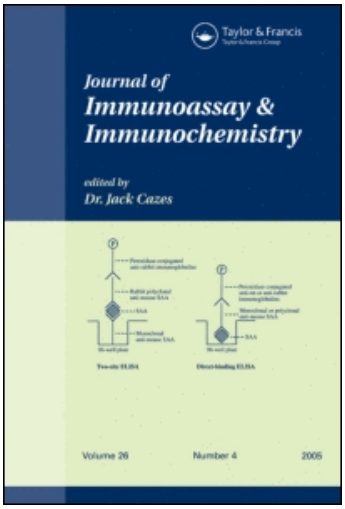

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Kenneth W. Hunter[a]; L. Patrick Smith[a]; G. Thomas Strickland[a]; Warren A. Blackburn[a]
[a] Departments of Pediatrics and Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD

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HYPERGAMMAGLOBULINEMIA AND ERYTHROCYTE AUTOANTIBODY COMPLICATE ENZYME IMMUNOASSAY OF ANTIMALARIAL ANTIBODY

Kenneth W. Hunter, L. Patrick Smith,
G. Thomas Strickland and Warren A. Blackburn
Uniformed Services University of the Health Sciences
Departments of Pediatrics and Medicine
4301 Jones Bridge Road, Bethesda, MD 20014

ABSTRACT

Enzyme immunoassay of specific antimalarial antibody in sera from Plasmodium yoelii-infected mice was complicated by hypergammaglobulinemia and erythrocyte (RBC) autoantibody. Malarious serum had higher immunoglobulin levels which resulted in a substantial nonspecific adsorption of antibody to antigen uncoated microtiter wells even in the presence of a surfactant. Since it was possible that some of the antibody binding to solid phase-bound malarial antigen was also due to nonspecific adsorption, the antibody binding of nonimmune serum was compared to that of malarial immune serum diluted to an equivalent immunoglobulin concentration. This allowed differentiation of specific antimalarial antibody binding from nonspecific adsorption of immunoglobulin. Although malarial antigen was partially purified from intraerythrocytic parasites, it bound a significant amount of rabbit anti-mouse RBC antibody, indicating the presence of residual RBC antigens. Serum from immune mice, but not nonimmune mouse serum, also showed substantial binding to RBC stroma antigen, suggesting that a component of the observed antibody binding to malarial antigen was anti-RBC antibody. Hypergammaglobulinemia and the induction of RBC autoantibody may both be related to the polyclonal activating property of the malarial parasite. The presence of RBC autoantibody in serum from malaria infected mice necessitates a more conservative interpretation of specific antimalarial antibody levels until a more purified malarial antigen is available.

INTRODUCTION

Since its introduction in 1971 (4,16), enzyme immunoassay (EIA) has become a widely used tool for measuring antibody to specific antigens (19,3). One of the earliest applications of EIA in infectious disease was for the detection of antibodies to human malarial parasites (17,18). We have re-

cently explored the use of EIA to study the specific antibody response to Plasmodium yoelii in mice. During the course of these investigations we encountered two major technical problems related to the biological characteristics of the malarial parasite. In this paper we describe the EIA procedure in detail and discuss the problems encountered.

MATERIALS AND METHODS

Mice and Parasites. Six to 8 week-old female C57BL/6 mice were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were housed in groups of 6 or fewer and given food and water ad libitum. A nonlethal strain of P. yoelii 17X was stored at -70°C as frozen parasitized mouse blood in Alsever's glycerin. This material was thawed and injected intraperitoneally into donor mice from which infecting inocula were prepared when parasitemia levels attained 10%.

Antibodies. Affinity purified rabbit anti-mouse IgG (RaMγ) and IgM (RaMμ) antibodies were prepared as previously described (10). Alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from Miles Laboratories, Elkhart, ID. Rabbit anti-mouse red blood cell (RaMRBC) stroma (see below) antibodies were produced by repeated immunizations with C57BL/6 RBC stroma in complete and incomplete Freund's adjuvant. Serum immunoglobulin (Ig) levels were measured by the method of Mancini (13).

Antigens. A partially purified malarial antigen (MAP) was prepared from intraerythrocytic P. yoelii according to Hunter et al. (10). To prepare mouse RBC stroma antigen, normal mouse blood was first passed over a column of cellulose powder to remove leukocytes. After two washes in phosphate buffered saline (PBS), the packed cells were resuspended in 20 volumes of distilled water. The RBC stroma was then washed 5 times in PBS by centrifugation in an Eppendorf microfuge (Brinkman Instruments Inc., Westbury,

NY). The protein concentration of MAP and mouse RBC stroma antigens were measured by the method of Bradford (2).

Test Sera. C57BL/6 mice were infected with 1.0×10^5 P. yoelii parasitized erythrocytes, followed by 3 similar inoculations at monthly intervals. After the primary infection (peak parasitemia 25%), only transient low levels of parasitemia were found indicating the development of solid immunity. A pool of immune serum was obtained 3 weeks following the final infection. A pool of nonimmune serum was obtained from age-matched C57BL/6 mice left unchallenged.

