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Role of *Escherichia coli* Alpha-Hemolysin and Bacterial Adherence in Infection: Requirement for Release of Inflammatory Mediators from Granulocytes and Mast Cells

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We investigated the role of bacterial mannose-resistant fimbriation of S fimbriae (Fim), mannose-resistant hemagglutination (S-Mrh), and hemolysin (Hly) production by an *Escherichia coli* parent and genetically cloned strains as regards (i) their effect on histamine release from rat mast cells and (ii) generation of the chemiluminescence response, leukotriene, and enzyme release from human polymorphonuclear granulocytes. These mediators are involved in the induction of inflammatory disease processes and lead, e.g., to the enhancement of vascular permeability, chemotaxis, aggregation of granulocytes (leukotriene B_4), lysosomal enzyme release, and smooth-muscle contraction (leukotrienes C_4, D_4, and E_4). The content of azurophilic and specific granules in polymorphonuclear granulocytes consists of highly reactive enzymes which amplify inflammatory reactions. Washed bacteria (*E. coli* 764 Hly$^{\pm}$, *E. coli* 21085 Hly$^{\pm}$, *E. coli* 536 Hly$^{\pm}$ Fim$^{\pm}$ Mrh$^{\pm}$), as well as their culture supernatants, were analyzed at various times during their growth cycle. No differences exist between parent and cloned or mutant strains with respect to their outer membrane proteins and lipopolysaccharide pattern. Washed bacteria [*E. coli* 764 and 21085(pANN202-312)] which produced hemolysin, unlike Hly$^-$ strains, induced high levels of histamine release from rat mast cells and led to a significant chemiluminescence response and enzyme and leukotriene release from human polymorphonuclear granulocytes. Bacterial culture supernatants from Hly$^+$ and secreting strains showed similar results with the exception of *E. coli* 21085(pANN202-312), which is a hemolysin-producing but not a secretory strain. Our data suggest a potent role for hemolysin as a stimulus for noncytotoxic mediator release from various cells. Furthermore, we showed that the presence of Fim and S Mrh potentiates mediator release. The simultaneous presence of Mrh and Fim [*E. coli* 535/21(pANN801-4)] increased mediator release compared with Mrh$^+$ Fim$^-$ strains [*E. coli* 536/21(pANN801-1)]. *E. coli* 536/21 (Msh$^-$ Mrh$^-$ Fim$^-$ Hly$^-$) did not induce mediator release.

Escherichia coli alpha-hemolysin is a protein that causes in vitro lysis of erythrocytes from several species of animals (6, 12, 16–18, 23). Hemolysin-producing *E. coli* strains occur only infrequently in the normal fecal flora of humans but are often isolated from patients with extraintestinal infections such as urinary tract infections, bacteremia, and septicemia (13, 22, 25, 36–38, 46–48). The high percentage of Hly$^+$ *E. coli* strains among isolates from patients with urinary tract infections suggested that hemolysin contributes to the virulence of *E. coli* strains. The role of hemolysin as a virulence factor has been recently demonstrated by using various animal models and cell cultures. Alpha-hemolysin is one of the very few proteins produced by members of the family *Enterobacteriaceae* that is released extracellulary. The genetic control of alpha-hemolysin production, transport, and release from cells is complex (24, 26, 30). At least four genes located on the bacterial chromosome or on large transmissible plasmids are required to elicit a cell-free hemolytic phenotype. Bohach and Snyder (6) suggested that the existence of alpha-hemolysin complexed with lipopolysaccharide may have important implications in the understanding of its biological effects.

In addition to hemolysin production, a variety of factors, e.g., fimbriae, expression of specific hemagglutination, and O and K antigens, may contribute to the virulence of *E. coli* strains (35, 44). In this respect, adherence to host cells represents the initial step in the course of bacterial infection. Adherence is mediated by fimbriae and agglutinins (4, 6, 15, 19–21, 42).

The adherence may or may not be inhibited by α-mannosides and is then termed mannose sensitive (MS) or mannose resistant (MR). Furthermore, the fimbriae can be subdivided according to their receptor requirement for adhesion. MS fimbriae, which have been termed common or type 1 fimbriae, recognize manno-oligosaccharide-containing glycoproteins. MR fimbriae can be subdivided according to their different receptor specificities: the P fimbriae of urinary-tract-infective *E. coli* strains recognize α-galactosyl-1,4-β-galactose as a receptor; S fimbriae recognize complex carbohydrates terminating in *o*-*N*-acetylneuraminyl-*N*-acetyl-galactosamine; MR fimbriae for which the receptors are not yet characterized are termed X fimbriae.

