INFECTION AND IMMUNITY, Jan. 1987, p. 181–186
0019-9567/87/010181-06$02.00/0
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Vol. 55, No. 1

A Membrane-Associated Neuraminidase in *Entamoeba histolytica* Trophozoites

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Received 17 July 1986/Accepted 14 October 1986

Trophozoites of the parasitic amoeba *Entamoeba histolytica* HM-1:IMSS possess a surface neuraminidase capable of liberating *N*-acetylneuraminic acid (NANA) from *N*-acetylneuramin–lactose (α2→3 or α2→6) or mucin in their medium. The neuraminidase was found to be membrane associated, with more than 50% of the yield being recovered in the plasma membrane fraction. The neuraminidase specific activity of the plasma membrane fraction was six times that of internal membrane fraction enzyme. The optimum pH and temperature for this enzyme were 6.7 and 37°C, respectively. Neuraminidase activity was inhibited by ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and the optimum Ca^{2+} concentration was 2 mM. The microfilament disruptor cytochalasin D (30 μg/ml) inhibited motility and neuraminidase activity of intact *Entamoeba* trophozoites. The cytochalasin D-induced loss of surface neuraminidase activity was explained in part by a redistribution of enzyme with a loss of plasma membrane enzyme and an increase in intracellular membrane enzyme. A qualitatively similar cytochalasin D effect was observed with two other membrane-associated enzymes, calcium-regulated ATPase and acid phosphatase. Membrane-associated enzyme was minimally affected by Triton X-100 and saponin. An *N*-acetylneuraminic acid aldolase, optimum pH, 7.4, was found in trophozoite homogenate supernatant fractions. NANA and NANA-containing compounds stimulated trophozoite-directed motility. This motility stimulation by NANA-containing compounds did not apparently require prior release of free NANA by the trophozoite surface neuraminidase. *Entamoeba* neuraminidase is one of a series of enzymes that may modify the mucus blanket and target cell surface and thereby play a role in the pathogenesis of amebiasis.

Entamoeba histolytica is a parasitic amoeba in humans. Trophozoites of this parasite may remain in a commensal form, primarily in the large bowel lumen, or may invade the intestinal mucosa to produce signs and symptoms of intestinal amebiasis (17). The invasive process involves attachment of trophozoites to host mucosal epithelial cell surface molecules via some amoeba surface lectin (14, 25), killing of the host cell (11), and phagocytosis (30); alternatively, it may involve the trophozoites penetrating between cells (29), perhaps at the site where normal epithelial cell sloughing occurs (20). Whatever the signals or intestinal lumen environmental factors involved in the transition of trophozoites from commensal to invasive organisms, the amoebae must first penetrate the host's mucus blanket. This is probably not easily done, as *Entamoeba* trophozoite motility has been shown to be greatly reduced when the amoeba is associated with intestinal mucus (15).

The physical and chemical properties of the mucus gel would be expected to play a role in determining whether or not a given *Entamoeba* trophozoite could gain access to the mucosal epithelium. We previously found that *N*-acetylneuraminic acid (NANA) stimulates *Entamoeba* motility, whereas L-fucose inhibits it (15). This is probably significant in determining how an *Entamoeba* trophozoite behaves in or adjacent to the mucus blanket, as these two molecules are major mucin carbohydrate end groups (27). This observation, together with the fact that the NANA content of a mucin gel is a major determinant of the charge characteristics of the gel (9), led us to investigate the possibility of *Entamoeba* trophozoites possessing a neuraminidase and whether the motility effects of NANA required the activity of such an enzyme.

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MATERIALS AND METHODS

Trophozoites. Trophozoites of the pathogenic strain of *E. histolytica*, HM-1:IMSS, were cultivated axenically in Diamond TP-S-1 medium (7). The trophozoites were grown in capped 10-ml tubes and harvested in late growth phase by chilling of the tubes in ice water for 5 min, followed by centrifugation at 500 × *g* for 2.5 min. Amoebae were washed in NaCl solutions buffered with 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) to pH 6.7, with an osmolarity of 344 mOsm.

