THE COLLAGENASE OF *ENTAMOEBA HISTOLYTICA**

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During the course of invasive intestinal amebiasis, *Entamoeba histolytica* actively penetrates the mucosa and submucosa of the large intestine (1–6). Electron microscopy studies of intestinal epithelium of in vivo and in vitro infected tissues in autopsies from humans and experimental animals reveal that the basal lamina and the connective tissue of the submucosa are primarily damaged (3, 5, 6). Since collagen is a major component of the basal lamina and the extracellular matrix of the intestine (7–9), we studied the presence of collagenolytic activity in *E. histolytica* when plated on native type I or III human collagen. Our results show that *Entamoebae* possesses a membrane-bound proteolytic enzyme that digests native type I and type III collagen fibers inside the helix when incubated at neutral pH at 37°C. The collagenase was more active against type I collagen.

Materials and Methods

Entamoeba Cultures. *E. histolytica* HM1:IMSS was cultured in TYI-S-33 medium (10). All the experiments were done using trophozoites harvested during logarithmic phase of growth.

Collagens. Collagen types I and III were extracted and purified following the procedure described by Rojkind et al. (11). A human placenta was sliced and incubated with 500 ml of 0.25 M acetic acid containing pepsin (0.25 mg/ml) at 4°C for 24 h. After centrifugation at 16,000 *g* for 40 min, the clear supernatant was decanted and neutralized to pH 7.0 to deactivate the pepsin. This digestion process was repeated twice, and the three supernatants pooled for purification of collagen types. Type III and I collagens precipitated out of solution by dialysis at 4°C against 0.05 M Tris-HCl buffer containing 1.7 M NaCl and 2.5 M NaCl, respectively. Precipitates were dissolved and dialyzed in 0.25 M acetic acid, lyophilized, and stored at 4°C until used.

Collagen Substrates. Collagen in 0.25 M acetic acid (15 ml) was dialyzed for 1 h against 1 liter of 0.05 M Tris-HCl buffer, pH 7.2, containing 0.005 M $CaCl_2$, and then the pH of the solution adjusted to 6.9 with 0.1 N NaOH. Reconstituted collagen gels for digestion assays were prepared on Falcon dishes (35 mm Diam, Falcon Labware, Becton, Dickinson & Co., Oxnard, Calif.) with 0.5 ml of collagen type I (10 mg/ml) and incubated at 37°C for 1 h, to allow fibrillar polymerization. The gels were incubated overnight at room temperature under an ultraviolet light source placed 20 cm above the dishes. To test cell adhesion, films were prepared as described above, except that 0.1 ml of a 1 mg/ml collagen solution was used per dish, and dried after polymerization at room temperature overnight.

Adherence of Amebas to Collagen Films. Films of collagen types I or III were covered with 2 ml of modified TYI-S-33 medium (without serum and L-cysteine), and 1 ml of the same medium containing 1×10^5 trophozoites was added. Cell adherence was evaluated after different incubation times at 37°C. Afterwards, unattached amebas were washed twice in modified TYI-S-33-medium, and adherent amebas were removed after incubation in 0.15 M cold NaCl for 10 min at 4°C. The amebas were collected by low speed centrifugation, resuspended in 0.25 ml

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phosphate-buffered saline for amebas and counted with a hemocytometer (one part 0.15 M phosphate buffer, pH 7.2, and nine parts 0.25 M NaCl).

