Desialylation of Lysosomal Membrane Glycoproteins by *Trypanosoma cruzi*: A Role for the Surface Neuraminidase in Facilitating Parasite Entry into the Host Cell Cytoplasm

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Summary

Trypanosoma cruzi enters host cells via formation of an acidic vacuole which is subsequently disrupted, allowing the parasite access to the cytoplasm. We show that in an acid environment, release of the parasite surface neuraminidase is enhanced, and this release is likely mediated by a phosphatidylinositol-specific phospholipase C (PIPLC), since antibodies to a carbohydrate epitope (CRD) revealed in glycosylphosphatidylinositol (GPI)-anchored proteins after PIPLC cleavage remove the great majority of the soluble neuraminidase activity from culture supernatants. The neuraminidase is active at acidic pH, and is capable of desialylating known vacuolar constituents, i.e., lysosomal membrane glycoproteins. Parasite escape into the cytoplasm is significantly facilitated in terminal sialylation-defective mutant Lec 2 cells, and enzymatically desialylated membranes are more susceptible to lysis by a parasite hemolysin previously implicated in vacuole membrane rupture. These findings provide evidence that terminal sialylation on carbohydrate moieties contributes to maintaining lysosomal membrane integrity, and indicate a role for a protozoan-derived neuraminidase in facilitating parasite entry into host cells. These observations raise the possibility that other microbial neuraminidases may serve a similar function in acidic intracellular compartments.

Trypanosoma cruzi, an obligate intracellular parasite that is the etiologic agent of Chagas' disease, invades a wide range of cell types in vitro. The early intracellular stages of parasite entry involve formation of a membrane-bound vacuole followed by disruption of the membrane to allow the parasite access to the cytoplasm (1–5). The vacuole is acidic, and agents that inhibit acidification block parasite escape into the cytoplasm (5). An acid-active, transmembrane pore-forming protein (Tc-TOX) that is antigenically crossreactive with complement component C9 is secreted by *T. cruzi* and has been implicated in the process of vacuole disruption (6).

Another protein implicated in *T. cruzi* invasion is a developmentally regulated neuraminidase (TcNA), which is released by the parasites in a soluble form (7, 8), and recently has been shown to also have *trans*-sialidase activity (9). This enzyme has been suggested to have an extracellular function either in preventing superinfection by inhibiting parasite attachment to target cells (10), in being associated with myotropism of different strains of *T. cruzi* (11), or in creating an epitope required for cell invasion (12). However, both high and low density serum lipoproteins inhibit the enzyme (13, 14), reducing the likelihood of an extracellular role in vivo. In addition, the pH optimum of TcNA activity has been reported to lie in the acidic range (7). These observations prompted us to consider an intracellular role for TcNA within an acidic compartment.

Lysosomes fuse with *T. cruzi*-containing vacuoles (1, 15–18), but in the absence of immunological activation, this event is not associated with intracellular killing of infective parasite stages. Because the luminal aspects of lysosomal membranes are lined by a "halo" of glycoconjugates (19) composed, at least in part, by tightly packed lysosomal membrane glycoproteins (20), we hypothesized that modification of these structures might facilitate parasite escape from the vacuole. Since lysosomal membrane glycoproteins are unusually rich in sialic acid moieties (20), we examined TcNA for its ability to function within the vacuole and to enhance membranolysis.

Materials and Methods

Parasites and Cells. Tissue culture trypomastigotes of the Y strain of *T. cruzi* (21) were maintained in monolayers of LLC-MK2 cells according to published protocols (22). Epimastigotes of the Silvio X10/4 clone (23) were grown to late log phase in liver infusion

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tryptase (LIT) medium (5) and allowed to further differentiate into culture-derived metacyclic trypomastigotes in Grace's medium (Gibco Laboratories, Grand Island, NY) according to published protocols (24).

Lec 2 and wt Chinese Hamster Ovary (CHO)[1] cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). They were cultured in alpha MEM (Gibco Laboratories) containing 3.5% (vol/vol) heat-inactivated (56°C, 30 min) FCS (J. R. Scientific, Woodland, CA). HeLa cells were also obtained from ATCC and cultured in DMEM containing 10% FCS.

Immunofluorescence Microscopy. Cells were plated on glass coverslips at a density of 2×10^4 cells/cm^2 and allowed to adhere for at least 12 h in a 37°C, 5% CO_2 incubator before use. Monolayers on glass coverslips were infected for 20 min with 100 trypomastigotes per host cell in DMEM 2% FCS, and then washed extensively with cold medium to remove extracellular parasites. Fresh warm medium was added, and the incubation at 37°C continued. Monolayers were washed in serum-free DMEM and fixed in 3% (wt/vol) paraformaldehyde for 30 min. They were then washed three times (5 min at room temperature) in PBS. Unreacted aldehyde groups were blocked by subsequently incubating in 50 mM NH_4Cl for 10–30 min. Coverslips were used immediately or stored at 4°C in buffer (TBS) containing 20 mM Tris, pH 7.6, 150 mM NaCl, 5 mM NaN_3, and 1 mM EDTA.

Tissue culture supernatant containing E9A, a murine mAb to a 95-kD lysosomal membrane glycoprotein of CHO cells (S. Schmidt and I. Mellman, unpublished results), was obtained from Dr. Ira Mellman (Yale University School of Medicine, New Haven, CT). Tissue culture supernatant containing 5G11 (25), a murine mAb to a 120-kD human lysosomal membrane glycoprotein, was provided by Dr. J. Thomas August (Johns Hopkins University School of Medicine, Baltimore, MD).

Cells were permeabilized with 0.005–0.01% (wt/vol) saponin (Sigma Chemical Co., St. Louis, MO) in TBS for 15 min, and then incubated for 30 min at room temperature with primary mAb in TBS containing saponin plus 10% (vol/vol) normal goat serum. Monolayers were washed three times with TBS containing saponin and normal serum, and then incubated for 30 min at room temperature in the dark in fluorescein-conjugated, affinity-purified goat anti-mouse Ig antibody (Zymed Laboratories, South San Francisco, CA). The coverslips were washed in TBS containing saponin, and incubated for 3 min with TBS containing saponin, and 10 μg/ml 4,6 diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) at room temperature in the dark (26). The coverslips were then washed, inverted, and mounted in pH 8.5 Mowiol (Calbiochem Corp., San Diego, CA). Coverslips were examined using a Nikon Microphot microscope (Nikon Inc., Natick, MA) equipped for fluorescence microscopy with excitation and barrier filters to minimize crossover epifluorescence. Parasites were identified on the basis of the morphology and fluorescence in the host cell cytoplasm exhibiting the characteristic appearance of the parasite nucleus and kinetoplast after DAPI staining.

Electron Microscopy. Ultrastructural investigation of the extent of parasitophorous membrane disruption in wild type and Lec 2 CHO cells infected at a 500:1 parasite/cell ratio was carried out on thin sections of Epon-embedded, glutaraldehyde-fixed cells (5). Sections were cut from different parts of the cell pellets and each cell in every section was systematically examined for the presence of *T. cruzi*. The parasitophorous vacuole in every infected cell was examined for structural integrity, and scored as being either intact or disrupted (see Fig. 7 legend). All five sections, or 200 parasites (whichever was reached first), were examined in this way.

