INFECTION AND IMMUNITY, Oct. 1994, p. 4611–4617
0019-9567/94/$04.00+0

Vol. 62, No. 10

Pore Formation by the *Escherichia coli* Alpha-Hemolysin: Role for Mediator Release from Human Inflammatory Cells

B. KÖNIG,[1] A. LUDWIG,[2] W. GOEBEL,[2] AND W. KÖNIG[1]*

Medizinische Mikrobiologie und Immunologie, AG Infektabwehr, Ruhr-Universität Bochum, Bochum,[1] and Theodor-Boveri-Institut für Biowissenschaften, Universität Würzburg, Würzburg,[2] Germany

Received 2 May 1994/Returned for modification 23 June 1994/Accepted 18 July 1994

The *Escherichia coli* alpha-hemolysin represents a potent stimulus for inflammatory mediator release (O_2^-, β-glucuronidase release, and leukotriene generation) from human polymorphonuclear granulocytes, for histamine release from a suspension of human lymphocyte/monocyte basophil cells (LMB), and for serotonin release and 12-hydroxyeicosatetraenoic acid generation from human platelets. In contrast, the *E. coli* alpha-hemolysin leads to a downregulation of cytokine release (interleukin-1β [IL-1β], IL-6, and tumor necrosis factor alpha) from human LMB. Recently, it became apparent that the *E. coli* alpha-hemolysin is composed of several functional structures. We analyzed the role of pore formation, pore stability, and calcium-dependent membrane binding for inflammatory mediator release by using washed bacteria as well as culture supernatants of isogenic recombinant *E. coli* strains expressing no hemolysin (Hly^-), the wild-type hemolysin (Hly^+), or hemolysin molecules deficient or modulated in defined functions (pore formation, calcium-dependent membrane binding, or pore stability). In human granulocytes and platelets, mutant hemolysin with enhanced pore stability did not lead to a further increase in induction; mutant hemolysin deficient in pore-forming activity or calcium-dependent membrane binding no longer induced leukotriene B_4 generation or β-glucuronidase release compared with the wild-type hemolysin. Similar results were obtained with regard to histamine release from human LMB. The induction of cytokine release from human LMB differed depending on the type of mutant *E. coli* alpha-hemolysin. The wild-type hemolysin, the mutant hemolysin with enhanced pore-forming activity, and, to a lesser degree, the mutant hemolysin deficient in pore-forming activity decreased cytokine release (IL-1β, IL-6, IL-8, and tumor necrosis factor) compared with untreated cells. In contrast, the mutant hemolysin deficient in calcium-dependent membrane binding led to an increase of up to 50% in cytokine release compared with that by unstimulated cells. Our results indicate that simultaneous expression of the pore-forming and calcium-dependent membrane-binding activities of the hemolysin molecule was necessary to obtain the full cellular inflammatory response pattern observed with the wild-type hemolysin.

Pathogenic *Escherichia coli* strains isolated from patients with extraintestinal infections, e.g., urinary tract infection, sepsis, and meningitis, generally exhibit several characteristics which contribute to their virulence, including alpha-hemolysin, an exotoxin (11, 19, 21, 33, 42).

The *E. coli* alpha-hemolysin was regarded primarily as a pore-forming exotoxin which exhibits calcium-dependent hemolysis of erythrocytes (4, 22). Recently, several different functional regions, responsible for pore formation, calcium-dependent membrane binding, and pore stability, have been detected in the hemolysin protein (HlyA) of *E. coli* (6, 7, 30, 31). For erythrocyte lysis, two functions of the hemolysin molecule are necessary, pore formation and Ca^{2+}-dependent membrane binding to erythrocytes (6, 7, 30, 31). There are currently no data on the role of the various functional regions of the hemolysin molecule with regard to release of mediators of inflammation from human cells.

Human polymorphonuclear granulocytes (PMN), monocytes, mast cells, basophils, and blood platelets actively participate in the inflammatory response to the *E. coli* alpha-hemolysin (22, 26, 28, 34). After interaction with hemolysin-producing *E. coli* bacteria or with soluble hemolysin, human inflammatory cells release mediators after degranulation of preformed constituents (e.g., serotonin, histamine, and enzymes) or after novel generation (e.g., 12-hydroxyeicosatetraenoic acid [12-HETE] and leukotrienes) (26, 28, 34). The *E. coli* alpha-hemolysin inhibits the release of cytokines (interleukin-1β [IL-1β], IL-6, and tumor necrosis factor [TNF]) from a suspension of human lymphocyte/monocyte/basophil cells (LMB) (25). In this regard, serotonin and histamine represent vasoactive (vasoconstriction and vasodilation) substances, and leukotriene B_4 (LTB_4) and 12-HETE are potent chemotactic components for PMN and may mediate leukocyte diapedesis through the vascular endothelium (17, 27, 36). Furthermore, platelet-derived 12-HETE can be rapidly incorporated into bystander cell populations (e.g., human leukocytes) and thereby function as a transcellular signal (28, 36).

