Title

Trypanosoma cruzi: Identification of a surface antigen restricted to the flagellar region of the infective form of the parasite

Journal

Experimental Parasitology, 70(4)

ISSN

0014-4894

Authors

Saborio, Jose L
Wrightsman, Ruth A
Kazuko, Sandra G
et al.

Publication Date

1990-05-01

DOI

10.1016/0014-4894(90)90125-v

EXPERIMENTAL PARASITOLOGY 70, 411–418 (1990)

Trypanosoma cruzi: Identification of a Surface Antigen Restricted to the Flagellar Region of the Infective Form of the Parasite

JOSE L. SABORIO, RUTH A. WRIGHTSMAN, SANDRA G. KAZUKO, BARBARA S. GRANGER, AND JERRY E. MANNING

Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

SABORIO, J. L., WRIGHTSMAN, R. A., KAZUKO, S. G., GRANGER, B. S., AND MANNING, J. E. 1990. *Trypanosoma cruzi:* Identification of a surface antigen restricted to the flagellar region of the infective form of the parasite. *Experimental Parasitology* 70, 411–418. A hybridoma cell line was derived from spleen cells of B6D2 mice infected with the Peru strain of *Trypanosoma cruzi.* The monoclonal antibody produced by this hybridoma, designated mAb20H1, reacts exclusively with molecular components of trypomastigotes, the infective form of the parasite. The results of indirect immunofluorescence and of immunoelectron microscopy with gold-tagged antibodies indicate that the 20H1 antigen is restricted to the surface of the part of the flagellum in contact with the cell body and to the surface of the cell body in the immediate vicinity of this organelle. Western blot analysis showed that the 20H1 antigen consists of four to five different molecules with sizes between 34 and 41 kDa, and that these molecules are glycoproteins with affinity for concanavalin A. In other strains of *T. cruzi,* mAb20H1 reacts with glycoproteins with apparent sizes that range between 37 and 43 kDa in the CL, Esmeraldo and Y strains, and between 41 and 45 kDa in the Silvio strain.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Trypanosoma cruzi;* Trypomastigote; Flagellar surface antigen; Immunocytochemical localization; Western blot analysis; Lectin affinity chromatography; Polyacrylamide gel electrophoresis (PAGE); Phosphate-buffered saline (PBS); Radioimmunoassay (RIA); Rhodamine (Rho); Fluorescein isothiocyanate (FITC); Concanavalin A (Con A); Dolicus biflorus agglutinin (DBA); Peanut agglutinin (PNA); Soybean agglutinin (SBA); Ulex europaeus agglutinin (UEA-I).

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas' disease, has a complex life cycle that includes phases of proliferation and differentiation in the intestinal lumen of the insect vector, and intracellular multiplication and differentiation in the mammalian host. Adaptation of *T. cruzi* to the conditions in these different environments has evolved into the development of specialized forms of the parasite, which exhibit distinct morphological, physiological, and biochemical characteristics (Vickerman and Preston 1976; De Souza 1984).

Among the transformations undergone by the parasite during its life cycle are remarkable changes in the organization of the flagellar apparatus. In trypomastigotes, the infective form of *T. cruzi,* the flagellum originates at the posterior end, has a portion attached to the entire length of the body, and a free portion that emerges from the anterior end. In comparison, in epimastigotes, the replicative form in the insect vector, the flagellum originates at the anterior end, attaches to the body for a short length, and emerges as a free organelle. Only traces of flagellum are found in amastigotes, the form of the parasite that replicates intracellularly in mammalian cells (Vickerman and Preston 1976; De Souza 1984).

Although it is generally acknowledged that the flagellum of *T. cruzi* is involved in cell locomotion, available information suggests that this organelle may also have a role in other vital events, such as the attachment of the parasite to different substrates, including the surface of cells in the

0014-4894/90 $3.00

intestinal tract of the vector and the surface of mammalian cells susceptible to invasion (Dvorak and Schmunis 1972; Vickerman and Preston 1976; De Souza 1984). Moreover, flagellar components have been associated with the induction of immunoprotection in mice (Segura *et al.* 1977) and more recently with the development of autoimmune pathology (Van Voorhis and Eisen 1989). Unfortunately, apart from the identification of the major components of the axonemic microtubules (Paulin *et al.* 1988) and the paraflagellar rod (Saborio *et al.* 1989), information on the molecular composition of this organelle is scarce. It is clear, however, that if functions as important as the induction of immunoprotection and autoimmune pathology may be associated with molecules that coexist in the flagellum, a thorough investigation of the flagellar components at different stages of the cell cycle will be necessary to understand the molecular and functional organization of this organelle.

