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Thiol-independent activity of a cholesterol-binding enterohemolysin produced by enteropathogenic *Escherichia coli*

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Abstract

Enterohemolysin produced by *Escherichia coli* associated with infant diarrhea showed characteristics similar to those of thiol-activated hemolysins produced by Gram-positive bacteria, including inactivation by cholesterol, lytic activity towards eukaryotic cells and thermoinstability. However, enterohemolysin activity was not inactivated by oxidation or by SH group-blocking agents (1 mM $HgCl_2$, 1 mM iodoacetic acid) and the hemolysin (100 μg/ml) was not lethal to mice, in contrast to the lethality of the thiol-activated hemolysin family to animals. Earlier reports showed that intravenous injection of partially purified streptolysin O preparations (0.2 μg) was rapidly lethal to mice. These results suggest that *E. coli* enterohemolysin is not a thiol-activated hemolysin, despite its ability to bind cholesterol, probably due to the absence of free thiol-group(s) that characterize the active form of the thiol-activated hemolysin molecule.

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Escherichia coli produces various types of hemolysins (1), the most extensively studied is α-hemolysin (2). A novel hemolysin produced by *E. coli* strains belonging to serogroups O26 and O111 and known as enterohemolysin (EHly) was first detected by Beutin et al. (3). In contrast to α-hemolysin, which is more frequent in *E. coli* strains causing extraintestinal infections in humans (2) and is associated with enteric disease (3,4), this EHly is not expressed by cultures grown without shaking, is not secreted into the culture medium by the bacteria, and can be detected only on blood-agar plates containing washed erythrocytes (3,4). *E. coli* enterohemolysin is structurally and genotypically unrelated to the α-hemolysin of this species, and is encoded by temperate phages. The gene structure of this hemolysin is still unknown. A 60-kDa outer membrane protein, present only in EHly-positive bacteria (5), may be the EHly itself, but no further information on the purification of this protein is available. In the present paper, we describe the purification of EHly and show that it shares several properties with thiol-activated hemolysins having the following general characteristics, in addition to being

inactivated by cholesterol: inactivation by oxidation and reactivation with reducing agents, lethality to mice, and lytic activity towards eukaryotic cells through the formation of pores in the cell membrane (6).

E. coli strain C3888 (O26: H^-; Stx^-), kindly provided by Lothar Beutin (Robert Koch Institute, BGA, Berlin, Germany), was used for EHly purification. The strain was maintained as frozen stock cultures at -70ºC in 20% glycerol plus trypticase soy broth (TSB; Difco, Detroit, MI, USA). *E. coli* strain C3888 was initially inoculated in 5 ml of TSB, followed by incubation for 18 h at 37ºC. One milliliter of the seed culture was then transferred to Erlenmeyer flasks containing 500 ml of fresh TSB with 12 µg/ml ethylenediamine hydroxyphenylacetic acid (EDDA) and incubated for 22 h at 37ºC, with constant shaking at 150 rpm (New Brunswick Scientific, Edison, NJ, USA). Cultures were centrifuged at 10,000 *g* for 15 min at 4ºC and the supernatant was discarded. The cell pellet was then suspended in 100 ml of 10 mM Tris-HCl buffer, pH 7.6, and disrupted by ultrasonication (10 cycles of 10 s each with a 50% energy pulse) in a cell disrupter (Vibra Cell, Sonics, Newtown, CT, USA). This was followed by centrifugation for 20 min at 12,000 *g* and the resulting supernatant was used as a source of crude EHly (3).

The supernatant was precipitated with ammonium sulfate to 45% saturation at 4ºC. After centrifugation, the supernatant was adjusted to 60% saturation at 4ºC. This second precipitate was collected by centrifugation as described above and dissolved in 20 ml of 10 mM Tris-HCl, pH 7.6. The solution was dialyzed against the same buffer at 4ºC for 18 h.

The resulting material (420 mg) was applied to a DEAE Sepharose fast flow column equilibrated with 10 mM Tris-HCl, pH 7.6 (4 cm x 34 cm; Pharmacia LKB Biotechnology, Buckinghamshire, England). Fractions (10 ml/tube) revealing the hemolytic activity were collected and concentrated to 2.5 ml by membrane ultrafiltration (PM-50 Diaflo, Amicon Corp., Lexington, MA, USA). The concentrated hemolysin (2.4 mg) was applied to a Superdex 200 column (1.6 cm x 90 cm; Pharmacia LKB) equilibrated with 10 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl. The material obtained by chromatography represented partially purified EHly and was used in the biological tests. As the final purification step, the material (0.23 mg) from the preceding step was applied to an HR 5/5 Mono-Q anion-exchange column (Pharmacia LKB) for fast protein liquid chromatography. The fractions with hemolytic activity were stored at -20ºC.

Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) was carried out using a slab gel system (7) in a BioRad Mini-Protean II apparatus (BioRad Laboratories, Hercules, CA, USA) with phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin as the standards and protein concentrations were determined by the method of Bradford using a BioRad Protein assay kit (BioRad Laboratories) with bovine serum albumin as the standard.