Cytokines are released from a variety of cells and are involved in the induction, persistence, and elimination of microbial infection (12, 15, 20, 40, 41). In contrast to the above-mentioned inflammatory mediators, IL-1β and IL-6 exert pyrogenic activities and are also responsible for the induction of acute-phase proteins (1, 10). TNF-α has been implicated in the development of cachexia and septic shock (12, 37, 41). TNF-α may also prime inflammatory effector cells for a subsequent response, and thereby enhanced mediator generation may occur (35). IL-8, a recently described 6- to 10-kDa protein, is produced by a variety of cells in vitro, including peripheral blood leukocytes, and represents a cytokine that induces chemotaxis, degranulation, respiratory burst,

* Corresponding author. Mailing address: Medizinische Mikrobiologie und Immunologie, AG Infektabwehr, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany. Phone: (049) 0234-700-6860. Fax: (049) 0234-7094122.

TABLE 1. *E. coli* 5K strains used

Strain no.	Plasmid	Specific hemolytic activity (U)	Pore formation	Ca^{2+}-dependent membrane binding	HlyA deletion	Reference(s)
1	None	0	NA[a]	NA	NA	30
2	pANN202-312*	100	Yes	Yes	None	16, 30
3	pANN202-312AL2*	0	No	Yes	Hydrophobic region DII	31
4	pANN202-312AL10-9*	0	Yes	No	Repeat region	30
5	pANN202-312AL13*	250	Enhanced	Yes	N-terminal amphiphilic region	31

[a] NA, not applicable.

adherence, shape change, Ca^{2+} mobilization, and upregulation of CD11b/CD18 glycoprotein in human PMN (2, 9, 14, 39, 43).

The purpose of our study was to analyze the role of the various functional regions of the hemolysin molecule mediating pore formation, pore stability, and calcium-dependent membrane binding with regard to inflammatory mediator release (LTB_4, enzymes, serotonin, 12-HETE, histamine, and cytokines). For this purpose, we used recombinant isogenic *E. coli* strains expressing no hemolysin, the wild-type hemolysin, or hemolysin molecules with site-specific deletions or modifications of the various functional structures.

MATERIALS AND METHODS

Materials. Brain heart infusion was obtained from Oxoid, Basingstoke, Hampshire, England. All other chemicals were purchased from Sigma Chemical Co., Deisenhofen, Germany.

Buffer solutions. The medium used for washing the leukocytes and the platelets was Dulbecco's modified phosphate-buffered saline (PBS; 0.137 M NaCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4, 27 mM KCl [pH 7.4]); for stimulation (enzyme and histamine release or LTB_4 and 12-HETE generation), 0.6 mM $CaCl_2$ and 1 mM $MgCl_2$ were added. For cytokine release experiments, cells were resuspended in RPMI 1640 medium (Gibco) containing 10% fetal calf serum and 1% antibiotics (complete medium).

Preparation of cells. Human leukocytes were obtained from heparinized blood from healthy donors, separated on a Ficoll-metrizoate gradient, and sedimented with dextran (8). The LMB fraction consisted of lymphocytes (85% ± 4.6%), monocytes (14.2% ± 4.1%), and basophilic granulocytes (1.2% ± 0.5%). Monocytes were isolated from LMB by adherence to plastic in complete medium for 60 min in a humidified atmosphere of 5% CO_2 in air. The monocytes were removed by gentle scraping with a rubber policeman, and a purity and viability of 96 to 98% were obtained (38). The PMN fraction consisted of 98% pure PMN. The cells were then washed at low speed (600 rpm) to remove platelets. The erythrocytes were removed by hypotonic exposure of the cell suspension. The PMN/LMB were resuspended to a final concentration of 2×10^7 cells per ml for enzyme and leukotriene release and to 1×10^7 cells per ml for histamine release; for cytokine release, LMB were adjusted to a concentration of 1×10^6/ml. For platelet preparation, EDTA-blood was centrifuged at $200 \times g$ for 25 min to obtain platelet-rich plasma. Platelets were obtained by centrifugation at 2,200 rpm at 4°C. The platelets were washed in PBS buffer and resuspended at about 2×10^8/ml. The platelet preparation was 98% pure.

Cell viability. Cell viability was assessed by the trypan blue exclusion test as well as by the analysis of lactate dehydrogenase (LDH) release from stimulated and nonstimulated cells. Analysis of LDH (EC 1.1.1.27) was carried out as described previously (26).

Bacterial strains. The characteristics of the *E. coli* strains are summarized in Table 1. Cloning and functional characterization of the plasmid-encoded determinants were performed at the Institut für Genetik und Mikrobiologie, Universität Würzburg, Würzburg, Germany (16, 30, 31). The hemolysin-negative strain *E. coli* 5K (Sm^r *lacY1 tonA21 thr-1 supE44 thi* $r^- m^+$) does not produce hemolysin and served as a hemolysin-negative (Hly^-) control. *E. coli* 5K was transformed with reference plasmid pANN202-312* to become hemolysin positive. The previously described plasmid pANN202-312 contains only the genes *hlyC*, *hlyA*, *hlyB*, and *hlyD* (16). In contrast, plasmid pANN202-312* carries the complete hemolysin determinant (*hlyR*, *hlyC*, *hlyA*, *hlyB*, and *hlyD*) of pHly152 cloned in vector pACYC184 as described previously (30, 31); the hemolysin is fully active and is called wild-type hemolysin. Plasmid pANN202-312AL2* is a deletion derivative of pANN202-312* in which the codons for amino acids 300 to 319 of HlyA have been deleted (30, 31). The hemolysin is secreted but is not hemolytic; it is deficient in pore-forming activity. Plasmid pANN202-312AL10-9* contains the *hlyRCABD* genes in which 5 of 12 repeated sequences in *hlyA* have been deleted (30, 31); this strain secretes a nonhemolytic hemolysin which is deficient in calcium-dependent membrane binding. Plasmid pANN202-312AL13* is a deletion derivative of pANN202-312 in which the codons for amino acids 9 to 37 have been deleted. The hemolysin is secreted and is 2.5-fold more hemolytic than wild-type hemolysin (30, 31).

