A TRYPANOSOMA CRUZI MONOCLONAL ANTIBODY THAT RECOGNIZES
A SUPERFICIAL TUBULIN-LIKE ANTIGEN

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A monoclonal antibody (MAB 10), obtained from mice infected with Trypanosoma cruzi, was found to recognize a superficial antigen in living or fixed parasites. It reacted more strongly with T. cruzi than with related parasites such as T. brucei and Leishmania. In immunoblots it recognized a single trypanosoma polypeptide and also brain tubulin, both of which had the same electrophoretic mobility. Further analysis suggested that the α -tubulin subunit contained the epitope recognized by MAB 10. These results suggest that a surface tubulin-like protein is present in T. cruzi. © 1986

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Trypanosoma cruzi is the ethiological agent of American trypanosomiasis (Chagas' disease) which affects an estimated 20 million individuals in the Western hemisphere (1). This disease leads to autoimmunity as shown by the presence in patients' sera of EVI antibodies against normal endocardium, vascular structures and the interstitium of cardiac muscle (2) together with others that react against Swann cells (3), Laminin (4) and antigens from striated muscle (5). In addition, some antibodies are produced against important surface proteins of T. cruzi such as the glycoproteins Gp 90 (6), Gp 85 (7) and Gp 72 (8). Of particular

Abbreviations used : ELISA, enzyme-linked immunoabsorbent assay; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

interest to the present study was the possibility that antibodies produced by infected animals might react with a superficial protein in T. cruzi that was also present in host cells. Our results suggested that a monoclonal antibody obtained from infected mice produced antibodies that recognized superficial tubulin-like antigens present in all morphologies of T. cruzi.

METHODS

Growth of parasites

The strain of T. cruzi used in this study, originally isolated from a clinical case, was obtained from Dr. Jorge Albar (Centro de Virología, Majadahonda, Madrid). The trypanosomes were maintained by subcultures in LIT medium (9). Metacyclic trypomastigotes were prepared by differential centrifugation of parasite cultures at 500 g for five minutes after which the supernatant was recentrifuged. This supernatant contained >95% metacyclic trypomastigote forms.

Preparation of monoclonal antibodies

Female Balb/c mice were immunized by injection with a mixture of morphologies of live T. cruzi (10^6 trypanosome per mouse) on the first (intraperitoneal), 15th (i.p.) and 27th (intramuscular) days. Five days after the last injection the spleen cells were removed and fused with P3/X63-Ag 8653 myeloma cells (10) as described previously (11). Successful hybrids were selected and screened for antibody production by enzyme-linked immunoabsorbent (ELISA) and indirect immunofluorescence assays. The selected hybrids were cloned three times.

Indirect immunofluorescence with the monoclonal antibody MAB 10

This technique was performed on either living or fixed parasites. For the latter, parasites were smeared onto glass slides, air-dried and fixed for 10 minutes at -20°C with acetone/methanol (1:1). Either fixed or living cells were incubated with the MAB 10 hybridoma ascites fluid (at a final dilution of 1/500 in PBS) for 1 hour at 4°C . Samples were then stained with a fluorescein-conjugated goat anti mouse IgG (Nordic, Tilburg, The Netherlands) diluted 1/100 in PBS for 1 hour at 4°C .

Enzyme-linked immunoabsorbent assay (ELISA)

The assay was adapted for use with intact cells (12) or extracts from "freeze-thaw" treatment with slight modifications. Briefly parasites (10^6 /ml of PBS) were heated at 50°C for 5 minutes before being washed and resuspended to 2.5×10^6 /ml of PBS. They were then applied to a 96-well polyvinyl chloride (PVC) plate, and incubated with serial dilutions of ascites fluid containing MAB 10, or control-ascites, for one hour at room temperature. A peroxidase-conjugated anti-mouse IgG was then added to each well, at a

final dilution of 1/500 in PBS/BSA, and incubated for one hour at 4°C. After reaction with the substrate (100 ml of distilled water, 0.47 g of citric acid, 0.73 g of Na_2HPO_4 , 40 mg of O-phenylenediamine and 30 μl of 30% H_2O_2), the coloured reaction product was measured with a multispectrophotometer (Titertek, Multiskan) at a wavelength of 405 nm.

Preparation of microtubule and trypanosome proteins

Pig brain microtubules were prepared by two cycles of temperature dependent recylation as described previously (13). Where indicated in Results, tubulin free of associated proteins was prepared by phosphocellulose chromatography (14) after which it was reduced and carboxymethylated (15), prior to electrophoresis. A protein extract was obtained from parasites, washed with PBS, by lysis with 0.5% w/v Nonidet P-40 (NP-40).

The NP-40 soluble proteins of *T. cruzi* and microtubule proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE) in an 8% polyacrylamide slab. Carboxymethylated tubulin was electrophoresed in a 5-15% gradient gel. Proteins were either visualized by staining with Coomassie blue or electrophoretically transferred to nitrocellulose membrane filters (16). The blots were incubated with the hybridoma culture supernatants (1/10 dilution) containing MAB 10 at 4°C overnight. They were then incubated with rabbit antimouse IgG (Nordic) followed by commercially iodinated protein A (100,000 cpm/ml -Amersham International, Amersham, U.K.).

