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Immunocytochemical Localization of Neuraminidase in *Trypanosoma cruzi*

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A polyclonal antibody obtained against neuraminidase purified from *Trypanosoma cruzi* was used for the localization of the protein in whole cells by immunofluorescence microscopy and in thin sections of parasites (epimastigote, amastigote, and trypomastigote forms) embedded at a low temperature in Lowicryl K4M resin. The intensity of labeling, as evaluated by the number of gold particles associated with the parasite, varied according to the protozoan developmental stage. In the noninfective epimastigote forms, labeling of the cell surface was very weak. However, an intense labeling of some cytoplasmic vacuoles was observed. Labeling of the surfaces of most of the trypomastigote forms was weak, while gold particles were seen in association with the flagellar pockets of these forms, which suggests that the enzyme is secreted through this region. Intense labeling of the surfaces of many, but not all, transition forms between trypomastigote and amastigote forms was observed. Amastigote forms found in the supernatant of infected cell cultures had their surfaces intensely labeled, while few particles were seen on the surfaces of intracellular amastigotes. The results obtained are discussed in relation to the role played by *T. cruzi* neuraminidase in the process of parasite-host cell interaction.

Trypanosoma cruzi, the causative agent of Chagas' disease, presents the following three morphologically and physiologically distinct developmental stages: (i) epimastigote, the multiplicative form found in the insect vector; (ii) amastigote, the multiplicative form within mammalian host cells; and (iii) trypomastigote, a highly infective nonmultiplicative form which can be found in the bloodstream of the mammalian hosts and in the posterior portion of the intestine of the invertebrate host (for a review, see references 4 and 8).

Studies carried out in recent years have shown that infective forms of *T. cruzi* enter the vertebrate cells by an endocytic process (13) after an initial step of parasite-host cell recognition in which surface components of both cells play an important role (1–3, 22–24). It has been shown that trypomastigote forms of *T. cruzi* contain neuraminidase activity and that living parasites can desialylate mammalian cells in vivo and in vitro (13, 16, 17). A possible role for neuraminidase in the process of invasion of host cells by *T. cruzi* has been suggested (6, 9, 10, 18). Recent biochemical studies led to the isolation and characterization of the neuraminidase of *T. cruzi* and production of polyclonal antibodies which recognize a single band of M_r 60,000 (11) or a set of proteins with high molecular weights (165,000 to 200,000) and two additional bands of 79,000 and 82,000, which may represent degradation products of the higher-molecular-weight proteins (6).

In order to have a clear picture of the localization of neuraminidase in *T. cruzi*, we used a polyclonal antibody generated against the enzyme, which was purified from membranes of the parasite 5,000-fold to apparent homogeneity and which migrated as an entity of M_r 60,000 under denaturing conditions (11), to locate the sites containing the protein in whole parasites by immunofluorescence microscopy and in parasites embedded at a low temperature in Lowicryl K4M and labeled with gold particles. The results are described in this paper.

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MATERIALS AND METHODS

Parasites. Epimastigote forms of *T. cruzi* (Y strain) were cultivated for 4 days at 24°C in brain heart infusion broth plus liver extract and 10% bovine serum. Tissue culture trypomastigotes and amastigotes were obtained from the supernatant of Vero or NIH 3T3 cell cultures previously infected with bloodstream forms and grown at 37°C in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) containing 10% fetal calf serum. Only parasites of the first intracellular cycle were employed. Metacyclics were obtained from Grace's insect medium or TAUP medium (7) 6 days after incubation. Pure metacyclics or tissue culture trypomastigotes diluted in phosphate-buffered saline (PBS) (pH 7.2) plus 1% glucose (PBSG) were purified by chromatography on DEAE cellulose equilibrated in PBSG.

Antibodies. Antisera were produced in rabbits as previously described by Harth et al. (11). The enzyme was purified from soluble membrane proteins of the Y strain of *T. cruzi*, and the antisera were produced in New Zealand White rabbits. Rabbits received subcutaneous injections of 10 μg of purified and extracted neuraminidase in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.), and 10 days later, rabbits received a second injection of 15 μg of neuraminidase in incomplete Freund adjuvant. Immunoglobulins were prepared from postimmune sera taken 10 and 30 days after the second injection, as well as from preimmune sera, by chromatography on protein A–Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N. J.). These antibodies were characterized in detail previously (11). Their specificity for the neuraminidase is demonstrated (see Fig. 13).

