POTENT LEUKOCIDAL ACTION OF *ESCHERICHIA COLI* HEMOLYSIN MEDIATED BY PERMEABILIZATION OF TARGET CELL MEMBRANES

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Approximately 50% of *Escherichia coli* strains causing extra-intestinal infections in humans elaborate a cytolysin, designated *E. coli* hemolysin (ECH)[1], which is responsible for the characteristic zones of β-hemolysis surrounding bacterial colonies on blood agar (1–6). Experimental support for a role of this cytolysin as a virulence factor derives from numerous studies with isogenic bacterial strains conducted in animal models (7–10). A considerable amount of molecular genetic data are now available on the toxin (10–15). The primary sequence of the 107,000-dalton protein is known (16), and sequence homologies have been detected with three other cytolysins, i.e., the cytolysin of *Pasteurella hemolytica* (17, 18) and the hemolysins of *Proteus* and *Morganella* species (19, 20).

In contrast to the large amount of molecular genetical data available on ECH, information on the mechanism of its cytolytic action and on its biological effects in a physiological environment has been scarce. Following earlier reports that together had suggested a membrane-perturbating effect of ECH on erythrocytes (21–24), we recently showed that ECH forms discrete hydrophilic transmembrane pores of ~2 nm effective diameter both in erythrocyte membranes (25) and in planar lipid bilayers (26). These pores are probably generated by the insertion of toxin monomers into the bilayer; in this respect, they differ from oligomerizing pore-formers including C5b-9 complement complexes (27), lymphocytolysins (28), and cytolysins of Gram-positive organisms (29–33).

The recognition that a bacterial cytolysin generates transmembrane pores does not in itself explain its pathogenetic role in bacterial infections. Many pore-formers including ECH apparently do not bind to cellular receptors, and they disrupt the integrity of protein-free liposomes and planar lipid bilayers (33). Poorly understood cell surface factors may influence the binding of such toxins to cell targets; as a consequence, various cell types may differ widely and unpredictably in their suscepti-

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[1] *Abbreviations used in this paper:* BCECF-AM, bis-carboxyethyl-carboxyfluorescein pentaacetoxymethylester; ECH, *E. coli* hemolysin; HDL, high density lipoprotein; HSA, human serum albumin; LDL, low density lipoprotein; MPO, myeloperoxidase; NHS, normal human serum; PMN, polymorphonuclear neutrophils.

bility toward a given toxin (33, 34). Additionally, the cytolytic capacity of an exotoxin may be subject to considerable inhibition or modification by human plasma proteins (e.g., naturally occurring antibodies). For these reasons, the necessity arises to identify those cells that represent preferred targets for toxin attack under physiological conditions. The simplest model that lends itself to such studies is the analysis of toxin interactions with blood cells in the presence of human plasma or serum.

Based on this reasoning, we recently conducted a study on the interaction of *Staphylococcus aureus* α-toxin with cells in human blood. Surprisingly, we found that α-toxin attacks human platelets in a selective fashion, thus promoting blood coagulation (35). Presumably due to the very rapid binding of the toxin to platelets, this process was not prevented by otherwise effective plasma inactivators of α-toxin.

In the present study, we sought to determine whether ECH would similarly attack a particular cell type in human blood. We found that several plasma proteins effectively protected erythrocytes and platelets against the action of this cytolysin. In contrast, protection of polymorphonuclear leukocytes by the same proteins was quite ineffective. As a result, ECH mounted a selective attack on granulocytes in human blood. We will present quantitative data on this phenomenon that collectively identify ECH as the most potent leukocidin known to date.

Materials and Methods

Cells. Granulocytes were isolated from heparinized blood following conventional procedures. In brief, 1 vol of gummi arabicum (10% wt/vol in isotonic phosphate buffer, pH 7.4) was added to 4 vol (20–40 ml) whole blood (20 U heparin/ml), and cells were allowed to sediment in tilted plastic centrifugation tubes (10 ml) for 60 min at 37°C. The erythrocyte-depleted supernatants were applied in 4-ml aliquots to 4-ml Ficoll-Hypaque gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged for 45 min at 400 *g*, 20°C. The cell pellets containing polymorphonuclear leukocytes (PMN) and contaminating erythrocytes were resuspended in 0.2–0.3 ml PBS, and the erythrocytes were lysed by addition of ice-cold distilled water (3 ml) under vigorous agitation for 30 s. Re-isotonization was achieved through addition of 1 ml 0.6 M KCL. The PMN were resedimented (100 *g*, 10 min) and washed once with PBS. They were resuspended to 1–5 × 10^7 cells/ml in PBS and kept on ice before use. The cell preparations contained <3% contaminating lymphocytes, and <4% nonviable cells as determined by staining with trypan blue.

Human erythrocytes were isolated from citrated blood and resuspended in PBS.

Plasma Proteins. Human albumin was obtained as a 5% solution from Plasmapharm Sera GmbH, Leimen, FRG. Plasma HDL and LDL were isolated from pooled human serum by centrifugation in KBr density gradients following published procedures (36). Protein concentrations were determined by the Folin method in the presence of 0.1% SDS. Concentrations of the lipoproteins were adjusted to levels contained in pools of normal human sera (LDL: ~1 mg protein/ml; HDL: ~2.5 mg protein/ml) by quantitative immunoelectrophoresis using specific antisera from Behringwerke, Marburg, FRG. Pooled human IgG preparations (50 mg/ml) were from Sandoz (Sandoglobulin^R; Basel, Switzerland).

E. coli Hemolysin. The preparation of ECH followed a procedure that has been described previously (25). The toxin preparations exhibiting the 107,000 mol wt ECH protein band (Fig. 1) were immediately shock frozen in liquid nitrogen and stored at −70°C before use. Each thawed vial was retitrated immediately before experiments were conducted, and all experiments were conducted within 1 h thereafter. The toxin was held on ice throughout the duration of experiments. Adherence to this protocol was essential since ECH lost activity even when kept at 0°C within a few hours. The applied doses of ECH will be referred to in hemolytic units (HU)/ml. By definition, 1 HU/ml is the toxin concentration evoking 60% lysis of a suspension containing 5 × 10^8 erythrocytes/ml (in PBS). As to be reported elsewhere, the protein content of different toxin preparations with the same hemolytic activity

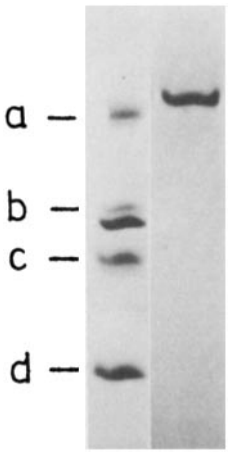

FIGURE 1. SDS-PAGE of an *E. coli* hemolysin preparation containing 100 μg/ml protein and exhibiting a hemolytic activity of 1,250 AU/ml. 100 μl (10 μg protein) were applied to the 10% gel (*right lane*). Coomassie Brilliant Blue staining revealed a single protein band of M_r 110,000. (*Left lane*) Marker proteins (Serva): (*a*) phosphorylase b (94,000); (*b*) BSA (67,000); (*c*) OVA (43,000); (*d*) carbonic anhydrase (30,000).

varies considerably because of the inevitable presence of inactive toxin. In the present study, toxin preparations were used in which 1 HU/ml corresponded to 50–100 ng protein/ml. Control experiments were also conducted with toxin preparations that had been inactivated by incubation at 37°C for 1–2 h; this resulted in spontaneous loss of 90–100% of hemolytic activity.

Hemolytic Titrations. Titrations were conducted such that direct comparisons could be made between hemolysis curves and granulocyte permeabilization data. To 100 μl of a given solute (see Fig. 2) we added 100 μl of human erythrocytes in PBS (5×10^7 cells/ml) in Eppendorf tubes. 20 μl of a serially diluted ECH solution (PBS) were then added and hemolysis read after 60 min at 37°C by measurement of hemoglobin absorbance at 412 nm in the supernatant.

Measurements of Platelet Aggregation. Preparation of platelet-rich plasma and assays for platelet aggregation and ATP-release from these cells were performed as described (35).

