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Immunology

Anti-gamete monoclonal antibodies synergistically block transmission of malaria by preventing fertilization in the mosquito

(malaria gamete antigens/hybridoma/agglutination)

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ABSTRACT **Experiments from our laboratory previously demonstrated that infected chickens immunized with gametes of the avian malaria parasite *Plasmodium gallinaceum* were no longer infectious to the mosquito vector *Aedes aegypti* and that this transmission-blocking immunity was mediated by antibody. To identify those antigens that are the targets of transmission-blocking immunity, hybrid mouse cell lines secreting monospecific antibodies to surface antigens on male and female gametes of *P. gallinaceum* have been produced. We describe two such anti-gamete antibodies, 10G3 and 11C7, which act synergistically both to agglutinate male gametes *in vitro* and to suppress infectivity of parasitized blood fed to mosquitoes. In the presence of a mixture of these antibodies, the male gametes became agglutinated during gametogenesis and failed to detach from the residual body of the gametocyte. In the presence of either antibody alone, the male gametes readily detached during gametogenesis. Neither antibody alone mediated more than a slight reduction in infectivity of gametocytes to mosquitoes. Although both 10G3 and 11C7 recognize surface antigens on both male and female gametes, agglutination and prevention of detachment of the male gametes from the residual body appears to be the primary mechanism by which the mixture of these antibodies prevents fertilization.**

Malaria persists in the tropical world because current methods of control, including the use of anti-malarial drugs, larvicides, and insecticides, are inadequate to eliminate the parasite or prevent its transmission to mosquitoes. A promising approach for the future is the development of vaccines against the three susceptible stages in the life cycle: sporozoites, asexual parasites, and gametes (1). Two of these stages occur in the vertebrate host: sporozoites, which transfer the disease from mosquito to host, and erythrocyte asexual parasites, which cause the clinical disease. Gametes, the third stage, are the sexual form of the parasite and are present only in the midgut of the mosquito. They develop from sexual erythrocytic forms, gametocytes, which are ingested by the mosquito during a blood meal. Immediately after gamete formation in the midgut, fertilization occurs. The zygote invades the gut wall and develops as an oocyst. Fourteen days later sporozoites are released from the oocysts and migrate to the salivary glands. The cycle of infection is completed when the sporozoites are injected into a new host during the next blood meal. Although vaccines against asexual erythrocytic parasites would surely reduce morbidity in the vaccinated individual, transmission of malaria in the community would not necessarily be prevented. Our interest has been in vaccines against male and female gametes, which would block gamete fertilization in the mosquito, thereby preventing further transmission of malaria to new hosts.

Working with *Plasmodium gallinaceum*, a malaria parasite of chickens transmitted in the laboratory by *Aedes aegypti* mosquitoes, we previously demonstrated that a parasitized animal immunized with gametes is no longer infectious to mosquitoes. This transmission-blocking immunity is mediated by anti-gamete antibodies, which prevent fertilization (2, 3). To isolate and characterize the antigens that are the targets of transmission-blocking immunity, we have produced hybrid cell lines secreting antibodies against surface antigens on male and female gametes. In the present report, we describe two anti-gamete antibodies, 10G3 and 11C7, which act synergistically to agglutinate male gametes and suppress transmission to mosquitoes.

MATERIALS AND METHODS

Production of Hybridoma Antibodies. Hybrid cells secreting antibody against antigens on male and female gametes of *P. gallinaceum* were produced by the methods of Köhler and Milstein (4). The hybrid cell lines used in this study were derived from the fusion of mouse plasmacytoma cell line P3-X63-Ag8 (4) with spleen cells from BALB/c mice that had been immunized by an intraperitoneal inoculum of 1×10^7 male and female gametes with Freund's complete adjuvant, followed 10 days later by an intravenous booster of 1×10^7 gametes. Four days later the cells were fused according to the method of Galfre *et al.* (5). The hybrid cells were grown in HY-HAT medium (6) in 2-ml-well Costar cluster plates and screened 14–18 days after fusion. Hybridoma cultures producing anti-gamete antibodies, as shown by indirect immunofluorescence, were cloned by limit dilution in the presence of feeder cells and subsequently grown as intraperitoneal ascites tumors in pristane-primed BALB/c mice. Antibodies derived from ascites fluids were precipitated with 40% saturated ammonium sulfate and dialyzed against glucose-containing Tris-buffered saline (SA solution) at pH 8.0 (10 mM Tris·HCl/150 mM NaCl/100 mM glucose). This buffer, when mixed with chicken serum, maintains gametocyte infectivity to mosquitoes.

