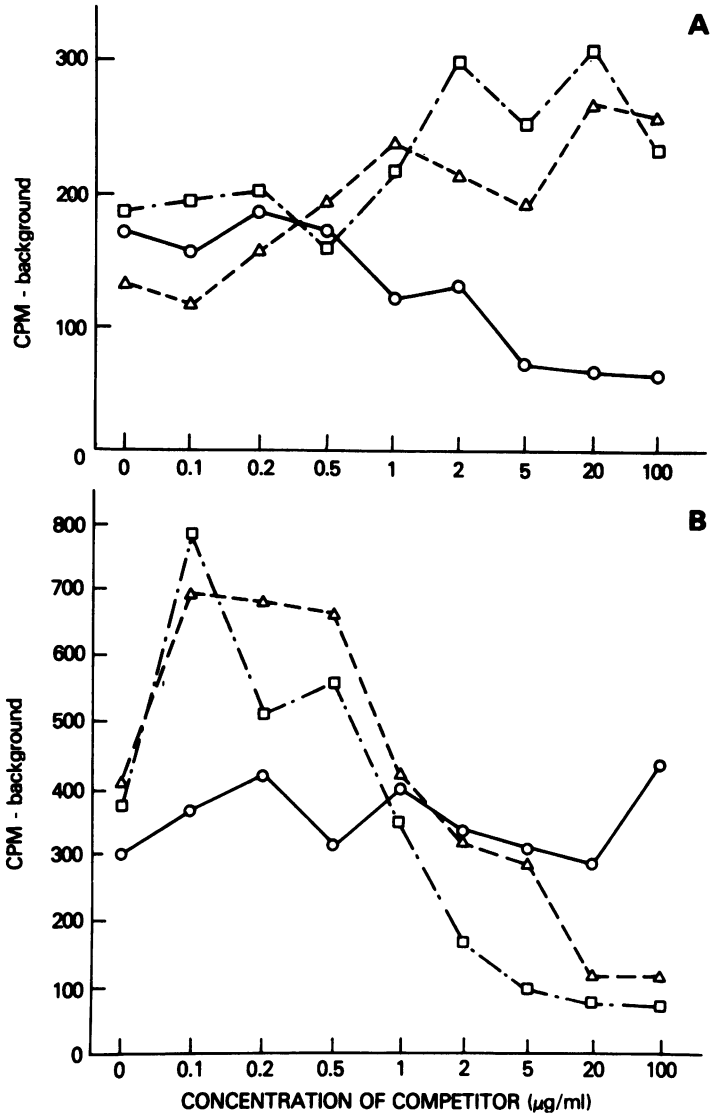

FIG. 2. Competition analysis by the one-site IRMA: (A) using ^{125}I-labeled IIC5-B10 (at 3 μg of protein A-purified Mab per ml) on 7G8 gametocyte-infected blood; (B) using ^{125}I-labeled IA3-B8 (at 1 μg of protein A-purified Mab per ml) on 7G8 gametocyte-infected blood. Binding of the ^{125}I-labeled Mab was competed with unlabeled ammonium sulfate-precipitated IIC5-B10 (○), IA3-B8 (□), or IID2-A10 (△). All values given are the mean of triplicate samples.

concentration similar to that of the ^{125}I-labeled IIC5-B10. The tendency for binding of ^{125}I-labeled IIC5-B10 to be enhanced in the presence of heterologous Mabs IA3-B8 and IID2-B10 was also confirmed. At concentrations above about 1 μg/ml the presence of either IA3-B8 or IID2-B10 increased binding of ^{125}I-labeled IIC5-B10 to about twice that in controls; below 0.5 μg/ml these Mabs had little effect on binding of ^{125}I-labeled IIC5-B10.