Immunoprecipitation and Two-dimensional Gel Electrophoresis. 6×10^5 HeLa cells were plated in 60-mm tissue culture dishes overnight. The following morning they were incubated for 60 min at 37°C in methionine-free DMEM containing 10% (vol/vol) dialyzed FBS (both from Gibco Laboratories), washed once in methionine and serum-free medium, and then incubated for 6 h more in the same medium to which 0.5 mCi Tran^{35}S-label (ICN Biomedicals, Inc., Costa Mesa, CA) had been added. The monolayers were then washed 3–5 times, covered with harvesting buffer (20 mM Tris, pH 7.8, 150 mM NaCl, 1 mM EDTA, 5 mM NaN_3, 50 μM p-nitrophenyl-p'-guanidinobenzoate (NPGB), 10 μg/ml leupeptin, 50 μM pepstatin, 1 mM iodoacetamide), scraped with a rubber scraper, and pelleted. Lysis buffer (harvesting buffer plus 1% (vol/vol) NP-40 and 1% (wt/vol) sodium desoxycholate (Sigma Chemical Co.) was added, and the incubation continued for 30 min. The lysates were centrifuged to remove insoluble debris, and the supernatants preabsorbed for 120 min with 100 μl packed volume recombinant protein G coupled to Sepharose (Zymed Laboratories, Inc., South San Francisco, CA). These lysates were then assayed for TCA-precipitable radioactivity.

For immunoprecipitations, 100 μl of tissue culture supernatant containing mAb (5G11) to lysosomal membrane glycoprotein (lgp) was added to lysate volumes containing equivalent amounts of TCA-precipitable radioactivity. The samples were then rotated end-over-end at 4°C overnight. 60 μl of a 50% slurry of recombinant protein G-Sepharose in lysis buffer was then added to each tube, and the incubation continued for another 4 h. The tubes were then gently centrifuged in a Sorvall microfuge (DuPont Co., Newton, CT) at 4°C for 3 min, the supernatants removed, and the Sepharose pellets washed three times by centrifugation through 1-ml lysis buffer, and then once through 1 ml 150 mM NaCl containing 20 mM Tris, pH 7.8. The bound antigens were then eluted by resuspending the pellet in 9 M urea containing 1% (vol/vol) NP-40 and 2% (vol/vol) 2-ME and incubating at 56°C for 30 min.

Two-dimensional gel electrophoresis was carried out according to published procedures (27). The pH gradient of the isoelectric focusing dimension was obtained by slicing a replicate tube gel, equilibrating the samples in water, and then reading the pH. In the second dimension, a 7.5% acrylamide gel was used. Completed gels were incubated in EN^{3}HANCE (New England Nuclear, Boston, MA), dried and exposed to Eastman Kodak (Rochester, NY) X-ray film at −80°C.

For immunoprecipitations of TcNA with anti-CRD antibodies (provided by Drs. M. Davitz, New York University and P. Englund, Johns Hopkins University), 3 h-culture supernatants of *T. cruzi* were prepared as described below. 50 μl of supernatant was mixed with 25 μl of buffer containing 15 μg of purified IgG and incubated for 30 min at 4°C. An equal volume of recombinant protein G-Sepharose was then added to each tube, and the incubation continued for an additional 60 min. Finally, the Sepharose was pelleted and the supernatants assayed for neuraminidase activity.

Preparation and Assay of T. cruzi Neuraminidase Enriched Supernatants. Tissue culture trypomastigote forms obtained from the supernatants of infected monolayers were resuspended at 10^8/ml in serum-free DMEM, and incubated for 3 h at 37°C. The medium pH was adjusted after addition of 10 mM sodium acetate (pH 4.5–6.0), or 10 mM Hepes (pH 6.5–7.0). The parasites were pelleted

[1] *Abbreviations used in this paper:* CHO, Chinese Hamster Ovary; CRD, crossreacting determinant; GPI, glycosylphosphatidylinositol; PIPLC, phosphatidylinositol-specific phospholipase C.

by centrifugation, and the supernatants collected and filtered through a 0.22-μm filter (Millipore Corp., Bedford, MA). Protease inhibitors (NPGB, leupeptin, pepstatin, and iodoacetamide) were added, and the supernatants stored at −80°C until further use.

Neuraminidase activity was assessed by cleavage of the fluorogenic substrate 4-methylumbelliferryl-*N*-acetylneuraminic acid (Sigma Chemical Co.) according to published procedures (8). Briefly, 20 μl of sample was mixed with 20 μl of 0.17 M sodium acetate buffer, pH 6.5, and 20 μl of 0.4 mM substrate diluted in serum acetate buffer, and the total mixture incubated at 37°C overnight. 2 ml of 0.085 M glycine buffer, pH 9.0, was then added to each mixture, and upon excitation of the sample at 450 nM, the emission at 365 nM measured in a fluorimeter. Neuraminidases from *Vibrio cholerae* or *Clostridium perfringens* (both from Calbiochem Corp.) were assayed as positive controls.

Metabolic label viability controls were performed by incubating parasites in methionine-free DMEM (Gibco Laboratories) pH 7.0 containing 10% dialysed FBS for 30 min at 37°C, and then for 2 h with 200 μCi/ml Tran^{35}S-label (ICN Biomedicals, Inc.). After washing, the parasite pellet was lysed in 0.5 N NaOH, TCA precipitated, and the protein-associated radioactivity determined in a scintillation counter.

To determine the total amount of surface protein and of TcNA released into the medium during preparation of the supernatants, washed trypomastigotes were treated with Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, IL) at 500 μg/ml for 20 min at 4°C, and washed with PBS twice before incubation in DMEM at different pHs as described above. Samples of the supernatant (50 μl in triplicate) were used immediately to determine neuraminidase activity, and the remaining (200 μl in duplicate) was dialysed against water, lyophilized, run on SDS-PAGE, and transferred to nitrocellulose. The membranes were then either treated directly with streptavidin coupled to alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, IN) or with 39, a mouse mAb to the *T. cruzi* neuraminidase/*trans*-sialidase, kindly provided by Dr. Victor Nussenzweig (New York University, NY) (9), followed by anti-mouse IgG coupled to alkaline phosphatase (Boehringer Mannheim Corp.), followed by color development using the appropriate substrates (Sigma Chemical Co.). After drying, the stained lanes were scanned in a Visage 2000 (Bio Image, Eastman Kodak).

Hemolytic Assays. Guinea pig erythrocytes (Colorado Serum Co., Denver, CO) were washed three times in PBS and resuspended in 150 mM NaCl, 10 mM acetate, pH 5.5 (acetate buffer) at 5 × 10^8/ml. Aliquots of 1 ml were incubated at 37°C after addition of 10 mU of *V. cholerae* neuraminidase (Calbiochem Corp.) or 25 μl containing 0.7 mU of an enriched, partially purified preparation of *T. cruzi* neuraminidase, prepared according to published procedures (8) (and kindly provided by G. Harth, Palo Alto Medical Research Foundation, Palo Alto, CA). After 2 h, the erythrocytes were washed twice with PBS and resuspended at 8 × 10^7/ml in acetate buffer. Amastigotes of *T. cruzi*, obtained by overnight incubation at 37°C, as described (4), were washed three times in PBS, and also resuspended at 8 × 10^7/ml in acetate buffer. Aliquots of 0.25 ml of the erythrocyte and amastigote suspensions were mixed together and incubated at 37°C after adding 20 mM NaN_3 to half the tubes (background controls). At the designated time points, the tubes were vortexed, centrifuged, and 0.1 ml of the supernatants transferred to a 96-well plate. The degree of hemolysis was calculated from the amount of hemoglobin released as determined by reading the absorbance at 450 nm in a microplate ELISA reader (Molecular Devices Corp., Menlo Park, CA).