We have undertaken the study of the molecular organization of the flagellum of *T. cruzi,* and have previously identified and characterized the major molecular components of the paraflagellar rod (Saborio *et al.* 1989). In this work we report on the identification of a surface antigen exclusively found in trypomastigotes and distributed along the part of the flagellar apparatus in contact with the cell body.

Materials and Methods

Parasites. Tissue culture-derived trypomastigotes from the CL, Esmeraldo, Peru, Silvio, and Y strains of *T. cruzi* were obtained from VA 13-infected cells as previously described (Beard *et al.* 1985).

Antibodies. B6D2 mice were infected with trypomastigotes of the Peru strain of *T. cruzi,* and the spleens of these animals were used to obtain antibody-producing hybridomas as described (Beard *et al.* 1985). Hybridomas producing antibodies with specificity for trypomastigote surface components were selected by a RIA assay, using as probe a preparation of trypomastigote surface antigens obtained by iminobiotinylation and chromatography on avidin–Sepharose (Beard *et al.* 1985).

Immunocytochemistry. Trypomastigotes were collected from tissue culture medium by centrifugation and were washed twice with PBS. The parasites were suspended at a density of 2×10^6 in 0.1 *M* phosphate buffer, pH 7.2, containing 4% carbodiimide. Aliquots of 125 μl of this suspension were pipetted onto glass coverslips that were kept at 4°C for 60 min.

For indirect immunofluorescence the cells were rinsed with PBS and were incubated with the antibodies with no further treatment or after fixation–permeabilization with methanol (10 min) and acetone (5 min) at −20°C. Incubation with primary antibodies or with Rho- or FITC-labeled secondary antibodies were done at room temperature for 30 min. After incubation with antibodies the preparations were mounted for microscopy as described (Saborio *et al.* 1989) and were observed with an epifluorescence microscope equipped with filters for the selective detection of rhodamine or fluorescein. Kokak Ektar 1000 film was used for the photography.

For immunoelectron microscopy (immuno EM), treatment with methanol–acetone was omitted. Incubation with antibodies was done as described, but in this case the secondary antibody was a gold-tagged (15-nm gold particles, Janssen Life Sciences Products) goat anti-mouse preparation. Cells were subsequently fixed with glutaraldehyde and processed for electron microscopy as described (Saborio *et al.* 1989).

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in one (1D-PAGE)- or two-dimensional (2D-PAGE) systems and Western blot analysis were done as described (Saborio *et al.* 1989).

Affinity chromatography. Chromatography on Con A-Sepharose and with other lectins was done as described (Katzin and Colli 1983).

Results

Identification of a surface antigen restricted to the flagellar region of trypomastigotes. An antibody-producing hybridoma, designated hyb20H1, was obtained from spleen cells of B6D2 mice infected with the Peru strain of *T. cruzi.* The antibody secretion by this hybridoma, designated mAb20H1, reacted specifically with trypomastigote surface proteins in a RIA assay.

The cellular distribution of the antigen reactive with mAb20H1 was studied by indirect immunofluorescence. *T. cruzi* trypomastigotes fixed with methanol–acetone were successively incubated with mAb20H1 and FITC-labeled goat anti-mouse antibodies. A positive reaction was obtained with

trypomastigotes of the CL, Esmeraldo, Peru, Silvio, and Y strains. A positive reaction, albeit slightly weaker, was also obtained when the methanol–acetone treatment was omitted. No positive reaction was detected in epimastigotes or amastigotes reacted with mAb20H1, nor when any of the forms of the parasite were reacted with mouse monoclonal antibodies not specific for *T. cruzi*-derived proteins. These observations, together with the proved selectivity of the procedures used for the isolation of hybridomas producing antibodies against surface antigens (Beard *et al.* 1985), indicate that the 20H1 antigen is a surface component exclusively found in the infective form of the parasite.

