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Metacyclic Neutralizing Effect of Monoclonal Antibody 10D8 Directed to the 35- and 50-Kilodalton Surface Glycoconjugates of *Trypanosoma cruzi*

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It was shown in this work that the infectivity of metacyclic forms of *Trypanosoma cruzi* was affected upon interaction with the monoclonal antibody (10D8), which reacts with a carbohydrate epitope of the 35- and 50-kilodalton (kDa) surface glycoconjugates. The invasion of Vero cells by metacyclic forms of strains Tulahuen and G was inhibited 50 to 67% in the presence of 10D8 (10 μg/ml), whereas a nonrelated monoclonal antibody to *Plasmodium berghei* had no such effect. In mice that were inoculated with metacyclic forms preincubated with 10D8 or that had passively received 10D8 before challenge with metacyclic forms, a considerable decrease in the parasitemia levels was observed. The 35- and 50-kDa antigens were detectable by the galactose oxidase and sodium boro[^{3}H]hydride procedure but not by surface iodination or metabolic labeling with [^{35}S]methionine, suggesting that they may be of glycolipid nature. The finding that the 35- and 50-kDa antigens are major bands recognized by sera of mice immunized with killed metacyclic forms and protected against acute infection, in addition to the results with 10D8, indicate that these glycoconjugates may play an important role in the metacyclic form-host cell association that initiates *T. cruzi* infection.

Infection by *Trypanosoma cruzi*, the agent of Chagas' disease, is initiated by the metacyclic trypomastigotes derived from the insect vector. After entering the host cells, the parasites multiply intracellularly as amastigotes and differentiate afterwards into trypomastigotes. These are released into the bloodstream and can invade new host cells, thus propagating the infection.

The determinants involved in the host cell-*T. cruzi* interaction that ultimately lead to infection are not as yet clear. Proteins and glycoconjugates present on the surfaces of infective-stage trypomastigotes are thought to play a key role in the process of recognition and invasion of host cells by the parasite. It has been claimed (1, 18) that one such *T. cruzi* surface component, a glycoprotein of 85 kilodaltons kDa; pI, 6.3 to 7.5), serves as a ligand that mediates the parasite-host cell recognition in vitro. According to Boschetti et al. (3), cell culture-derived trypomastigotes contain an 83-kDa protein (pI, 8.1 to 8.6) which binds to the surface of fibroblasts in vitro. Another surface molecule that may be associated with the infective capacity of *T. cruzi* is a metacyclic-stage-specific 90-kDa polypeptide (pI, 4.6) identified by the monoclonal antibody 1G7 (11), which is capable of reducing the metacyclic infectivity in mice (2) and of inhibiting the adhesion and penetration of metacyclic trypomastigotes in Vero cells (J. C. Gonzalez and N. Yoshida, unpublished data).

We report in this paper that the metacyclic surface glycoconjugates of approximately 35 and 50 kDa identified by the monoclonal antibody 10D8, which partially neutralizes the parasite infectivity, may also be implicated in host cell recognition by *T. cruzi*.

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MATERIALS AND METHODS

Parasites. Two strains of *T. cruzi* were used: strain G, isolated from an opossum by Mena Barreto in the Amazon (15), and Tulahuen, isolated from *T. infestans* in Chile (8). The parasites have been maintained alternately in mice and in liver infusion tryptose medium or in triatomine vectors.

Preparation of metacyclic trypomastigotes. The parasites grown in liver infusion tryptose medium were harvested at late stationary phase, when the cultures are enriched in metacyclic forms, and centrifuged at 2,000 × *g* for 5 min. After two washings with phosphate-buffered saline (PBS) containing 5.4% glucose (PSG), pH 8.0, the pellet was resuspended in PSG and passed through a 4-cm-high DEAE-cellulose column packed in a 10-ml plastic syringe. The PSG eluate contained 99 to 100% metacyclic forms. Insect-derived metacyclic trypomastigotes were obtained as follows. *T. infestans* infected with *T. cruzi* were fed on mice, and soon after the blood meal, most triatomines eliminated clear transparent drops of liquid (urine) which were individually examined under a phase-contrast microscope for the presence of parasites. Samples containing pure trypomastigotes, or metacyclic forms mixed with a small number of epimastigotes, were pooled so that the final preparation contained more than 95% trypomastigotes.

