Evidence That Hyaluronidase Is Not Involved in Tissue Invasion of the Protozoan Parasite \textit{Entamoeba histolytica}

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As previous reports suggested that a hyaluronidase is involved in tissue invasion of \textit{Entamoeba histolytica}, we searched for such an activity in trophozoite extracts. A hyaluronidase activity was not detectable in long-term cultures or in amoebae freshly passaged through a gerbil liver, as evidenced by four different techniques.

\textit{Entamoeba histolytica} is the causative agent of human amebiasis. Worldwide the protozoan parasite causes about 50 million cases of colitis or extraintestinal abscesses, and the disease results in at least 50,000 fatalities annually (33). The reproductive forms, the trophozoites, inhabit the cavity of the lower intestine in humans. For unknown reasons, they invade the mucosa of the colon and can enter several extraintestinal organs via the blood circulation system. Several factors are considered to play a crucial role in destroying the colonic epithelium: the adherence to cells is mediated by a Gal- and GalNac-specific lectin (24, 26). Subsequently, cells are killed by pore-forming peptides termed amoebaporens (19, 20). Extracellular matrix proteins are degraded by the proteolytic activity of several isoforms of cysteine proteinases (3, 14, 27, 30). Conceptually, for the effective spread of the parasite, amoebae should exert hyaluronidase-like activities. Therefore, the presence of such an enzyme in trophozoite extracts may be postulated.

The substrate of a hyaluronidase is hyaluronic acid (hyaluronan), a nonsulfated glucosaminoglycan that consists of repeating disaccharide units of \(\alpha\)-glucuronic acid and \(N\)-acyethyl-\(\alpha\)-glucosamine and which is a major structural component of the interstitial matrix of connective tissue in the lamina propria of the gastrointestinal tract (18). Hyaluronidases are broadly distributed enzymes and have been implicated in tissue invasion (5, 7, 17). Parasite-associated hyaluronidases are thought to participate in the infective process. In two parasitic nematodes, \textit{Ancylostoma caninum} and \textit{Anisakis simplex}, hyaluronidase activity is present in the gastrointestinal invasive stage and may facilitate tissue histolysis and mucosal invasion (11, 12). Hyaluronidase activity in \textit{E. histolytica} extract has been described previously (2, 16, 32). Activity was detected by measuring the loss of viscosity or turbidity of hyaluronic acid and the release of \(N\)-acyethylglucosamine after incubation with crude trophozoite extract. However, the results are equivocal. It is reported that hyaluronidase activity is present only in amoeba isolates freshly passaged through a hamster liver. After a few weeks of cultivation, the activity is lost (2). Considering that hyaluronidase is expressed only during the invasion process and is rapidly lost when the appropriate stimulus is absent, the finding that hyaluronidase activity is present in stock cultures of \textit{E. histolytica} without passing through the livers of hamsters is controversial (16).

Despite the fact that the existence of hyaluronidase activity in \textit{E. histolytica} was reported decades ago and the possibility that such a factor plays an essential role in tissue penetration, a parasite protein with hyaluronidase activity has not been characterized. This intrigued us to search for hyaluronidase activity in trophozoite extracts in order to characterize the enzyme at the molecular level. For our survey, we have used very simple assays and highly elaborate techniques to detect hyaluronidase specifically.

Comparison of several hyaluronidase assays. The sensitivities of common hyaluronidase assays, which are neither species nor phylum specific (13), were compared using hyaluronidase from bovine testis (Sigma) as a standard (Table 1).

(i) Zymographic (substrate-gel) assay. In the zymographic (substrate-gel) assay (9), samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a 10% separation gel containing 0.17 mg of hyaluronan per ml. After electrophoresis, the gel was washed in 3% Triton X-100 and subsequently incubated in 0.15 M NaCl–0.1 M sodium formate, pH 4.6, at 37°C overnight. Undigested substrate was stained with the dye Alcian blue, and the position of a protein with hyaluronidase activity was indicated by a clear band. In this assay, recombinant human hyaluronidase I was also used as a positive control (see Fig. 1).

