

# Effects of *Escherichia coli* Hemolysin on Human Monocytes

## Cytocidal Action and Stimulation of Interleukin 1 Release

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### Abstract

This study reports on the potent cytotoxic and interleukin-1 releasing properties of *Escherichia coli* hemolysin (ECH) on human monocytes. Nanomolar concentrations of purified ECH (250–2,000 ng/ml) caused rapid and irreversible depletion of cellular ATP to levels below 20% of controls within 60 min. Subcytotoxic doses (10–200 ng/ml) of ECH induced rapid release within 60–120 min of large amounts of interleukin 1 $\beta$  (IL-1 $\beta$ ) from cultured monocytes. IL-1 $\beta$  release occurred in the presence of actinomycin D and cycloheximide, and was thus probably due to processing and export of intracellular IL-1 $\beta$  precursor. Incubation of toxin-producing *E. coli* at ratios of only 0.3–3 colony-forming units per monocyte evoked ~ 50% depletion of total cellular ATP within 90 min. Toxin producers also stimulated synthesis and release of large amounts of interleukin 1, but not of tumor necrosis factor within the same time span. In contrast, non-toxin producers caused neither cell death nor rapid interleukin 1 release. Stimulation of rapid interleukin 1 release coupled with potent cytotoxic effects on cells of monocytic origin may represent pathogenetically significant events incurred by bacterial strains that produce ECH and related cytotoxins. (*J. Clin. Invest.* 1990; 85:1746–1753.) cytotoxin • membrane permeabilization • cytokine processing

### Introduction

*Escherichia coli* hemolysin (ECH)<sup>1</sup> is produced by approximately 50% of *E. coli* isolates causing extraintestinal infections in man, and is thus one of the most prevalent bacterial protein toxins encountered by humans (1–7). The pathogenetic relevance of ECH has been established in several animal models (8–10). The cytotoxin has recently been identified as a novel prototype of a pore-forming protein that may insert as a monomer into target lipid bilayers to generate a functional transmembrane lesion of 1–2 nm effective diameter (11–14). The cytotoxins of *Proteus*, *Morganella*, and *Pasteurella haemolytica* exhibit marked structural homologies with ECH, and probably constitute a toxin family sharing similar functional and immunological properties (15–18).

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1. Abbreviations used in this paper: ECH, *E. coli* hemolysin; LPS, lipopolysaccharide; NHS, normal human serum; PMB, polymyxin B.

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A deeper understanding of the mechanisms responsible for the pathogenetic role of any bacterial cytotoxin requires resolution of two basic questions. First, cells that represent targets for toxin attack under physiological conditions must be identified. Second, the major consequences of toxin attack on these cells should be recognized. In vitro, bacterial cytotoxins will generally damage a broad spectrum of mammalian cells as well as artificial lipid bilayers (14). However, this does not mean that they will mount an unselective attack on all cells they encounter in the host organism. On the one hand, certain cell types may possess high-affinity binding sites and thus be particularly vulnerable. On the other hand, naturally occurring antibodies and lipoproteins generally exhibit the capacity to neutralize membrane-damaging cytotoxins. Successful attack thus requires that toxin-binding membrane perturbation occur more rapidly than the inactivation processes. For these reasons, we are conducting model studies on the cytotoxic action of several major pore-forming bacterial cytotoxins on human cells in the presence of plasma proteins. First results obtained with *S. aureus*  $\alpha$ -toxin and ECH revealed an unexpected divergence in their action. Although both toxins attack protein-free liposomes and planar lipid bilayers to produce transmembrane pores of similar size,  $\alpha$ -toxin mounted a selective attack on blood platelets (19), whereas ECH exhibited potent cytotoxic action towards granulocytes (20). Despite their classical designation as hemolysins, neither toxin induced lysis of human erythrocytes unless applied at extremely high concentrations.

We have now investigated the action of ECH on monocytes utilizing purified, LPS-depleted ECH, as well as a series of *E. coli* strains. The results extend brief earlier reports (21, 22) and demonstrate that ECH displays a high cytotoxic potency towards monocytes. In addition, toxin attack induces very rapid release of IL-1 $\beta$  from cultured cells and from cells confronted with viable *E. coli*. Release of IL-1 $\beta$  by cells of monocytic origin following attack by very low doses of ECH and, probably, other members of this toxin family could contribute significantly to the pathogenesis of local and systemic lesions during gram-negative infections.

### Methods

**Preparation of ECH.** A detailed description of the procedure for isolating ECH in lipopolysaccharide (LPS)-depleted form from culture supernatants of *E. coli* strain LE 2001 (23, 24) is to be given separately (Muhly, M., U. Zähringer, and S. Bhakdi, manuscript in preparation), and only the major steps will briefly be recounted here. 200 ml of prewarmed Todd-Hewitt-Broth (THB; Difco Laboratories, Detroit, MI) supplemented with 15 mM CaCl<sub>2</sub> were inoculated with 1 ml of an overnight bacterial culture and maintained at 37°C for ~ 3 h until the pH of the culture dropped to 6.0–6.1. Cells were pelleted by centrifugation (5 min, 25,000 g, Sorvall centrifuge RC 2B, rotor SS 34; Sorvall Instruments Div. Du Pont Co., Newton, CT) and to the supernatants

were added 22 g polyethyleneglycol 4000 (PEG) per 100 ml fluid. After stirring for 20 min, 4°C, the PEG precipitates were collected by centrifugation, dissolved in 5 ml of distilled water, and sterile filtered. The filtered toxin solutions were subjected to a 60 min centrifugation in linear 10–50% wt/vol glycerol density gradients using a vertical rotor type VTi 65.1 (Beckman Instruments, Inc., Fullerton, CA). Fractions exhibiting peak hemolytic activity (2,000–4,000 HU/ml) and containing nonaggregated, LPS-depleted toxin were pooled and used in the experiments. The preparations contained 300–400 µg/ml protein as determined by amino acid analyses and ELISA, and 1–1.5 µg/ml LPS as determined with a Limulus assay (KabiVitrum Diagnostica, Stockholm, Sweden). The toxin was stored in aliquots at –70°C and fully retained its cytolytic activity for > 3 mo. For control experiments, ECH was inactivated by heating at 56°C for 60 min.