Preparation of epimastigotes, amastigotes, and blood trypomastigotes. The various developmental stages of *T. cruzi* were obtained essentially as previously described (11). Trypomastigotes derived from 3T3 mouse fibroblast cell cultures were kindly provided by Sérgio Schenkman.

Production and purification of the monoclonal antibody 10D8. The anti-*T. cruzi* hybridoma 10D8 was obtained as previously described (11). Briefly, spleen cells from a BALB/c mouse immunized with killed strain G metacyclic forms were fused with P3U1 plasmacytoma cells. The hybridoma supernatants were screened by the indirect immunofluorescent-antibody test using formaldehyde-fixed strain

G metacyclic trypomastigotes as the antigen, and the antibody-producing hybridomas were cloned twice by the limiting-dilution procedure. Cloned hybridoma cells secreting the monoclonal antibody 10D8 were inoculated into BALB/c mice previously injected with pristane to induce ascites formation. Purification of 10D8 from ascitic fluid was carried out in a protein A-Sepharose column (Pharmacia Fine Chemicals, Piscataway, N.J.). Fab fragments were prepared by papain (Sigma Chemical Co., St. Louis, Mo.) digestion and purification by protein A-Sepharose chromatography.

Surface membrane labeling of metacyclic forms by using galactose oxidase and sodium boro[^{3}H]hydride. Metacyclic trypomastigotes were washed in RPMI 1640 medium and resuspended to a density of 10^8 parasites in 100 μl of medium. They were treated for 45 min at room temperature with 1 U of galactose oxidase (Sigma) and then washed three times in medium and resuspended in 150 μl of medium containing 750 μCi of NaB^3H_4 (16 Ci/mmol; Research Products International Corp., Mt. Prospect, Ill.). After incubation at room temperature for another 45 min, the parasites were washed three more times with medium, lysed with 1% Nonidet P-40 in the presence of protease inhibitors, and processed for immunoprecipitation as described previously (11). The immunoprecipitates dissolved in boiling sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 10% SDS and 10% 2-mercaptoethanol were electrophoresed in 6 to 16% polyacrylamide linear gradient microslab gels prepared by the method of Matsudaira and Burgess (7).

Surface iodination and metabolic labeling with [^{35}S]methionine were carried out essentially as previously described (11).

Immunoblotting. Immunoblotting was carried out essentially as described by Towbin et al. (12). After electrophoresis, proteins were transferred to nitrocellulose sheets (0.45-μm pore size; Millipore Corp., Bedford, Mass.) for at least 2 h at 200 mA. The transferred polypeptides and molecular weight markers (low-molecular-weight range; Pharmacia) were visualized by staining the sheets with 0.1% (wt/vol) Ponceau-S in 10% acetic acid. The sheets were subsequently soaked in blot buffer (150 mM NaCl, 1 mM EDTA, 30 mM Tris hydrochloride, pH 7.3, 0.25% gelatin, 0.05% NaN_3) for at least 30 min to block the remaining protein-binding sites. After incubation with ascitic fluid of P3U1 cells or hybridomas secreting 10D8, diluted 1:30 in blot buffer, for 1 h at room temperature, the sheets were subjected to three washes of 15 min each in blot buffer containing 0.01% Tween 20 under constant motion. Bound immunoglobulins were visualized after incubation for 1 h with the appropriate anti-immunoglobulin coupled to horseradish peroxidase (Dako Corp., Santa Barbara, Calif.) following three 10-min washes in PBS and reaction with diaminobenzidine (0.2 mg/ml) and H_2O_2 (5 μl of a 30% solution in 30 ml of PBS). Anti-actin monoclonal antibody (6) was obtained from Amersham International PLC, Buckinghamshire, England. Anti-cross-reactive-determinant (anti-CRD) antibody and partially purified sVSG (soluble form of *T. brucei* variant surface glycoprotein) were gifts from M. L. C. de Almeida from Escola Paulista de Medicina, Sao Paulo, Brazil.

