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Monoclonal Antibodies to Stages of *Trypanosoma cruzi*: Characterization and Use for Antigen Detection

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Monoclonal antibodies to the amastigote and epimastigote stages of *Trypanosoma cruzi* were produced and characterized by immunoglobulin class and subclass. Of the 17 monoclonal antibodies, 14 were of the immunoglobulin M (IgM) class and 2 were of the IgG2 and 1 was of the IgG1 subclass of IgG. Five of the monoclonal antibodies recognized the antigens of amastigotes only, two recognized the antigens of epimastigotes only, and ten recognized an antigen(s) common to both stages of *T. cruzi*. By using an immunofluorescence test with monoclonal antibodies, it was possible to visually localize amastigote- or epimastigote-specific antigens and the antigens common to both. Antigens specific for epimastigotes were noted on the flagellum or in spots over the entire body of the parasite. The antigens common to both amastigotes and epimastigotes were on one of the extremities of the amastigotes and on the region of the flagellar pouch of the epimastigotes. Four of the monoclonal antibodies were capable of detecting *T. cruzi* antigen in serum from mice infected with the parasite and in the supernatant of infected cell cultures, suggesting that monoclonal antibodies may be useful for antigen isolation and diagnostic methods.

Chagas' disease is a chronic, debilitating illness which affects millions of people in South and Central America (7). The causative agent, *Trypanosoma cruzi*, undergoes transformation from the epimastigote stage to the metacyclic trypomastigote stage in the arthropod vector and to the blood trypomastigote and the intracellular amastigote stages in the vertebrate host (17). These parasitic forms differ in their susceptibility to lysis by human or animal serum and their fate within phagocytic cells (9, 14). Recent publications have focused on the study of the cell membrane of the parasite to elucidate the mechanisms involved in these differences. Proteins and glycoproteins distinct for each form of the parasite have been identified, and a number of them have been shown to be recognized by antibodies in the sera of infected animals and humans (1, 5, 6, 22, 23, 25). In addition, different strains of *T. cruzi* have different tropisms for various cells or tissues in the host (16) and may cause different types of disease (19). In this context, a number of attempts have been made to correlate certain characteristics, such as the isoenzyme and kinetoplast DNA patterns of particular strains of *T. cruzi*, to the type of disease observed in endemic areas (18, 20, 21). As part of our ongoing effort to isolate and study pure antigens of *T. cruzi* which may be relevant for immunization and diagnosis, we report here the development and characterization of monoclonal antibodies to antigens of the amastigote and epimastigote stages of the parasite. Some of the monoclonal antibodies were specific for the antigens of amastigotes or epimastigotes only, and some recognized an antigen common to both stages. The use of monoclonal antibodies in an immunofluorescent antibody assay (IFA) allowed the visual localization of epimastigote-specific antigen and of an antigen common to both amastigotes and epimastigotes. Because a number of reports have presented evidence for the presence of circulating antigens of *T. cruzi* in the sera of animals (2, 10, 12) and possibly of humans (4, 12) infected by the parasite, some of the monoclonal antibodies were examined in an enzyme assay designed for the detection of such antigens. A number of the monoclonal antibodies tested to date were capable of detecting antigen in the serum of animals infected with *T. cruzi*, suggesting their potential for use in the diagnosis of infections caused by this parasite.

MATERIALS AND METHODS

Growth of epimastigotes and amastigotes of *T. cruzi*. The Y strain of *T. cruzi* was cloned by the method of Goldberg and Chiari (11). Epimastigotes were grown in the liquid medium described by Bone and Steinert (8) supplemented with 3% calf serum. Cultures were kept at 28°C for 5 days, and 100% of the organisms were epimastigotes as determined by microscopy of preparations examined fresh and after staining with Giemsa stain. Amastigotes were obtained from cul-

tures of L929 cells infected with blood trypomastigotes 7 days before being harvested (6). Contamination of the amastigotes with trypomastigotes was less than 0.01%.