Motility. Trophozoite directed motility was measured at 37°C as previously described (15). Briefly, this method counts the number of times any part of a trophozoite, usually a pseudopodium, intersects a side of a 1/16-mm hemacytometer grid square in 5 min. Trophozoites were harvested and washed three times in PIPES (20 mM)-buffered saline, pH 6.7, 344 mOsm. They were resuspended in the test solution (this saline solution contained the solute to be tested for motility stimulation), and the motility of representative trophozoites was immediately measured. All reagents were purchased from Sigma Chemical Co., St. Louis, Mo. Several concentrations of NANA (type IV) and equivalent NANA concentrations of *N*-acetylneuramin–lactose (from bovine colostrum, primarily α2→3) and mucin (type I) were used. In experiments in which cytochalasin D was used, this agent was first solubilized in dimethyl sulfoxide (DMSO) and added to the NaCl solution to a final DMSO concentration of 0.5%. The viability of trophozoites incubated in solutions containing 30 μg of cytochalasin D per ml for 60 min

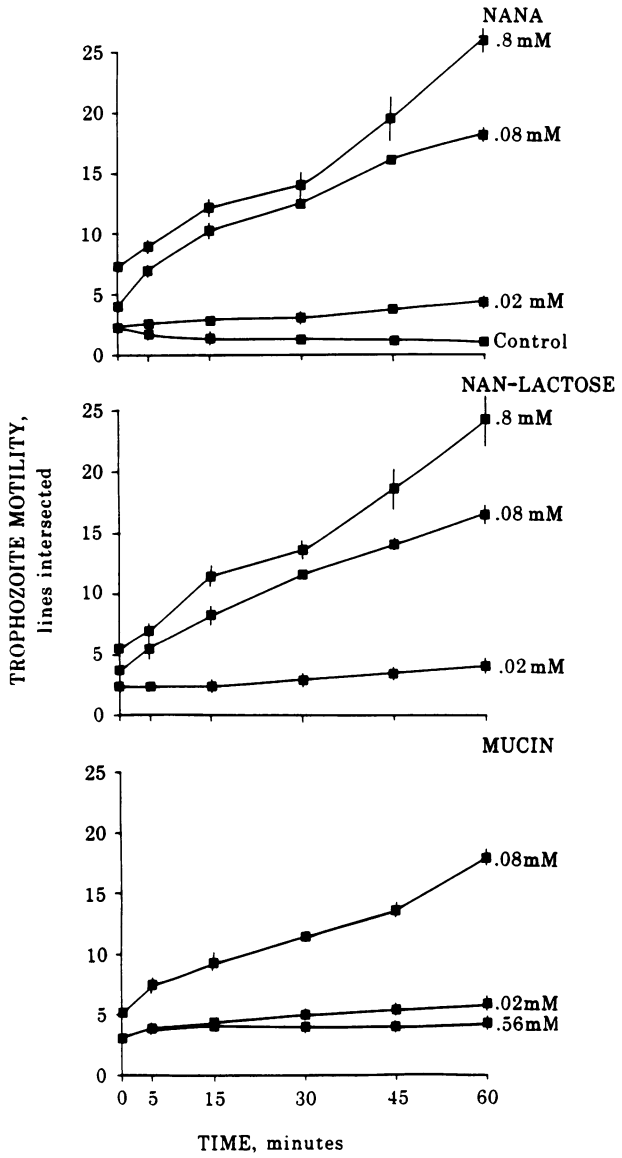

FIG. 1. *E. histolytica* trophozoite motility measured in a PIPES-buffered saline solution (pH 6.7, 344 mOsm) containing NANA, *N*-acetylneuramin–lactose (NAN-lactose), or mucin. The mucin concentration is given as equivalent to its NANA concentration. Motility was scored as the number of times the lines of a 1/16-mm-square hemacytometer grid was intersected by any part of a trophozoite in a 5-min period. The values are means ± SEM.

remained at or above 95%, provided the DMSO concentration was not greater than 0.5%.

Enzyme assays. Neuraminidase was assayed by measuring the release of NANA from an appropriate substrate: *N*-acetylneuramin–lactose (from bovine colostrum, primarily α2→3, or human colostrum, primarily α2→6) and mucin (Sigma; type I). Free NANA was determined by the thiobarbituric acid assay of Warren (31). When the NANA content of mucin samples was to be determined, the samples were first hydrolyzed in 0.2 N H_2SO_4 at 80°C for 1 h to liberate sialic acid before its measurement.