^{14}C Labeling of Collagen. Acetylation of collagen with ^{14}C-labeled acetic anhydride was carried out by the method of Gisslow and McBride (12) as modified by Johnson-Wint (13). Purified collagen type I (2 mg/ml, 125 ml) in 0.01% acetic acid was brought to pH 8.4 by the addition of 1 M K_2HPO_4. The acetylating agent [1-^{14}C]acetic anhydride (10 mCi/mmol, 1 mCi, New England Nuclear, Boston, Mass.) was added dropwise in 1.5 ml of benzene to the collagen solution over a period of 30 min at 10°C. The reaction mixture was stirred for 1 h; to remove benzene and [^{14}C]acetic acid, collagen was precipitated at 4°C by dialysis against 0.05 M Tris-HCl, pH 7.3, containing 2.5 M NaCl, for 24 h (11). Collagen type III was precipitated with the same buffer containing 1.7 M NaCl. The precipitates were centrifuged at 16,000 *g* for 40 min at 4°C, resuspended in 0.5 M acetic acid, and dialyzed exhaustively against 0.5 M acetic acid. The acetylated collagens were lyophilized and stored at 4°C until needed. The specific activity of collagen type I was 1.3×10^5 dpm/mg, and that of type III 1.6×10^5 dpm/mg.

Preparation of Collagen Films for Assay Plates. Films of [^{14}C]acetate-labeled collagen were prepared as described by Johnson-Wint (13) with the following modifications: aliquots of type I (25 μl; 10,000 cpm) or type III (25 μl; 12,000 cpm) [^{14}C]acetate-labeled collagen solutions (3.6 mg/ml) were added to each well of tissue culture multiwell plates (model 76-003-05 96 flat-bottomed wells, Diam 0.6 cm, Linbro, Chemical Co., Hamden, Conn.). The plates were incubated at 37°C for 1 h to allow polymerization of collagen in a fibrillar form. The amount of collagen bound to the dish was determined by the radioactivity retained in the wells.

Collagenase Assay. Two different procedures were used to assay for collagenase activity. (*a*) Collagen gels, reconstituted type I collagen gels were covered with 0.25 ml of an ameba suspension containing 5×10^5 trophozoites, 50 μg/ml gentamicin, and 2.76 ml of modified TYI-S-33 medium. Because of the retraction of the gel during sterilization, the trophozoites tended to accumulate in the center of the gel. The dishes were incubated at 37°C for 24 h; after incubation, the collagen gels were fixed in 4% wt/vol formaldehyde and stained with picrosirius solution (0.1% sirius red in saturated aqueous picric acid) for 1 h (14). Excess dye was washed out three times with 0.1 M HCl for 14 h, and the area of lysis was measured by two independent methods with identical results. One method consisted of direct measurement of the area of lysis and another by gravimetric analysis of the area of lysis cut-out from a print. As shown in Fig. 2 the area of lysis is devoid of collagen and no staining is obtained with the picrosirius red dye. (*b*) [^{14}C]Acetate-labeled collagen films for the assay plates, type I and type III ^{14}C-labeled collagen films containing 90 μg protein each, and 10,000 and 12,000 cpm for I and III, respectively, were covered with 0.2 ml of modified TYI-S-33 medium containing 5×10^4 trophozoites. The plates were incubated at 37°C for 3–24 h. The supernatant (0.1 ml), containing collagen-digested products, was then collected, transferred to a scintillation vial containing 5 ml of aquasol, and counted in a Packard Tri-Carb liquid scintillation spectrometer (model 3003, with an automatic control, model 574, Packard Instrument Co., Downers Grove, Ill.) with an efficiency of 83%. To detect denatured collagen, a sample containing 0.01% (wt/vol) trypsin was also included. In several assays the radioactivity released with trypsin was 1,144 ± 200 dpm (9.5 ± 2%) and 1,400 ± 320 dpm (10 ± 2%) from collagen type I and III, respectively. We defined a unit of collagenase as the enzymatic activity able to digest 1 μg (110 cpm) of collagen/min at 37°C (13). Each assay carried out at least in triplicate.

Identification of Collagen Fragments. Collagen fragments resulting from amebic collagenase digestion were analyzed by electrophoresis in 7.5% polyacrylamide gels (15); radioactive polypeptides were detected by autoradiography (16). For this assay, collagen type I was radiolabeled with ^{125}I (17); 0.1 ml of modified TYI-S-33 medium containing 10^6 cells was mixed with 0.1 ml of ^{125}I-collagen type I (specific activity, 1.5×10^7 cpm/mg), and incubated at 37°C for 3, 6, or 24 h. At the end of each incubation period, the reaction was terminated by the addition of 0.01 M EDTA, 0.01 M *N*-ethylmaleimide (NEM),[1] and 2–3% sodium dodecyl sulfate (final concentration).