Preparation of monoclonal antibodies to *T. cruzi*. Female BALB/c mice were injected intraperitoneally with membrane-rich preparations of either amastigotes or epimastigotes. The membrane preparations were obtained by disrupting 10^8 parasites by four pulses of sonication lasting 30 s each. Sonication was performed in an ice bath in a Biosonik apparatus (Bronwill Scientific Inc., Rochester, N.Y.) set at 60 cycles/s. Two cycles of centrifugation at 1,500 × *g* for 20 min and at 37,000 × *g* for 2 h were performed after sonication of the organisms. The sediment of the final centrifugation was suspended and adjusted to 1 mg of protein per ml in phosphate-buffered saline (PBS; pH 7.2). This preparation (0.2 ml) was injected intraperitoneally without adjuvants into each mouse weekly for 3 weeks. A fourth immunization was performed 4 days before the spleen cells were harvested for fusion with the NS−1 variant of the P3 (MOPC2) myeloma cell line. The cells were fused by the method of Kohler and Milstein (15), and the antibody-secreting hybridomas were cloned by the soft-agar technique of Sharon et al. (24).

Screening and characterization of the monoclonal antibodies. Antibody production by the hybrid cells was assessed between days 14 and 21 after fusion by an enzyme immunoassay (EIA). Briefly, antigens from the sonicated amastigotes and epimastigotes were diluted to 50 μg of protein per ml in 0.1 M sodium carbonate buffer (pH 9.6), and 100 μl of the solution was dispensed into each well of a U-bottom, 96-well polyvinyl chloride plate. A 1 mg/ml solution of carbodiimide in sodium carbonate buffer (10 μl) was added to each well, and the plates were incubated overnight at 4°C. The plates were then washed with PBS containing 0.05% Tween-20 (PBS-Tween) and incubated for 30 min at room temperature with 0.1 M ammonium chloride to bind the residual groups. After the plates were washed with PBS-Tween, the hybridoma culture supernatants were added to the wells and incubated for 2 h at 37°C. Serum from mice chronically infected with *T. cruzi* and supernatant from the NS-1 cell culture medium were used as positive and negative controls, respectively. The plates were again washed with PBS-Tween, 100 μl of diluted conjugate [sheep $F(ab')_2$ anti-mouse immunoglobulins conjugated with β-galactosidase] was added to each well, and the plate was incubated for at least 2 h at 37°C. The plates were washed again, and the substrate (*p*-nitrophenyl-β-D-galactoside), prepared in a solution containing 2-mercaptoethanol, was added. The reaction was read in an automatic enzyme-linked immunosorbent assay reader set at 405 nm.

Immunoglobulin characterization of monoclonal antibodies. Characterization of the monoclonal antibodies by their immunoglobulin class or subclass was performed by an indirect IFA in which fluorescent-labeled rabbit anti-mouse immunoglobulin M (IgM), IgG1, IgG2, and IgG3 conjugates specific for heavy chains were used (13). Before conjugation, the rabbit antisera were absorbed with various myeloma immunoglobulins and found to be specific by double diffusion in agar and by immunofluorescence (13). Formalin-killed amastigotes and epimastigotes obtained as described above and trypomastigotes obtained from acutely infected mice were fixed on IFA microscope slides and treated with the supernatant of the hybridoma cell cultures for 1 h at 37°C. After being washed with PBS and distilled water, the slides were incubated with the specific conjugates for 1 h at 37°C, washed again, mounted with 1% glycerol in carbonate buffer (pH 9.6), and examined under a fluorescent microscope with incident UV light.

Detection of circulating antigens. A double-sandwich EIA (3) was used to determine the ability of monoclonal antibodies to detect circulating antigens of *T. cruzi* in serum from mice infected with the parasite and antigen in the supernatant of infected cell cultures. Microtiter plates were coated overnight at 4°C with 0.1 ml of each monoclonal antibody (protein concentration, 100 μg/0.1 ml) in carbonate buffer (pH 9.6). The monoclonal antibodies used to coat the microtiter plates were purified from culture supernatants by precipitation with ammonium sulfate. The plates were washed with PBS-Tween, and filtered serum from mice acutely infected with *T. cruzi* or the supernatant of infected cell cultures was added to the coated wells. The plates were incubated at 37°C for 1 h and then overnight at 4°C. After the plates were washed with PBS-Tween, 0.1 ml of a conjugate prepared by coupling rabbit anti-*T. cruzi* IgG antibodies to alkaline phosphatase was added to each well, and the plates were incubated at 37°C for 1 h. Another wash was followed by the addition of the substrate (*p*-nitrophenylphosphate disodium). Filtered serum from normal mice and the supernatant of noninfected cell cultures were used as controls (3). The reaction was read in an automatic enzyme-linked immunosorbent assay reader set at 405 nm after 1 h of incubation at room temperature. Readings at least two times higher than the average of eight negative control well readings were considered positive.