The reciprocal experiment was conducted, using ^{125}I-labeled IA3-B8 at 1 μg/ml (Fig. 2B). As competitors the homologous Mab IA3-B8 reduced binding by 50% at about 1 μg/ml and the heterologous Mab IID2-A10 did so at about 5 μg/ml. Below 0.5 μg/ml, however, both IA3-B8 and IID2-A10 enhanced binding of ^{125}I-labeled IA3-B8. At the concentrations tested, IIC5-B10 neither significantly enhanced nor reduced binding of ^{125}I-labeled IA3-B8.

Two-site IRMA. The two-site IRMA was conducted as described in Materials and Methods. Polystyrene wells were incubated with dilutions of a "ground" Mab in serum-free PBS, washed, and blocked with solutions containing 10% bovine serum or 0.05% Tween 20 in PBS. The Mab-treated wells were incubated with Triton X-100 extracts of *P. falciparum* 7G8 gametocyte-infected RBCs (the antigen) and then washed and incubated with an ^{125}I-labeled Mab diluted in 10% bovine serum in PBS. After final washing each well was counted in a gamma counter. Results in which IA3-B8, IID2-A10, or IIC5-B10 was used as the ground Mab and IA3-B8 or IIC5-B10 was used as the ^{125}I-labeled Mab are given in Table 1; four different experiments are represented. The values are given after subtraction of counts in controls from which antigen was omitted. The values thus represent labeled antibody specifically bound to antigen. Four to 10 times more ^{125}I-labeled IIC5-B10 reacted with antigen bound by ground Mab IA3-B8 or IID2-A10 than reacted with antigen bound by the homologous IIC5-B10. The reverse was also true as at least three times more ^{125}I-labeled IA3-B8

TABLE 1. Two-site IRMA[a]

^{125}I-labeled Mab	Expt	Ground Mab		
		IIC5-B10	IID2-A10	IA3-B8
IIC5-B10	a	6	61	67
	b	19	85	252
	c	11	50	93
	d	83	ND	492
IA3-B8	a	261	63	78
	b	361	53	70
	c	882	117	117
	d	364	ND	76

[a] Results of the two-site IRMA, using IA3-B8, IID2-A10, or IIC5-B10 as ground Mab, 0.5% Triton X-100 extracts of 7G8 gametocyte-infected RBCs as antigen, and ^{125}I-labeled IIC5-B10 or IA3-B8 as second antibody. The results of four separately conducted experiments are given (cpm). In each experiment results are given as the mean of triplicate samples and represent the amount of ^{125}I-labeled Mab bound after subtraction of control samples in which 10% bovine serum in PBS was substituted for antigen. The results thus represent ^{125}I-labeled Mab specifically bound to antigen. The conditions of the experiments were as follows. In experiments a, b, and c, a ground Mab concentration of 20 μg of ammonium sulfate-precipitated protein per ml, ^{125}I-labeled IIC5-B10 at 3 μg/ml, and ^{125}I-labeled IA3-B8 at 1 μg/ml were used. Three different lots of 7G8 gametocyte antigen preparation were used: a and b, 10^7 gametocytes per ml; c, 3×10^6 gametocytes per ml. In experiment d, a ground Mab concentration of 10 μg of protein A-purified protein per ml, ^{125}I-labeled IIC5-B10 at 1.5 μg/ml, ^{125}I-labeled IA3-B8 at 1.5 μg/ml, and 7G8 gametocytes at 10^7 per ml were used. ND, Not done.

reacted with antigen bound by IIC5-B10 than with antigen bound by IID2-A10 or the homologous IA3-B8.

These results indicate that antigen bound to the plate by one Mab excludes further reaction with the homologous Mab but may permit binding by a heterologous Mab. This is consistent with IIC5-B10 and IA3-B8 recognizing distinct epitopes, each of which is represented once on the bound antigen. IID2-A10 behaves as though it reacts with the same epitope as IA3-B8.

The two-site IRMA was tested for sensitivity in detecting gametocyte antigen (Fig. 3). IIC5-B10 was used as the ground Mab at three different dilutions (10, 1, and 0.1 μg/ml), and Triton X-100 extracts of 7G8 gametocytes were used equivalent to 1×10^6 down to 3×10^4 gametocytes per ml. At a ground Mab concentration of 10 μg of IIC5-B10 per ml, 7G8 gametocytes were readily detected at concentrations down to 3×10^5 per ml.