Determination of Collagenolytic Activity Released by Trophozoites. To test whether the ameba secreted collagenase, blind-well chambers (Nucleopore Corp., Pleasonton, Calif., model 441200)

[1] *Abbreviations used in this paper:* NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride.

separated by polycarbonate membranes (pore size, 5 μm; Unipore, Bio-Rad Laboratories, Richmond, Calif., No. 341-1001) were used. The lower compartment contained a [^{14}C]collagen film (11,000 cpm, 100 μg), and the upper compartment was filled with a suspension of 2×10^5 trophozoites in modified TYI-S-33 medim with 20 μg of nonlabeled collagen. After 14 h incubation at 37°C, 200 μl of supernatant was collected from each compartment, and the radioactivity measured as described.

Results

Kinetics of Adherence and Release of E. histolytica from Collagen Substrates. Trophozoites in modified TYI-S-33 medium adhered to films of collagen types I and III with the same efficiency. After 1 h of incubation at 37°C, 50% of the added trophozoites adhere to either type of film, as shown in Fig. 1. The rate of cell detachment, however, was different for each collagen type. There was a considerable release of ameba from collagen type I that occurred wtihin 4 h, and only a poor release from type III films

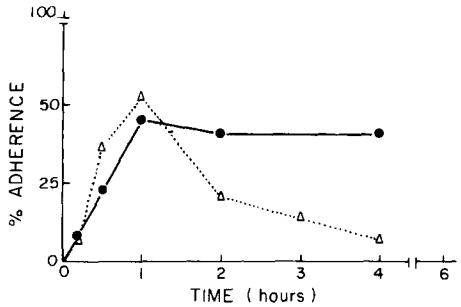

FIG. 1. Kinetics of detachment of *E. histolytica* bound to collagen films. Trophozoites adhered to collagen types I (△) and III (●) are actively dissociated from both collagens with different velocities.

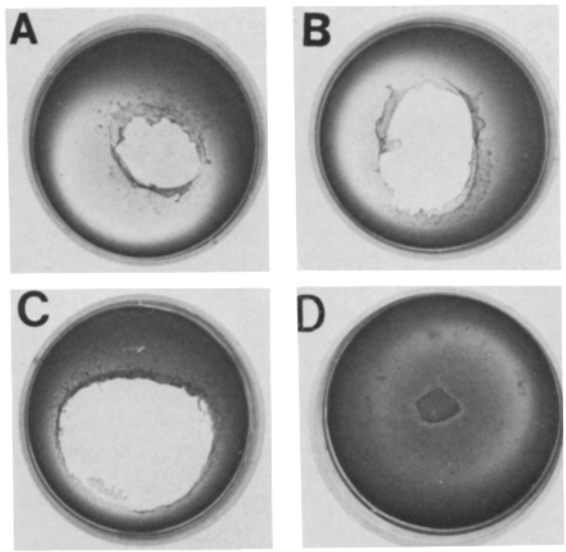

FIG. 2. Collagen digestion by *E. histolytica*. Trophozoites in modified TYI-S-33 medium (without L-cysteine and serum) were overlayed on reconstituted collagen gels at densities of 0.5, 1, or 2×10^6 amebas (A, B, and C, respectively). Fetal calf serum (15% vol/vol) and 10^6 trophozoites (D).