Results

During the Early Stages of Infection of Mammalian Cells, the Vacuole Membrane Surrounding T. cruzi Contains lgp. Fibroblast CHO cells, or a human epithelial cell line, HeLa, were infected with tissue culture-derived trypomastigotes. During the early stages of infection of CHO cells (i.e., 20 min), intracellular parasites were located at the cell periphery (Fig. 1, *a–d*), and over 80% of them exhibited circumferential staining for lgp (Fig. 1 *c*). With longer incubation times, parasites often appeared in a more central, perinuclear location, and there was a gradual loss of lgp staining (see Figs. 1, *e–h*, and 5), compatible with previous observations that *T. cruzi* disrupts the vacuolar membrane to enter the cytoplasm (1, 6). Similar results were obtained when HeLa cells were infected with tissue culture-derived trypomastigotes (Fig. 2).

Lgps are incorporated into parasitophorous vacuoles during their formation. This is demonstrated in Fig. 2, *a–d*. Arrows point to portions of partially internalized trypomastigotes that clearly lie external to the cell and do not stain for the presence of circumferential lgp. In contrast, the majority of the body of the parasite (*arrowheads*) that is already inside the cell is clearly circumscribed by the anti-lgp labeling. The open arrow in Fig. 2 *a* indicates an extracellular parasite that does not stain for the presence of lgp. These results indicate that lgp incorporation into the parasitophorous vacuole occurs in the periphery of the host cell and before complete parasite internalization.

TcNA Release Is Enhanced under Acidic Conditions. Since the parasitophorous vacuole surrounding *T. cruzi* is acidic (5), we sought to determine whether *T. cruzi* neuraminidase functions in a comparable acidic milieu. The *T. cruzi*-derived neuraminidase has been reported by Pereira (7) to function over a broad pH range, with an optimal activity occurring around pH 6.0–6.5. Conditions promoting the release of TcNA into the surrounding milieu, however, were not known. To determine whether exposure of extracellular *T. cruzi* to acid pH was associated with increased TcNA activity in the supernatant, parasites were incubated for 3 h in media adjusted to pH between 4.5 and 7.0. Supernatants were collected and assayed under uniform conditions (pH 6.5) as described in Materials and Methods. As shown in Fig. 3 and Table 1, neuraminidase activity in the supernatant increased as the pH of the incubation medium dropped from 7.0 to 5.5, then declined as the pH was lowered to 4.5. Incorporation of [^{35}S]methionine into parasites incubated at the various pHs eliciting increased neuraminidase activity remained constant (Fig. 3), indicating that parasite metabolic viability was unaffected between pH 6.5 and 5.5. Furthermore, the same number of intact parasites was recovered after incubation at the different conditions, as determined by protein assay of the detergent lysates (not shown).

Two possible explanations for the observed increase in TcNA activity in response to exposure to acid pH were considered. The simplest was that the increased enzyme activity reflected an increased concentration of enzyme protein. We also, however, considered the possibility that the increased TcNA ac-

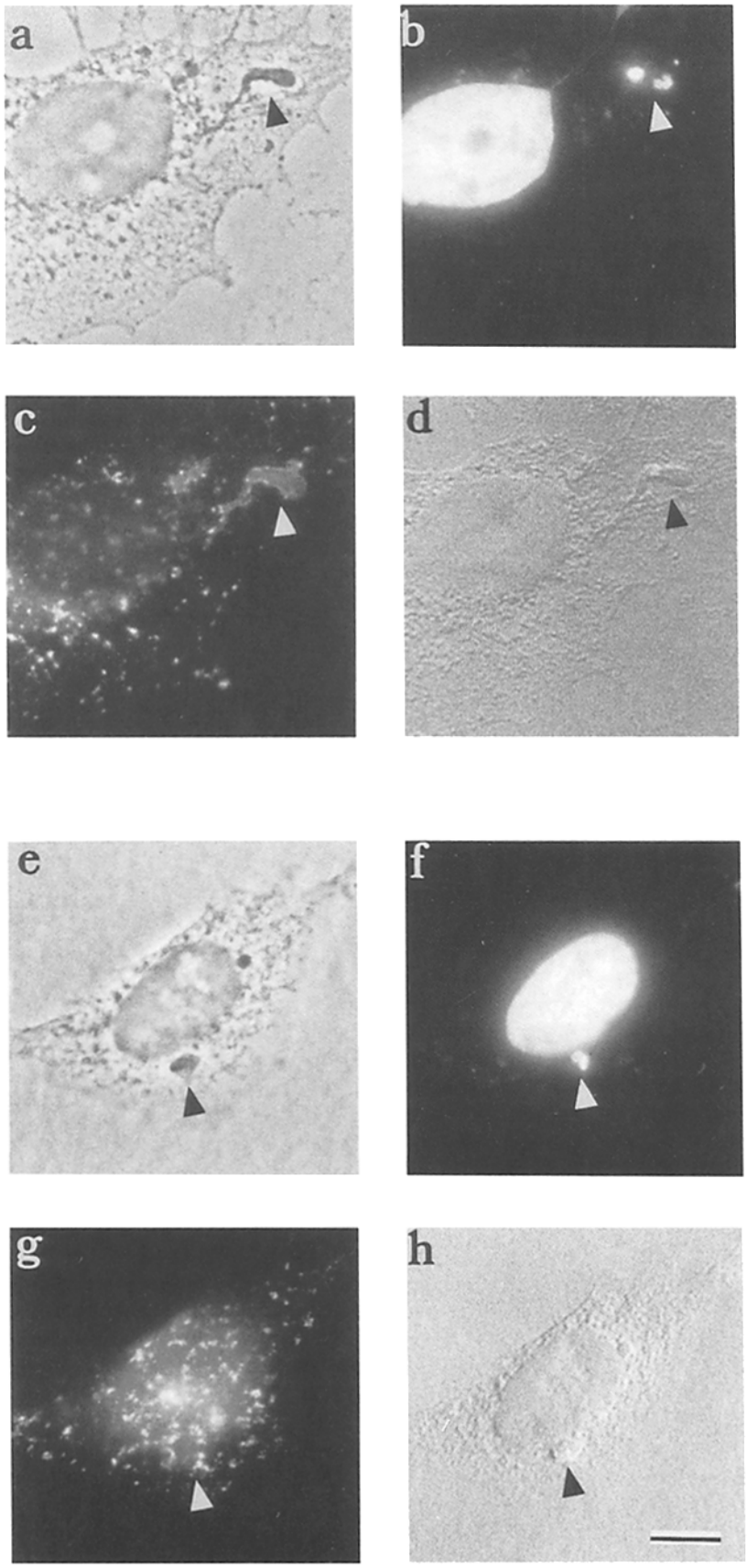


Figure 1. Immunofluorescent staining of lysosomal membrane glycoproteins in *T. cruzi*-infected CHO cells. Infections were carried out for 20 min, and the cells processed as described in Materials and Methods. (*a* and *e*) Phase contrast micrographs of intracellular *T. cruzi*; (*b* and *f*) fluorescence of intracellular *T. cruzi* after staining with DAPI: note the characteristic morphology of the parasite nucleus and kinetoplast; (*c* and *g*) immunofluorescent staining of lgp in infected cells; (*d* and *h*) differential interference contrast micrograph of infected cells. (*Arrowheads*) Intracellular parasites. (*a–d*) show a parasite still enclosed in a lgp-positive vacuole; (*e–h*) show a parasite free in the cytosol. Bar, 5 μm.

tivity observed after incubation at low pH might be due to conversion of the enzyme to a more active state when exposed to acidic conditions. To test this latter hypothesis, neuraminidase-containing supernatants were initially prepared at pH 7.0, and then the pH of representative aliquots was adjusted into the acidic range. The aliquots were incubated for 3 h at 37°C, and then assayed under standard conditions (pH 6.5). As shown in Table 1, transiently shifting the pH of the supernatant to 5.5 had no effect on the relative activity of the neuraminidase. In contrast, incubating whole parasites at pH 5.5 resulted in a threefold increase in activity. Transiently shifting the pH of the supernatant to 5.0 or lower resulted in a loss of enzymatic activity. Taken together, these results indicate that the neuraminidase is not activated by exposure to an acidic pH, and that the increased activity seen after parasite incubation most likely stems from an increase in neuraminidase concentration in the supernatant.