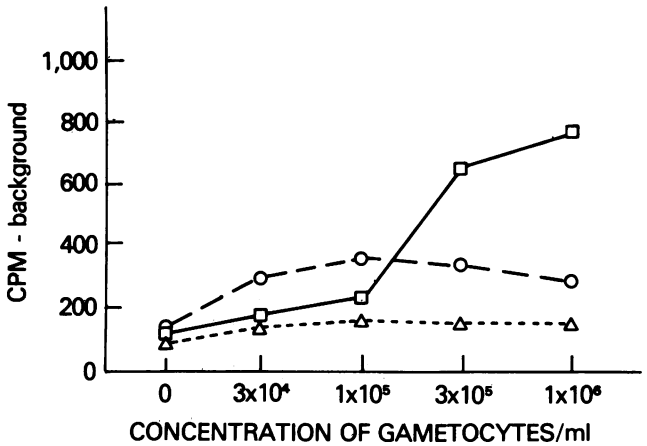

FIG. 3. Sensitivity of two-site IRMA to gametocyte antigen concentration and to concentration of ground Mab. Ammonium sulfate-precipitated protein A-purified IIC5-B10 was used as the ground Mab at concentrations of 10 (□), 1 (○), and 0.1 (△) μg/ml. Antigen was extracted as usual in 0.5% Triton X-100 and diluted in 10% bovine serum–0.05% Tween 20 in PBS. ^{125}I-labeled protein A-purified IA3-B8 was used at 0.5 μg/ml in 10% bovine serum in PBS.

