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Monoclonal Antibodies to *Trypanosoma cruzi* Inhibit Motility and Nucleic Acid Synthesis of Culture Forms

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Monoclonal antibodies were raised against the surface of epimastigotes and metacylic trypomastigotes of *Trypanosoma cruzi*, as shown by electron microscopy, agglutination, and immunofluorescence. The antibodies were stage specific but not strain specific. A deleterious effect of the antibodies on *T. cruzi* culture forms was shown by the drastic reduction of parasite motility and incorporation of nucleic acid precursors. Some fraction of the parasite population, however, was viable and replicated and infected mouse macrophages in culture. The antibodies were found to also mediate complement-induced lysis of culture forms of *T. cruzi*.

Circulating antibodies have been detected in humans and animals suffering from Chagas' disease, which is caused by the protozoan flagellate *Trypanosoma cruzi*. The role of these antibodies in host resistance to the parasite has been established by passive transfer and by neutralization experiments (reviewed in reference 3); however, neither the exact mechanisms nor the antigens involved in this protection have been characterized.

The surface components of *T. cruzi* have been the focus of a number of recent studies (9), and some of the corresponding antigens have been identified. A major surface membrane component characterized as a lipopeptidophosphoglycan (7) was found to be a poor immunogen, posing difficulties for its immunological analysis (2).

Two glycoproteins of approximate molecular weight 75,000 (75K) and 90K appear to be the main parasite components recognized by both human and mouse immune sera (10). The 75K glycoprotein has been detected only in epimastigotes and metacylic stages, i.e., culture forms. The 90K glycoprotein, according to Snary and Hudson (15), can be detected in all developmental stages of *T. cruzi*, although the distribution has not been corroborated by others (10). Antibodies raised against the 90K glycoprotein conferred partial protection to mice and resulted in lower parasitemia and longer survival times upon challenge with blood forms of *T. cruzi* (12, 15).

Recently, Snary et al. (14) obtained monoclonal antibodies to a glycoprotein of 72K present in culture forms. Immunization with this glycoprotein, however, failed to protect mice against challenge with blood forms.

The fact that some of the main surface antigens recognized by immune sera confer little or no protection against challenge suggests the possibility that minor antigenic components play an important role in the immune response to *T. cruzi*. Therefore, the use of hybridoma technology in this system appears to be a potentially fruitful approach. The present report contains our initial results, obtained through the use of monoclonal antibodies, in this area.

MATERIALS AND METHODS

T. cruzi. *T. cruzi* Y was isolated from an acute human case of Chagas' disease (13), and the CL strain was isolated from a naturally infected vector (4). Culture cells of *T. cruzi* were grown in liver infusion-tryptose (Difco Laboratories, Detroit, Mich.) (LIT) medium (5) supplemented with 10% fetal calf serum (GIBCO, Grand Island, N.Y.), penicillin (200 μg/ml), and streptomycin (100 U/ml). Cultured metacyclic trypomastigotes were purified from epimastigote forms by chromatography on DE 52 resin (1) (Whatman Ltd., Kent, Great Britain) as follows. Cells (10^9) were overlaid on a DE 52 column (1.5 by 3 cm) and eluted with phosphate-buffered saline (PBS) (pH 7.2). Fractions of 1 ml were collected, and those containing only trypomastigotes were pooled (>97% trypomastigotes). Blood form trypomastigotes were isolated from BALB/c mice (Texas Inbred Mice Co., Houston, Tex.) which were previously irradiated (600 rad) and infected with 5×10^5 blood forms. The trypomastigotes were separated as previously described (8). Amastigotes were obtained from cultured peritoneal macrophages infected with culture forms of *T. cruzi* (multiplicity of 1:6).

Monoclonal antibodies. BALB/c mice were immunized every other week intraperitoneally with increasing doses of live *T. cruzi* (10^2, 10^3, and 10^4 culture forms, followed by 10^5 and 10^6 blood forms). Three weeks later, the mice were given an intravenous booster with 10^8 sonicated culture forms. The absence

TABLE 1. Stage and strain specificity of the *T. cruzi* surface antigens recognized by monoclonal antibody B10/1 determined by immunofluorescence

Glutaraldehyde-fixed[a] parasites	*T. cruzi* strain	Immunofluorescence assay titer[b]
Epimastigotes	Y	1:160
Metacylic	Y	1:320
Amastigote	Y	Negative
Blood forms	Y	Negative
Epimastigotes	CL	1:80
Metacyclic	CL	1:80
Blood forms	CL	Negative

[a] Parasites were fixed with 0.2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 20 min at room temperature.