There is only a small amount of information about the membrane biochemical events induced by adherent and hemolysin-producing bacteria. We recently provided evidence that mediators of inflammation, e.g., histamine and leukotrienes, are released from rat mast cells and human granulocytes on interaction with hemolysin-producing *E. coli* strains (43). The simultaneous presence of MR$^+$ (type Vb), pili, and hemolysin production led to an increase in

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mediator release as compared with MR^- Hly^+ strains. Leukotriene B_4 (LTB_4) was shown to be chemotactic for human neutrophils and eosinophils (7, 9, 41). LTC_4, LTD_4, and LTE_4 were identified as the slow-reacting substance of anaphylaxis, which is involved in bronchoconstriction and mucus production. Granulocytes, mononuclear cells, and macrophages release large amounts of leukotrienes on activation with various stimuli, e.g., the calcium ionophore A 23187, bacterial exo- and endotoxins, and phagocytosis (10–13, 33, 39). Hyperemia, increase in vascular permeability, and granulocyte accumulation and activation are prominent signs during bacterial infections and may well be attributed to the release of the above-described mediators (10–13, 33). In our studies, human polymorphonuclear neutrophils (PMNs) were used as target cells for the analysis of the respiratory burst, as well as lipoxygenase transformation products of arachidonic acid; rat mast cells were studied as the source of histamine release (32, 43). The purpose of the present investigation was the analysis of the role of hemolysin as a pathogenicity factor and the definition of the properties which induce mediator release from the cells. Our previous observations were further extended by analysis of the role of fimbriae and MR hemagglutination with regard to mediator generation from the various cells.

(This research was conducted in partial fulfilment of the Ph.D. thesis of B. König, Ruhr-Universität Bochum, Bochum, Federal Republic of Germany)

MATERIALS AND METHODS

Buffers. Unless stated otherwise, the medium used for washing the cells and for mediator release was a 0.025 M Tris buffer (pH 7.35) containing 120 mM NaCl, 4 mM KCl, 0.6 mM $CaCl_2$, and 1 mM $MgCl_2$. This buffer is referred to as TCM buffer (Tris, calcium, magnesium) (41). Bacteria were grown in brain heart infusion broth (Oxoid Ltd.), which consisted of calf brain heart infusion solids (12.5 g), beef heart infusion solids (5.0 g), Proteose Peptone (20.0 g), sodium chloride (5.0 g), glucose (2.0 g), and disodium phosphate (2.5 g). Distilled water was added to 1,000 ml.

Preparation of cells. Human leukocytes were obtained from heparinized blood of healthy donors and separated on a Ficoll-metrizoate gradient followed by dextran sedimentation (8). This method leads to >90% pure PMNs. The cells were then washed at low speed (600 rpm) three times to remove the platelets. Less than 2% of the platelets were detected. The purity of the PMN fraction was determined by light microscopy. The erythrocytes were removed by hypotonic exposure of the cell suspension. For the collection of rat peritoneal cells, Wistar rats were bled and then received an intraperitoneal injection of Tyrode buffer (32). Their abdomens were massaged, and the buffer was recovered. After centrifugation (20 min at 600 rpm), the cells from several rats were pooled. The cell suspension contained 3 to 5% mast cells.

Bacterial strains. Cloning and functional characterization of the plasmid-encoded determinants was performed at the Institut für Genetik und Mikrobiologie, Universität Würzburg, Würzburg, Federal Republic of Germany (24, 26–30). *E. coli* 536 was the parent strains for the *E. coli* 536 variants. *E. coli* 536 (O6:K15:H31) was isolated from a patient with urinary tract infection; this strain exhibits MR (type Vb) and MS (type I) hemagglutination and produces hemolysin. Strain 536/21 is a spontaneous mutant of strain 536 that has lost the ability to produce hemolysin, the Mrh phenotype, and the MS hemagglutination. *E. coli* 536/21 was transformed with recombinant plasmids to *E. coli* 536/21 (pANN801-1), expressing only MR hemagglutination but no visible fimbriae, and also to *E. coli* 536/21(pANN801-4), which showed MR hemagglutination and production of S fimbriae. *E. coli* 536/31(pANN202-312) showed the MR and MS phenotypes, was Hly^+, and expressed fimbriae.

E. coli 764/2 is a mutant of *E. coli* 764 which has lost only the hemolysin determinant. The strain expresses MR hemagglutination of type VIc. *E. coli* 21085 (Hly^-) was transformed with plasmid pANN202-312 (*hlyC*, *hlyA*, *hlyB*, *hlyD* from pHly152) to *E. coli* 21085(pANN202-312) (Hly^+). Owing to a mutation in the bacterial chromosome, this strain has lost the ability to release hemolysin into the medium.