Periodate oxidation of carbohydrate residues. We used the procedure based on the method described by Woodward et al. (14). Briefly, after SDS-PAGE of metacyclic lysates, transfer to nitrocellulose, and visualization with Ponceau-S, the nitrocellulose strips were washed with 50 mM sodium acetate, pH 4.5, for 10 min, followed by 1 h of incubation, at room temperature and in the dark, with 10 mM sodium *m*-periodate (Sigma) in 50 mM sodium acetate, pH 4.5. Control strips were incubated without periodate. The strips were subsequently washed in PBS and incubated with 50 mM sodium borohydride in PBS for 30 min. Excess borohydride was removed with PBS and the strips were processed for immunoblotting as described above.

Cell cultures and parasite invasion assay. Vero cells were grown in plastic bottles at 37°C in RPMI 1640 supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) in a humidified 5% CO_2 atmosphere. For the parasite invasion assay, Vero cells were pretreated with trypsin (2 mg/ml for 15 min at 37°C) and 10^5 cells were seeded onto each well of 24-well plates containing sterile 13-mm-diameter round glass coverslips. After overnight incubation at 37°C, the cells were infected with 5×10^5 purified metacyclic trypomastigotes previously incubated at room temperature for 1 h in RPMI 1640 with or without monoclonal antibody (the monoclonal antibody 3D11 against *Plasmodium berghei* sporozoites was provided by F. Zavala from New York University). After 3 h, the duplicate coverslips were washed three times with PBS and stained by May Grünwald-Giemsa. The rate of infection was calculated by counting the number of intracellular parasites in a total of 500 cells.

RESULTS

Reactivity of 10D8 with different *T. cruzi* developmental stages. We investigated whether the monoclonal antibody 10D8, which was raised against metacyclic trypomastigotes (strain G), recognized other developmental stages. By indirect immunofluorescent-antibody test using formaldehyde-fixed parasites, 10D8 was found to react with metacyclic trypomastigotes from triatomine or from culture as well as with epimastigotes but not with tissue amastigotes or blood trypomastigotes (data not shown). By immunoblot analysis, doublet bands of approximately 35 and 50 kDa were detected by 10D8 in culture- or insect-derived metacyclic forms and in epimastigotes, but these antigens appear to be lacking both in tissue culture-derived and blood trypomastigotes (Fig. 1).

Metacyclic surface antigen(s) identified by 10D8. Since the immunoblot technique does not discriminate between internal and surface antigens, we carried out labeling experiments using strain G trypomastigotes to identify the 10D8-reactive metacyclic surface components(s). Iodination of metacyclic forms, followed by immunoprecipitation with 10D8 and SDS-PAGE, repeatedly failed to reveal any specific band, and this was not due to technical error since the precipitation of the same labeled metacyclic preparation with 1G7 resulted in a strong band (Fig. 2B). In addition, the possibility that 10D8 does not bind to *Staphylococcus* protein A used in the immunoprecipitation was ruled out since this antibody could be purified through protein A-Sepharose. Precipitation of metacyclic forms metabolically labeled with [^{35}S]methionine with 10D8 also gave negative results (not shown). On the other hand, when metacyclic forms were labeled by the galactose oxidase and sodium boro[^{3}H]hydride procedure, two bands around 35 and 50 kDa were detected in the total extract and were specifically precipitated by 10D8 (Fig. 2A).

