CYTOTOXIC ACTIVITY OF PARTIALLY PURIFIED *ESCHERICHIA COLI* ALPHA HAEMOLYSIN

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SUMMARY. Alpha haemolysin, produced by *Escherichia coli*, grown in a chemically defined medium, was purified 19-fold and the endotoxin content reduced 2176-fold by ultrafiltration and glycerol-gradient ultracentrifugation. Immunodiffusion of purified α haemolysin (PH) against antiserum to crude haemolysin (CH) revealed only one precipitation line. PH was cytotoxic in nanogram amounts for mouse-fibroblast 3T3 cells, and the cytotoxicity exhibited proportional dose-response and time-course kinetics. The cytotoxic and haemolytic activities of PH were neutralised by immunoglobulins to CH. A mutant, produced by treating the haemolytic wild type with mitomycin C, possessed all of the biochemical characteristics of the wild type with the exception that the extracellular products of the mutant were non-haemolytic and non-cytotoxic.

INTRODUCTION

The ability of *Escherichia coli* to cause disease in man and in animals is well recognised. Probable virulence factors include enterotoxins, endotoxin, and attachment and adherence factors. Some strains of *E. coli* are also haemolytic (Smith and Linggood, 1971; Minshew *et al.*, 1978*a* and *b*) and can disrupt renal lysosomes *in vitro* whereas non-haemolytic strains do not (DePauw, Gill and Fried, 1971). Injection of haemolytic *E. coli* into rats resulted in mitochondrial degeneration in the kidney cells, in the absence of intrarenal bacteria (Fry, Fried and Goven, 1975). Fried and Wong (1970) also produced pyelonephritis in normal rats by intravenous injection of haemolytic *E. coli* but not by similar injection of non-haemolytic *E. coli*. Chaturvedi *et al.* (1969) demonstrated that haemolytic culture filtrates from *E. coli* were cytotoxic for chick-embryo fibroblasts, but they were able to destroy haemolytic activity and retain cytotoxicity by heating at 56°C for 1 h.

Smith (1963) described two separate haemolysins produced by *E. coli*. One, α haemolysin, was filterable whereas the other, β haemolysin, was cell bound. Calcium was required for the activity of both haemolysins (Snyder and Koch, 1966).

Purification of α haemolysin from *E. coli* grown in meat-infusion media has been attempted by several workers. Zwadyk and Snyder (1971) reported a 20-

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to 30-fold purification by precipitation with 20–25% ethanol and re-precipitation by 30% $(\text{NH}_4)_2\text{SO}_4$, but dialysis resulted in loss of activity. Rennie and Arbuthnott (1974) achieved a 4×10^3 -fold purification by a multi-step process including 50% $(\text{NH}_4)_2\text{SO}_4$ precipitation, re-precipitation in acetate buffer at the isoelectric point and Sephadex-G200 chromatography. Williams (1979) did not confirm these results but obtained a 900-fold purification by slightly different methods.

The molecular weight of α haemolysin has been estimated by several workers and ranges from as low as 120 000 (Williams, 1979), to as high as 600 000 (Rennie and Arbuthnott, 1974). These differences may reflect the existence of various forms of a multimeric molecule.

The absence of studies on the purification and cytotoxic activity of the haemolysin produced in a chemically-defined medium (CDM) prompted us to purify the haemolysin from *E. coli* grown in CDM (Snyder and Koch, 1966) and determine its cytotoxic potential for mouse-fibroblast 3T3 cells.

MATERIALS AND METHODS

E. coli strain and cultural conditions. *E. coli* type O55 was a clinical isolate from a patient with a urinary-tract infection at West Virginia University Hospital (Morgantown, WV). Isolated haemolytic colonies were identified as *E. coli* by gram staining and biochemical tests (Edwards and Ewing, 1972). These included fermentation of glucose, lactose, maltose, mannitol, inositol, salicin and sucrose; decarboxylation of lysine, arginine and ornithine; production of indole, hydrogen sulphide, urease and acetylmethylcarbinol; motility and methyl-red test. The standard inoculum was prepared and cultured in CDM as previously described (Snyder and Koch, 1966).