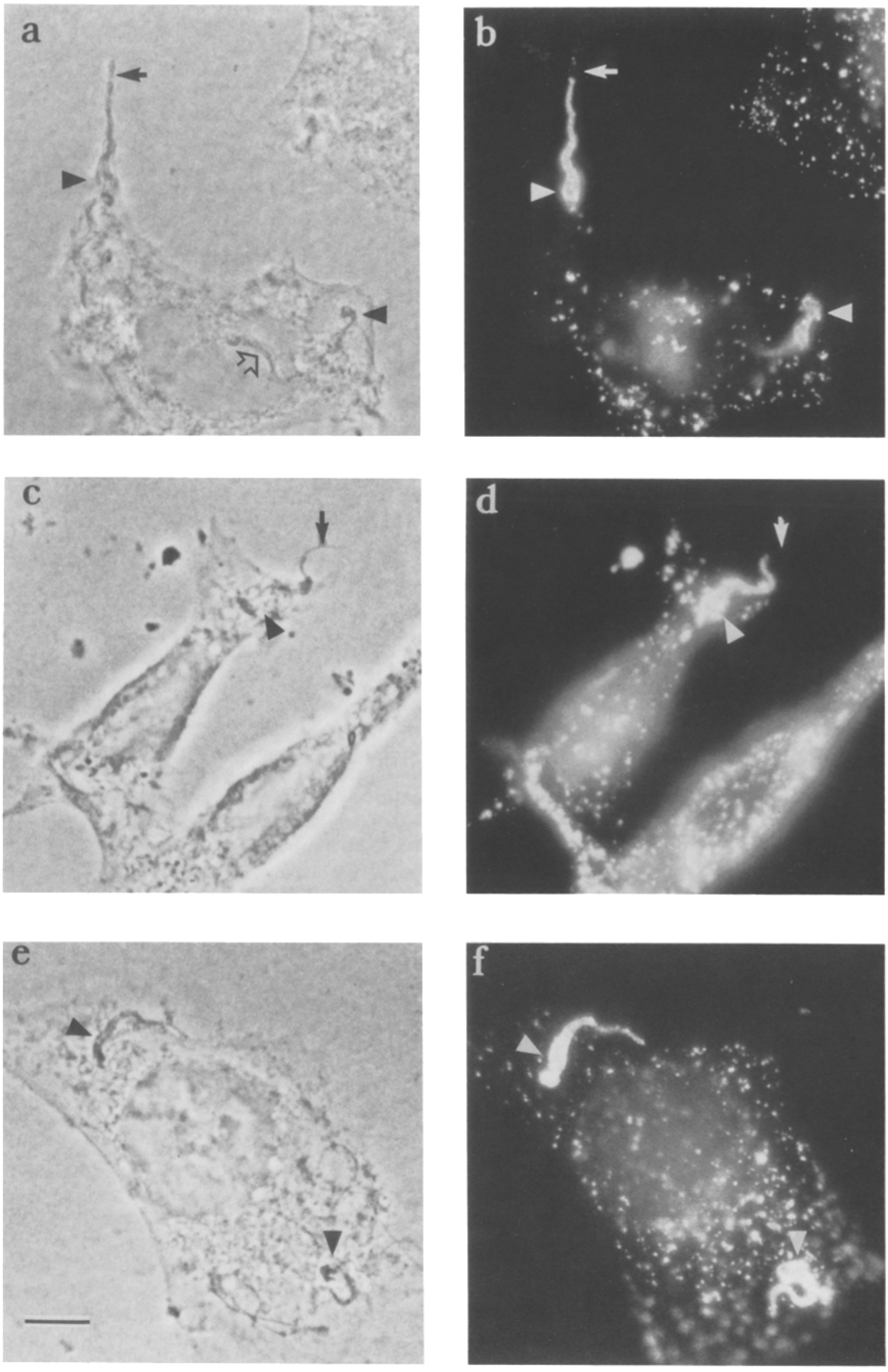
The conclusion above was confirmed by the experiment shown in Fig. 4. Trypomastigotes were surface biotinylated and incubated in medium adjusted to pH 7.0, 6.5, 6.0, or 5.5 for 3 h at 37°C. The total biotinylated proteins released into the medium were analyzed by SDS-PAGE, transferred to nitrocellulose, and revealed by alkaline phosphatase reaction (Fig. 4, *bottom right*). The amount of TcNA present in equivalent samples of each supernatant was determined by treatment of the membrane with the mAb 39 before detection with goat anti-mouse IgG coupled to alkaline phosphatase (Fig. 4, *bottom left*). The integrated optical density of the stained lanes shows that while the total amount of protein released from the surface of the parasites decreases from pH 7.0 to 5.5 (Fig. 4, *top right*), an inverted relationship is observed with TcNA. Both the amount of protein recognized by the mAb 39 and the neuraminidase activity increase as the pH of the supernatant is lowered (Fig. 4, *top left*). The less significant increase in released TcNA (as compared with Fig. 3) was typical of biotinylated parasites.

Surface proteins of trypanosomes are commonly anchored in the parasite membrane by a covalently attached glycosylphosphatidylinositol (GPI) moiety (for review, see reference 28). Such proteins can be released by a PIPLC, resulting in the appearance of a new carbohydrate epitope (the crossreacting determinant, CRD). Schenkman et al. (9) have reported detection of the CRD epitope in immunoblots of the *T. cruzi* neuraminidase from culture supernatants, and Rosenberg et al. (29) showed that treatment with *Trypanosoma brucei* PIPLC releases neuraminidase activity from *T. cruzi* trypomastigotes. We therefore asked whether PIPLC cleavage was the predominant mechanism for neuraminidase release from the trypanosome surface. If this were the case, the great majority of the neuraminidase present in the supernatants should be removed with anti-CRD antibodies. As shown in Table 2, IgG of a rabbit anti-CRD antiserum removed >90% of the neuraminidase activity from a *T. cruzi* supernatant when compared with IgG of a rabbit immunized against Tc-TOX.

lgp Are Substrates for TcNA. We next addressed whether the lgp, vacuolar membrane constituents known to be extremely rich in sialic acid, could function as substrates for the parasite-derived neuraminidase. Lgp 120 were immunoprecipitated from HeLa cells metabolically labeled with [^{35}S]methionine. The protein G-Sepharose beads bearing lgp-containing immune complexes were then exposed for 3 h at 37°C to supernatants from *T. cruzi* cultures known to contain neuraminidase activity. Incubations were always performed in the presence of protease inhibitors, at pH 6.0. Compared with lgp treated with medium alone (see Fig. 5 *A*), lgp exposed to neuraminidase-containing supernatants exhibited a significantly different pattern upon isoelectric focusing. The most acidic spot (*arrowhead*, Fig. 5 *A*) was dramatically reduced in intensity, the centrally located spots were increased in intensity, and two new spots with more basic pI (*arrowheads*, Fig. 5 *B*) appeared. Immunoprecipitated lgp treated with purified *V. cholerae* neuraminidase (Fig. 5 *C*) exhibited even more extensive desialylation with the majority of the lgp species migrating more cathodally. Similar results were obtained when lgp were exposed to supernatants of culture-derived metacyclic trypomastigotes, the infective insect stages of *T. cruzi* (data not shown).

