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Bacterial Adherence and Hemolysin Production from *Escherichia coli* Induces Histamine and Leukotriene Release from Various Cells

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We investigated the role of bacterial adherence and hemolysin production from *Escherichia coli* parent and genetically cloned strains as to their effects on histamine release from rat mast cells and leukotriene generation from human polymorphonuclear granulocytes. These mediators were involved in the induction of inflammatory disease processes and led, for example, to enhancement of vascular permeability, chemotaxis (leukotriene B_4 [LTB_4]), chemoaggregation, lysosomal enzyme release, and smooth muscle contraction, (LTC_4, LTD_4, and LTE_4). Washed bacteria (*E. coli* K-12 MS^+ $Hly^\pm$; *E. coli* 536 MS^+ $MR^\pm$ $Hly^\pm$) as well as their culture supernatants were analyzed. Washed *E. coli* K-12 (Hly^+), unlike Hly^- strains, induced high amounts of histamine release from rat mast cells and chemotactic activity from human polymorphonuclear granulocytes. Significant leukotriene release was obtained with washed *E. coli* K-12 Hly^+ strains and their bacterial culture supernatants. Leukotriene induction was dependent on the amount of hemolysin activity present in the supernatant. However, additional soluble factors should also be considered. The presence of hemolysin appeared to accelerate and enhance the rate of phagocytosis of bacteria by neutrophils. When *E. coli* 536 (MS^+ $MR^\pm$ $Hly^\pm$) strains were analyzed, the simultaneous presence of MR^+ pili and hemolysin production led to an increase in histamine release as compared with MR^- Hly^+ strains. The genetically cloned MR^+ Hly^+ *E. coli* 536 strain induced higher amounts of leukotrienes as compared with the wild-type strain. Our data suggest a potent role for adhesins and hemolysin as virulence factors in inducing the release of inflammatory mediators.

The role of hemolysin in the virulence of *Escherichia coli* has been recently demonstrated in various animal models and cell cultures (6–8, 13, 20, 29, 33, 39). In a hematogenous, nephropathogenic mouse model, the nephropathogenicity of a nonhemolytic, avirulent *E. coli* strain was increased by simultaneous injection with its hemolytic, nephropathogenic parent (26, 37). In general, hemolytic *E. coli* strains may be isolated from a variety of sources (15, 17). In humans, the normal fecal flora contains about 12% Hly^+ *E. coli* compared with 35 to 50% causing extraintestinal infections such as bacteremia, septicemia, and urinary tract infections (10, 11, 18, 28). Among *E. coli* strains causing urinary tract infections, the production of hemolysin is often associated with MR hemagglutination caused by specific protein pili and specific O and K antigens (2, 9, 19, 21, 25, 35). These additional factors induce the adherence of bacteria to epithelial cells and resistance to serum and phagocytosis (32). Hly^+ *E. coli* strains isolated from humans usually carry the Hly determinant on the chromosome, unlike *E. coli* strains from animal sources (1, 30, 38); these genes have been shown to be located on large transmissible plasmids (14, 18). Quite recently, it has been shown that cloned hemolysin genes from *E. coli* that cause urinary tract infection determine different levels of toxicity in mice (15). The authors described that deletion of the chromosomal hemolysin (Hly) determinant in an *E. coli* O6:K15:H31 urinary tract infection isolate led to a significant reduction in toxicity for mice (16); its reintroduction in a recombinant plasmid partially restored the original toxicity. Although the introduction of the wild-type plasmid pHly152-encoded Hly determinant into the Hly^- *E. coli* O6 mutant strain increased toxicity by only a marginal degree, transformation with the cloned chromosomal Hly determinants from two *E. coli* strains of serotypes O18ac:K5:H^- and O75:K95:H? resulted in marked toxicity even exceeding that of the original Hly^+ *E. coli* O6 wild-type strain.

Only few data exist that describe by which mechanism the pathological reactions of hemolysin-producing *E. coli* strains are initiated. It has been suggested that Hly^+ *E. coli* in the presence of plasma are cytotoxic for various cells (13). Welch et al. reported that an isolated, DNA sequence-encoding hemolysin added by recombinant DNA technology to avirulent, nonhemolytic, fecal *E. coli* results in strains having enhanced virulence as measured in a rat peritonitis model (39). In view of the fact that adherence as well as toxin production might induce the release of inflammatory mediators, we analyzed $Hly^\pm$ MS^+ $MR^\pm$ *E. coli* strains (14–17) with regard to their effect on rat mast cells and human granulocytes.