Preparation of crude α haemolysin (CH). Two 2-L flasks, each containing 500 ml of sterile CDM, were inoculated with 5 ml each of a standard inoculum, and incubated at 35°C for 10 h with shaking. The cultures were centrifuged at 4°C, the supernate was sterilised by filtration and the filtrate concentrated to 25 ml at 4°C by an ultrafiltration pressure-dialysis system (Amicon Corp., Lexington, MA) with a XM-300 membrane (exclusion limit mol. wt 300 000). The concentrated CH was sterilised by filtration.

Preparation of purified α haemolysin (PH). Glycerol solutions (5% and 30%) were made up in a medium (MCDM) consisting of MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$ and $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ in the same concentrations as in CDM and adjusted to pH 5.4. The gradients (32 ml) were poured into 1 $\times$ 3.5-inch centrifuge tubes and 5 ml of CH were layered on top of each gradient. The tubes were centrifuged at 4°C for 20–21 h at 89 100 *g* in a SW27 rotor (Beckman Instruments, Inc., Palo Alto, Ca). One-ml fractions were collected by siphoning and assayed for haemolytic activity. Fractions containing high haemolytic activity were pooled, dialysed in MCDM and sterilised by filtration.

Assay for haemolytic activity. A preliminary titration was performed as previously described (Snyder and Koch, 1966), except that 50% haemolysis was the endpoint, as judged visually. A quantitative assay was done by the use of 0.1-ml increments of the highest dilution of haemolysin that caused at least 50% lysis in the preliminary test. The volume was adjusted to 1 ml with calcium saline (Snyder and Koch, 1966) and 1 ml of a 1% suspension of thrice-washed sheep red-blood cells (SRBC) in saline was added. After 1 h at 37°C the unlysed cells were removed by centrifugation and the supernate decanted into a tube containing 1.5 ml of saline. The optical density at 545 nm was converted to percentage lysis by reference to a standard curve. The final titre was calculated by dividing the titre in the preliminary assay by the volume of the dilution that gave 50% lysis in the quantitative assay (HU50).

Chemical analyses. Protein concentrations were measured by the microassay method (Bradford, 1976) with bovine-serum albumin (Sigma Chemical Co., St Louis, MO) as a standard.

Endotoxin concentrations were measured by the limulus-lysate technique (Ward and Hill, 1972). *E. coli* O26:B6 lipopolysaccharide (Difco Laboratories, Detroit, MI) was used as the standard.

Carbohydrate was measured by the phenol-sulphuric acid method of Dubois *et al.* (1956), heptoses by the method of Osborn (1963) and 2-Keto-3-deoxyoctonate (KDO) by the method of Waravdekar and Saslaw (1959).

Lipids were detected by thin-layer chromatography (TLC) as described by Kates (1972). The sample was extracted with chloroform:methanol (2:1 v/v). The chloroform layer was removed, evaporated to dryness in nitrogen and redissolved in diethyl ether. The extract was spotted on a 5 × 20-cm strip of chromatography medium (ITLC type, Gelman Instrument Co., Ann Arbor, MI). Ascending TLC was run in petroleum ether:diethyl ether:acetic acid (50:10:1) and chloroform:methanol:acetic acid:water (85:15:10:4) solvent systems to detect individual lipids and total lipids respectively. After drying, the strips were sprayed with 50% H₂SO₄, heated at 230°C for 10 min, and observed for spots.

Molecular weight was approximately determined by passing PH through a 8 mm × 150-mm column of Sephadex G200 (Sigma) at room temperature. MCDM was used as the eluant and the void volume was determined with 1.5% dextran blue 2000. Fractions (0.5 ml) were collected and screened for haemolytic activity.