To analyze the function of the wild-type hemolysin as well as the mutant hemolysins, hemolysis of sheep erythrocytes and a lipid bilayer assay were used as described previously in detail (31). The wild-type hemolysin and the hemolysin mutants were secreted by *E. coli* 5K to a similar degree, as was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent silver staining. The amounts of secreted wild-type as well as mutant hemolysin were between 5 and 10 μg/ml of culture supernatant. Storage of the culture supernatants for 24 to 48 h at 4°C influenced neither the activity nor the integrity of the wild-type or mutant hemolysins (data not shown) (30, 31). Thus, experiments were carried out with comparable amounts of intact hemolysins.

Bacterial growth. Brain heart infusion broth (10 ml) was inoculated with 100 μl of an overnight culture of *E. coli* 5K containing either the reference plasmid pANN202-312 or one of the mutant *hly* plasmids; the culture was incubated at 37°C on a shaker (150 rpm) until the late logarithmic growth phase was reached (26). Cell culture supernatants were obtained by centrifuging the cultures at 4,000 rpm for 20 min at 4°C and filtering the supernatant through hydrophilic filters (pore size, 0.22 μm; Millipore Corp.). The cell pellets were washed once and adjusted to a concentration of 5×10^9/ml. The hemolytic titers of the washed bacteria and the HlyA preparations were determined as described below.

Hemolysin assay. A quantitative alpha-hemolysin assay was performed as described previously (26). In brief, culture

supernatants or washed bacteria (200 μl) of recombinant *E. coli* 5K cultures containing either the reference plasmid pANN202-312 or one of the mutant *hly* plasmids were incubated with 800 μl of a PBS solution containing 2% sheep erythrocytes, 1 mM $MgCl_2$, and 0.6 mM $CaCl_2$ for 30 min at 37°C. Subsequently, the erythrocytes were pelleted, and the amount of released hemoglobin in the supernatant was determined photometrically at 540 nm. The results are expressed as the percentage of the total hemoglobin content released (optical density at 540 nm = 2.0).

Cellular activation. To study enzyme and histamine release and leukotriene and 12-HETE generation, human leukocytes (2×10^7/ml) or human platelets (2×10^8/ml) were stimulated in the presence of 0.6 mM Ca^{2+} and 1 mM Mg^{2+} with 100 μl of buffer (control) or with 100 μl of undiluted or appropriately diluted culture supernatants of *E. coli* 5K (Hly^-), recombinant strain *E. coli* 5K(pANN202-312*) (Hly^+), or the isogenic recombinant *E. coli* 5K strains expressing the hemolysin mutants (Table 1) for the indicated times at 37°C. In cytokine release experiments, cells (10^6/ml) were stimulated in complete medium (RPMI 1640 medium with 10% fetal calf serum and 1% antibiotics). The appropriate supernatants of stimulated cells (1,000 μl) were analyzed for LTB_4, 12-HETE, histamine, enzyme, serotonin, or cytokines. The hemolysin-containing culture supernatants were assessed by immunoblot for quantitation (expression levels) and for integrity.

β-Glucuronidase release from human PMN. β-Glucuronidase (EC 3.2.1.31) content was determined as described previously (26). Released β-glucuronidase is expressed as a percentage of the total β-glucuronidase content determined by cell lysis.

Histamine release. Human LMB (10^7/ml) in PBS were stimulated with buffer or with the appropriate stimulus in the presence of 0.6 mM Ca^{2+} and 1 mM Mg^{2+} for the indicated times at 37°C. Cells were centrifuged for 10 min at 300 × *g*; the supernatant was deproteinized by the addition of 2 ml of 2% $HClO_4$ and centrifuged at 1,000 × *g* for 10 min, and histamine content was analyzed by the fluorophotometric analyzer technique (26). Released histamine is expressed as a percentage of the total histamine content determined by cell lysis. Cells in the presence of buffer and bacterial supernatants by themselves at the appropriate dilutions served as controls.