T. cruzi Escapes from Parasitophorous Vacuoles into the Cytoplasm in Sialylation-defective Mutant Lec 2 Cells Faster than in Wild Type CHO Cells. Having established that *T. cruzi* neuraminidase could function under vacuolar conditions to desialylate lgp, we wished to examine the effect of desialylation on parasite escape from the vacuole into the cytoplasm. The rate of *T. cruzi* escape from the parasitophorous vacuole was compared in wild type CHO cells and in mutant Lec 2 cells. Lec 2 cells have defect in the CMP-sialic acid transporter so that glycoproteins are deficient in terminal sialic acid content (30). Their lysosomes, however, are normal in hydrolase content, ATP-dependent acidification, and turnover of membrane glycoproteins (20). As shown in Fig. 6, after 20 min of infection, ~80% of parasites in both wild type and Lec 2 cells stained positively for the presence of lgp in parasitophorous vacuoles. Over the ensuing 40 min, however, the percentage of parasites exhibiting circumferential lgp staining decreased to <10% in the sialylation-defective Lec 2 cells. Over the same time period, the percentage of parasites with circumferential lgp staining in the wild type CHO cells also declined, but to a much lesser extent. To determine that the absence of circumferential lgp staining correlated with vacuolar membrane disruption, the integrity of the vacuolar membrane was examined by transmission electron microscopy after a 20-min infection followed by a 30-min incubation at 37°C (Fig. 7). Although the majority of the parasites were either all in or all out (Fig. 7, *C* and *D*), in the few cases in which there was ambiguity parasites residing in vacuoles with >50% membrane intact were scored as being "in" (Fig. 7 *A*), whereas those with <50% vacuolar membrane intact were scored as being "out" (Fig. 7 *B*). When scored in a single blinded fashion, 73% of parasites in wild type CHO cells were found to still be in, whereas only 34% of those in Lec 2 cells were still considered to be in.

Desialylation Renders Membranes More Susceptible to Parasite-derived, Acid-activated Hemolysin, Tc-TOX. It has previously been suggested that the ability of *T. cruzi* to rupture the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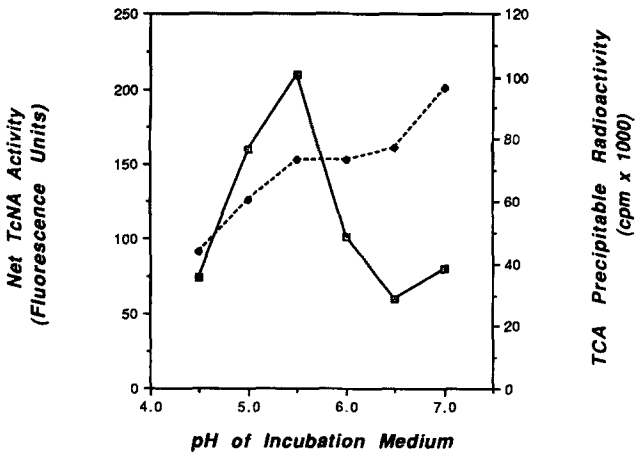

Figure 3. Neuraminidase activity in supernatants from *T. cruzi* trypomastigotes incubated under distinct pH conditions. Trypomastigotes were incubated for 3 h at 37°C in DMEM adjusted to the indicated pH. The parasites were then centrifuged, and the supernatants collected and assayed for TcNA activity (——) under standard conditions (pH 6.5) as described in Materials and Methods. Equivalent parasite samples were metabolically labeled with [^{35}S]methionine (- - - -) as described in Materials and Methods. Points were determined in duplicate and values differed by <15%.

Table 1. *Comparison of Acid Induction and Acid Sensitivity of TcNA Activity*

	Relative Activity			
Acid induction*				
pH	**7.0**	**5.5**	**5.0**	**4.5**
	100	339	198	88
		(n = 4)	(n = 2)	(n = 2)
Acid sensitivity‡				
pH Shift	**7.0→7.4**	**7.0→5.5**	**7.0→5.0**	**7.0→4.5**
	100	101	84	66
		(n = 2)	(n = 2)	(n = 2)

* Trypomastigotes were incubated for 3 h in DMEM adjusted to the indicated pH, and the neuraminidase activity in the supernatant was assayed at pH 6.5, as described in Materials and Methods. TcNA activity is expressed relative to the activity observed at pH 7.0 (100%). Values are a mean of independent experiments (n = number of experiments), and varied by <20% between experiments.

‡ Parasite supernatants prepared at pH 7.0 as described in Materials and Methods were shifted to the indicated pH by addition of Tris or sodium acetate buffers and reincubated for 3 h at 37°C. TcNA activity at pH 6.5 was determined and the results expressed as described above.

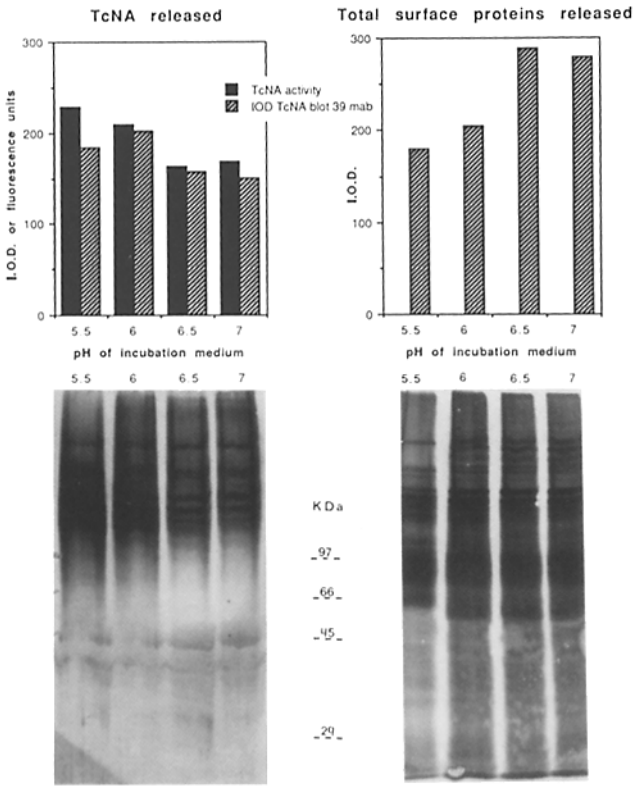

Figure 4. Neuraminidase released into the medium at different pHs, compared with total surface proteins released. *T. cruzi* trypomastigotes were surface biotinylated and incubated in DMEM adjusted to different pHs, as described in Materials and Methods. The proteins transferred to nitrocellulose were detected by reaction with streptavidin-alkaline phosphatase ([*right*] total surface proteins released) or by treatment with the antineuraminidase mAb 39 followed by anti-mouse IgG-alkaline phosphatase ([*left*] TcNA released). The (*Top, hatched columns*) integrated optical density values for each lane (IOD) obtained by densitometry. (*Left, black columns*) Neuraminidase activity present in the samples was assayed as described in Materials and Methods.

parasitophorous vacuole is due to Tc-TOX, an acid-active, C9-related transmembrane pore-forming protein (6). Our data indicate that desialylation in the parasitophorous vacuole facilitates parasite escape into the cytoplasm. We therefore asked whether membrane desialylation might facilitate the action of Tc-TOX.