Bacterial growth. Brain heart infusion broth (10 ml) was inoculated with 100 μl of an overnight culture; bacterial growth proceeded for 3.5 h at 37°C on a shaker (150 rpm) unless otherwise stated. Subsequently, the bacteria were centrifuged at 4,000 × *g* for 15 min, separated from the culture supernatant, and washed in TCM buffer. For the actual experiments, washed bacteria at the stated concentrations, as well as the bacterial culture supernatants obtained during the late logarithmic phase of bacterial growth, were studied. The bacterial culture supernatant was analyzed for its hemolysin activity and protease and endogenous histamine contents.

Hemolysin assay. The production of hemolysin was tested on sheep blood agar plates. Strains having a clear halo after overnight culture at 37°C were defined as hemolysin positive. A quantitative hemolysin assay was performed as described previously (24, 43). Cells were grown in brain heart infusion broth (10 ml; Oxoid) at the appropriate temperature in a shaking water bath up to 2.5×10^9/ml. The cells were pelleted at the late logarithmic phase or, as indicated in Results, by centrifugation at 4,000 × *g* and separated from the supernatant. The cells (2.5×10^{10}) were suspended in 500 μl of 10 mM Tris buffer (pH 7.4) with 25% sucrose–40 mM EDTA–10 μg of lysozyme per ml. After 30 min on ice, the cells were pelleted and the supernatant (labeled periplasmic) was removed. The pellet was resuspended in 500 μl of buffer and lysed by sonication (five 10-s bursts with a Branson ultrasonifier). For the hemolysin assay, a 10 mM Tris buffer (pH 7.4) with 20 mM $CaCl_2$–106 mM NaCl in a final volume of 1,000 μl was used; 2% washed sheep erythrocytes and 10 μl of sonic extract (cytoplasmic) or EDTA-lysozyme supernatant (periplasmic) or 200 μl of culture supernatant (extracellular) were incubated at 37°C. Samples were removed after 1, 3, 5, 10, and 60 min and placed on ice. Unlysed erythrocytes were removed by centrifugation (1 min in an Eppendorf centrifuge). The optical density of the supernatant at 420 nm was determined. Hemolysin activity is expressed as the increase in optical density per minute or, when indicated, as percent lysis compared with the control (e.g., water lysis of erythrocytes). Hemolysin activity present in the outer membranes of washed bacteria was determined by the amount of hemoglobin released from erythrocytes. The reaction mixture (total, 1 ml) contained 800 μl of 10 mM Tris buffer (pH 7.4) with 20 mM $CaCl_2$, 160 mM NaCl, 2% washed sheep erythrocytes, and 200 μl of bacterial suspension (5×10^8/ml). The assay tubes were incubated at 37°C for 30 min. The amount of released hemoglobin was measured as described above.

Leukotriene release from human PMNs. Human PMNs (2×10^7) were suspended in 1,000 μl of TCM buffer. For stimulation, bacterial cell suspension (100 μl; 10^9 bacteria) or bacterial culture supernatant (50 μl) was added to the cells

and incubated as described in Results. The supernatant of stimulated cells (1,000 μl) was analyzed for leukotrienes by high-pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) (1, 31, 41).

Analysis of 5-lipoxygenase activity. The reaction mixture contained PMNs (10^7 cells in 500 μl of phosphate-buffered saline), 50 μl of 2 mM $CaCl_2$, 50 μl (0.1 μCi) of [^{14}C]arachidonic acid, and 50 μl of the stimulus (45). Incubation proceeded for 1, 5, 10, and 15 min at 37°C. The reaction was terminated by addition of 2 ml of acidified methanol (0.01% acetic acid, vol/vol). Denaturated protein and cell debris were removed by centrifugation, and the clear supernatants were extracted twice with 3.5 ml of chloroform. To minimize losses of radioactive material that was poorly extractable with methanol, the pellet was additionally extracted with 1 ml of chloroform-methanol (2:1, vol/vol). The losses of radioactivity within the final pellet and the aqueous phase determined by liquid scintillation accounted for 4 to 5% (Packard Tri-Carb liquid scintillation spectrometer 3244; external standardization). The pooled extracts were evaporated to dryness under nitrogen; the residues were dissolved in 80 μl of chloroform and spotted onto a silica gel thin-layer plate (250 μm, Kieselgel 60; E. Merck AG, Darmstadt, Federal Republic of Germany). Arachidonic acid, 5-hydroxyeicosatetraenoic acid (5-HETE), LTB_4, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol served as references. The plates were developed once with solvent system 1 (organic phase, ethyl acetate–2,2,4-trimethyl pentane–acetic acid–water [55:25:10:50, vol/vol]). Solvent system 2 (chloroform-methanol-acetic acid-water [90:10:1:0.65, vol/vol]) was used for rechromatography of LTB_4 and 5-HETE. Radioactivity was detected with an Isomess Radio-Dünnschicht-Analysator IM 3,000 (Isotopenmessgeräte GmbH, Straubenhardt, Federal Republic of Germany).

