Isolation, Purification, and Partial Characterization of an Enterotoxin from Extracts of Entamoeba histolytica Trophozoites

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Soluble cell-free extracts of pathogenic Entamoeba histolytica, as well as serum-free minimal media in which trophozoites are incubated, contain substances that cause the rapid rounding up and detachment of tissue-cultured monolayers of mammalian cells (cytopathic activity) and induce fluid secretion in ligated intestinal loops of indomethacin-pretreated rats (enterotoxic activity). A semiquantitative assay for the determination of the cytopathic activity based on the rate of detachment of tissue-cultured baby hamster kidney cells was developed. Two peaks containing cytopathic activity were obtained upon gel filtration of the soluble extracts: peak I, with over 60% of the activity, emerged in the 30,000 to 50,000 molecular weight region, and peak II, containing the remaining activity, was in the 15,000 to 25,000 molecular weight region. The activity of peak I was found to be heat labile and inhibited by sialoglycoproteins such as fetuin and mucin (5 mg/ml), as well as by sialic acid. Protease inhibitors such as antitrypsin, pepstatin, phenylmethylsulfonyl fluoride, metalloprotease inhibitors, and bacitracin had no effect on the cytopathic activity. Marked inhibition of cytopathic activity was observed, however, with iodoacetamide and p-chloromercuribenzoate, which affect sulphydryl groups. The toxic material in peak II was found to have ionophoric activity and was not inhibited by sialic acid-containing compounds. The materials from both peaks had enterotoxic activity in intestinal ligated loops. The active substance from peak I was further purified (200×) on an agarose-fetuin affinity column, yielding one major protein band with an apparent molecular weight of ca. 30,000 on sodium dodecyl sulfate. Amino acid analysis revealed that the protein was very poor in sulfur amino acids. The sialic acid-sensitive toxic activity was higher in known virulent strains such as HM-1:IMSS and could be markedly augmented after preincubation of the trophozoites with certain Escherichia coli strains.

Entamoeba histolytica trophozoites invade the intestinal mucosa to produce various clinical symptoms (7, 29). Pathogenic strains of Entamoeba histolytica have been shown to be capable of killing cultured mammalian cells on contact and causing hepatic abscesses in hamsters (3, 5, 12, 14–16, 26, 29, 33). In addition, a variety of soluble substances that are toxic to mammalian cells are known to be released from amoeba trophozoites (2, 19, 23). Incubation of cell-free extracts of axenically cultured Entamoeba histolytica trophozoites with tissue-cultured mammalian cells in serum-free medium caused the rounding up and detachment of the target cells (19, 23). Furthermore, the amoebic cell-free extracts were reported to have enterotoxic activity when tested in rat ileal loops after pretreatment with indomethacin (35), and were shown to contain proteins that form ion channels in membranes (20, 38). Recent reports have also shown that cell-free extracts of Entamoeba histolytica have a proteolytic activity (18) as well as a serotonin effect on intestinal electrolyte transport (25).

Virulence of Entamoeba histolytica has also been recognized to be related to the association of the amoeba with suitable bacterial cells (5, 30, 37). The interaction between trophozoites and bacterial cells was found to be mediated by lectins which specifically recognize certain carbohydrate moieties on the cell surface of the bacteria and the trophozoite (4, 27). Association of axenically cultured trophozoites with certain bacteria has been shown to augment their virulence, as evidenced by their enhanced ability to cause hepatic lesions in hamsters (37) and to destroy monolayers of tissue-cultured mammalian cells (5, 27).

The different reports on the various toxic activities found in the crude extracts or partially purified fractions of Entamoeba histolytica presented a complex picture that required better resolution and characterization of the components involved.

In the present study, we purified and partly characterized an amoebic enterotoxin that also has cytopathic activity and is a protein that is specifically inhibited by fetuin and N-acetyl neuraminic acid and does not have ionophoric activity. Furthermore, we investigated the effect of association of amoebae and bacteria on the level of cytopathic and enterotoxic activities of the parasite. These enterotoxic activities were found to be markedly augmented after ingestion of certain bacterial strains by Entamoeba histolytica trophozoites.

MATERIALS AND METHODS

Cultivation of Entamoeba histolytica. Trophozoites of Entamoeba histolytica strains HM-1:IMSS, HK-9, and 200-NIH were axenically grown in TYI-S-33 medium by the methods of Diamond et al. (10). Trophozoites were grown for 72 h (exponentially growing culture) and harvested by chilling in an ice water bath for 10 min. The trophozoites were washed twice in saline (0.9%) by low-speed centrifugation (600 × g, 5 min) and resuspended in saline to a final concentration of 10⁶ amoebae per ml. Counting of the amoebae was done under a microscope with a hemacytometer. Viability of trophozoites was determined by trypan blue exclusion (22).