Immunofluorescence. Immunofluorescence was carried out by the method of Wofsy et al. (25). Live parasites were harvested from culture media and centrifuged in a polypropylene tube at 1,000 × *g* for 10 min at 0°C. Parasites were washed two times in 2 ml of PBSG and centrifuged as before. Live parasites (5×10^6 parasites per ml) were incubated with antineuraminidase immunoglobulin G (1:20) for 30 min at 4°C in a cold room. Cells were then centrifuged as before, washed twice in PBSG, and incubated with fluorescein-

labeled goat anti-rabbit immunoglobulin G (Tago Immunologicals) (1:20) for 30 min in ice. Parasites were washed and centrifuged as described above after incubation with the primary antibody, washed once with PBS and once with PBS plus 5% bovine serum albumin, suspended in 100 μl of filtered 100% fetal calf serum, and then streaked out on a glass slide. Slides were dried at room temperature for 30 min. Samples were fixed in 95% ethanol for 20 s and allowed to dry at room temperature for 15 min; then, one drop of glycerol-PBS (9:1) was added. A cover slip was sealed to the slide with mounting medium. Fixed immunofluorescence assay was done with 1% formaldehyde prior to the incubation with the primary antibody. The rest of the procedure was identical to the one described for live IFA. Positive controls were done with Chagasic sera and anti-gp72 monoclonal antibodies against epimastigotes and tissue culture trypomastigotes. Negative controls were done with the corresponding preimmune sera. Prints were made in a Nikon Microphot Fx microscope with a magnification of ×1,200, oil immersion, and Ektachrome 400 film.

Electron microscopy. Two methods were used for localization of antigenic sites. First, the parasites were collected by centrifugation (2,000 × *g*) for 10 min at 4°C, washed twice with PBS, and fixed for 60 min in a solution containing 0.1% glutaraldehyde and 2% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer (pH 7.2). After the parasites were fixed, they were washed twice with PBS, incubated for 60 min with PBS containing 5% nonfat milk and 0.01% Tween 20 (pH 8.0) (PBS-milk-Tween), and then incubated for 60 min in the presence of PBS-milk-Tween containing a 1:5 dilution of antineuraminidase antibody. Cells were washed twice with PBS containing 1% bovine serum albumin and 0.01% Tween 20 (pH 8.0) (PBS-bovine serum albumin-Tween) containing a 1:20 dilution of gold-labeled goat anti-rabbit antibody (10 nm; E-Y Laboratories, San Mateo, Calif.). Cells were washed in PBS, post-fixed in osmium tetroxide, dehydrated in acetone, and embedded in Epon. Thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 100 CX electron microscope. Second, the parasites were collected and fixed in glutaraldehyde-formaldehyde as described above, dehydrated in 30 to 90% methanol, and embedded in Lowicryl K4M resin at −20°C. Thin sections were collected on 300-mesh nickel grids, incubated for 60 min at room temperature in the presence of PBS-milk-Tween (pH 8.0), and then incubated in the presence of PBS-milk-Tween containing a 1:5 dilution of antineuraminidase antibody. After the grids were incubated, they were rinsed in PBS-bovine serum albumin-Tween and then incubated for 60 min at room temperature in the presence of PBS-milk-Tween (pH 8.0) containing a 1:20 dilution of gold-labeled goat anti-rabbit antibody. After this step, the grids were washed once with PBS and distilled water, stained with uranyl acetate and lead citrate, and observed with an electron microscope. As controls, the sections were incubated in the presence of preimmune antineuraminidase sera and other well-characterized antibodies or in the presence of the second gold-labeled antibody only.

RESULTS

Labeling of trypomastigotes. Immunofluorescence microscopy of live metacyclics or tissue culture-derived trypomastigotes showed that 10% of the total population of these infectious forms (about 1,000 were examined) were labeled. The immunofluorescence label was not equally distributed over the bodies of the parasites but appeared to be patchy. In all cases examined, the flagella of these parasite forms were negative for fluorescence (Fig. 1). Approximately 40% of all infectious forms were positive, with fluorescing immunocomplexes. About 75% of these immunocomplexes were visible on the surface membrane and 25% were in the process of being sloughed off of the parasites. About half of all infectious forms appeared negative. Preimmune sera did not react with these parasites (Fig. 1c). If live immunofluorescence is done at room temperature or at 37°C, capping and shedding of immunocomplexes are even more rapid than they are at 4°C. In the latter case, only 1% of all trypomastigote forms evaluated showed the presence of immunocomplexes on their surface membranes (data not shown). When the trypomastigotes were fixed with 1% formaldehyde before being incubated with the primary antibody, all of the organisms were labeled (Fig. 2). The intensity of labeling, however, varied from cell to cell, being stronger in short trypomastigotes and transition forms from trypomastigotes to amastigotes.