Enzyme Immunoassay Procedure. Polystyrene microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 50 µl of MAP or mouse RBC stroma (10 µg/ml protein concentration) in 0.1 M $NaHCO_3$ buffer, pH 9.6, for 24 hrs at 4°C. After removing the coating antigen, the wells were washed with the coating buffer, emptied, and filled to the top with blocking buffer consisting of 5% bovine serum albumin (BSA) in PBS. Preliminary experiments revealed the need for blocking antigen uncoated sites on the polystyrene surface. After 1 hr incubation at 4°C the plates were washed with PBS containing 0.2% Tween 20 (washing buffer). Plates were used immediately, but other experiments (data not shown) indicated that they could be stored in PBS-Tween containing 0.02% sodium azide for up to 1 week without significant loss of activity. Test serum diluted in PBS-Tween was then added (50 µl) and incubated for 30 min at 4°C. The plates were again washed by 5 cycles of filling and emptying each well with washing buffer. The next step was to add 50 µl of either RaMγ or RaMµ (10 µg/ml antibody protein in PBS-Tween) followed by a 30 min incubation at 4°C. After another wash cycle each well received 50 µl of alkaline phosphatase-conjugated goat anti-rabbit IgG (used at 1:500 dilution of stock). After 30 min at 4°C, the plates were washed as above and 100 µl of enzyme substrate (p-nitrophenyl phosphate; Sigma Chemical Co., St. Louis, MO) was added. The reaction was allowed to proceed for 30 min at room temperature,

then each plate was scanned at 405 nm using a Multiscan micro-ELISA reader (Flow Laboratories, Rockville, MD). When plates could not be read immediately, the enzymatic reaction was stopped by adding 25 µl of 3N NaOH.

RESULTS AND DISCUSSION

Titers of IgG anti-P. yoelii antibody were calculated from the dilution curves of the test sera. In this procedure, at least 4 dilutions of test serum were evaluated and the absorbance plotted against the log reciprocal serum dilution (Fig. 1). Titration curves generated in this manner allowed comparisons to be made on the linear portion of the curves where differences relate in a linear manner. This also avoided situations where antibody excess would mask differences between sera. An absorbance value for titration was chosen arbitrarily, but always represented a point near the middle of the linear portion of the majority of curves (in this case, 0.7 absorbance units). The relative antibody titer was defined as the reciprocal serum dilution which produced an absorbance of 0.7 units. In the case of this particular immune serum, the relative titer was 54. Nonimmune serum showed a curve which was substantially below that of immune serum, but the exact titer could only be called less than 1:10 since the maximum absorbance value was less than the selected titration value of 0.7. However, if the nonimmune serum curve was linear at 1:10, it was possible to extrapolate back to the point where the line intersects the titration curve for immune serum.

In preliminary experiments it was observed that antigen-uncoated wells bound Ig from dilutions of test serum (data not shown), even in the presence of a surfactant like PBS-Tween. This nonspecific adsorption could be reduced substantially by blocking the wells with BSA-PBS. Even so, immune serum dilutions incubated in BSA-coated wells gave a lower but consistent background absorbance which actually titrated (Fig. 1). Since malarial infections induce polyclonal activation and the production of antibodies of

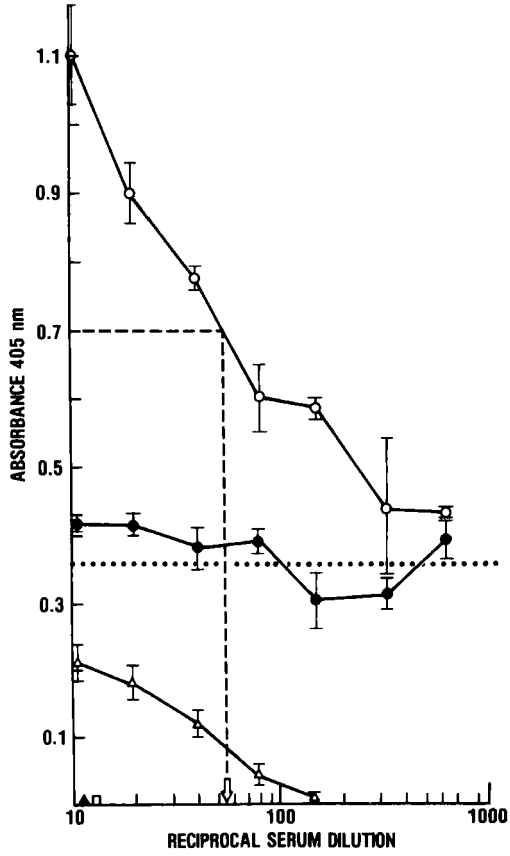

Fig. 1. EIA titration curves for IgG antimalarial antibody in immune (o-o) and nonimmune (●-●) sera. Each point represents the mean of triplicate determinations. The dotted line indicates the chosen titration point (0.7 units) near the mid point of the linear portion of the titration curve. The arrow indicates the relative IgG titer of 54. The solid horizontal line represents background absorbance in MAP-coated wells not incubated with serum. Note also the apparent titration of immune serum incubated in BSA-coated wells (Δ-Δ). BSA-coated wells incubated with nonimmune serum (▲) or without serum (□) were negative.

many specificities (5,7), it is possible that a component of the low level binding of Ig to BSA-coated wells was due to specific anti-BSA antibodies.