It has been established in the past that histamine and leukotrienes are important mediators of inflammatory reactions induced by immunological and nonimmunological stimuli (3–5, 24, 31). Leukotriene B_4 (LTB_4), unlike LTB_4 isomers, has been shown to be chemotactic for human neutrophils and eosinophils. LTC_4, LTD_4, and LTE_4 were identified as the slow-reacting substance of anaphylaxis which is involved in bronchoconstriction and mucus production (24). Human polymorphonuclear granulocytes (PMNs) release high amounts of leukotrienes upon activation with various stimuli, e.g., the calcium ionophore A 23187 and bacterial exo- and endotoxins (3–6), during phagocytosis (24, 31). In rat serosal mast cells, the major arachidonic acid metabolites are not the leukotrienes but prostaglandin D_2. Since the above-mentioned target cells can be obtained without difficulties, rat mast cells were used as the source for histamine (23), and human PMNs were used as the target cell for leukotrienes (31). It was the purpose of the present investigation to study the role of bacterial adherence and

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hemolysin production with regard to the release of preformed (histamine) and newly generated (leukotrienes) mediators of inflammation.

MATERIALS AND METHODS

Buffers. The medium used for washing the cells and for mediator release unless stated otherwise was a Tris buffer (pH 7.35, 0.025 M) containing NaCl (120 mM), KCl (4 mM), $CaCl_2$ (0.6 mM), and $MgCl_2$ (1 mM) (3–5). This buffer is referred to as TCM buffer (Tris, calcium, magnesium). Bacterial growth was carried out in brain-heart infusion broth (Oxoid) which consisted of calf brain infusion solids (12.5 g), beef heart infusion solids (5.0 g), protease peptone (20.0 g), sodium chloride (5.0 g), dextrose (2.0 g), and disodium phosphate (2.5 g). Distilled water was added up 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 (24, 34). This method leads to more than 97% 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. As target cells for the chemotactic assay, guinea pig peritoneal cells rich in eosinophils (30 to 80%) were used by injecting human serum at weekly intervals. Human neutrophils as target cells for the chemotactic assay were obtained by the above-described procedure.

For the collection of rat peritoneal cells, Wistar rats were bled, and they then received an intraperitoneal injection of Tyrode buffer. The abdomen was massaged, and the buffer was recovered. After centrifugation (20 min at 600 rpm), the cells of several rats were pooled. The cell suspension contained 3 to 5% mast cells (23).

Bacterial strains. The cloning and functional characterization of the plasmid-encoded determinants was performed at the Institut für Genetik und Mikrobiologie, Universität Würzburg (13–17). *E. coli* K-12(pHly152) carries the 68-kilobase IncJ2 plasmid of an animal-pathogenic strain, which comprises at least four cistrons (*hlyC*, *hlyA*, $hlyB_a$, and $hlyB_b$). The hemolysin determinants are present within *E. coli* K-12(pANN202-312) in cloned form. The plasmids pANN5211, pANN5311, and pANN5411 express the hemolysin determinants of bacterial strains pathogenic for humans. They are derived from the bacterial chromosome. *E. coli* 536, isolated from a patient suffering from urinary tract infection, was obtained from the Institut für Hygiene und Mikrobiologie, Universität Würzburg. This strain belongs to the serotype O6:K15:h31 and is Mrh Vb^+, causing mannose-resistant hemagglutination of bovine erythrocytes and a delayed hemagglutination with human and guinea pig erythrocytes. The spontaneous mutant Nr.21 of the strain 536 has lost the ability to produce hemolysin and the Mrh^+ phenotype (Hly^- Mrh^-), owing to a deletion in the chromosome of more than 30 kilobases. Strain 536/21 was transformed with Hly^+ recombinant plasmids; in essence, the recombinant plasmids carried the following cloned *hly* gene; pANN202-312 = *hlyC*, *hlyA*, $hlyB_a$ and $hlyB_b$ from pHly152; pANN5211-06 = *hlyC* from pHly152 and *hlyA*, $hlyB_a$, and $hlyB_b$ from *E. coli* 536 (O6:K15:H31); pANN5311-O18ac = *hlyC* from pHly152 and *hlyA*, $hlyB_a$, and $hlyB_b$ from *E. coli* 764 (O18ac:K5:H^-); pANN 5411-075 = *hlyC* from pHly152 and *hlyA*, $hlyB_a$, and $hlyB_b$ from *E. coli* 341 (O75:K95:H) (15, 16).

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). 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 supernatant obtained at the late logarithmic phase of bacterial growth was studied. The bacterial culture supernatant was analyzed as to its hemolysin content. At the dilutions used, the supernatant was not chemotactic for neutrophils or eosinophils by itself.

Adherence and phagocytosis. [^{3}H]thymidine (15 μCi) was added to a 10-ml bacterial culture after 1 h of incubation; the culture was incubated for an additional 2.5 h. Bacteria were washed twice in TCM (15 min, 4,000 × *g*); 5×10^8 bacteria were present in 1 ml. For the detection of phagocytosis, bacteria (50 μl) were added to 500 μl of PMNs and incubated at 37°C. Subsequently, the reaction mixture was placed on ice, and the cells were separated by centrifugation at 300 × *g* for 10 min. Adherent bacteria were removed from the granulocytes by incubating them on ice with Tris buffer (pH 7.35, 25 mM) containing NaCl (120 mM), KCl (4 mM), EDTA (40 mM), and lysozyme (100 μg/ml). By repeated washing in TCM and subsequent lysis in distilled water, the percentage of phagocytosed bacteria was determined. Labeled bacterial suspension (50 μl) served as a control. For the analysis of adherent bacteria, lysozyme treatment was omitted. The percentage of adherence was calculated as the difference between the lysozyme-treated and untreated granulocyte suspensions (36).