We used pre- and postembedding methods for the localization of antigenic sites by electron microscopy. Although labeling was observed with both methods, the best results were obtained with the postembedding method.

In view of previous studies showing that tissue culture-derived trypomastigotes presented the highest level of neuraminidase activity, tissue culture-derived trypomastigotes were selected for ultrastructural immunocytochemistry (11). Typical trypomastigotes of *T. cruzi* can be identified by the morphology of the kinetoplast-DNA network, which appears as a rounded structure formed by filaments that are not tightly packed.

Our observations showed that very few trypomastigotes had their surfaces labeled when incubated in the presence of antineuraminidase antibodies (Fig. 4). In addition, the labeling was not intense, as indicated by the number of gold particles seen. However, in most of the trypomastigotes and transition forms, labeling, which in some cases was very intense, was seen in the membrane lining of the flagellar pocket region as well as within the pocket (Fig. 5 through 7). Some cytoplasmic vesicles were also labeled (Fig. 6 and 8). Some parasites, which had a kinetoplast characteristic of trypomastigote forms but located close to the nucleus, showed intense labeling of the cell surface and some particles that are associated with the nuclear membrane and that are in small cytoplasmic vesicles (Fig. 8). However, no labeling of the cell surface of other trypomastigotes with a similar morphology was observed. In all cases, very light labeling or no labeling at all was observed in the flagellar membrane.

Labeling of amastigotes. Amastigote forms can be morphologically characterized by their rounded or oval shape and by the structure of the kinetoplast-DNA network which appears as a slightly concave disk formed by a filamentous material arranged in a tightly packed row of fibers perpendicularly oriented in relation to the longitudinal axis of the protozoan.

All amastigotes found within vertebrate cells showed labeling of the surface. However, the labeling was not intense (Fig. 9). In contrast, amastigotes found in the supernatant of vertebrate cells previously infected with trypomastigote forms had their surfaces intensely labeled (Fig. 10 and 11).

Labeling of epimastigotes. Immunofluorescence microscopy of epimastigotes showed that live parasites are negative on prints and extremely weak upon inspection. Formaldehyde-fixed epimastigotes presented a weak visible fluorescence in 100% of the parasites (Fig. 3). In addition, epimastigotes obtained from axenic cultures were immuno-