When trophozoite membrane neuraminidase was to be measured, the membrane sample was incubated for 30 min in 150 mM NaCl–2 mM $CaCl_2$–20 mM PIPES (pH 6.7)–0.2 mM *N*-acetylneuramin–lactose (bovine), with a final membrane protein concentration of 0.01 to 0.04 mg/ml.

Entamoeba membrane calcium-regulated ATPase (Ca-ATPase) was measured by the method of McLaughlin and Muller (19) with a final calcium concentration of 55 μM, a pH of 8.8, and incubation for 10 min at 30°C. P_i liberated from the ATP substrate was measured by the method of McLaughlin and Meerovitch (18).

Membrane *p*-nitrophenylphosphatase (acid phosphatase) was measured by the method of Serrano et al. (26) with incubation at 37°C for 2 min and an incubation medium pH of 5.0.

NANA aldolase was measured in homogenates of washed trophozoites by an adaptation of the method of Barnett et al. (3). Samples were incubated in 100 mM potassium phosphate buffer for 30 min at 37°C. After color development, trichloroacetic acid was added to blanks, standards, and samples to a final concentration of 4%. Calcium carbonate was then added to excess, and the A_{585} of the filtrate was measured. Sample protein concentrations were measured by the method of Bradford (4) with bovine serum albumin as a standard.

***Entamoeba* membrane isolation.** *Entamoeba* trophozoites were washed and suspended in phosphate-buffered saline containing 1 mg of concanavalin A per ml for 5 min. Cell lysis and membrane fraction isolation were performed by the method of Aley et al. (2). This method yields three membrane fractions referred to as plasma membranes, internal vesiculated membranes, and internal nonvesiculated membranes (including debris). Before any enzyme assay, these membrane fractions were exhaustively dialyzed against 10 mM Tris hydrochloride, pH 6.7, at 4°C.

Statistical analyses were performed by paired Student *t* tests.

RESULTS

Figure 1 illustrates the effects of NANA, *N*-acetylneuramin–lactose (bovine), and mucin on the motility of *Entamoeba* trophozoites. Each point represents the mean motility value of 10 trophozoites (2 trophozoites measured in five replicate experiments). The mucin concentrations are given as equivalent NANA concentrations. The motility stimulation seen with NANA was the same as that seen with an equivalent concentration of *N*-acetylneuramin–lactose. Equivalent concentrations of lactose did not stimulate motility. Mucin, at equivalent NANA concentrations of up to 0.08 mM, similarly stimulated *Entamoeba* trophozoite motility. At higher concentrations of mucin, motility decreased until, by an equivalent NANA concentration of 0.56 mM, motility was completely inhibited.

When intact *Entamoeba* trophozoites were incubated in unbuffered NaCl, pH 6.7, containing *N*-acetylneuramin–lactose (of bovine or human colostrum origin) or mucin, free NANA was detected in the medium. No free NANA could be detected in the medium of trophozoites suspended in the NaCl solution alone, suggesting the existence of an *Entamoeba* surface neuraminidase.

Figure 2 illustrates the effects of varying the medium calcium concentration on trophozoite motility, the 30-min neuraminidase activity of intact trophozoites, and the neuraminidase specific activity of the isolated *Entamoeba* plasma membrane fraction. Removing medium and cell surface calcium with 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) significantly inhibited motility and neuraminidase activity when

compared with Ca^{2+}-free medium alone. Motility and both intact trophozoite and *Entamoeba* plasma membrane neuraminidase activities exhibited optimum medium calcium concentrations. Whereas these three optimum concentrations were not identical, they were all in the range of 2 to 5 mM.

The optimum pHs for trophozoite homogenate and *Entamoeba* membrane neuraminidase activities were the same. Figure 3 illustrates the case for *Entamoeba* plasma membrane fractions in the presence of an optimum Ca^{2+} concentration and in 0.2 mM EGTA. The optimum pH was 6.7. The optimum temperature for the same membrane fraction neuraminidase was 37°C.