Isolation and purification of the cell-free Entamoeba histolytica cytopathic activity. Washed Entamoeba histolytica trophozoites at a concentration of 5 × 10⁶ per ml of water...
were disrupted by freezing in an acetone-dry ice bath (10 min), thawing in 37°C water, and sonication in a bath sonifier (30 s). The trophozoite lysate was sedimented at 100,000 x g for 1 h at 4°C to remove membrane and cellular debris. The soluble supernatant fraction obtained was referred to as the crude amoebic extract (fraction A).

**Ammonium sulfate precipitation.** The amoebic cell extract (fraction A, 10 ml) derived from the 10^6 trophozoites was precipitated with ammonium sulfate. The precipitate, obtained at 30 to 70% saturation by the addition of solid ammonium sulfate, according to Dixon’s nomogram (11), was resuspended in water at a final volume of 2 ml (fraction B).

**Gel filtration.** Fraction B was fractionated on an Ultragel AcA-54 column (LKB, France). Material obtained from 10^6 trophozoites was applied to a column (80 cm long by 2.0 cm diameter) at 4°C, and fractions of 1.7 ml were collected at a flow rate of 0.02 ml/min. The column was eluted with 0.05 M Tris-hydrochloride buffer (pH 7.5). Aliquots (250 µl) were taken from each fraction, and the cytopathic activity was determined on monolayers of tissue-cultured BHK cells (see below). Calibration of the columns was done with bovine serum albumin, ovalbumin, and lysozyme.

**Affinity chromatography.** The cytopathic component obtained after gel filtration of fraction B was further purified on an affinity column of agarose-fetuin (Sigma Chemical Co.). Amoebic protein (5 mg/ml) was loaded on the affinity column (0.7 by 30 cm) with 0.05 M Tris-hydrochloride buffer (pH 7.5) at room temperature. Fractions of 1 ml were collected. The elution of the material retained on the column was done with 0.2 M ammonia solution (pH 10.5). The eluted fractions were lyophilized (to remove the dilute ammonia) and resuspended in water to the original volume. Aliquots were taken from fractions, and the cytopathic activity was determined on BHK cells (see below). The protein content was determined by Bradford’s method (6). Sodium dodecyl sulfate (SDS)-acylamide gradient (10–15%) gels were prepared by the method of Laemmli (17). Phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (Pharmacia Fine Chemicals, Uppsala, Sweden) were used as markers.

**Release of the enterotoxin from intact trophozoites.** Trophozoites (10^6/ml) were incubated in saline at 37°C for 2 h. After incubation, the trophozoites were spun down (600 x g, 5 min) and the supernatant was tested for cytopathic activity on BHK cells. The remaining intracellular activity was measured as described above.

**Amino acid analysis.** The amino acid composition of the amoebic enterotoxin was determined with a Dionex D502 amino acid analyzer after acid hydrolysis in HCI (6 M) for 20 h at 105°C in sealed tubes under vacuum.

**Collagenase activity.** Collagenase activity was tested with Azocoll (Calbiochem) as substrate. Azocoll (20 mg) was incubated with the amoebic samples tested (50 µg of protein) in 0.05 M Tris-hydrochloride buffer (pH 7.5) (total volume, 1 ml) at 37°C for 3 h. After the incubation, the tubes were centrifuged at 600 x g for 15 min and the supernatant was measured optically at 520 nm.

**Bacterial casein.** Proteolytic activity was tested with 125I-labeled casein as a substrate. 125I-labeled casein (50,000 cpm/µg) was incubated in 100 µl of Dulbecco modified Eagle medium with amoebic samples obtained from 10^6 trophozoites for 2 h at 37°C. After incubation, the reaction mixture was applied to SDS-acylamide electrophoresis (12%) as described previously (17), and the gels were exposed to autoradiography. The disappearance of the 125I-labeled casein band (43 kilodaltons [kDa]) served as an indication of proteolytic activity.

