Mammalian Cell Sialic Acid Enhances Invasion by
Trypanosoma cruzi

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We have used a Chinese hamster ovary cell mutant (Lec2) that express much less sialic acid on the surface than the parental cell line (Pro5) to investigate whether sialic acid plays a role during cell invasion by Trypanosoma cruzi. Trypomastigotes derived from a tissue culture (corresponding to bloodstream trypomastigotes) and metacyclic trypomastigotes (corresponding to infective stages of the insect vector) invaded the Lec2 mutant less efficiently than the parental cell line. Invasion of the Lec2 mutant cells could be restored to the Pro5 level by resialylation of the mutant cells with T. cruzi trans-sialidase and sialyllactose. Conversely, pretreatment of the Pro5 parental cells with bacterial neuraminidase decreased invasion. These results indicate that sialic acid associated with the host cell contributes to invasion by T. cruzi.

Trypanosoma cruzi, an obligatory intracellular protozoan parasite that causes Chagas’ disease in humans, invades many cell types and infects hosts belonging to several mammalian species (4, 6, 29). The stages of T. cruzi that actively enter mammalian cells are the metacyclic trypomastigotes, found in the hind gut of Reduviidae insects, and the bloodstream trypomastigotes (20).

Several observations suggest that sialic acid plays a role in cell invasion by bloodstream and metacyclic forms. As reviewed by Pereira (9), he and coworkers identified a neuraminidase on the surface of T. cruzi trypomastigotes and showed that the inhibition of this enzyme by monoclonal antibodies (MAbs) (13) or by serum lipoproteins (14) increased the entry of trypomastigotes. They further found that the addition of exogenous bacterial neuraminidase abrogated this enhancement. On the basis of these findings, they suggested that in order to invade, T. cruzi recognizes a sialic acid-containing receptor and that the removal of this carbohydrate by the parasite neuraminidase negatively controls the level of infection.

We have found that the neuraminidase described by Pereira is in fact a trans-sialidase (TS) (16, 19, 24). The transfer of sialic acid was detected originally in noninfective epimastigotes by Previo et al. (12) and later in trypomastigotes by Zingales et al. (28). The enzyme transfers α(2,3)-linked sialic acid from host glycoproteins and glycolipids to parasite acceptors consisting of terminal β-galactosyl units. In the absence of acceptors, TS transfers sialic acid from a donor to water in a typical sialidase reaction (16).

Trypomastigotes growing in medium without sialo-glycoproteins contain low levels of sialic acid but are still able to invade cells. During invasion, the sialic acid is transferred from the host cell to the parasite, resulting in the sialylation of a trypomastigote surface antigen, recognized as stage-specific epitope 3 (Ssp-3) (1, 19). On the basis of the finding that MAbs to Ssp-3 prevent cell invasion (15, 17), we have reasoned that the transfer of sialic acid from the host cell to the parasite could be required for invasion. To test this hypothesis, we have studied T. cruzi invasion of a mutant (Lec2) derived from Chinese hamster ovary cells and resistant to wheat germ agglutinin (22). This mutant has little sialic acid on its surface because of a defect in the transfer of CMP-sialic acid into the Golgi complex (5).

MATERIALS AND METHODS

Trypanosomes. T. cruzi trypomastigotes, strain Y (21), were derived from supernatants of cultures of LCCMK2 cells (ATCC CCL-7; American Type Culture Collection, Rockville, Md.) grown in low-glucose Dulbecco’s modified Eagle’s medium with penicillin and streptomycin (DMEM; GIBCO, Grand Island, N.Y.) and 10% fetal bovine serum (FBS) at 37°C in 5% CO2. Subconfluent cultures of LCCMK2 cells were infected with 5 × 10^4 trypomastigotes. Free parasites were removed after 24 h, and the cultures were maintained in 10% FBS–DMEM. 10% FBS–DMEM was removed on the third day following infection, the monolayers were washed twice with phosphate-buffered saline (PBS), and DMEM containing 0.2% bovine serum albumin (BSA) (ultrapure; Boehringer Mannheim, Indianapolis, Ind.) and 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (Sigma) (pH 7.4) (0.2% BSA–DMEM) was added. The parasites in the cell supernatants were used directly in some experiments, whereas in others, the slender trypomastigotes were purified from contaminating amastigotes or intermediate forms. For this purification, parasite suspensions were centrifuged at 2,000 × g for 5 min and incubated at 37°C. The motile trypomastigotes swam up from the pellet into the medium and were collected with the supernatant after 2 h. The contamination of this fraction with amastigotes or intermediate forms was less than 1%. Metacyclic trypomastigotes, strain CL (2), were obtained from aged cultures of epimastigotes in liver infusion trypomos medium containing 10% FBS at 28°C. When the cultures started to differentiate, the parasites were centrifuged and resuspended in Grace’s medium (GIBCO). After 5 to 7 days, 50 to 60% of the parasites were metacyclic trypomastigotes and were separated from epimastigotes by passage through a DE-52 column (Whatman, Maidstone, United Kingdom).