The mitogenic effect of malarial infections also leads to hypergammaglobulinemia (1,14). If the higher Ig content of immune serum also resulted

in more nonspecific "stricking" to MAP-coated wells, a component of the difference in antimalarial antibody titers between immune and nonimmune serum would be artifactual. To approach this question, the total IgG content of immune and nonimmune sera was determined by single radial immunodiffusion. Immune serum had approximately 2.9 times the total IgG as nonimmune serum (1670 mg/dl vs. 580 mg/dl). Based on the fold increase in total IgG, a comparison was made (on the titration curve) between the absorbance of 1:10 nonimmune serum and immune serum diluted to an equivalent IgG concentration (e.g., 1:10 nonimmune serum x fold increase in IgG (2.9) = 1:29). At a dilution of 1:29, the absorbance of immune serum was still higher than 1:10 nonimmune serum (Fig. 1), indicating that at least this difference was due to specific binding of antimalarial antibody. This method was also successfully applied to IgM and subclasses of IgG. It is important therefore to routinely ascertain whether the higher titration curve observed in an immune serum is due to higher levels of specific antimalarial antibody or to greater sticking of Ig in a hypergammaglobulinemic serum.

We have endeavored to remove contaminating RBC stroma from MAP by anion exchange chromatography (10). To demonstrate the immunological purity of MAP, we compared the binding of RaMRBC antibody to MAP and RBC stroma antigen. The results of this experiment indicated that although more antibody bound to RBC stroma, MAP-coated wells bound a substantial amount of RaMRBC antibody (Table 1). It thus appeared that our efforts to remove RBC stroma from MAP had not been totally effective. This finding also raised the question of whether some of the observed antibody binding to MAP in malarial immune serum was due to anti-RBC antibody rather than antimalarial antibody. To test the specificity of the anti-MAP response, microtiter plates were coated with MAP or RBC stroma antigen and tested with malarial immune serum. It was discovered that IgG antibodies (IgM antibodies also bound, data not shown) from immune serum bound to MAP and RBC stroma (Table 2). Although it is

TABLE 1

Comparison of the Binding of RaMRBC Antibody to MAP and RBC Stroma Antigen

Serum	Relative IgG Titer (Reciprocal) MAP	RBC Stroma	BSA
RaMRBC	50	185	<10
Normal Rabbit	<10	<10	<10

RaMRBC and normal rabbit sera were titered at 0.9 absorbance units.

TABLE 2

Comparison of the Binding of Antibody from malarious Serum to MAP or RBC Stroma Antigen

Serum	Relative IgG Titer (Reciprocal) MAP	RBC Stroma	BSA
Immune	65	27	<10
Nonimmune	<10	<10	<10

Immune and nonimmune sera were titered at 0.7 absorbance units.

difficult to assess quantitative relationships, it appeared that about half as many antibodies bound to the stroma. These findings suggest that malarial immune serum contains antibodies with specificities for both malarial antigens and RBC stroma antigens, whereas nonimmune serum contains very little antibody to either of these antigens. As mentioned above, malaria is a polyclonal activator capable of inducing the proliferation of B cells bearing receptors for a multitude of antigenic specificities (6,9). Malarial infections have been demonstrated to induce the production of antibodies to determinants on RBC which are exposed by bromelain treatment (15), similar to the way bacterial lipopolysaccharide, a very potent B cell mitogen, stimulates the production of anti-RBC antibodies (8). In addition, our previous studies (11) and the studies of Lustig et al. (12) have documented the binding of Ig to noninfected RBC during the course of murine malarial

infections. It seems probable that the antibody in immune serum which binds to RBC is indeed RBC autoantibody. Therefore, a component of the observed antibody binding to MAP may represent RBC autoantibodies reacting with RBC stroma contaminants.

The correlation between depressed antibody binding to MAP and increased P. yoelli parasitemia levels and mortality in CBA/N mice with an X-linked B lymphocyte defect (10) argues that this assay reflects protective immunity. Moreover, we have recently shown that the IgM and IgG anti-MAP response is higher and more prolonged in genetically resistant AKR/J mice than in sensitive C57BL/6 mice (Hunter et al., unpublished observations). Antibodies bound to the surface of parasitized RBC, whether specific for malarial parasite determinants or RBC determinants, would still sensitize the cell for destruction and thus contribute to the protective host immune response.

The nonspecific binding of Ig and RBC autoantibody would also compromise specific antimalarial antibody determination using radioimmunoassay if the antigens employed are derived from intraerythrocytic parasites. It is also possible that other widely used serologic tests such as hemagglutination and immunofluorescence would be influenced by RBC autoantibody.

In conclusion, two problems have been defined which affect EIA for antimalarial antibody: hypergammaglobulinemia and RBC autoantibody. The first is easily solved by testing malarious sera at dilutions which contain Ig concentrations equivalent to control sera. The second is less easily dealt with and necessitates a more restricted interpretation of antimalarial antibody levels until a more purified antigen preparation is obtained.

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