Indirect Immunofluorescence Assays (IFA). Immunofluorescence assays were performed with air-dried male and female gametes, or with live female gametes. Live male gametes were not used for IFA because of their fragility. For this reason, hybridoma antibodies specific for surface determinants on male gametes were assayed by agglutination (see below). Male and female gametes of *P. gallinaceum* were prepared by the methods of Carter *et al.* (7) and suspended in SA solution at pH 7.4 plus 10% fetal bovine serum. All of the following procedures were carried out at room temperature. Gametes were applied to eight-well toxoplasmosis slides (Bellco Glass) and air-dried overnight. The slides were stored at −70°C until use. After hydration in phosphate-buffered saline, the antigens were allowed to react with culture supernatants or ascites fluids diluted

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Abbreviation: IFA, immunofluorescence assay.

1:4 with phosphate-buffered saline, followed by fluorescein-conjugated goat anti-mouse κ chain reagent (0.5 mg of protein per ml; 3.4 mol of fluorescein per mol of protein), a kind gift of R. Asofsky, diluted 1:40 with phosphate-buffered saline.

IFA with live female gametes was performed as follows. A suspension of gametes in SA solution plus 10% fetal bovine serum was mixed with equal amounts of culture supernatants or diluted ascites fluids and incubated for 5 min. The samples were spun in an Immufuge (Dade, Miami, FL) at 1000 × *g* for 1 min. The pellets were washed in phosphate-buffered saline and then resuspended in fluorescein-conjugated anti-mouse antibody diluted 1:20 in phosphate-buffered saline for 5 min. After two washes, the gametes were resuspended in 10 μl of phosphate-buffered saline and observed as wet mounts.

Classes and subclasses of antibodies bound to live female gametes were determined by their staining properties with fluorescein-conjugated anti-mouse IgG_1, IgG_2, IgM, or IgA reagents (gift of R. Asofsky).

Agglutination of Male Gametes. Precipitated and dialyzed ascites proteins, individually or in combinations, were diluted with SA solution, pH 8.0, to final protein concentrations of 6 mg/ml. Serial dilutions were made to 1:1000. Male gametes were suspended in SA solution plus 10% fetal bovine serum to 10^8 per ml. Equal volumes of protein solutions and the gamete suspensions were mixed and incubated for 10 min and observed with a phase-contrast microscope.

Gametogenesis. The effect of the antibodies on gametocytes undergoing gametogenesis was observed by phase-contrast microscopy. A suspension of *P. gallinaceum*-infected blood was stimulated to undergo gametogenesis by mixing 10 μl of the blood suspension at a 20% hematocrit in SA solution, pH 7.4, with 20 μl of SA solution supplemented with 25 mM $NaHCO_3$ and 10% fetal bovine serum at pH 8.4; 25 μl of ascites protein at 6 mg/ml in pH 8.0 SA solution was immediately added to the activated suspension; 25 μl of pH 7.4 SA solution was added to controls.

Determination of Infectivity. Chicken blood at a parasitemia of 10–15% was drawn in a heparinized syringe and washed immediately with 10 vol of pH 7.4 SA solution. The cells were resuspended to 5 times their original volume in SA solution, pH 7.4, and 1-ml aliquots were spun for 2 min in an Immufuge at 1000 × *g*. The pellets were resuspended in 200 μl of a solution that consisted of 150 μl of heat-inactivated normal chicken serum and 50 μl of hybridoma antibodies at a concentration of 6.0 mg/ml in pH 8.0 SA solution. Control samples were prepared by substituting SA solution for hybridoma fluids. Each suspension was supplemented with 0.15 mM ADP as a phagostimulant for mosquitoes and fed to *A. aegypti* mosquitoes through Swiss nylon monofilament screening, pore size 183 μm (Tetko, Elmsford, NY) warmed to 40°C by a glass water jacket. Fed mosquitoes were dissected 7 days later and the numbers of oocysts (products of fertilization) on their midguts were determined as a measure of infectivity. Impaired ability of gametes to undergo fertilization was reflected by reduced numbers of oocysts.