[b] The monoclonal antibody used in these reactions (B10/1) was partially purified by precipitation with 50% ammonium sulfate.

of intact parasites was ascertained microscopically. After 4 days, the spleen cells from the boostered mice were fused with the P3U1 myeloma line (6). Positive hydridomas were selected by indirect immunofluorescence with glutaraldehyde-fixed cells, cloned by limiting dilution, expanded, and injected into pristane-primed CD2F1 mice (Cumberland Farms, Clinton, Tenn.). The antibodies were purified from ascites fluid or culture supernatants by precipitation with 50% ammonium sulfate, followed by filtration through Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The antibody class was determined by double diffusion in agar with specific antisera for mouse immunoglobulin subtypes (Litton Bionetics, Kensington, Md.).

Eleven hydridomas were cloned, and all were found to react with both culture forms, i.e., epismastigotes and metacyclic stages of *T. cruzi*. Several of these monoclonal antibodies incubated with unfixed, viable culture stages were found to produce immunofluorescence of the parasite's membrane and agglutination of the cells.

Effects of the monoclonal antibodies on the synthesis of nucleic acid by *T. cruzi*. For each experimental point, 10^7 culture cells in 0.5 ml of LIT medium or LIT medium containing monoclonal antibodies (50 or 500 μg/ml) were incubated at 28°C for 4, 8, 24, or 120 h. As controls, an equal number of parasites were incubated with culture medium (LIT) alone or after addition of an unrelated monoclonal antibody (2C11). After incubation, the cells were washed with PBS and resuspended in Dulbecco modified Eagle medium (GIBCO) containing 2 μCi of [5,6-^{3}H]uridine or [*methyl*-^{3}H]thymidine per ml (New England Nuclear Corp., Boston, Mass.), 10% fetal calf serum, penicillin (200 μg/ml), and streptomycin (100 U/ml). After 3 h at 28°C, the cells were centrifuged, and the radioactivity precipitated by 5% trichloroacetic acid was determined in a liquid scintillation counter. The same experiment was done with Dulbecco modified Eagle medium instead of LIT medium. All experiments were done in duplicate. Anti-*Plasmodium falciparum* monoclonal antibody (2C11) was used as a control, as was medium alone. Under the conditions described, the incorporation of [^{3}H]uridine and [^{3}H]thymidine was proportional to the number of *T. cruzi* culture cells present (approximately 1×10^7 to 4×10^7 cells).

Agglutination assay. Culture cells of *T. cruzi* were washed three times with PBS and resuspended in the same buffer. Cells (5×10^5/ml) were incubated with monoclonal antibodies for 30 min at room temperature, and the agglutination was followed microscopically. Unrelated monoclonal antibodies (anti-*Plasmodium cynomologi* and anti-*Plasmodium berghei* sporozoites) were used as controls.

***T. cruzi* lysis.** *T. cruzi* cells were labeled with [^{3}H]uridine as described above and washed with PBS (three times). Labeled cells (3×10^5) were incubated in 50 μl of PBS or PBS upon addition of monoclonal antibodies at the described concentration. After 1 h at 28°C, fresh or heat-inactivated human serum (56°C for 1 h) was added to a final concentration of 10%, and the cells were incubated for another 30 min at 28°C. The cells were then spun down ($1{,}000 \times g$ for 15 s), and 30 μl of the supernatant fluid was counted in a liquid scintillation counter. The percentage of lysed cells was related to the radioactivity released by the same amount of cells treated with 1% Nonidet P-40 (20 min at room temperature) and taken as 100%.

Macrophage infection by *T. cruzi*. Marrow-derived macrophage monolayers were obtained as described earlier (11). Culture forms (10^6) of *T. cruzi* were treated with monoclonal antibody or with LIT medium and incubated with the macrophages. Free parasites were removed by washing, the macrophages were incubated for an additional 24 h, and the cultures were fixed in methanol and stained with Giemsa. The macrophages containing three or more intracellular parasites were scored as infected. At least 200 macrophages were scored per cover glass. Duplicate determinations were used for each incubation time.

Electron microscopy. *T. cruzi* was incubated with serum from mice bearing the myeloma tumour for 1 h at room temperature, washed, and fixed in 2.5% glutaraldehyde–0.1 M cacodylate buffer (pH 7.3)–4% sucrose. The preparation was postfixed in 1% osmium tetroxide for 1 h, dehydrated, and embedded in Epon 812. The sections were examined with a Siemens Elmiskop 101 electron microscope.