**Cytopathic activity assay.** The cytopathic activity assay was based on the rate of detachment of the target cells from their substratum after exposure to the amoebic extract. The amoebic extract concentration was determined by the number of trophozoites from which it was prepared per unit of volume, as well as from protein contents, as determined by Bradford’s method (6). Strain HM-1:1MSS contained ca. 1 mg of protein per 10^6 trophozoites. The cytopathic activity of amoebic extracts was assayed on monolayers of BHK cells. In some experiments, confluent monolayers of HeLa cells were grown and gave essentially the same results. Confluent monolayers of BHK cells (3 x 10^6 per well) were washed (2 x) with saline (0.9%). Aliquots of the amoebic extract were diluted in Dulbecco modified Eagle medium (without serum) to a final volume of 0.5 ml and added to the tissue cultures. The samples were incubated for 2 h at 37°C with 5% CO2. After incubation, the plates were washed with saline and fixed with formaldehyde (4% in phosphate-buffered saline, pH 7.2) for 10 min. After this treatment, the cells were washed (2 x) with saline and stained for 10 min with 0.1% methylene blue dissolved in 0.01 M boric acid-borax buffer (pH 8.6). The dye retained by the cells was extracted with 0.1 M HCl at 37°C for 30 min. The density of the color was optically determined after appropriate dilutions with 0.1 M HCl at 660 nm. The retention of methylene blue by the tissue-cultured cells was proportional to the amount of cells attached to the plate (8). The amount of dye extracted from control monolayers that had no amoebic extract was defined as 0% cytopathic activity, whereas maximal destruction of monolayers (defined as 100% activity) was determined by staining plates from which all (>99%) mammalian cells were removed. The lowest concentration of amoebic extract (strain HM-1:1MSS) that caused the removal of 50% of the mammalian cells (after 120 min of incubation) and the extracted dye that afforded approximately half the optical density of nonreacted control monolayers was defined as one unit of cytopathic activity.

**Treatment of tissue-cultured cells with neuraminidase.** Monolayers were preincubated with 10^–2 U of neuraminidase per ml from *Vibrio cholerae* (Behringwerke AG, Marburg, West Germany) in phosphate-buffered saline for 6 h at 37°C. After incubation, the cells were washed with PBS (2 x) and exposed to the amoebic material.

**Trypsinization of the amoebic extract.** The amoebic cell-free extract was treated with trypsin (Worthington Diagnostics) for 30 min at 37°C at an amoebic protein-to-trypsin ratio of 25:1. The reaction was stopped with soybean trypsin inhibitor (Sigma) at an inhibitor/trypsin ratio of 2:1. Soybean trypsin inhibitor had no damaging effect on the tissue culture cells and did not inhibit the activity of the amoebic extract.

**Inhibitors of cytopathic activity.** Soybean, peanut, and wheat germ agglutinins, as well as fetuin, mucin, thyroglobulin, and N-acetyl-neuraminic acid were obtained from Sigma. Asialo-fetuin was prepared by the method of Van Lenten and Ashwell (36). Concanavalin A was obtained from Miles Yeda, Rehovoth, Israel. Preincubation of BHK cells with lectin was for 45 min at 37°C, with a solution of 2 mg/ml using 0.5 ml per well (3 x 10^4 BHK cells). After incubation, the cells were washed (3 x) with prewarmed Dulbecco modified Eagle medium without serum and exposed to the amoebic soluble extract. Glycoproteins and sugars of various concentrations were preincubated with constant amounts of amoebic protein (45 min at 0°C) before addition to the cells. Bacitracin was obtained from Nutritional Biochemi-
cals Corp., Cleveland, Ohio. Phenylmethylsulfonyl fluoride, anti-trypsin, and pepstatin were purchased from Sigma. Carbobenzoxyglycylglyoxylate and theoacetyl-leucyl-phenylalanine were obtained from S. Blumberg, Weizmann Institute, Rehovoth, Israel. p-Chloromercuribenzoic acid was purchased from Sigma, and iodoacetamide was from Fluka, Buchs, Switzerland.

**Enterotoxic activity assay.** Inhibition of prostaglandin synthesis by indomethacin has been demonstrated to predispose animal intestines to enterotoxigenic factors (34). Female rats (SPD) (6 to 8 months old; 200 g), starved for 24 h, were intraperitoneally injected with indomethacin (0.1 mg/kg) by the procedure of Udezulu et al. (35). Thirty minutes before the experiment, the animals were anesthetized with ether and the intestine was exposed after midline laparotomy. Colonic loops ca. 1.5 cm in length were formed in the ascending colon region and injected with soluble amoebic extract (originating from 2 × 10⁶ amoebae) in a total volume of 0.2 ml in saline. Four hours after the operation, the animals were sacrificed and the volume of the fluid in the colon loops was carefully removed and measured. The enterotoxic effect was determined by the volume of the fluid accumulated per centimeter of loop. V. cholerae toxin (50 μg; Sigma), whereas saline introduced in adjacent loops served as controls.