Hemolysin assay. Hemolysin assay was performed as described by Goebel et al. (14). 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 by centrifugation at 4,000 × *g* and separated from the supernatant. The cells (2.5×10^{10}) were suspended in 500 μl of Tris buffer (pH 7.4, 10 mM) with sucrose (25%)–EDTA (40 mM)–lysozyme (100 μg/ml). After 30 min on ice, the cells were pelleted, and the supernatant (labeled periplasmic) was removed. The cell 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 Tris buffer (pH 7.4, 10 mM) with $CaCl_2$ (20 mM)–NaCl (160 mM) in the 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) was incubated at 37°C. Samples were removed after 1, 3, 5, and 10 min and placed on ice; unlysed erythrocytes were removed by centrifugation (1 min in an Eppendorf microfuge). The absorption of the supernatant at 530 nm was determined. Hemolysin activity is expressed as the increase in optical density per minute.

Chemotaxis. The method for measuring eosinophil and neutrophil migration has been described (3, 4, 24, 34). Briefly, 2.5×10^6 guinea pig peritoneal cells containing 35 to 70% eosinophils were placed above a nitrocellulose filter (pore size, 12 μm; diameter, 13 mm; Sartorius Membranfilter, GmbH., Göttingen, Federal Republic of Germany) in a modified Boyden chamber. The chemotactic factor was placed below the filter. After 3 h of incubation, eosinophils that had migrated through the filter were counted at a magnification of ×400. Five high-power fields were evaluated. Neutrophil chemotaxis was performed with 3-μm filters and human PMNs as target cells. Incubation was

carried out for 2 h. Cells and buffer (no stimulated cell supernatants) below the filter served as the control.

Leukotriene release from human PMNs. Human PMNs (1×10^7 or 2×10^7) were suspended in a volume of 1,000 μl of TCM buffer. The supernatant of stimulated cells (500 μl) was assayed for chemotaxis with either guinea pig or human neutrophils as target cells (3, 4, 24). The supernatant of stimulated cells was also analyzed for leukotrienes by high-pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) studies.

Analysis of leukotriene release. For analysis of leukotriene release, the supernatants of the stimulated cells were deproteinized by the addition of 2 volumes of acidified methanol (methanol-acetic acid, 1,000:1) overlaid with argon and frozen at −70°C for 12 h (22). 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) for reverse-phase HPLC; HPLC analysis was performed with a Nucleosil C18 column (5-μm pore size, 4 by 200 mm; Machery Nagel, Düren, Federal Republic of Germany) with methanol-water-acetic acid (64:36:0.98, pH 5.9; titer 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 absorbance of the volume effluent was monitored with a variable UV detector (LDC Spectromonitor III 1204 A) adjusted to 280 nm. 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 19.60 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 the determination of the retention time and comparison with external standards of synthetic leukotrienes (gift from J. R. Rokach, Merck Frosst, Quebec, Canada), RIA, and UV absorbance (see below). The area integration of the absorption peaks allows the quantitative analysis of the substances. With the described extraction procedure, the recovery rates of leukotrienes from 250 μl of cell supernatant were 80 to 85% for LTC_4, LTD_4, and LTE_4 and 90 to 95% for LTB_4. The overall recovery was obtained from subcellular fractions adding the respective leukotrienes to equal portions of the fraction, subsequently followed by the addition of acidified methanol to avoid the leukotriene conversion. The standard curve of 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 (5, 22).

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 (5). A 200-μl sample of the supernatants suspended in 30% methanol was 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 anti-plasma dilution as well as synthetic LTC_4, LTB_4 at concentrations from 10 ng to 25 pg, or unknown samples were added to tubes containing ^{3}H-LTB_4 or ^{3}H-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 quantities detected were approximately 50 pg for LTC_4 and 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, cross-reactivity was <2%. The RIA for LTB_4 was obtained from Wellcome Research Laboratories (Beckenham, England). The cross-reactivity against LTC_4 was 2%. The LTB_4 antiserum reacted with the isolated LTB_4 isomer. 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 the LTB_4.

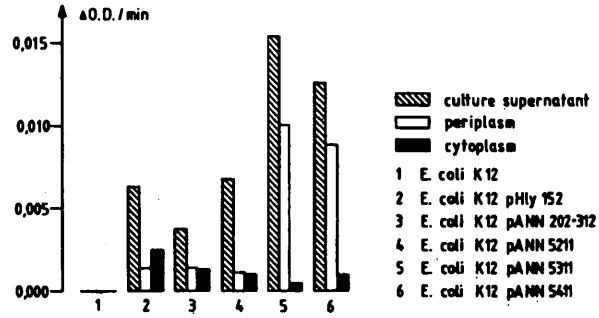

FIG. 1. Analysis of hemolysin activity produced by *E. coli* K-12 strains. The culture medium, cytoplasm, and periplasm were analyzed.