### Genetically related *E. coli* strains

*E. coli* 2980 and derivatives thereof were used in this study. *E. coli* 2980 of serotype O18A:K5:H5 produced mannose-resistant P-fimbriae of serotype F8 and mannose-sensitive type 1 fimbriae (now termed F1). The strains and the properties relevant here are listed in Table I.

*E. coli* 20397 (F8<sup>-</sup>), a mutant lacking the P-fimbriae was obtained by mutagenesis of strain 2980 (25). *E. coli* 20469 (Hly<sup>-</sup>) was a spontaneous hemolysin-negative mutant of strain 2980. The capsule-negative (K5<sup>-</sup>) strains 20400 and B2313 were selected from the parents 20397 and 20469, respectively, as mutants resistant to coliphage K5 which is specific for the K5 antigen (26).

In addition, an *rfb* defective mutant having the complete LPS core but unable to produce O-specific polysaccharides was obtained by introduction of the *his*-linked defective *rfb* region of an *E. coli* K-12 Hfr donor into *E. coli* 2980. The rough strain B2249 (O18A<sup>-</sup>:K5<sup>-</sup>) was selected therefrom as a mutant resistant to coliphage K5.

All the strains listed in Table I still produced the F1 fimbriae which have been shown to play a role in adherence to rat peritoneal macrophages and human polymorphonuclear granulocytes (27).

**Other reagents.** LPS were from two sources. First, we used preparations of active or inactive ECH containing the measured amounts of LPS. Second, LPS from *E. coli* O55:B5 were obtained from Sigma (Münich, FRG) and suspended in sterile saline at 10 mg/ml. Polymyxin B (PMB) was obtained from Serva (Heidelberg, FRG) and used in cell cultures at a final concentration of 2 µg/ml. All buffer solutions were autoclaved before use. Normal human serum (NHS) was obtained from the respective donors and used immediately or stored at –20°C for no longer than 7 d. Three NHS samples were depleted of IgG by one passage over a 1-ml protein G-Sepharose column (Pharmacia, Uppsala, Sweden, equilibrated in saline). Over 95% IgG was removed as ascertained by radial immunodiffusion. Pooled human IgG was obtained from Sandoz Laboratories (Basel, Switzerland).

### Preparation of monocytes

Blood was drawn in heparin (50 U/ml final concentration; obtained from Hoffmann-La Roche, Basel, Switzerland) and 5-ml aliquots were applied to 4 ml Ficoll-Hypaque gradients (Pharmacia Fine Chemicals) in sterile plastic tubes. After 45 min centrifugation at 900 g, 20°C (Heraeus Minifuge RF; Hereens Sepatech GmbH, Ostende, FRG) cells

from the intermediate phase were recovered, washed twice with RPMI medium at 500 g for 5 min (20°C), and resuspended in 8 ml of this buffer. 200-µl aliquots were applied to 96-well culture plates (Nunc, Wiesbaden, FRG) for 1 h in an incubator (7% CO<sub>2</sub>, 37°C). Thereafter, one of two protocols was followed. To obtain highly purified monocytes, the supernatants containing nonadherent cells were aspirated, and the plates were thoroughly washed by three consecutive applications of 200 µl RPMI. The buffer was applied under vigorous pressure using a 12-channel distributor. Alternatively, the supernatants were aspirated and adherent cells were used without further washing. The latter procedure permitted considerably higher recovery of monocytes that were contaminated with lymphocytes and platelets. Since both types of cell preparations yielded similar results with respect to IL-1β responses, most experiments were performed following the second protocol. Cell numbers were approximated by counting 25 fields per well through a calibrated microscopic objective. Each culture well typically contained approximately 10<sup>5</sup> monocytes in routine experiments (without thorough washing). Monocytes were identified by staining for nonspecific esterase and they typically accounted for ~ 50% of total cells. Vigorous washing resulted in monocyte preparations that were 90–95% pure, but cell numbers were then in the order of 1.5–2 × 10<sup>4</sup> per well.

The adherent monocytes were either used directly, or they were cultured overnight in RPMI containing 10% FCS, 0.1 mg/ml streptomycin and 100 U/ml penicillin (without PMB) and used on the following day. When indicated, cycloheximide or actinomycin D was added to the medium at a final concentration of 2 or 10 µg/ml, respectively.

### Treatment of monocytes with stimuli

The supernatants were aspirated and cells were immersed in 100 µl of either 10% FCS or 10% autologous serum in RPMI 1640 medium (Biochrom, Berlin, FRG). 10 µl of appropriately diluted LPS or ECH were then added to yield the required final concentration of each stimulus. Incubations with LPS were for 2, 6, and 24 h. Incubation periods with ECH varied between minutes and 24 h, as described in the individual experiments. PMB was added at a final concentration of 2 µg/ml whenever indicated.