As shown in Fig. 6, when guinea pig erythrocytes were incubated at pH 5.5 in the presence of *T. cruzi*, hemolysis occurred in a time-dependent manner. Specific hemolysis was

Figure 2. Immunofluorescent staining of lysosomal membrane glycoproteins in *T. cruzi*-infected HeLa cells. Infections were carried out for 20 min and the cells processed as described in Materials and Methods. (*a, c, e*) Phase contrast micrographs of intracellular *T. cruzi*. (*b, d, f*) Immunofluorescent staining of lysosomal membrane glycoproteins in infected cells. (*Arrowheads*) Intracellular parasites; (*arrows*) portions of entering *T. cruzi* that are still external to the cells and do not stain for the presence of lysosomal membrane glycoprotein; *open arrow* in *a* indicates an extracellular parasite that does not exhibit circumferential staining with antibody to lysosomal membrane glycoprotein. Bar, 5 μm.

Table 2. *Removal of TcNA Activity with Anti-CRD Antibodies*

Treatment	Neuraminidase activity	Percent activity remaining after treatment
	Fluorescence U, mean ± SD	
Control IgG	211.0 ± 0.6	100
Anti-CRD IgG	75.0 ± 4.6	6.7

Supernatants containing TcNA were prepared, immunoprecipitated with control rabbit or anti-CRD antibodies, and assayed for neuraminidase activity as described in Materials and Methods, in triplicate. Background levels of fluorescence (untreated medium, not containing neuraminidase) were determined to be 65.2 ± 0.6 U. Percent activity remaining after treatment was calculated by the following formula:
100 × [(sample fluorescence − 65.2)/(control fluorescence − 65.2)].

abrogated in the presence of sodium azide, which blocks secretion of Tc-TOX (31). When guinea pig erythrocytes were pretreated with *V. cholerae* neuraminidase (see Fig. 8 *A*), the rate of lysis increased, as demonstrated by the leftward shift of the curve. Incubation of neuraminidase-treated cells in the presence of sodium azide reduced the level of lysis to that seen in control cells incubated with sodium azide, indicating that neuraminidase treatment by itself was not sufficient to destabilize the erythrocyte membrane. Similar effects were seen by pretreating the erythocytes with a highly enriched, partially purified preparation of TcNA (see Fig. 8 *B*). Since sodium azide had no effect on release of the *T. cruzi* neuraminidase (not shown), but had previously been shown to inhibit release of Tc-TOX (31), it is likely that the lytic event requires the presence of Tc-TOX and is facilitated by removal of sialic acid moieties.

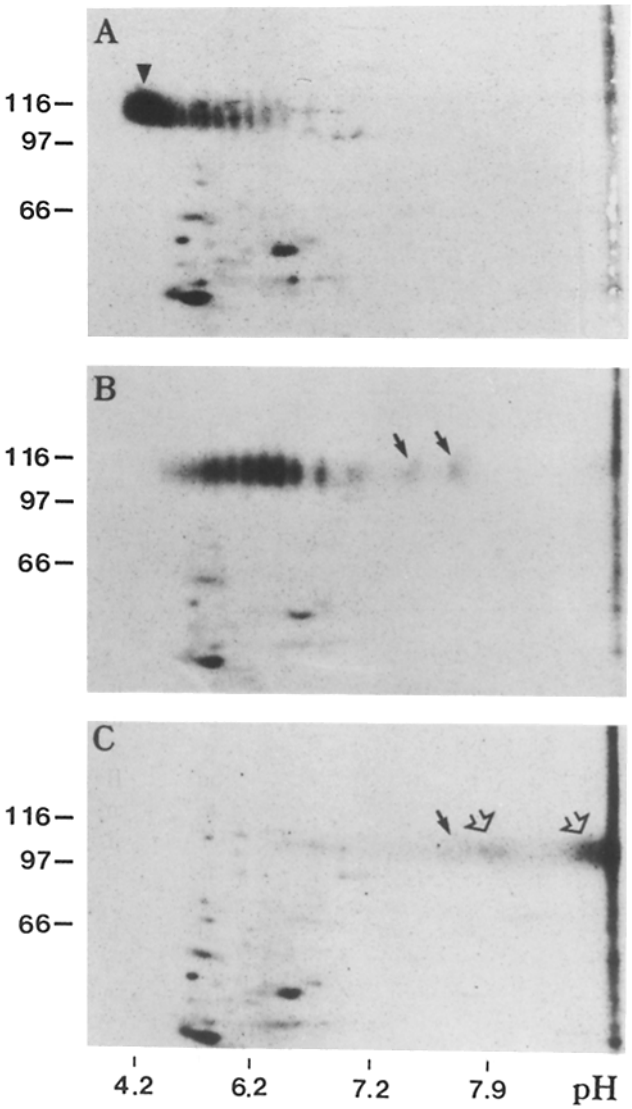

Figure 5. Desialylation of lysosomal membrane glycoproteins. Lgp were immunoprecipitated from Tran^{35}S-labeled HeLa cells as described in Materials and Methods, and then the protein G-Sepharose beads treated for 3 h at 37°C with medium alone (*A*), TcNA-containing, *T. cruzi*-conditioned medium (*B*), or medium containing 10 mU *V. cholera* neuraminidase (*C*). The beads were then washed, and the bound material eluted and analyzed by two-dimensional gel electrophoresis and fluorography. (*Horizontal axis*) Isoelectric focussing gradient; and (*vertical axis*) molecular mass standards (kD). (*Arrowhead*) Predominant, highly acidic species of untreated lgp. (*Solid arrows*) New, more basic species of lgp appearing after treatment with TcNA. (*Open arrows*) Most basic species of lgp appearing after treatment with *V. cholerae* neuraminidase.

Discussion

Although previous investigators have focused on an extracellular role for the *T. cruzi* neuraminidase, the data presented here are consistent with its function within the acidic environment of the parasitophorous vacuole. TcNA exhibits significant activity in the pH 5.5–6.5 range (7, 9). Trypsinization experiments indicate that virtually all of the relevant enzyme is initially located at the parasite surface (12, and our unpublished results), in keeping with prior immunofluorescent (32) and immunocytochemical studies (33, 34). We show here that at pH 5.5, TcNA is released from the surface of the parasites into the surrounding milieu and could therefore function with increased efficiency on the components of the vacuolar membrane.