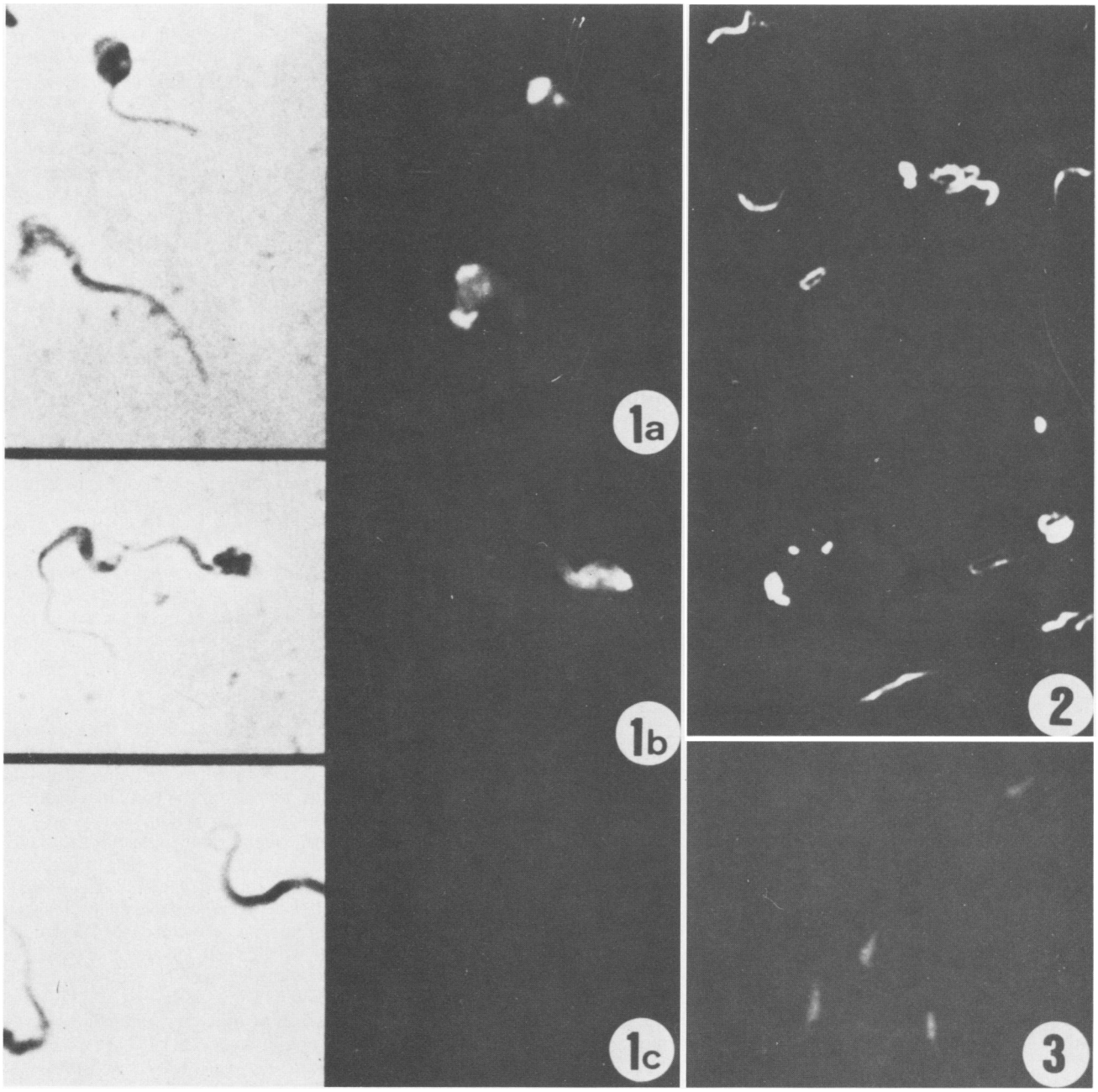

FIG. 1 through 3. Immunofluorescence microscopy. (Fig. 1) Phase-contrast (left panel) and immunofluorescence (right panel) microscopy of living tissue culture-derived trypomastigotes labeled with antineuraminidase antibody at 4°C. Parasites showed patchy labeling as well as capping and shedding of immunocomplexes (a and b) occurring in 40% of the total population of organisms. Trypomastigotes incubated with preimmune immunoglobulin G were negative (c). Magnification, ×1,200. (Fig. 2) Immunofluorescence microscopy of fixed tissue culture-derived trypomastigotes. All parasites were labeled, although the intensity of labeling varied from cell to cell. Magnification, ×600. (Fig. 3) Immunofluorescence microscopy of fixed epimastigotes. A weak fluorescence was observed. Magnification, ×600.

cytochemically labeled with antibodies associated with colloidal gold and were analyzed by electron microscopy. The results showed a faint labeling of the cell surface. In contrast, we found an intense labeling of several intracellular vacuoles located close to the flagellar pocket region and in the posterior region of the epimastigote form (Fig. 12).

DISCUSSION

Since the initial description of developmentally regulated neuraminidase activity in *T. cruzi* (16), considerable attention has been given to its possible role in the process of *T. cruzi* host cell interaction and in the pathogenesis of Chagas' disease. The studies carried out up to now with living parasites in contact with erythrocytes (10, 16, 19), myocardial cells, and endothelial cells (13) show that sialic acid is removed from the surfaces of the vertebrate cells. This observation suggests that the neuraminidase of the parasite is either released from the parasite or associated with the plasma membrane and exposed on the parasite surface.