Experiments with genetically related strains of *E. coli* were conducted as follows. Bacteria from overnight cultures were inoculated into fresh broth and brought into log-phase growth by culture for 3–3½ h at 37°C. The cells were then pelleted (Eppendorf table-top centrifuge, 10,000 g, 2 min), washed twice with sterile saline, and suspended to approximately 3 × 10<sup>8</sup> CFU/ml in PBS. No free toxin was detectable by functional or immunoassays. Cell dilutions were prepared from these stock suspensions and added to RPMI/10% FCS to yield the final, required ratios of bacteria to monocytes (0.03, 0.3, 3, 30, and 300 CFU/monocyte). After 90 min, 37°C, supernatants were harvested and given 10 µg/ml PMB to halt bacterial growth. Hemolytically active toxin was never detectable in any of these supernatants.

**Measurements of cellular ATP.** At the end of the incubation periods, cell supernatants were collected and the microculture dishes were placed on ice. Measurements of cellular ATP were performed by lysing the cells with 0.2 ml of ice-cold 0.1% (vol/vol) Triton X-100 (aqueous solution) and pipetting 70–100 µl of the solubilisate into a prewarmed (2 min, 37°C) mix of firefly reagent (ATP-bioluminescence CLS from Boehringer, Mannheim, FRG; 100 µl) plus 200 µl PBS. Bioluminescence measurements were performed using a Lumi-Aggro-Meter (model 400; Chrono-Log Corp., Coulter Electronics, Krefeld, FRG). These ATP-measurements proved sensitive, rapid, and reliable. The cellular ATP content ranged from 70 to 160 pmol/well, dependent on donor and cell preparation. The relative bioluminescence peaks determined for the control cultures (without toxin) were taken as 100% values for each series. The heights of the bioluminescence peaks varied linearly with the amount of ATP in a given sample. Bacterial ATP was not detected with the described assay. Control assays conducted with ATP (from Boehringer) excluded that ECH or a product of its interaction with monocytes interfered with the luciferase assay.

Table I. *E. coli* Strains and Relevant Properties

Strain No.	O18A	K5	F8	Hly
2980	+*	+	+	+
20397	+	+	–	+
20400	+	–	–	+
20469	+	+	+	–
B2313	+	–	+	–
B2249	–	–	+	+

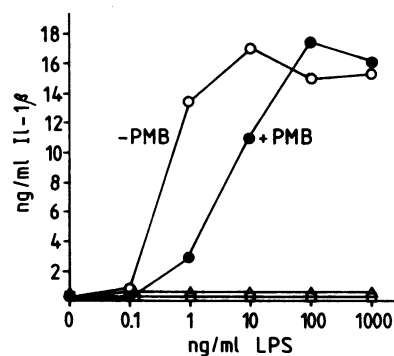
\* +, present; –, absent

**Measurements of monokines.** IL-1 $\beta$  and TNF- $\alpha$  were assayed in cell culture supernatants with the use of radioassays (obtained from IRE Diagnostics, Düsseldorf, FRG). Additionally, IL-1 $\beta$  determinations were also performed with the use of an ELISA from Paesel (Frankfurt, FRG). Both assays yielded equivalent results. Concentrations were expressed in nanograms per milliliter and calculated from calibration curves obtained with the standards supplied in each kit. In three experiments, attempts were also made to measure intracellular IL-1 $\beta$ . After removal of the cell supernatants, cell adhering to the culture dishes were immersed in PBS and lysed by three cycles of freeze-thawing. These samples were then analyzed by radioimmunoassay and ELISA.

## Results

**Release of monokines in response to LPS.** Although the ECH preparations utilized in this study contained less than 1 mol LPS per 40 mol cytolysin, it was essential to exclude that any detected monokine-releasing effects might derive from contaminating endotoxin. The production of monokines in response to LPS-stimulation of monocytes was therefore first studied and the following observations were made. First, it was found that freshly isolated monocytes responded to LPS at a concentration threshold of  $\sim 1$  ng/ml with the production of large amounts of TNF- $\alpha$  and IL-1 $\beta$ . The levels of TNF- $\alpha$  measured after an incubation of 6 h generally was in the order of 25–30 ng/ml cell supernatant. The levels of IL-1 $\beta$  remained stationary 2 h after addition of LPS, and then rose markedly to reach levels of  $\sim 15$ –20 ng/ml after 6 h incubation (Fig. 1). These results were in good accord with published data (28). The addition of 2  $\mu$ g/ml PMB to cell cultures almost totally inhibited the cytokine-stimulatory action of 1 ng/ml, but suppressed to a lesser extent the effect of 10 ng/ml (Fig. 1). The addition of LPS to cell cultures did not evoke any depletion of cellular ATP.

Adherent monocytes that had been cultured overnight presented an entirely different response to LPS, irrespective of the source of endotoxin. These cells continued to exhibit an essentially identical dose-response behavior with respect to production of TNF- $\alpha$ . However, the IL-1 $\beta$  response dropped to levels essentially indistinguishable from background and no significant IL-1 $\beta$  production could be elicited even with very high doses of LPS (Fig. 1). These results corroborated a recent