(ii) Substrate-agarose plate assay. In the substrate-agarose plate assay (29), samples were added to wells, 4 mm in diameter, that had been punched into a plate of 1% agarose containing 0.4 mg of hyaluronan per ml in 50 mM sodium phosphate, pH 5.5. After incubation at 37°C overnight, clear zones were visible around wells containing hyaluronidase activity.

(iii) Stains-all assay. In the stains-all (Sigma) assay (1), samples were incubated at 37°C with 60 \(\mu\)g of hyaluronan per ml in 0.1 M sodium acetate, pH 6.0, for 1 h. The dye stains-all changed the color from red to blue upon binding to hyaluronan, while digested substrate was not bound by the dye. Extinction was measured with 640-nm-wavelength light. This assay was found to be quite sensitive, but in the presence of salts, the test resulted in false positives.

(iv) ELISA-like assay. In the enzyme-linked immunosorbent assay (ELISA)-like assay (31), a microtiter plate was coated with hyaluronan. Samples were added and incubated in different reaction buffers (0.1 M sodium acetate [pH 3.5 to 5.0] or 0.1 M sodium formate [pH 4.5 to 7.0], both with 0.15 M NaCl and 0.2 mg of bovine serum albumin per ml) at 37°C for 5 to 16 h, followed by several washing steps with phosphate-buffered saline containing 0.05% Tween 20. Nonspecific binding sites for developing reagents were blocked by incubating with 300 \(\mu\)l of ELISA blocking reagent (Boehringer Mannheim) per
well at 37°C for 30 min. Residual substrate was detected using a biotinylated hyaluronan binding protein diluted in a buffer containing 25 mM sodium phosphate, 0.15 M NaCl, 0.3 M guanidine-HCl, 0.08% bovine serum albumin, and 0.02% sodium azide, pH 7.0, and incubated at 37°C for 1 h. Subsequently, the plate was washed with phosphate-buffered saline containing 0.05% Tween 20 and incubated with peroxidase-conjugated streptavidine (1 μg/ml in 10 mM sodium phosphate, 0.25 M NaCl, pH 7.6), and the assay was completed using 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) as a substrate. Absorbance at 405 nm was read.

Quantification of hyaluronidase activity using the ELISA-like assay was not affected by salts and was the most sensitive assay used (Table 1).

*E. histolytica* extracts do not exert hyaluronidase activity. Extracts of *E. histolytica* strain HM1:IMSS from long-term axenic cultures (6) and from this strain freshly passaged through a gerbil liver (HM1:IMSS G1) were tested for hyaluronidase activity. Trophozoites were extracted by three freeze-thaw cycles, either in phosphate-buffered saline including proteinase inhibitors (21) or in 50 mM sodium acetate, pH 5.0. After centrifugation at 150,000×g, the supernatants, referred to hereafter as trophozoite extracts, were analyzed by all four assays discussed above, particularlyzymography (Fig. 1) and the ELISA-like assay with a pH ranging from 4.5 to 7.0 (Fig. 2). For the latter two assays, trophozoite extracts were also lysed by various detergents (2% octylglucoside, 1% Brij 35, 3% Triton X-100, or 1% SDS) in 150 mM NaCl. Furthermore, extracts obtained by freeze-thaw cycles were subjected to anion- or cation-exchange chromatography, and fractions were analyzed by the ELISA-like assay.