To verify that the 10D8-reactive epitope is a carbohydrate, we performed the periodate oxidation test. The strain G metacyclic lysates were electrophoresed, blotted onto nitrocellulose sheets, and then treated with periodate. As shown in Fig. 3, the ability of 35- and 50-kDa antigens to bind 10D8 was abolished upon periodate treatment, whereas the reac-

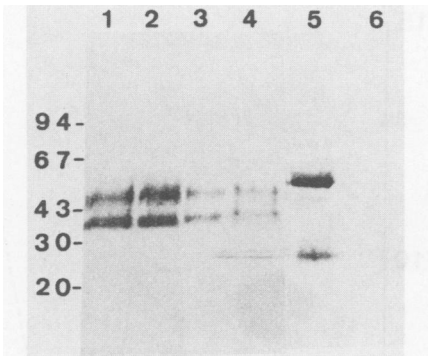

FIG. 1. Reactivity of monoclonal antibody 10D8 with different developmental stages of *T. cruzi* (strain G) as determined by immunoblotting. Reaction of 10D8 with 5×10^6 metacyclic trypomastigotes (lane 1) or epimastigotes (lane 2) from axenic cultures identified the antigens around 35 and 50 kDa, which were also detected in extracts of 1×10^6 culture-derived (lane 3) or insect-derived (lane 4) metacyclic trypomastigotes. No specific bands were revealed in lysates of 5×10^6 trypomastigotes from infected mice (lane 5) or from tissue culture cells (lane 6). The bands in lane 6 correspond to the immunoglobulin heavy and light chains from the blood of infected mice. Molecular sizes (in Kilodaltons) are on the left.

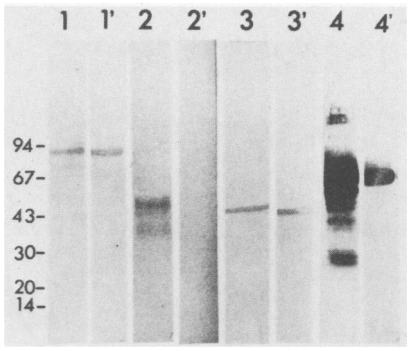

FIG. 3. Effect of periodate treatment on the 10D8-reactive epitope. Cell lysates of metacyclic trypomastigotes (strain G) were subjected to SDS-PAGE, blotted onto nitrocellulose, and then treated with periodate. Both untreated (lanes 1, 2, and 3) and treated (lanes 1', 2', and 3') nitrocellulose strips were incubated with the monoclonal antibodies 1G7 (lanes 1 and 1') or 10D8 (lanes 2 and 2') or with an anti-actin antibody (lanes 3 and 3'). The reaction of *T. brucei* sVSG, untreated (lane 4) or treated (lane 4') with periodate, is also shown. Molecular sizes (in Kilodaltons) are on the left.

tivity of 1G7, the monoclonal antibody to the metacyclic-form-specific surface polypeptide of 90 kDa, and that of an anti-actin monoclonal antibody remained unaffected. The periodate treatment also markedly reduced the reactivity of anti-CRD antibody, which is directed to a carbohydrate epitope of the variant surface glycoprotein of *T. brucei* (13).

Of note was the finding that the 35- and 50-kDa antigens form one of the major bands revealed by the sera of mice immunized with killed metacyclic trypomastigotes (Fig. 4) and shown previously to be protected against acute *T. cruzi* infection (16). Broad antigen doublets, of slightly different size, were also detected by 10D8 in strain Tulahuen metacyclic forms (Fig. 4).

Metacyclic trypomastigote-neutralizing effect of 10D8. To determine whether the monoclonal antibody had any effect on the infectivity of metacyclic trypomastigotes, a series of in vivo and in vitro experiments were carried out. Metacyclic forms of strain Tulahuen were incubated with the serum of mice bearing hybridoma 10D8 for 45 min at room temperature, and 10^5 parasites were then injected intravenously into mice. As shown in Fig. 5, the parasitemia levels in mice that received metacyclic forms pretreated with 10D8 were much lower than those in control animals. Fab fragments of 10D8 were as effective as the intact immunoglobulin; mice inoculated with Tulahuen metacyclic forms incubated with 10D8 Fab fragments (1 mg/ml) in PBS containing 1% bovine serum albumin displayed reduced parasitemia levels (Fig. 5). Although less effective than the in vitro neutralization, the inhibitory effect of 10D8 on the infectivity of the metacyclic forms was also observed by passive transfer of antibody; 60% of mice transfused with 10D8 (0.5 mg/ml) developed very slight parasitemia and the course of infection of the animals with severe parasitemia was distinct from that of the