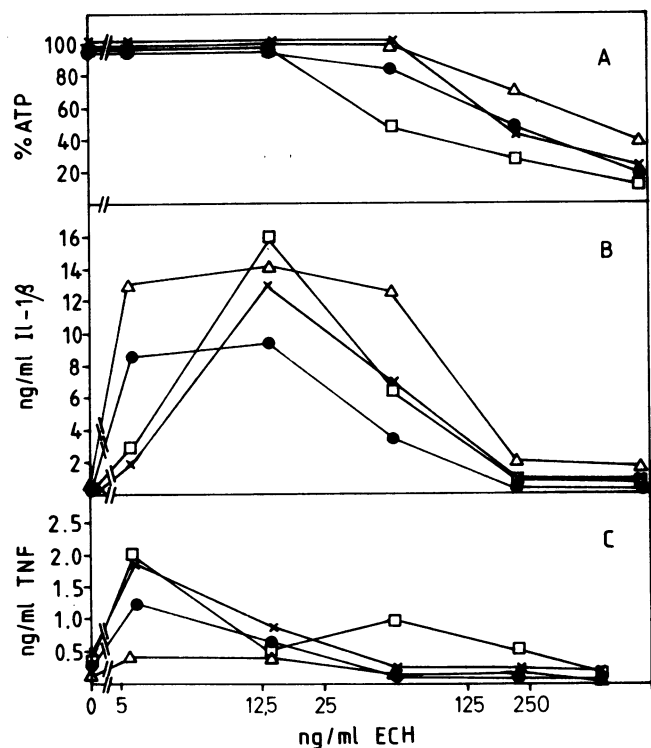
**Figure 1.** Production of IL-1 $\beta$  by freshly isolated human monocytes (circles) or cultured monocytes (triangles) in the presence or absence of 2  $\mu$ g/ml PMB induced by LPS from *E. coli*. Approximately  $10^5$  cells were treated with LPS for 24 h in the presence of 10% FCS, and IL-1 $\beta$  was quantified in the cell supernatants (0.1 ml) by radioimmunoas-

say. Cultured monocytes (20 h, 37°C) did not respond to the LPS stimulus with IL-1 $\beta$  production (triangles; lower two plots depict results obtained with and without PMB). PMB at the given concentration almost totally inhibited the stimulatory action of 1 ng/ml LPS on freshly isolated cells.

report that showed monocytes to lose their ability to produce IL-1 $\beta$  in response to an LPS stimulus after a culture period of 48 h (29). Data on the response of monocytes cultured for shorter periods were not presented in that communication.

**Depletion of cellular ATP by ECH.** The cytosidal effects of ECH could most conveniently be detected and quantified by measurements of cellular ATP. This parameter has previously been applied in the study of ECH action on renal tubular epithelial cells (30). As shown in Fig. 2A, nanomolar concentrations of ECH evoked depletion of ATP in monocytes. The same ATP-depleting effects were observed with both freshly isolated and cultured cells, and they were irreversible since no replenishment of ATP-content was observed if cells were further cultured for up to 24 h. By light microscopy, ATP-depleted cells were found to present a swollen appearance and they also stained positively with trypan blue. 50% ATP-depletion occurred at toxin concentrations of  $\sim 250$  ng/ml in the presence of FCS. The kinetics of ATP-depletion were very rapid: ECH at a dose of 2,000 ng/ml effected  $> 90\%$  depletion within 45 min (Fig. 3). If monocytes were suspended in 10% autologous serum, the dose-response curves shifted to the right. The extent of this shift varied somewhat dependent on the donor (Fig. 4A). ATP depletion generally commenced at  $\sim 250$  ng/ml ECH, and 60–80% depletion was observed at the highest tested toxin dose of 2,000 ng/ml.

**ECH stimulates release of IL-1 $\beta$  from cultured monocytes.** Subcytotoxic doses of ECH, defined as doses that effected no



**Figure 2.** Cytokine-stimulatory action and ATP-depleting effects of ECH on cultured monocytes in the presence of 10% FCS. (A) Cellular ATP-content, expressed as percent of non-toxin-treated controls. The mean ATP-content in the four controls was 90 pmol/well. (B, C) Concentrations of IL-1 $\beta$  and TNF in the cell supernatants. The incubation period with ECH was 6 h at 37°C in the presence of 2  $\mu$ g/ml PMB. The plots depict results obtained in four different experiments.

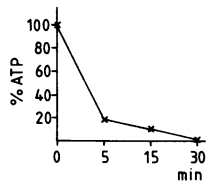


Figure 3. Kinetics of ATP-depletion in cultured monocytes treated with 2,000 ng/ml ECH in the presence of FCS and 2  $\mu$ g/ml PMB. The results were reproduced twice. The 100% ATP-value in this experiment corresponded to 110 pmol ATP.

or only minimal ATP depletion, evoked release of large amounts of IL-1 $\beta$  by cultured monocytes suspended in FCS (Fig. 2 B). This behavior was noted irrespective of donor, with peak IL-1 $\beta$  concentrations in the range of 10–15 ng/ml in cell supernatants. These levels were comparable to those found in supernatants of freshly isolated cells stimulated with 1–100 ng/ml LPS (28). In all experiments, cells were immersed in medium containing 2  $\mu$ g/ml PMB immediately before toxin application. That the IL-1 $\beta$  releasing effect of ECH might be due to contaminating LPS was excluded by four findings. First, ECH failed to provoke production of large amounts of TNF- $\alpha$ . Levels of this cytokine were generally raised to 1–2 ng/ml at subcytotoxic concentrations (Fig. 2 C). In contrast, monocytes stimulated with LPS inevitably presented a stronger TNF- $\alpha$ -response, relative to IL-1 $\beta$  production. Second, ECH provoked production of IL-1 $\beta$  only in cultured monocytes; freshly isolated cells did not produce substantial amounts of IL-1 $\beta$  at any of the tested toxin concentrations. This response behavior was thus reciprocal to that observed with LPS-stimulation. Third, inactivated toxin preparations that had lost their cytocidal properties failed entirely to stimulate IL-1 $\beta$  production. Fourth, toxin-dependent release of IL-1 $\beta$  in cultured monocytes displayed extremely rapid kinetics, peak levels in cell supernatant approaching maximal values within 1–2 h after toxin application (Fig. 5). As stated