Enzymes and other reagents. TS was affinity purified from the trypomastigote supernatants as described previously...
(16). In brief, the trypomastigotes released from infected LLCMK₂ cells were centrifuged for 10 min at 3,200 x g, and the supernatants were filtered through 0.45-μm-pore-size filters to remove remaining parasites or any other cellular debris. The filtered supernatants were concentrated by precipitation with 50% ammonium sulfate, and the precipitates were resuspended in PBS, dialyzed against PBS, and passed through a Tresyl-Sepharose (Schleicher & Schuell) column containing immobilized MAb 39 (prepared in accordance with manufacturer instructions). The column was washed with PBS and then with 10 mM sodium phosphate (pH 6.5), and TS was eluted with 3.5 M MgCl₂, 10 mM sodium phosphate (pH 6.0). The fractions eluted from the column were immediately filtered through Sephadex G-25 equilibrated with 20 mM Tris-HCl (pH 8.0) to remove the MgCl₂. The enzyme was stored in a sterile manner at 4°C. MAb 39 reacts specifically with TS that is more than 95% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis silver staining, as shown previously (20). Vibrio cholerae neuraminidase (protease free), biotin-labeled Maackia amurensis lectin (MAA), and sialyllactose from bovine colostrum [65% α(2-3)-linked sialic acid] were obtained from Boehringer. Clostridium perfringens neuraminidase (type X, with less than 0.002 U of protease activity per mg of casein) was obtained from Sigma. Anti-T. cruzi rabbit antiserum was prepared as described previously (17). Heparin sulfate was kindly provided by Helena Nader, Escola Paulista de Medicina, São Paulo, Brazil.

Cell invasion experiments. Lec2 cells (ATCC CRL 1736) and the parental Pro5 cells (ATCC CRL 1781) were plated in 24-well plates with or without 12-mm-diameter glass coverslips and containing alpha-MEM (GIBCO) plus 10% FBS. When plated directly on plastic, 5 x 10⁴ cells were plated per well and used after 48 h. When glass coverslips were used, 1.5 x 10⁵ cells were plated per well and used after 72 h to achieve identical spreading for both cell lines. Prior to infection, the cells and the parasites were washed twice with 0.2% BSA-alpha-MEM and treated as indicated in each experiment. The parasites were incubated with the cells for 15 to 120 min at various concentrations in a final volume of 0.25 to 0.5 ml. The infections were quantitated either by counting microscopically the number of parasites stained by immunofluorescence and associated with the cells or by measuring the TS activity of intracellular parasites after the fifth day of infection. In the first method, after incubation of the parasites with the cells, the coverslips were washed three times with medium 199 and one time with PBS and fixed with 4% paraformaldehyde in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 in PBS, and the parasites were visualized after being stained by indirect immunofluorescence with a polyclonal anti-T. cruzi antibody. The total number of parasites and the number of infected cells were counted for at least 500 cells in triplicate experiments. When intracellular TS activity was used as a measure of infection, cells were washed five times with 0.2% BSA–DMEM and incubated in 10% FBS–alpha-MEM for 5 days at 37°C. Cell monolayers were washed twice with PBS, removed from the culture wells by trypsin-EDTA treatment, suspended in DMEM-BSA, and transferred to Eppendorf tubes. After centrifugation, the cells were washed with PBS and lysed in 100 μl of 50 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40, 0.1 M EDTA, 1 mM phenylmethysulfonyl fluoride, and 0.5 μg each of antipain, leupeptin, and pepstatin (Sigma) per ml. TS activity was determined by incubation of the lysates in 20 mM HEPES buffer (pH 7.2) in the presence of sialyllactose and D-glucose-[1-¹⁴C]lactose (60 mCi/mmol) (Amersham, Arlington Heights, Ill.) as described previously (16). This method provides results very similar to those provided by the first method. All experiments were performed in triplicate, and the results are expressed as the mean ± the standard error of mean. P values were calculated by use of an unpaired two-tailed t test.

RESULTS AND DISCUSSION

T. cruzi trypomastigotes derived from LLCMK₂ cells and metacyclic trypomastigotes growing in liver infusion trypsin medium were incubated for 30 min with Pro5 and mutant Lec2 cells. As shown in Fig. 1, tissue culture trypomastigotes and metacyclic forms invaded Lec2 cells less efficiently, as reflected by the total number of parasites associated with the cells and by the percentage of infected cells.