The results of the immunofluorescence assay indicate that the 20H1 antigen is distributed along a sharp, thread-like longitudinal region of the parasite's body (Fig. 1a). In an attempt to determine whether this staining pattern reveals a restricted area of the body, *T. cruzi* trypomastigotes were simultaneously incubated with mAb20H1 (mouse antibody) and polyclonal antibodies against tubulin (rabbit antibodies), followed by incubation with a mixture of Rho-labeled goat anti-rabbit and FITC-labeled goat anti-mouse antibodies. The results of this experiment indicate that, as expected, the entire cell body and the flagellum of the parasite are stained by the reaction of anti-tubulin antibodies with axonemic and subpellicular microtubules (Fig. 1a'). In contrast, the pattern obtained with mAb20H1 (Fig. 1a) is reminiscent of the specific flagellar staining previously observed in this laboratory with antibodies against paraflagellar proteins (Saborio *et al.* 1989). The results of a parallel experiment, in which trypomastigotes were simultaneously reacted with anti-tubulin antibodies and the paraflagellar specific mAbPAR 2 (Saborio *et al.* 1989), reveal that the latter antibody stains a restricted region of the cell body (Figs. 1b and 1b'), similar to the one recognized by mAb20H1. However, while mAbPAR 2 reacts with the entire length of the flagellum, mAb20H1 appears to react only with the flagellar structure associated with the body of the parasite, suggesting that the 20H1 antigen is localized to the flagellar region in contact with the cell body.

In order to determine whether the 20H1 antigen does indeed have a more restricted distribution than that of the known flagellar components, Par 1 and Par 2 (Saborio *et al.* 1989), a different double-labeling experiment was performed. Trypomastigotes were simultaneously incubated with mAb20H1 and a polyclonal antibody (rabbit) against the paraflagellar protein PAR 1 (pcAbPAR 1), followed by incubation with a mixture of Rho-labeled goat anti-rabbit and FITC-labeled goat anti-mouse antibodies. The results of this experiment indicate that, as previously observed (Saborio *et al.* 1989), pcAbPAR 1 reacts along the entire length of the flagellum (Fig. 1c'). However, while the 20H1 antigen has exactly the same distribution as PAR 1 in the portion of the flagellum that is attached to the cell body, this protein, unlike the Par protein, does not extend to the free portion of this organelle (Fig. 1c).

Ultrastructural localization of the 20H1 antigen. The distribution of the 20H1 antigen at a level of higher resolution was determined by immuno EM. *T. cruzi* trypomastigotes were fixed with 4% carbodiimide, incubated with antibodies, and prepared for electron microscopy as described under Materials and Methods. As shown in Figure 2a, which corresponds to a longitudinal section of a parasite that includes the entire length of the cell body and a small portion of free flagellum, the 20H1 antigen is concentrated along a narrow region of the body that extends from the posterior end to the region where the free flagellum starts. Most of the gold particles are on or next to structures distinguishable as axonemic microtubules (MT) or paraflagellar filaments (PAR), the two major flagellar