**Ionophoric activity assay.** Ionophoric activity of the materials obtained after gel filtration of amoebic extracts (fraction B) was determined by Ian Rosenberg, Weizmann Institute, in artificial bilayer membranes as described by Lynch et al. (20).

**Cultivation of bacteria.** Escherichia coli serotype O115 (strain 7343) or serotype O55 was grown overnight at 37°C in a medium containing yeast extract (1%), peptone (0.5%; Difco Laboratories, Detroit, Mich.), and NaCl (0.5%) (4, 5). Bacteria were harvested by centrifugation at 9,000 × g for 10 min, washed (3×) with saline, and suspended in saline to a concentration of 10¹⁰ bacteria per ml. Bacterial cells were disrupted in the cold (4°C) with a French press (American Instruments Co., Inc., Silver Spring, Md.) at 15,000 lb/in².

**Interaction between amoebae and bacteria.** Bacteria (10⁶) were incubated with Entamoeba histolytica trophozoites (10⁶) in a total volume of 1 ml of saline solution for 1.5 h at 37°C. The incubation mixture was slowly rotated to prevent settling of the amoebae. After the incubation period, the mixture was disrupted by freeze-thawing and sedimented at 100,000 × g as described above. The supernatants were analyzed for cytopathic and enterotoxic activities (see above). As controls, the freeze-thaw extracts of the same number of trophozoites (without bacteria) and those of bacteria alone were also tested.

**RESULTS**

**Cytotoxic activity of the cell-free soluble extracts of Entamoeba histolytica.** Cell-free extracts of Entamoeba histolytica have been shown to cause rounding up and detachment of tissue-cultured mammalian cells (2, 19). This assay was used to determine the cytotoxic activity in fractions in cell-free extracts of various Entamoeba histolytica strains. Increasing amounts of cell-free extracts of the three Entamoeba histolytica strains were found to have different cytotoxic activity as determined by their rates of tissue monolayer destruction (Fig. 1). The lowest concentration of the amoebic extract (from strain HM-1:1MSS) causing 50% detachment of tissue-cultured cells after 2 h of incubation was equivalent to the material obtained from 10⁶ amoebae per ml and was defined as one cytotoxic unit. Virulent strains of Entamoeba histolytica such as HM-1:1MSS, which are known to readily produce hepatic abscesses in hamsters (29), and the highest amount of cytotoxic activity (Fig. 1).

**Release of cytotoxic activity from intact trophozoites.** The cytotoxic activity was found to be gradually released by Entamoeba histolytica trophozoites (strain HM-1:1MSS) upon incubation in saline (Fig. 2). The supernatant obtained from 5 × 10⁶ trophozoites (after 2 h of incubation in saline at 37°C at a concentration of 10⁶/ml) contained ca. 60% of the

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**FIG. 1.** Cytotoxic activity of different strains of Entamoeba histolytica. Strains HM-1:1MSS (×), 200-NIH (△), and HK-9 (○) were harvested after axenic growth for 72 h. The supernatants, after freeze-thawing of the trophozoites, were tested for cytotoxic activity on BHK cell monolayers. The BHK cultures were exposed to the extracts for 2 h.

**FIG. 2.** Time-dependent secretion of Entamoeba histolytica toxin. Trophozoites (strain HM-1:1MSS) were incubated at 37°C at a concentration of 10⁶/ml in saline (0.9% NaCl). After the incubation, the trophozoites were sedimented (600 × g, 5 min). The cytotoxic activity of the supernatant, as well as that remaining in the sedimented trophozoites and subsequently released by freeze-thaw, was determined. Symbols: ○, extracellular toxin; •, intracellular toxin.

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cytopathic activity originally present in the cell-free extracts of nonincubated trophozoites. Cell-free extracts after freeze-thawing and sonication of the sedimented trophozoites contained the remaining cytopathic activity (>35%). Over 90% of the amoeba trophozoites remained fully viable after the incubation time, as determined by trypsin blue exclusion. The small amounts (10%) of lysed amoebae could not be responsible for the cytopathic activity released into the incubation media.