Assay for serotonin release. Washed human platelets were resuspended at a concentration of 10^9/ml in buffer containing 10 mM glucose and [^{3}H]serotonin (5-hydroxy[^{3}H]tryptamine; 0.2 μCi/10^8 cells). The suspension was incubated for 30 min at 37°C; during this time, 75% of the 5-hydroxy[^{3}H] tryptamine was incorporated. The labeled platelets were washed twice with buffer and finally suspended at a concentration of 2×10^8 platelets per ml. The release of [^{3}H]serotonin was determined after mixing the platelet suspension with 0.5 ml of 3% paraformaldehyde in buffer. The formaldehyde-treated samples were centrifuged at 1,200 × *g* for 10 min, and the supernatant was analyzed for ^{3}H radioactivity by liquid scintillation spectrometry. Released [^{3}H]serotonin is expressed as a percentage of the total platelet-bound [^{3}H] serotonin.

Analysis of leukotriene, LTB_4, and 12-HETE generation. Cell supernatants were analyzed for leukotrienes and 12-HETE as described before (26, 28). Two milliliters of methanol-acetonitrile (50:50, vol/vol) was added to the culture supernatants. After centrifugation at 1,900 × *g* for 15 min (Cryofuge 6-4; Heraeus Christ), the supernatants were evaporated to dryness by lyophilization (Modulyo; Edwards-Kniese, Marburg, Germany). The residues were dissolved in 600 μl of methanol-water (30:70), and 200 μl was analyzed by reversed-phase high-pressure liquid chromatography (HPLC). The column (4.6 by 200 mm) was packed with Nucleosil C_{18} (particle size, 5 μm; Macherey-Nagel, Düren, Germany). HPLC equipment consisted of a CM4000 pump, an SM4000 detector (both from Laboratory Data Control/Milton Roy, Hasselroth, Germany), and an automatic sample injector (WISP 710B; Waters, Eschborn, Germany). Leukotrienes were analyzed using a mobile phase consisting of methanol-water-acetonitrile-phosphoric acid (48:24:28:0.03, vol/vol) including 0.04% EDTA and 0.15% K_2HPO_4, pH 5.0. The flow rate was maintained at 0.9 ml/min. The A_{280} of the column effluent was determined. For the analysis of 12-HETE, the mobile phase was a mixture of a K_2HPO_4 solution (17 mM) containing 0.05% EDTA, acetonitrile, and methanol (1:0.75:0.75, by volume) adjusted to pH 5.0 with phosphoric acid. The A_{230} of the column effluent was determined. Quantification and identification of leukotrienes and 12-HETE were performed with synthetic standard solutions. LTB_4 generation was calculated as the combined amounts of LTB_4 and the LTB_4 omega oxidation products 20-hydroxy-LTB_4 and 20-carboxy-LTB_4.

Cytokine assays. For IL-1β, IL-6, and TNF-α analysis, 100 μl of the cell supernatants was applied to a commercial solid-phase enzyme-linked immunosorbent assay (ELISA) for IL-1β, IL-6, or TNF-α by the sandwich technique (R&D Systems, Minneapolis, Minn.). The assay was carried out as suggested by the manufacturer. Each ELISA recognizes both natural and recombinant cytokines (IL-1β, IL-6, or TNF-α) and demonstrates no measurable cross-reactivity with other recombinant human cytokines. The minimum detectable concentrations of IL-1β, IL-6, and TNF-α are 4, 5, and 5 pg/ml, respectively. The corresponding IL-1β, IL-6, and TNF-α concentrations were calculated from calibration curves obtained with the standards supplied in each kit.

IL-8 release was determined by a sandwich ELISA by the method of Ceska as published previously (2). Each well of a 96-well plate (Nunc Maxisorb) was coated overnight at 4°C with 100 μl of PBS-Tween 20 (0.1%) containing anti-IL-8 antibodies at a concentration of 5 μg/ml. The plates were washed three times with PBS-Tween, the appropriate sample or the IL-8 standard was added, and incubation proceeded for 2 h at 37°C. Thereafter, alkaline phosphatase-linked anti-IL-8 antibody was added. After addition of *p*-nitrophenylphosphate (15 mg/ml), an ELISA reader was used for quantification, and Mikrotek software (SLT Labinstruments, Crailsheim, Germany) was used for calculation.

Statistics. Data show mean values ± standard deviations (SD) for at least three individual experiments with cells from different donors. Significance was examined with Student's *t* test for independent means.

RESULTS

Release of preformed (β-glucuronidase, histamine, and serotonin) and newly generated (LTB_4 and 12-HETE) mediators from human inflammatory cells. Preformed and newly generated mediators play an important role in the inflammatory process. We analyzed the role of the structural regions for pore formation, pore stability, and calcium-dependent membrane binding of the *E. coli* alpha-hemolysin for the release of β-glucuronidase and generation of LTB_4 from human PMN, for histamine release from human basophils, and for serotonin release and 12-HETE generation from human platelets. The assay conditions were chosen from previous experiments (26, 27). Human PMN (2×10^7/ml), LMB (1×10^7/ml), or platelets (2×10^8/ml) were stimulated either with buffer (100 μl) or with cell culture supernatants (100 μl) of the recombinant *E. coli* 5K strains (Table 1), either undiluted (1:1) or down to a 1:10

TABLE 2. Release of preformed and newly generated mediators from human inflammatory cells[a]