Measurements of ATP Release from PMN. Continuous measurements of ATP-release from PMN were performed with the firefly assay (37) using a Lumi-Aggro-Meter (model 400; Chrono-Log Corp., Coulter Electronics, Krefeld, FRG; references 38, 39). The apparatus was equipped with an Omni-Scribe II recorder (Coulter Electronics) for continuous recordings of optical measurements (38, 39). In these experiments, 150 μl of PBS, or of a solution mix containing physiological concentrations of albumin (40 mg/ml), LDL (1 mg protein/ml), HDL (2.5 mg protein/ml), and pooled IgG (15 mg/ml), or of pooled normal human serum were pre-warmed to 37°C. 20–30 μl of PMN cell suspensions ($2–5 \times 10^7$ cells/ml) were added together with 170 μl of firefly reagent (ATP-bioluminiscence CLS from Boehringer, Mannheim, FRG), and toxin applied at the given doses 60 s after incubation of the total mix at 37°C.

In neutralization experiments, ECH was first pre-incubated with normal human serum (NHS) or a solution mix for 20 min at 0°C. These solutions were then added to PMN and firefly reagent, and ATP was measured as described.

Detection of Influx of Propidium Iodide into Toxin-treated PMN. These studies were conducted with the use of a FACSCAN (Becton Dickinson & Co., Heidelberg, FRG) flow-cytometer with computer-assisted evaluation of data (Consort 30 software). PMN were suspended at 2×10^6 cells/ml in PBS in the presence of 4 μg/ml propidium iodide (Sigma Chemical Co., Münich, FRG) at 37°C. Toxin was added at varying concentrations, and the ensuing influx of propidium iodide was detected after 10 min by measurements of cell fluorescence. This assay is based on the fact that intact cell membranes are virtually impermeable to propidium iodide, and viable cells therefore exhibit low background fluorescence. In contrast, permeabilized membranes allow propidium iodide to diffuse to the nucleus, where firm binding to nucleic acids occurs with concomitant appearance of bright cell fluorescence (40, 41).

Measurement of Entrapment of BCECF. These assays were based on the principle that biscarboxyethyl-carboxyfluorescein pentaacetoxymethylester (BCECF-AM; Calbiochem-Behring

Corp., La Jolla, CA), a water-soluble esterified derivative of carboxyfluorescein, spontaneously diffuses through biological membranes and is cleaved by cytoplasmic esterases to yield the fluorescent product BCECF that diffuses only slowly back to the extracellular space (42–44). The experiments were conducted in order to assess whether repair of membrane pores could occur after attack of PMN by ECH. Cells were suspended in PBS and treated with 1–5 HU/ml ECH for 10 min at 37°C. 1 vol of NHS was then added and incubation continued for another 30 min. Aliquots were withdrawn at the given time intervals, given 20 μM BCECF-AM, and cells were pelleted after an additional 15 min of incubation at 37°C. The BCECF that was trapped in the cells was released by suspending the cell pellets in 1 ml of distilled water and assessed fluorimetrically using a Hitachi-Perkin-Elmer Fluorescence Spectrophotometer 204 (Tokyo, Japan). Cells not treated with ECH were handled in the same way, and fluorescence measurements of these control lysates were taken as 100% values.

Release of Granule Constituents from PMN. Myeloperoxidase (MPO) and elastase were taken as markers for primary lysosomes (45, 46). Vitamin B 12 binding protein was considered a marker for secondary lysosomes, and glucosaminidase was chosen as a marker of tertiary granules (47). Measurements of MPO, vitamin B 12 binding protein, and glucosaminidase release were performed exclusively in protein-free buffer (HBSS or PBS). Measurements of elastase release were conducted both with cells suspended in protein-free buffer and in whole blood. In the former experiments, cell suspensions were treated with ECH for 5–10 min at 37°C, and autologous serum was subsequently added to the samples. The released elastase was measured immunologically as a complex of elastase and serum proteinase inactivator using a commercial ELISA kit from Merck Laboratories, Darmstadt, FRG (PMN-Elastase Immunoassay, No. 15689). MPO, vitamin B 12 binding protein, and glucosaminidase were quantified according to described procedures (47). The maximal release (100%) values were those obtained by sonication of cell aliquots (15 s, 50 W).

Measurements of Superoxide Generation by PMN. These assays were performed according to a standard procedure (48).

Quantification of ECH Bound to PMN. 0.5-ml aliquots of cell suspensions containing 2×10^7 PMN were treated with 2.5–20 HU/ml ECH for 4 min at 37°C. Cells were then pelleted, washed thrice in PBS, and solubilized by suspension in 100 μl of 130 mM *n*-octyl β-D-glucopyranoside (Sigma Chemical Co., Münich, FRG; henceforth referred to as octylglucoside). ELISA measurements for ECH were performed using a sandwich technique in which monoclonal antitoxin antibodies were used to capture the antigen, and polyclonal rabbit IgG antibodies were used as second antibodies. The principle and performance of this assay is to be described in detail elsewhere (Eberspächer, B., F. Hugo, and S. Bhakdi, manuscript in preparation).

Assays for Phagocytic Killing by PMN. The phagocytic killing capacity of PMN was assayed by a simple conventional procedure by measuring the percentage of survival of *S. aureus* after a 60-min incubation with granulocytes in the presence of 20% NHS (49). PMN (10^6 cells in 250 μl PBS) were incubated with 100 μl NHS, 100 μl (10^7 cells) *S. aureus* Wood 46 and 50 μl of ECH at various final concentrations. The *S. aureus* cells were obtained from an overnight culture in tryptic soy broth (Difco Laboratories, Detroit, MI). Bacteria were washed twice in PBS, suspended in this buffer, and briefly sonicated before use. Parallel incubations of *S. aureus* in 20% NHS plus ECH (2.5 and 50 HU/ml) without PMN served as 100% growth controls. All samples were prepared in 1 ml Eppendorf tubes and rotated end-on-end for 60 min at 37°C. Thereafter, the cell suspensions were briefly sonicated (10 s, 50 W; Branson Sonifier B-12), appropriately diluted in ice-cold saline, and plated out on Cled-agar plates (Difco Laboratories) for determination of colony-forming units. Alternatively, PMN were suspended in PBS and pre-incubated with ECH for 5 min at 37°C, after which NHS (20% final concentration) and *S. aureus* cell suspensions were added, and phagocytic killing was similarly determined after 60 min.

Results

Human Plasma Proteins Inhibit Lysis of Erythrocytes by ECH. The ECH preparations used in our study exhibited high hemolytic activity of 2,500–10,000 HU/ml. As de-

termined by the ELISA, 1 HU/ml, defined using a 10% erythrocyte suspension (10^9 cells/ml), corresponded to 50–100 ng/ml of toxin (Eberspächer, B., F. Hugo, and S. Bhakdi, manuscript submitted for publication). When conducted with dilute cell suspensions (2.5×10^7 cells/ml final concentration in the assay), titration curves shown in Fig. 2 were obtained. In PBS, 60% hemolysis was then registered at toxin concentrations of ~0.25 HU/ml.

It was found that human serum albumin (HSA), high density lipoprotein (HDL), low density lipoprotein (LDL), and IgG antibodies reduced the hemolytic capacity of ECH. The addition of these plasma proteins to PBS at physiologic concentrations resulted in the depicted shifts in titration curves. When the four plasma components were present together (solution mix in Fig. 2), 60% hemolysis was elicited only at toxin levels of >100 HU/ml. In the presence of whole serum, protection of erythrocytes was even more pronounced, and 60% hemolysis could not be induced at the highest toxin concentration tested (500 HU/ml). In all these experiments, toxin was applied directly to the cell suspensions without preincubation with the inactivating proteins. Since ECH titers of >50 HU/ml are rarely reached by toxin-producing *E. coli* strains under optimal conditions in broth culture, we conclude that in vivo lysis of human erythrocytes by ECH probably never occurs in whole blood or protein-rich body fluids and exudate.

Lack of Effect of ECH on Platelets in Platelet-rich Plasma (PRP). ECH was added to PRP and the behavior of platelets monitored by measurements of aggregation and ATP release. It was found that ECH at doses of 100 HU/ml entirely failed to provoke platelet aggregation or effect ATP release from platelets in PRP.

ECH Provokes Rapid ATP Release from PMN. The release of ATP from PMN evoked by ECH was first monitored in protein-free buffer (Fig. 3). These assays generated information on the susceptibility of these cells towards the toxin and on the kinetics of membrane permeabilization. Human PMN responded to attack by ECH by rapid release of cellular ATP which commenced within seconds after application of moderate toxin doses (5–20 HU/ml). As doses decreased, lag phases increased to a maximum of ~90 s (Fig. 3 *A*).