RESULTS

Of the 11 hybridomas cloned, 2, B10/1 and B2/5, were chosen for more extensive characterization. They were found to be of the immunoglobulin G1 and G3 subclasses, respectively, with κ light chains and produced the same pattern of uniform membrane fluorescence with glutaraldehyde-fixed epimastigotes and trypomastigotes of both the Y and CL strains of *T. cruzi*. However, both monoclonal antibodies failed to react with either amastigotes or blood forms of these two parasite strains (Table 1). A certain variability in the fluorescent staining of individual culture forms was detected when viable cells were incubated with the monoclonal antibodies.

The incubation of live culture forms of *T. cruzi* with monoclonal antibodies B10/1 and B2/5 was found to result in strong agglutination of both

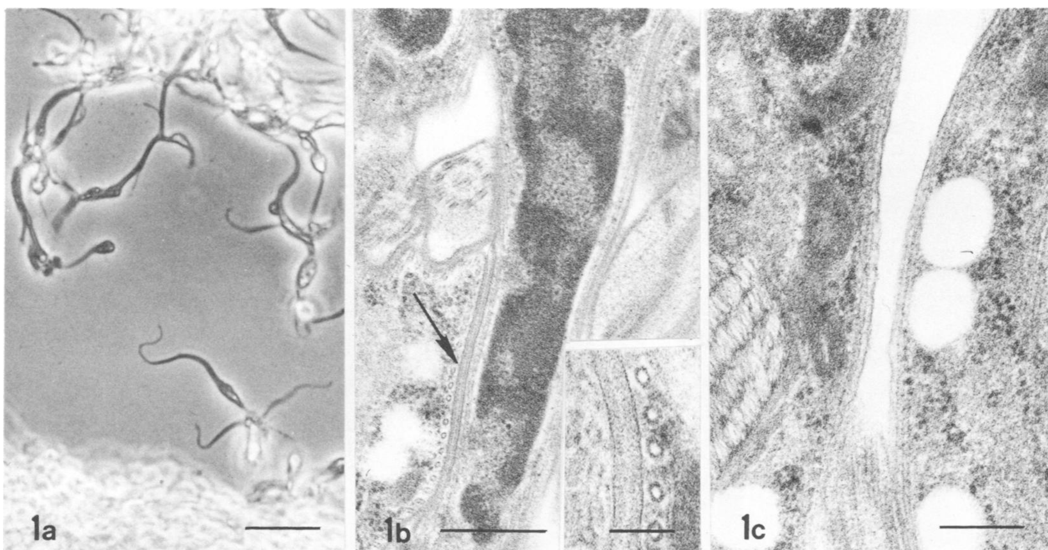

FIG. 1. (a) Light micrograph of the culture forms of *T. cruzi* incubated with hybridoma-derived monoclonal antibody (B2/5). Bar, 10 μm. (b) Electron micrograph of the metacyclic trypomastigotes of *T. cruzi* incubated with hybridoma-derived monoclonal antibody (B2/5). Note agglutination of the parasite (arrow). Bar, 1.5 μm. (Inset) Higher magnification micrograph showing fibrillar structure which adheres to these parasites. Bar, 0.1 μm. (c) Electron micrograph of the metacylic trypomastigotes of *T. cruzi* incubated with anti-*Plasmodium knowlesi* antibody. No agglutination took place between the parasites. Bar, 0.25 μm.

trypomastigotes and epimastigotes, as well as intermediate forms (Fig. 1). Agglutination of culture forms (5×10^5) was observed upon incubation of the cells with >10 μg of the purified monoclonal antibodies per ml. Incubation of the parasites with higher concentrations (>200 μg/ml) resulted almost immediately in a drastic reduction of parasite motility. In fact, most of the parasites rapidly become immobilized, interfering with clump formation. This interaction is stage and species specific; it did not occur upon incubation of the monoclonal antibodies with blood forms of *T. cruzi* or with promastigotes of *Leishmania mexicana amazonensis*. Unrelated monoclonal antibodies (anti-*P. berghei* and anti-*P. cynomologi*) failed to induce immobilization, agglutination, or immunofluorescence of epimastigotes or trypomastigotes of *T. cruzi* under identical experimental conditions.