To verify whether the absence of sialic acid was responsible for the lower level of infection of Lec2 cells, we sialylated the cells by incubating them with affinity-purified T. cruzi TS in the presence of an appropriate sialic acid
donor, sialyllactose. The transfer of sialic acid to the Lec2 cells was corroborated by the incubation of TS-treated cells with biotin-labeled MAA and then with fluorescein isothiocyanate (FITC)-streptavidin. We found that MAA, which recognizes α(2,3)-sialyl-galactosyl units (26), bound to Lec2 cells treated with TS and sialyllactose but not to untreated cells, as seen by use of FITC-streptavidin (Fig. 2). As shown in Fig. 3A, the level of invasion of Lec2 cells that had been pretreated with TS and sialyllactose was similar to the level of invasion of Pro5 cells. This effect was not due to the presence of TS, since pretreatment with TS but without sialyllactose had no effect on the invasion of Lec2 cells (Fig. 3B). Similar results were obtained when the TS activity of parasites 5 days after invasion was measured. As shown in Table 1, TS activity on the fifth day after infection was significantly higher in Lec2 cells that had been resialylated by pretreatment with TS and sialyllactose.

The level of invasion of the Lec2 cell line was consistently lower than that of the Pro5 cell line, and the deficiency for the Lec2 cells was overcome following sialylation. The simplest explanation for these results is that sialylation of the Lec2 cells was responsible for the increase in invasion. Nevertheless, we cannot exclude the possibility that TS in the presence of sialyllactose had an effect other than the sialylation of Lec2 cells. For example, TS could act as an adhesive protein and increase the attachment of trypomastigotes to Lec2 cells; TS is a complex molecule that contains, in addition to the catalytic site, type III modules of a fibronectin domain that could interact with the target cell surface (10). To obtain further evidence for the participation of sialic acid in invasion, we pretreated the Lec2 and Pro5 cell lines with neuraminidase before invasion under two different sets of conditions. In the first treatment, the cells were incubated with 0.1 U of *V. cholerae* neuraminidase per ml for 40 min at pH 7.0, while in the second treatment, the cells were incubated with the same concentration of neuraminidase but at pH 6.5 for 50 min. As shown in Fig. 4A, treatment at pH 7.0 diminished infection of Pro5 cells only. Incubation with neuraminidase at a lower pH also decreased the invasion of Lec2 cells (Fig. 4B), suggesting that even the small amounts of sialic acid present on the surface of Lec2 cells (10% of the amounts present on the surface of parental cells [23]) could mediate invasion by *T. cruzi*.

The fact that the invasion of Lec2 cells is still significant even after neuraminidase treatment suggests that other molecules mediate attachment and/or invasion of *T. cruzi* independently or in combination with sialic acid. For example, matrix components have been implicated in attachment of and invasion by *T. cruzi*, and it is known that trypomastigotes bind to collagen (8) and fibronectin (25). Trypomastigotes also express a heparin-binding molecule, named pentrin, that is able to prevent the invasion of *T. cruzi* and, when expressed in *Escherichia coli*, confers invasive capabilities on the bacteria (7). Therefore, in addition to sialic
acid, heparan sulfate could mediate adhesion and invasion. Under our assay conditions, however, preincubation of tissue culture trypomastigotes, metacyclic trypomastigotes, or the target cells with up to 50 µg of heparan sulfate per ml had no effect on the invasion of either Lec2 or Pro5 cells (data not shown).

In the experiments described above, we used trypomastigotes released from LLCMK₂ cells growing in medium containing BSA. Under these conditions, the parasites are weakly labeled by anti-Ssp-3 antibody and contain small amounts of sialic acid (19). The amounts of sialic acid greatly increase when the parasites are released into medium containing serum or are pretreated with sialylactose. In Table 1, we compare invasion by parasites with low levels of sialic acid with invasion by those fully sialylated. Trypomastigotes preincubated with sialylactose still invaded Pro5 cells better than Lec2 cells. Similar results were obtained in many experiments when invasion was estimated after 30 min of contact between cells and parasites or when we used parasites released by LLCMK₂ cells grown in medium containing FBS (data not shown). In all of these situations, only a small increase in invasion was obtained for sialylated parasites.