Identification of [^{14}C]arachidonic acid products. The synthesis of [^{14}C]arachidonic acid metabolites identified as 5-HETE and LTB_4 was completely inhibited by known inhibitors of lipoxygenase activity such as BW 755 C (30 μg/ml), esculetin (0.5 mM), and caffeic acid (0.1 mM) (34, 45). The cyclooxygenase inhibitor indomethacin (0.1 μM) did not show any effect. Values of the 50% effective dose obtained by dose-response curves correlated well with those published elsewhere. 5-HETE and LTB_4 were further characterized by cochromatography with authentic 5-[^3H]HETE and [^3H]LTB_4 with solvent system 1 and rechromatography with solvent system 2.

Determination of marker enzymes and protein. Lysozyme (EC 3.2.1.17), β-glucuronidase (EC 3.2.1.31), myeloperoxidase (EC 1.11.1.7), lactate dehydrogenase (LDH) (EC 1.1.1.27) (3, 12), and protein were determined as previously described (41).

Analysis of leukotriene release. For analysis of leukotriene release, the supernatant of the stimulated cells was deproteinized by the addition of 2 volumes of acidified methanol (methanol-acetic acid [1,000:1, vol/vol]), overlaid with argon, and frozen at −70°C for 12 h. After centrifugation at 3,000 × *g*, the supernatants were evaporated to dryness under a stream of nitrogen and suspended in 400 μl of methanol-water (30:70, vol/vol) for reverse-phase HPLC (10–13, 31, 41); HPLC analysis was performed with a Nucleosil C 18 column (5-μm pore size, 4 by 200 mm; Macherey Nagel, Düren, Federal Republic of Germany) with methanol-water-acetic acid (64:36:0.98, vol/vol/vol; pH 5.9); the titer was determined with ammonia as the eluent. Consta Metric pumps I and III (LDC Laboratory Water Control; Milton Roy) and the automatic sample injection module WISP 710 B (Waters Associates, Inc., Milford, Mass.) were used. The A_{280} of the volume effluent was monitored with a variable UV detector (LDC Spectromonitor III 1204 A). The peak area or height was calculated with an LDC Computing Integrator 301. The chromatograms were recorded with a printer plotter (LDC). Under these conditions, the retention times were 19.50 to 20.00 min for LTB_4, 11.30 to 11.70 min for LTC_4, 17.20 to 17.60 min for LTD_4 and 6-*trans* LTB_4, and 20.60 to 21.50 min for LTE_4. Identification of leukotrienes was assessed by determining the retention time and comparing it with those of external standards of synthetic leukotrienes (gift from J. R. Rokach, Merck Frosst, Quebec, Canada), by RIA, and by UV absorbance (see below). The area integration of the absorption peaks allows quantitative analysis of the substances. By using the described extraction procedure, the recovery rates of leukotrienes from 250 μl of cell supernatant were determined to be 80 to 85% for LTC_4, LTD_4, and LTE_4 and 90 to 95% for LTB_4 (31, 41). The standard curve for the individual leukotrienes was obtained with five different concentrations (5 to 125 ng) and showed the following correlations: LTC_4, 0.985; LTD_4, 0.995; LTE_4, 0.985; LTB_4, 0.999; and 6-*trans* LTB_4, 0.998. The minimum detectable quantity was 1 ng for the various leukotrienes.

RIA for LTC_4 and LTB_4. In addition to HPLC analysis, the cell supernatants were studied by RIA for LTC_4 and LTB_4 (1, 41, 43). A 50-μl sample of the supernatants was suspended in 30% methanol and evaporated to dryness under nitrogen. The material was then suspended in 100 μl of Tris buffer (0.1 M) containing 0.1% gelatin. An appropriate antiplasma dilution, as well as synthetic LTC_4, LTB_4 at concentrations from 10 ng to 25 pg, or unknown samples, was added to tubes containing [^3H]LTB_4 or [^3H]LTC_4 in a total volume of 0.6 ml. After incubation at 4°C overnight, antibody-bound and free ligands were separated with 0.5 ml of charcoal suspension (20 mg/ml) for 2 h at 37°C. After charcoal precipitation by centrifugation, 0.9 ml of the supernatants was added to 9 ml of Scintigel (Roth, Karlsruhe, Federal Republic of Germany). The radioactivity was determined in a liquid scintillation counter. The minimal quantity detected was approximately 20 pg for LTB_4. For the LTC_4 determination, the cross-reactivity with LTD_4 was <35%; for LTB_4 and LTE_4, the cross-reactivity was <2%. The RIA for LTB_4 was obtained from Wellcome Research Laboratories, Beckenham, England. The antiserum reacted with the isolated LTB_4 isomers. The correlation of the results obtained by HPLC analysis and RIA was determined to be $r = 0.75 \pm 0.1$ for LTC_4 and $r = 0.74 \pm 0.08$ for LTB_4. In addition to the biochemical and radioimmunological analyses, the leukotrienes in selected fractions were identified by their characteristic UV spectra (Lambda 5 UV Vis spectrophotometer; The Perkin-Elmer Corp., Norwalk, Conn.), as well as by bioassay. LTC_4, LTD_4, and LTE_4 (slow-reacting substance of activity) in selected fractions were analyzed by their capacity to contract guinea pig ileum, as described above.