RESULTS

We obtained 17 hybridomas producing antibodies to *T. cruzi*, as detected by EIA IFA, and characterized them by immunoglobulin class (Table 1). Of these, 11 were derived from mice immunized with the amastigote preparation and 6 from mice immunized with the epimastigote preparation. Of the 11 hybridomas derived from mice immunized with the amastigote preparation, 6 produced antibody which also recognized an antigen of epimastigotes. Similarly, three of the hybridomas derived from mice immunized with the epimastigote preparation produced antibody which also recognized an antigen of amastigotes.

Of the 17 antibody-producing clones, 14 produced antibody of the IgM class, 2 produced the IgG2 subclass, and 1 produced the IgG1 subclass.

The pattern of fluorescent staining in the IFA with Formalin-killed amastigotes or epimastigotes varied from bright rims or spots over the entire organism to a stain located in a particular area of the parasite. A representative set of the various patterns is shown in Fig. 1. The mono-

TABLE 1. Screening and characterization of monoclonal antibodies to amastigotes and epimastigotes of *T. cruzi* by EIA and IFA

Immunizing antigen	Clone	Antibody detected by:				Isotype
		EIA		IFA		
		Bound to amastigote	Bound to epimastigote	Bound to amastigote	Bound to epimastigote	
Amastigote	VP6AD2-1	+	−	+	−	IgM
Amastigote	VP6AD2-2	+	−	+	−	IgM
Amastigote	VP6AD2-3	+	−	+	−	IgM
Amastigote	VP6AB1-1	+	+	+	+	IgM
Amastigote	VP6AB4-2	+	−	+	−	IgM
Amastigote	VP6AB6-1	+	−	+	−	IgM
Amastigote	VP6AD4-2	+	+	+	+	IgM
Amastigote	VP6BB1-6	+	+	+	+	IgM
Amastigote	VP9BC6-1	+	+	+	+	IgM
Amastigote	VP9CB2-3	+	+	+	+	IgM
Amastigote	VP9CB2-3A	+	+	+	+	IgG2
Epimastigote	VP7BC4-2A	+	+	+	+	IgM
Epimastigote	VP7BC4-3A	−	+	−	+	IgG1
Epimastigote	VP7BC4-3B	−	+	−	+	IgG2
Epimastigote	VP7BD4-5	+	+	+	+	IgM
Epimastigote	VP7DC1-7	+	+	+	+	IgM
Epimastigote	VP9CB2-2	+	+	+	+	IgM

clonal antibody VP7BC4-3B, derived from mice immunized with the epimastigote preparation and of the IgG2 subclass, was specific for epimastigotes and bound to an antigen which was localized in discrete spots over the entire parasite (Fig. 1A). On the other hand, monoclonal antibody VP7BC4-3A, similarly derived from mice immunized with the epimastigote preparation but of the IgG1 subclass, was also specific for epimastigotes and bound to an antigen localized on the flagellum of the parasite (Fig. 1B). The use of a monoclonal antibody directed against an antigen common to both amastigotes and epimastigotes allowed visual localization of the antigen in both stages of *T. cruzi*. Thus, monoclonal antibody VP7DC1-7, derived from mice immunized with the epimastigote preparation and of the IgM class, bound to an antigen located on one of the extremities of the amastigotes (Fig. 1C), whereas in the epimastigotes the same antibody bound to an antigen located in the flagellar pouch and in the flagellum of the organism (Fig. 1D). The IFA test with Formalin-killed organisms does not provide information on whether the antigens are surface or internal antigens. However, preliminary results of experiments in which the IFA test was performed with live organisms revealed that one monoclonal antibody (VP7BC4-3B) recognizes an antigen which is in the cytoplasm of the epimastigotes.