To determine whether membrane fraction vesicle formation had masked some of the neuraminidase activity by restricting access of enzyme to substrate, membrane fractions were sonicated three times for 1 min at 20 W or treated with Triton X-100 over a concentration range of 0.1 to 0.5%. Such treatment only resulted in a minimal (less than 10%) increase in neuraminidase specific activity in some preparations. Similarly, treatment of *E. histolytica* membrane fractions with 0.15% saponin, had no significant effect on neuraminidase activity.

The microfilament disruptor cytochalasin D inhibited *Entamoeba* trophozoite motility in a dose-dependent manner (Fig. 4). At a concentration of 30 μg/ml, cytochalasin D completely inhibited *N*-acetylneuramin–lactose-stimulated motility. The neuraminidase activity of intact trophozoites was also significantly inhibited by this concentration of cytochalasin D. Figure 4 also illustrates the release of free NANA into the trophozoite medium when intact trophozoites were incubated with medium containing *N*-acetylneuramin–lactose. In the DMSO carrier control experiment, this release of NANA was relatively constant for the duration of the experiment, whereas in the case of cytochalasin D-treated cells no further release of NANA occurred after 30 min, suggesting complete inhibition of surface neuraminidase.

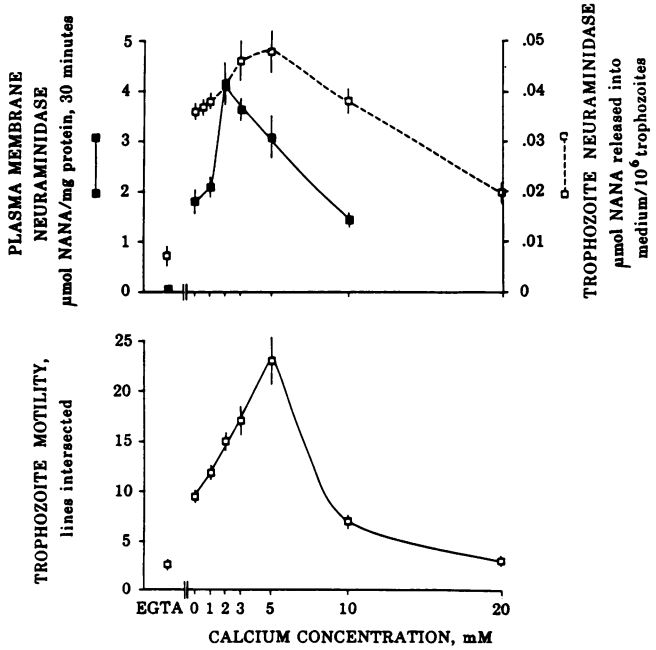

FIG. 2. *E. histolytica* trophozoite motility, surface neuraminidase activity of intact trophozoites, and trophozoite plasma membrane fraction neuraminidase specific activity plotted as a function of the medium calcium concentration. The EGTA points refer to a calcium-free medium in which the EGTA concentration was 0.2 mM. The values are means ± SEM.

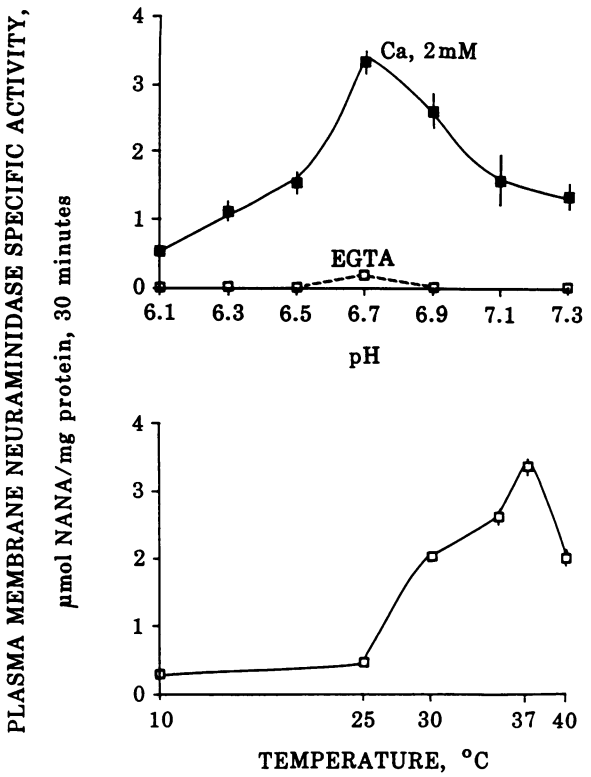

FIG. 3. Effects of pH and temperature on neuraminidase specific activity of plasma membrane fractions of *E. histolytica* trophozoites. Conditions of optimum pH (6.7) and calcium concentration (2 mM) were used when measuring the effects of temperature on enzyme activity. EGTA refers to a calcium-free medium in which the EGTA concentration was 0.2 mM. The values are means ± SEM.

