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Entamoeba histolytica Cytotoxin from Infected Liver: Purification, Physico-chemical Characterization and Virulence

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A heat-labile cytotoxin was isolated from amoebic liver abscess produced in hamster by a virulent strain of *E. histolytica*. Extract from the abscessed liver possessed marked cytotoxic activity against rabbit RBC and HeLa cell as compared to normal liver. Cytotoxin was partially purified by ammonium sulphate precipitation and gel filtration. Cytotoxic activity was stable in a narrow *p*H range (7-7.2). The cytotoxic activity was inhibited at *p*H 2-6 and 8-10. Specific immune rabbit serum inhibited the cytotoxic effect against rabbit RBC cell lysis. The cytotoxin was active at temperature 56°C and inactive at 60-100°C. The virulent strain of *E. histolytica*, therefore, appears to possesses an immunogenic cytotoxin protein which is important in the pathophysiology of amoebiasis. Amoebic abscessed liver is considered as the source of high degree of *E. histolytica* cytotoxin.

Key Words: ***Entamoeba histolytica*, Cytotoxin characterization**

Introduction

A search for the toxic substance from *E. histolytica* as suggested by Eaton et al. 1970 proved unsuccessful till Lushbaugh et al. (1979) reported the isolation of a heat-labile cytotoxin from *E. histolytica*. Later, cytotoxic activity in cell-free extracts of *E. histolytica* was confirmed (McGawn et al. 1980). The virulence of *E. histolytica* is defined as its capacity to produce liver abscess in appropriate experimental animals (Mattern & Keister 1977). *In vitro* cytotoxic activity of intact and cell-free extracts of *E. histolytica* has been shown by Prasad et al. (1982).

The objectives of the present study were: (i) to purify the cytotoxin derived from liver abscess, experimentally induced by *E. histolytica*, (ii) its lysis of RBC and mammalian cell lines (HeLa cell), *in vitro*, (iii) to characterise its physicochemical properties (temperature, immune serum and *p*H), and (iv) to correlate it with virulence, determined by liver abscess model in hamsters.

Materials and Methods

Method of Xenic Cultivation of ***E. histolytica*** (H-39)

Virulent strains of *E. histolytica* (H-39) were maintained in Boeck and Drobohlav (1925) medium. The medium contained inactivated bovine serum [diluted 1/8th with M/40 phosphate buffer in 0.85% (w/v) NaCl, *p*H 7.0], rice starch and gentian violet (1/10,000, w/v) or acriflavine (1/10,000 w/v). The stock strains of amoebae were used for the development of hepatic amoebiasis models.

Experimental Amoebic Liver Abscess in Hamsters

For hepatic infection in golden hamsters (40 to 50g) about 0.5cm long incision in the abdominal

skin was made a little behind xiphisternum. Connective tissue, underlying the skin, was gently removed to expose the muscular layer of the abdomen. With the help of a pair of forceps, 0.05 to 0.08ml of inoculum containing 25,000 to 50,000 trophozoites of *E. histolytica* were dropped in the peritoneal cavity near the liver with a tuberculin syringe through the muscular layer. The amoebic liver abscess developed within 4 to 6 days after inoculation.

Infected liver pieces showing active trophozoites were inoculated directly into liver and continuously maintained through liver to liver passage in order to increase virulence of the amoebae.

Isolation of Cytotoxin from ***E. Histolytica****-Infected Liver Pieces*

Amoebic liver abscess was produced in golden hamster by highly virulent strain of *E. histolytica* (H-39) maintained in Boeck and Drbohlav medium. Infected liver pieces were separated, homogenized in normal saline, sonicated and centrifuged at 40,000 rpm for 60 min at 4°C. The supernatant was the source of the crude cytotoxin.

Normal hamster liver pieces were also homogenized, sonicated and centrifuged (40,000 rpm for 60 min at 4°C) and the supernatant was used as normal control in the cytotoxicity test.

Protein contents of Normal and Infected liver supernatants were estimated (Lowry et al. 1951) with bovine serum albumin as the standard. The cytotoxic activities of these supernatants were measured on RBC lysis (Prasad et al. 1982) using 20% suspension of rabbit RBC in PBS-*p*H 7.2.

Purification by Ammonium Sulphate Precipitation

Saturated ammonium sulphate precipitation technique (Dixon 1953) was used for the purification of the cytotoxin. Normal and Infected liver supernatants were reconstituted with 0.85% NaCl (20ml) and subjected to differential precipitation with 30, 50, 70 and 100% (w/v) concentrations of ammonium sulphate, allowed to stand overnight at 4°C, centrifuged, washed (2X) and finally suspended in ammonium sulphate solutions. Each fraction was dialysed extensively against 1N NaCl, lyophilized and reconstituted in NaCl. Protein of each fraction was estimated (Lowry et al. 1951). Cytolytic and cytotoxicity activities of all these fractions were carried out on rabbit RBCs and HeLa cell monolayers respectively.

