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Identification of Antibody Classes and Fc Receptors Responsible for Phagocytosis of *Trypanosoma musculi* by Mouse Macrophages

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The phagocytosis of *Trypanosoma musculi* by macrophages in the presence of specific antibodies was investigated. In 14-day-infected mice, opsonic antibodies were detected in serum, and phagocytosis of parasites by peritoneal macrophages was observed. The mechanism of *T. musculi* phagocytosis was analyzed. The binding of trypanosomes to peritoneal macrophages and J774 cells was observed in the presence of serum from hyperimmune mice and from mice infected 14 or 28 days earlier, but not in the presence of control mouse serum or sera from 7-day-infected mice. Binding was partially inhibited by mouse monoclonal immunoglobulins G1 (IgG1) or IgG2a and almost completely inhibited by a mixture of both. Binding was also partially inhibited by the anti-Fcγ1/γ2b receptor monoclonal antibody 2.4G2. Binding of *T. musculi* was also induced by fractions of serum from 28-day-infected mice obtained by protein A-Sepharose chromatography. Only the IgG1-rich fraction eluted at pH 6.0 and the IgG2a-rich fraction eluted at pH 4.5 promoted binding which could be almost completely inhibited by monoclonal IgG1 and IgG2a. These data indicate that (i) IgG1 and IgG2a anti-*T. musculi* antibodies are responsible for the phagocytosis of *T. musculi* by mouse macrophages and (ii) both Fcγ2a and Fcγ1/γ2b receptors are involved. Such a mechanism is likely to account for the elimination of parasites in *T. musculi*-infected mice.

Trypanosoma musculi, a nonpathogenic protozoan hemoflagellate, produces a self-limiting infection in mice which lasts about 20 to 25 days (25). A long lasting immunity to reinfection develops (26). This trypanosomiasis is therefore a useful model for studying host immune mechanisms leading to parasite killing. Wechsler and Kongshavn have recently shown that immune plasma contains curative activity which is presumably mediated by an immunoglobulin (28). Vargas et al. have demonstrated that, in B-cell-deficient mice, the initial control of parasitemia is relatively unaffected by this deficiency, but that parasites cannot be eliminated from the blood (23). These data support the hypothesis that antibodies participate in the elimination of parasites. Nevertheless, the immunoeffector mechanisms involved remain unclear.

Among other effector systems (1, 11, 24), mononuclear phagocytes seem to play an important role in the elimination of trypanosomes from blood (7, 8, 15). As shown by Taliaferro and Pavlinova (20) and by Brooks and Reed (2), the early control of *T. musculi* parasitemia may be due, at least in part, to macrophages. In a previous work, we reported changes in macrophage numbers and functions during *T. musculi* infection in mice (27). In the present study, we investigate the role of antitrypanosome antibodies in mediating phagocytosis and killing of *T. musculi* by macrophages. We analyze the mechanism of immunoadherence, and we demonstrate that only two immunoglobulin G (IgG) antibody subclasses, among other antibodies, induced parasite attachment and phagocytosis, involving two separate macrophage Fc receptors (FcR).

MATERIALS AND METHODS

Mice. Female 6-week-old Swiss mice were purchased from FEASL (St Denis de Pile, France) and used for 3 months.

Parasites. The Partinico II strain of *T. musculi* used in this investigation was originally obtained from the London School of Hygiene and Tropical Medicine (12). Mice were infected intraperitoneally by injecting normal recipients with 5×10^4 *T. musculi* freshly isolated from the blood of mice infected 9 days previously. In some experiments, trypanosomes were collected from mice that had been X-irradiated (450 R) 10 days previously.

Peritoneal *T. musculi* could be collected and counted after extensive washings of the peritoneal cavity.

Macrophages. The peritoneal cavity of each mouse was washed with 5 ml of RPMI 1640 (Eurobio, Paris, France) containing 10 U of heparin per ml, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. The cells were collected in siliconized centrifuge tubes. After centrifugation and cell counting, 2×10^5 macrophages in 0.2 ml of the same medium were allowed to adhere to each chamber of an eight-chamber Lab-Tek slide (Lab-Tek 4808; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). After vigorous washings, adherent cells were cultured in RPMI 1640 containing 100 U of penicillin per ml, 100 μg of streptomycin per ml, 25 mM HEPES buffer, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (GIBCO Europe, Paisley, Scotland).