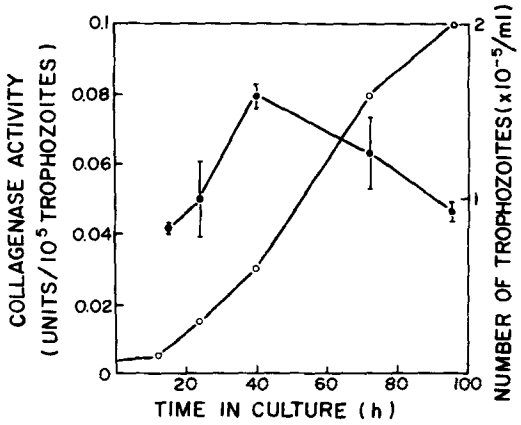

FIG. 3. Collagenase activity of trophozoites harvested at different phases of growth. 5 × 10^4 trophozoites obtained at different stages of the growth curve were overlayed on [^{14}C]collagen films, and then incubated for 10 h at 37°C. Collagenolytic activity was expressed as units of enzyme activity per 10^5 trophozoites (●). 1 unit equals 1 μg (110 cpm) of collagen digested per minute. Each assay was carried out using at least three samples. Counting efficiency was 83% (○), number of trophozoites (× 10^{-5}/ml).

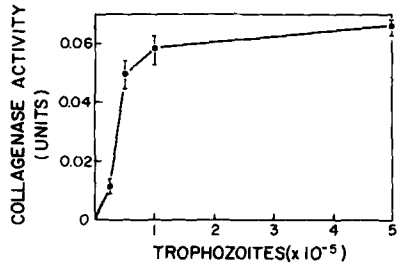

FIG. 4. Effect of cell density on the release of radioactive peptides. Trophozoites in modified TYI-S-33 medium were overlayed on collagen gels at densities of 2.5, 5, 10, or 50 × 10^4 cells, and were incubated for 7 h at 37°C.

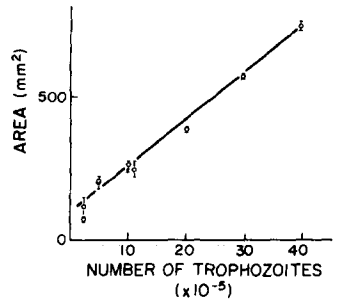

FIG. 5. Effect of cell density on area of lysis of reconstituted collagen gels. Reconstituted type I collagen was covered with a suspension of 0.5, 1, 2, 3, or 4 × 10^6 trophozoites as described, and was incubated for 24 h. The activity was measured by the area of lysis after staining with the picrosirius solution (14). Values are arithmetic means of three independent experiments with duplicate assays.

(Fig. 1). This result suggested that detachment from the gels could be due to different degrees of digestion of the collagens by ameba.

Adherence of amoeba to plain culture dishes in the absence of serum and L-cysteine

was very low. 6 h after plating, 60% of the amebas had died and were floating. In contrast, 90% of the trophozoites plated on collagen gels were alive after 6 h and 80% after 24 h.

Collagen Digestion by E. histolytica. To test for collagenase activity in *E. histolytica*, reconstituted gels of collagen type I were layered with a suspension of 0.5, 1, or 2 × 10^6 trophozoites in the same volume of modified TYI-S-33 medium and incubated for 24 h at 37°C. A well-defined area of lysis was observed in the gel (Fig. 2). This area of lysis was proportional to the number of trophozoites used in the assay. No lysis was observed when dishes were incubated with medium alone or with medium containing trypsin.

Collagenase Activity as a Function of the Growth Phase of Trophozoites. A suspension of 5 × 10^4 trophozoites obtained at different stages of growth (lag, log, or stationary phase) of *E. histolytica* was incubated for 10 h with [^{14}C]collagen films on tissue culture multiwell plates as described above. Fig. 3 illustrates that collagenase activity increased during the logarithmic phase of growth and declined after 42 h, reaching the original values after 100 h. Optimum collagenase activity was observed at logarithmic phase. Therefore, all further experiments were done at 42 h of culture using a cell density of 1.5 × 10^5 trophozoites/ml.