Our immunocytochemical observations show that in most

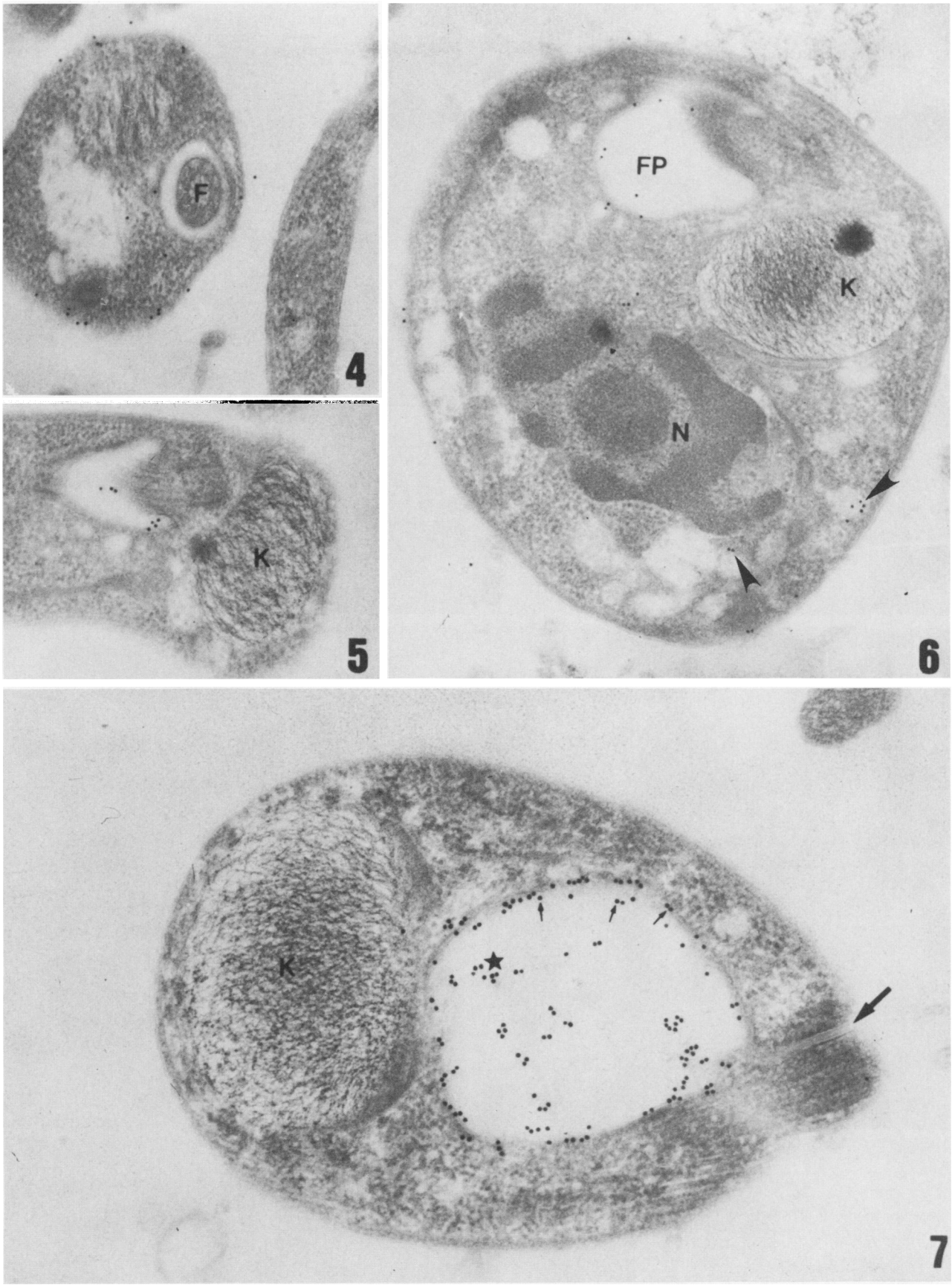

FIG. 4 through 7. Transmission electron microscopy of thin sections of Lowicryl-embedded parasites obtained from the supernatant of vertebrate cells previously infected with trypomastigote forms of *T. cruzi* and sequentially incubated with antineuraminidase antibodies and a second gold-labeled antibody. Typical trypomastigotes (Fig. 4 and 5) and trypomastigote-to-amastigote transition forms (Fig. 6 and 7) are seen. Labeling of the cell surface was not intense. In some parasites, the membrane lining the flagellar pocket (small arrows in Fig. 7) or the interior of the pocket (star in Fig. 7) was intensely labeled. Abbreviations: F, flagellum; FP, flagellar pocket; K, kinetoplast; N, nucleus. Large arrow in Fig. 7 indicates the region of the cell body-flagellum attachment. Arrows in Fig. 6 show labeling in a cytoplasmic vesicle. Magnifications: Fig. 4 and 6, ×40,000; Fig. 5, ×47,000; Fig. 7, ×60,000.