Various mechanisms of release of *T. cruzi*-derived proteins into the surrounding supernatant have been described, including budding of vesicles from the cell surface (35), secretion (31), and PIPLC cleavage of GPI-anchored surface proteins (36). The observation that TcNA release is increased at low pH in spite of an overall reduction in the total amount of surface protein released (Fig. 4) argues against an adverse effect of acidic conditions on the cells and suggests the existence of a mechanism preferentially controlling the release of TcNA. At present, we believe that the principal mechanism of release of the neuraminidase is PIPLC cleavage of the membrane-bound protein, since >90% of TcNA activity released into the supernatant can be removed with anti-CRD antibodies (Table 2). Neither depletion of ATP stores with 2-deoxyglucose and sodium azide, nor inhibition of protein

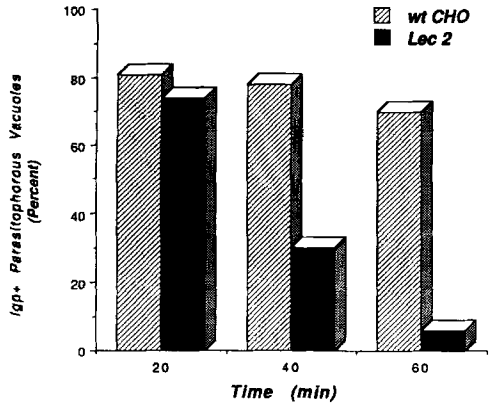

Figure 6. Kinetics of vacuole rupture in wt CHO vs. Lec 2 cells. Percentage of intracellular *T. cruzi* circumscribed by intact vacuolar membranes at different time points after infection, as assessed by circumferential immunofluorescent staining of intracellular *T. cruzi* with anti-lgp antibodies. More than 100 cells were examined at each point, as described in Materials and Methods.

synthesis with puromycin, has any effect on the amounts of TcNA released (data not shown). In keeping with these observations, it was shown recently that soluble TcNA reacts with anti-CRD on immunoblots (9), and that PIPLC from *T. brucei* releases TcNA activity from *T. cruzi* trypomastigotes in vitro (29). Furthermore, cloned *T. cruzi* neuraminidase sequences contain hydrophobic stretches characteristic of GPI-anchored proteins (37, 38).

Phosphatidylinositol-linked sialidases of pig brain (39), as well as other GPI-anchored enzymes of protozoan and mammalian cells (reviewed in reference 28) have previously been described. PIPLC have been described in both *T. cruzi* (36) and *T. brucei* (40–42). Although the *T. cruzi* PIPLC has not yet been purified, enzymatic cleavage of the GPI anchor of membrane form variant surface glycoprotein (VSG) of *T. brucei* by *T. cruzi* lysates was found to be significant at pH 6 and below (43). It remains to be determined whether the acid-sensitive enhancement of releasee of TcNA is due to direct acid activation of the *T. cruzi* PIPLC. pH-dependent expression of virulence factors has been described in microbial pathogens (44).

Lysosomal membrane glycoproteins are susceptible substrates for TcNA as demonstrated by in vitro desialylation of lgp (see Fig. 5). Two lines of evidence indicate this process is important for the enhanced function of the *T. cruzi* hemolysin (Tc-TOX). First, the experiments comparing the rates of parasite escape from the vacuole in the sialylation-defective Lec 2 cells and in wild-type CHO cells demonstrate that the absence of terminal sialic acids enhances vacuolar membrane disruption (see Fig. 6). Second, neuraminidase pretreatment of target guinea pig erythrocytes renders them more susceptible to Tc-TOX-mediated hemolysis (see Fig. 8).

Only a small percentage of total cellular lgp is incorporated into parasitophorous vacuoles (see Figs. 1 and 2), and under the short-term infection conditions required for synchronization of infection, typically only 50–60% of the cells are infected. It is therefore not surprising that it has not been possible to directly demonstrate the appearance of desialylated lgp species in infected cells, particularly in view of the considerable heterogeneity exhibited by these proteins upon isoelectric focusing (see Fig. 5). Large scale isolation of parasitophorous vacuoles will probably be required for the direct verification of TcNA's proposed intravacuolar role.

A developmentally regulated neuraminidase activity of *T. cruzi* was described nearly a decade ago (7). Subsequent studies, however, suggested extensive polymorphism of the enzyme, with different laboratories reporting strikingly different molecular weights (8, 32, 45). The basis for this polymorphism is just now beginning to be understood. Recent molecular genetic studies have revealed conserved sequences of bacterial and viral neuraminidases in a *T. cruzi* 85-kD surface antigen large gene family (37, 46), and also in a 180-kD protein expressing neuraminidase activity (38). Recent observations (9, 47) indicate that the neuraminidase and a previously described sialyltransferase activity (12, 48, 49) can be mediated by the same group of 120–220 kD *T. cruzi* surface proteins, previously described as SAPA antigen (50). The mAb 39 used in our studies recognizes multiple bands in this size range, and removes both neuraminidase and *trans*-sialidase activities from parasite supernatants (9).

Taken together, the observations discussed above suggest that multiple surface *T. cruzi* neuraminidase/transialidase-related enzymes encoded by distinct members of a gene family may exist. It remains to be clarified which specific polypeptides are responsible for the activities observed. To date, however, only a role in extracellular phases of infection has been considered for these enzymes. Here we propose an alternative model in which a primary biological role of the neuraminidase activity would be intracellular. Entry into a host cell and formation of a membrane-bound vacuole surrounding the parasite would have several consequences. First, the restricted volume would prevent dilution of *T. cruzi* neuraminidase released into the vacuolar space, and would isolate the enzyme from its serum inhibitors. Second, as acidification ensued, an enhanced release of the enzyme from the parasite surface into the vacuole would occur. Desialylation of vacuolar membrane constituents, notably lysosomal membrane glycoproteins and possibly glycolipids, would occur. Third, as acidification continued into the pH 5.5 range, Tc-TOX (the acid-active pore-forming protein) activity would increase. Active Tc-TOX would diffuse and insert more efficiently into the lipid bilayer of the desialylated vacuolar membrane, resulting in membrane disruption.

We do not know, at the molecular level, how *T. cruzi* neuraminidase facilitates the action of Tc-TOX. Or, put another way, we do not know how sialic acids impede the action of Tc-TOX. A coat of glycoconjugates on the luminal aspect of lysosomes has been described (19) and has been suggested to protect the lysosomal membrane from degradation by the luminal enzymes (51). One possibility is that by presenting an array of highly negatively charged, heavily glycosylated moieties, the matrix of lysosomal membrane glycoproteins prevents access to the lipid bilayer. Once the negative charges are removed, however, aggregation of the membrane glyco-