Chemiluminescence. Chemiluminescence was measured at 37°C in a Lumacounter M 2080 (Lumac). Samples for chemiluminescence were obtained by adding a PMN suspension (0.05 ml, 10^6 cells) to polypropylene tubes containing TCM (300 μl) and luminol (20 μl, 0.25 mM). The tubes were placed in the lumacounter and allowed to stand until a stable background was obtained (15 min). To activate the system, 50 μl of the stimulus (whole bacteria) was added. The tubes were stirred, and the light emission was recorded continuously.

TABLE 1. Histamine release from rat mast cells and enzyme release and leukotriene generation from human PMNs by *E. coli* strains

Strain	Hemolysin release (%)	% Release of:				Generation (ng) of:		Histamine release (%)	Chemiluminescence (10^4 cpm)
		β-Glucuronidase	Lysozyme	LDH	Peroxidase	LTC_4[a]	LTB_4[a]		
E. coli 764 (Hly^+) (O18:K5:H)	64.8	7.04	24.8	6.7	21.0	12.6	4.9	41.7	2.5
E. coli 764/2 (Hly^-) (O18:K5:H)	0	2.6	12.0	8.3	10.5	0	0.8	14.8	0
E. coli 21085 (Hly^-) (O75:K:H5)		5.0	20.0	6.0	7.5	0.15	0	11.7	0
E. coli 21085(pANN202-312) (Hly^+ Cm^+) (O75:K:H5)	68.0	12.0	58.0	1.0	30.0	15.0	5.3	52.8	7.8
E. coli 536/31(pANN202-312) (Mrh^+ Hly^+ Fim^+) (O6:K15:H31)	87.0	0.98	24.0	2.8	43.5	22.9	5.1	61.4	8.7
E. coli 536/21(pANN801-4) (Mrh^+ Hly^- Fim^+) (O6:K15:H31)	0	0.44	22.0	4.3	23.9	7.9	4.9	24.8	0
E. coli 536/21(pANN801-1) (Mrh^+ Hly^- Fim^-) (O6:K15:H31)	0	0.55	19.0	5.0	22.8	3.6	1.5	10.3	0

[a] Leukotriene analysis was carried out by HPLC and RIA; the standard deviation of the RIA ranged between 7 and 11%. The experiments were carried out three times in duplicate; a similar pattern was obtained for each experiment. One representative experiment is shown.

Histamine release. Rat peritoneal cells (5 to 10% mast cells) (31) were suspended in 2 to 20 μl of TCM buffer, and 100 μl of bacterial suspension was added. Incubation proceeded for 30 or 60 min at 37°C. Cells were centrifuged for 10 min at 300 × *g*; the supernatant was removed and deproteinized by the addition of 2 ml of 2% $HClO_4$. The supernatant was subsequently centrifuged at 1,000 × *g* for 10 min, and the histamine content was analyzed by the fluorophotometric analyzer technique (43). Cells in the presence of buffer and bacterial supernatant at the appropriate dilutions served as controls.

RESULTS

Characterization of hemolysin activity and functional properties. The hemolysin activity of the various *E. coli* strains within the bacterial culture supernatant (extracellular) and in the periplasm and cytoplasm was determined at various times during cell growth. An accumulation of extracellular hemolysin was obtained up to the mid-logarithmic phase; the amount of hemolysin then decreased and reached a low level when the cells entered the stationary phase. The hemolysin activity within the medium exceeded the amounts present in the periplasm and cytoplasm when the hemolysin-producing *E. coli* 764 and 536/31(pANN202-312) were analyzed (data not shown). In contrast, *E. coli* 764/2, 21085, 536/21(pANN801-4), and 535/21(pANN801-1) expressed no hemolysin activity in the culture supernatant (Table 1).

Studies on chemiluminescence and lysosomal enzyme release. Washed bacteria were also studied with regard to their capacity to induce chemiluminescence from human granulocytes; the maximal responses obtained after a kinetic stimulation were plotted. Hemolysin-producing bacteria induced chemiluminescence from human polymorphonuclear granulocytes to a remarkably higher degree than did Hly^- bacteria (Table 1). The induction of chemiluminescence was dependent on the time and on the bacterial cell count (data not shown).