These results indicate that complete sialylation and the expression of large amounts of Ssp-3 are not primary requirements for invasion. The same conclusion was obtained in other experiments in which sialic acid was transferred from Ssp-3 to a sialic acid acceptor in the incubation medium. Preincubation of trypomastigotes in 10 mM lactose or the addition of lactose during invasion did not interfere with entry for either the Lec2 or the Pro5 cell line. Similar results were obtained with other cell lines, such as BALB/C 3T3 fibroblasts and LLCMK₂ cells (data not shown).

These conclusions are in apparent contradiction with prior findings. Cell invasion is inhibited by Fab fragments of MAbs against Ssp-3 (17, 19), which is the major sialic acid acceptor of trypomastigotes. Also, MAbs to the glycoconjugates of 35- and 50-kDa antigens, which are the major sialic acid acceptors of metacyclic trypomastigotes (15a, 27), prevent cell invasion by metacyclic forms (15). That Ssp-3-containing molecules are involved in invasion is also supported from experiments in which trypomastigotes were opsonized with anti-Ssp-3 antibodies and then incubated with nonphagocytic cells that had been engineered to express Fc receptors. In sharp contrast to opsonization with antibodies against other parasite surface antigens, opsonization of trypomastigotes with anti-Ssp-3 antibodies did not enhance but rather inhibited invasion (18). Others have also provided data that suggest that sialylated molecules in the parasites have a role in invasion. For example, Piras et al. (11) have shown that incubation of trypomastigotes with sialic acid-containing glycoproteins increases their invasiveness, whereas Couto et al. (3) have found sialic acid in an 85-kDa glycoprotein of trypomastigotes that is involved in invasion.

One possible explanation for these contradictory results is that sialic acid-dependent mechanisms for parasite invasion require minimal amounts of sialic acid, which could be provided by the LLCMK₂ cells in which the trypomastigotes were grown. Alternatively, the Ssp-3 precursor molecules from the parasite surface may accept sialic acid from and

| TABLE 1. Invasion of Lec2 and Pro5 cells, measured 5 days after infection
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*Trypomastigotes released from LLCMK₂ cells were preincubated with 1 mM sialyllactose (SL) (⁺) or medium alone (⁻) and incubated with Chinese hamster ovary cells also pretreated with 1 mM SL and 10 µg of TS per ml or medium alone for 2 h. Parasites and cells were washed and coincubated for 15 min at 37°C. Unbound parasites were aspirated, the cells were washed five times with 0.2% BSA–alpha-MEM, and the infectivity was measured on the basis of the intracellular production of TS 5 days postinfection. Results are given as the mean ± the standard error of the mean for triplicate experiments. Similar results were obtained in two independent experiments. The value for Lec2 cells alone was 175 ± 45 cpm. The difference between the invasion of Lec2 cells and that of Lec2 cells pretreated with TS and SL was significant in the case of nonsialylated trypomastigotes (\(P = 0.004\)) or sialylated trypomastigotes (\(P = 0.003\)). The difference between sialylated and nonsialylated trypomastigotes was not significant (\(P > 0.05\)).

FIG. 4. Neuraminidase treatment of target cells diminishes invasion by T. cruzi. Pro5 and Lec2 cells were treated with 0.1 U of azide-free V. cholerae neuraminidase per ml in 0.2% BSA–alpha-MEM (pH 7.0) for 40 min (A) or in the same medium but at pH 6.5 for 50 min (B). In the control experiments, the cells were treated with the same medium and buffer lacking neuraminidase. The cells were then washed and incubated with 2.5 × 10⁷ trypomastigotes per ml in 0.2% BSA–alpha-MEM for 30 min. Open bars correspond to the infection of untreated cells, and hatched bars correspond to the infection of cells treated with neuraminidase. The difference in the invasion of ProS cells treated or not treated with neuraminidase was significant (\(P = 0.0071\) in panel A and \(P = 0.011\) in panel B). The difference in the invasion of Lec2 cells treated or not treated with neuraminidase was not significant in panel A (\(P = 0.47\)) but was significant in panel B (\(P = 0.01\)).
donate sialic acid to the host during invasion. This sialic acid exchange could facilitate the movement of the parasites into the cells and prevent an irreversible attachment. This hypothesis would explain why cells with less sialic acid are invaded to a lesser degree. In the presence of exogenous acceptors, such as lactose, the transfer would occur from the target cell both to the exogenous saccharide and to the Ssp-3 precursor, thus explaining the lack of inhibition of penetration upon the addition of lactose to the incubation medium.

It follows that the effect of antibodies to Ssp-3 could be to prevent the exchange of sialic acid. However, the direct demonstration that sialic acid transfer is necessary for invasion by *T. cruzi* requires the development of powerful TS inhibitors or the construction of *T. cruzi* strains lacking TS.

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