In addition to the biochemical and radioimmunological analysis, the leukotrienes in selected fractions were also identified by their characteristic UV spectra (Perkin-Elmer Lambda 5 UV Vis spectrophotometer) as well as by bioassay. LTB_4 activity was analyzed with regard to its chemotactic properties (3, 4, 24). LTC_4, LTD_4, and LTE_4 (slow-reacting substance activity) in selected fractions were analyzed by their capacities to contract guinea pig ileum, as previously described (4).

Histamine release. Rat peritoneal cells (5 to 10% mast cells) were suspended in 200 μl of TCM buffer (23), and 100 μl of bacterial suspension was added. Incubation proceeded for 60 min at 37°C. Cells were centrifuged for 10 min at 300 × *g*, and supernatant was removed and deproteinized by the addition of $HClO_4$ (2%, 2 ml). The supernatant was subsequently centrifuged at 1,000 × *g* for 10 min, and the histamine content was analyzed by the fluorophotometric analyzer technique. Cells in the presence of buffer and bacterial supernatant at the appropriate dilutions served as controls.

RESULTS

Chemotactic factor induction by *E. coli* K-12. Hemolysin activity of *E. coli* K-12 strains was determined in the culture supernatant, periplasm, and cytoplasm of bacteria. It is apparent from Fig. 1 that, in contrast to *E. coli* K-12, the *E. coli* K-12(pANN202-312) strain expresses weak hemolysin activity. *E. coli* K-12(pHly152)(pANN5211) expressed intermediate activities, and *E. coli* K-12(pANN5311)(pANN5411) expressed high hemolysin activities. The activities within the medium exceeded the amounts present in the periplasm and cytoplasm. Experiments were then carried out to analyze the release of chemotactic factor activity from human granulocytes. For this purpose, human PMNs were stimulated with washed bacteria (2.5×10^7) or 50 μl of bacterial culture supernatant.

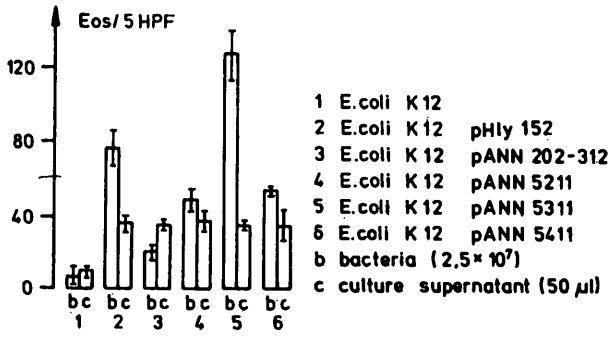

FIG. 2. Induction of chemotactic factor activity from human granulocytes on incubation of the cells with washed *E. coli* K-12 strains and their culture supernatants (eosinophils per 5 high-power fields). Supernatant (500 µl) from the stimulated granulocytes was analyzed in a Boyden chamber. The experiment was performed three times in triplicate. The figure represents an individual experiment. A similar pattern was seen in the other experiments.

The supernatant of the stimulated granulocytes was then analyzed for chemotactic activity in a Boyden chamber with guinea pig eosinophils as target cells. Figure 2 demonstrates that the highest chemotactic activity was obtained when granulocytes were stimulated with washed *E. coli* K-12(pHly152) and *E. coli* K-12(pANN5311)(pANN5211)(pANN5411). The correlation of hemolysin-producing strains and chemotaxis induction by washed bacteria was $r = 0.85$ ($n = 5$). The bacterial culture supernatant was equally or less active. Similar results were obtained with human neutrophils as target cells (data not shown). In general, the strains with hemolysin acitivity induced higher chemotactic activity as compared with the non-hemolysin-producing *E. coli* K-12 strain. In kinetic studies, it was shown that the release of chemotactic factor activities occurred in the first 10 to 15 min of incubation and then decreased at later times (data not shown). These data correlated with previously published results, e.g., with alveolysin from *Bacillus alvei* as the stimulus (3).

Leukotriene and histamine release by various *E. coli* K-12 strains. Neutrophils (2×10^7/ml) stimulated for 15 min at 37°C with either bacteria (5×10^7 in 100 µl of TCM) or bacterial culture supernatant (100 µl) were then analyzed for the capacity to release LTB_4, 6-*trans* LTB_4, LTC_4, and LTD_4. With *E. coli* K-12 as the stimulus, the leukotriene levels (LTC_4, LTD_4, LTB_4, and 6-*trans* LTB_4) were below the detection limits of reverse-phase HPLC (Table 1). With all hemolysin-producing bacterial strains, LTC_4 was readily detected. Significant amounts of LTD_4 and 6-*trans* LTB_4 were generated when granulocytes were stimulated with the bacterial culture supernatants. LTB_4 was only obtained upon incubation of neutrophils with the washed bacteria *E. coli* K-12(pANN5311) and *E. coli* K-12(pANN5411). The bacterial culture supernatants proved to be highly active with the exception of the supernatant obtained from *E. coli* K-12 Hly^-. The culture supernatant of *E. coli* K-12(pANN202-312) with weak hemolysin activity proved to be more potent than that of *E. coli* K-12(pANN5411) which expressed high hemolysin activity. Although the bacterial culture supernatant was not biochemically characterized, the mediator release seems to be dependent on the hemolysin content (Fig. 3).