In an attempt to identify the mechanism of interaction of monoclonal antibodies with parasites, we investigated the long-term effects of these antibodies on the nucleic acid synthesis by culture forms of *T. cruzi*.

Figure 2 shows that both hybridomas, B10/1 and B2/5, were strongly inhibitory, significantly reducing the uridine and thymidine incorporation of the parasites as compared with the controls. The values obtained for both controls, i.e., parasites incubated in medium alone or after addition of an unrelated monoclonal antibody, were very similar. There were quantitative differences, however, in the effects produced by B10/1 and B2/5. At equivalent concentrations, B2/5 was more active. When *T. cruzi* was incubated with the monoclonal antibodies for 4 to 8 h, the inhibitory effect was clearly detected by measuring uridine incorporation. A drastic reduction of incorporation of both precursors was seen after 24 and 48 h of incubation with either of the hybridomas. During this period, large numbers of nonmotile, apparently nonviable parasites were observed. Determination of the percentage of nonviable culture forms was precluded by the extensive antibody-induced agglutination. After 5 days of incubation in the presence of monoclonal antibodies, some of the parasites appeared to escape this inhibitory effect, indicating the presence of a viable parasite population.

The persistence of viable, infective culture forms upon incubation of the parasites with the monoclonal antibodies was also confirmed by exposing cultured mouse macrophages to antibody-treated parasites. Preincubation of the parasites with 500 μg of monoclonal antibody B10/1 per ml for 1, 48, or 96 h resulted in 80, 36, and 50% infected macrophages, respectively.

In a different set of experiments in which the parasites and monoclonal antibodies (B10/1 or B2/5) were incubated simultaneously with macrophages, parasite interiorization was enhanced

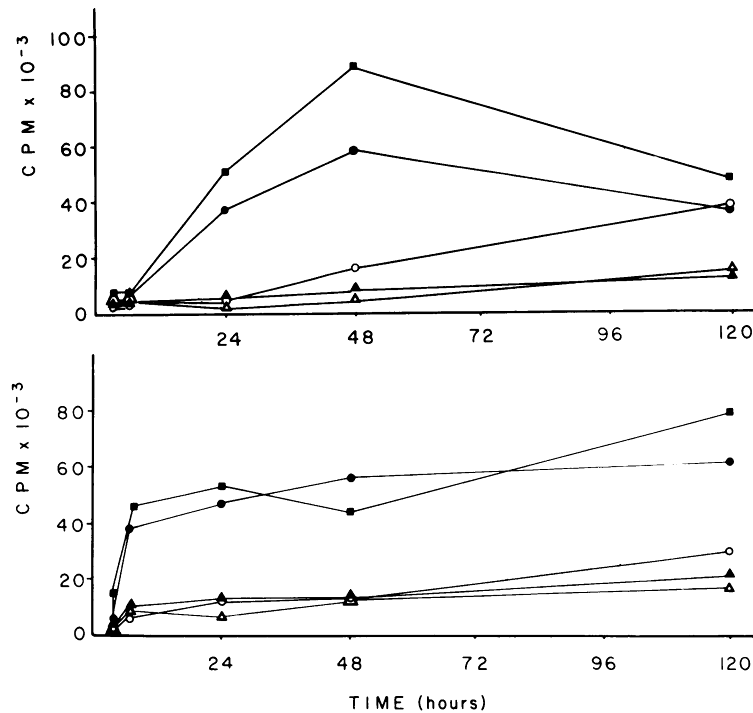

FIG. 2. Incorporation of [^{3}H]thymidine and [^{3}H]uridine by culture forms of *T. cruzi* preincubated with monoclonal antibodies (B10/1 or B2/5) or control medium. Abscissa, period of incubation with antibodies or control medium; ordinate, radioisotope incoporation in trichloroacetic acid precipitates (counts per minute). Radioactive precursors were given 3 h after incubation with antibodies. Upper graph, [^{3}H]thymidine; lower graph, [^{3}H]uridine. Symbols: ■, LIT medium; ▲, B2/5, 500 μg/ml; ○, B10/1, 500 μg/ml; △, B2/5, 50 μg/ml; ●, B10/1, 50 μg/ml.

as compared with the controls. The intracellular development appeared to be unaltered. After 3 to 4 days, however, free epimastigotes were absent in the surrounding culture medium, as if parasite development had been arrested at the amastigote stage due to the presence of the monoclonal antibody. This was in direct contrast to the control cultures, in which epimastigotes were abundant at this time.