**Augmentation of Entamoeba histolytica cytopathic and enterotoxic activities by Escherichia coli bacteria.** Both the virulent strain of Entamoeba histolytica Hm-1:IMSS and the less virulent strain HK-9 were interacted with Escherichia coli cells (4). After the interaction, a clear augmentation in the cytopathic activity of the cell-free extracts was obtained (Fig. 3). The amount of cytopathic activity released upon incubation of intact trophozoites was also augmented after the interaction with bacteria. The augmentation was dependent on the ratio between the trophozoites and bacterial cells and occurred with Escherichia coli (serotypes O55 and O115) (4, 5). The level of augmentation was dependent on the incubation time of the amoebae and bacteria. Maximal activity was observed after 90 to 120 min of incubation. Trophozoites and bacteria were not incubated for more than 120 min due to increasing lysis of the amoebae.

Sonicated or heat-inactivated bacteria had no effect on the amoebic cytopathic activity. In addition to augmentation of the cytopathic activity, the preincubation of the trophozoites with the Escherichia coli cells also resulted in an increase in their enterotoxic activity (Table 1).

**Purification of the Entamoeba histolytica cytopathic/enterotoxic activity: gel filtration.** The soluble fraction of disrupted trophozoites, obtained after 100,000 × g centrifugation and ammonium sulfate (30 to 70%) precipitation (fraction A) was chromatographed on an Ultrogel AcA-54 column and eluted with 0.05 M Tris-hydrochloride buffer (pH 7.5) (Fig. 4). Two peaks with cytopathic activity were observed. The first (peak I), which contained most of the cytopathic activity (>70%), had an apparent molecular weight of between 30,000 and 50,000, and the second peak (peak II), which had the rest of the activity, had an apparent molecular weight of 15,000 to 25,000.

**Properties of the toxic activities in peaks I and II.** The material from peak I and peak II originating from extracts of 10⁴ trophozoites of strain Hm-1:IMSS were introduced into intestinal ligated loops of indometacin-pretreated rats. After 4 h, the fluid accumulation in the colonic loops was 0.3 ml/cm for peak I and 0.27 ml/cm for peak II. Controls with cholerae toxin (50 μg) showed fluid accumulation of 0.54 ml/cm (Table 1). Both the cytopathic and enterotoxic activities of the amoebic cell-free extracts were found to be heat labile. After 30 min of incubation at 70°C, the toxic effect was lost completely. Trypsin digestion of the amoebic cell-free extract also resulted in the disappearance of the toxic effect. Addition of protease inhibitors such as antipyrin, pepstatin, phenylmethylsulfonyl fluoride, carbobenzoxy-glycyl-hydroxamate, thioacetetyl-leucyl-phenylalanine, and bacitracin, even at high concentrations (2 mg/ml), had no effect on the toxic activity of the material in peak I. On the other hand, p-chloromercuribenzoate and iodoacetamide totally inhibited the cytotoxic effect. Among different lectins, glycoproteins, and sugars tested, the activity of peak I was partially inhibited by preincubating the tissue-cultured cells with concanavalin A and completely inhibited by wheat germ agglutinin (Table 2). Other lectins, such as peanut agglutinin, phytohemagglutinin, and soybean agglutinin, had no effect on the cytopathogenicity of the soluble extract. The cytopathic activity was also strongly inhibited by fetuin or mucus (5 mg/ml) or calf serum (1%). Among the sugars tested, the toxic effect was specifically inhibited by sialic acid (10 mg/ml; Table 2); other sugars had no effect. After oxidation of the sialic acid residues of fetuin by sodium periodate (36), the glycoprotein lost most of its inhibitory effect (Fig. 5). Furthermore, pretreatment of target tissue-cultured HeLa cells with neuraminidase (0.06 U/ml for 6 h) made them less sensitive to the cytopathic activity (40% destruction), as compared with the untreated control cells (97% destruction).

**TABLE 1.** Enterotoxic activity of Entamoeba histolytica Hm-1:IMSS

<table>
<thead>
<tr>
<th>Amoebic fraction</th>
<th>Enterotoxic activity* (ml/cm of colonic loop per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.05</td>
</tr>
<tr>
<td>Supernatant from freeze-thawed trophozoites centrifuged at 100,000 × g</td>
<td>0.55</td>
</tr>
<tr>
<td>Supernatant from freeze-thawed trophozoites preincubated with Escherichia coli O115 and centrifuged at 100,000 × g</td>
<td>1.27</td>
</tr>
<tr>
<td>Peak I from Ultragel AcA-54</td>
<td>0.81</td>
</tr>
<tr>
<td>Peak II from Ultragel AcA-54</td>
<td>1.3</td>
</tr>
<tr>
<td>Eluate after agarose-fetuin column chromatography</td>
<td>1.05</td>
</tr>
<tr>
<td>Control: V. cholerae toxin (50 μg)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*Enterotoxicity was determined in colonic loops of indometacin-treated (0.1 mg/kg) SPD rats. The protein content of the administered samples was determined by Bradford's method (6). The preincubation of the Hm-1 trophozoites with the Escherichia coli bacteria was done as described in the text. Results are given in ml fluid accumulated per cm of colonic loop.
The cytopathic activity of peak II was not inhibited by fetuin or sialic acid. The material found in peak II contained ionophoric activity (L. M. Rosenberg, personal communication) as measured by changes in conductance in artificial bilayers, as described by Lynch et al. (20). In contrast, the material in peak I did not show any ionophoric activity in the same system. Properties of peak I and II are summarized in Table 3.

