BRIEF NOTE

Blockage of Immune Serum-Mediated Inhibition of Growth of *Plasmodium falciparum* by Absorption With *P. falciparum* Antigens[1]

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ABSTRACT. This report describes a microtiter system that should be useful for making preliminary determinations of which components of plasmodia may be useful as vaccines. The system is based on antigen-mediated blockage of inhibition of plasmodial growth in culture by immune serum. The necessity for a quantitative approach to the analysis is emphasized if reproducible results are to be obtained.

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INTRODUCTION

An important avenue of research for the development of a vaccine to combat malaria is identification of the antigens to which the host must respond to become immune. This must finally be done by immunizing and challenging susceptible primates and human volunteers with the candidate vaccines, but an *in vitro* test of immunogenicity would be a valuable adjunct to any vaccine development program.

Immune serum inhibits the growth of *Plasmodium falciparum in vitro* (Green et al. 1981, Jensen et al. 1982, Brown et al. 1983). Inhibition has been considered to be by antibody-mediated mechanisms (Cohen et al. 1969, Khusmith et al. 1982), and also by nonantibody-mediated mechanisms (Clark et al. 1976, Ockenhouse and Shear 1984). The nonantibody-mediated mechanisms appear to be a consequence of macrophage activation (Clark et al. 1981) and thus are not antigen-specific and do not have an anamnestic component.

The nonantibody-mediated mechanisms of culture inhibition are irrelevant to the present study except to the degree that if they were operative in the immune serum used, they would interfere with the demonstration of antigen blockage of antibody-mediated growth inhibition. This note is a report on preliminary research in this area.

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MATERIALS AND METHODS

IMMUNE SERUM. A pool of immune serum was obtained from owl monkeys (*Aotus trivigatus*) that were inoculated intraperitoneally repeatedly with *P. falciparum* that had been carried continuously in culture according to published procedures (Trager and Jensen 1976). The parasitized human erythrocytes that were used for immunization were washed several times in physiological saline before being injected into the monkeys. The monkeys had parasitemias following the first two or three injections but recovered after chloroquin sulfate treatment. Subsequent inoculations did not result in parasitemias. Serum was collected from the monkeys after they had become immune, well after the chloroquin treatments were completed. The serum was pooled, heat-inactivated, and adsorbed repeatedly with human erythrocytes before being used.

TEST SYSTEM. A microtiter system was developed to test the inhibitory properties of the immune serum. The parasite cultures were propagated in 60-mm plastic Petri plates using RPMI 1640 with 10% human serum and type O human erythrocytes at a 5% hematocrit. When the cultures attained about 4 to 6% infected erythrocytes, the infection level was adjusted to approximately 1% with fresh erythrocytes, and the cultures were allowed to develop overnight in a candle jar at 37° C. The cells were then suspended in fresh medium, and 0.10 mL was seeded into wells of a 96-well, flat bottom, microtiter plate. Thin films were prepared immediately from several wells to determine the parasitemia at T_O; 0.1 mL of the serum to be tested was added to the appropriate wells. The plates were incubated in a candle jar for 36 h. The test was terminated, and films were prepared from each well, fixed in methanol, and stained with Giemsa.

ANTIGEN PREPARATION. The *Plasmodium falciparum* antigen used to block antibody-mediated inhibition of plasmodial growth was prepared from a pool of red cells harvested from cultures that, when combined, had a parasitemia of 6.1%. The cells were washed once in RPMI 1640, and the pellet was suspended in four times its volume of RPMI. The suspension of infected erythrocytes was dispensed into 1 mL glass vials and immersed directly into liquid nitrogen. A preparation of normal, uninfected, human erythrocytes was treated in the

same manner and frozen. Just before use, the antigens were disrupted by freeze-thawing.

PROCEDURE FOR ABSORPTION OF IMMUNE SERUM. The absorption of the sera was accomplished by adding a volume of antigen preparation, either lysed, infected erythrocytes (IRBC) or lysed, normal erythrocytes (NRBC), to an equal volume of the serum to be tested and incubating the mixture overnight at 4°C. The sera were then centrifuged to remove any particulate matter, and 0.1 mL of the supernatant fluid was added to the microtiter wells. After 36 h of incubation, thin smears were prepared and the percentages of parasitized erythrocytes were determined by examining at least 500 erythrocytes in thin films made from each of the wells. In addition to the wells containing immune monkey serum (IMS) absorbed with IRBC and NRBC antigen, wells containing normal human serum (NHS) and no supplemental serum served as parasite growth controls.

DATA ANALYSIS. Standard independent two-sample tests for differences between means and variances were used to determine whether the two treatments, (i.e., absorption with normal erythrocyte antigen or infected erythrocyte antigen) produced significant differences (Remmington and Schork 1985).