To assess the degree of protection afforded by serum protein components, experiments were conducted using either a solution mix (containing HDL, LDL, HSA, and IgG) or NHS (compare with Fig. 2). In the presence of the solution mix, ECH still evoked ATP release from PMN at concentrations that were far below the hemolytic threshold. As shown in Fig. 3 *B*, 5 HU/ml ECH caused rapid and maximal ATP release, and a toxin effect was still observed at concentrations below 1 HU/ml

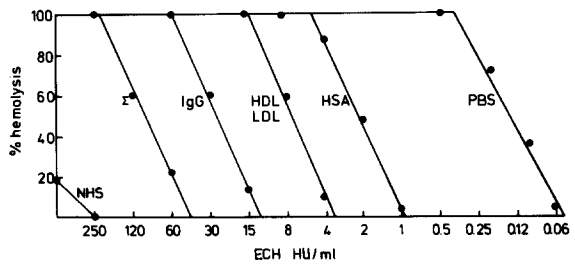

FIGURE 2. Hemolytic titrations of ECH using human erythrocytes suspended in protein-free buffer (PBS); in PBS supplemented with physiological concentrations of HSA, HDL, LDL, IgG; or in a solutions mix of these four protein components (●); or in 50% autologous serum (NHS). Note the effective protection of erythrocytes by these plasma protein components.

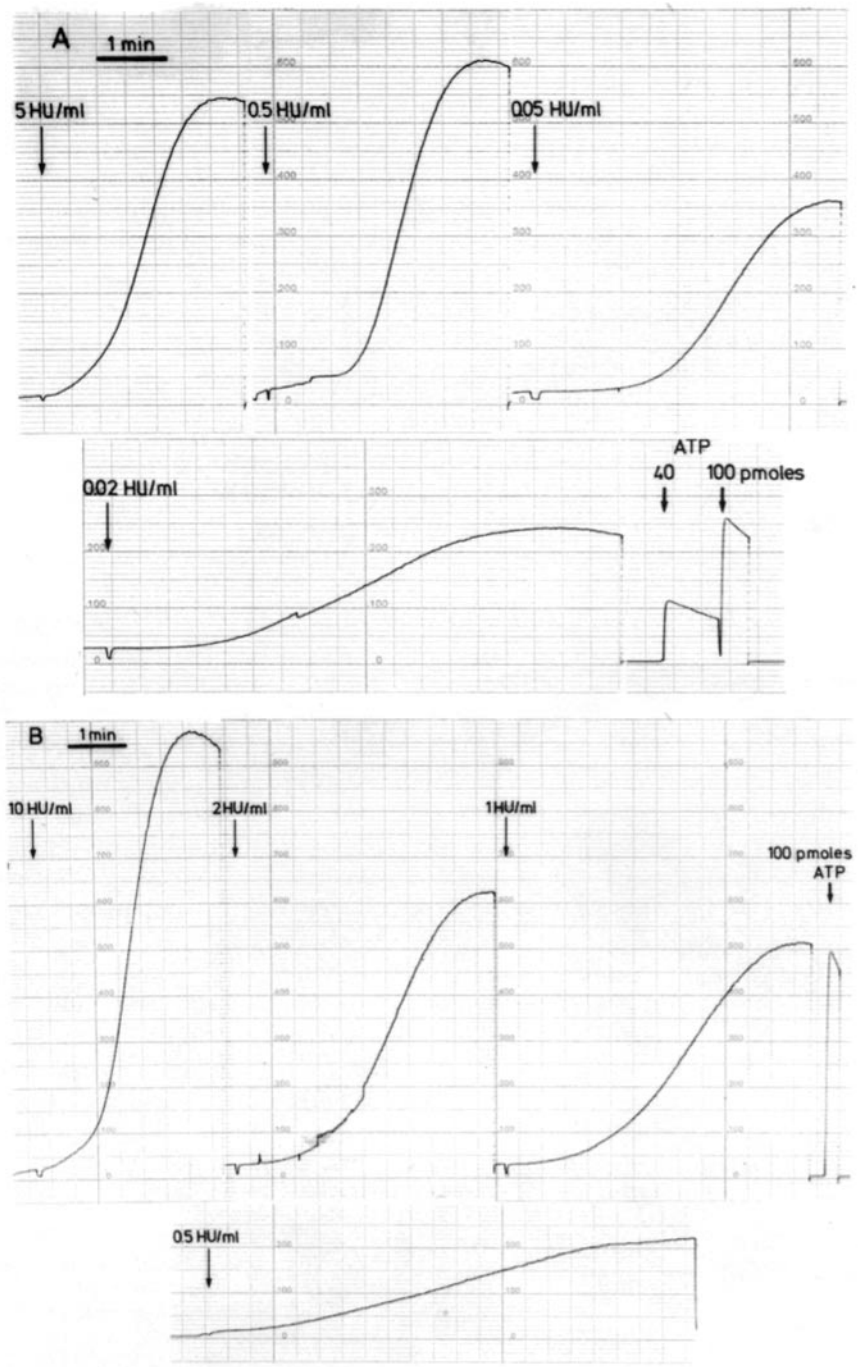

FIGURE 3. Release of ATP from PMN (10^6 cells in 400 μl) suspended in PBS (*A*) or in a solution mix containing physiological concentrations of HSA, HDL, LDL, and IgG (*B*) induced by ECH. ATP was measured continuously using the firefly assay. The final concentrations of ECH applied are given in HU/ml. The assays were calibrated with ATP as depicted. Note the relatively poor protection of PMN against toxin action afforded by the plasma protein components.

(≤50 ng/ml). Somewhat higher toxin doses were required to elicit the same effects when PMN were suspended in normal human serum (NHS). In this case, ATP release was initiated at toxin doses around 5 HU/ml.

Preincubation with Plasma Proteins Leads to Neutralization of ECH. ECH was effectively inactivated by NHS or the protein mix if incubated on ice with the respective inactivators before gaining contact with PMN. In the experiment of Fig. 4, 10 HU/ml toxin were completely inactivated by preincubation with the protein solution mix, and no ATP release ensued if PMN were added thereafter. However, subsequent application of only 1 HU/ml toxin to the same cell suspension resulted in release of ATP. Fig. 4 *B* depicts ATP release evoked by 100 HU/ml toxin in NHS; note the extremely rapid leakage of ATP commencing seconds after application of this toxin dose. If 50 HU/ml toxin were preincubated with NHS for 20 min on ice, however,