J774, a murine macrophage cell line maintained in the same culture medium, was used (17). Cultured J774 cells provide a convenient source of large numbers of pure, homogeneous macrophages devoid of serum immunoglobulins and having the same phagocytic properties and Fc R as peritoneal macrophages (17, 22).

Sera. Normal mouse serum and sera from mice infected 8, 14, or 28 days earlier were collected. Hyperimmune mice were injected intraperitoneally with 5×10^4 parasites 3 times at monthly intervals, and sera were collected 4 days after the last injection and kept at −80°C before use.

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Sera were assayed for antitrypanosomal antibodies by using indirect-fluorescent-antibody titration, as described by Viens et al. (26), with fluorescein-labeled goat anti-mouse immunoglobulins (Nordic Laboratories, Tilburg, The Netherlands).

Fractionation of mouse serum on protein A-Sepharose. Serum from mice infected 28 days previously was fractionated by affinity chromatography by using staphylococcal protein A convalently linked to Sepharose 4B (Pharmacia, Uppsala, Sweden), by the technique described by Ey et al. (5).

After elution with buffers of decreasing pH, the fractions were neutralized, dialyzed, and concentrated to 5 mg/ml. The immunoglobulin content of each fraction was analyzed by double immunodiffusion by using antisera to mouse immunoglobulins (Meloy Laboratories, Springfield, Va.).

Adherence and phagocytosis of *T. musculi*. The assay was carried out in Lab-Tek chambers (Miles Laboratories, Inc.) by the methods described by Takayanagi et al. (19) and Greenblatt et al. (10) with either peritoneal macrophages or J774 cells. Adherent cells were washed, and 0.2 ml of *T. musculi*, which was previously purified by DEAE cellulose chromatography (14) and adjusted to 8×10^6/ml in RPMI, was introduced into each chamber. Normal serum, immune serum, or immunoglobulin fraction was then added. Cells were incubated for 30 min at 37°C in a 5% CO_2 atmosphere. The Lab-Tek chambers were observed by using an inverted microscope. A cell with five or more attached trypanosomes was scored as positive for adherence. Glass slides were then stained by using May Grünwald Giemsa stains.

Inhibition of adherence. The murine cells that were adherent in Lab-Tek chambers were washed. They were incubated at 4°C for 1 h with 150 μl of medium containing the following monoclonal immunoglobulins (with trade names in parentheses): from Bionetics, Charleston, S.C., IgG1 (MOPC 21), IgG2a (UPC 10), IgG2b (MOPC 195), IgG3 (FLOPC 21), IgA (MOPC 315), and IgM (MOPC 104E); from Immunotech, Marseille, France, IgG1 (ALB 1), IgG2a (A 50), and IgG2b (B 9.4). A trypanosome suspension (50 μl; 3.2×10^7/ml) was then added, together with immune serum or immunoglobulin fraction without washing. After 30 min of incubation at 37°C, the percentage of macrophages with attached parasites was determined by using an inverted microscope.

Inhibition experiments were also performed by treating macrophages with 2.4G2, a monoclonal antibody directed against mouse Fcγ1/γ2b receptor (22). The cells were preincubated with dilutions of 2.4G2 ascitic fluid at 4°C for 1 h. After being washed, the macrophages were assessed for the binding of trypanosomes in the presence of immune serum.

RESULTS

Time course of *T. musculi* infection and antibody response. Experimental mice were infected with 5×10^4 *T. musculi* intraperitoneally. Parasites developed in the peritoneum during the first 2 weeks, and, by day 13 of infection, an average of 7×10^6 trypanosomes were enumerated in the peritoneal cavity. Within the following 2 to 4 days, the number of parasites declined rapidly, and phagocytosis of *T. musculi* by peritoneal macrophages could occasionally be seen. This spontaneous phenomenon could be mimicked by injecting mice, infected 13 days earlier, intraperitoneally with anti-*T. musculi* hyperimmune serum. A dramatic decrease in the number of peritoneal parasites was observed as early as 2 h after the injection, and phagocytosis of *T. musculi* by macrophages was observed at different stages from adherence to complete ingestion of parasites; by contrast, mice injected with phosphate-buffered saline or normal mouse serum contained similar numbers of parasites to those in untreated mice.