To show that hemolysin-containing bacterial supernatant is able to trigger leukotriene release, supernatant fractions with various hemolysin contents from *E. coli* K-12(pANN 5411) were incubated with human PMNs. An increase in hemolysin activity induced higher amounts of LTB_4 and LTC_4 release from neutrophils. In comparison, human neutrophils (10^7) from donors ($n = 5$) stimulated with the calcium ionophore A 23187 (7.3×10^{-6} M), opsonized zymosan (2 mg), or *N*-formyl-methionyl-leucyl-phenylalanine (*N*-fMet-Phe-Leu) (1.9×10^{-5} M) led to the levels of leukotriene release shown in Table 2. *N*-fMet-Phe-Leu is a rather weak inducer of leukotriene release from human cells (Table 2).

Our previous results with washed bacteria ($Hly^{\pm}$) suggested that mediator release might be due to the perturbation of the cell membrane. Therefore, experiments were carried out to analyze the rate of adherence to and phagocytosis of human granulocytes for the various bacteria. It became apparent that *E. coli* K-12 expressed an early peak in adherence which declined after 5 min. At its maximum, 18% of the bacteria adhered to the granulocytes. The phagocytosis rate steadily increased after 5 to 15 min of incubation; 22% appeared within the granulocyte after 15 min of incubation. When the strains with hemolysin production [*E. coli* K-12(pANN5311) and *E. coli* K-12(pANN5411)] were studied, the rate of phagocytosis was higher than the rate of adherence. The rate of phagocytosis had reached its maximum after 5 min of incubation; only a low amount of adherence (2 to 4%) was determined (data not shown).

Further experiments were carried out to study histamine release from rat peritoneal cells with washed bacteria. Rat peritoneal cells containing mast cells (5×10^4 in 200 µl of TCM) were incubated with washed bacteria (5×10^6 in 100 µl of TCM). As can be seen, the strains with high hemolysin activity induced high amounts of histamine, whereas *E. coli* K-12 and *E. coli* K-12(pANN202-312) with weak hemolysin activity gave only background values (Fig. 4). The correlation between hemolysin production and histamine release amounted to $r = 0.94$ ($n = 5$).

Effect of MR^+ adherent strains and hemolysin production

TABLE 1. Generation of leukotrienes from granulocytes upon stimulation with bacteria or bacterial culture supernatant[a]

E. coli strain	Stimulant[b]	Leukotriene release (ng/2×10⁷ PMNs)		
		LTC_4	LTD_4 + LTB_4 isomer	LTB_4
K-12	C	2.0	3.4	1.0
	SUP	2.0	3.4	1.0
K-12(pHly152)	C	9.6	3.4	1.0
	SUP	14.4	11.7	8.1
K-12(pANN202-312)	C	5.6	3.4	1.0
	SUP	16.1	13.5	13.8
K-12(pANN5211)	C	10.5	3.4	1.0
	SUP	16.3	12.3	18.6
K-12(pANN5311)	C	5.8	3.4	2.6
	SUP	20.7	15.6	5.9
K-12(pANN5411)	C	10.7	5.1	5.2
	SUP	9.0	7.9	10.0
	TCM only	2.0	3.4	1.0

[a] The experiment was repeated twice. Standard deviation, 8 to 12%. Analysis was performed by reverse-phase HPLC.
[b] Abbreviations: C, washed bacteria; SUP, bacterial culture supernatant.