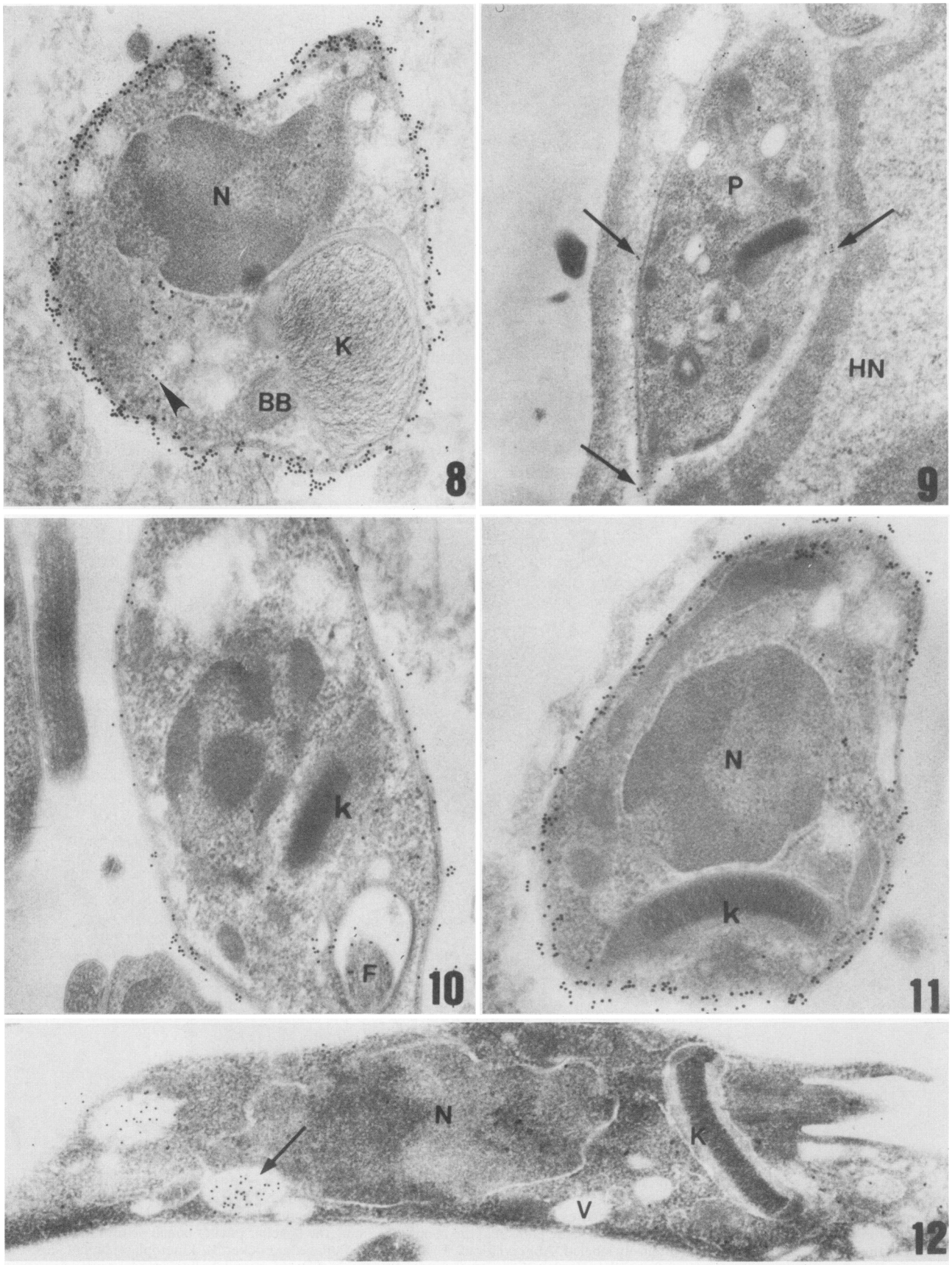

FIG. 8 through 12. Immunogold labeling of transition and amastigote forms. (Fig. 8, 10, and 11) Transition (Fig. 8) and amastigote (Fig. 10 and 11) forms of *T. cruzi* obtained from the supernatant of cell cultures previously infected with trypomastigotes. The surfaces of some transition and of most of the amastigote forms were intensely labeled. Arrow in Fig. 8 shows labeling in cytoplasmic vesicles. Abbreviations

of the trypomastigotes, gold particles indicative of the presence of neuraminidase are located mainly in the membrane lining of the flagellar pocket region and within the pocket. It is important to point out that in trypanosomatids, the flagellar pocket is the only region where there are no subpellicular microtubules and the only place where endocytic and exocytic events take place (8). Therefore, the finding of neuraminidase only at this region suggests that this enzyme is secreted by the parasite through the flagellar pocket.