These observations suggested that the spontaneous clearance of parasites after week 2 of infection with *T. musculi* might have been caused by in vivo antibody-mediated phagocytosis, which was made possible by the development of a specific humoral immunoresponse. Anti-*T. musculi* antibody production in infected mice was therefore examined. The sera of infected mice were collected at days 7, 14, and 28 of infection, and the titers of anti-trypanosome antibodies were determined by indirect-immunofluorescence assay. The sera of mice infected 7 and 14 days earlier had average titers of 1/20 and 1/40, respectively. For mice infected 28 days earlier and hyperimmune mice, the titers were 1/250 and 1/1,250, respectively.

Antibody-dependent adherence and phagocytosis of *T. musculi* by macrophages in vitro. To examine the possibility that antibodies appearing in the serum of infected mice could mediate parasite elimination, the ability of antibodies in the serum of infected mice to induce adherence and phagocytosis was investigated in vitro by using normal mouse macrophages or J774 cells. Sera from mice infected 14 or 28 days earlier and from hyperimmune mice all mediated adherence and phagocytosis of *T. musculi* by macrophages (Fig. 1) and by J774 cells (data not shown). Neither adherence nor phagocytosis was induced by serum from 7-day-infected mice. The titers were 1/40 for 14-day serum, 1/120 for 28-day serum, and 1/360 for hyperimmune-mouse serum.

The mechanism by which parasites were phagocytized by mouse macrophages in the presence of immune serum was investigated by separately incubating target cells and effector cells with immune serum. Mouse anti-*T. musculi* serum was added to the macrophages, and incubation was performed at 37°C for 40 min. The macrophages were carefully washed, and the trypanosomes were added; after another 40-min incubation at 37°C, the binding of parasites to macrophages was examined. Very few trypanosomes bound to 5% of macrophages only. The same was seen with normal serum instead of immune serum. When trypanosomes were first incubated with anti-*T. musculi* serum for 40 min, washed, and added to macrophages for a 40-min incubation at 37°C, 96% of macrophages bound parasites. If trypanosomes were incubated with normal serum instead of immune serum, less than 5% of macrophages bound parasites.

Antibody classes and FcR mediating adherence and phagocytosis of *T. musculi*. To determine the class(es) of antibodies responsible for the adherence of phagocytosis of *T. musculi*, serum from 28-day-infected mice was fractionated by affinity chromatography on protein A-Sepharose. The pH 8.0 effluent and fractions, eluted at pH 6.0, 4.5, and 2.8, were neutralized, dialyzed, and examined for immunoglobulin content by immunodiffusion. The pH 8.0 fraction contained IgM and IgA, the pH 6.0 fraction contained IgG1 and IgG3, the pH 4.5 fraction contained IgG2a and IgG3, and the pH 2.8 fraction contained IgG2b. All fractions were adjusted to the same protein concentration (5 mg/ml) and were examined for anti-*T. musculi* antibodies by indirect-immunofluorescence assay. Their titers were 1/50 for the pH 8.0 fraction, 1/80 for the pH 6.0 fraction, 1/150 for the pH 4.5 fraction, and 1/60 for the pH 2.8 fraction. The fractions were assayed for adherence and phagocytosis with peritoneal macrophages