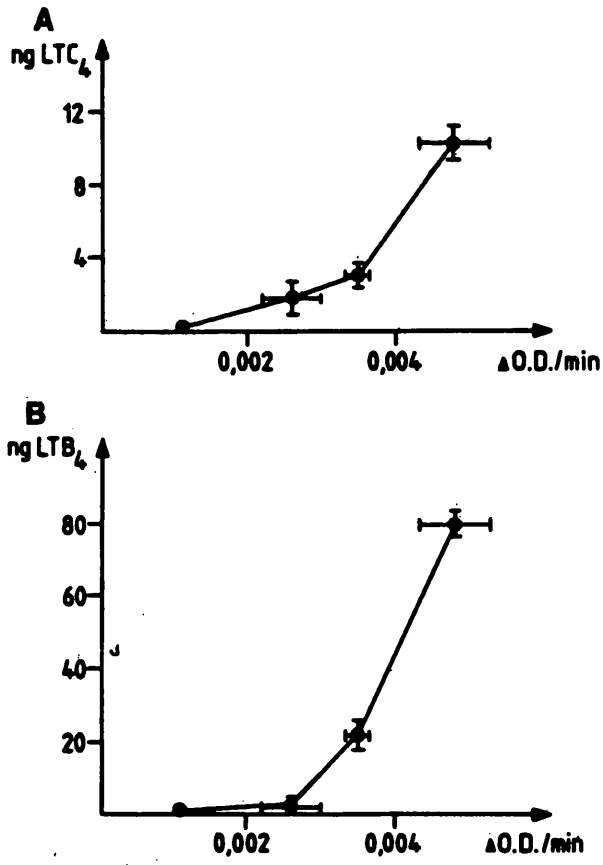

FIG. 3. Generation of leukotriene release (LTB_4 [A] and LTC_4 [B]) by hemolysin-containing bacterial culture supernatant obtained form *E. coli* K-12(pANN5411). Data are for one representative experiment done in triplicate. Leukotrienes were analyzed by reverse-phase HPLC and RIA.

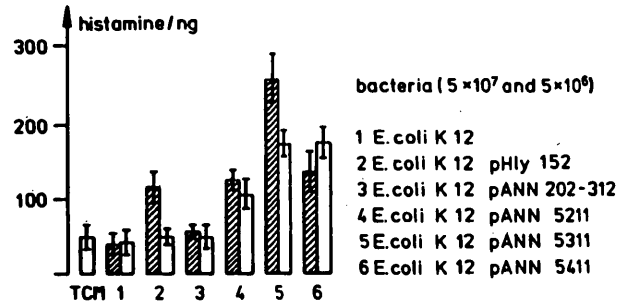

FIG. 4. Histamine release from rat peritoneal mast cells with 5×10^7 (▨) and 5×10^6 (□) *E. coli* K-12 cells. The experiment was performed three times and consistently showed a similar pattern.

on mediator release. We next studied the role of MR^+ strains on mediator release in the various systems. With regard to hemolysin activities, *E. coli* 536/21(pANN5211)(pANN5311) (MR^-) as well as *E. coli* 536/31(pANN5211)(pANN5311) (MR^+) expressed similar values as did the parent strain *E. coli* 536 (MR^+). *E. coli* 536/21 (MR^-) and *E. coli* 536/31 (MR^+) had lost the ability to produce hemolysin. The experimental design for histamine release was the same as has been described for the *E. coli* K-12 MS^+ strains. As is apparent, the MR^+ Hly^+ *E. coli* 536 bacteria induced a higher amount of histamine release as compared with the Hly^+ MR^- strain [*E. coli* 536/21(pANN5211)(pANN 5311)] (Fig. 5). In contrast, the culture supernatant of the hemolysin-producing strains (MR^+) with high hemolysin activity revealed about the same amounts of histamine release from rat peritoneal mast cells (~65% of total histamine). Mediator release occurred under noncytotoxic conditions as was assessed by the lack of lactate dehydrogenase release from the cells (data not shown).

For leukotriene release, *E. coli* 536 (MR^+ Hly^+), *E. coli* 536/31 (MR^+ Hly^-), *E. coli* 536/21 (MR^- Hly^-), and *E. coli* 536/31(pANN5311) were studied. Human neutrophils (2×10^7/ml of TCM) were stimulated for 15 min at 37°C with either the various washed *E. coli* 536 strains (5×10^7 in 100 μl of TCM) or the respective bacterial culture supernatant (100 μl). The supernatant obtained from the stimulated neutrophils was then analyzed for leukotriene release by either reverse-phase HPLC or RIA. The MR^+ strain [*E. coli* 536/31(pANN5311)] transformed with the cloned chromo-

TABLE 2. Generation of LTB_4 and LTC_4 release from human PMNs (10^7/ml) by the calcium ionophore A 23187, opsonized zymosan, and *N*-fMet-Phe-Leu[a]

Stimulant (amt)	Mean leukotriene release (ng/10^7 PMNs) ± SD	
	LTB_4	LTC_4
Ca ionophore A 23187 (7.3×10^{-6} M)	190 ± 26	53 ± 11
Opsonized zymosan (2 mg)	22.1 ± 0.6	2.5 ± 0.98
N-fMet-Phe-Leu (1.9×10^{-5} M)	10.2 ± 5.2	1.3 ± 0.4

[a] Incubation proceeded for 15 min at 37°C. Analysis was carried out by reverse-phase HPLC and RIA. Values are the means of five experiments.

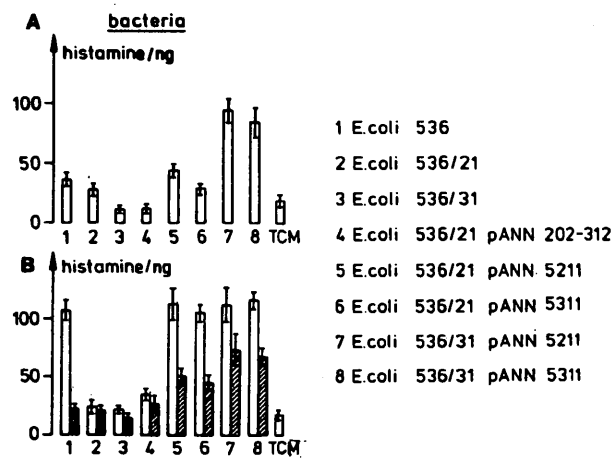

FIG. 5. Histamine release from rat peritoneal mast cells by *E. coli* 536 strains. Panel A, 5×10^6 washed bacteria. Panel B, 20 μl (□) and 2 μl ( ) of bacterial culture supernatant diluted up to 200 μl in TCM buffer. Data are for one representative experiment done in triplicate.