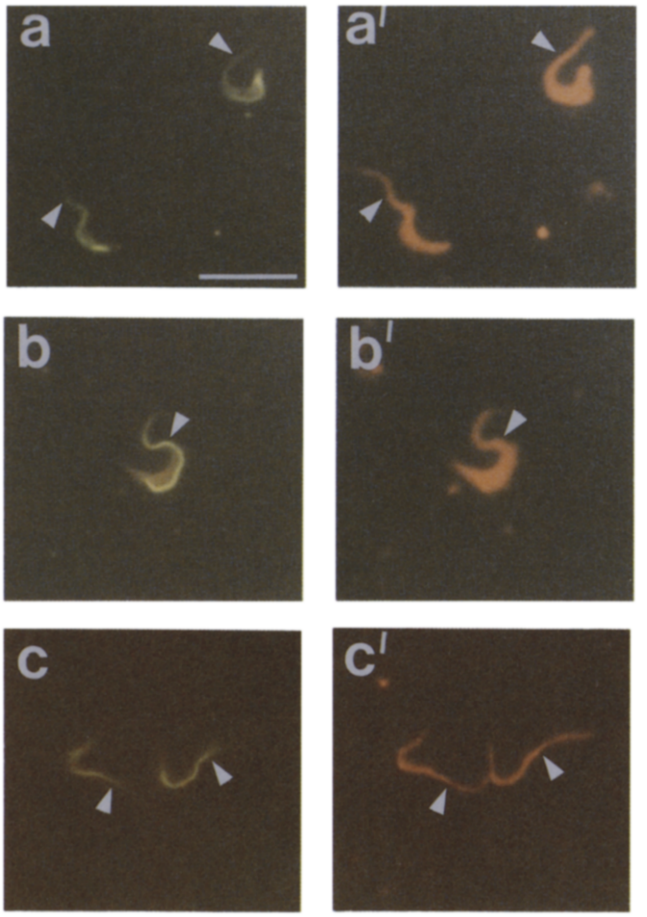

FIG. 1. Localization of the 20H1 antigen by indirect immunofluorescence. Trypomastigotes of the Peru strain of *T. cruzi* were fixed on glass coverslips with 4% carbodiimide in 0.1 *M* PO_4 buffer for 60 min at 4°C and with methanol (10 min) and acetone (5 min) at −20°C. The cells were then incubated with the following combinations of primary antibodies: mAb20H1 plus anti-tubulin (a and a'); mAbPAR2 plus anti-tubulin (b and b'); and mAb20H1 plus pcAbPAR1 (c and c'). mAb20H1 and mAbPAR2 are mouse and pcAbPAR1 and anti-tubulin are rabbit antibodies. After rinsing with PBS, the three preparations were incubated with a mixture of Rho-labeled goat anti-rabbit plus FITC-labeled goat anti-mouse antibodies. The cells were mounted for microscopy as described (Saborio *et al.* 1989). Photography was done with an epifluorescence microscope equipped with filters for the selective detection of rhodamine or fluorescein. Kodak Ektar 1000 film was used. The arrowheads signal the point of origin of the free portion of the flagella. The bar in (a) corresponds to 10 μm and also applies to the other panels in this figure.

components. However, in agreement with the results of the immunofluorescence experiments, no gold particles are detected in the visible part of the free flagellum. In cross sections of trypomastigotes, gold particles are found concentrated in a region of the cell surface located in the immediate vicinity of the flagellum (Fig. 2b) and in the membrane of that organelle (Fig. 2c), also in agreement with the distribution revealed by immunofluorescence.

Identification of the 20H1 antigen. Trypomastigote and epimastigote whole cell lysates from the Peru strain of *T. cruzi* were fractionated by 1D-PAGE and the proteins transferred to nitrocellulose paper were