RESULTS

Of the hybridoma cell lines produced, two, 10G3 and 11C7, were chosen for detailed study. 10G3 and 11C7 are IgM and IgG_1, respectively, and bind to surface antigens of male and female gametes. We describe here some special properties of these antibodies relating to their ability to cause male gamete agglutination and to block transmission of malaria to mosquitoes.

Agglutination of Male Gametes. In the presence of either SA solution plus 10% fetal bovine serum or of hybridoma antibodies that did not bind to male gametes as determined by IFA, gametes swam freely and did not attach to each other (Fig. 1*a*). In the presence of 10G3 (Fig. 1 *b* and *c*) or 11C7 (not shown), the gametes agglutinated into stellate clusters of actively motile cells. Individual gametes attached to each other at a single point (Fig. 1*b*), although multiple clusters were not uncommon (Fig. 1*c*). Stellate agglutination was observed with 10G3 at protein concentrations ranging from 3 to 0.02 mg/ml and with 11C7 from 3 to 1.5 mg/ml. When 10G3 and 11C7 were mixed in equal amounts and added to male gametes, a different form of gamete agglutination was observed (Fig. 1*d*). In this reaction, gametes adhered to one another along their entire lengths and formed long, ropelike bundles, three or four gametes thick. Such ropelike agglutination was observed with mixtures of equal amounts of 10G3 and 11C7 with final protein concentrations ranging from 3 to 0.02 mg/ml. At no concentrations did 10G3 or 11C7 alone cause the ropelike agglutination characteristic of the mixture. It appears, therefore, that two distinct specificities against male gametes are represented by antibodies 10G3 and 11C7 which, when mixed, act synergistically to produce a unique form of agglutination.

The action of 10G3 and 11C7 on gametocytes of *P. gallinaceum* undergoing gametogenesis *in vitro* was observed by phase-contrast microscopy. In the presence of either antibody alone male gametes developed and readily escaped into the medium as they did in controls to which no antibody was added. In the presence of a mixture of 10G3 and 11C7, however, the male gametes formed from each gametocyte became agglutinated in a ropelike bundle and failed to detach from the residual body of the gametocyte.

Prevention of Infectivity. Antibodies from 10G3 and 11C7 were tested for their effects on the infectivity of gametocytes to mosquitoes by the membrane feeding assay. Antibodies from either 10G3 or 11C7 caused a slight and inconsistent suppression of infectivity as judged by a reduction of oocysts (Table 1). However, when the antibodies were fed to mosquitoes in equal

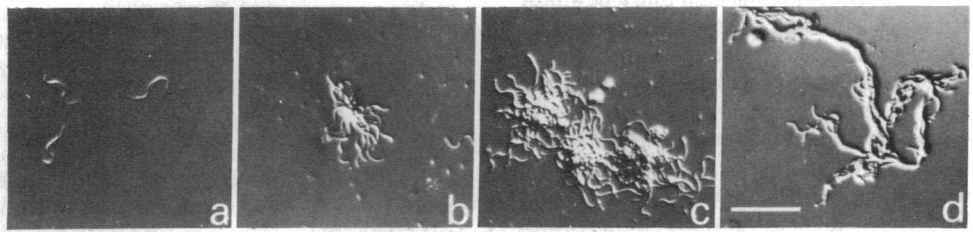

FIG. 1. Agglutination reactions of male gametes of *P. gallinaceum* in the presence of anti-gamete antibodies 10G3 and 11C7. Hybridoma antibodies 10G3 and 11C7 were added separately or mixed in equal amounts to preparations of male gametes. (*a*) Male gametes in the presence of SA buffer plus 10% fetal bovine serum were free swimming. (*b*, *c*) Male gametes in the presence of 10G3 agglutinated into stellate clusters of motile cells. (*d*) Male gametes in the presence of 10G3 plus 11C7 agglutinated into long ropelike bundles. The preparations were fixed with 1% glutaraldehyde and photographed with an inverted microscope (Leitz Diavert) equipped with Smith differential interference optics. Bar, 20 μm.