Experiments were then carried out to study the release of granular (lysozyme, peroxidase, β-glucuronidase) and cytosolic (LDH) enzymes from human polymorphonuclear granulocytes. PMNs (10^7) were incubated with 50 μl of washed bacteria (5 × 10^8/ml) obtained at the beginning of the late-logarithmic phase. When the release of β-glucuronidase was studied, enzyme activity in the supernatant of stimulated granulocytes correlated well with the presence of hemolysin-producing strains (Table 1). With regard to peroxidase generation, Hly^+ strains in general produced more peroxidase than did Hly^- strains; however, the presence of the Mrh phenotype appeared to favor peroxidase release. *E. coli* 536/21(pANN801-4) (Mrh^+ Hly^- Fim^+), as well as *E. coli* 536/21(pANN801-1) (Mrh^+ Hly^- Fim^-), generated nearly as much peroxidase as did Hly^+ *E. coli* 764. When the release of lysozyme was studied, a similar pattern as that for peroxidase generation was observed. While clear differences were obtained for the Hly^+ strains [764, 21085(pANN202-312)] as compared with the Hly^- strains (764/2, 21085), different results were observed for *E. coli* 536/31(pANN202-312), 536/21(pANN801-4), and 536/21(pANN801-1). The last three strains induced similar amounts of lysozyme activity independent of their hemolysin production. Similar results were obtained with *E. coli* 21085 (Hly^-). Negligible amounts of LDH were liberated from the neutrophils, suggesting that the release process occurs under noncytotoxic conditions. No differences existed between Hly^+ and Hly^- strains.

Release of 5-HETE and leukotrienes. Human PMNs labeled with [^{14}C]arachidonic acid were incubated for various times with washed bacterial cells or with the culture supernatant

TABLE 2. Kinetics of [^{14}C]arachidonic acid metabolism

Incubation time (min)	Generation[a] (pmol/assay) of 5-HETE and LTB_4 from strain:							
	E. coli 21085		*E. coli* 21085(pANN202-312)		*E. coli* 764/2		*E. coli* 764	
	5-HETE	LTB_4	5-HETE	LTB_4	5-HETE	LTB_4	5-HETE	LTB_4
1	5.45	1.80	3.0	3.02	4.83	1.7	3.01	4.77
5	12.5	2.35	1.0	5.30	12.5	2.45	1.28	15.86
10	3.9	1.13	2.0	7.22	3.4	1.33	2.84	12.47
15	1.80	0.66	2.22	3.66	2.2	0.86	2.48	6.30

[a] Each value represents the mean of three experiments; the standard error of the mean ranged from 4 to 8%.

TABLE 3. Hemolysin-dependent leukotriene generation by washed *E. coli* cells

Incubation time (h)	*E. coli* 21085(pANN202-312)			*E. coli* 764			*E. coli* 536/31(pANN202-312)		
	Hemolysin activity (%)[a]	Generation[b] (ng) of:		Hemolysin activity (%)	Generation (ng) of:		Hemolysin activity (%)	Generation (ng) of:	
		LTC_4	LTB_4		LTC_4	LTB_4		LTC_4	LTB_4
2.5	61.90	12.0	2.5	48.57	4.3	5.0	87.00	22.9	5.1
3.0	68.09	15.0	3.2	64.76	12.6	5.0	73.00	21.5	4.5
3.5	44.76	7.0		31.21	0.0	1.1	72.00	19.9	5.0

[a] Hemolysin activity is expressed as a percentage of the control (water lysis of erythrocytes).
[b] Leukotriene levels were analyzed by HPLC (below 2 ng, by RIA). The experiment was repeated three times in duplicate; the data represent one individual experiment. The standard deviation of HPLC or RIA ranged between 4 and 11%.

(Table 2). As is apparent, the Hly^- *E. coli* strains generated significant amounts of 5-HETE and low amounts of LTB_4. The Hly^+ strains such as *E. coli* 764 and 21085(pANN 202-312) induced significant amounts of LTB_4 and low amounts of 5-HETE. These data were also confirmed when bacterial culture supernatants were analyzed. Again, the Hly^+ strains induced more LTB_4 than 5-HETE (data not shown).