Gel filtrations of the material released into saline upon incubation of intact trophozoites (see above) revealed that material from both peak I and peak II was released (Fig. 4).

As mentioned above, interaction of trophozoites and bacteria caused an increase in the soluble cytopathic activity (Fig. 3). Comparison of the gel filtration pattern of cell-free extracts from trophozoites (strain HM-1:IMSS) interacted and noninteracted with bacteria demonstrated that the activity of peak I was increased, whereas the activity of peak II seemed to remain unchanged. The increased activity of peak I was almost totally inhibited by fetuin (Fig. 3) as well as by sialic acid itself.

Separation between the two types of cytopathic material (peaks I and II) could also be achieved by high-speed centrifugation (150,000 X g) of the amoebic soluble fraction.

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**TABLE 2. Inhibition of the cytopathic activity of Entamoeba histolytica**

<table>
<thead>
<tr>
<th>Substance added</th>
<th>Final concn (%)</th>
<th>Destruction of amoebic monolayer: % release of BHK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>WGA</td>
<td>0.1</td>
<td>7 ± 1.5</td>
</tr>
<tr>
<td>Con A</td>
<td>0.1</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>PNA</td>
<td>0.1</td>
<td>100 ± 0.5</td>
</tr>
<tr>
<td>PHA</td>
<td>0.1</td>
<td>100 ± 0.5</td>
</tr>
<tr>
<td>SBA</td>
<td>0.1</td>
<td>100 ± 0.5</td>
</tr>
<tr>
<td>Fetuin</td>
<td>0.5</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>1.0</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Human serum</td>
<td>1.0</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

The cytopathic fraction (supernatant 150,000 X g) of freeze-thawed trophozoites of strain HM-1:IMSS [10⁸ amoebae] caused total detachment of the BHK monolayers after 2 h incubation was defined as 100% activity. The amount of dye retained by tissue-cultured cells in controls without amoebic material was defined as 0% activity. The BHK cells were preincubated with the lectins (45 min at 37°C, followed by washing with Dulbecco modified Eagle medium) before exposure to the amoebic extract. The other inhibitors were preincubated with the amoebic sample (30 min at 0°C) before addition to the cells.

Abbreviations: WGA, wheat germ agglutinin; Con A, concanavalin A; PNA, peanut agglutinin; PHA, phytohemagglutinin; SBA, soybean agglutinin.

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**FIG. 4.** Gel filtration of the soluble components of Entamoeba histolytica trophozoites (10⁸, strain HM-1:IMSS) on an Ultrogel AcA 54 column (2.0 by 80 cm). Samples (250 µl) of the eluted fractions (1.7 ml) were tested for their cytopathic activity on monolayers of tissue-cultured cells. Two peaks with cytopathic activity were obtained. Peak I eluted between 30 and 50 kDa, and peak II eluted at 15 to 25 kDa.

**FIG. 5.** Inhibition of the cytopathic activity by fetuin and asialofetuin (36). The cytopathic activity of HM-1:IMSS (supernatant from amoebic material centrifuged at 150,000 X g) was checked on monolayers of BHK cells. The amoebic material (from 5 x 10⁸ cells) was preincubated with the inhibitors (45 min at 0°C) and interacted with BHK cells. Symbols: ·, fetuin; ○, asialofetuin.
TABLE 3. Properties of peaks I and II from the Ultrogel AcA-54 column