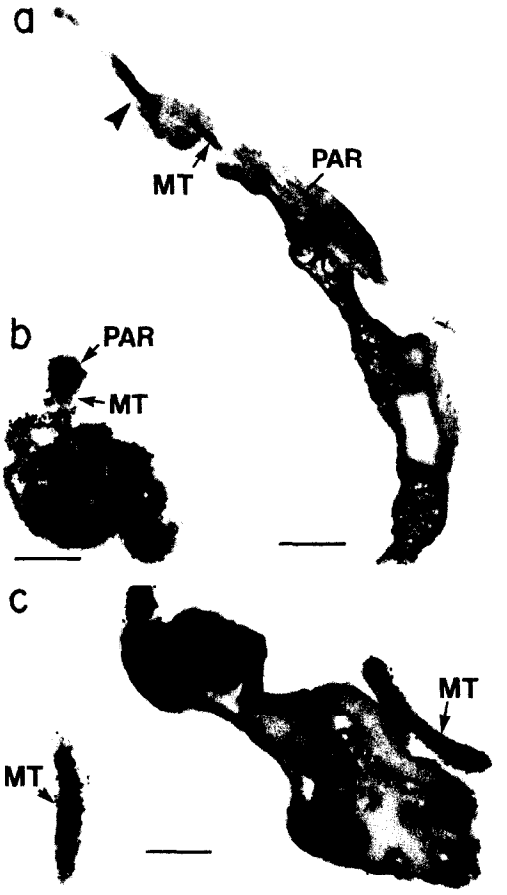

FIG. 2. Localization of the 20H1 antigen by immuno EM. Trypomastigotes of the Peru strain of *T. cruzi* were fixed on glass coverslips with 4% carbodiimide in 0.1 *M* PO4 buffer for 60 min at 4°C. The cells were then successively incubated with mAb20H1 and gold-tagged (15-nm gold particles) goat anti-mouse antibodies. After these incubations the preparations were fixed with glutaraldehyde, flat-embedded, and prepared for electron microscopy as described (Saborio *et al.* 1989). The different panels show: (a) longitudinal section of a parasite that includes the entire length of the body and a small segment of free flagellum. Axonemic microtubules (MT) and paraflagellar filaments (PAR), the major flagellar components, are identifiable in this and the other panels. The arrowhead in this panel signals the point of origin of the free portion of the flagellum; (b) cross section of a parasite in which the cell body, axonemic microtubules, and paraflagellar filaments are distinguishable; and (c) oblique sections of a cell body and two flagella in which axonemic microtubules are clearly seen. The bars in (a), (b), and (c) correspond to 750, 390, and 485 nm, respectively.

probed with mAb20H1. As shown in Fig. 3A, a broad region, corresponding to M_r between 34 and 41 kDa and in which four to five diffuse bands can be distinguished, is detected by the monoclonal antibodies in trypomastigote (lane c) but not in epimastigote (lane b) lysates. Western blot analysis of trypomastigote proteins fractionated by 2D-PAGE indicate that the proteins reactive with mAb20H1 separate into a major group with a p*I* value of 6.5 and a minor group of p*I* 6.8. Both groups, however, contain the same four to five different molecular components (Fig. 3B).

The broad and diffuse electrophoretic patterns (Figs. 3A and 3B), together with the results of the studies on cellular distribution (Figs. 1 and 2), suggested that the 20H1 antigen may correspond to a family of surface glycoproteins. To test for the presence of sugar residues, lysates from trypomastigotes of the Peru strain were chromatographed on five different lectins coupled to solid supports: Con A, DBA, PNA, SBA, and UEA-I. The results of these experiments indicate that all the molecular components reactive with mAb20H1 in whole cell lysates are retained on and are specifically released from Con A-Sepharose columns with an elution buffer containing a mixture of mannoside and glucoside (Fig. 3C, lane c). None of the other lectins tested showed any affinity for the molecules reactive with mAb20H1.

To test whether the 20H1 antigen is also present in other strains of *T. cruzi*, trypomastigote lysates from the CL, Esmeraldo, Silvio, and Y strains of the parasite were fractionated by 1D-PAGE and the proteins transferred to nitrocellulose paper were probed with mAb20H1. As shown in Fig. 3D, the 20H1 antigen is present in all the strains tested, although the electrophoretic patterns are not identical to that of the Peru strain. The patterns of the CL (lane c), Esmeraldo (lane d), and Y (lane g) strains are very similar to each other and consist of four to five diffuse bands with apparent mo-