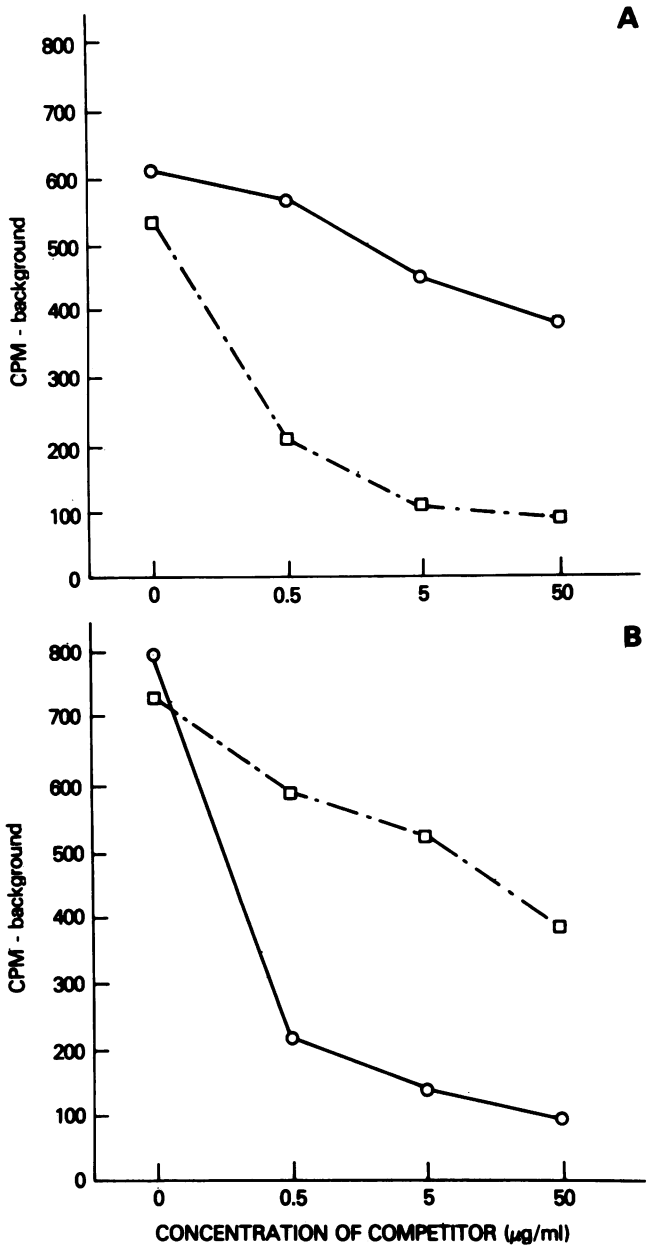

FIG. 4. Competition analysis by the two-site IRMA. (A) The ground Mab was protein A-purified IA3-B8 at 7 μg/ml, antigen was 0.5% Triton X-100 extract of 7G8 gametocytes diluted in 10% bovine serum in PBS to 10^6 gametocytes/ml, and ^{125}I-labeled protein A-purified IIC5-B10 was at 1.5 μg/ml in 10% bovine serum in PBS. (B) The ground Mab was protein A-purified IIC5-B10 at 7 μg/ml, antigen was 0.5% Triton X-100 extract of 7G8 gametocytes diluted in 10% bovine serum in PBS to 10^6 gametocytes per ml, and ^{125}I-labeled protein A-purified IA3-B8 was at 1.5 μg/ml in 10% bovine serum in PBS. Binding of ^{125}I-labeled Mab was competed with protein A-purified IIC5-B10 (□) or IA3-B8 (○). All points are the means of triplicate samples.

Competition analysis with two-site IRMA. The two-site IRMA was used as a competition assay as follows. After incubation with ground Mab and gametocyte antigen, the wells were incubated first with various concentrations of the homologous or heterologous Mabs followed by incubation with the heterologous ^{125}I-labeled Mab. The ^{125}I-labeled Mab was effectively competed by its homolog and relatively ineffectively by the heterologous Mab (Fig. 4A and B). At no concentration tested (down to 0.1 μg/ml; data not shown) did either homologous or heterologous competition give enhanced binding of the ^{125}I-labeled Mab.

DISCUSSION

The Mabs used in these studies include those previously shown to block infectivity of gametocytes of *P. falciparum* to *A. freeborni* mosquitoes (8). Two of the Mabs, IIC5-B10 and IA3-B8, interacted synergistically to mediate suppression of infectivity. Both Mabs reacted by the indirect immunofluoresence assay with the surface of extracellular gametes of *P. falciparum* and each immunoprecipitated a complex of proteins of 260,000, 59,000, and 53,000 M_r from Triton X-100 extracts of surface-radioiodinated gametes. Among several isolates of *P. falciparum* tested, gametocytes of one African parasite, L.E5, failed to react by indirect immunofluorescence assay or by immunoprecipitation with Mab IA3-B8 but did react with IIC5-B10 (6), indicating that the Mabs recognized distinct epitopes on the target antigen(s). In the present study we have analyzed the interactions of the Mabs and their target epitopes by IRMA.

Our results have confirmed that IA3-B8 and IIC5-B10 react with two distinct epitopes on a single Triton X-100-extractable, *P. falciparum* gametocyte-specific antigen or antigen complex. They also indicate that each epitope is represented once only on the antigen. This was demonstrated in the two-site IRMA in which antigen bound by one Mab, (e.g., IA3-B8) was able to react with the other (e.g., IIC5-B10) but no further antigenic sites were available to bind the homologous Mab (IA3-B8). The reciprocal situation was also true. A third Mab, IID2-A10, although mediating significantly lower binding of IIC5-B10, behaved in an analogous manner to IA3-B8, suggesting that these two Mabs recognized the same epitope, a result also in accord with previous findings (6).

Although the results of the two-site IRMA are consistent with the Mabs recognizing nonrepeated epitopes, the data, while suggestive, cannot be considered conclusive. For instance, a small number of repeated epitopes clustered in a single region of a native protein molecule might be rendered sterically unavailable to further binding when one of them reacts with a plate surface-bound antibody. It is unlikely, however, that a highly repeated epitope could be masked in this way in a native molecule.