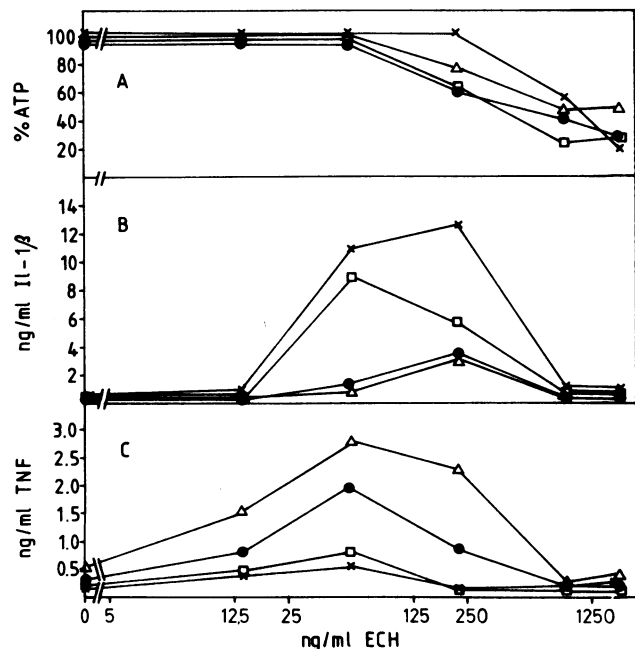


Figure 4. Action of ECH on cultured monocytes in the presence of 10% autologous serum and 2  $\mu$ g/ml PMB. Each plot represents data obtained with one individual. There was a marked variation in the IL-1 $\beta$  responses. The mean 100% ATP-content per well in the four experiments was 100 pmol.

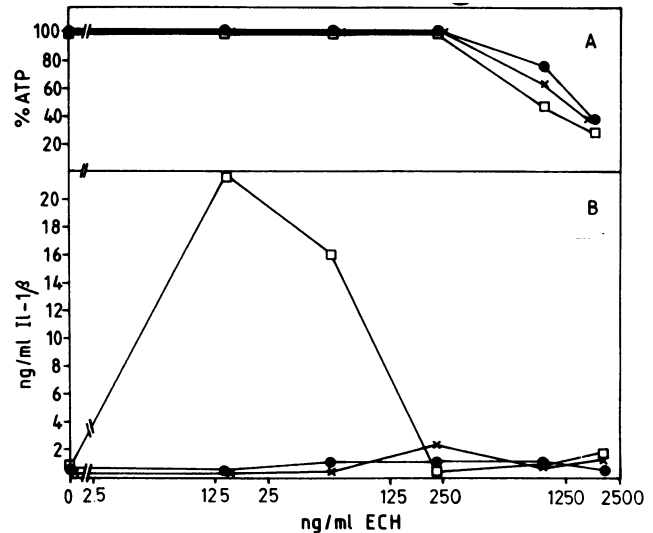


Figure 5. ATP-depletion (A) and IL-1 $\beta$  production (B) in cultured monocytes treated with ECH in the presence of autologous serum (○); IgG-depleted autologous serum (□); and the latter serum reconstituted with pooled human IgG at an approximately twofold concentration of physiological plasma IgG levels (×). Note the abrogation of the IL-1 $\beta$  response in the presence of IgG, but the lack of protection afforded by the antibodies against attack by higher ATP-depleting toxin doses. A similar set of data was obtained with cells and serum from a second individual. 2  $\mu$ g/ml PMB was present during all incubations with ECH.

above, no increase in IL-1 $\beta$  levels was observed 2 h after stimulation with LPS.

If FCS was replaced by autologous serum, an essentially similar response pattern with respect to IL-1 $\beta$  release was noted. However, marked interindividual differences in the peak levels of the cytokine were now encountered (Fig. 4 B), with values ranging from 1 to 13 ng/ml. Low and varying levels of TNF- $\alpha$  were also detected (not shown).

It was reasoned that varying antibody titers against ECH might be responsible for the observed variation in IL-1 $\beta$  release. To test this hypothesis, serum samples from two donors presenting very low or moderate release were depleted of IgG and used in another set of experiments. Indeed, removal of IgG caused the appearance of a uniform, strong IL-1 $\beta$  release with cytokine levels now exceeding 20 ng/ml (Fig. 6). Upon addition of pooled human IgG to these sera, the IL-1 $\beta$  release returned to minimum levels. A similar suppression was noted if the sera were reconstituted with autologous IgG eluted from

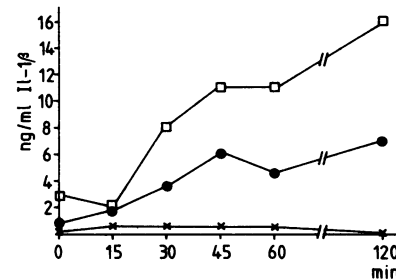


Figure 6. Kinetics of IL-1 $\beta$  release induced by ECH. Cultured monocytes were treated with 0 (○), 1.25 ng/ml (●) or 2.5 ng/ml ECH (□) in the presence of 10% FCS and 2  $\mu$ g/ml PMB, and the levels of IL-1 $\beta$  quantified in the cell supernatants at the depicted times. Note