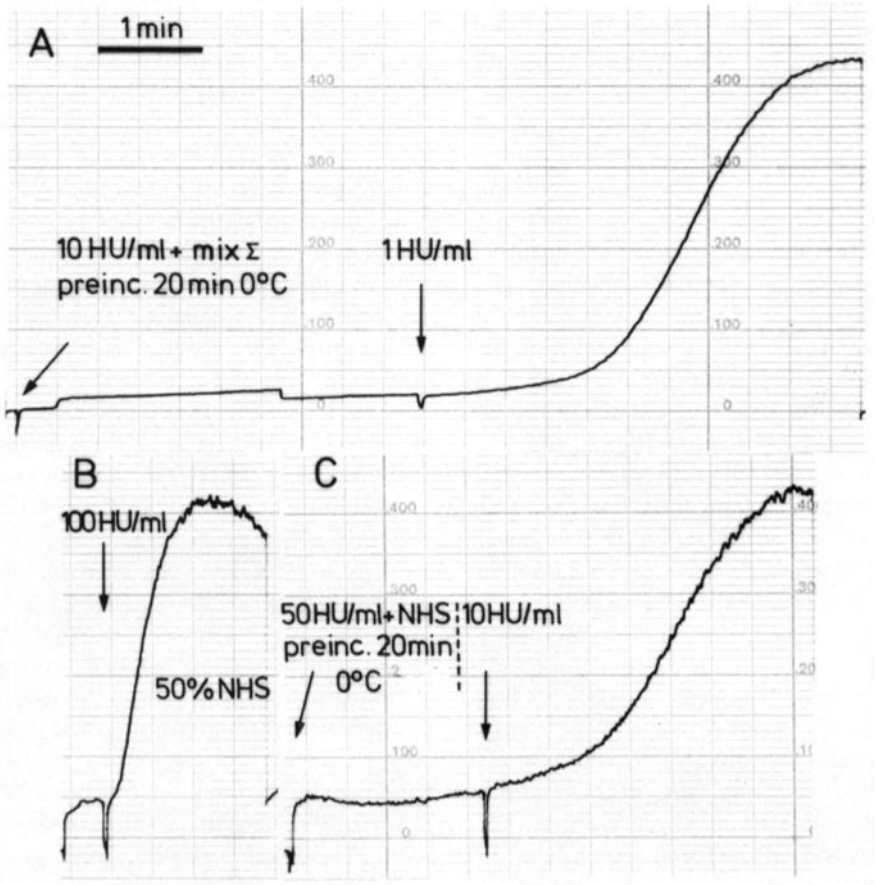

FIGURE 4. Preincubation of ECH with plasma inactivators results in toxin inactivation. (*A*) ECH (10 HU/ml) was preincubated with the protein solution mix of Fig. 3 for 20 min at 0°C. Application of this solution to PMN failed to elicit ATP efflux. However, subsequent application of only 1 HU/ml ECH to these cells led to cell membrane permeabilization. (*B*) PMN suspended in 50% NHS responded to 100 HU/ml ECH with immediate release of ATP. (*C*) Preincubation of 50 HU/ml ECH with 50% NHS (20 min, 0°C) led to toxin inactivation. Again, subsequent application of 10 HU/ml ECH to the cells resulted in efflux of ATP.

cells added subsequently remained intact (Fig. 4 *C*). Again, application of another 10 HU/ml toxin to the cell suspension caused ATP efflux. These results underline the fact that seemingly effective plasma inactivators may fail to protect cells against attack by a cytolysin under physiological conditions, i.e., when the toxin is simultaneously confronted with both inactivator and its cell target.

Influx of Propidium Iodide into Toxin-treated Cells. An alternative means to document membrane permeabilization was to assess the degree of cellular fluorescence deriving from transmembrane diffusion and binding of propidium iodide to nucleic acids. Cells were treated with ECH in the presence of propidium iodide. After 10 min, automated analyses were conducted in a flow cytometer, and typical results are depicted in Fig. 5. Toxin doses around 0.02–0.05 HU/ml evoked uptake of the dye, as evident from an increase in fluorescence intensity of the cells. Toxin doses of 0.2 HU/ml led to permeabilization of all cells and dye uptake within the same time period (Fig. 5 *A*). Concomitantly, enlargement of the cells indicative of a swelling reaction was noted, as evident from an increase in cell volume (forward scatter, Fig. 5 *B*).

Inability of PMN to Repair ECH Lesions. Two types of experiments were performed in order to test whether PMN would be able to repair permeabilized membrane areas. The first experiments were conducted with cells suspended in PBS without serum proteins, and utilized measurements of propidium iodide influx as a parameter for membrane integrity. Cells were treated with similar doses of toxin shown in Fig. 5 in the absence of propidium iodide. After 15 min, the marker was added and dye uptake was assessed. Control experiments showed that ECH at the low concentrations used spontaneously lost its hemolytic and membrane-permeabilizing properties within 10–15 min at 37°C. Therefore, any permeability defects detectable after 15 min must have been generated within the first 10 min of incubation with the toxin. In none of these experiments could a reduction in dye uptake be detected, i.e., toxin-treated cells remained equally permeable to propidium iodide 15–20 min after toxin attack had been initiated.

The second set of experiments exploited the use of BCECF-AM, an acetylated carboxyfluorescein derivative that diffuses across intact membranes but becomes trapped intracellularly after its cleavage by cytoplasmic esterases. The amount of trapped marker can be assessed fluorimetrically after lysis of the cells. Control intact cells (incubated with PBS) were suspended in serum and BCECF-AM added 30 min thereafter. After a 20-min incubation with BCECF-AM at 37°C, the cells were pelleted, washed once in PBS, and trapped dye was released by hypotonic cell lysis in water. The measured fluorescence in this lysate was taken as the 100% value. In parallel, cells were treated with 1 or 5 HU/ml ECH for 10 min in buffer, and then suspended in one volume of NHS. No residual membrane-permeabilizing toxin activity could be detected in the samples after addition of the serum. At the depicted times (Fig. 6), aliquots were removed, given BCECF-AM for 20 min, and the amount of trapped dye was subsequently measured. Again, it was found that the cells remained unable to retain fluorescent dye despite incubations in serum for periods up to 30 minutes (Fig. 6).

Degranulation of PMN Induced by ECH. Leukocyte elastase and MPO present in primary azurophilic granules were released from cells attacked by ECH. The release of elastase was measured in serum-free buffer or in whole citrated blood (Fig. 7 *A*). Elastase release from cells in buffer commenced at toxin concentrations of

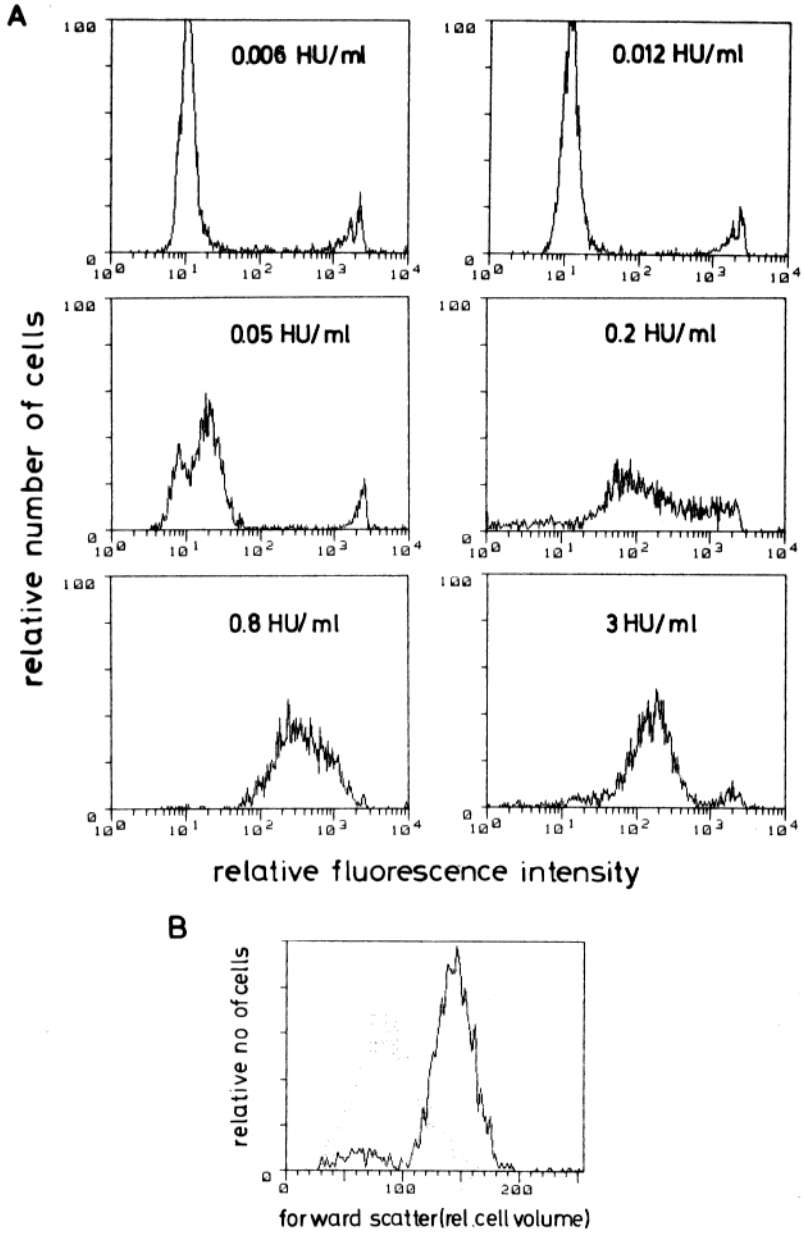

FIGURE 5. Flow cytometric analysis of propidium iodide influx into PMN treated with various concentrations of ECH. Excitation with a 488-nm argon laser, fluorescence measured at 585 nm wavelength. 2,000 cells were counted in each experiment. Histograms revealed a dose-dependent increase in fluorescence intensity (*A*), and swelling of ECH-treated PMN as measured by forward scatter (*B*). Dotted line in Fig. 5 *B* represents size distribution of cells treated with 0.006 HU/ml ECH; an indistinguishable curve was found with control, non-toxin-treated cells. Solid line: size distribution of cells treated with 3 HU/ml ECH.