Collagenase Activity as a Function of Cell Density. The collagenase activity of amebas, as measured by either one of the two procedures used in this study was proportional to the amount of trophozoites used in the assay, as shown in Figs. 4 and 5. However, in the assay that used ^{14}C-labeled collagen films, the correlation between enzymatic activity and number of trophozoites was linear up to ~10^5 cells (Fig. 4), whereas in the other assay the correlation was linear up to 4 × 10^6 cells (Fig. 5).

Partial Characterization of Collagenase in E. histolytica. The enzymatic activity corresponds to a collagenase because the enzyme works on native collagen at neural pH and physiological temperature under conditions that nonspecific proteases (trypsin) will release only 9.5 ± 2% of counts. The former conclusion is also supported by the results of the electrophoretic analysis of the fragments of collagen generated during

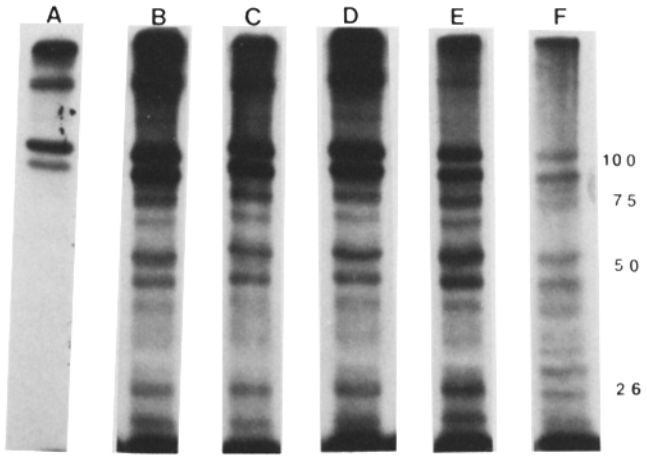

FIG. 6. Fluorograph of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of collagen digested by amebas. Native type I collagen (A) and fragments at 3 (B, C), 6 (D, E), and 24 h (F) of incubation with 1 × 10^6 trophozoites. A was a control incubated for 24 h. B and D contained 10 mM NEM in addition to the culture medium.

TABLE I

*Effect of Enzyme Inhibitors on Collagenase Activity of E. histolytica HM1**

Inhibitor	Concentration	Activity‡	Viability (10 h)§
		%	%
None	—	100 ± 7	75
L-Cysteine	0.006 M	47 ± 14	80
EDTA	0.01 M	0	0
Soybean trypsin inhibitor	100 μg/ml	75 ± 16	82
Calf serum	16%	17 ± 7	25
PMSF	0.001 M	92 ± 14	20
NEM	0.001 M	80 ± 15	0

* Trophozoites (5×10^4) of *E. histolytica* HM1 were incubated at 37°C in [^{14}C]collagen films coated on multiwell tissue culture plates and incubated for 10 h.

‡ 5×10^4 trophozoites were adhered onto collagen substrate for 3 h, then incubated with inhibitors for 7 h. The activity is expressed as a percentage of solubilized [^{14}C]collagen of the control without inhibitors, which showed 45% solubilization. The values are the means of three or more assays ± SD.

§ Cell density of refractile trophozoites showing membrane integrity. This was used as the main parameter of cell viability, because most of the killed amebas appeared as ghosts or were disrupted.

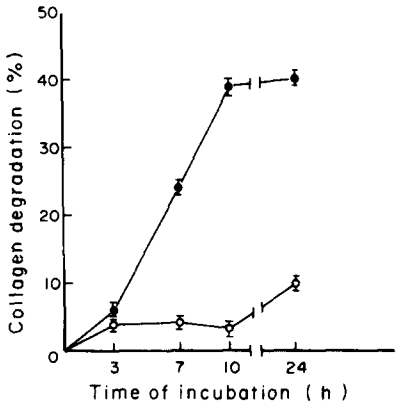

FIG. 7. Specificity of collagenase activity. 10^5 trophozoites containing a total collagenolytic activity of 0.085 U (5.1 μg of type I collagen degraded per h of incubation at 37°C) were added to type I (●) and type III (○) [^{14}C]collagen films and incubated for 3, 7, 10, and 24 h at 37°C. The results are expressed as a percentage of the total radioactivity released above the trypsin control. The total [^{14}C]collagen in the assay was 12,000 ± 500 dpm for collagen type I and 14,000 ± 600 dpm for collagen type III. The trypsin controls only released 9.5 and 10% radioactivity from collagen type I and III, respectively.