Preparation of 20% RRBCs Suspension for Cytolytic Activity

Five ml rabbit cardiac blood was collected in a heparinized vial, kept at 4°C for 2 hr and then centrifuged at 2,000 rpm for 15 min. Erythrocytes were separated and washed thrice, with PBS 7.2 by centrifugation. Finally 20% suspension of erythrocytes was made in PBS 7.2. Equal volume of each fraction (containing equal volume of protein and equal volume of 20% RBCs suspension) was incubated for 4 hr at 37°C and over night at 4°C for the cytolytic activity. After incubation samples were centrifuged and 100µl supernatant was added in 2.5ml of PBS *p*H 7.2 separately and optical density (OD) was measured at 440nm by Bausch and Lomb spectrophotometer (Spectronic 20) for haemolysis of RBCs.

Cytotoxicity Testing: Maintenance of HeLa Cell Culture in Microtitre Plate

The HeLa cell culture was obtained from NICED, Calcutta. For subculturing, the medium MEM *p*H 6.9 was removed from the bottle containing a confluent monolayer of cells. The bottle was rinsed with two to three changes in PBS. Two ml of 0.05% trypsin and 3ml of 0.02% EDTA solution was added to the bottle and allowed to react for 1 min. The trypsin-EDTA solution was discarded from the bottle. The bottle was left in the incubator (37°C) for 10 min. After 10 min of incubation the monolayer came out of the bottle in small fragments into the bottom of the bottle. Five ml fresh medium was added to the bottle and equal quantity of cell suspension was taken into each well of microtitre plate with the help of adjustable volumetric digital pipette.

The toxigenicity of each fractions were assessed *in vitro* by microscopic observations of their activity on HeLa cell line culture in microtitre plate. The criterion for a positive cytopathogenic effect (CPE) was rounding and aggregation of 50% of the cells. Appropriate controls containing only PBS or hamster liver lysate were tested for toxicity by the same method.

Preparative Poly-acrylamide Gel Electrophoresis - (PAGE)

Cytotoxic fractions obtained by 0-30, 30-50, 50-70 and 70-100% ammonium sulphate precipitation, were lyophilized and reconstituted to 10mg/ml concentration in NaCl. Test fractions (10mg of each) were loaded on 7.5% polyacrylamide gel over two preparative slabgels and electrophoresis was run at 30mA for 3 hr.

Physico-chemical Characterisation of Cytotoxic Activity

Effect of temperature: Heat treatment of 50-70% ammonium sulphate fraction at 56°, 60° and 100°C for 30 min was carried out in a constant temperature water bath and then allowed to cool to room temperature. Prior to cytotoxicity testing, untreated supernatants from the same preparative batch was used as control.

Effects of serum: Normal (preimmune) and immune serum produced by subcutaneous injection of 50-70% ammonium sulphate fraction in complete Freund's adjuvant, in rabbits, were used to find out their effect on cytotoxin of *E. histolytica* by using haemolysis of cells. Both sera used in these experiments were inactivated at 56°CS for 30 min and then cooled to room temperature prior to cytotoxicity testing.

Effect of pH: Liver extracts were placed in solutions of different *p*H 2-10, adjusted with 1N NaOH and 1N HCl. After incubation at various *p*H and temperatures, the samples were adjusted to *p*H 7.0 by addition of 0.2M phosphate buffer (*p*H 7.2). Cytotoxic activity was carried out by RRBCs lysis.

Results

Complete lysis of cells was found in 50-70% fractions in 2 to 4hr duration and partial lysis in 30-50% and 70-100% fractions in 4hr and no significant change was found in the fraction of 0-30% (table 1).