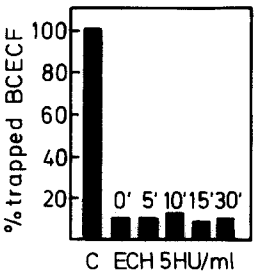

FIGURE 6. Inability of PMN to trap BCECF after treatment with ECH. PMN were permeabilized with 5 HU/ml ECH for 5 min at 37°C and then suspended in 50% NHS. Control cells (C) received no toxin. After 0, 5, 10, 15, and 30 min at 37°C, aliquots of the toxin-treated cells received 20 μM BCECF-AM for 20 min, 37°C, and the amount of trapped BCECF was subsequently measured. Control cells received BCECF-AM after a 30-min incubation in serum, and the fluorescence measured in the lysates of these cells was expressed as 100%. Toxin-treated cells did not regain their ability to entrap the fluorescent marker. The same results were obtained with cells treated with 1 HU/ml ECH.

~1 HU/ml (50–100 ng/ml) and approached maximal values at ~5 HU/ml. In whole blood, release commenced at ~10 HU/ml and was maximal at 200–500 HU/ml ECH. These results indicate that ECH induces degranulation of primary granules; however, the process is initiated at toxin levels above those that are required to promote ATP release or influx of propidium iodide.

Fig. 7 *B* summarizes the results of further assays for granule constituents released by toxin-treated PMN in protein-free solution. Release of MPO, another component of primary granules, also commenced predominantly at toxin levels ~1 HU/ml. Additionally, small amounts of this marker were liberated at toxin concentrations in the range of 0.1–1 HU/ml. In some contrast to the above findings, vitamin B12-binding protein, a constituent of secondary granules, was released at lower toxin concentrations. The dose-response relationship here closely resembled that of ATP release (compare with Fig. 3). Release of glucosaminidase, regarded as a component of tertiary granules, exhibited a dose-response behavior similar to elastase and MPO

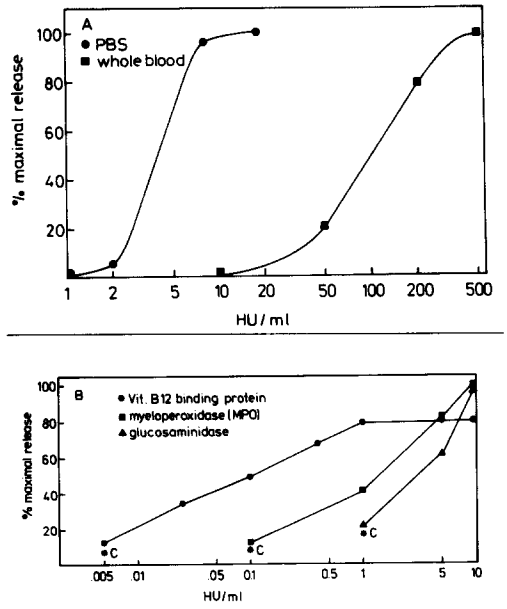

FIGURE 7. Release of granule constituents from PMN induced by ECH. (*A*) Release of elastase from cells suspended in PBS or in whole blood. (*B*) Release of other granule constituents from cells suspended in PBS. Asterisks denote values measured in the respective, non-toxin-treated controls (*C*). Results are representative of three similar experiments performed on separate days.

(Fig. 7 *B*). Collectively, these data indicate that degranulation of secondary granules occurs at similar toxin doses that evoke ATP leakage and propidium iodide influx, whereas the release of primary and tertiary granule constituents requires attack by higher toxin doses.

Superoxide Generation by Toxin-treated PMN. Toxin doses between 0.5 and 20 HU/ml were found to induce only slight increases in superoxide anion generation, in the order of 5% above basal levels.

Antiphagocytic Effects of ECH. PMN were suspended in 20% NHS and incubated with *S. aureus* in the presence or absence of ECH. Phagocytic killing of staphylococci was assessed after a 60 min incubation by colony counting. These experiments demonstrated that low doses of ECH depressed phagocytic killing by PMN. Whereas 15–20% bacterial survival was found in non-toxin-treated controls, survival rates increased already at ECH doses of ~1 HU/ml, which corresponded to the dose required to initiate ATP release in 20% NHS. Total survival of bacteria was noted at toxin levels of 10–25 HU/ml (Fig. 8 *A*).

If cells were preincubated with toxin for 5 min in the absence of serum, marked antiphagocytic activity was noted already at toxin doses around 0.02 HU/ml (Fig. 8 *B*). These results again correlated very well with the observations on ATP release from PMN in protein free solution, and indicated that the process of membrane permeabilization and ATP loss was directly paralleled by a loss of phagocytic killing capacity. It is noted that phagocytosis assays in serum were conducted over a period of 60 min. The antiphagocytic effects of relatively low toxin doses thus further indicated that permeabilized cells were unable to recuperate from toxin attack.

Quantitation of Toxin Binding to PMN. The amounts of cell-bound toxin that elicited biological effects were below the detection limit of the available assay system, and estimates could be made only by extrapolation. PMN (2×10^7 cells in 0.5 ml PBS) were treated with 2.5–20 HU/ml of toxin, and cell-bound toxin was quantified by ELISA after 4 min incubation at 37°C. This brief incubation was chosen because pilot experiments indicated that cell-bound toxin was rapidly degraded, possibly by elastase released from PMN, and could later not be detected immunologically. Fig. 9 depicts the results of these determinations. Assuming a linear correlation between the amount of toxin offered and the number of cell-bound molecules at lower toxin concentrations (dotted line, Fig. 9), we estimated that treatment of 2×10^7 cells

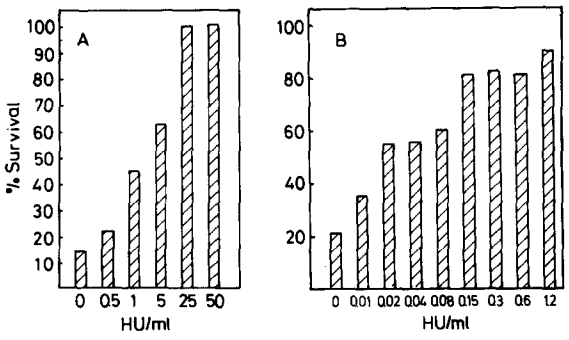

FIGURE 8. Inhibition of phagocytic killing of *S. aureus* by ECH. (*A*) PMN suspended in 20% NHS were incubated with *S. aureus* in the presence of the depicted final doses of ECH; data represent mean values obtained from four similar experiments. (*B*) PMN were suspended in PBS and treated with ECH at the depicted doses for 5 min, 37°C, after which 20% serum and *S. aureus* were added; data represent mean values obtained in three similar experiments.

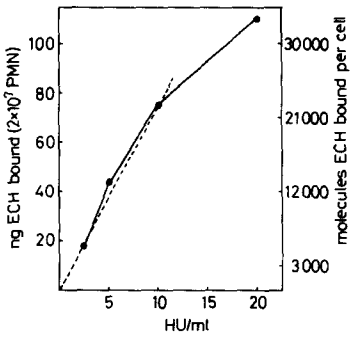

FIGURE 9. Quantitation of ECH bound to PMN. Cells (2×10^7/ml) were suspended in PBS and treated with the depicted doses of ECH for 5 min at 37°C. Bound toxin was quantified by ELISA. The dotted line shows the extrapolation of results to low toxin doses. Data are mean values from three similar experiments.

with 0.2 HU of toxin in a 1-ml aliquot should result in average binding of ~300 molecules ECH/cell (Fig. 9). This corresponded approximately to the toxin dose that was previously found to initiate ATP release (compare with Fig. 2 *A*: 0.02 HU/ml ECH evokes ATP release from 2×10^6 PMN/ml).

Leukocidal Effects of ECH Are Dependent on its Pore-forming Capacity. Toxin preparations rapidly lost their capacity to lyse erythrocytes and form pores in planar lipid bilayers upon incubation at 37°C for 1–2 h. This loss of pore-forming capacity was paralleled by a loss in the capacity of the toxin to evoke all of the above described processes in PMN.