Isolation of a non-haemolytic mutant of E. coli. The haemolytic strain of *E. coli* was inoculated into 10 ml of sterile CDM containing mitomycin C 0.40 µg/ml (ICN Pharmaceuticals Inc., Cleveland, OH), and incubated with shaking in a 37°C water bath. After 24-h and 48-h, samples were plated onto blood-agar plates. Non-haemolytic colonies were selected and characterised by biochemical tests as described above for the haemolytic strain.

Preparation of antiserum and immunoglobulins. New Zealand rabbits were given intradermal injection thrice weekly for 2 weeks with 0.25 ml of CH. Serum was obtained 7 days after the last injection. Serum from immunised rabbits was absorbed with whole, heat-killed (100°C for 30 min), washed, non-haemolytic *E. coli* cells. Equal volumes of serum and packed cells were mixed, incubated for 5 h at 40–45°C and overnight at 4°C, then separated by centrifugation.

Immunoglobulins were precipitated from serum by addition of an equal volume of saturated (NH₄)₂SO₄ and were resuspended in saline to one-fifth of the original volume. Serial twofold dilutions of immunoglobulin or serum samples to be assayed for antihemolytic activity were made in calcium saline such that the final volume was 0.5 ml. A volume of CH or PH sufficient to cause 100% lysis of 1 ml of 1% SRBC was brought up to 0.5 ml and then added to each tube. The tubes were incubated at 37°C for 15 min. One ml of 1% SRBC was then added to each tube and the tubes were reincubated for 1 h. The dilution of immunoglobulin or serum that neutralised 50% of the haemolysis was taken as the titre.

Immunodiffusion. Immunodiffusion agar was prepared as described by Burrell (1979) except that 0.01% sodium azide was added. Agar was poured into 60 × 15-mm plastic dishes to a depth of 3 mm and the wells were cut 5 mm apart. Immunodiffusion was done at 37°C for 2 days after which the wells were refilled with 3.5% polyethylene glycol to precipitate the immune complexes, and reincubated at 37°C for 1 day.

Assay of cytotoxic activity. Mouse-fibroblast 3T3 cells were grown in Dulbecco's modified Eagle medium (DME) with 10% fetal calf serum (Gibco, Grand Island, NY). Incubation was at 37°C in 5% CO₂. Confluent monolayers were detached with trypsin and washed once with an equal volume of DME with 50% serum. After washing, the cell pellet was resuspended in DME and seeded either diluted or undiluted onto new plates.

For measurement of cytotoxic activity, detached 3T3 cells were washed and resuspended in DME. Trypan blue was added to a portion of the cell suspension and viable cells counted in a haemocytometer. A known number of cells (10⁵–10⁶ cells) was added to vials and the volume was brought up to 4.5 ml with DME. The vials were gassed with a CO₂-air mixture and incubated with shaking (200 rpm) at 37°C for 2 h. Viable-cell counts were then done on each vial, the test sample was added and the vials were reincubated. Viable counts were done at intervals up to 60 min.

RESULTS

Purification of α haemolysin

Fractionation of the crude haemolysin on a glycerol gradient resulted in a marked separation of endotoxin and other proteins from the haemolysin (fig. 1). A single protein peak was associated with haemolytic activity. The consistent appearance of a shoulder on the haemolytic-activity curve (see arrow, fig. 1) suggests the existence of two species of haemolysin.

A non-haemolytic mutant of *E. coli*, obtained by treatment of the haemolytic parent with mitomycin C, was identical to the parent in all biochemical characteristics tested. Concentrated culture filtrates from the mutant were fractionated on glycerol gradients, and the major protein peak that coincided with peak haemolytic activity in the parent strain (fig. 1) was

FIG. 1.—Fractionation of crude haemolysin on a 5–30% glycerol gradient. — = Protein concentration; . . . = haemolytic activity; --- = endotoxin concentration. The arrow indicates a shoulder suggesting the existence of two species of haemolysin.

absent. In all other respects the protein curves for the two strains were identical (data not shown).