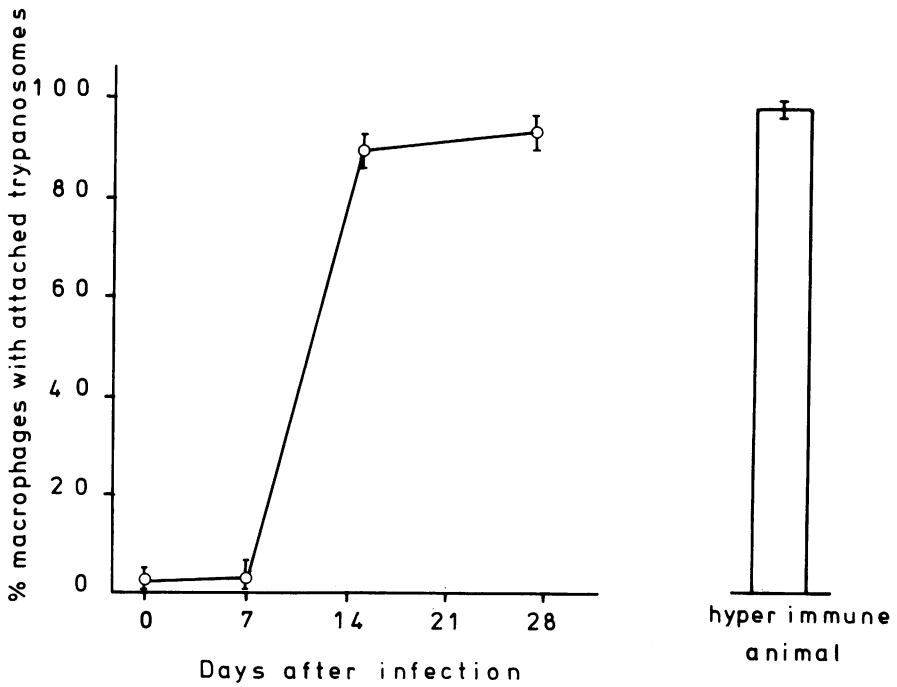

FIG. 1. Kinetics of antibody production mediating trypanosome binding. Trypanosomes were added to peritoneal macrophages. Normal sera, hyperimmune sera, or sera from 7-, 14-, 21-, and 28-day-infected mice were then added (final serum dilution, 1/20). After cells were incubated for 30 min at 37°C, the percentage with attached trypanosomes was evaluated for each serum. Each result represents the mean value and standard deviation of four experiments.

and J774 cells. Only the pH 6.0 and 4.5 fractions induced significant activity with either type of effector cell (Table 1).

Since several FcR, especially three separate FcγR, have been observed on mouse macrophages (4), we examined whether such FcR and which one(s) are involved in the adherence and phagocytosis of *T. musculi*. For this purpose, peritoneal macrophages (Fig. 2) or J774 cells (Fig. 3) were preincubated at 4°C for 1 h with various concentrations of purified monoclonal immunoglobulins of different classes. Trypanosomes and immune serum were then added without washing, and incubation was performed at 37°C for 30 min. IgA, IgM (data not shown), and IgG3, up to 2 mg/ml, failed to inhibit adherence to trypanosomes, but IgG1, IgG2a, and, to a lesser extent, IgG2b inhibited adherence (Fig. 2 and 3). High concentrations (1 mg/ml) of IgG were required, and with both effector cells, inhibition was not complete. However, when a mixture of IgG1 and IgG2a was used, adherence was completely inhibited, and as little as 0.25 mg of each immunoglobulin per ml in the mixture was more inhibitory than 1 mg of either IgG1 or IgG2a per ml. By contrast, a mixture of either IgG1 plus IgM or IgG1 plus IgG3 was no more inhibitory than IgG1 alone; a mixture of IgG2a plus IgG2b was more inhibitory than IgG2a alone, whereas a mixture of either IgG2a plus IgM or IgG2a plus IgG3 was no more inhibitory than IgG2a alone (data not shown). When inhibition experiments were performed with 2.4G2, the binding of trypanosomes was only partially inhibited (45 to 55% inhibition for ascitic fluid diluted 1/1,000). Altogether, these data strongly suggested that the two different FcγR, which are known to bind IgG1 and IgG2a, were involved in immune serum-induced adherence. To demonstrate this possibility, experiments of the same design were repeated on J774 cells, with the two active fractions of 28-day serum fractionated over protein A-Sepharose; i.e., the pH 6.0 fraction, containing IgG1 and IgG3, and the pH 4.5 fraction, containing IgG2a and IgG3. Monoclonal IgG1 completely inhibited adherence induced by the pH 6.0 fraction, but was totally ineffective in inhibiting the activity of the pH 4.5 fraction (Fig. 4). Conversely, monoclonal IgG2a completely inhibited adherence induced by the pH 4.5 fraction, whereas it failed to inhibit the activity of the pH 6.0 fraction.

TABLE 1. Binding activity of protein A-eluted fractions of immune serum collected 28 days after infection

Serum or fraction	% Cells[a] with attached trypanosomes	
	Macrophages	J774 cells
Normal mouse serum[b]	4 ± 3	≤4
Whole immune serum[b]	97 ± 3	98 ± 1
pH 8 fraction[c]	6 ± 5	≤4
pH 6 fraction[c]	85 ± 4	98 ± 1
pH 4.5 fraction[c]	90 ± 3	98 ± 1
pH 2.8 fraction[c]	9 ± 3	≤4

[a] Mean and standard deviation from four experiments.
[b] Serum dilution, 1/40.
[c] Of each fraction, 10 μl (5 mg/ml) was added to each chamber medium (200 μl; final concentration, 250 μg/ml).