Discussion

Previous investigations have demonstrated a cytotoxic effect of ECH on isolated PMN (50–53), but the primary cause of cell damage has not been defined, nor are quantitative data on this phenomenon available. The present study addresses these questions and probes the possible biological relevance of the process through the construction of experiments that reflect in vivo situations. The collective results identify ECH as the most potent leukocidin known to date.

When tested in protein-free solution, it was found that release of ATP from PMN occurred at similar toxin concentrations as those required to cause hemolysis. Immunological measurements of cell-bound toxin led to an estimate that membrane permeabilization occurred upon binding of ≤300 molecules ECH/cell. This number is in the same order of magnitude as that of ~100 molecules previously estimated to be required for formation of a cytolytic lesion in an erythrocyte (Eberspächer, B., F. Hugo, and S. Bhakdi, manuscript in preparation). The process of cell binding and membrane permeabilization by ECH in PMN is very rapid, ATP release commencing within a few seconds after application. The rapidity of toxin binding to PMN was probably responsible for the surprising inefficiency of plasma protein inactivators to neutralize ECH unless they were preincubated with the toxin.

Pilot experiments showed that erythrocytes suspended in NHS were resistant to lysis by even high doses of ECH (250 HU/ml). LDL, HDL, HSA, and IgG were identified as four major plasma components that contributed towards this protective effect. LDL has previously been found to inactivate α-toxin (54), and IgG antibodies to ECH are present in plasma of all healthy adults (55). A solution mix was prepared that contained all four protectants in physiological concentrations. In the presence

of these inactivators, a remarkable divergence in susceptibility between PMN and erythrocytes became apparent, and toxin doses that were entirely nonhemolytic continued to provoke rapid ATP release from PMN. Whole serum contained further, as yet unidentified inactivators, and the toxin doses required to elicit ATP release rose to 5 HU/ml (200–500 ng/ml), a level that we still consider low enough to be of potential relevance in vivo.

Preincubation of ECH with plasma protein inactivators or whole serum for 20 min on ice led to effective toxin inactivation. In most laboratory assays for neutralizing antibodies, antigen is similarly preincubated with serum, and target cells are added subsequently. High neutralization titers are then generally thought to reflect immunity of the host organism towards the action of the respective agent. Our experience with ECH and α-toxin (35) indicates that this conclusion is not invariably justified. It is noteworthy that in a single previous study, ECH was diluted in serum and then applied to granulocytes. No adverse effects were detected, and the conclusion was drawn that secreted ECH would probably not be able to damage leukocytes in vivo (53).

Factors responsible for the rapid binding of ECH to leukocytes (and of α-toxin to platelets) have yet to be defined. The presence of a specific toxin receptor for ECH on PMN has not been excluded, but positive experimental evidence is not available. Binding studies with pore-forming cytolysins are beset with intrinsic problems since these proteins become irreversibly cell bound once they enter the lipid bilayer (32, 33). There is presently no method for dissociating the process of cellular binding of ECH from that of insertion.

Membrane permeabilization by ECH was further demonstrated through assessment of propidium iodide uptake into the cells. This process exhibited an essentially identical dose-response behavior as the release of ATP. At the same time, the analyses revealed that toxin-treated cells exhibited a swelling reaction while retaining their gross cellular contours, i.e., frank dissolution of the attacked cells did not occur. Influx of propidium iodide continued to take place 15–20 min after toxin application and we thus obtained no evidence for the existence of an effective cellular repair mechanism. In another set of experiments, toxin-treated cells were suspended in serum, and their capacity to trap BCECF examined thereafter. Again, we obtained no indication that PMN could repair the toxin lesions in a physiological environment.

Many previous investigations have collectively shown that passive flux of calcium ions across toxin and complement pores can provoke secondary reactions (32, 33, 56–59) and trigger exocytotic processes in nucleated cells (60–62). Our present finding that toxin attack elicits release of granule constituents from PMN is therefore not surprising. We have found that ECH permeabilizes PMN in the presence of 10 mM EGTA, but that granule exocytosis does not occur in the presence of this chelator (data not shown). Release of granule constituents from PMN probably contributes to local effects during infections with toxin-producing *E. coli* strains.

Finally, the effects of ECH on the capacity of PMN to mount a bactericidal phagocytic response were investigated. The results extended previous data obtained in a serum-free system (51) by providing quantitative data in a more physiological assay. It was found that ECH applied at doses that evoked ATP release also reduced the phagocytic killing capacity of PMN. Thus, ECH counteracts the basic microbicidal function of PMN. This process could be particularly relevant when toxin production occurs in the immediate vicinity of the target cells, e.g., when serum-resistant

toxin-producers gain intimate contact with phagocytes. One previous study reported that incubation of toxin-producing *E. coli* with leukocytes indeed caused the latter to lose viability (53). It was concluded that cytotoxicity was probably "initiated by the local effect of alpha-hemolysin on the plasma membrane of those leukocytes to which bacteria are in close proximity" (53). Our present data fully support this contention.

In keeping with a unitarian hypothesis of toxin action, we envisage ECH to form hydrophilic pores in the plasma membrane of PMN. The pores permit passive efflux of intracellular molecules including ATP that are essential for phagocyte function. At the same time, Ca^{2+} flux into the cells triggers exocytosis of granule constituents. Since repair mechanisms are absent or relatively ineffective, these processes culminate in an overt leukocidal effect of ECH, paralleled by liberation of biologically active substances and mediators from the dying cells.

A brief comparison between ECH and other previously described bacterial leukocidins is warranted. The mechanism of cell damage by the classical leukocidin of *S. aureus* appears more complex and has not been fully elucidated (63–65). This toxin inflicts lethal injury on isolated PMN in a similar concentration range as ECH (65). However, we are not aware of quantitative studies on the leukocidal action of this toxin performed in the presence of serum proteins, a statement that equally applies to all other leukocidins. The cytolysin of *Pasteurella haemolytica* has been designated a leukocidin because it can damage isolated rodent PMN (66–70). Quantitative data on the cytocidal efficiency of this protein are not available. The cytotoxin of *Pseudomonas aeruginosa*, also termed Pseudomonas leukocidin (71–76), appears to attack isolated PMN at concentrations at least two orders of magnitude above those found for ECH (74). This cytolysin appears to be a poreformer (76). Finally, although other pore-forming cytolysins including streptolysin-O (33, 77) and *S. aureus* α-toxin (34) can damage isolated PMN, these toxins are very ineffective against cells suspended in human plasma because of their inactivation by antibodies and lipoproteins. From these considerations, ECH now emerges as the most potent of all known leukocidins. It may be anticipated that similar leukocidal properties are shared by related cytolysins including those of *Proteus*, *Morganella*, and *Pasteurella haemolytica*.

In sum, this and a previous study (35) have together demonstrated how bacterial cytolysins may evoke entirely different pathophysiological effects despite close similarities in their primary action. Both *S. aureus* α-toxin and ECH probably belong to the category of "receptorless" pore formers that generate lesions of similar dimensions in target membranes. When added to human blood however, the action of α-toxin will be directed towards platelets, whereas ECH will mount an attack on leukocytes. This realization is fundamental to the understanding of pathological processes that may be triggered by these major, medically important bacterial exotoxins.

Summary

The contribution of *Escherichia coli* hemolysin (ECH) to bacterial virulence has been considered mainly in context with its hemolytic properties. We here report that this prevalent bacterial cytolysin is the most potent leukocidin known to date. Very low concentrations (~1 ng/ml) of ECH evoke membrane permeability defects in PMN (2–10 × 10^6 cells/ml) leading to an efflux of cellular ATP and influx of propidium iodide. The attacked cells do not appear to repair the membrane lesions. Human serum albumin, high density and low density lipoprotein, and IgG together

protect erythrocytes and platelets against attack by even high doses (5–25 μg/ml) of ECH. In contrast, PMN are still permeabilized by ECH at low doses (50–250 ng/ml) in the presence of these plasma inactivators. Thus, PMN become preferred targets for attack by ECH in human blood and protein-rich body fluids. Kinetic studies demonstrate that membrane permeabilization is a rapid process, ATP-release commencing within seconds after application of toxin to leukocytes. It is estimated that membrane permeabilization ensues upon binding of ~300 molecules ECH/PMN. This process is paralleled by granule exocytosis, and by loss of phagocytic killing capacity of the cells. The recognition that ECH directly counteracts a major immune defence mechanism of the human organism through its attack on granulocytes under physiological conditions sheds new light on its possible role and potential importance as a virulence factor of *E. coli*.