Immunodiffusion analysis of haemolysin preparations revealed one precipitation line between PH and unabsorbed anti-haemolysin serum, and this line demonstrated identity with a single precipitation line seen between PH and absorbed antiserum. Immunodiffusion between unabsorbed antiserum and CH revealed two precipitation lines, one of which demonstrated identity with the precipitation line between PH and unabsorbed antiserum. The other was removed by absorption. Precipitation lines were not observed between CH or PH and preimmune serum.

Table I shows the data on the samples and fractions obtained during purification. The reason for the almost twofold increase in activity after ultrafiltration of the haemolytic culture filtrate is unclear. The use of a glycerol gradient for further purification resulted in a final product with a 19.4-fold increase in specific haemolytic activity and a recovery of 111% of the initial activity. The total endotoxin content of the purified haemolysin was lowered from 740 μ g in the culture filtrate to 0.34 μ g in the purified haemolysin, a reduction of more than 2000-fold. The fraction that contained the highest haemolytic titre and the fraction on each side were pooled, dialysed and used in the cytotoxicity experiments.

Recovery of haemolytic activity after dialysis ranged from 75% to 100%. The activity of PH was stable for months at -20°C and for several days at 4°C .

Chemical analysis of PH did not reveal detectable lipid, heptoses or KDO. However, carbohydrate 75 μ g/ml was detected in a sample of PH containing protein 6.2 μ g/ml. The molecular weight of PH exceeded 300 000 because it was excluded by a 300 000 molecular-weight cut-off filter and appeared in the void volume after chromatography on Sephadex G200.

Cytotoxicity of purified haemolysin to mouse-fibroblast 3T3 cells

In preliminary experiments, PH was added directly to 3T3-cell monolayers.

TABLE I
Purification of E. coli α haemolysin

Preparation	Total volume (ml)	Total protein (mg)	Total haemolytic activity (HU)	Specific haemolytic activity (HU/mg)	Relative specific activity	Percentage yield of total activity	Total endotoxin (μ g)	Fold decrease in endotoxin
Culture filtrate	740	5.92	69 560	11 750	1	100	740	...
Concentrated culture filtrate	24	1.10	129 336	117 578	10	186	24	31
Haemolytic fractions from glycerol gradient*	7	0.34	77 448	227 788	19.4	111	0.34	2176

HU50 = 50% haemolytic units.

* All data in this row are from one gradient and have been adjusted (except the volume) to the 24-ml total volume of concentrated culture filtrate of which 5 ml per gradient was applied.

Cytotoxicity in the form of cell detachment and death was observed. Because this response was difficult to measure, cell suspensions were used, and 3T3 cells were incubated for 1 h with different concentrations of PH. The dose-response curve was approximately linear over a range of 0–10 HU50/ml of PH (fig. 2). The curve levelled off at higher doses with 100% mortality occurring at 25 HU50/ml of PH. As little as 10 HU50/ml of PH (23 ng of protein killed more) than 90% of 3T3 cells—as many as 4.3×10^5 cells/ml.

To determine the effect of time on the progression of cytotoxicity, 3T3 cells were incubated at 37°C with 100 HU50/ml and assayed at several time intervals (fig. 3). More than 70% of 3T3 cells were killed after 15 min, and killing was approximately linear during 0–30 min. A cytotoxic effect (15% mortality) over control values (5% mortality) was found after 5 min of incubation. Complete killing (99–100% mortality) occurred after 45–60 min— 4.9×10^5 cells/ml killed. Control values (haemolysin not added) remained at or near 0% mortality throughout the incubation period of 60 min.