TABLE 3. Generation of leukotrienes by *E. coli* strains[a]

Bacteria	Stimulant[b]	Leukotriene release (ng/2 × 10^7 cells)			
		LTB_4	LTD_4 + LTB_4 isomer	LTE_4	LTC_4
E. coli 536	C	1.0	1.0	1.0	0.4
	SUP	190	27.3	62.5	54.7
E. coli 536/31(pANN5311)	C	65.5	12.1	16.1	28.7
	SUP	190	18.0	36.2	49.7
E. coli 536/21	C	1.0	1.0	1.0	0.3
	SUP	1.0	1.0	1.0	ND
E. coli 536/31	C	1.2	ND[c]	ND	0.3
	SUP	0.9	ND	ND	0.9

[a] The experiment was performed three times. Standard deviation, 7 to 15%. Analysis was carried out by reverse-phase HPLC and RIA for LTC_4 and LTB_4.
[b] See Table 1, footnote *b*.
[c] ND, Not determined.

somal Hly determinant was highly potent in inducing leukotriene release as compared with the original Hly^+ *E. coli* O6 wild-type strain (*E. coli* 536) (Table 3). In comparison, the culture supernatants of both strains were about equally active. *E. coli* 536/21 (MR^- Hly^-) as well as *E. coli* 536/31 (MR^+ Hly^-) expressed similar activities whether washed bacteria or the culture supernatant was analyzed.

In kinetic studies, it was shown that the supernatant of *E. coli* 536 induced a time-dependent release of leukotrienes (Fig. 6). A maximum in LTB_4 generation was obtained after 5 min of incubation. A similar profile with a sharp increase and a decrease at later times was observed for LTC_4, LTE_4, LTD_4, and 6-*trans* LTB_4. Kinetic experiments were also performed with *E. coli* 536/31(pANN5311) as well as the washed bacteria. LTB_4 was the most pronounced product, with the peak after 5 to 10 min of incubation. At its maximal release, 77 and 200 ng were obtained with washed bacteria and the culture supernatant, respectively. LTD_4, 6-*trans* LTB_4, and LTE_4 showed a maximum after 10 to 15 min of incubation, with a steady decrease at later times.

DISCUSSION

Our data clearly demonstrate that Hly^+ *E. coli* strains as well as bacterial supernatants trigger various cells for mediator release, i.e., histamine from mast cells and leukotrienes from human PMNs. These results were supported by experiments in which the release of chemotactic factor from human PMNs was studied upon stimulation with bacteria or bacterial culture supernatant. Hly^+ strains induced chemotactic factor activity to a greater extent than Hly^- strains. Previously, evidence has been obtained that the chemotactic factor released from human PMNs which attracts neutrophils and eosinophils is due to lipoxygenase transformation products of arachidonic acid, especially LTB_4 (24, 34). *E. coli* K-12 bacteria as well as their culture supernatant barely induced chemotactic factor activity from human PMNs.

In general, Hly^+ *E. coli* strains (K-12 as well as 536) were more effective in inducing mediator release as compared with Hly^- strains. However, exceptions are noted, e.g., with regard to *E. coli* 536 (Hly^+ MR^+) wild-type strain. Washed bacteria induced significant amounts of histamine release from mast cells above the control and less than 1 ng of leukotrienes from human PMNs. In contrast, the culture supernatant was highly active in inducing LTB_4, 6-*trans* LTB_4, LTC_4, LTD_4, and LTE_4 release as well as histamine from mast cells. The amount of hemolysin in the culture supernatant appeared to be responsible for mediator induction. As was shown, higher amounts of hemolysin within the supernatant proved to be more potent in leukotriene release from human PMNs (Fig. 3). One has to consider that hemolysin activity is labile and decays rapidly within 60 min of incubation at 37°C. Leukotriene release by hemolysin-containing supernatants is induced after 15 min, whereas histamine release was analyzed after 60 min of incubation. In addition, our data also show that the culture supernatant also contains factors which may interfere with the amount of released mediators. In this regard, the culture supernatant of *E. coli* K-12(pANN202-312) with weak hemolysin activity proved to be more active in leukotriene release than did the supernatant of *E. coli* K-12(pANN5411) with high hemolysin activity (Table 1). These data clearly indicate that the induction of leukotriene release by bacterial culture supernatants is multifactorial. Thus, in the crude supernatant, the hemolysin content most likely represents one component among others which are able to induce leukotriene release.