<table>
<thead>
<tr>
<th>Property</th>
<th>Value or presence (+) or absence of (-) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak I</td>
</tr>
<tr>
<td>Mol wt</td>
<td>30,000–50,000</td>
</tr>
<tr>
<td>Solubility (150,000 × g)</td>
<td>+</td>
</tr>
<tr>
<td>Cytopathic activity</td>
<td>+</td>
</tr>
<tr>
<td>Enterotoxic activity</td>
<td>+</td>
</tr>
<tr>
<td>Ionophoric activity</td>
<td>–</td>
</tr>
<tr>
<td>Secretion into media by intact trophozoites</td>
<td>+</td>
</tr>
<tr>
<td>Inhibition by fetuin or sialic acid</td>
<td>+</td>
</tr>
<tr>
<td>Activity augmented by bacteria</td>
<td>+</td>
</tr>
</tbody>
</table>

obtained after the first centrifugation (100,000 × g) and ammonium sulfate (30 to 70%) precipitation (fraction B). Fractionation of the 150,000 × g soluble fraction on the Ultrogel column revealed only one peak containing cytopathic/enterotoxic activity. This peak was eluted in the 30,000 to 50,000 range as peak I. It contained ca. 80% of the total cytopathic activity and was inhibited by fetuin or sialic acid. The material that sedimented at high speed contained ca. 20% of the total cytopathic activity and had ionophoric activity (I. A. Rosenberg, personal communication).

Affinity chromatography. The cytopathic activity that emerged from gel filtration in peak I was further purified on an agarose-fetuin affinity column (Fig. 6). The first peak, containing over 95% of the protein, did not display any cytopathic activity. The cytopathic activity was eluted upon changing of the pH of the eluant to pH 10.5 with dilute ammonia solution. The recovery of activity in the eluted peak varied somewhat in different preparations and was, on average, 20%. The purified material had no collagenase activity when tested with Azocoll. On the other hand, it slowly digested 125I-labeled casein, and this degradation was inhibited in the presence of fetuin or by p-chloromercuribenzoate.

The material containing the cytopathic activity recovered from the fetuin affinity chromatography column was also tested for enterotoxic activity in indomethacin-treated rats. About 200 ng of the purified toxin (originally obtained from 8 × 10^7 HM-1 trophozoites) induced after 4 h a fluid accumulation of 0.83 ml/cm.

SDS-acrylamide gels of the material containing the cytopathic activity revealed a major protein band that migrated with an apparent molecular weight of ca. 30,000 (Fig. 7). Protein recovery of the purified enterotoxin from 10^7 trophozoites (strain HM-1:1MSS) was 25 ng, or 0.25%. Amino acid analysis of the purified enterotoxin showed that the protein was devoid of cysteine and contained only small amounts of methionine (58 nmol/mg).

DISCUSSION

The soluble fraction obtained from homogenates of pathogenic strains of amoebae has been shown to possess substances that are toxic for a variety of mammalian cell lines (2, 19, 24, 25, 33). The different reports on cytopathic, enterotoxic, and ionophoric activities found in crude extracts or partially purified fractions of Entamoeba histolytica, which most likely contribute to the pathogenesis of the parasite, have created an unclear picture of the molecular identity of the different activities. The most commonly observed effect is the rounding up and detachment of tissue-cultured cells. The first report of an amoebic cytotxin-enteroxin was made by Lushbaugh et al. (19). The cytotoxicity of amoebic sonic extract was found to reside in proteins of between 25 and 35 kDa that were inhibited by serum and were reported to have enterotoxic activity in rabbit ileal loops (19). Bos and Van de Griend found that the 35-kDa serum-sensitive amoebic cytotoxin was heat labile and could be released into the media by the intact trophozoites. Mattern et al. (23) reported that rounding up and detachment of BHK cells by crude extracts of Entamoeba histolytica was inhibited by serum and fetuin. McGowan et al. (26) partially purified a cell-free cytotoxin which on gel electrophoresis migrated as four bands (at 25, 58, 65, and 68 kDa), and strains with higher virulence contained more cytopathic activity.

UdezuLu et al. (35), using low concentrations of indomethacin to impair prostaglandin-mediated mucosal cytotoxicity, found enterotoxic effects in soluble fractions of amoebic sonic extracts. The enterotoxic effect was demonstrated in rabbit ileum and rat colon, and was heat labile and inhibited in the presence of fetuin.

The amoeba have also been reported to contain an ionophore activity. Lynch et al. (20) reported that pore-forming components, which were present in higher amounts in the more virulent strains, were released into the growth media and had a subunit molecular weight of ca. 13,000. The presence of an ionophore material with similar properties, but with a molecular weight of 30,000, was also reported by Young et al. (38).