The neuraminidase activity of *Entamoeba* trophozoites was found to be associated with membrane fractions. Table 1 summarizes the yield and specific activity data obtained when trophozoite membranes were fractionated by the method of Aley et al. (2). Most of the membrane-associated neuraminidase was found in the plasma membrane fraction. The specific activity of this plasma membrane neuraminidase was approximately six times that of the intracellular membranes. The total neuraminidase activity of the membrane fractions was twice that of the homogenate from which they were derived, suggesting that NANA released by the neuraminidase was subsequently metabolized by some cytoplasmic aldolase.

To determine whether the microfilament disruptor cytochalasin D inhibited the neuraminidase activity of intact trophozoites by causing enzyme redistribution, neuraminidase and two other membrane-associated enzymes, Ca-ATPase and acid phosphatase (*p*-nitrophenylphosphatase), were measured in membrane fractions of trophozoites treated for 45 min with 30 μg of cytochalasin D per ml or DMSO carrier alone. With the exception of the plasma membrane fraction, there were differences in membrane neuraminidase specific activities when control preparations (Table 1) were compared with DMSO carrier preparations (Table 2). DMSO might be expected to have some effect on the distribution of membranes within the cell since this agent

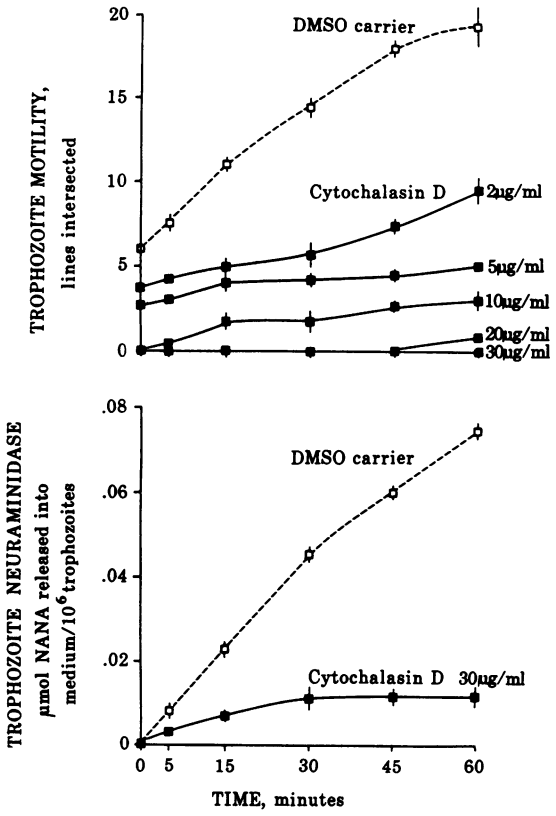

FIG. 4. Effects of cytochalasin D in DMSO carrier and carrier alone on the motility and neuraminidase activity of intact *E. histolytica* trophozoites. In the neuraminidase experiments, trophozoites were incubated in DMSO carrier medium or cytochalasin D medium for 15 min before the addition of substrate.

is known to increase synaptic vesicle fusion, even at a concentration of 0.5% (10). There was a feature common to all three membrane-associated enzymes when values from cytochalasin D-treated trophozoites were compared with values from DMSO carrier-treated trophozoites. With each enzyme, cytochalasin D significantly reduced the specific activity and percent yield in the plasma membrane fraction and increased these values in the internal vesiculated membrane fraction. The cytochalasin D effect in nonvesiculated internal membrane fractions was variable. Taken together, these data suggested that cytochalasin D reorganized membrane-associated enzymes so that they were increased in intracellular membranes at the expense of their distribution at the membrane surface. Cytochalasin D did result in a total reduction in recovered membrane-associated enzyme. However, when homogenate enzyme activities were measured, no cytochalasin D effect was observed (Table 2).