E. coli 5K strain no.	Plasmid	Dilution[b]	Release[c] (% of total)			Generation[d] (ng)	
			β-Glucuronidase	Serotonin	Histamine	LTB_4	12-HETE
1	None	1:1	0	1 ± 1	0	0	2 ± 2
		1:2	0	0	0	0	3 ± 2
		1:5	0	0	0	0	0
		1:1	0	0	0	0	0
2	pANN202-312*	1:1	94 ± 6	81 ± 14	30 ± 4	44 ± 6	73 ± 11
		1:2	38 ± 9	81 ± 15	15 ± 5	32 ± 9	46 ± 12
		1:5	14 ± 5	65 ± 11	9 ± 3	9 ± 7	28 ± 9
		1:10	7 ± 3	35 ± 6	5 ± 3	ND[e]	ND
3	pANN202-312AL2*	1:1	3 ± 2	0	0	0	2 ± 2
		1:2	0	0	0	0	4 ± 2
		1:5	0	0	0	0	0
		1:10	0	0	0	ND	ND
4	pANN202-312AL10-9*	1:1	2 ± 1	0	0	0	3 ± 2
		1:2	0	0	0	0	2 ± 3
		1:5	0	0	0	0	0
		1:10	0	0	0	ND	ND
5	pANN22-312AL13*	1:1	79 ± 12	79 ± 14	25 ± 9	57 ± 7	66 ± 13
		1:2	63 ± 15	74 ± 8	14 ± 4	38 ± 12	35 ± 12
		1:5	21 ± 8	55 ± 7	6 ± 6	17 ± 5	26 ± 7
		1:10	17 ± 6	6 ± 3	5 ± 7	ND	ND

[a] Human PMN (2×10^7/ml), LMB (2×10^7/ml), or platelets (2×10^8/ml) were incubated in the presence of buffer (PBS) or in the presence of culture supernatants of the appropriate recombinant *E. coli* strains at the indicated dilutions for 30 min (β-glucuronidase, serotonin, and LTB_4) or for 60 min (histamine and 12-HETE) at 37°C.

[b] The biological hemolytic activities of the undiluted (1:1) and 1:2, 1:5, and 1:10 diluted culture supernatants were 82, 77, 38, and 12% of that of the wild-type hemolysin, respectively.

[c] Values are expressed as the percentage of total β-glucuronidase, [^{3}H]serotonin, or histamine content released; values for the buffer control were subtracted. Data are means ± SD for seven independent experiments.

[d] Values are expressed per 10^7 cells (total LTB_4 is LTB_4 plus omega products) or per 10^8 cells (12-HETE); values for the buffer control were subtracted. Data are means ± SD for five independent experiments.

[e] ND, not determined.

dilution, for up to 60 min at 37°C. As is apparent from Table 2, the dose-dependent release of β-glucuronidase, serotonin, and histamine and the generation of LTB_4 and 12-HETE correlated well with the capacity to lyse erythrocytes. Thus, the combination of pore formation and of calcium-dependent membrane binding activities was necessary for the release of the above-mentioned preformed and newly generated mediators. An increase in pore stability did not further enhance the capacity for inflammatory mediator release. The data presented in Table 2 show the maximal release and generation of the mentioned preformed and newly generated mediators after an incubation time of 30 min (β-glucuronidase, serotonin, and LTB_4) or 60 min (histamine and 12-HETE). The biological hemolytic activities of the undiluted and diluted culture supernatants of the reference strain *E. coli* 5K(pANN202-312) were 82, 77, 38, and 25% when approximately 600 ng (undiluted) down to 60 ng (1:10 dilution) of the various hemolysins was applied to the cells. Negligible amounts of LDH were liberated from the PMN and platelets as well.

Cytokine release from human LMB and monocytes. The effect of the *E. coli* alpha-hemolysin and the various mutant hemolysins on the ability of LMB as well as of monocytes to produce IL-1β, IL-6, TNF-α, and IL-8 was examined. Previously, we have shown that the *E. coli* alpha-hemolysin suppresses IL-1β, IL-6, and TNF-α release from human LMB (25). In a first set of experiments, human LMB (10^6/ml) were cultured with complete medium or with culture supernatants of the recombinant *E. coli* strains (Table 1) for 30, 60, 2, or 6 h at 37°C. The supernatants were analyzed for IL-1β, IL-6, TNF-α, and IL-8 by the respective ELISA. The data for IL-1β, IL-6, TNF-α, and IL-8 release are presented in Fig. 1 for a biological hemolytic activity of 85% ± 15% (1:1) and 25% ± 10% (1:5 dilution) of the reference hemolysin [*E. coli* 5K(pANN202-312)] and an incubation time of 3 h. The amounts of hemolysin were approximately 600 ng (1:1) and 50 ng (1:5 dilution). The data show that both activities, pore formation and calcium-dependent membrane binding, are necessary to fulfill the biological activities of the wild-type hemolysin. However, it is evident from Fig. 1 that calcium-dependent membrane binding alone led to a decrease in cytokine release [strain 3, *E. coli* 5K(pANN202-312AL2*)]. Nonetheless, the effects were less pronounced than with the wild-type hemolysin.