To obtain more evidence that cytotoxicity was due specifically to PH, a preparation containing 20 HU50/ml was incubated at 37°C for 15 min with a quantity of absorbed antihaemolysin immunoglobulins sufficient to neutralise

FIG. 2.—Dose-response kinetics of 3T3 cells to purified α haemolysin.

FIG. 3.—Time-course kinetics of 3T3 cells exposed to 100 HU50 of purified haemolysin/ml of cell suspension (●) or an equal volume of MCDM (○). See *Materials and methods* for composition of MCDM.

haemolytic activity. The same concentration of PH was also incubated with the same amount of immunoglobulin from preimmune serum. After incubation, the samples were brought up to 1 ml with MCDM and added to 3 ml of 3T3-cell suspension (7.4×10^5 cells/ml). PH treated with antihaemolysin immunoglobulin was not cytotoxic whereas PH treated with the same concentration of immunoglobulin from preimmune serum retained its cytotoxicity. Heat treatment also reduced the cytotoxic (table II) and haemolytic activities of PH threefold and tenfold respectively.

In addition, concentrated culture filtrates from the haemolytic parent were haemolytic for SRBC and cytotoxic for 3T3 cells whereas concentrated culture filtrates from the non-haemolytic mutant were neither haemolytic nor cytotoxic (data not shown).

To rule out the possibility that endotoxin caused the cytotoxicity, 3T3 cells were incubated with six times the amount of endotoxin found in 20 HU50 of PH (2.5×10^{-4} $\mu\text{g/ml}$). No effect on 3T3 cells was obtained.

TABLE II

Neutralisation of the cytotoxic effect of purified α haemolysin on mouse-fibroblast 3T3 cells by antibody and heat

Treatment	Percentage mortality
PH 20, HU 50/ml	88.3
PH 20, HU 50/ml + anti-haemolysin Ig	14.5
PH, 20 HU 50/ml + preimmune Ig	81.4
Anti-haemolysin Ig	7.1
Heated* PH, 20 HU 50/ml	30.9
Buffer	17.6

PH = purified α haemolysin; Ig = immunoglobulin preparation (see *Methods*).

* 100°C for 15 min.

DISCUSSION

The aims of this study were to purify *E. coli* α haemolysin and determine its cytotoxicity. These objectives are important for further definition of the role of α haemolysin in the virulence of *E. coli*.

A 19-fold increase in specific activity of the haemolysin was obtained by glycerol-gradient ultracentrifugation of concentrated culture filtrates of haemolytic *E. coli*. This is of the same order as the 23-fold purification reported by Zwadyk and Snyder (1971), but is much lower than the values reported by others (Rennie and Arbuthnott, 1974; Williams, 1979). This may be due to the fact that these workers grew the organisms in meat-infusion or nutrient-broth medium which, because of large amounts of protein present, results in a low initial specific activity and the appearance of greater purification. However the relative final specific activity of our PH, approximately 228 000 HU/50 mg, is comparable with the 10^6 HU/mg reported by Rennie and Arbuthnott (1974) and 109 000 HU/mg reported by Williams (1979).

Other workers (Short and Kurtz, 1971; Zwadyk and Snyder, 1971; Rennie and Arbuthnott, 1974; Williams, 1979) did not include a determination of endotoxin concentration or purity by immunological assays. In our study, purification of α haemolysin resulted in a 2176-fold reduction of the endotoxin content, and immunodiffusion analysis suggests the presence of only one antigen. Attempts to electrophorese α haemolysin preparations on conventional polyacrylamide gels were unsuccessful, even though the gel concentrations were varied to allow for entry of the molecule.

Fractionation of CH on glycerol gradients occasionally yielded a haemolytic curve with one or more shoulders, suggesting the presence of more than one species of haemolysin. This is in agreement with the findings of Short and Kurtz (1971) who reported the recovery of two peaks with haemolytic activity after fractionation of haemolytic filtrates on Sephadex G200, and Rennie and Arbuthnott (1974) who found that fractionation of α haemolysin on Sephadex G200, in conditions that reduced aggregation, resulted in two peaks of haemolytic activity. The protein profile of concentrated culture filtrates of our

non-haemolytic mutant did not contain a protein peak in the area where haemolytic activity was found in protein profiles of fractionated CH, suggesting that this protein peak is haemolysin.

