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Naturally Occurring Antibodies to an Epitope on *Plasmodium falciparum* Gametes Detected by Monoclonal Antibody-Based Competitive Enzyme-Linked Immunosorbent Assay

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The antibody response to an epitope on gamete antigens of *Plasmodium falciparum* in persons naturally exposed to malaria has been investigated by competitive enzyme-linked immunosorbent assay. The assay detects antibodies to an epitope on the 48/45-kilodalton (kDa) gamete surface antigen by competition with horseradish peroxidase-labeled monoclonal antibody IIC5-B10. Five sera previously shown to immunoprecipitate the 230- and 48/45-kDa antigens significantly inhibited IIC5-B10 binding to an average of 24.2% of control. The one serum which precipitated only the 48/45-kDa antigen did not inhibit IIC5-B10 binding. For 26 sera which were negative by immunoprecipitation, mean binding in the assay was 112.7% of control (pooled London nonimmune sera). Recognition of both 230-kDa and 48/45-kDa antigens was associated with a titer of 1:9 or greater (reciprocal geometric mean titer, 27.6) for inhibition to more than 2 standard deviations from the mean of the negative sera. The results show that the IIC5-B10 binding site is a naturally immunogenic epitope recognized by the majority of persons who had antibodies to the 48/45-kDa protein. An additional finding was enhancement of binding of IIC5-B10 to an average of 154.4% of control by five sera which recognized only the 230-kDa antigen, presumably due to conformational alteration of the gamete antigen complex.

Gametocytes of the human malaria parasite *Plasmodium falciparum* synthesize antigens of 230 and 48/45 kilodaltons (kDa) which are later exposed on the surface of the gametes when they emerge from the erythrocytes following uptake in a mosquito bloodmeal (9, 13). A third antigen of 25 kDa is synthesized predominantly after gametogenesis (13). All three antigens are the targets of monoclonal antibodies (MAbs) which block transmission of parasites to mosquitoes (10–12).

The 230- and 48/45-kDa antigens are present in a membrane-bound complex (8), and several MAbs (e.g., IIC5-B10 of Rener et al. [11]) immunoprecipitate them together from ^{125}I-surface-labeled gametes. However, the target epitope of IIC5-B10 has been shown by blotting studies to be on the 48/45-kDa antigen (R. Carter, N. Kumar, I. A. Quakyi, M. F. Good, K. N. Mendis, P. M. Graves, and L. H. Miller, Progr. Allergy, in press; N. Kumar, personal communication). There are at least three separate epitopes on the 48/45-kDa antigen (3, 14) and at least two on each of the 230- and 25-kDa antigens (10; Carter et al., in press). Variation among strains of *P. falciparum* in at least one of the 48/45-kDa epitopes has been demonstrated (7).

Antibody responses to the 230- and 48/45-kDa gamete antigens have been shown to occur in individuals naturally exposed to malaria in Papua New Guinea (6). However, the observed pattern of response is not a simple one. Although all adults in a hyperendemic area show strong responses to asexual-stage antigens through repeated exposure, only a minority respond to gamete antigens, with no apparent correlation between responsiveness and age. Furthermore, the response to particular antigens varies among individuals. Immunoprecipitation with protein A-Sepharose from ^{125}I-surface-labeled gametes by individual sera revealed responses either to the 230-kDa antigen only, to both the 230- and 48/45-kDa antigens, or (in one serum) to the 48/45-kDa antigen only (6). The ability of sera to block transmission of *P. falciparum* from in vitro cultures to mosquitoes correlated with the responses to the 230-kDa antigen. Such antigamete antibodies provide a possible explanation for the failure of many gametocyte carriers to infect mosquitoes (5).

Previous studies have demonstrated only that some immune sera react with gamete antigens of *P. falciparum* but give no information on the specific epitope recognized. In view of the existence of strain variation in epitopes and the differential recognition of antigens by sera, a competitive assay has been developed which measures the response to a particular epitope, that recognized by IIC5-B10, on the 48/45-kDa protein. Similar assays have been used to measure natural immune responses to particular epitopes of hepatitis B virus (1), flavivirus (2), and schistosomes (4). Results in the IIC5-B10 assay have been compared with the response of each serum to the 230- and 48/45-kDa gamete antigens as previously determined by immunoprecipitation with protein A (6).