Monoclonal antibodies B2/5 and B10/1 were found to mediate complement-induced lysis of culture forms of *T. cruzi* (Table 2). Since the epimastigotes are lysed by complement in the absence of antibody, the experiment was done at

TABLE 2. Hybridoma-mediated complement-dependent lysis of culture forms of *T. cruzi*[a]

Monoclonal antibody (μg/ml)	% Lysis[b]			
	Metacyclic trypomastigotes		Epimastigotes	
	Hus	IHuS	Hus	IHuS
	15	8	26	25
B2/5 (500)	87	14	40	23
B2/5 (50)	76	15	33	27
B2/5 (5)	42	19	27	24
B10/1 (500)	78	17	65	37
B10/1 (50)	30	18	57	27
B10/1 (5)	28	17	45	28
2C11[c] (500)	25	28	27	24

[a] Parasites were metabolically labeled with [^{3}H]uridine for 3 h at 28°C.

[b] 100% lysis corresponds to the radioactivity released by parasites incubated with 1% Nonidet P-40. HUS, Human serum; IHus, heat-inactivated human serum.

[c] Anti-*P. falciparum* hybridoma.

28°C rather than the standard 37°C to minimize these effects. Complement activation appeared to be mediated by the alternative pathway, since it was inhibited by 1 mM EDTA but remained unaltered in the presence of 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and 3 mM $MgCl_2$. Under these experimental conditions, even upon incubation with high concentrations of antibodies, lysis was never complete. This indicates the presence of a relatively resistant parasite population, which may correspond to a particular developmental stage.

DISCUSSION

We obtained monoclonal antibodies which not only reacted with glutaraldehyde-fixed *T. cruzi*, but also bound, agglutinated, and immobilized viable culture forms. Agglutination and immobilization of *T. cruzi* were not reported in a recent paper on this subject, which also involved the use of hybridoma technology (14). This may be due to different specificities or to differences in the binding affinity and isotype of the respective antibodies, or both.

Our results indicate that monoclonal antibodies against *T. cruzi* distinguish stage-specific antigens and might provide useful markers to separate this parasite from other related hemoflagellates. It will be important to evaluate the reactivity of the monoclonal antibodies with the metacyclic infective forms found in the insect vectors. Although these monoclonal antibodies cannot distinguish between Y and CL strains, screening of other *T. cruzi* strains appears desirable.

The properties of the two monoclonal antibodies appear qualitatively similar. Differences in antibody titers or in inhibitory effects on motility and metabolism may depend on different binding affinities. However, conclusions as to the specificity of the antibodies must await the characterization of the relevant antigens.

Prolonged incubation with the antibodies was required for inhibition of parasite metabolism and replication in cell-free cultures, as well as for decrease of the percentage of infected macrophages. The results were identical whether we used LIT or Dulbecco modified Eagle medium, or whether the fetal calf serum had or had not been heat inactivated, indicating that the effects on the parasites were neither complement mediated nor determined by the presence of a particular component of the culture media.

The inhibitory effects of the monoclonal antibodies on nucleic acid synthesis, macrophage-parasite interaction, and antibody-induced, complement-mediated lysis were partial. Although different mechanisms may be involved in these effects, the presence of viable parasites upon prolonged incubation with antibody might have one common explanation. Since we used a heterogeneous, noncloned, asynchronous parasite population, our results could be explained if a fraction of the population were genetically distinct. Under these conditions, some of the parasites could either lack or express only minimal amounts of the relevant surface antigen which is present on the remaining population. Alternatively, the antigen may only be expressed on a well-defined developmental stage. Another possibility is that different parasite populations might have different rates of removal of antigen or antigen-antibody complexes or both from their surface. Isolation of parasites resistant to the effect of monoclonal antibodies might enable us to distinguish between these different "escape" mechanisms.

The deleterious effect of the monoclonal antibodies on the parasites might be due to membrane cross-linking and agglutination. In fact, we found the the F(ab) fragments of both monoclonal antibodies failed to agglutinate and to inhibit uridine and thymidine incorporation by *T. cruzi* (data not shown).

Finally, the failure of the antibodies to affect the entire parasite population may explain the parasitemia and death of mice inoculated with antibody-treated cultures of *T. cruzi* (data not shown). Taken together, these results indicate that, although the protective antigen(s) of *T. cruzi* still remains unidentified, these as well as other monoclonal antibodies will contribute to the delineation of the antigenic repertoire of the parasite and the mechanism(s) of the interaction of the antibody with the parasite.

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