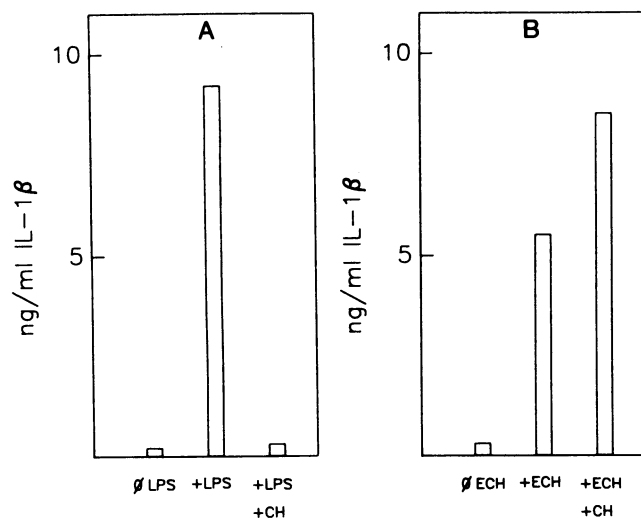
the very rapid release of IL-1 $\beta$  induced by the toxin. Similar response patterns were found in two separate experiments.

the Protein G-Sepharose column (not shown). No consistent alterations in TNF-responses were noted following removal of IgG from NHS.

Preincubation of ECH with human serum for 15 min at 0°C led to complete loss of all ATP-depleting and cytokine-stimulatory effects. Hence, neutralizing plasma components were efficient if permitted to react with ECH before confrontation with the sensitive cells occurred. Heat inactivation of ECH (30 min, 56°C) totally destroyed all ATP-depleting and cytokine-releasing properties. The ability to effect membrane insertion and form transmembrane pores thus appeared directly related to the cytokine-releasing action of ECH.

*Toxin-dependent IL-1 $\beta$  release does not involve de novo protein synthesis.* In a set of control experiments, highly purified, freshly isolated monocytes were stimulated with 0.1  $\mu$ g/ml LPS in the presence of actinomycin D or cycloheximide. No IL-1 $\beta$  could be detected in these cell supernatants after 6–24 h, confirming that the inhibitors had effectively suppressed de novo production of the cytokine (Fig. 7 A). In analogous experiments, highly purified monocytes were first cultured overnight and then immersed in medium containing inhibitor or a combination of both inhibitors for 30 min, after which ECH was applied. In these cases, unaltered or even slightly higher levels of IL-1 $\beta$  were found in the supernatants (Fig. 7 B). These findings indicated that the toxin affected processing and export of intracellularly accumulated IL-1 $\beta$  precursor, rather than inducing genuine de novo cytokine synthesis.

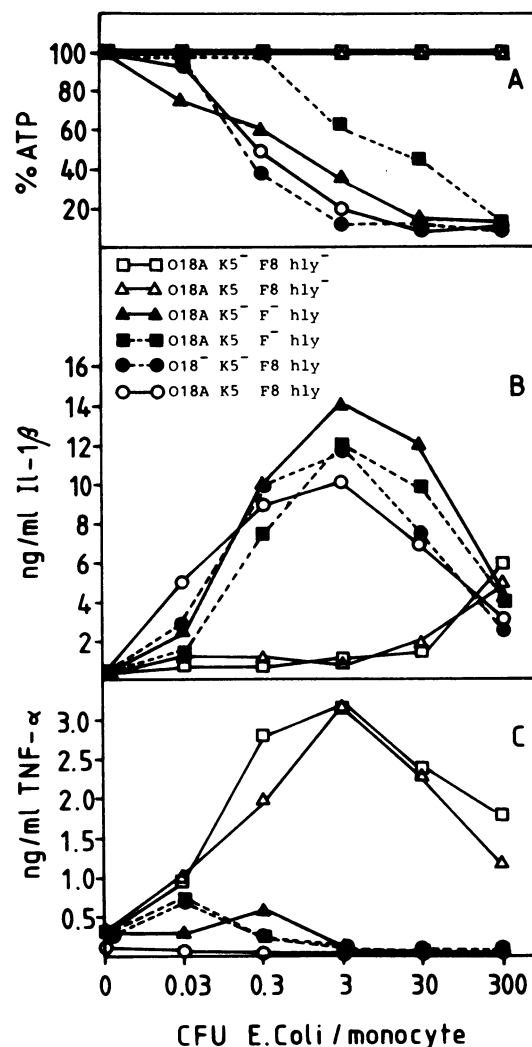
Attempts were then undertaken to directly show that IL-1 $\beta$  had accumulated in the cultured cells. These were lysed by freeze-thawing in 100  $\mu$ l of medium, and the samples analyzed by radioimmunoassay and ELISA. Lysates of freshly isolated



**Figure 7.** (A) Highly purified monocytes were cultured for 20 h in medium containing 10% FCS without LPS, with 100 ng/ml LPS, or with 100 ng/ml LPS + 2  $\mu$ g/ml cycloheximide (CH). IL-1 $\beta$  was quantified in cell supernatants; CH fully inhibited the de novo synthesis of the cytokine. (B) Highly purified monocytes were first cultured overnight in 10% FCS and then treated with 80 ng/ml ECH for 6 h, 37°C in the presence or absence of 2  $\mu$ g/ml CH. Release of the cytokine occurred despite the presence of the inhibitor. The same results were obtained when ECH was applied in the presence of 10  $\mu$ g/ml actinomycin D.

cells were found to contain 0.1–0.2 ng/ml IL-1 $\beta$ , whereas cells cultured overnight contained 1.5–2.0 ng/ml IL-1 $\beta$ , as determined with these assays. Hence, although slight rises in intracellular IL-1 $\beta$  were found after culture, they did not account for the very marked levels of IL-1 $\beta$  (generally above 10 ng/ml) recovered in the supernatants of cells after treatment with ECH.