E. histolytica NANA aldolase obtained from the homogenate supernatant fraction after centrifugation at 150,000 × *g* for 30 min had an optimum pH of 7.4 and was completely inhibited by 5 mM sodium borohydride in the presence of pyruvate but not EDTA (3). The mean (± the standard error of the mean [SEM]) NANA activity of five samples in the presence of 10 mM EDTA was 189 ± 17 μmol of *N*-acetylmannosamine per mg of protein after 30 min, and the control value was 193 ± 16 μmol/mg. When the neuraminidase activity of trophozoite homogenates of DMSO- and cytochalasin D-treated trophozoites was measured while so inhibiting the aldolase, values of 0.062 ± 0.001 μmol of NANA per mg of protein (30 min) were obtained for both preparations, approximately double the value seen in the absence of aldolase inhibition. Thus, in a preparation in which NANA degradation was inhibited, we were able to confirm that cytochalasin D did not affect whole amoeba neuraminidase activity but only affected cellular enzyme distribution. Both of the products of NANA aldolase, pyruvate and *N*-acetylmannosamine, caused modest stimulation of trophozoite motility (one-third that of an equivalent concentration of NANA), making it unlikely that NANA stimulation of motility was mediated by either of these products of its metabolism.

DISCUSSION

NANA increased the directed motility of *E. histolytica* trophozoites maintained in saline solution at 344 mOsm and pH 6.7. This motility stimulation also was observed when NANA was associated with another sugar (Fig. 1, *N*-acetylneuramin–lactose) or was an end group sugar in the oligosaccharide of a glycoprotein (Fig. 1, mucin equivalent to 0.08 mM NANA). The motility effect of NANA did not require the sialic acid to be free. Equivalent concentrations of NANA increased motility whether the NANA was free or not, and the neuraminidase activity of intact trophozoites could not have generated sufficient free NANA from the substrates to account for the observed results. At higher concentrations of mucin (e.g., equivalent to 0.56 mM), physical retardation of trophozoite motility by the mucin gel masked any NANA-induced stimulation (15).

Entamoeba trophozoite motility and neuraminidase activity and the specific activity of *Entamoeba* trophozoite plasma membrane neuraminidase all exhibited optimum calcium concentrations between 2 and 5 mM. There was also an absolute requirement for Ca^{2+}, as motility and neuraminidase activity were almost completely inhibited by EGTA. This range of optimum Ca^{2+} concentrations is the same order of magnitude as that found in normal human extracellular fluid and is lower than that normally found in large-bowel luminal fluid (32). The optimum pH (6.7) of *Entamoeba* plasma membrane neuraminidase is also within the range normally found in the large-intestinal luminal content of humans (32).

Cytochalasin D inhibited *E. histolytica* trophozoite motility and intact trophozoite neuraminidase activity. This, together with the fact that this microfilament disruptor caused the activity of the neuraminidase and two other membrane-associated enzymes, Ca-ATPase and acid phosphatase, to be reduced in plasma membrane fractions while being increased in internal membrane fractions, suggested that there is a continuous turnover of the surface neuraminidase as the amoeba membrane turns over (1). As no

TABLE 1. Neuraminidase activity in membrane fractions of *E. histolytica*

Membrane type	Mean (±SEM) % yield (n = 5)	Mean (±SEM) sp act (μmol of NANA/mg of protein, 30 min; n = 5)
Plasma	54.8 ± 1.5	3.52 ± 0.15
Internal vesiculated	34.5 ± 0.8	0.58 ± 0.05
Internal nonvesiculated	8.9 ± 0.8	0.67 ± 0.08

TABLE 2. Effect of cytochalasin D (30 μg/ml) on specific activities of *E. histolytica* membrane-associated and homogenate enzymes