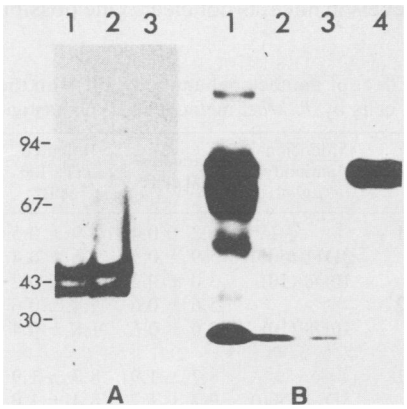

FIG. 2. Metacyclic surface antigens recognized by monoclonal antibody 10D8. Trypomastigotes (strain G) from axenic cultures were labeled by the galactose oxidase and sodium boro[^{3}H]hydride procedure (A) or with ^{131}I by the Iodogen method (B). The parasite lysates were precipitated with 10D8 (lanes 2), normal mouse serum (lane 3B), or 1G7 (lane 4B). The pattern of the labeled metacyclic extract is shown in lane 1. Molecular sizes (in kilodaltons) are on the left.

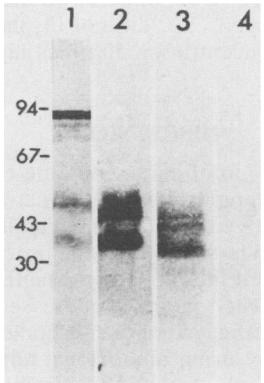

FIG. 4. Immunoblot analysis of 10D8-reactive antigens of *T. cruzi* strains. Lysates of 10^7 metacyclic trypomastigotes of strain G (lane 2) and of strain Tulahuen (lane 3) were subjected to SDS-PAGE and then processed for immunoblotting. Note that the size of the doublet of strain Tulahuen detected by 10D8 (lane 3) is slightly different from that of strain G (lane 2). Lane 1, Profile of strain G metacyclic antigens recognized by sera of mice immunized with killed metacyclic forms; lane 4, reactivity pattern of normal mouse serum. Molecular sizes (in Kilodaltons) are on the left.

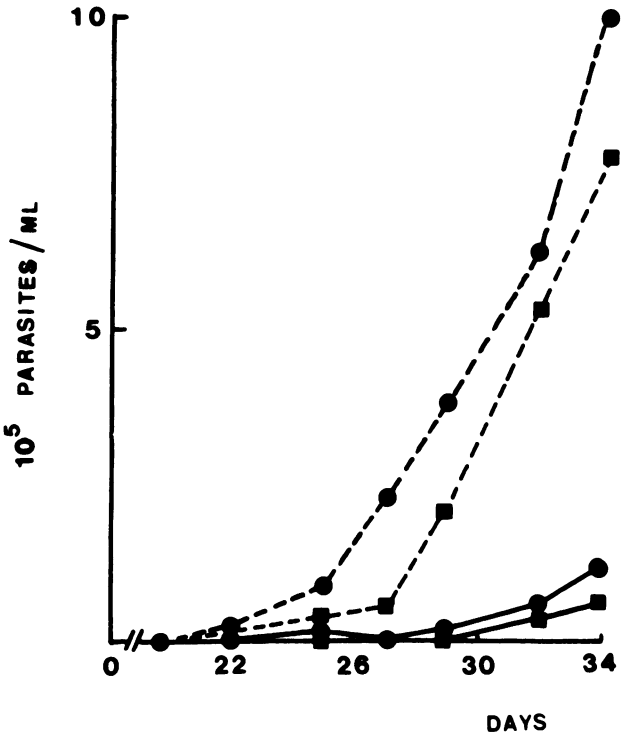

FIG. 5. Inhibition of infectivity of *T. cruzi* metacyclic trypomastigotes by the monoclonal antibody 10D8. Mice injected intravenously with 10^5 Tulahuen metacyclic forms preincubated with normal mouse serum (●-----●), sera of mice bearing hybridoma 10D8 (●——●), PBS–1% bovine serum albumin (■-----■), or Fab fragments of 10D8 in PBS–1% bovine serum albumin (■——■). The parasitemia was measured by examining 5-μl blood samples under a phase-contrast microscope. Values are the means of five animals.