*Cytokine-stimulatory and cytotoxic action of viable E. coli on monocytes.* The next experiments were performed in order to assess the effects evoked upon confrontation of monocytes with viable, toxin-producing bacteria. As shown in Fig. 8 A, incubation of freshly isolated monocytes with each of the four toxin-producing strains led to rapid ATP-depletion within 90 min. An average ratio of only 0.3–3 CFU/monocyte sufficed to cause ~ 50% loss of total ATP. At the same time, all toxin-



**Figure 8.** Effects of incubation of viable *E. coli* with freshly isolated human monocytes in the presence of FCS. Six strains were used with the given characteristics and at the given, approximate bacteria to cell ratios. Measurements of cellular ATP (A) and of IL-1 $\beta$  (B) and TNF- $\alpha$  (C) in the cell supernatants were performed after 70 min incubation at 37°C. All strains of toxin-producers evoked ATP-depletion and IL-1 $\beta$  production, but depressed TNF-production presumably due to their cytotoxic action. The 100% ATP-content corresponded to ~ 140 pmol ATP per well.

producing strains elicited IL-1 $\beta$  responses which were no longer dissociated from the ATP-depleting effects (Fig. 8 B). However, IL-1 responses were reduced at the highest bacteria:cell ratio (300:1), presumably due to ultra-rapid killing of the monocytes. In contrast, neither of the two nontoxin producers evoked either ATP-depletion or IL-1 production within the incubation period of 90 min.

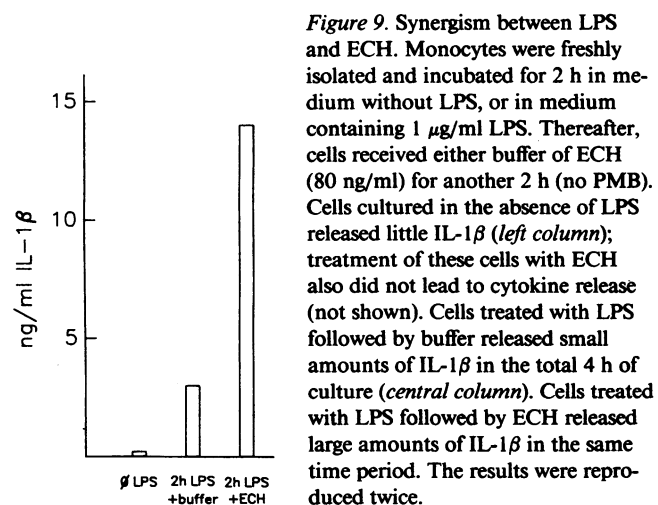
TNF-responses were measured after 2 h incubation. As shown in Fig. 8 C, toxin-dependent cytotoxic activity was paralleled by a sharp decrease in TNF-production; hence, there was a clear reciprocity between production of TNF vs. IL-1 $\beta$ . Non-toxin producers evoked release of considerable amounts of TNF into the supernatants.

When cultured monocytes (20 h) from the same donor were used in the same experimental system, the cytotoxic activity of hemolytic *E. coli* strains remained comparable, and there was a slightly enhanced IL-1 $\beta$  response compared to freshly isolated cells at all tested bacteria: monocyte ratios. The TNF-response pattern was essentially the same as in Fig. 8 C (data not shown). When cultured monocytes were incubated with toxin-producing bacteria in the presence of actinomycin D, IL-1 $\beta$  release was again registered (peak values: 10–12 ng/ml).

**Synergism between LPS and ECH in the provocation of IL-1 $\beta$  release.** All the previous data indicated that ECH provoked or accelerated release of IL-1 $\beta$  when the cytokine precursor was present in cells. In a final set of experiments, freshly isolated monocytes were incubated with 1  $\mu$ g/ml LPS for 2 h in 10% FCS. Thereafter, one set of cell cultures received ECH, and the controls received medium. After another 2 h, IL-1 $\beta$  was quantified in cell supernatants. Whereas the control cells released only small amounts of IL-1 $\beta$  in this time period, cells additionally treated with ECH liberated large quantities of IL-1 $\beta$  (Fig. 9). Hence, ECH was found to synergize with LPS to effect rapid liberation of IL-1 $\beta$  from primed cells.

## Discussion

This study shows that in addition to PMN, monocytes represent sensitive targets for the cytotoxic action of ECH. Subnanomolar concentrations of purified toxin effect rapid, irreversible depletion of cellular ATP, a process that is paralleled by cellular swelling and trypan blue staining positivity. As pre-



**Figure 9.** Synergism between LPS and ECH. Monocytes were freshly isolated and incubated for 2 h in medium without LPS, or in medium containing 1  $\mu$ g/ml LPS. Thereafter, cells received either buffer or ECH (80 ng/ml) for another 2 h (no PMB). Cells cultured in the absence of LPS released little IL-1 $\beta$  (left column); treatment of these cells with ECH also did not lead to cytokine release (not shown). Cells treated with LPS followed by buffer released small amounts of IL-1 $\beta$  in the total 4 h of culture (central column). Cells treated with LPS followed by ECH released large amounts of IL-1 $\beta$  in the same time period. The results were reproduced twice.

viously found for PMN, naturally occurring plasma protein inhibitors are unable to effectively suppress this cytotoxic effect, which we assume is due to formation of transmembrane pores in the target plasma membranes.