the assay, which suggest that the cleavage is inside the helix. Three major groups of ^{125}I-collagen fragments, 75,000, 50,000, and 25,000 daltons, were obtained after 3 h of incubation (Fig. 6). After 6 h of incubation, a large proportion of the collagen fragments were small peptides (Fig. 6E). However, in the presence of 10 mM NEM, this further proteolysis of collagen fragments was partially inhibited (Fig. 6D). These results could suggest a subsequent participation of other proteases from the ameba in the degradation of the partially hydrolyzed collagen fragments. After 24 h of incubation most radioactive collagen was degraded (Fig. 6F).

The enzymatic activity demonstrated shows a pattern of inhibition similar to that reported for other well-characterized collagenases. Serum and the metal ion chelator EDTA inhibited the activity completely. Cysteine inhibited the reaction by 60%, whereas inhibitors of serine proteinases such as soybean trypsin inhibitor, phenylmethanesulfonyl-fluoride (PMSF), and the thiol-blocking reagents such as NEM had no effect (Table I). Inhibition of the activity was unrelated to the toxicity of the agent. Thus, cell death after the amebas were attached to the substrate did not influence the assay.

Moreover, the activity appears to be more specific toward type I collagen films as compared with type III. As shown in Fig. 7, *E. histolytica* (80% viability) digested three and a half times more type I than type III collagen after 24 h of incubation.

Analysis of Collagenolytic Activity in the Cell and Medium. After disruption of the ameba by freezing and thawing three times, and centrifugation at 16,000 *g*, all the activity was recovered in the particulate fraction. Ameba-free supernatant medium, obtained after incubation of collagen and ameba for 24 h, was devoid of collagenase activity. Amebas plated on top of collagen gel on a polycarbonate filter of pores of 5-μm Diam did not digest underlying collagen. To test whether collagenase production could be induced by exposure of ameba to low collagen doses, 2×10^5 trophozoites containing soluble collagen (25 μg/ml) were incubated 14 h at 37°C in the upper compartment of blind well chambers, separated by a polycarbonate membrane (5-μm pore) from [^{14}C]collagen films contained in the lower compartment. There was no digestion of the radiolabeled collagen, although there was free diffusion of hemoglobin as a soluble marker. These results could suggest that a close cell-substrate interaction is required for collagenolysis to occur and that specific collagenolytic activity is located on the membrane of ameba.

Discussion

Tissue damage of the large intestine during invasive amebic infection requires active penetration of trophozoites through the intestinal mucosa and submucosa (1–6). Several enzymes may be involved in this invasive process. Since collagen is one of the major components of both the basal lamina (type IV) and the extracellular matrix of the intestine (types I, III, and V), we studied the presence of a collagenolytic activity in *E. histolytica*.

The differences in the rates of detachment and of collagen digestion of amebas adhered in vitro to type I collagen as compared with type III collagen suggests a greater specificity of this enzyme for type I collagen. This result is of interest since different cells appear to contain collagenases with variable specificities for different collagen types. Tadpole and human skin collagenase have a broad specificity and digest most collagen types (18, 19). Neutrophil collagenase is specific for type I collagen, a metastatic murine tumor collagenase degrades type IV collagen, and a recently isolated collagenase from macrophages cleaves type V collagen (20–22).

Collagenase activity was measured by two independent methods: the area of lysis produced on a type I reconstituted collagen gel, or the release of ^{14}C-fragments from a ^{14}C-labeled collagen film. In both instances the activity was proportional to the number of cells plated. However, in the radioactivity release assay a smaller number of amebas were used due to the reduced surface area of the dishes.