MATERIALS AND METHODS

Preparation of antigen and conjugated MAb. *P. falciparum* gametocytes of strain NF54 were grown in culture in a continuous-flow apparatus or in flasks as previously described (11). Gametocytes were purified on Nycodenz (12) or Percoll gradients and extracted in 0.5% Nonidet P-40 with protease inhibitors. Before use, the extract and a control extract of uninfected erythrocytes were dialyzed extensively against phosphate-buffered saline (PBS) (pH 7.4) at 4°C and stored at −70°C.

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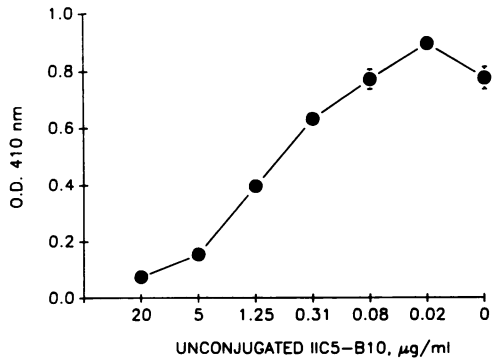

FIG. 1. Inhibition of binding of HRP-conjugated IIC5-B10 (2 μg/ml) to plates precoated with gametocytes at 1.6×10^5 per well by unconjugated IIC5-B10 in fourfold serial dilution. Mean and SD of two replicates.

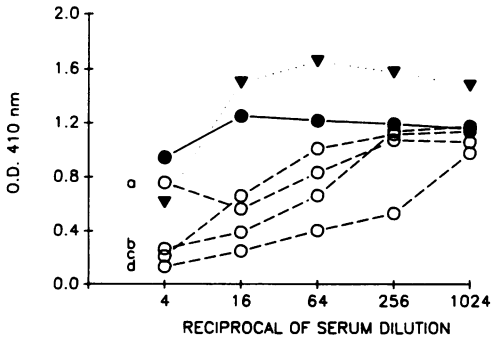

FIG. 2. Binding of HRP-conjugated IIC5-B10 in the presence of human sera in fourfold serial dilution starting at 1:4. Symbols: ●, London pool, mean of two replicates; ○, sera reacting with both 230- and 48/45-kDa antigens (a = A5, b = JMS2.49, c = A21, d = A67); ▲, serum (A41) reacting with 48/45-kDa antigen only. Antigen and HRP-MAb concentrations were as for Fig. 1.

The MAb IIC5-B10 (11) was purified and conjugated to horseradish peroxidase (HRP) by Kirkegaard and Perry Labs Inc., Gaithersburg, Md.

Competitive assay. Polyvinylchloride microtiter plates (Flow Labs) were coated with 50 μl of antigen at the appropriate dilution (usually 1:40 from the stock solution of 1.25×10^8 gametocytes per ml, i.e., 1.56×10^5 gametocytes per well) in PBS for 5 to 8 h at room temperature.

Wells were aspirated dry and filled for 1 h with blocking buffer (PBS [pH 7.4] with 0.5% casein, 1.0% bovine serum albumin, 0.01% thimerosal, and 0.002% phenol red). After removal of blocking buffer, the competitor (human serum or unconjugated MAb) at 2 times final dilution in PBS + 0.05% Tween was added in a volume of 25 μl per well. Then 25 μl of the HRP-conjugated MAb (HRP-MAb) at 4 μg/ml in blocking buffer (i.e., final concentration, 2 μg/ml) was added. Control wells containing no competitor and two replicates containing London pool control serum were included on each plate as well as a blank well (PBS only). The plates were incubated overnight at room temperature and then washed three times with PBS-Tween, and 100 μl of 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate) (ABTS) peroxidase substrate (Kirkegaard and Perry Laboratories; two-component) was added per well. Plates were read after 15 to 60 min on a Dynatech microplate reader at 410 nm.