The hemolytic activity of partially purified EHly was assayed by incubating 100 µl of pure sample or dilutions (1:2, v/v) with an equal volume of 2% sheep erythrocytes washed three times with PBS, pH 7.2, for 1 h at 37ºC in round-bottom 96-well microtiter plates (8). One hemolytic unit was defined as the amount of hemolysin causing 50% hemolysis of 100 µl of a 2% suspension of sheep erythrocytes after 60 min at 37ºC.

The influence of cholesterol on hemolytic activity was tested as described by Geoffroy et al. (9). An alcoholic solution of cholesterol, 10 mg/ml, was diluted serially (twofold dilutions) on 96-well microtiter plates to a concentration of 50 µg/ml, after which the cholesterol was incubated for 60 min at 37ºC with aliquots of purified EHly followed by 18 h at 4ºC. The hemolytic activity was detected by incubating the above mix-

ture with a 1% suspension of erythrocytes for 60 min at 37ºC. Purified EHly was also incubated for 20 min at 37ºC with some SH group-blocking agents (1 mM $HgCl_2$, 1 mM iodoacetic acid), with H_2O_2 (30, 20 and 10 v) and with some reducing agents (1 mM dithiothreitol, 2 mM cysteine and 1 mM glutathione) (10). The residual hemolytic activity was determined by incubating the mixtures with sheep erythrocytes at 37ºC for 60 min.

The susceptibility of Vero, HeLa, CHO, 3T3, HEp-2 and Caco-2 cells to the cytotoxic or cytostatic activity of purified EHly was assayed as reported by Konowalchuk et al. (11). All cells were grown in Eagle's minimal essential medium (EMEM; Seromed, Biochrom KG, Berlin, Germany) with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), as described by MacLeod and Gyles (12). Monolayers were established on 36-well plastic plates, using 0.1 ml of cell culture (2 x 10^5 cells/ml). For Caco-2 cells, 24-well plates containing 1 ml of cell culture were used. The plates were incubated at 37ºC for 7 days in a 5% CO_2 atmosphere. Purified EHly (100 µg/ml) was serially diluted in two-fold dilutions with EMEM before adding to the wells. The plates were incubated at 37ºC in a 5% CO_2 atmosphere and observed at 5-h intervals for 72 h.

Mouse lethality was assayed in 6-week-old male Swiss and BALB/c mice (N = 5) inoculated intravenously and intraperitoneally with 100 µl of purified EHly (100 µg/ml). Five mice were also used in the control group. Deaths were recorded over a 7-day period. EHly was purified about 100-fold with a recovery of 0.06% and a molecular mass estimated at 60 kDa by SDS-PAGE (data not shown). The storage of purified material at -20ºC overnight also led to complete loss of hemolytic activity. The hemolytic activity of partially purified EHly was stable for as long as two months with freezing and was inactivated after heating at 60º and 100ºC for 5 min. There was no inactivation by SH group-blocking agents and H_2O_2 or alteration by reducing agents, but cholesterol concentrations of ≥2.5 mg/ml fully inhibited total hemolysis, whereas concentrations of 1.25-2.3 mg/ml produced only partial inhibition (Table 1). Partially purified EHly (100 µg/ml) obtained by Superdex 200 chromatography was cytotoxic to HEp-2, HeLa, Caco-2, Vero, 3T3 and CHO cells, and produced cell rounding and detachment of the cell layer after 24 h. The effect ob-

Table 1. Inhibition of the hemolytic activity of enterohemolysin by cholesterol.

Cholesterol (mg/ml)	Inhibition of hemolysis
10	Total
5	Total
2.5	Total
1.25	Partial
0.65	Partial
0.3	None[a]
0.15	None
0.05	None

[a]Total hemolysis.

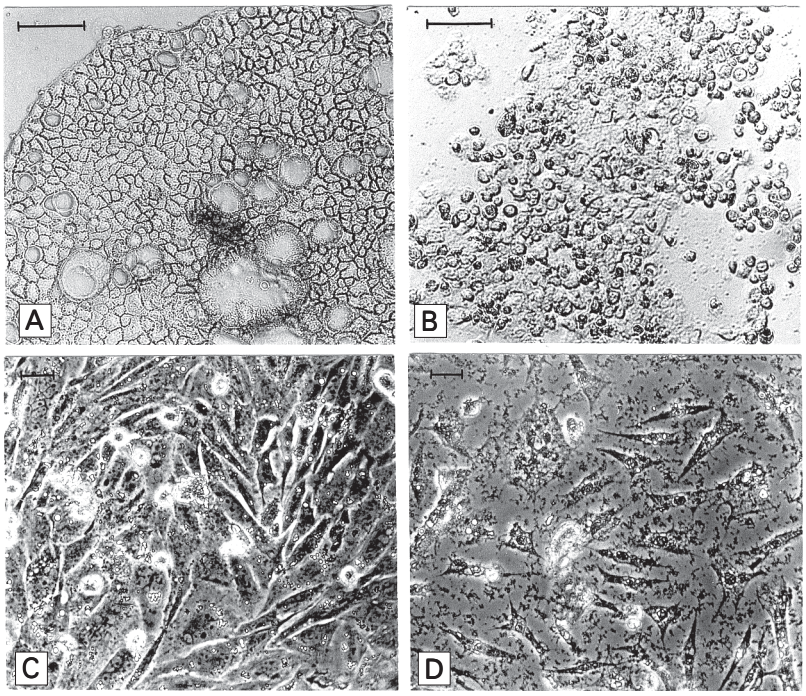

Figure 1. Effect of *Escherichia coli* enterohemolysin (EHly) on Caco-2 and CHO cells. *A*, Control Caco-2 cells; *B*, Caco-2 cells exposed to EHly; *C*, control CHO cells; *D*, CHO cells exposed to EHly. The bar corresponds to 20 µm.