The presence of collagen-bound collagenase in our collagen substrates (23) was

ruled out because the control incubation medium and the assays containing trypsin showed neither significant substrate digestion products nor area of lysis. Furthermore, the negative results obtained with trypsin indicate the native structure of the substrate used. These results are important because it has been shown that collagen type III can be hydrolyzed by trypsin (24), by a bacterially derived metalloprotease (25), and by an elastase derived from human neutrophils (26).

The behavior of the collagenase of *E. histolytica* in the presence of several enzyme inhibitors resembled the behavior of mammalian collagenases. It was inhibited by serum, EDTA, and cysteine, and was not affected by PMSF, soybean trypsin inhibitor, or NEM. These results explain in part the lack of success in early attempts to show collagenase activity in ameba (27) because large concentrations of serum (15%) were used in the incubation assay. As demonstrated in this communication and by others (28–31), collagenases are inhibited by serum.

The major ^{125}I-collagen fragments of this collagenolytic activity after incubation for 3–6 h were 75,000, 50,000, and 25,000 daltons, but other proteolytic enzymes derived from ameba further hydrolyzed these polypeptides after prolonged incubation times. This latter hydrolysis was inhibited by NEM. The presence of other proteases in *E. histolytica* has been reported (32–37); they include a gelatinase (34) and a caseinase (35).

The enzymatic activity reported here is apparently localized on the plasma membrane of the ameba. The experiments that support this suggestion are the following: (*a*) most of the collagenolytic activity was recovered in the particulate fraction obtained after lysis of the ameba by freezing and thawing; (*b*) the supernatant collected from areas of collagen lysis after 24 h of incubation of viable ameba on type I collagen gels did not contain any measurable collagenase activity; (*c*) ameba plated on polycarbonate membranes (5-μm pore size) and placed on top of collagen gels did not show any signs of lysis under the membrane; and (*d*) trophozoites included in the upper section of a chemotaxis chamber in the presence of radioactive collagen in the bottom compartment did not release any radioactivity. Under the assay conditions, diffusion of large molecular weight proteins from the upper to the lower chamber is not inhibited by the pore size used.

The results presented in this communication suggest that collagenolytic activity may play some role during active penetration of ameba through the intestinal mucosa and submucosa. Therefore, the relationship of this enzymatic activity with the virulence of several strains of ameba should prove to be of interest in the understanding of the mechanisms of amebic invasion.

Summary

The present work was designed to investigate the capacity of trophozoites of *Entamoeba histolytica* to adhere to and digest human collagen types I and III in vitro. The time-course of binding of ameba to both human collagen types I and III was similar. However, the kinetics of detachment were different for each collagen type. Trophozoites of *E. histolytica* cultured on heat-reconstituted type I collagen gels produced a well-defined area of lysis. Quantitative studies using ^{14}C-labeled collagen revealed that after 24 h of incubation, *Entamoeba* digested three and a half times more type I than type III collagen, thus suggesting the presence of a collagenase with higher specificity for type I collagen. This activity was optimum with trophozoites harvested

after 42 h in culture (1.5×10^5 trophozoites/ml). The digestion of type I collagen was a function of the number of trophozoites, and was inhibited by EDTA, L-cysteine, and serum, but not by soybean trypsin inhibitor, phenylmethanesulfonyl fluoride, or *N*-ethylmaleimide (NEM). Electrophoretic analysis of the type I collagen fragments revealed three main classes of polypeptides of 75,000, 50,000, and 25,000 daltons. Subsequent proteolysis of these collagen fragments was probably carried out by other proteases derived from trophozoites. This activity was inhibited with 10 mM NEM. Collagenase activity appeared to be located at the plasma membrane and direct contact of the ameba with the substrate is required for collagen digestion. The results suggest that collagenase activity of *E. histolytica* may play an important role in tissue invasion.

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