RESULTS

The optimal concentrations of capture antigen and HRP-MAb for the competitive assay were determined in pilot studies. Using antigen at the equivalent of 1.6×10^5 gametocytes per well (3.125×10^6/ml), the HRP-MAb concentration of 2 μg/ml was chosen as giving >60% of maximal binding of HRP-IIC5-B10 and an optical density (OD) reading of >1.0 at 30 min. No binding was observed between the HRP-MAb and either control uninfected erythrocytes at equivalent concentration or the plate only. Competition of unconjugated IIC5-B10 in serial dilution is shown in Fig. 1, which indicates the sensitivity of the assay.

Papua New Guinea sera with known antibody to the gamete antigens when tested by immunoprecipitation with protein A were initially assayed in serial fourfold dilution at final concentrations ranging from 1:4 to 1:1,024. The control was the mean of two replicates of a pool of 10 sera from a London blood bank. At 1:4 dilution, but not at 1:16, the London pool gave some nonspecific inhibition of binding of IIC5-B10 (Fig. 2). Shown in Fig. 2 are the responses of four of the sera which immunoprecipitate both the 230- and 48/45-kDa gamete antigens (A5, A21, A67, and JMS/2.49). Serum A41, which reacts with only the 48/45-kDa antigen, did not inhibit the binding of IIC5-B10 at 1:16 or greater dilution.

Subsequent experiments were performed with sera in threefold serial dilution starting at 1:3. The nonspecific inhibition observed with the London pool was negligible by 1:9 dilution. Inhibition curves for three of the sera reacting with both 230- and 48/45-kDa antigens are shown in Fig. 3. The binding is expressed as a percentage of the control binding to allow for comparison between assays run in different experiments. Comparison of results for sera A5 and A67 in Fig. 2 and 3 demonstrates the repeatability of the responses from one experiment to another. Certain sera, particularly those which immunoprecipitated the 230-kDa antigen only, enhanced the binding of IIC5-B10 in the assay above the control 100% level. Responses of three anti-230-kDa sera, of which two enhanced, are shown in Fig. 3.

The sera were classified into groups according to their protein A immunoprecipitation results (6). The range of percentage of antigen precipitated by the Papua New Guinea sera (amount precipitated as a percentage of the antigen presented in the reaction) varied from 0 to 43.3% for the

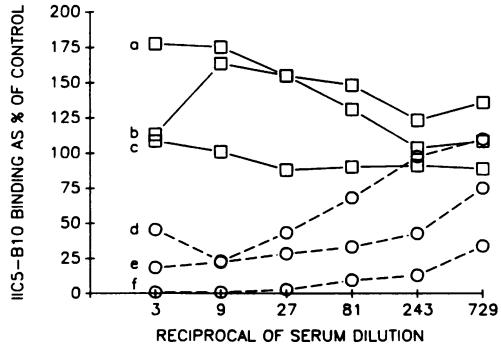

FIG. 3. Binding of HRP-conjugated IIC5-B10 in the presence of human sera in threefold serial dilution starting at 1:3, expressed as percentage of mean binding in the presence of London pool serum at the same dilutions. Symbols: □, sera reacting with 230 antigen only (a = JMS2/40, b = T447, c = T426); ○, sera reacting with both 230 and 48/45 antigens (d = A5, e = A67, f = A29). Antigen and HRP-MAb concentrations were as for Fig. 1.

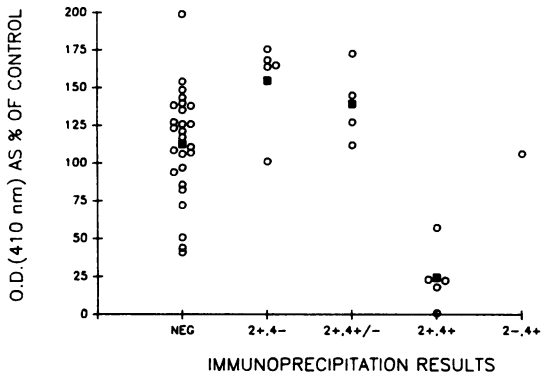

FIG. 4. Binding of HRP-conjugated IIC5-B10 in the presence of Papua New Guinea serum at 1:9 dilution, expressed as percentage of mean binding by London control. Sera were classified as positive (+), weak positive (+/−), or negative (−) to the 230-kDa antigen (2) or the 48/45-kDa antigen (4) according to criteria given in the Results section. Solid squares indicate the mean for each group. Antigen and HRP-MAb concentrations were as for Fig. 1.