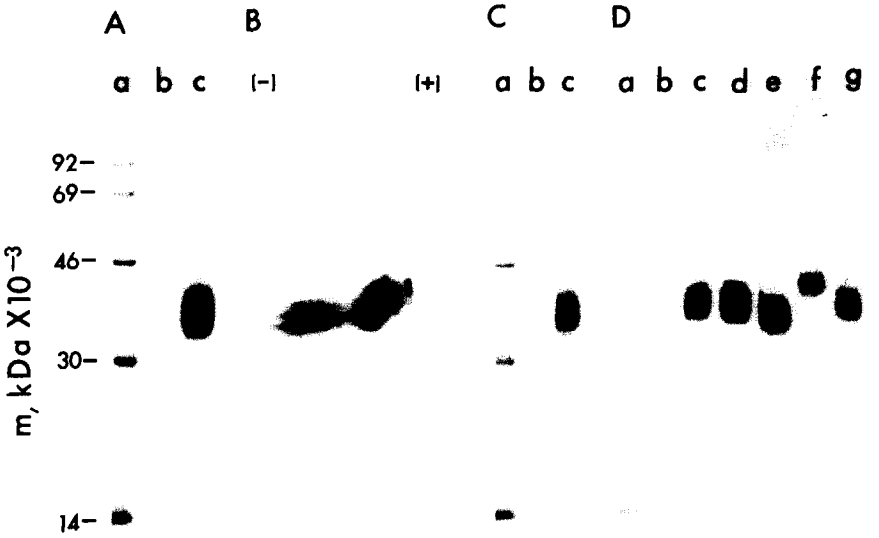

FIG. 3. Western blot analysis of different preparations of the 20H1 antigen. *T. cruzi* whole cell lysates (A, B, and D) or the material selected by affinity chromatography on Con A-Sepharose (C) were fractionated by 1D (A, C, and D)- or 2D-PAGE (B). The proteins transferred to nitrocellulose paper were probed with mAb20H1. The different panels correspond to: (A) Lane (a), size markers (phosphorylase b, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; and lysozyme, 14 kDa); lane (b), epimastigote lysate (Peru strain); lane (c), trypomastigote lysate (Peru strain). (B) Trypomastigote lysate (Peru strain) fractionated by 2D-PAGE. (C) Lane (a), size markers; lane (b), eluate from a Con A-Sepharose column loaded with trypomastigote lysate (Peru strain) and extensively washed with loading buffer; lane (c), material eluted from the Con A-Sepharose column with 0.1 *M* mannoside–0.1 *M* glucoside. (D) Lane (a), size markers; lane (b), epimastigote lysate; and trypomastigote lysates from the CL (lane c), Esmeraldo (lane d), Peru (lane e), Silvio (lane f), and Y (lane g) strains of *T. cruzi*.

lecular weights between 37 and 43 kDa, while the pattern of the Silvio strains is unique and corresponds to two to three diffuse bands with M_r between 41 and 45 kDa (lane f). As in the case of the Peru strain, the polypeptides reactive with mAb20H1 in the CL, Esmeraldo, Silvio, and Y strains can also be selected by affinity chromatography on Con A-Sepharose (not shown).

DISCUSSION

A *T. cruzi* antigen, exclusively found in trypomastigotes, the infective form of the parasite, was identified in this work with a monoclonal antibody (mAb20H1) produced by a hybridoma derived from spleen cells of mice infected with the Peru strain of *T. cruzi*. The results of immunocytochemical experiments, at the optical (Fig. 1) and electron microscopy (Fig. 2) levels, indicate that this antigen, designated 20H1, is restricted to the surface of the part of the flagellum attached to the cell body and to the surface of the cell body in the immediate vicinity of this organelle.

The possibility that the flagellar membrane of *T. cruzi* may correspond to a highly specialized region, with a protein composition qualitatively and quantitatively different from that of the cell body membrane, was clearly suggested by freeze–fracture studies on the surface of *T. cruzi* (De Souza *et al.* 1978). Recently, Van Voorhis and Eisen (1989) identified a 160-kDa polypeptide that, based on data from immunofluorescence and surface labeling experiments, was identified as a trypomastigote-specific antigen restricted to the

flagellar surface of the parasite. Although, to the best of our knowledge, the 20H1 antigen described in this paper is the first one unambiguously identified as a surface component of the flagellar region of *T. cruzi* by direct ultrastructural localization, based on the available information, it is reasonable to anticipate that other such molecules will be identified.