Previous biochemical studies indicated that trypomastigotes presented the highest neuraminidase activity, epimastigotes presented low enzyme activity, and amastigotes were devoid of enzyme activity. Our present observations by immunofluorescence microscopy show that when parasites previously fixed with formaldehyde were incubated in the presence of the antineuraminidase antibody, all of the parasites had their surfaces labeled. In contrast, incubation of living cells in the presence of the antibodies showed the presence of labeled and unlabeled parasites. This ability to show the presence of labeled and unlabeled parasites is due to capping of the antigen-antibody complex, which was extensively discussed for other *T. cruzi* proteins (15). This process is dependent on the incubation temperature; this process is very rapid at room temperature and at 37°C, leading to a tremendous decrease in detectable fluorescence (15). In some preparations, we could see that slender trypomastigotes showed a less intense fluorescence than that observed in stout trypomastigotes and in some rounded forms. By using an immunocytochemical approach associated with electron microscopy in which the protein, and not its enzyme activity, is detected, very few gold particles were observed in direct association with the plasma membrane of typical trypomastigote forms. These forms were identified by electron microscopy on the basis of the position of the kinetoplast relative to the nucleus and on the basis of the array of the kinetoplast-DNA network. The high resolution of this technique, however, allowed us to localize the gold particles to the flagellar pocket region in most of these parasite forms. Other trypomastigotes representing early transition forms from trypomastigotes to amastigotes showed intense labeling of their surfaces. It is possible that these forms represent a subpopulation in a phase of transformation. These forms are designated spheromastigotes and are highly infectious for vertebrate cells in vitro (20). Taken together, both methods demonstrated the increase of neuraminidase expressed in trypomastigote forms compared with that expressed in epimastigote forms. Future experiments are designed to answer the question of whether this increase in neuraminidase expression is due to increased transcription of the neuraminidase gene or increased stability of the mRNA in trypomastigote forms.

Parasites that are found in the supernatant of cell cultures previously infected with bloodstream forms and that show a morphology similar to that observed for intracellular amastigotes had their surfaces intensely labeled when incubated in the presence of the antineuraminidase antibody. It is important to point out that in this case, all parasites presented a kinetoplast-DNA network characteristic of the intracellular multiplicative form of the parasites and that all of them had their surfaces labeled. Similar forms found within the vertebrate cells were also labeled, although not so intensely as in the case of the parasites found in the supernatant. These immunocytochemical observations are in contradiction with previous biochemical studies indicating that amastigotes are devoid of neuraminidase activity (11). One possibility is that amastigotes synthesize the protein in an enzyme-inactive form. We have some evidence that this might be the case. After the trypomastigote-to-amastigote transition, the parasite loses its neuraminidase activity, although a very small amount of the protein remains detectable immunologically. Late-stage amastigotes, which are about to convert back to trypomastigotes, regain enzymatic activity, and the protein increases to previously determined levels in trypomastigotes. This holds true for both intracellular amastigotes and amastigotes found in culture supernatants. The main protein band detected with the antineuraminidase antibodies is of M_r 60,000 approximately. Additional protein bands at slightly higher molecular weights were very faint and could represent precursors of the active enzyme. It is not known if these faint bands are related or even identical to the neuraminidase bands described by Cavallesco and Pereira (6). Furthermore, it still needs to be demonstrated whether these bands are related to the set of proteins of M_r 165,000 to 200,000 or whether they represent precursors of the active enzyme of M_r 60,000. It is important to point out that the amastigote forms are infective both in vitro and in vivo (5, 12).

The surfaces of epimastigotes, which are not infective, were not significantly labeled when incubated in the presence of antineuraminidase antibodies, an observation which is in agreement with biochemical studies showing that the epimastigotes possess very low enzyme activity. However, an intense labeling of cytoplasmic structures which may represent lysosomes or reservosomes was observed.