A simple concentration-dependent competition occurred between homologous but not between heterologous combinations of IA3-B8 and IIC5-B10 in the two-site IRMA. Competition studies with the one-site IRMA further confirmed that IA3-B8 and IIC5-B10 reacted with distinct epitopes and that IID2-A10 appeared to recognize the same epitope as IA3-B8. Binding of ^{125}I-labeled Mab to the acetone-fixed gametocytes in the one-site IRMA was effectively competed by equivalent or higher concentrations of the homologous Mab. Unexpected, however, was the finding that binding of ^{125}I-labeled IA3-B8 was enhanced when preincubated with low concentrations of IA3-B8 or with its apparent analog IID2-A10; this observation is hard to explain. It was also unexpected that binding of IIC5-B10 to the acetone-fixed gametocytes was enhanced by high concentrations of the heterologous Mabs IA3-B8 or IID2-A10. Enhanced binding in the presence of heterologous Mabs in the one-site assay is of interest in view of the synergistic suppression of infectivity of gametocytes to mosquitoes by IA3-B8 and IIC5-B10 (8). That a heterologous combination of Mabs enhanced the binding of individual Mabs in the one-site assay where antigen was presented as dried, acetone-fixed cells (but not in the two-site assay, with antigen extracted in Triton X-100) may indicate that the phenomenon is dependent upon the location of antigen in the cell membrane. Mutually enhanced binding of Mabs against distinct nonrepeated epitopes has been recorded in studies with human gonadotrophic hormone as antigen (4).

Further studies are being undertaken to develop Mabs against other epitopes on the *P. falciparum* gamete and gametocyte-specific proteins of 260,000, 59,000, and 53,000 M_r and to analyze their properties and effects on gametocyte infectivity.

ACKNOWLEDGMENTS

We thank David Read for valuable discussions and suggestions during the development of the immunoradiometric assays and Karen Symons for excellent technical assistance. We also thank Wilma J. Davis and Brenda B. Martin for typing and editorial assistance.

LITERATURE CITED

1. **Burkot, T. R., J. L. Williams, and I. Schneider.** 1984. Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. Trans. R. Soc. Trop. Med. Hyg. **78**:335–8.
2. **Carter, R., and L. H. Miller.** 1979. Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. Bull. W.H.O. **57**(Suppl. 1):37–5.
3. **Chen, P., G. Lamont, T. Elliot, C. Kidson, G. Brown, G. Mitchell, J. Stace, and M. Alpers.** 1980. *Plasmodium falciparum* strains from Papua New Guinea: culture characteristics and drug sensitivity. Southeast Asian J. Trop. Med. Public Health **11**:435–40.
4. **Ehrlich, P. H., W. R. Moyle, Z. A. Marstala, and R. E. Canfield.** 1982. Mixing two monoclonal antibodies yields enhanced affinity for antigen. J. Immunol. **128**:2709–2713.
5. **Fraker, P. J., and J. C. Speck.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide 1, 3, 4, 6-textrachloro 3α, 6α-diphenyl glycoluril. Biochem. Biophys. Res. Commun. **80**:849–857.
6. **Graves, P. M., R. Carter, T. R. Burkot, J. Rener, D. C. Kaushal, and J. L. Williams.** 1985. Effects of transmission-blocking monoclonal antibodies on different isolates of *Plasmodium falciparum*. Infect. Immun. **48**:611–616.
7. **Ifediba, T., and J. P. Vanderberg.** 1981. Complete *in vitro* maturation of *Plasmodium falciparum* gametocytes. Nature (London) **294**:364–6.
8. **Rener, J., P. M. Graves, R. Carter, J. L. Williams, and T. R. Burkot.** 1983. Target antigens of transmission-blocking immunity on gametes of *Plasmodium falciparum*. J. Exp. Med. **158**:976–981.
9. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193**:673–5.
10. **Zavala, F., A. H. Cochrane, E. H. Nardin, R. S. Nussenzweig, and V. Nussenzweig.** 1983. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. J. Exp. Med. **157**:1947–57.