Dilution of the culture supernatants of *E. coli* 5K(pANN202-312) (wild type) down to 1:50 led to a 90% suppression of TNF-α as well as of IL-8 release from human LMB. Since monocytes are the main producers of cytokine release, identical experiments were performed with monocytes (purified by plastic adherence) for IL-8 release. Monocytes (2×10^5/ml) synthesized up to 90% of total IL-8 released from unstimulated LMB (1×10^6/ml). As is apparent from Table 3, monocytes challenged with the culture supernatant of *E. coli* 5K (pANN202-312) showed results similar to those with LMB. LMB stimulated with *E. coli* lipopolysaccharide (LPS, 1 μg/ml) served as a control for cytokine release. The response induced by LPS exceeded that obtained from unstimulated cells by up to 100-fold (IL-6 and IL-1β) or 10-fold (TNF-α); IL-8 release was only slightly enhanced in the presence of *E. coli* LPS (maximally by 20%).

DISCUSSION

Previously, the *E. coli* alpha-hemolysin was characterized as a pore-forming toxin (13, 14, 29). It is now well accepted that

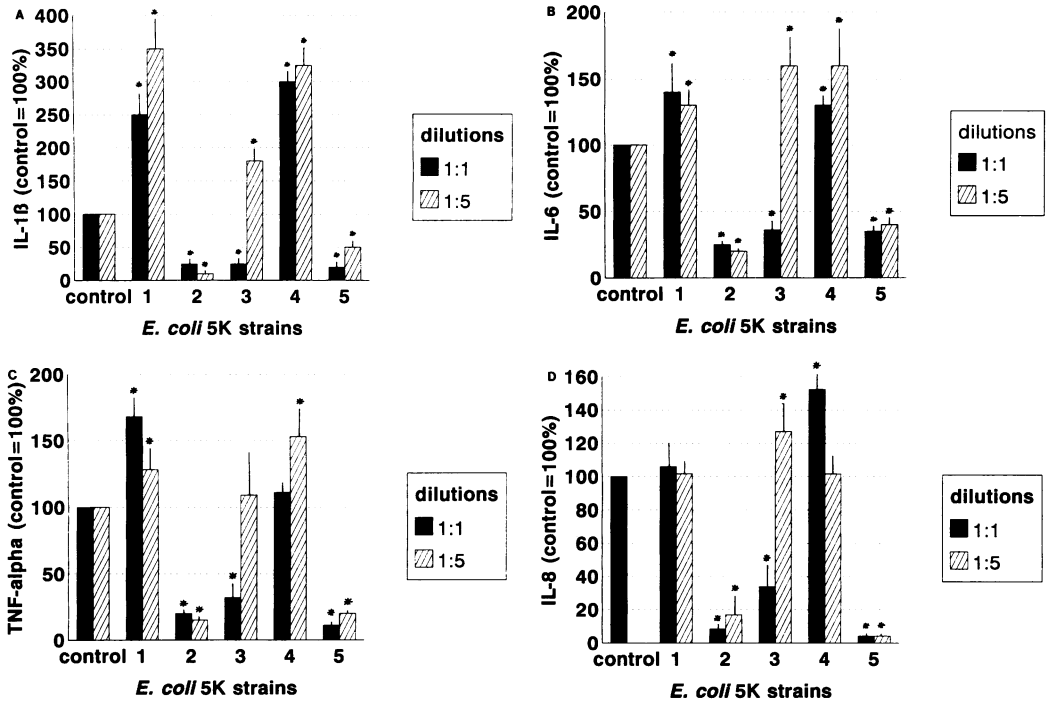

FIG. 1. Cytokine secretion by human LMB. LMB (10^6/ml) were incubated with complete medium (control) or with culture supernatants of the appropriate recombinant isogenic *E. coli* strains expressing no hemolysin (strain 1), the wild-type hemolysin (strain 2), or the various mutant hemolysins mutants (strains 3, 4, and 5; see Table 1) at the indicated dilutions for 3 h at 37°C. The biological hemolysin activities of the undiluted and diluted culture supernatants of the wild-type hemolysin [*E. coli* 5K(pANN202-312*)] were 85% ± 15% and 25% ± 10%, respectively. The cell supernatants were analyzed for (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) IL-8 by the corresponding ELISA. The amount of IL-1β-, IL-6, IL-8, and TNF-α released from unstimulated cells was set at 100%. Data are means ±SD for seven independent experiments. *, significantly different from the control ($P < 0.05$).

the hemolysin molecule is composed of several structural units which fulfill distinct functions (6, 7, 30, 31). For erythrocyte lysis, two functions of the hemolysin molecule are necessary, pore formation and Ca^{2+}-dependent binding to erythrocytes (6, 7, 30, 31). The present data demonstrate that the combined action of the pore-forming and calcium-dependent membrane binding activities is required to express the biological responses observed with the wild-type hemolysin after interaction with human leukocytes and platelets. In this regard, our results clearly show that the capacity of the *E. coli* alpha-hemolysin to induce inflammatory mediator release from human effector cells (neutrophilic and basophilic granulocytes and platelets) correlates with its hemolytic activity. Importantly, however, the inhibition or suppression of cytokine release appears to be controlled differently.