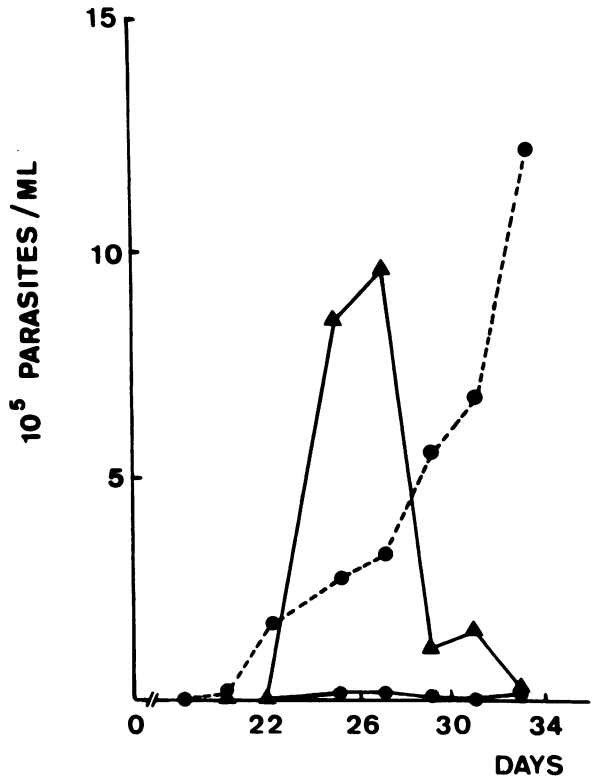

FIG. 6. Time course of *T. cruzi* infection in mice inoculated with the monoclonal antibody 10D8. Mice received intravenously PBS (●-----●) or 0.5 mg of the monoclonal 10D8 (●——●, ▲——▲) 30 min before challenge with 10^5 Tulahuen metacyclic forms. The parasitemia was measured by examining 5-μl blood samples under a phase-contrast microscope. The values are the means of two (▲——▲), three (●——●), and five (●-----●) animals.

controls (Fig. 6). In another set of experiments we used an in vitro tissue culture system to test the ability of 10D8 to block cell invasion by metacyclic forms. The invasion of Vero cells by Tulahuen and G metacyclic trypomastigotes preincubated with 10D8, at a nonagglutinating concentration (10 μg/ml), was inhibited by more than 50% (Table 1). The unrelated monoclonal antibody 3D11, directed to the *P. berghei* sporozoite surface antigen (17), did not display any inhibitory effect, even at a concentration 50 times higher than that of 10D8 (Table 1).

DISCUSSION

The characterization of cell membrane components of *T. cruzi* metacyclic trypomastigotes, the vector-borne developmental stages infective to the mammalian host, is of critical importance to an understanding of the process by which the first parasite-host cell interaction is established.

In the past few years, we have addressed the question of which metacyclic surface components could be implicated in *T. cruzi* infection by using monoclonal antibodies to metacyclic surface antigens. Among the possible candidates is a metacyclic-stage-specific 90-kDa polypeptide, identified by the monoclonal antibody 1G7 (11), which was shown to reduce the metacyclic form infectivity in mice (2) and to inhibit the adhesion and interiorization of metacyclic forms in cultured Vero cells (J. C. Gonzalez and N. Yoshida, unpublished data).