Table 1 *HeLa cell interaction with different ammonium sulphate fractions of* ***E. histolytica*** *at 1,2,3 and 4 hr observations (Average of 4 readings)*

		Hours			
		1	2	3	4
1.	*Normal liver extract* (control)	Cells intact	Cells intact	Cells intact	Cells intact
	Normal liver 0-30; 30-50; 50-70; 70-100% ammonium sulphate fractions	Cells intact	Cells intact	Cells intact	Cells intact
2.	*Infected liver extract*	Complete lysis of cells	Complete lysis of cells	Complete lysis of cells	Complete lysis of cells
	Ammonium sulphate 0-30% fraction (I)	No significant change	No significant change	Cells intact	Cells intact
	30-50% Ammonium sulphate fraction (II)	No significant change	Cells intact	Partial lysis of cells	Partial lysis of cells
	50-70% Ammonium sulphate fraction (III)	Partial lysis of cells	Moderate lysis of cells	Complete lysis of cells	Complete lysis of cells
	70-100% Ammonium sulphate fraction (IV)	No significant change	No significant change	No significant change	Partial lysis of cells

Table 2 *Effect of temperature on haemolytic activity of* ***E. histolytica*** *cytotoxin (O.D at 400nm) and protein content*

	Control	56°C	60°C	100°C
Normal liver	0.0685±0.0049	0.0685±0.0049	−0.0375±0.0035	−0.008±0.0042
Relative haemolysis (%)*				
Protein	10.95 ±0.3535	10.95 ±0.3535	5.5 ±0.4243	0.7 ±0.2828
Normal liver 50-70%	0.0565±0.0049	0.0565±0.0049	−0.0305±0.0035	−0.005±0.0042
Relative haemolysis (%)*				
Protein	7.55 ±0.4950	7.55 ±0.4950	4.05 ±0.3536	0.45 ±0.3536
Infected liver	0.0595±0.0035	0.0605±0.0049	−0.028 ±0.0042	−0.006±0.0042
Relative haemolysis (%)*				
Protein	8.8 ±0.4243	8.8 ±0.4243	5.15 ±0.3536	0.50 ±0.2828
Infected liver 50%-70%	0.0455±0.0049	0.0455±0.0049	−0.027 ±0.0042	−0.004±0.0042
Relative haemolysis (%)*				
Protein	6.4 ±0.4243	6.4 ±0.4243	3.35 ±0.4950	0.3 ±0.2828

Data are mean ± SD of three repetitions
* haemolytic activity assayed using 250μg ml^{-1} protein

Tables 3 *Effect of immune and non-immune sera on Haemolytic activity of* ***E. histolytica*** *cytotoxin (OD at 440nm)*

	Control	Non-immune sera	Immune sera
Normal liver extract	0.0857±0.0060	0.0537±0.1874	0.0083±0.0060
Normal liver 50-70%	0.0623±0.0061	0.0353±0.0075	0.0035±0.0030
Infected liver extract	0.0763±0.0055	0.0317±0.0060	0.0050±0.0066
Infected liver 50-70%	0.0517±0.0080	0.0267±0.0060	0.0027±0.0050

Data are mean ± SD of three repetitions
Haemolytic activity assayed using 250 μg ml^{-1} protein

Table 4 *Effect of pH 2 to 10 on* ***E. histolytica*** *cytotoxin derives from Normal liver and 50-70% Ammonium sulphate fractions (Relative haemolysis values) OD at 440nm at 37°C*

*p*H	Control	Infected liver	Activity
2	0.027 ±0.0066	−0.0100±0.0066	(Nil) − ve
4	0.046 ±0.0075	−0.0217±0.0065	(Nil) − ve
6	0.067 ±0.0066	−0.0580±0.0075	(Nil) − ve
7.0 (NL)	0.0757±0.0093	+0.0697±0.0084	(Positive)+ ve
7.0 (50-70%)	0.0573±0.0096	+0.0723±0.0070	(Positive)+ ve
7.1 (NL)	0.0773±0.0091	+0.0713±0.0081	(Positive)+ ve
7.1 (50-70%)	0.0573±0.0096	+0.0687±0.0099	(Positive)+ ve
7.2 (NL)	0.0757±0.0093	+0.0697±0.0084	(Positive)+ ve
7.2 (50-70%)	0.0570±0.0105	+0.0683±0.0092	(Positive)+ ve
8	0.062 ±0.0076	−0.0750±0.0060	(Nil) − ve
10	0.048 ±0.0065	−0.0497±0.0090	(Nil) − ve

Data are mean ± SD of three repetitions
Haemolytic activity assayed using 250 μg ml^{-1} protein