Studies were then carried out to analyze the release of leukotrienes from human PMNs induced by the various strains. Bacteria cultured overnight were further grown on a shaker for 2.5, 3.0, and 3.5 h. A sample of the washed bacteria was then analyzed for hemolysin activity, as well as for its capacity to induce LTC_4 and LTB_4 release from neutrophils (Table 3). For this purpose, human PMNs were stimulated for 15 min at 37°C. As is shown for *E. coli* 764, bacteria obtained after 3 h expressed more hemolysin activity than did bacteria after 2.5 and 3.5 h, respectively. LTC_4 and LTB_4 levels markedly decreased when bacteria expressed lower amounts of cell-bound hemolysin activity. Similar results were obtained with *E. coli* 21085(pANN202-312). Bacteria obtained after 3.5 h of growth expressed low hemolysin activity. As is apparent, the capacity to release leukotrienes decreased significantly compared with that of the samples obtained after 2.5 and 3 h of growth. With *E. coli* 536/31(pANN202-312), the activity of the cell-bound hemolysin remained constant over the various time points; as is shown, the bacteria obtained after various times of growth induced similar amounts of leukotrienes.

Kinetic experiments on leukotriene release were performed with *E. coli* 536/31(pANN202-312), 536/21 (pANN801-4), and 536/21(pANN801-1) (Table 4). Washed bacteria or bacterial culture supernatant were applied as stimuli. Optimal LTB_4 release from human PMNs already occurred after 5 min of incubation and further declined over time. LTC_4 release steadily increased up to 10 and 15 min of incubation. When bacterial culture supernatants were analyzed, Hly^+ strains induced leukotriene release. The supernatants of Hly^- strains, e.g., *E. coli* 536/21(pANN801-4) and *E. coli* 536/21(pANN801-1), expressed no activity (Table 4).

Studies on histamine release. Our studies were then directed toward the analysis of histamine release from rat mast cells. For this purpose, the various *E. coli* strains or their bacterial culture supernatants were incubated with rat peritoneal mast cells (2.5×10^5). It became apparent that increasing the amounts of cell-bound hemolysin led to an enhanced release of histamine (data not shown).

Table 1 summarizes the various parameters. Histamine release, enzyme secretion, and leukotriene generation induced by washed bacteria were analyzed from mast cells and human PMNs. In general, it is obvious that Hly^+ strains induce higher amounts of histamine and leukotrienes than do Hly^- strains, as is shown for *E. coli* 764 and *E. coli* 764/2. Our studies also suggest that the presence of bacterium-bound hemolysin activates the various cells for enzyme and mediator release, as is shown for *E. coli* 21085(pANN202-312) and *E. coli* 21085 (Table 1). The simultaneous presence of MR hemagglutination and S fimbriae [*E. coli* 536/21(pANN801-4) (Mrh^+ Hly^- Fim^+)] enhanced mediator release as compared with the situation for *E. coli* 536/21(pANN801-1) (Mrh^+ Hly^- Fim^-), which expressed no fimbriae. *E. coli* 536/21 (Msh^- Mrh^- Hly^-) was inactive in all assays and did not induce mediator release (data not shown). When the bacterial culture supernatants of the various strains under study were analyzed, it became apparent that only hemolysin-secreting bacteria were potent inducers for histamine and leukotriene release (data not shown).

DISCUSSION

Our data extend the previous observations that the alpha-hemolysin of *E. coli* represents a potent pathogenicity factor by inducing the release of inflammatory mediators from various cells (43). The simultaneous presence of Mrh and

TABLE 4. Kinetics of leukotriene release from human PMNs by bacteria and supernatants[a]

Incubation time (min)	Amt (ng) released by *E. coli* 536/31(pANN202-312)				Amt (ng) released by *E. coli* 536/21(pANN801-4) bacteria[b]		Amt (ng) released by *E. coli* 536/21(pANN801-1) bacteria[b]	
	Bacteria		Supernatant					
	LTC_4	LTB_4	LTC_4	LTB_4	LTC_4	LTB_4	LTC_4	LTB_4
1	1.2	5.3	0.3	8.0	0.25	1.2	0.1	0.25
5	10.3	45.2	12.0	39.2	2.3	7.1	1.2	4.1
10	28.2	38.4	18.3	20.1	5.2	4.94	2.3	3.0
15	25.5	15.3	18.5	6.1	5.0	4.0	1.5	2.64

[a] Analysis was carried out by HPLC (RIA if the values were lower than 2 ng). The standard deviation of the RIA was 7 to 11%. The experiment was repeated three times in duplicate; the data represent one individual experiment.
[b] Release by supernatant was not determined.

MR-S fimbriae enhanced mediator release. We also provide evidence that cell-bound hemolysin, as well as the hemolysin within the bacterial culture supernatant, is active. *E. coli* 21085(pANN202-301), which expressed cell-bound hemolysin activity but did not release the molecule owing to a mutation, was already a potent stimulus; these results suggest that the cell-bound hemolysin is expressed in its biological form.