DISCUSSION

The results described here demonstrate that mononuclear phagocytes can ingest and kill *T. musculi* in the presence of specific IgG1 or IgG2a antibodies which mediate adherence to macrophages by two Fc receptors, Fcγ1/γ2b and Fcγ2a.

The role of the macrophagic system on immunity to various trypanosomes has been investigated with particular emphasis on *T. brucei* and *T. lewisi* infections (6, 7, 9, 15, 16). In vivo and in vitro studies have demonstrated that the

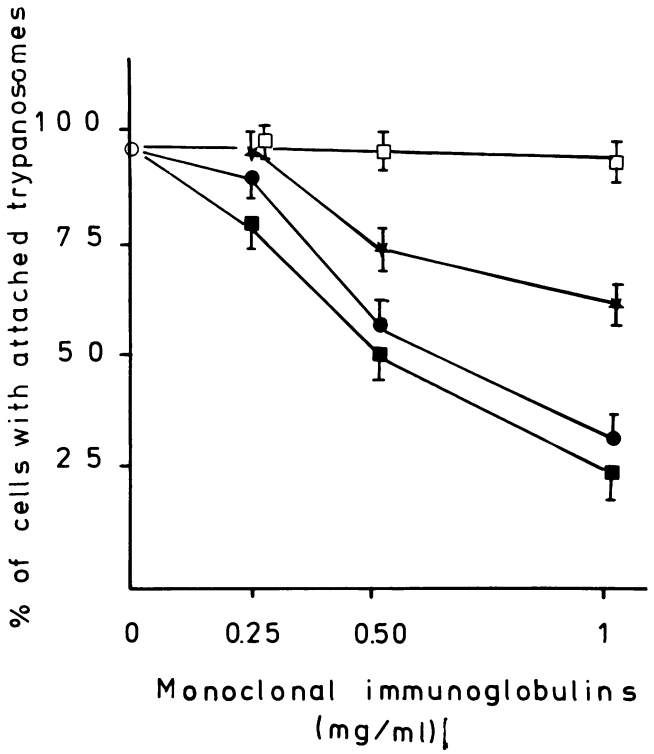

FIG. 2. Inhibition of *T. musculi*-binding activity of immune serum to macrophages by monoclonal immunoglobulins. Peritoneal macrophages were preincubated with monoclonal immunoglobulins IgG1 (■), IgG2a (●), Ig2b (★), or IgG3 (□) at the concentration indicated on the abscissa. Trypanosomes and immune serum from 28-day-infected mice were then added. After incubation, the percentage of macrophages with attached parasites was determined. Each point represents the mean value of three or four chambers plus or minus the standard deviation.

killing of extracellular trypanosomes is accomplished by phagocytosis. By using *T. brucei* labeled with ^{75}Se-methionine, it was found that the removal of blood parasites is largely accomplished by antibody-mediated hepatic phagocytosis (15). In *T. musculi*-infected mice, quantitative and functional changes in peritoneal macrophages have been observed to be maximal around day 14 of infection, at a time when parasites are eliminated from the peritoneal cavity. These changes are due neither to an inflammatory process nor to the presence of parasites, but they might be the consequence of the phagocytosis of parasites (27). The involvement of antibodies in the phagocytosis of parasites by macrophages has been reported in several studies with *T. gambiense*, *T. rhodesiense*, and *T. lewisi* (7, 10, 18, 19). In vitro phagocytosis of *T. musculi* by mouse peritoneal macrophages has been reported by Chang and Dusanic (3). In the present investigation, we have observed spontaneous and passive antibody-induced phagocytosis of *T. musculi* in the peritoneal cavity, and we have developed an in vitro system to analyze the characteristics of *T. musculi* phagocytosis and the role of cells, antibodies, and immunoglobulin classes.