Enzyme	Mean (±SEM) activity (n = 5) with:		
	DMSO carrier	Cytochalasin D	Difference (P)
Plasma membranes			
Neuraminidase (μmol of NANA/mg of protein, 30 min)	3.326 ± 0.185	0.458 ± 0.027	<0.001
Ca-ATPase (μmol of P_i/mg of protein, 10 min)	394.1 ± 24.2	165.4 ± 2.7	<0.001
p-Nitrophenylphosphatase (μmol/mg of protein, 2 min)	20.30 ± 1.23	10.51 ± 0.30	<0.001
Internal vesiculated membranes			
Neuraminidase (μmol of NANA/mg of protein, 30 min)	0.095 ± 0.019	0.125 ± 0.023	<0.05
Ca-ATPase (μmol of P_i/mg of protein, 10 min)	104.9 ± 3.7	147.0 ± 2.7	<0.01
p-Nitrophenylphosphatase (μmol/mg of protein, 2 min)	1.63 ± 0.08	2.10 ± 0.05	<0.01
Internal nonvesiculated membranes			
Neuraminidase (μmol of NANA/mg of protein, 30 min)	0.256 ± 0.048	0.168 ± 0.032	>0.05
Ca-ATPase (μmol of P_i/mg of protein, 10 min)	188.8 ± 22.8	149.6 ± 11.9	>0.05
p-Nitrophenylphosphatase (μmol/mg of protein, 2 min)	11.08 ± 1.04	8.93 ± 0.63	<0.05
Whole cell homogenate			
Neuraminidase (μmol of NANA/mg of protein, 30 min)	0.031 ± 0.001	0.032 ± 0.001	>0.05
Ca-ATPase (μmol of P_i/mg of protein, 10 min)	35.2 ± 0.09	37.7 ± 1.5	>0.05
p-Nitrophenylphosphatase (μmol/mg of protein, 2 min)	1.88 ± 0.04	1.85 ± 0.02	>0.05

neuraminidase activity was recovered from intact trophozoite supernatant solutions, neuraminidase must be exported to the surface of the amoeba and then returned to the intracellular membrane pool by pinocytosis. Presumably, cytochalasin D inhibited this export of enzyme to the cell surface more than it inhibited enzyme uptake into intracellular vesicles. Cytochalasin D had qualitatively, but not quantitatively, the same effect on the three membrane-associated enzymes studied.

Neuraminidases are widely distributed in bacteria and eucaryotic cells (21). In pathogenic organisms, attempts have been made to determine whether neuraminidase is a pathogenic factor, e.g., contributing to bacterial adherence or mucus degradation in the case of enteropathogens. In the case of *Vibrio cholerae*, these attempts have been inconclusive (28), and although there is an apparent correlation between *V. cholerae* strain neuraminidase activity and the severity of the clinical signs and symptoms of cholera, many other known enteropathogens lack the enzyme (12).

Other parasitic protozoa also possess neuraminidase activity. Notable among these is *Trypanosoma cruzi*, which varies its neuraminidase activity with its life cycle (24). As is the case of *E. histolytica*, the neuraminidase in *T. cruzi* is found on the trypomastigote surface, has an optimum pH of approximately 6.5, and is calcium dependent (23).

Most, but not all, organisms that possess a neuraminidase possess an NANA aldolase (21). *E. histolytica*, however, possesses both a membrane neuraminidase and a soluble aldolase (type 1 [21]). *Entamoeba* trophozoites probably do not contain NANA on their cell surface. In mammalian cells, NANA carboxyl groups make a significant contribution to the negative surface charge of the cell surface (6). In protozoa, e.g., *Naegleria gruberi* (13) and *Trypanosoma lewisi* (8), the surface negative charge is unaffected by neuraminidase. However, in *Amoeba proteus*, neuraminidase inhibits phagocytosis (5), which may indicate the presence of NANA in this amoeba.

Whereas no role has yet been demonstrated for *E. histolytica* surface neuraminidase in the pathogenesis of amebiasis, it may be that this enzyme is one of a series of calcium-activated, membrane-associated enzymes (19), one of several that may be involved in the destruction of mucin, cell surface glycoproteins, and extracellular matrix during amebic invasion (16, 22).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant RR-8006 from the General Research Support Branch, Division of Research Resources, National Institutes of Health, Bethesda, Md. I.A.U. was a recipient of a Nigerian Government Scholarship while a student in the Department of Biology, Atlanta University, Atlanta, Ga.

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