TABLE 3. IL-8 release from human LMB and monocytes[a]

Dilution of strain 2	IL-8 release (% of control)	
	Monocytes	LMB
1:1	7.91 ± 1.2	4.52 ± 2.5
1:5	6.32 ± 2.2	2.82 ± 3.2
1:10	3.93 ± 1.8	2.99 ± 2.2
1:50	11.9 ± 4.2	7.82 ± 3.2

[a] Human LMB (1×10^6/ml) or monocytes (2×10^5/ml) were incubated with the culture supernatant of *E. coli* 5K(pANN202-312) (strain 2) for 3 h at 37°C. The biological hemolytic activities of the undiluted (1:1) and diluted culture supernatants ranged from 80% (1:1) down to 4% (1:50 dilution). Values are expressed as a percentage of IL-8 release by unstimulated cells (100%).

In order to study the significance of pore formation, pore stability, and Ca^{2+}-dependent binding for inflammatory mediator release, we used isogenic recombinant *E. coli* 5K strains expressing the wild-type hemolysin or mutant hemolysins (HlyA) with deletions in different functional regions of HlyA (30, 31). Like Ludwig et al. (30, 31), we observed that the amount of secreted HlyA protein in all the transformants was similar to that of wild-type HlyA (5 to 10 μg/ml of culture). The secretion of active hemolysin followed identical kinetics (26, 28), as verified by SDS-PAGE (30, 31). However, as is apparent from the Results, small differences in hemolysin concentrations up to a factor of 2 were not relevant in the assay systems. Purified mutant hemolysin was found to be very unstable in aqueous salt solution, in contrast to culture supernatant; therefore, the results presented were performed predominantly with cell-free culture supernatants of strain *E. coli* 5K containing either the reference plasmid pANN202-312 or one of the mutant *hly* plasmids (30, 31). The wild-type hemolysin and the mutant hemolysins were stable for up to 24 to 48 h at 4°C (data not shown) without degradation. An LPS effect may be excluded since LPS leads to neither downregulation of cytokine release nor generation of LTB_4 or 12-HETE.

Furthermore, the culture supernatants differed markedly in their activation profile towards the target cells (PMN, platelets, LMB, and monocytes). Therefore, one may rule out LPS as an active principle in the respective culture supernatants, since isogenic *E. coli* strains do not differ in their LPS pattern. Furthermore, effects by bacterium-target cell contact were excluded. Nonetheless, similar results were obtained in exper-

iments in which whole recombinant *E. coli* 5K bacteria were used.

The asolectin lipid bilayer, consisting mainly of plant glycolipids, has recently been shown to be an excellent model system for studying pore formation induced by various hemolysins (13, 31). By introduction of site-specific deletions, distinct regions in HlyA which appear to be involved in pore formation by the *E. coli* hemolysin were identified. To study the role of pore formation for mediator release, three different approaches were undertaken. (i) Deletion of amino acids 9 to 37 at the N terminus led to a hemolysin (strain 5) which had an almost 2.5-fold-higher specific hemolytic activity than wild-type hemolysin and formed pores in an artificial asolectin lipid bilayer with a much longer lifetime (50-fold) than those produced by the wild-type hemolysin (31). Nonetheless, an increase in pore stability, represented by the recombinant *E. coli* strain 5K (pANN202-312AL13) (strain 5), did not lead to a further increase in inflammatory mediator release within a concentration range from 85% ± 15% down to 14.2% hemolytic activity. (ii) Deletions within hydrophobic region DII or DIII [*E. coli* 5K(pANN202-312AL2), strain 3] completely abolished the pore-forming activity of the *E. coli* alpha-hemolysin on erythrocytes and artificial membranes (31). This mutant protein no longer induced the generation of LTB_4 or 12-HETE or the release of enzymes, histamine, or serotonin from inflammatory cells. (iii) When a hemolysin mutant deficient in calcium-dependent membrane binding activity [*E. coli* 5K(pANN202-312AL10-9), strain 4] was used for target cell stimulation, again, no inflammatory mediator release was observed. Surprisingly, mutant hemolysins with deletions (30) in the repeat domain of HlyA, which is involved in the Ca^{2+}-dependent binding of erythrocyte target membranes (6, 7, 29, 30), were still able to form ion-permeable pores in the artificial asolectin lipid bilayer membrane.

It is well established that cytokines play a pivotal role in the inflammatory process induced by bacteria as well as bacterial products. In contrast to the above-described mediators (O_2^- metabolites, leukotrienes, histamine, and serotonin), the *E. coli* alpha-hemolysin suppresses spontaneous as well as induced cytokine release from human LMB in a time- and dose-dependent manner (25). Our recent data extend these findings and show that IL-8 release is also suppressed by the *E. coli* alpha-hemolysin molecule. Again, the alpha-hemolysin-induced effects on cytokine release from human monocytes have been related to the pore-forming activity (5). Our data clearly show that the pore-forming activity of the *E. coli* alpha-hemolysin alone is not responsible for the suppression of cytokine release from human LMB. Moreover, with regard to cytokine release, we may conclude that the calcium-dependent membrane binding activity is primarily responsible for the observed suppressive effects of the alpha-hemolysin on cytokine release. In this regard, mutant hemolysin deficient in pore-forming activity but with intact calcium-dependent membrane binding activity (strain 3) led to a significant suppression of cytokine release from human LMB. However, suppression of cytokine release was less pronounced than with the wild-type hemolysin. Thus, concomitant to inflammatory mediator release from human granulocytes and platelets, the different structures of the hemolysin molecule fulfill distinct functions on LMB as well. To rule out cross-reacting activities of T and B cells on monocytes treated with the *E. coli* alpha-hemolysin, we performed identical experiments with monocytes (obtained by plastic adherence). As is shown for IL-8 release, identical results were obtained with monocytes as with an LMB suspension.