IgG antibodies of the four subclasses were found to be produced during *T. musculi* infection. The titers of specific IgG1, IgG2a, and IgG2b, as determined by indirect immunoflurorescence assay in protein A fractions, were of the same order of magnitude. Surprisingly, however, when examined for their biological activity, not all IgG subclasses were capable of inducing adherence and phagocytosis of *T. musculi* to macrophages or J774 cells. The pH 2.8 fraction containing IgG2b antibodies produced no detectable effect, and only two fractions, containing either IgG1 and traces of IgG3 (pH 6.0 fraction) or IgG2a and IgG3 (pH 4.5 fraction), induced parasite attachment. To determine which of these isotypes was (were) operating in the two biologically active fractions, competition experiments were performed with purified mouse monoclonal immunoglobulins. IgG3 failed to inhibit parasite binding. It is therefore unlikely that IgG3 antibodies played a major role. By contrast, both IgG1 and IgG2a immunoglobulins were inhibitory. Since adherence induced by the pH 6.0 and 4.5 fractions was completely inhibited by IgG1 and IgG2a, respectively, one could assign to the antitrypanosome antibodies of corresponding classes responsibility for the whole activity in the fractions. This further confirmed the limited effect, if any, of IgG3 antibodies, especially in the pH 4.5 fraction. By contrast with the adherence induced by protein A fractions, serum-induced adherence was only partially inhibited by IgG1 and IgG2a, individually, but was almost totally abolished by a mixture of IgG1 and IgG2a. This indicates that the serum activity could be assigned entirely to antibodies of the IgG1 and IgG2a subclasses. Competition experiments also provided information on macrophage FcR involved in the binding of parasites. Three FcγR were identified on mouse macrophages as being

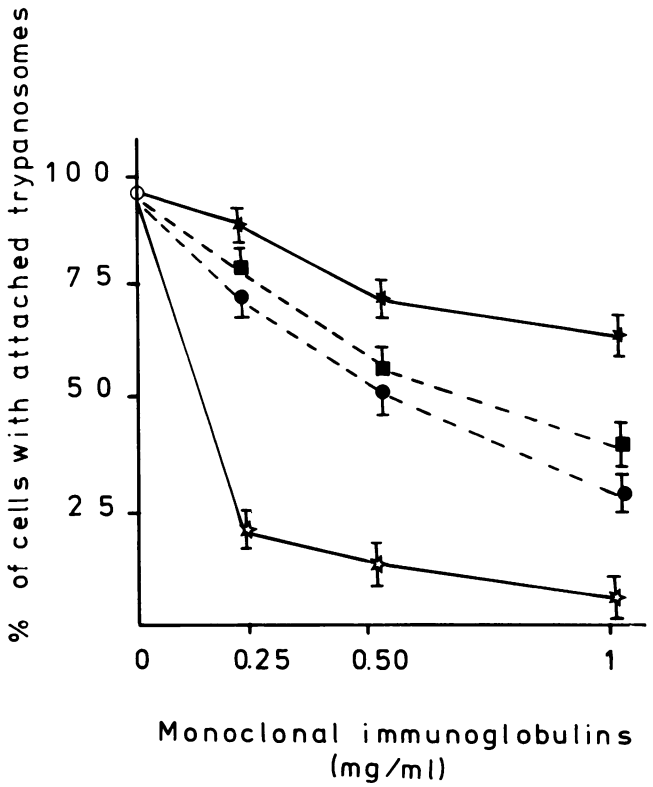

FIG. 3. Inhibition of *T. musculi*-binding activity of immune serum to J774 by monoclonal immunoglobulins. J774 cells were preincubated with IgG1 (■), IgG2a (●), IgG2b (★), or IgG1 plus IgG2a (☆) at concentrations indicated on the abscissa. Trypanosomes and immune serum from 28-day-infected mice were then added. After incubation the percentage of cells with attached trypanosomes was determined. Each point represents the mean value of three or four chambers plus or minus the standard deviation.

capable of binding monomers of IgG2a (Fcγ2aR), aggregates of IgG1 and IgG2b (Fcγ1/γ2bR), or IgG3 (Fcγ3R) (4). Two of these FcR mediated the immunoadherence of *T. musculi*. Fcγ2aR were involved in the pH 4.5 fraction-induced binding, since only monoclonal IgG2a was inhibitory. Fcγ1/γ2bR were involved in the pH 6.0 fraction-induced binding since monoclonal IgG1 was inhibitory, although monoclonal IgG2a was not. Both Fcγ2aR and Fcγ1/γ2bR were involved in immune serum-induced binding, since IgG1, IgG2b, IgG2a, or the anti-Fcγ1/γ2bR monoclonal antibody 2.4G2 was partially inhibitory when a mixture of IgG1 and IgG2a inhibited adherence completely.