A second, novel finding relates to the liberation of large quantities of IL-1 $\beta$  from monocytes attacked by very low doses of ECH. In addition, small quantities of and TNF- $\alpha$  were detected. Since levels of the latter monokine were low, we confined our present studies to a closer analysis of the IL-1 $\beta$  response. Several considerations collectively excluded that this derived from contaminating LPS. First, ECH-dependent IL-1 $\beta$  release was observed only in cultured monocytes. In contrast, LPS induced IL-1 secretion only in freshly isolated cells (29, and this report). Second, the amounts of LPS contaminating the ECH preparations were far below the threshold required for induction of IL-1 $\beta$  synthesis in the given presence of 2  $\mu$ g/ml PMB. In this context, heat-inactivated ECH also failed to provoke liberation of the cytokine. Third, LPS-induced IL-1 $\beta$  secretion was totally abrogated in the presence of actinomycin D or cycloheximide, whereas neither inhibitor suppressed the appearance of IL-1 $\beta$  in the supernatants of ECH-treated cells. Thus, in contrast to LPS, ECH apparently did not stimulate de novo monokine synthesis. By exclusion, toxin-dependent release of IL-1 $\beta$  must have derived from processing and export of its 31K precursor that presumably accumulates intracellularly during cell culture. If correct, this assumption would also explain why ECH failed to induce IL-1 $\beta$  release from freshly isolated cells.

Although experimental data to directly prove the latter hypothesis are not yet available, recent reports indicate that the concept is basically tenable. It is known that LPS first induces accumulation of IL-1 precursors in the cell cytoplasm, processing and export then following in a second, temporally dissociated step (31–33). Secretion of the active 17K IL-1 $\alpha$  and IL-1 $\beta$  molecules probably occurs via a novel mechanism that is currently under investigation (34). Production of both IL-1 $\alpha$  and IL-1 $\beta$  31K precursors is apparently triggered when cells become adherent and during short-term culture, whereby the cytokines remain predominantly cell-associated (35–38). Cell-bound IL-1 has previously been quantified mainly with the use of bioassays. Since only the precursor of IL-1 $\alpha$  binds to the IL-1 receptor, such assays may not have detected the IL-1 $\beta$  precursor (39). In this connection, our inability to measure significant levels of cell-associated IL-1 $\beta$  by radioassay and ELISA could indicate that the IL-1 $\beta$  precursor is also poorly reactive in the commercial immunoassays. Clearly, there is presently a demand for reliable assays to quantify the IL-1 $\beta$  precursor.

When experiments were conducted with genetically related strains of *E. coli*, toxin producers were found to exhibit a truly remarkable cytotoxic potency that was independent of cellular factors including capsule, O-antigen and P-fimbriae. Confrontation of freshly isolated monocytes with very low numbers of toxin-producing bacteria resulted in rapid release of large amounts of IL-1 $\beta$ . These findings would be compatible with the concept that very small amounts of ECH produced by viable bacteria provoke rapid processing and release of intracellular IL-1 $\beta$ , the production of which can be due both to LPS-stimulation (in freshly isolated monocytes) and to cell cultivation. The notion that ECH can synergize with LPS in evoking IL-1 $\beta$  release was subsequently directly corroborated (Fig. 9).

The mechanisms responsible for IL-1 release from ECH-treated cells await clarification. High toxin doses applied either as isolated protein or as dense bacterial suspensions led to a decrease in measurable IL-1 $\beta$ . Therefore, IL-1 $\beta$  export may be unable to occur if cells are rapidly killed. At the same time, these results speak against simple leakage of intracellular IL-1 $\beta$  through toxin pores. According to previous estimates, such pores would indeed be too small to permit passage of such a large molecule (11–13). Possibly, low and subcytotoxic doses of ECH transiently generate a few transmembrane pores, permitting ion fluxes to take place that may be important in triggering the export machinery. IgG antibodies appear to be able to suppress the action of such low toxin doses and they may thus exert beneficial, protective effects in this regard.

The possibility that lymphocytes contaminating the monocyte preparations released lymphokines in response to attack by ECH, this in turn stimulating IL-1 $\beta$  production and liberation, appears highly unlikely. First, IL-1 $\beta$  release from cultured monocytes occurred in the presence of cycloheximide and actinomycin D, which should have effectively suppressed production of lymphokines (IL-2 or GM-CSF) by lymphocytes. Second, the kinetics of IL-1 $\beta$  release were very rapid even in the presence of these inhibitors; it is improbable that a time span of only 60 min would suffice not only to stimulate lymphokine production but also for these lymphokines to act on the monocytes. Finally, IL-1 $\beta$  release was also noted in experiments using very pure preparations of monocytes (Fig. 7).

The possibility may further be considered that the failure of freshly isolated monocytes to secrete IL-1 $\beta$  in response to ECH was due to IL-1 $\beta$  release during cell isolation, followed by unresponsive of the cells to stimuli for a few hours thereafter. However, this appears highly unlikely because monocytes isolated in our experiments readily responded to LPS stimulation (Fig. 1). Furthermore, freshly isolated monocytes also rapidly produced and released IL-1 $\beta$  when they were confronted with viable, toxin-producing *E. coli* (Fig. 8).

In sum, hemolytic *E. coli* are endowed with a remarkable capacity to kill both polymorphonuclear and mononuclear phagocytes. Toxin production paired with serum resistance thus provides the micro-organisms with uniquely effective mechanisms for counteracting major components of the host immune defence system. The cytotoxic action of ECH may extend to several other cell types including endothelial cells (40) and renal tubular epithelial cells (30). Confrontation of monocytes with just a few bacteria will result in rapid production and release of IL-1 $\beta$ . Cells previously primed to synthesize IL-1 $\beta$  precursor will probably be induced to release their content of the cytokine. It is possible that other biologically active polypeptides sharing the same pathway of secretion are liberated simultaneously. In view of the known local and systemic effects of IL-1 (41, 42), and its synergism with TNF (43, 44), such processes are probably relevant to the pathogenesis of disease caused by hemolytic strains of *E. coli* and other medically important *Enterobacteriaceae* that produce functionally related cytotoxins.

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