The molecular heterogeneity of the 20H1 antigen is intriguing. In our view there are at least three possible explanations for this heterogeneity: (a) mAb20H1 reacts with sugar residues common to a family of different polypeptides, (b) mAb20H1 reacts with a single polypeptide at different stages of post-translational processing, and (c) mAb20H1 reacts with common epitopes in a family of closely related but different polypeptides. The results indicating that the molecules reactive with mAb20H1 correspond to mannose-containing glycoproteins, specifically selected by affinity chromatography on Con A-Sepharose (Fig. 3C), are compatible with these three possibilities. However, although mannose residues are ubiquitous in *T. cruzi* glycoproteins (Doyle *et al.* 1986), in all the strains analyzed mAb20H1 reacts exclusively with molecules with an apparent M_r from 34 to 45 kDa (Fig. 3D), which indicates that if mAb20H1 was a carbohydrate-specific antibody, the residues involved would have to be other than mannose and restricted to a family of different polypeptides but all within a very narrow M_r range. This latter possibility seems unlikely. Moreover, fusion proteins with immunological determinants recognized by mAb20H1 have been detected in our laboratory during the screening of λ gt11 expression libraries (unpublished results). This observation supports the view that mAb20H1 is a polypeptide-specific antibody, but does not allow us to discriminate between the second and third possibilities. Likewise, we have no explanation for the differences in the electrophoretic patterns of the 20H1 antigen exhibited by the different strains of *T. cruzi*.

Currently we have no clues about the function of the 20H1 antigen. However, its presence in all the strains of *T. cruzi* analyzed suggests that 20H1 may be a conserved antigen associated with a vital function. Moreover, the observations reported raise important questions in connection with the sorting and anchoring mechanisms responsible for the restricted localization of the 20H1 antigen and as to whether these mechanisms may be shared by other trypanosomatides.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grant AI 18873 from the National Institutes of Health and the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases.

REFERENCES

BEARD, C. A., WRIGHTSMAN, R. A., AND MANNING, J. E. 1985. Identification of monoclonal antibodies against the trypomastigote stage of *Trypanosoma cruzi* by use of iminobiotinylated surface polypeptides. *Molecular and Biochemical Parasitology* 16, 199–212.

DE SOUZA, W. 1984. Cell biology of *Trypanosoma cruzi*. *International Review of Cytology* 86, 197–283.

DE SOUZA, W., MARTINEZ-PALOMO, A., AND GONZALEZ-ROBLES, A. 1978. The cell surface of *Trypanosoma cruzi;* cytochemistry and freeze-fracture. *Journal of Cell Science* 33, 285–299.

DOYLE, P., DE LA CANAL, L., ENGEL, J. C., AND PARODI, A. J. 1986. Characterization of the mechanism of protein glycosylation and the structure of glycoconjugates in tissue culture trypomastigotes and intracellular amastigotes of *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology* 21, 93–101.

DVORAK, J. A., AND SCHMUNIS, G. A. 1972. *Trypanosoma cruzi:* Interaction with mouse peritoneal macrophages. *Experimental Parasitology* 32, 289–300.

KATZIN, A. M., AND COLLI, W. 1983. Lectin receptors in *Trypanosoma cruzi:* An N-acetyl-D-glucosamine-containing surface glycoprotein specific for the trypomastigote stage. *Biochimica et Biophysica Acta* 727, 403–411.

PAULIN, J. J., KEITH, C. H., AND TARLETON, R. L. 1988. A monoclonal antibody to alpha tubulin rec-

ognizes host cell and *Trypanosoma cruzit* tubulins. *Journal of Protozoology* **35**, 123–129.

SABORIO, J. L., HERNANDEZ, J. M., NARAYANSWAMI, S., WRIGHTSMAN, R., PALMER, E., AND MANNING, J. 1989. Isolation and characterization of paraflagellar proteins from *Trypanosoma cruzi*. *Journal of Biological Chemistry* **264**, 4071–4075.

SEGURA, E. L., VAZQUEZ, C., BRONZINA, A., CAMPOS, J. M., CERISOLA, J. A., AND GONZALEZ CAPPA, S. M. 1977. Antigens of the subcellular fractions of *Trypanosoma cruzi*. II. Flagellar and membrane fraction. *Journal of Protozoology* **24**, 540–553.

VAN VOORHIS, W. C., AND EISEN, H. 1989. F1–160: A surface antigen of *Trypanosoma cruzi* that mimics mammalian nervous tissue. *Journal of Experimental Medicine* **169**, 641–652.

VICKERMAN, K., AND PRESTON, T. M. 1976. Comparative cell biology of the kinetoplastid flagellates. *In* "Biology of Kinetoplastida" (W. H. R. Lumsden and D. E. Evans, Eds.), pp. 35–130. Academic Press, New York.

Received 14 August 1989; accepted with revision 20 November 1989