The data presented clearly demonstrate the stage specificity of the neuraminidase (Fig. 13). To date, it is not known if the neuraminidase is shed from the flagellar pocket of the parasites right after entry into the bloodstream of a newly infected individual or at a later stage of the infection. Furthermore, there are no data available on the mode of neuraminidase action. However, because the enzyme is expressed at greatly elevated levels in the infectious forms, it could, for example, play a role in the process of establishing an infection by cleaving sialic acid residues from target molecules on host cell surfaces. Another mode of action would involve cleaving sialic acid moieties from host cell glycoproteins in order to mask and modify parasite surface molecules. This latter hypothesis would require the presence of a sialyltransferase (21, 26). Immunological studies with sera from Chagasic patients proved that infected individuals generate antibodies against the neuraminidase (11). As demonstrated by live immunofluorescence, antibodies against the enzyme are ineffective in blocking reinfection of cells by the parasite, most likely because of the ability of the parasite to slough off immunocomplexes (8, 15). Late-stage amastigotes express the neuraminidase, yet they are protected from the immune system of the host because they reside inside host cells. Therefore, a third hypothesis on the role of the neuraminidase would be that the enzyme allows the parasite, after the conversion from the amastigote to the

for Fig. 8, 10, and 11: BB, basal body; K, kinetoplast; N, nucleus; F, flagellum. Magnifications: Fig. 8, ×42,000; Fig. 10, ×36,000; Fig. 11, ×47,000. (Fig. 9) Amastigote form located within the vertebrate cell showing a significant, although not intense, labeling (arrows). Abbreviations for Fig. 9: HN, host cell nucleus; P, parasite. Magnification, ×25,000. (Fig. 12) Epimastigote form obtained in axenic cultures. Very few gold particles are seen on the cell surface. Large cytoplasmic vacuoles (V), mainly located at the posterior portion of the protozoan, were intensely labeled. Magnification, ×30,000.

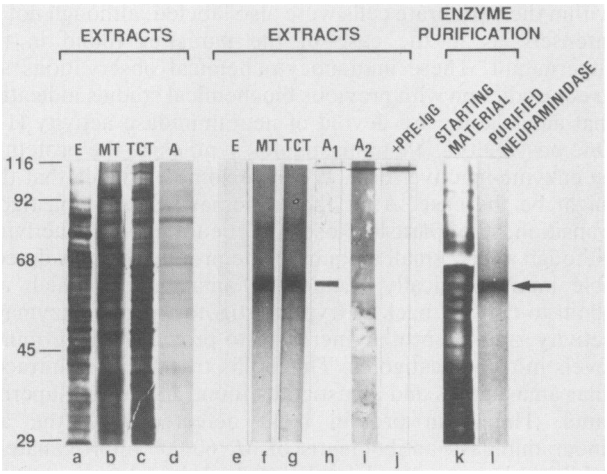

FIG. 13. Immunological detection of neuraminidase in *T. cruzi* extracts. Approximately 10 μg of protein from lysed parasites was electrophoresed on a denaturing 10% polyacrylamide gel and probed on a Western blot (immunoblot) with the monospecific, polyvalent antineuraminidase antibodies at a 1:250 dilution (11). Secondary antibody, alkaline phosphatase-labeled goat anti-rabbit immunoglobulins (Promega Biotec, Madison, Wis.) were applied at a 1:7,500 dilution. Lanes: a through d, silver-stained extracts from epimastigotes (E), metacyclic trypomastigotes (MT), tissue culture trypomastigotes (TCT), and amastigotes (A); e through h, the same extracts as in lanes a through d probed with antineuraminidase antibodies; lane i, the same extract as in lane h except that the blot shown in lane i was reacted with secondary antibody (A_2) at a 1:10,000 dilution instead of a 1:7,500 dilution to allow for a 2-h developing time with substrates compared with 15 min for the other lanes (e through h); lane j, extract from all four parasite forms probed with the corresponding preimmune antibodies (PRE-IgG, preimmune immunoglobulin G); lane k, starting material for neuraminidase purification (10 μg); lane l, homogeneous neuraminidase preparation (1 μg). Molecular weight markers (10^3) are indicated on the left. The arrow points to neuraminidase.

trypomastigote form, to rupture the infected host cell and enter the bloodstream. The generation of specific probes, such as the purified neuraminidase and antineuraminidase antibodies, will help to elucidate the function of neuraminidase in the process of infection.

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