230-kDa antigen and from 0 to 28.0% for the 48/45-kDa antigen. These percentages have been classified into three categories for each antigen, representing positive (precipitated >8% of antigen added), weak positive (4 to 8%), and negative reactions. Figure 4 shows the OD reading obtained with each serum at 1:9 dilution expressed as a percentage of the mean OD of the control serum.

For 26 sera which were negative to both antigens by immunoprecipitation, the mean OD at 1:9 dilution was 112.7% of control, with a standard deviation (SD) of 36.0%. There were four obvious outliers among the negative sera (Fig. 4): three which inhibited binding to less than 60% of control (A65, T380, and T384) and one which enhanced binding to 199% of control (T315). If these are excluded, the mean of the remaining 22 negative sera was 118.0% of control with an SD of 22.4%.

A panel of 40 nonimmune sera from the Brisbane blood bank were tested individually to investigate the range of inhibitions observed. The mean OD observed with these sera was 94.3% of control with an SD of 11.2%. All the sera fell in the range from 80.4 to 126.5%, and there were no obvious outliers as in the case of the Papua New Guinea sera.

Inhibition of IIC5-B10 binding was observed with those sera which strongly immunoprecipitated both the 230- and 48/45-kDa antigens (Fig. 4). The OD for this group of five sera averaged 24.2% of control and was significantly lower than the mean binding of the group of 26 negative sera (t = 5.289, $P < 0.0001$). There was no inhibition or enhancement with the one serum which was positive to the 48/45-kDa antigen only.

The nine sera which were positive against the 230-kDa antigen but negative or weakly positive against the 48/45-kDa antigen had mean binding of 147.5% of control with an SD of 28.0%. This enhancement is significant when compared either with the pool of 26 negative Papua New Guinea sera (t = 2.626, P = 0.013) or with the pool of 22 which excludes outliers (t = 3.089, P = 0.004). Dividing the nine sera into two groups, one containing five sera which recognized only the 230-kDa antigen and the other composed of four sera which were also weakly positive against the 48/45-kDa antigen, showed significant enhancement only for the former group (t = 2.421, P = 0.022) and not for the latter (t = 1.385, P = 0.177).

TABLE 1. Reciprocal titer of Papua New Guinea sera at particular levels of inhibition or enhancement of binding of HRP-conjugated IIC5-B10

Serum[a]	Immunoprecipitation results[b]		IIC5-B10 ELISA[c] titer			
			Inhibition		Enhancement	
	230 kDa	48/45 kDa	>2 SD	>1 SD	>2 SD	>1 SD
A5	+	+	9	81		
A21	+	+		9		
A29	+	+	729	729		
A67	+	+	81	729		
JMS2.49	+	+	27	81		
A3	+	+/−	3	3		
A34	+	+/−	4	4		
T510	+	+/−				
T547	+	+/−				9
T378	+/−	+/−	3	9		
T353	+	−				9
T426	+	−				
T447	+	−				27
JMS2.39	+	−				64
JMS2.40	+	−				27
T452	+/−	−				
A41	−	+		3		
A68	−	+/−	3	9		
A20	−	−				
A40	−	−		3		
A65	−	−		27		
T314	−	−		3		
T315	−	−			9	81
T333	−	−				
T336	−	−				
T350	−	−		3		
T362	−	−		729		
T370	−	−				
T373	−	−				
T374	−	−				
T375	−	−				
T376	−	−				
T377	−	−				
T380	−	−	9	729		
T381	−	−				9
T384	−	−	3	81		
T400	−	−				
T427	−	−				
T438	−	−				
T448	−	−				
T449	−	−				
T450	−	−				
T451	−	−		3		
T489	−	−		3		

[a] All sera were tested in threefold serial dilution, starting at 1:3, except A34 and JMS2.39, which were tested in fourfold dilution starting at 1:4.

[b] Data taken from Graves et al. (6). +, Positive (precipitated >8% of antigen added); +/−, weak positive (precipitated 4 to 8% of antigen added); −, negative (precipitated <4% of antigen added).