It has been suggested that at least four genes are required to elicit a cell-free hemolytic phenotype (6, 16, 17, 24, 26, 30). The hemolysin structural gene (*hlyA*) codes for a 106,000- to 110,000-dalton protein that can be detected within the cytoplasm of *E. coli* cells. Quite recently, it was shown that active alpha-hemolysin had a larger size and lower pI than that predicted by analysis of the *hlyA* gene sequence, thus suggesting that the *hlyA* protein is complexed with other bacterial products. In fact, lipopolysaccharide was detected in purified hemolysin complex preparations (6).

The release of mediators induced by cell-bound hemolysin, as well as Hly^+ bacterial culture supernatant, occurs under noncytotoxic conditions, as was assessed by the absence of LDH release from the cells. When the kinetics of mediator release were studied, it became apparent that for histamine, plateau values occurred after 60 min of incubation.

Interestingly, Hly^- strains led to an activation of the 5-lipoxygenase pathway leading to the stable product 5-HETE, which expresses less chemotactic activity on a molar basis than does LTB_4. It has been shown that the 5-lipoxygenase enzyme transforms arachidonic acid into 5-HPETE, which is reduced to 5-HETE (7, 9, 33). 5-HETE is metabolized by an LTA_4 synthetase into LTA_4. LTA_4 is converted via an LTA_4 hydrolase into LTB_4 and via a glutathione-*S*-transferase into LTC_4. LTC_4 is then transformed into LTD_4 and LTE_4 by a γ-glutamyltranspeptidase and dipeptidase, respectively (41). These metabolites exhibit a different pattern as regards the smooth-muscle response. Since the sensitivity of HPLC is in the range of 1 to 2 ng for LTD_4 and LTE_4, these metabolites, probably as a result of their low concentrations and lack of specific antisera, were not detected in the stimulated-cell supernatants. However, we recently demonstrated that the enzymes transforming the peptido-leukotriene LTC_4 into LTD_4 and LTE_4 are released into the supernatant of stimulated granulocytes (12, 41).

Our data thus provide evidence that Hly^+ strains activate the LTA_4 hydrolase and glutathione-*S*-transferase for the generation of LTB_4 and LTC_4, respectively. For LTB_4 release, a maximal response was obtained after 5 min of incubation. At later times, a sudden decline occurs, which is explained by the conversion of the highly chemotactic LTB_4 into the less active or inactive 20-hydroxy- and 20-carboxy-LTB_4. These compounds exhibit a retention time by HPLC analysis which is different from that of LTB_4. For LTC_4, plateau values were obtained after 10 to 15 min of incubation. Thus, it is obvious that LTC_4 levels exceeded the amount of LTB_4 at later times of stimulation (15 versus 5 min). The total amount of LTB_4 is about one-third of that generated by the Ca ionophore and exceeds the values obtained with opsonized zymosan or N-form-Met-Phe-Leu. It is unlikely that in our experiments this bacterial chemotactic peptide is of importance (43). The Hly^- bacterial culture supernatants were inactive in leukotriene release. Whether the LTB_4 and LTC_4 released are solely derived from the neutrophil or eosinophil granulocyte is under study (41).

Our results therefore suggest that the interaction of Hly^- *E. coli* strains with human polymorphonuclear granulocytes leads to a less pronounced activation of the cells with the release of the less chemotactic factor 5-HETE. This chemotactic event may already be sufficient to eliminate Hly^- bacteria by phagocytosis. Furthermore, the Hly^- strain *E. coli* 764/2, which expresses MR fimbriae, induced an insignificant chemiluminescence response, if any, from human PMNs. Similar results have been published previously for Mrh^- strains (5, 40). In contrast, *E. coli* 764, which has the same genetic background as *E. coli* 764/2 but is Hly^+ leads to a pronounced chemiluminescence response. A similar pattern was obtained when the release of lysosomal enzymes (lyoszyme, β-glucuronidase, peroxidase) was studied. Thus, Hly^+ *E. coli* strains are potent inducers for the release of inflammatory mediators. This release occurs under noncytotoxic concentrations. The highly chemotactic LTB_4 is generated from neutrophils, thus providing a further influx of granulocytes. The concomitant release of LTC_4 may potentiate an inflammatory event such as an increase in permeability. Histamine and the lysosomal enzymes will certainly contribute to inflammation and tissue destruction. These events are obviously more pronounced when bacteria possess MR adhesins and express S fimbriae. Thus, although hemolysin by itself already represents a potent pathogenicity factor, additional properties of the bacteria such as adherence may substantially potentiate the effect of hemolysin. The fact that the decay rate of cell-bound hemolysin is much slower than that of the secreted molecule provides the bacteria with the property to induce an inflammatory response, thus also facilitating bacterial invasion. It is of particular interest to evaluate whether these above-described assay systems will permit further insight into the event of colonization and bacterial invasion.

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