Altogether, these data demonstrate that IgG1 and IgG2a anti-*T. musculi* antibodies found in the serum of 28-day-infected mice induced trypanosome adherence. It remains to be understood why IgG3 and especially IgG2b antibodies failed to induce parasite attachment. The simplest explanation can only be quantitative. Protein A fractions were adjusted to the same immunoglobulin concentration, but their antibody content could only be grossly evaluated by the indirect immunofluorescence assay used. IgG1 and IgG2a might therefore constitute the bulk of the IgG response during *T. musculi* infection, as these immunoglobulins do in the response to many antigens. Alternatively and not exclusively, IgG2b antibodies might indeed be unable to mediate parasite binding, because their Fc portion might not be available for binding to macrophage FcγR. This could be the consequence of (i) the degradation of IgG2b Fc portions by proteases secreted by parasites, as demonstrated for *T. cruzi* (13), (ii) the complexation of IgG2b Fc portions by subclass-specific immunoglobulin-binding factors, or (iii) the binding of IgG2b Fc portions to parasite FcR, as has been described for schistosomes (21). Either of these possibilities could be efficient mechanisms devised by the parasites to escape the host immunoresponse, and they deserve further examination.

In spite of such possible escape mechanisms, antitrypanosome antibodies seem to be operative in vivo during *T. musculi* infection. Antibodies play a role in the elimination of *T. musculi* in infected mice. Wechsler and Kongshavn have shown that injections of *T. musculi*-infected mice with protein A-Sepharose fractions of immune serum induce parasite elimination from blood (29). The curative effect was found essentially in the fraction eluted at pH 4.5 and containing IgG2a and IgG3.

In the present work, we have found that antibodies of two IgG subclasses are capable of promoting phagocytosis of *T. musculi* by macrophages, through the binding of two separate FcγR. Other elements of the immune system may also be involved. As observed for *T. brucei*, C3 may induce phagocytosis of *T. musculi* through C3b, since C3 depletion of mice during peak parasitemia results in a reduced rate of parasite elimination (11). Platelets may also act as effector cells against *T. musculi* (24). The importance of these different defense mechanisms in the spontaneous cure of *T. musculi* infection remains to be delineated. In a given mouse strain, one or another mechanism may play a major role depending on the site, and this mechanism could be more or less efficient in different strains. Finally, it is likely that the fate of the parasite depends on cooperation among several components of the immunoresponse system. We suggest that, among these mechanisms, antibody-dependent phagocytosis of *T. musculi* is of prime importance in the in vivo elimination of parasites in infected mice. Indeed, phagocytosis was observed at day 14 of infection when opsonizing antibodies were detectable in the serum. Furthermore, we were able to mimic parasitic destruction, as early as day 12 of infection, by injecting passive anti-*T. musculi* antibodies.

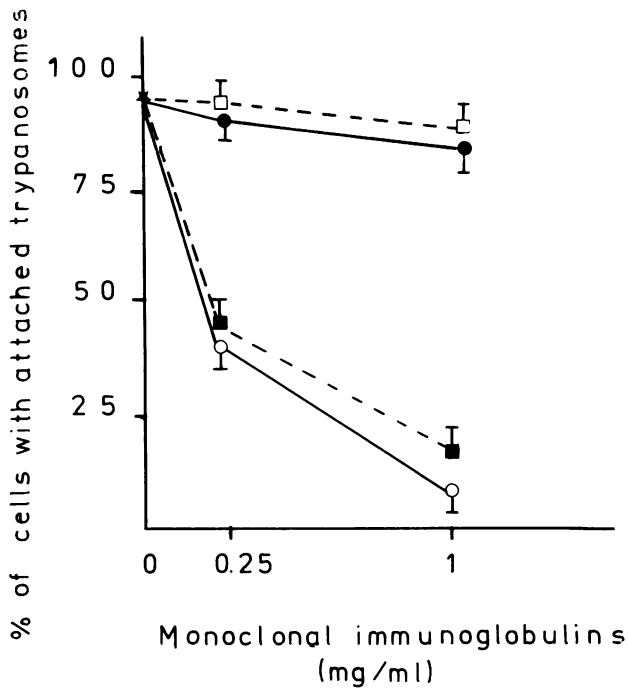

FIG. 4. Inhibition of *T. musculi*-binding activity of immune serum fractions to J774 cells by monoclonal immunoglobulins. J774 cells were preincubated with IgG1 before addition of pH 6 (■) or 4.5 fraction (□) or with IgG2a before addition of pH 6 (●) or 4.5 fraction (○) at concentrations indicated on the abscissa. Trypanosomes were then added. After incubation, the percentage of cells with attached trypanosomes was determined. Each point represents the mean value of three or four chambers plus or minus the standard deviation.

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