RESULTS

Several monoclonal antibodies were obtained after infecting mice with *T. cruzi*. One of these, denominated MAB

Fig. 1. Indirect immunofluorescence assay of live epimastigote forms of *T. cruzi* with MAB 10. The photograph in (a) shows the diverse morphologies found in the *T. cruzi* cultures visualized by phase contrast microscopy. In (b) is shown a phase contrast micrograph of the same population after incubation with MAB 10. An immunofluorescence micrograph (c) of the same field shown in (b) indicates the superficial localisation of the epitope recognized by MAB 10. The agglutination of the parasites is also suggestive of superficial labelling with MAB 10.

Fig. 2. Indirect immunofluorescence assay of metacyclic trypomastigote forms of T. cruzi with MAB 10. The phase contrast micrograph and staining with Giemsa in (a) and (b), respectively, indicate that the population used was highly enriched in trypomastigote forms (>90%) of T. cruzi. The indirect immunofluorescence micrograph shown in (c) shows high surface labelling with MAB 10 in these infectious forms.

10, was the subject of the present study. When a live T. cruzi culture was incubated with MAB 10 followed by fluorescein-labelled rabbit anti mouse IgG, a strong fluorescence was observed that was maintained for long time periods, suggesting that the epitope recognized by this antibody was superficial and stable (Fig. 1). To show that the natural infectious forms (trypomastigotes) expressed this epitope, a preparation of T. cruzi enriched in these forms was fixed and analyzed by immunofluorescence with MAB 10. As shown in figure 2, the preparation, which contained mostly infectious forms, as determined by phase-contrast microscopy (a) and staining with Giemsa (b), was preferentially stained on the cell surface.

The specificity of MAB 10 for live T. cruzi was then determined by ELISA. As can be seen in Figure 3, the antibody had a higher affinity for intact T. cruzi than for an extract of lysed T. cruzi. Its affinity for intact Leishma-

Fig. 3. Measurement of the specificity of MAB 10 for *T. cruzi* and related parasites using ELISA. Ascites fluids containing MAB 10 were incubated with fixed (intact) *T. cruzi* (○), a lysed *T. cruzi* extract produced by a cycle of freeze-thawing in H₂O (□) (extr. Tc), fixed promastigote forms of *Leishmania mexicana* (●) (L.m.), and bloodstream (△) (TbT) or epimastigote (■) (TbE) forms of *T. brucei*. The results are plotted as absorbance (A 405 nm) of coloured reaction against log dilution of ascites fluid. The shaded area represents the limits of the different controls of the non-immune ascites.

Fig. 4. Immunoblotting analysis of trypanosome and microtubule proteins. Extracts of NP-40 soluble trypanosome proteins and twice cycled microtubule proteins were separated by SDS-PAGE in either an 8% gel (a) or a 5-15% (b) gradient gel. Panel (a) shows Coomassie blue staining of trypanosome proteins (1) and microtubule proteins (2) and the autoradiographs of their corresponding immunoblots (3 and 4, respectively) with MAB 10. In panel (b) is shown the Coomassie blue staining gel of phosphocellulose-purified tubulin (1) and its corresponding immunoblot (2). Arrows indicate the position of the tubulin band (55 kDa). The upper arrow in the Coomassie gel in panel (b) indicates the α -tubulin subunit and the lower arrow represents β -tubulin.

nia and bloodstream forms of T. brucei was three to four orders of magnitude lower.

To analyze the nature of the antigen recognized by MAB 10 at the molecular level, immunoblotting analyses were performed. MAB 10 recognized a protein of molecular weight about 50 kDa in NP-40 soluble extracts of T. cruzi (Fig. 4A). Brain tubulin, which had the same electrophoretic mobility as the trypanosome antigen, was also recognized by this antibody. When a further blot was performed on tubulin purified by phosphocellulose chromatography and carboxymethylated prior to electrophoresis, the antibody specifically recognized the α -subunit (Fig. 4B).

DISCUSSION

The results of the present study suggest that an antigen similar to α -tubulin is exposed on the surface of both infective and non-infective forms of T. cruzi. However, at this stage we cannot rule out the possibility that this may represent a cross-reaction of MAB-10 with the native form of the antigen from trypanosome extracts and α -tubulin in immunoblots. Nevertheless, native brain tubulin competes with the binding of MAB-10 to its natural epitope in ELISA experiments (unpublished results).

The antigenic determinant recognized by MAB 10 is much more abundant (or more specific) in T. cruzi than in other parasites belonging to the same family (L. mexicana) or genus (T. brucei) which showed a titre with MAB 10 of three or four orders of magnitude lower.

Interestingly, antibodies to tubulin have been found in sera from patients with Leishmania (17), although they could have been produced against tubulin released from lysed

parasites. However, patients infected with T. cruzi produced antibodies that cross-reacted with both laminin and T. cruzi (4).

The fact that MAB 10 recognizes both brain tubulin and a superficial T. cruzi antigen with the same molecular weight, suggests that the latter may represent a surface membrane-associated form of tubulin. In this respect, a protein similar in electrophoretic mobility to tubulin has been described in cultured neurons (18) and leukemic lymphocytes (19), the latter being a modified form of α -tubulin.

Taken together, these findings and the results of the present study would be consistent with the idea that some antibodies produced against T. cruzi in infected animals may recognize superficial tubulin in the membrane of host cells. We are currently investigating this possibility.

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