Lipids were not detected in samples of PH. However, a significant amount of carbohydrate was detectable. This is in contrast to the findings of Rennie and Arbuthnott (1974) and Zwadyk and Snyder (1971) who did not find carbohydrates in haemolysin produced by *E. coli* grown in meat-infusion media. The possibility exists that the presence of this carbohydrate is due to endotoxin contamination. However, the small amount of endotoxin in PH as detected by the limulus test and the absence of heptoses and KDO, argue against this hypothesis. Whether this carbohydrate represents contamination or is actually part of the haemolysin molecule is unknown and will require more research. Unpublished data from our laboratory show that haemolytic activity of PH is destroyed by proteases, suggesting that protein is required for activity.

The high molecular weight of PH estimated by its exclusion from Sephadex G200 and its retention by a 300 000 molecular-weight filter is in agreement with the findings of Rennie and Arbuthnott (1974).

Little work has been done on the toxicity of purified *E. coli* α haemolysin for cells other than erythrocytes. Our study revealed that 3T3 cells in monolayers exposed to PH were killed and became detached, but this response was difficult to measure. In preliminary experiments with trypsinised washed-cell suspensions, our results were not reproducible and a proportional dose-response relationship could not be established. However, incubation of trypsinised and washed-cell suspensions in cell-culture medium for 2 h before the addition of PH resulted in a consistent dose-response curve, suggesting that the receptor sites on the cell membrane are destroyed or modified during the trypsinisation. Others have noted that trypsinised human embryonic intestinal cells are insensitive to staphylococcal enterotoxin B, but that the cells became sensitive again after further incubation (Schaeffer, Gabliks and Calitis, 1967).

The dose-response curve for 3T3 cells treated with PH was linear over a range of 0–10 HU50. Similar data were reported for the cytotoxic effects of other haemolysins (Snyder and Hamilton, 1963; Wiseman, 1968; Thelestam, Möllby and Wadström, 1973; Möllby, Thelestam and Wadström, 1974). In addition, the dose-response kinetics for 3T3-cell cytotoxicity were similar to those reported for erythrocyte lysis by Rennie, Freer, and Arbuthnott (1974). As little as 10 HU50 (23 ng of protein) of PH killed 90% or 4.3×10^5 3T3 cells and even 2 HU50 showed significant killing. By comparison, 10 HU of streptolysin S is the minimum amount that kills 10^5 McCoy cells, with as little as 0.5 HU reducing the viable cell counts to 25% of control values (Snyder and Hamilton, 1963).

The time kinetics for erythrocyte lysis (Rennie *et al.*, 1974) and for 3T3-cell killing by *E. coli* α haemolysin are linear and depend on haemolysin concentration. The kinetics of 3T3-cell cytotoxicity were consistent with the single-hit hypothesis for α haemolysin proposed by Short and Kurtz (1971) and Jorgensen, Hammer and Wu (1980).

Neutralisation of the cytotoxic and haemolytic activity of PH was achieved

by heat and by immunoglobulins from immune sera. In addition, cells treated with endotoxin did not show a loss of viability. Singer and Dutton (1979) have shown that it takes endotoxin at a concentration of at least 100 $\mu\text{g/ml}$, 10^6 -fold more than is present in PH samples, to cause cytotoxicity for mouse fibroblasts. Furthermore, filtrates from a non-haemolytic mutant were neither haemolytic nor cytotoxic. All these data suggest that the haemolytic and cytotoxic activities are caused by the same molecule.

We have shown that purified *E. coli* α haemolysin is cytotoxic and further work will be devoted to elucidating its mechanism of action and to defining its role in disease.

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