Table 1. Infectivity of gametocytes of *P. gallinaceum* to *A. aegypti* mosquitoes in the presence of hybridoma antibodies (10G3 and 11C7) specific for surface determinants on male and female gametes

Exp.	Infectivity, % of control		
	10G3	11C7	10G3 and 11C7
1	33 ± 16	109 ± 40	7 ± 4
2	97 ± 14	78 ± 15	21 ± 5
3	127 ± 42	41 ± 19	14 ± 4

Three replicate experiments were performed. In each series, a minimum of 10 and a maximum of 50 mosquitoes were dissected for each mixture of fluids fed. The mean numbers of oocysts per gut in control feedings in which SA buffer was substituted for hybridoma antibodies were 19, 41, and 87 in experiments 1, 2, and 3, respectively. Infectivity in the experimental feedings containing the hybridoma antibodies is expressed as a percentage of the values of the control feedings and is given as a mean ± 1 SEM. In all three experiments synergism between 10G3 and 11C7 was estimated to account for a reduction in infectivity of 65–70% out of a total reduction of 79–93%. The probabilities by a one-tailed t test that the observed reduction in infectivity by the mixture was due only to additive and not synergistic effects of 10G3 and 11C7 were <0.025, <0.01, and <0.05 for experiments 1, 2, and 3, respectively.

amounts in combination, there was a consistent reduction of gametocyte infectivity to mosquitoes by 79–93% below control levels. Statistical analysis confirmed that this suppression could be attributed mainly to synergistic action by the two hybridoma antibodies.

Antibody from hybridoma line 4D5, which did not react with surface antigens on gametes, had no effect on the infectivity of gametocytes to mosquitoes either by itself or in combination with 10G3 or 11C7 (data not shown).

DISCUSSION

As described earlier, ingestion of gametocyte-containing blood by a mosquito results in gamete formation, followed by fertilization and formation of oocysts. Immunization of the host with gametes prior to infection has been shown to prevent transmission of malaria to mosquitoes in avian, rodent, and primate models (2, 8–10). The resultant immunity does not affect the gametocytes while they remain within erythrocytes in the circulation of the host. However, after their formation in the mosquito midgut, extracellular gametes rapidly become neutralized by interaction with anti-gamete antibodies ingested with the blood meal.

The present results from experiments with hybridoma antibodies show that a mixture of two monospecific antibodies against surface determinants shared on male and female gametes inhibit fertilization. When gametocytes were fed to mosquitoes in the presence of these antibodies, the number of oocysts subsequently formed was reduced by 79–93% below control levels. Either of these monospecific antibodies when administered alone mediated only slight, inconsistent, reduction in oocyst numbers.

Because these hybridoma antibodies reacted with determinants on both male and female gametes, it is possible that the antibodies could have reduced fertilization by binding to gametes of either or both sexes. However, the association of their unique ropelike agglutination of male gametes with their synergistic inhibition of transmission suggests that the targets of these transmission-blocking antibodies were the male gametes. Observation of gametocytes undergoing gametogenesis in the presence of a mixture of these antibodies revealed that the male gametes became agglutinated in ropelike bundles before they were able to detach from the residual body of the gametocyte. In the presence of either antibody alone gamete agglutination did not occur, and many male gametes detached from the residual bodies to fertilize female gametes. Agglutination of the male gametes before they can detach from the residual body is clearly a severe impediment to fertilization and is probably the main mechanism whereby the mixed antibodies reduced gametocyte infectivity to mosquitoes. However, male gamete agglutination must occur within a few seconds if fertilization is to be prevented. The high concentrations of antibodies required in our studies may have been necessary to achieve sufficiently rapid agglutination.

The synergistic action of 10G3 and 11C7 in the ropelike agglutination of male gametes may be due to crosslinking by each monoclonal antibody of a different component on the gamete surface or crosslinking of different determinants on the same component. Because cooperation by two monoclonal antibodies is required for effective gamete agglutination, it is possible that the most effective agglutination requires the action of yet further antibody specificities. The failure of 10G3 and 11C7 to completely prevent infectivity to mosquitoes may be due to the requirement for polyspecific antibodies as in immune sera. Alternatively, effective suppression of infectivity may require the action of antibodies in addition to those involved in gamete agglutination. Such antibodies might be necessary to block receptors for fertilization on male and female gametes.

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