We failed to detect any hyaluronidase activity in crude or fractionated extracts and concluded that *E. histolytica* lacks hyaluronidase activity. The activity of the standard bovine enzyme did not decrease in the presence of amoebic extracts obtained by freeze-thaw cycles in 50 mM sodium acetate, pH 5.0, indicating that no inhibitory activity was present in the extracts (Fig. 2). Finally, viable trophozoites of *E. histolytica* were cultivated axenically overnight in hyaluronan-coated microtiter plates under anaerobic conditions in serum-free culture medium. No decrease in the amount of substrate could be observed as determined by the ELISA-like assay (not shown). This observation differed from those of previous studies (2, 16, 32). The activities observed, i.e., the decrease in the viscosity or turbidity of hyaluronan and the release of N-acetylgalcosamine, possibly were exerted by glycosidases other than hyaluronidase including exoglycosidases such as N-acetylgalcosaminidases. We tested two isoforms of a lysozyme-like glycosidase purified from *E. histolytica* trophozoite extracts (15, 22) for their ability to digest hyaluronan nonspecifically, but none of the isoforms were active towards the substrate (Fig. 1). Nonetheless, the data reported from the previous studies may be explained by the nonspecific detection methods used at that time. Another explanation for the finding of hyaluronidase activity in two of the previous studies (2, 16) might be that they were conducted prior to the availability of axenic cultures and that the active protein originates from the bacteria cocultured with the amoebae. In the only report in which axenic cultures were used for the study (32), it was emphasized that the hyaluronidase activity detected was exceedingly low, making its involvement in amoebic pathogenicity very unlikely.

Interestingly, it has been demonstrated that *E. histolytica* binds hyaluronan and aggregates in the presence of hyaluronan (28). The binding to Caco-2 epithelial cells is attributed to a CD44-like hyaluronan binding protein on the surface of the amoeba. After treatment of the target cells with hyaluronidase, the adhesion of amoebae is inhibited. This mechanism is rem-

### Table 1. Sensitivities of different hyaluronidase activity assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Stain</th>
<th>Incubation buffer</th>
<th>Sensitivity (U)</th>
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<tbody>
<tr>
<td>Substrate-gel agarose plate</td>
<td>Alcian blue</td>
<td>Sodium formate, pH 4.6</td>
<td>2.6</td>
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<tr>
<td>Substrate-gel agarose plate</td>
<td>Sodium phosphate, pH 5.5</td>
<td>0.7</td>
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<tr>
<td>Stains-all</td>
<td>Stains all</td>
<td>Sodium acetate, pH 6.0</td>
<td>0.18</td>
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<tr>
<td>ELISA-like</td>
<td>Peroxidase</td>
<td>Sodium acetate, pH 5.0</td>
<td>0.01</td>
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* Titrated by the use of hyaluronidase from bovine testis.

FIG. 2. Test for hyaluronidase activity in amoebic extracts using the ELISA-like assay. Hyaluronidase from bovine testis was used as a standard, and 0.01 U of the enzyme in 50 mM sodium acetate, pH 5.0, was detectable. Fifty percent of the substrate was digested by 1 U of the standard enzyme and was set at 1. Bars: 1, standard enzyme (1 U) in the presence of *E. histolytica* HM1:IMSS G1 trophozoite extract (44 mg of protein/ml); extract obtained by freeze-thaw cycles in 50 mM sodium acetate, pH 5.0); 2, trophozoite extract of *E. histolytica* HM1:IMSS from long-term cultures; 3, extract from trophozoites freshly passaged through a gerbil liver (HM1:IMSS G1). Trophozoite extracts (up to 2 mg of protein applied) did not exert hyaluronidase activity no matter which extraction method (as described) was used, and the same results were obtained at other pHs ranging from 3.5 to 7.0.
ininsent of the CD44-mediated mechanism of tissue invasion by cancer cells (28). The occasional observed paradox that both hyaluronidase and its substrate are associated with invasion (5) is apparently not evident in _E. histolytica_. Taking into account that invasion is a dead end for the parasite, because tissue trophozoites cannot transform into cysts and therefore are not transmittable (4), it may be assumed that this behavior is an accidental event for _E. histolytica_. This notion is strengthened by the finding that proteins essential for pathogenicity are present in the evolutionarily related but nonpathogenic _Entamoeba dispar_ as well (3, 23, 25). It has been suggested that coincidental selection for some other functions maintains genes encoding tissue-damaging proteins in _E. histolytica_ (8, 10), and this may explain why _E. histolytica_ does not possess a true hyaluronidase. However, as host cells are abundant at all steps of amoebic invasion, the possibility that host-cell-derived hyaluronidases promote dissemination of the parasite cannot be excluded.

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REFERENCES


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