Eight of the seventeen monoclonal antibodies were examined for their ability to detect antigens of *T. cruzi* present in either the circulating blood of infected mice or the supernatant of infected cell cultures (Table 2). Of the five monoclonal antibodies derived from mice immunized with the amastigote preparation, four detected antigens in the serum from infected mice, but only three of them detected antigen in the supernatant of infected cell cultures. Two monoclonal antibodies derived from mice immunized with the epimastigote preparation detected antigens only in the supernatant of infected cell cultures. Two monoclonal antibodies, one derived from mice immunized with antigens of the amastigote and the other from mice immunized with antigens of the epimastigote stage of *T. cruzi*, did not detect antigens in either the serum from infected mice or the supernatant of infected cell cultures.

DISCUSSION

The results presented in this report show that there are antigens which are specific for the amastigote and epimastigote stages of *T. cruzi* and antigens which are shared by both stages. Because amastigotes change to epimastigotes or trypomastigotes in culture, the results suggest that there are antigens which are acquired or lost as the parasite undergoes transformation from one stage to another and antigens which are retained throughout the transformation process. It is interesting to note that none of the monoclonal antibodies tested to date reacted with antigens of trypomastigotes obtained either from the blood of infected mice or from infected cell cultures (data not shown). On the other hand,

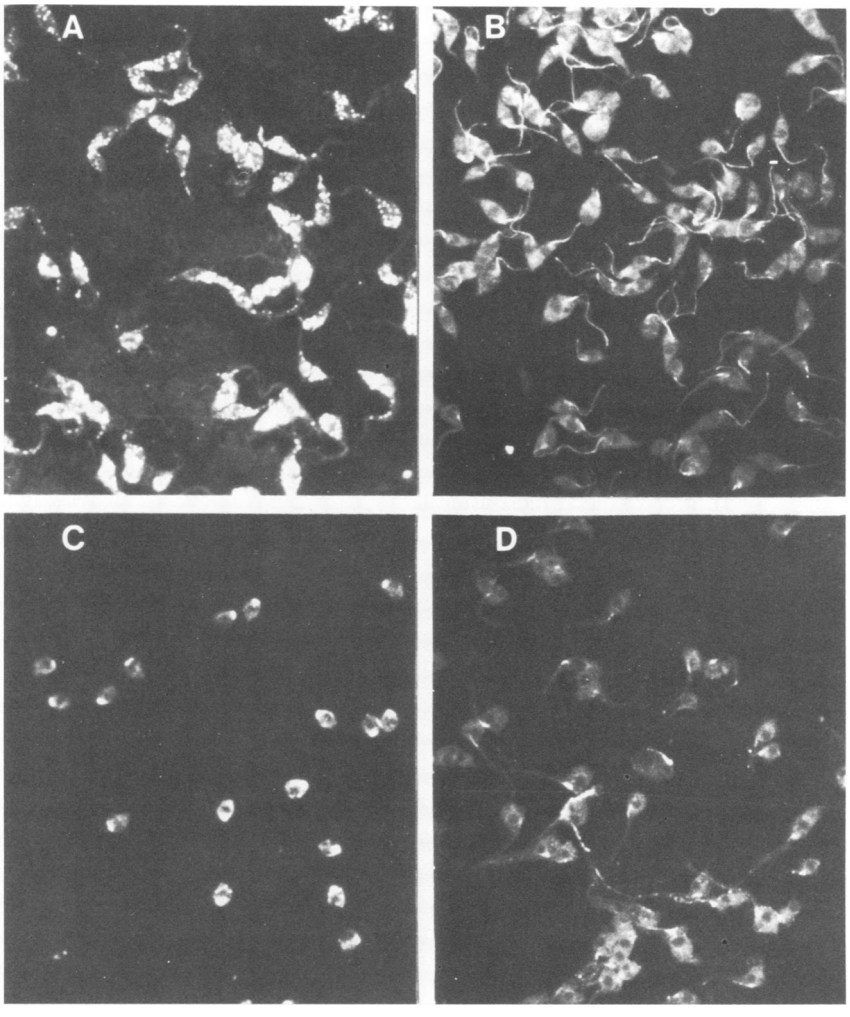

FIG. 1. IFA with monoclonal antibodies to antigens of amastigotes and epimastigotes of *T. cruzi*. (A) Epimastigotes treated with monoclonal antibody VP7BC4-3B. Fluorescent staining, represented by bright spots over the entire organism, was noted only with anti-IgG2. (B) Epimastigotes treated with monoclonal antibody VP7BC4-3A. Fluorescent staining was localized on the flagella of the parasites and was noted only with anti-IgG1. (C and D) Amastigotes and epimastigotes, respectively, treated with monoclonal antibody VP7DC1-7. Fluorescent staining occurred with anti-IgM only and was represented by a bright spot in one of the extremities of the amastigotes (C) and in the epimastigotes by a bright spot in the region of the flagellar pouch (D). In some organisms the fluorescent staining extended over the flagellum.

one monoclonal antibody, VP6AD2-1, which reacted in the IFA with an antigen of amastigotes of the Y strain of *T. cruzi*, was tested and reacted similarly with amastigotes of the Tulahuen, CL, and MR strains of this parasite. These strains are from widely different geographic areas: strain Y is from Central America, strains CL and MR are from South Brazil, and strain Tulahuen is from Chile. Moreover, they present distinct morphological characteristics in the blood stage (9) and in their tropisms for different tissues of the vertebrate host (16). The fact that monoclonal antibody VP6AD2-1 reacted with amastigotes of these four strains indicates that common antigens exist among distinct populations of *T. cruzi*.