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References

1. Cooke, E. M., and S. P. Ewins. 1975. Properties of strains of *Escherichia coli* isolated from a variety of sources. *J. Med. Microbiol.* 8:107.
2. Minshew, B. H., J. Jorgensen, G. W. Counts, and S. Falkow. 1978. Association of hemolysin production, hemagglutination of human erythrocytes, and virulence for chicken embryos of extraintestinal *Escherichia coli* isolates. *Infect. Immun.* 20:50.
3. Brooks, H. J. L., F. O'Grady, M. A. McSherry, and W. R. Cattell. 1980. Uropathogenic properties of *Escherichia coli* in recurrent urinary tract infection. *J. Med. Microbiol.* 13:57.
4. DeBoy, J. M., I. K. Wachsmuth, and B. R. Davis. 1980. Hemolytic activity in enterotoxigenic and non-enterotoxigenic strains of *Escherichia coli*. *J. Clin. Microbiol.* 12:836.
5. Evans, D. J., Jr., D. G. Evans, C. Hohne, M. A. Noble, E. V. Haldane, H. Lior, and L. S. Young. 1981. Hemolysin and K antigens in relation to serotype and hemagglutination type of *Escherichia coli* isolated from extraintestinal infections. *J. Clin. Microbiol.* 13:171.
6. Hughes, C., J. Hacker, A. Roberts, and W. Goebel. 1983. Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by *Escherichia coli*. *Infect. Immun.* 39:546.
7. Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow. 1981. Haemolysin contributes to virulence of extra-intestinal *E. coli* infections. *Nature (Lond.).* 294:665.
8. Waalwijk, C., J. F. van den Bosch, D. M. MacLaren, and J. de Graaff. 1982. Hemolysin plasmid coding for the virulence of a nephropathogenic *Escherichia coli* strain. *Infect. Immun.* 35:32.
9. Hacker, J., C. Hughes, H. Hof, and W. Goebel. 1983. Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. *Infect. Immun.* 42:57.
10. Cavalieri, S. J., G. A. Bohach, and I. S. Snyder. 1984. *Escherichia coli* α-hemolysin: characteristics and probable role in pathogenicity. *Microbiol. Rev.* 48:326.
11. Goebel, W., and J. Hedgepeth. 1982. Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. *J. Bacteriol.* 151:1290.
12. Welch, R. A., R. Hull, and S. Falkow. 1983. Molecular cloning and physical characterization of a chromosomal hemolysin from *Escherichia coli*. *Infect. Immun.* 42:178.
13. Mackman, N., and I. B. Holland. 1984. Functional characterization of a cloned haemolysin determinant from *E. coli* of human origin, encoding information for the secretion of a 107K polypeptide. *Mol. Gen. Genet.* 196:123.
14. Mackman, N., J. M. Nicaud, L. Gray, and I. B. Holland. 1986. Secretion of hemolysin

by *E. coli*. *Curr. Top. Microbiol. Immunol.* 125:159.
15. Ludwig, A., M. Vogel, and W. Goebel. 1987. Mutations affecting activity and transport of haemolysin in *Escherichia coli*. *Mol. Gen. Genet.* 206:238.
16. Felmlee, T., S. Pellet, and R. Welch. 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J. Bacteriol.* 163:94.
17. Strathdee, C. A., and R. Y. Lo. 1987. Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-hemolysin of *Escherichia coli*. *Infect. Immun.* 55:3233.
18. Lo, R. Y. C., C. A. Strathdee, and P. E. Shewen. 1987. Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. *Infect. Immun.* 55:1987.
19. Koronakis, V., M. Cross, B. Senior, E. Koronakis, and C. Hughes. 1987. The secreted hemolysins of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. *J. Bacteriol.* 169:1509.
20. Welch, R. A. 1987. Identification of two different hemolysin determinants in uropathogenic *Proteus* isolates. *Infect. Immun.* 55:2183.
21. Zwadyk, P., and I. S. Snyder. 1971. Purification and kinetic studies of the hemolysin from *Escherichia coli*. *Can. J. Microbiol.* 17:741.
22. Rennie, R. P., J. H. Freer, and J. P. Arbuthnott. 1974. The kinetics of erythrocyte lysis by *Escherichia coli* haemolysin. *J. Med. Microbiol.* 7:189.
23. Jorgensen, S. E., R. F. Hammer, and G. K. Wu. 1980. Effects of a single hit from alpha-hemolysin produced by *Escherichia coli* on the morphology of sheep erythrocytes. *Infect. Immun.* 27:988.
24. Jorgensen, S. E., P. F. Mulcahy, G. K. Wu, and C. F. Louis. 1983. Calcium accumulation in human and sheep erythrocytes that is induced by *Escherichia coli* hemolysin. *Toxicon.* 21:717.
25. Bhakdi, S., N. Mackman, J. M. Nicaud, and I. B. Holland. 1986. *E. coli* hemolysin may damage target cell membranes by generating transmembrane pors. *Infect. Immun.* 52:63.
26. Menestrina, G., N. Mackman, I. B. Holland, and S. Bhakdi. 1987. *E. coli* hemolysin forms voltage-dependent ion channels in lipid membranes. *Biochim. Biophys. Acta.* 905:109.
27. Bhakdi, S., and J. Tranum-Jensen. 1983. Membrane damage by complement. *Biochim. Biophys. Acta.* 737:343.
28. Henkart, P. 1985. Mechanism of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 3:31.
29. Füssle, R., S. Bhakdi, A. Sziegoleit, J. Tranum-Jensen, T. Kranz, and H. J. Wellensiek. 1981. On the mechanism of membrane damage by *S. aureus* α-toxin. *J. Cell Biol.* 91:83.
30. Bhakdi, S., R. Füssle, and J. Tranum-Jensen. 1981. Staphylococcal α-toxin: oligomerisation of hydrophilic monomers to form amphiphilic hexamers induced through contact with deoxycholate detergent micelles. *Proc. Natl. Acad. Sci. USA.* 78:5475.
31. Bhakdi, S., J. Tranum-Jensen, and A. Sziegoleit. 1985. Mechanism of membrane damage by streptolysin-O. *Infect Immun.* 47:52.
32. Bhakdi, S., and J. Tranum-Jensen. 1987. Damage to mammalian cells by proteins that form transmembrane pores. *Rev. Physiol. Biochem. Pharmacol.* 107:148.
33. Bhakdi, S., and J. Tranum-Jensen. 1988. Damage to cell membranes by pore-forming bacterial cytolysins. *Prog. Allergy.* 40:1.
34. McCartney, C., and J. P. Arbuthnott. 1978. Mode of action of membrane-damaging toxins produced by staphylococci. *In* Bacterial Toxins and Cell Membranes. J. Jeljaszewicz and T. Wadström, editors. Academic Press, New York. 89–127.
35. Bhakdi, S., M. Muhly, U. Mannhardt, F. Hugo, K. Klappetek, C. Mueller-Eckhardt, and L. Roka. 1988. Staphylococcal alpha-toxin promotes blood coagulation via attack on human platelets. *J. Exp. Med.* 168:527.
36. Poumay, Y., and M.-F. Ronveaux Dupal. 1985. Rapid preparative isolation of concentrated low density lipoproteins and of lipoprotein-deficient serum using vertical rotor gradient ultracentrifugation. *J. Lipid Res.* 26:1477.