RESULTS AND DISCUSSION

In preliminary experiments, we determined that the variation in parasite counts between replicate wells in treatment groups was greater if medium was changed than if no medium change was made. We also determined that the cultures grew for 36 h if no medium change was made, thus eliminating the necessity of replacing the growth medium and reducing variation and culture manipulation (data not shown).

The pool of immune monkey serum was shown to inhibit parasite growth in the microtiter system (Table 1, Trial 1). Wells containing non-immune monkey serum and human serum served as controls. The immune monkey serum was strongly inhibitory, as there was no increase in percent infected erythrocytes in the wells containing 0.1 mL of the immune monkey serum.

The results obtained in the first absorption experiment were encouraging (Table 1, Trial 2). The wells that received 0.1 mL of IMS absorbed with IRBC antigen had slightly higher reaction levels; that is, less inhibition than those which received 0.1 mL IMS absorbed with NRBC antigens. The results, although not statistically significant, encouraged us to persist. We theorized that while some of the inhibitory antibodies were removed from the immune serum by the absorption with IRBC antigen, perhaps there was insufficient antigen to remove them all. We next titrated our antibody to determine the minimum inhibitory dose, reasoning that if this amount of antibody was absorbed with the plasmoidal antigen a significant effect should be seen.

Dilutions of the immune monkey serum from 1:4 to 1:256 were prepared, absorbed with either NRBC lysate or IRBC lysate, and 0.1 mL was added to wells in a microtiter plate (Table 1, Trial 3). The difference in percent infected erythrocytes at 36 h between the wells containing IMS absorbed with IRBC lysate and those containing IMS absorbed with NRBC lysate increased with increasing dilution through 1/64 and then decreased at 1/256 at which point there was no inhibition to block. The absorption study was then repeated twice with serum diluted 1:64 (Table 1, Trials 4 and 5). One-tenth mL of a 1:64 dilution of immune monkey serum absorbed with IRBC antigen was significantly ($P < 0.01$) less inhibitory than an equivalent amount of immune monkey serum absorbed with NRBC antigen.

In conclusion, once the immune serum was diluted to the minimum inhibitory level, the effects of antigen absorption became evident. An absorption test such as this is like a complement fixation test and will give meaningful results only if all the reactants are properly titrated before use. We have thus demonstrated that, if properly run, a microtiter test can be used to evaluate antigens for their ability to block immune serum-mediated inhibition of *P. falciparum* growth in culture. This assay may possibly provide an *in vitro* correlate for vaccine evaluation and will provide another tool for study of the antigenic makeup of *P. falciparum.*

TABLE 1.
Effects of dilution and absorption of immune serum with antigens on its ability to inhibit the growth of P. falciparum in culture.

Treatment	Trial 1*	Trial 2	Trial 3	Trial 4	Trial 5
T_0	1.8 ± 0.18(3)**	1.0 ± 0.16(3)	1.1 ± 0.04(3)	1.2 (1)	1.2 ± 0.06(3)
No serum added	4.0 ± 0.45(3)	3.3 ± 0.07(3)	3.2 ± 0.50(3)	2.8 ± 0.05(3)	4.2 ± 0.05(3)
Normal Human Serum (NHS)	6.2 ± 0.28(3)	31. ± 0.07(3)	2.9 ± 0.14(3)	5.2 ± 0.73(3)	4.1 ± 0.22(3)
NHS absorbed with lysed NRBC		4.2 ± 0.20(3)			
NHS absorbed with lysed IRBC		3.3 ± 0.20(3)			
Normal Monkey Serum (NMS)	3.1 ± 0.08(2)				
Immune Monkey Serum (IMS)	1.6 ± 0.31(9)		0.20 ± 0.01(3)	0.21 ± .0.01(3)	
IMS absorbed with lysed NRBC		0.76 ± 0.27(9)			
IMS absorbed with lysed IRBC		1.1 ± 0.14(9)			
1/4 IMS absorbed with lysed NRBC			0.70 ± 0.08(3)		
1/16 IMS absorbed with lysed NRBC			1.3 ± 0.23(3)		
1/64 IMS absorbed with lysed NRBC			2.2 ± 0.51(3)	2.0 ± 0.43(10)***	2.2 ± 0.24(10)***
1/256 IMS absorbed with lysed NRBC			3.1 ± 0.15(3)		
1/4 IMS absorbed with lysed IRBC			0.9 ± 0.23(3)		
1/16 IMS absorbed with lysed IRBC			1.8 ± 0.56(3)		
1/64 IMS absorbed with lysed IRBC			2.9 ± 0.27(3)	3.3 ± 0.28(10)***	3.7 ± 0.28(10)***
1/256 IMS absorbed with lysed IRBC			3.3 ± 0.01(3)		

*See text for explanation of trials.
**$\overline{X}$ ± SD of % infected erythrocytes in wells (numbers in parentheses) at T_0 or after 36-h incubation.
***There is significantly ($P < 0.01$) less inhibition after absorption with lysed IRBCs than after absorption with lysed NRBCs in Trials 4 and 5.

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