We have found in this work that the infectivity of *T. cruzi* metacyclic forms in mice decreased upon interaction with the monoclonal antibody 10D8 or its Fab fragments (Fig. 5). This antibody reacted with glycoconjugates of approximately 35 and 50 kDa present on the parasite cell surface (Fig. 2). The mechanism by which the antibody 10D8 interferes with the metacyclic infectivity, leading to reduced parasitemia levels in mice, is not clear. One possibility is that

TABLE 1. Effect of monoclonal antibody 10D8 on the invasion of Vero cells by *T. cruzi* metacyclic trypomastigotes[a]

Strain	Expt	Monoclonal antibody (μg/ml)	Parasites/100 cells[b]	Infected cells/100 cells[b]	Inhibition (%)
G	1		6.2 ± 0.4	5.9 ± 0.5	0
		3D11 (500)	5.9 ± 0.5	5.6 ± 0.4	5.1
		10D8 (10)	3.0 ± 0.2	2.6 ± 0.2	55.9
	2		5.6 ± 0.6	5.4 ± 0.6	0
		10D8 (10)	2.0 ± 0.6	1.8 ± 0.4	66.6
Tulahuen	1		8.7 ± 1.9	8.7 ± 1.9	0
		3D11 (500)	9.2 ± 1.2	8.4 ± 1.0	3.5
		10D8 (10)	3.3 ± 0.7	3.3 ± 0.7	62.1
	2		9.6 ± 2.4	8.8 ± 2.0	0
		10D8 (3)	4.6 ± 1.8	4.4 ± 1.8	50.0

[a] Metacyclic forms preincubated in RPMI 1640 with or without antibody were seeded onto Vero cells and cultured for 3 h. The infection of cells was determined by counting the number of infected cells and the number of intracellular parasites in a total of 500 cells stained by May Grünwald-Giemsa.

[b] The values are the means ± the standard deviations of duplicate samples.

the binding of 10D8 to the metacyclic surface antigens blocks the parasite entry into host cells, as suggested by the in vitro assay showing that the monoclonal antibody 10D8 inhibited the adhesion and invasion of Vero cells by metacyclic forms (Table 1).

Several pieces of evidence indicate that the 35- and 50-kDa antigens may be glycolipids. Unlike the 90-kDa protein, which was intensely labeled with ^{131}I or with [^{35}S]methionine (11), the 35- and 50-kDa antigens were not detected by any of these procedures, being revealed only by immunoblot (Fig. 1) or by immunoprecipitation of metacyclic forms surface labeled with galactose oxidase and sodium boro[^{3}H] hydride (Fig. 2). Furthermore, in preliminary experiments, we have found that 10D8 recognized the 35- and 50-kDa antigens on an immunoblot of glycolipids isolated from culture-derived parasites by the hexane-isopropanol procedure (data not shown).

The metacyclic-form-neutralizing effects of 10D8 and 1G7 (2) suggest that there is a specific receptor(s) for these antigens on the host cell surface. Whether the 90-kDa protein (2, 11) and the 35- and 50-kDa antigens recognize different receptors or interact with distinct sites on the same component remains to be established.

It is interesting that the 35- and 50-kDa antigens, together with the 90-kDa polypeptide, constitute one of the major bands detected by the sera of mice immunized with killed metacyclic trypomastigotes (Fig. 4), which were shown to be protected against acute *T. cruzi* infection (16). This finding reinforces the notion that the 35- and 50- and the 90-kDa antigens may represent the main surface molecules of metacyclic trypomastigotes capable of associating with the host cell to initiate *T. cruzi* infection. This resembles the interaction of another group of trypanosomatids, the *Leishmania* species, with macrophages. In *Leishmania* promastigotes, two major glycoconjugates have been identified as receptors for macrophages: a lipophosphoglycan (4) and a glycoprotein of 63 kDa (10). It has recently been reported that these antigens, incorporated into liposomes, induce protective immunity against cutaneous leishmaniasis (9).

Whether *T. cruzi* surface molecules involved in the process of host cell invasion are of immunoprophylactic value, as seems to be the case in *Leishmania* spp. (5, 9), remains to be investigated. Among the molecules to be examined for that purpose we include the 35- and 50- and 90-kDa antigens, which are immunogenic and appear to be important elements of the repertoire of metacyclic surface components associated with *T. cruzi*-host cell interaction.

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