served in Caco-2 and CHO cells is shown in Figure 1. EHly was not lethal to mice when injected intraperitoneally or intravenously.

In the present study, we purified the EHly produced by *E. coli* strain C3888. Attempts to increase the production of EHly by varying the culture medium, incubation time and iron chelant levels were ineffective. Since a slight increase in toxin production was obtained using TSB containing EDDA, a medium was used to provide material for purification. The specific activity and the relative activity increased approximately 100 times. EHly lost considerable activity within a few hours during the final steps of purification and different solutions and protease inhibitors in Tris-HCl buffer containing 0.1 M $CaCl_2$ and 0.3 M Na_2SO_4 were used to minimize this effect (13,14). Although some stability was obtained, the hemolytic activity of purified EHly could still not be maintained. This difficulty justifies the use of the Superdex fractions (partially purified EHly) in the biological tests. The purified material yielded a single protein band of approximately 60 kDa by SDS-PAGE (data not shown), a result consistent with that obtained by Beutin et al. (5), who described a membrane protein of 60 kDa associated with EHly. Stroeher et al. (15) found outer membrane proteins of 33-35 and 65 kDa associated with EHly for both the $EHly^+$ and $EHly^-$ strains, with the 65-kDa protein possibly being an aggregate or dimer of the 33- to 35-kDa protein. These results suggest that EHly could be a member of the thiol-activated hemolysin family, a prominent group of bacterial toxins of which streptolysin O is the prototype.

Concentrations of cholesterol higher than 2.5 mg/ml completely inhibited the hemolytic activity of EHly, while concentrations of 1.25-2.3 mg/ml only partially inhibited this activity (Table 1). These results suggest that the factor responsible for the inhibition of enterohemolytic activity in agar blood-containing non-washed erythrocytes may be cholesterol, which is known to inactive thiol-activated hemolysin. However, these hemolysins require small amounts of free cholesterol to inhibit their lytic activity and EHly requires a concentration of cholesterol 100 times higher to inhibit hemolytic activity *in vitro*. These results were similar to those described by Miyake et al. (16) for inhibition of the hemolytic activity of *Vibrio metschnikovii* hemolysin and suggest that the two proteins were not thiol-activated hemolysins.

The foregoing conclusion was supported by the observation that the lytic activity of EHly was not inactivated by H_2O_2 and SH group-blocking agents or enhanced by reducing agents. The results obtained in the assays with SH group reagents (thiols) and reducing agents differed in some features from the thiol-activated hemolysin family in which the lytic activity is enhanced by reducing agents but suppressed by oxidation (17). Thiol-activated hemolysin activity is inhibited by sulfhydryl-blocking agents and oxidation because of the existence of free thiol group(s) (reduced form), which are characteristic of the active form essential for the lytic activity of thiol-activated hemolysin. These toxins lose their activity through the oxidation of thiol groups to disulfide bonds (6,17). Our results suggest that EHly has no free thiol group(s) to form the active site of the molecule, as in *Vibrio vulnificus* hemolysin (10).

EHly was cytotoxic to HEp-2, HeLa, Vero, 3T3, Caco-2 and CHO cells, with morphological changes including cell rounding and detachment of the cell layer, as described for verotoxin by Konowalchuk et al. (11). These results differ from those reported by Beutin et al. (3,4), who found no cytotoxic action of EHly on Vero, HeLa and HEp-2 cells. Beutin (1) reported that EHly affected human cultured cells, although the cell type was not specified. EHly was not lethal to mice, in contrast to the lethality of the thiol-activated hemolysin family to animals (17). The concentration of EHly used (100 μg/ml) was not lethal to mice, whereas

intravenous injection of partially purified streptolysin O preparations killed mice, rats, rabbits, guinea pigs and cats (18). The average LD_{50} for streptolysin O given *iv* is 0.2, 6 and 2 μg in the mouse, guinea pig and rabbit, respectively.

Since the first report of EHly in *E. coli* strains isolated from children with diarrhea (3), EHly has been investigated intensively in enterohemorrhagic *E. coli* strains that produce verotoxin and have caused outbreaks of diarrhea, especially in Germany and the United States (19,20). The biochemical and structural aspects of EHly have not yet been investigated as thoroughly as those of *E. coli* α-hemolysin. Further studies with EHly are necessary in order to establish the importance of this toxin as a virulence factor and its possible relation to other proteins produced by *E. coli.*

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