[c] ELISA, Enzyme-linked immunosorbent assay.

Table 1 shows the titer at which each serum inhibited or enhanced the binding of IIC5-B10 to greater than 1 or 2 SD from the mean of the 26 negative sera. There was a strong association between recognition of both the 230-kDa and 48/45-kDa antigens and a titer of 1:9 or greater (geometric mean reciprocal titer, 27.6) for inhibition to >2 SD from the mean binding of negative sera (i.e., <40.7%). There was also an association between recognition of only the 230-kDa antigen and a titer of 1:9 or greater (geometric mean reciprocal titer, 13.8) for enhancement to >1 SD from the mean of negative sera (i.e., >148.7%).

Of the 26 PNG sera which were completely negative to the gamete antigens, 1 (T380) inhibited IIC5-B10 binding to >2 SD below the mean (i.e., <40.7%) at 1:9 dilution and 3 did so to >1 SD (i.e., <76.7%). One serum (T315) enhanced binding by >2 SD (i.e., >184.7%), and one (T381) enhanced by >1 SD (i.e., >148.7%) at the same dilution. There were no cases in which the same serum enhanced and inhibited binding at different dilutions.

DISCUSSION

The natural antibody response to an epitope on the 48/45-kDa gamete antigen of *Plasmodium falciparum* has been investigated by a MAb-based competitive enzyme-linked immunosorbent assay. Both inhibition and enhancement of binding of the MAb were observed. Both phenomena have previously been observed in competitive radioimmunoassays comparing different MAbs (3).

Assay results were compared with previous studies on the antibody specificities in the sera. All five of the sera that had been previously shown to react strongly against the same isolate of *P. falciparum* by immunoprecipitation of both the 230- and 48/45-kDa antigens inhibited IIC5-B10 binding, thus showing that the IIC5-B10 binding site is an important immunogenic epitope recognized by the majority of individuals who respond to the 48/45-kDa protein. IIC5-B10 itself immunoprecipitates both antigens as a complex (8), despite being directed against an epitope on the 48/45-kDa antigen. The one serum recognizing the 48/45-kDa antigen but not inhibiting IIC5-B10 (presumably because it responded to a different epitope) did not immunoprecipitate the 230-kDa antigen.

Three of 32 sera which were negative to the 48/45-kDa antigen by immunoprecipitation inhibited IIC5-B10 binding to less than 60% of control when tested at 1:9 dilution. While these sera could be regarded as false-positives in the enzyme-linked immunosorbent assay caused by nonspecific binding, another possible explanation is that the sera contain antibodies of isotypes (e.g., immunoglobulin M) not recognized by protein A, which was used for the immunoprecipitations. This possibility is supported by the normal distribution of responses (with no outliers) observed among a panel of Brisbane blood bank sera.

Four of the five sera which were strong positives against only the 230-kDa antigen enhanced the binding of IIC5-B10, as did two of the negative sera. Presumably in these cases recognition of other epitopes causes conformational changes in the gamete antigen complex, resulting in increased binding of IIC5-B10.

We have previously demonstrated a correlation between recognition of the 230-kDa antigen by immune sera and suppression of infectivity of cultured *P. falciparum* gametocytes to mosquitoes (6). In view of this it is not surprising that there is no direct relationship between inhibition of binding of IIC5-B10 observed in this assay and the previously reported transmission-blocking activity in a serum. However, it is of interest that the anti-230-kDa sera, which tended to enhance IIC5-B10 binding, were all strong transmission blockers with infectivity averaging 9.4% of control (6). Those sera which recognized both 230- and 48/45-kDa antigens (and inhibited IIC5-B10 binding in this assay) reduced infectivity to an average of 24.3%, whereas the mean of the negative sera was 77.3% (6). Mechanisms of transmission blocking by these immune sera could involve at least two phenomena, (i) direct reaction of antibodies with epitopes on the 230- or 48/45-kDa antigens and (ii) enhanced binding of antibodies to the 48/45-kDa antigen as an indirect effect of antibodies binding to the 230-kDa protein. Extension of the assay to all the known gamete epitopes will provide more extensive information on the nature of the responses to these important targets of malaria transmission-blocking immunity.

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