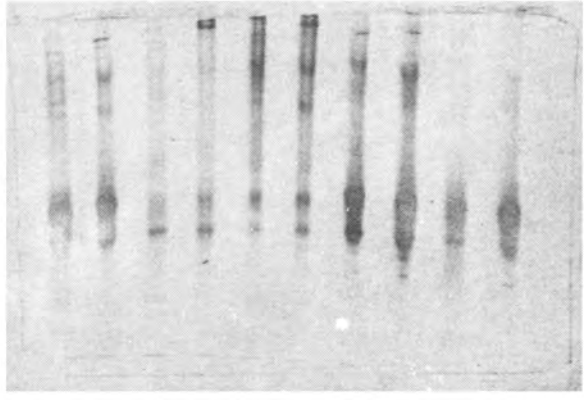

Figure 1A

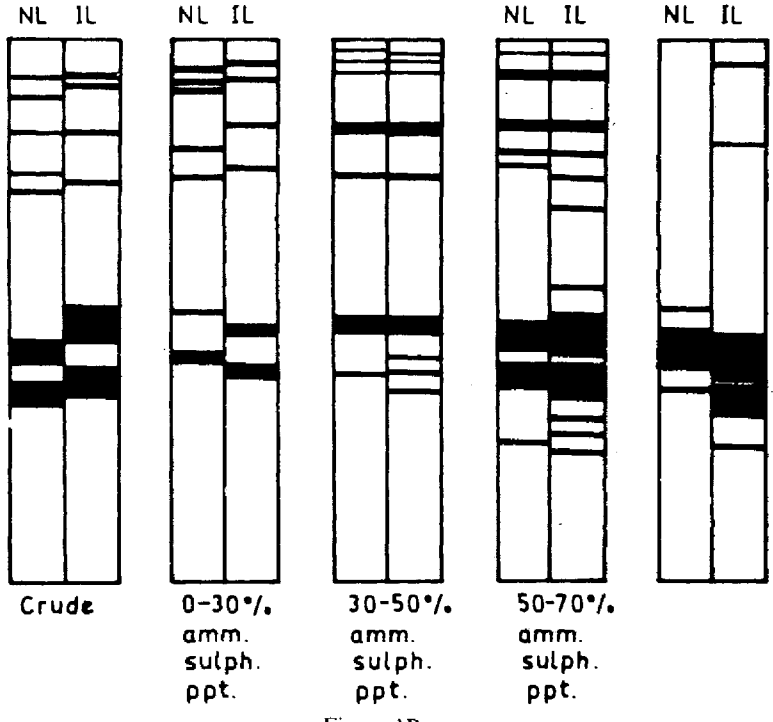

Figure 1B

PAGE of all the fractions are shown in figure 1a, 1b. Fraction 50-70% shows maximum number of bands in comparison with other fractions.

Heat treatment of cytotoxin at 56°C for 30 min did not alter the cytotoxic effect. However, heating of liver extract at 60-100°C for 30 min resulted in complete loss of cytotoxic activity (table 2).

Immune as wll as non-immune sera inhibited the cytotoxic activity of 50-70% ammonium sulphate fractions. However, degree of inhibition of immune sera was greater than normal sera against lysis of RBCs (table 3) by the cytotoxin.

*p*H 2-6 and B-10 inhibited the cytotoxic activity but maximum activity was noticed at *p*H 7-7.2 (table 4).

Discussion

The cytotoxic substances characterised by McGawn et al. (1982) were found to be heat-labile and stables over a narrow *p*H range (6-7.2). Our findings also confirm that the cytotoxic substances prepared from *E. histolytica* infected hamster liver was found to be heat-labile and stable at a narrow *p*H ranges of (7-7.2). These findings contrast with the more heat-stable cytotoxin characterised by Bos and Van Den Eijik, (1980). Their cytotoxin was stable over a broad range of *p*H 4-10, and was degradable by trypsin. Biological relevance of the amoebic cytotoxin is suggested by the fact of virulence *in vivo* a determined in an animal model of baby hamster. Our study confirms the previously reported results (Lushbaugh et al. 1979, McGawn et al. 1980, Bos & Van de Griend 1977, Mattern et al. 1980) that the inhibition of cytotoxic activity occurs by both immune and non-immune sera. Inhibition of CPE by immune serum was greater than the inhibition by non-immune rabbit serum. Thus, the cytotoxic protein appeared to be immunogenic and induced protective antibody in the rabbit. There is evidence that supports the speculation that the cytotoxin is related to the pathogenicity of *E. histolytica* infection. Cytopathic effect of the amoebae on mammalian tissue can not be attributed to a single function of the parasite but it is a combined results of various chemical and mechanical properties (Martinez-palomo 1982, 1986). It is clear from the study that destructive effect of pathogenic amoebae in the mammalian host may not be due exclusively to the action of the parasite, but could be the result of the host-parasite interactions. Infected and abscessed liver release quiet a good quantity of *E. histolytica* cytotoxin as compared to minute quantity of cytotoxin that can be obtained from sonicated *E. histolytica* trophozoites. Another point is that the cytotoxic substance may be located intracellularly within close proximity to the amoebal plasma membrane and likely to be shed by exocytosis in high concentration at contact sites with host cell. In this way the cytotoxic substance could escape inactivation by serum factors.

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