37. De Luca, M., and W. D. McElroy. 1978. Purification and properties of firefly lucifrase. *Methods Enzymol.* 57:3.
38. Detwiler, T. C., and R. D. Feinman. 1973. Kinetics of the thrombin-induced release of adenosine triphosphate by platelets. *Biochemistry.* 12:2462.
39. Feinman, R. D., J. Lubowsky, I. Charo, and M. P. Zabinski. 1977. The lumi-aggregometer: a new instrument for simulataneous measurement of secretion and aggregation by platelets. *J. Lab. Clin. Med.* 90:125.
40. Tanke, H. J., P. W. G. van der Linden, and J. Langerak. 1982. Alternative fluorochromes to ethidium bromide for automated read out of cytotoxicity tests. *J. Immunol. Methods.* 52:91.
41. Lakhanpal, S., N. J. Gonchoroff, J. A. Katzmann, and B. S. Handwerger. 1987. A flow cytofluorometric double staining technique for simultaneous determination of human mononuclear cell surface phenotype and cell cycle phase. *J. Immunol. Methods.* 96:35.
42. Bruning, J. W., M. J. Kardol, and R. Arentzen. 1980. Carboxyfluorescein fluorochromasia assays. I. Non-radioactively labeled cell mediated lympholysis. *J. Immunol. Methods.* 33:33.
43. Rink, T. J., R. Y. Tsien, and T. Pozzan. 1982. Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell Biol.* 95:189.
44. Kolber, M. A., R. R. Quinones, R. E. Gress, and P. A. Henkart. 1988. Measurement of cytotoxicity by target cell release and retention of the fluorescent dye bis-carboxyethyl-carboxy-fluorescein (BCECF). *J. Immunol. Methods.* 108:255.
45. Bentwood, B. J., and P. M. Henson. 1980. The sequential release of granule constituents from human neutrophils. *J. Immunol.* 124:855.
46. Dewald, B., U. Bretz, and M. Baggliolini. 1982. Subcellular localisation and heterogeneity of neutral proteinases in neutrophilic polymorphonuclear leucocytes. *J. Exp. Med.* 141:102.
47. Steadman, R., N. Tropley, D. E. Jenner, M. Davies, and J. D. Williams. 1988. Type 1 fimbriate *Escherichia coli* stimulates a unique pattern of degranulation by human polymorphonuclear leukocytes. *Infect. Immun.* 56:815.
48. Markert, M., P. C. Andrews, and B. M. Babior. 1984. Measurement of O_2 production by human neutrophils. *Methods Enzymol.* 105:358.
49. Leijh, P. C., R. van Furth, and T. L. van Zwet. 1986. *In vitro* determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. *In* Handbook of Experimental Immunology. Vol. 2. D. M. Weir, editor. Blackwell Scientific Publications, Oxford, England. 46.1.
50. Cavalieri, S. J., and I. S. Snyder. 1982. Cytotoxin activity of partially purified *Escherichia coli* alpha haemolysin. *J. Med. Microbiol.* 15:11.
51. Cavalieri, S. J., and I. S. Snyder. 1982. Effect of *Escherichia coli* alpha-hemolysin on human peripheral leukocyte viability in vitro. *Infect. Immun.* 36:455.
52. Gadeberg, O. V., and I. Orskov. 1984. In vitro cytotoxin effect of α-hemolytic *Escherichia coli* on human blood granulocytes. *Infect. Immun.* 45:255.
53. Gadeberg, O. V., I. Orskov, and J. M. Rhodes. 1983. Cytotoxic effect of an alpha-hemolytic *Escherichia coli* strain on human blood monocytes and granulocytes in vitro. *Infect. Immun.* 41:358.
54. Bhakdi, S., R. Füssle, G. Utermann, and J. Tranum-Jensen. 1983. Binding and partial inactivation of *S. aureus* α-toxin by human plasma low density lipoprotein. *J. Biol. Chem.* 258:5899.
55. Hugo, F., M. Arvand, J. Reichwein, N. Mackman, I. B. Holland, and S. Bhakdi. 1987. Identification of hemolysin produced by clinical isolates of *E. coli* with monoclonal antibodies. *J. Clin. Microbiol.* 25:26.
56. Seeger, W., M. Bauer, and S. Bhakdi. 1984. Staphylococcal α-toxin elicits hypertension in isolated rabbit lungs due to stimulation of the arachidonic acid cascade. *J. Clin. Invest.* 74:849.
57. Suttorp, N., W. Seeger, E. Dewein, S. Bhakdi, and L. Roka. 1985. Staphylococcal α-toxin

stimulates synthesis of prostacyclin by cultured endothelial cells from pig pulmonary arteries. *Am. J. Physiol.* 248:C127.

58. Imagawa, D. K., N. E. Osifchin, W. A. Paznekas, M. L. Shin, and M. M. Mayer. 1983. Consequences of cell membrane attack by complement: release of arachidonate and formation of inflammatory derivates. *Proc. Natl. Acad. Sci. USA.* 80:6647.
59. Seeger, W., N. Suttorp, A. Hellwig, and S. Bhakdi. 1986. Noncytolytic terminal complement complexes may serve as calcium gates to elicit leukotriene B4 generation in human polymorphonuclear leukocytes. *J. Immunol.* 137:1286.
60. Ahnert-Hilger, G., S. Bhakdi, and M. Gratzl. 1985. Minimal requirements for exocytosis: a study PC 12 cells permeabilized with staphylococcal α-toxin. *J. Biol. Chem.* 260:12730.
61. Bader, M. F., D. Thierse, D. Aunis, G. Ahnert-Hilger, and M. Gratzl. 1986. Characterization of hormone and protein release from α-toxin permeabilized chromaffin cells in primary culture. *J. Biol. Chem.* 261:5777.
62. McEwen, B. F., and W. J. Arion. 1985. Permeabilization of rat hepatocytes with *Staphylococcus aureus* α-toxin. *J. Cell. Biol.* 100:1922.
63. Woodin, A. M., and A. A. Wieneke. 1966. The interaction of leucocidin with the cell membrane of the polymorphonuclear leucocyte. *Biochem. J.* 99:479.
64. Noda, M., I. Kato, T. Hirayama, and F. Matsuda. 1982. Mode of action of staphylococcal leukocidin: effects of the S and F components on the activities of membrane-associated enzymes of rabbit polymorphonuclear leukocytes. *Infect. Immun.* 35:38.
65. Noda, M., T. Hirayama, F. Matsuda, and I. Kato. 1985. An early effect of the S-component of staphylococcal leukocidin on methylation of phospholipid in various leukocytes. *Infect. Immun.* 50:142.
66. Kaehler, K. L., R. J. F. Markham, C. C. Muscoplat, and D. W. Johnson. 1980. Evidence of species specificity in the cytocidal effects of *Pasteurella haemolytica. Infect. Immun.* 30:615.
67. Baluyut, C. S., R. R. Simonson, W. J. Bemrick, and S. K. Maheswaran. 1981. Interaction of *Pasteurella haemolytica* with bovine neutrophils: identification and partial characterization of a cytotoxin. *Am. J. Vet. Res.* 42:1920.
68. Himmel, M. E., M. D. Yates, L. H. Lauerman, and P. G. Squire. 1982. Purification and partial characterization of a macrophage cytotoxin from *Pasteurella haemolytica. Am. J. Vet. Res.* 43:764.
69. Shewen, P. E., and B. N. Wilkie. 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infect. Immun.* 35:91.
70. Lo, R. Y. C., P. E. Shewen, C. A. Strathdee, and C. N. Greer. 1985. Cloning and expression of the leukotoxin gene of *Pasteurella haemolytica* A1 in *Escherichia coli* K-12. *Infect. Immun.* 50:667.
71. Scharmann, W. 1976. Purification and characterization of leucocidin from *Pseudomonas aeruginosa. J. Gen. Microbiol.* 93:292.
72. Scharmann, W. 1976. Cytotoxic effects of leukocidin from *Pseudomonas aeruginosa* on polymorphonuclear leukocytes. *Infect. Immun.* 13:836.
73. Scharmann, W. 1976. Interaction of purified leukocidin from *Pseudomonas aeruginosa* on polymorphonuclear leukocytes. *Infect. Immun.* 13:1046.
74. Hirayama, T., and I. Kato. 1984. Mode of cytotoxic action of Pseudomonal leukocidin on phosphatidylinositol metabolism and activation of lysosomal enzyme in rabbit leukocytes. *Infect. Immun.* 43:21.
75. Hirayama, T., M. Noda, F. Matsuda, M. Nagamori, and I. Kato. 1984. Binding of Pseudomonal leukocidin to rabbit polymorphonuclear leukocytes. *Infect. Immun.* 46:631.
76. Lutz, F., S. Grieshaber, and K. Schmidt. 1982. Permeability changes of Ehrlich mouse ascites tumor cells induced by cytotoxin from *Pseudomonas aeruginosa. Arch. Pharmacol.* 320:78.
77. Alouf, J. E. 1980. Streptococcal toxins (streptolysin-O, streptolysin-S, erythrogenic toxin). *Pharmacol. & Ther.* 11:661.