Most of the monoclonal antibodies were of the IgM class. The reason for the high prevalence of hybridomas producing antibodies of this class is not clear; it may be due to the antigen employed to immunize the mice or to the immunization protocol. This is suggested by the fact that mice infected with live blood trypomastigotes formed antibodies of the IgM class and of the IgG1, IgG2, and IgG3 subclasses. These antibodies appeared at different times during the infection:

TABLE 2. Detection of antigens of *T. cruzi* by EIA with monoclonal antibodies

Immunizing antigen	Monoclonal antibody	Antigen detected in:	
		Serum from infected mice	Supernatant of infected cell cultures
Amastigote	VP6AD2-1	+	+
Amastigote	VP6AD2-2	+	+
Amastigote	VP6AB1-1	+	+
Amastigote	VP6AB4-2	−	−
Amastigote	VP6AD4-2	+	−
Epimastigote	VP7BC4-2A	−	−
Epimastigote	VP7BD4-5	−	+
Epimastigote	VP9CB2-2	−	−

IgM after 4 days, IgG1 after 28 days, IgG2 after 12 days, and IgG3 after 20 days of infection (Araujo et al., manuscript in preparation).

Immunization procedures other than those used in the present study are now being employed in an effort to increase the yield of hybridomas that produce antibodies of other immunoglobulin classes.

Our results also showed that monoclonal antibodies may be used to detect circulating antigens of *T. cruzi* in the sera or other body fluids of patients infected with the parasite. None of the three monoclonal antibodies derived from the spleen cells of mice immunized with the epimastigote preparation and used for antigen detection were capable of detecting circulating antigens in the sera of infected mice. This observation is of interest because epimastigotes such as those that are obtained in large numbers in acellular cultures are not observed in infected vertebrate hosts. Because of the exquisite specificity of monoclonal antibodies, diagnostic methods based on antigen detection may be developed which use these antibodies. Also, the isolation of specific cell membrane or cytoplasmic antigenic components will be made possible by the use of monoclonal antibodies. The isolation of pure antigens will be an important step which will allow us to examine the antigens which stimulate a protective immune response, thereby providing information for the formulation of a vaccine against Chagas' disease. Moreover, monoclonal antibodies may be helpful in determining the exact role that antibodies play in resistance to *T. cruzi* and the mechanisms by which the parasite survives in the hostile environment of the host.

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