Our results show that the parasite has at least two sets of toxic substances which appear to be distinct from each other. The two sets of toxic substances were separated either on a gel filtration column or by high-speed centrifugation. Upon passage of the soluble free extract of trophozoites on an Ultrogel AcA-54 column, we obtained two peaks (peak I, 30 to 50 kDa; peak II, 15 to 25 kDa) with cytopathic and enterotoxic activities. Peak II was shown to have ionophore activity and probably contained the amoebapore that has already been described (20, 38).

Our interest focused on the component of peak I which contained most of the cytopathic activity, was augmented by Escherichia coli bacteria, and had no ionophoric effect. The activities of peak I, including its weak proteolytic activity on casein, were inhibited by fetuin. This is most likely in agreement with the observations of Lushbaugh et al. (18), Mattern et al. (23), and UdezuLu et al. (35), who reported on cytopathic and enterotoxic activities in Entamoeba histolytica crude extracts that could be blocked by fetuin.

Our results indicate that the inhibitory effect of fetuin and other sialoglycoproteins (such as mucins) is due to their sialic acid residues; the activity was also blocked by sialic acid itself. This is also supported by the facts that asialofetuin was not an inhibitor and neuraminidase-treated mammalian cells lose their sensitivity to the cytopathogenic factor. Moreover, we have recently shown that tumor cells in which the cell surface sialic acid content is increased after incubation with retinoic acid are more sensitive to the amoebic cytopathic substance (13). Since carbohydrates have been shown to serve as receptors for various enterotoxins of microbial origin (1, 9, 28, 31), it is possible that in the case of the amoeba toxin, sialic acid serves as a recognition marker or receptor for the enterotoxin on target cells.

The enterotoxin was purified on an agarose-fetuin affinity column with acceptable recovery of the activity. The purified cytopathic enterotoxin appears to have a main protein band corresponding to a molecular weight of ca. 30,000.
Unfortunately, affinity chromatography of the toxin does not purify it to homogeneity, and minor bands can also be observed. Further work on its purification is in progress, and we hope this will cast further light on any possible multi-unit composition of the toxic substance.

The mechanism of action of the amoebic enterotoxin is presently under investigation. Preliminary experiments with homogenates of human colonic mucosal biopsies (32) indicate that it activates adenylate cyclase and induces the production of cyclic AMP (D. Rachmilewitz, personal communication), but has no apparent ADP-ribosylation activity (M. Woolkalis, personal communication).

Different observations by several investigators (30, 37) have indicated that interactions between amoeba and bacteria are important for the expression of pathogenicity in Entamoeba histolytica. Association of axenically grown trophozoites with bacteria has been shown to increase their ability to produce hepatic abscesses in hamsters (37) and to destroy tissue-cultured monolayers (5, 27). Ingested bacteria stimulate amoebic virulence apparently by enhancing electron donor reactions and activating the respiratory chain, causing an increase in the redox potential of the amoeba (5). In view of the above, we decided to study whether association of amoeba and bacteria may also affect the level of cytopathic and enterotoxigenic activities of the parasite. After preincubation of the Entamoeba histolytica trophozoites with certain Escherichia coli bacteria with which the trophozoites were known to interact (5, 27), for example serotypes O115 and O55, the cytotoxic activity of the amoebae was significantly increased. Only intact bacteria caused the increase in the toxic activity, whereas heat-inactivated or bacterial sonic extracts did not. Moreover, bacteria that were not ingested by the trophozoite, such as Staphylococcus aureus or Shigella flexneri (27), did not stimulate the toxic activity. These results suggest that an increase in the redox conditions of the trophozoite activates or helps preserve the activity of the toxic substance. Fractionation of the cell-free extracts of trophozoites that had been interacted with bacteria for 90 min before their disrup-
tion and gel filtration revealed that the activity of peak 1 was the one augmented. Both the cytotoxic and enterotoxic activities were significantly inhibited by cetuximab and sialic acid. Our results suggest that (i) the expression of *Entamoeba histolytica* virulence may depend on the microenvironment of the parasite and (ii) the symptomatic or asymptomatic phenomena in amoebiasis might in part be related to interaction of the trophozoite with suitable bacteria of the intestinal flora of the host (5).

The virulence of *Entamoeba histolytica* appears to be complex and to involve more than one substance or mechanism. The cytotoxic activity, i.e., rounding up and detachment of amebic cells, might facilitate the removal and subsequent phagocytosis of target cells by the trophozoites (21). It remains to be determined, however, which of all the toxic activities of the parasite is the most important in the development of the disease, and whether any one of them could serve as a suitable candidate to elicit effective intestinal immune protection.

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LITERATURE CITED