Recently, it has been shown that the bacterial chemotactic peptide *N*-fMet-Phe-Leu (27, 31) as well as phospholipases are able to trigger cells for mediator release (34). However, when the leukotriene-inducing stimuli (e.g., the calcium ionophore A 23187, opsonized zymosan, and *N*-fMet-Phe-Leu) were compared with each other, it became evident that the latter stimulus, which is known as a chemoattractant, is a weak inducer of leukotriene release. Thus, it appears unlikely that the amount of *N*-fMet-Phe-Leu within the supernatant is a decisive component for leukotriene generation. Similar results for the effect of *N*-fMet-Phe-Leu on human PMNs have been described (31). Our data provide sufficient evidence that the release of these mediators plays a role in the pathogenicity of the bacterial strains.

In fact, Hacker et al. analyzed the influence of the transformed, cloned Hly determinants on toxicity for mice (15). Intraperitoneal infection with 2.5×10^8 *E. coli* 536/21 (Hly^-), 536/21(pANN202-312) (Hly^+), 536/21(pANN5211) (Hly^+), 536/21(pANN5311) (Hly^+), and 536/21(pANN5411) (Hly^+) cells killed 1, 8, 30, 90, and 100% of the mice, respectively. According to their data, endotoxin appears to have no influence on the change in toxicity for mice, because all derivatives of 536/21 strains showed the same amount of O6 antigen as did the wild-type strain. The above data correlate well when histamine release was analyzed with either washed *E. coli* 536 bacteria or culture supernatants. Hly^+ cells and culture supernatants were more active as compared with Hly^- bacteria or the culture supernatant with low hemolysin activity.

The induction and release of mediators with washed bacterial cells as well as culture supernatant occurred under noncytotoxic conditions as was assessed by the absence of lactate dehydrogenase release and trypan blue exclusion studies (data not shown). It has been described by Gadeberg et al. that α-hemolytic *E. coli* strains exert cytotoxic effects on human blood monocytes and granulocytes in vitro in the presence of fresh autologous plasma (13). In these experiments, monocytes and granulocytes underwent marked morphological changes during incubation with the bacteria, and the percentage of intact phagocytes decreased progressively. In our experiments, washed bacteria were incubated with rat peritoneal mast cells in the absence of serum components.

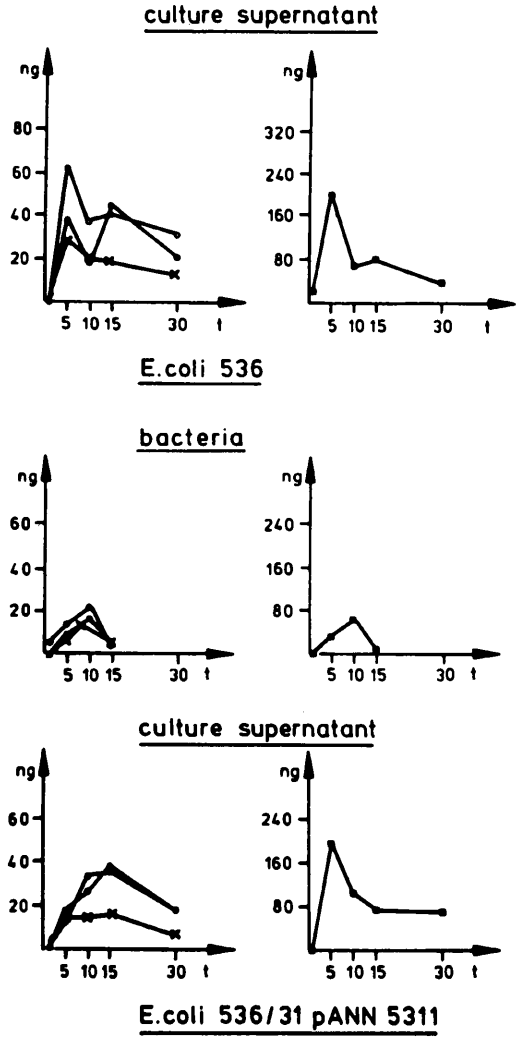

FIG. 6. Kinetics of leukotriene release from human granulocytes by *E. coli* 536 culture supernatant and by *E. coli* 536/31(pANN5311) bacteria and culture supernatant. The experiment was repeated three times; range of standard deviation, 7 to 15%. Analysis was carried out by reverse-phase HPLC. Symbols: ●, LTC_4; ×, LTD_4 + LTB_4 isomer; ○, LTE_4; ■, LTB_4.

Previously, it was demonstrated that phagocytosis leads to leukotriene release from human PMNs, as was assessed by the generation of chemotactic factor activity (4, 24, 31). When the rates of phagocytosis and adhesion were evaluated in our experiments, it appeared that the presence of hemolysin impaired adhesion but enhanced phagocytosis. A functional and structural disorganization of plasma membrane by bacterial hemolysin and phospholipases has been described (8, 29). Further studies will be carried out to determine the exact membrane biochemical mechanisms required for mediator release by bacteria-cell interaction. Thus, our studies suggest that MR^+ adherence and hemolysin production favor inflammatory mediator release which facilitates bacterial invasion and spreading within the inflamed tissue.

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