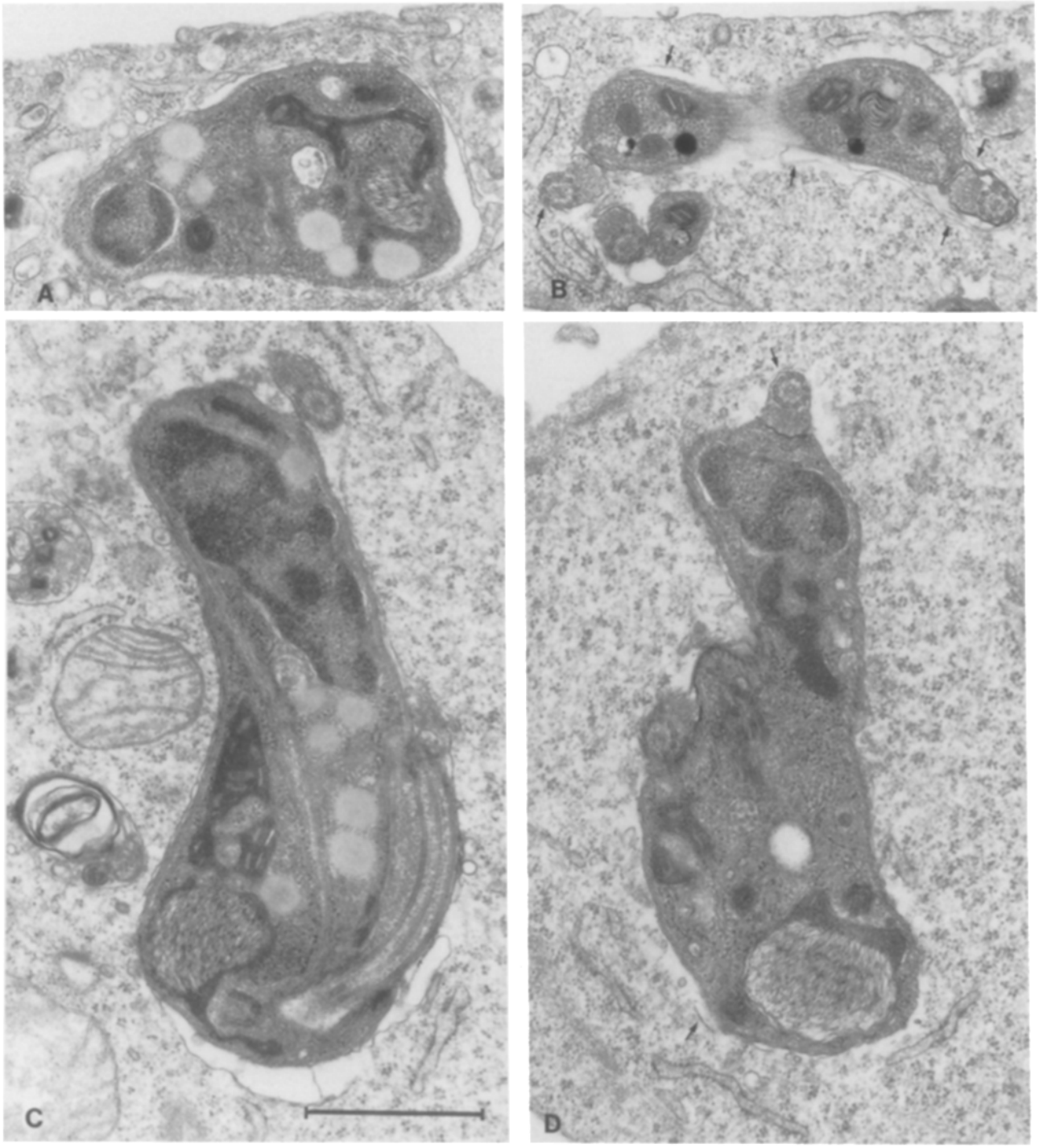

Figure 7. Sections through *T. cruzi*-infected Lec 2 cells showing various stages of escape from the surrounding parasitophorous vacuole. (*A*) Enough of the parasitophorous vacuole membrane is present around the parasite to count the vacuole as intact. (*B*) Although vacuole membrane is present (*arrows*), it is fragmented. This vacuole was counted as being broken. (*C*) Most of the membrane around the parasite is still present. (*D*) Most of the host membrane (*arrows*) has disappeared. Bars, 0.5 μm.

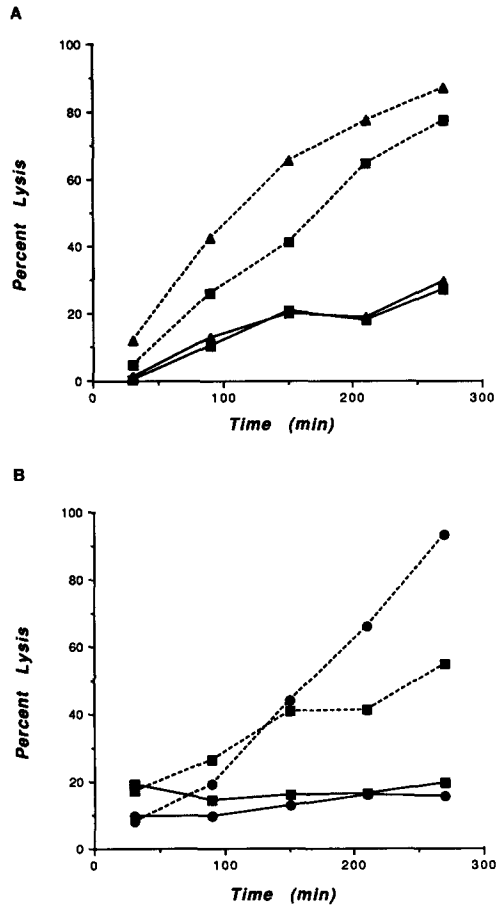

Figure 8. Neuraminidase pretreatment renders erythrocytes more susceptible to lysis by Tc-Tox. (*A*) Kinetics of hemolysis after pretreatment of target erythrocytes with *V. cholerae* neuraminidase. (*B*) Kinetics of hemolysis after pretreatment of target erythrocytes with partially purified *T. cruzi* neuraminidase. Assays were carried out as described in Materials and Methods. (——) Samples in which amastigotes were treated with NaN_3 to block release of Tc-TOX; (- - - -) untreated samples. (■) Samples in which control untreated erythrocytes were used as targets; (▲; *V. cholerae* neuraminidase or ●, *T. cruzi* neuraminidase) samples in which erythrocytes were pretreated with neuraminidase. Points were determined in triplicate and SD was <10%.

proteins may occur, creating regions where the lipid bilayer might be accessible to Tc-TOX. Alternatively, since Tc-TOX has a pI of 8.6–8.9 (N. W. Andrews, unpublished results), sialic acid moieties might bind Tc-TOX directly via electrostatic interactions, preventing its insertion into the membrane.

Our observations and those of others (12) raise the intriguing possibility that acquisition of sialic acid moieties by *T. cruzi* enhances parasite survival in a susceptible host. Sialic acids have been suggested to function as biological masks, reducing immunogenicity (52). Sialic acids on pathogenic bacteria control complement activation (53), and at least in the case of *Neisseria gonorrheae*, the sialic acids are acquired from the host (54, 55). Neuraminidase treatment has also been reported to convert trypomastigotes of *T. cruzi* into activators of the alternative pathway of complement (56).

In view of the increased sensitivity of desialylated membranes to lysis by Tc-TOX, the acquisition by *T. cruzi* of sialic acids from serum donor molecules may also represent a form of developmental preadaptation for parasite entry into host cells. Sialic acid previously has been shown to be important for the function of cell surface inhibitors of perforin, and neuraminidase treatment of cytotoxic T cells enhances their binding of perforin (57). Since Tc-TOX and perforin are functionally and antigenically related (6), *T. cruzi* may render itself resistant to the lytic action of Tc-TOX through its acquisition of sialic acid in an analogous manner to that in which sialic acid moieties inhibit lysis of CTL by perforin.

In conclusion, we have demonstrated that the neuraminidase/*trans*-sialidase of *T. cruzi* is likely to function optimally within the newly formed, acidic parasitophorous vacuole. We propose that this enzyme acts in concert with a parasite-derived, transmembrane pore-forming protein to facilitate parasite escape from the vacuole into the cytoplasm. Although the exact interplay between host cell sialic acid moieties, and parasite-derived neuraminidase and Tc-TOX remains to be elucidated at the molecular level, the hypothesized roles are amenable to experimental testing and are currently under investigation in our laboratories. Since a wide variety of bacterial and viral pathogens exhibit neuraminidases active at low pH (reviewed in reference 58), our observations may have broad implications for a number of microbial pathogenic processes.

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