Cell perturbation with the generation of functional transmembrane lesions of 1 to 2 nm has been suggested as basic mechanism for the alpha-hemolysin interaction with target cells (platelets and leukocytes). Thus, the pore formation leading to calcium influx was thought to be the active principle for inflammatory mediator release from human target cells. Previously, we have shown that the *E. coli* alpha-hemolysin leads to inflammatory mediator release from human leukocytes and platelets via a distinct signal transduction cascade including G proteins, calcium influx, and protein kinase C activation (23, 28). Furthermore, it is established that the hydrolysis of the membrane inositol phospholipids represents one major step in the cellular signalling process by the *E. coli* alpha-hemolysin (18, 24, 32). Our results support and extend the previous findings and show that neither the pore-forming activity nor the calcium-dependent membrane binding activity by itself is sufficient for the induction of a cellular response. Thus, our data indicate novel aspects of the molecular mechanism underlying the cellular activation of human inflammatory cells by the *E. coli* alpha-hemolysin. Furthermore, one may conclude from our results that at least two different signalling pathways induced by the pore-forming and the calcium-dependent membrane binding activities result in an activating process for the induction or suppression of inflammatory mediator release from human leukocytes and platelets. Indeed, preliminary experiments indicate that the different structural units of the *E. coli* alpha-hemolysin differ in their interaction and activation pattern of key elements (GTP-binding proteins and tyrosine kinases) of the signal transduction pathway (data not presented).

In summary, our data present novel aspects of the molecular mechanism underlying the cellular activation of human inflammatory cells by the *E. coli* alpha-hemolysin. However, precise analysis of the mechanism by which the bacterial exotoxin triggers cellular activation and deactivation is currently under study.

ACKNOWLEDGMENT

W. König was supported by the Minister für Wissenschaft und Forschung, NRW.

REFERENCES

1. **Baumann, H., and J. Gauldie.** 1990. Regulation of hepatic acute phase plasma protein genes by hepatocyte stimulating factors and other mediators of inflammation. Mol. Biol. Med. **7**:147–159.
2. **Bazzoni, F., M. A. Cassatella, F. Rossi, M. Ceska, B. Dewald, and M. Baggiolini.** 1991. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. J. Exp. Med. **173**:771–774.
3. **Benz, R., A. Schmid, W. Wagner, and W. Goebel.** 1989. Pore formation by the *Escherichia coli* hemolysin: evidence for an association-dissociation equilibrium of the pore-forming aggregate. Infect. Immun. **57**:887–895.
4. **Bhakdi, S., N. Mackman, J.-M. Nicaud, and I. B. Holland.** 1986. The *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. Infect. Immun. **52**:63–69.
5. **Bhakdi, S., M. Muhly, S. Korom, and G. Schmidt.** 1990. Effects of *Escherichia coli* hemolysin on human monocytes—cytocidal action and stimulation of interleukin-1 release. J. Clin. Invest. **85**:1746–1753.
6. **Boehm, D. F., R. A. Welch, and I. S. Snyder.** 1990. Calcium is required for binding of *Escherichia coli* hemolysin to erythrocyte membranes. Infect. Immun. **58**:1951–1958.
7. **Boehm, D. F., R. A. Welch, and I. S. Snyder.** 1990. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. Infect. Immun. **58**:1959–1964.
8. **Boyum, A.** 1976. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. **5**:9–15.
9. **Brom, J., and W. König.** 1992. Cytokine-induced (interleukin-3, -6

and -8 and tumour necrosis factor-beta) activation and deactivation of human neutrophils. Immunology **75**:281–285.

10. **Castell, J. V., M. J. Gomez-Lection, M. David, T. Andus, T. Geiger, R. Trullenque, R. Fabra, and P. C. Heinrich.** 1989. Interleukin-6 is the major regulator of the acute phase protein synthesis in adult human hepatocytes. FEBS Lett. **242**:237–239.
11. **Cavalieri, S. J., G. A. Bohach, and I. S. Snyder.** 1984. *Escherichia coli* alpha-hemolysin: characteristics and probable role in pathogenicity. Microbiol. Rev. **48**:326–343.
12. **Cerami, A., and B. Beutler.** 1988. The role of cachectin/TNF in endotoxin shock and cachexia. Immunol. Today **9**:28–33.
13. **Chakraborty, T., A. Schmid, S. Notermans, and R. Benz.** 1990. Aerolysin of *Aeromonas sobria*: evidence for formation of ion-permeable channels and comparison with alpha-toxin of *Staphylococcus aureus*. Infect. Immun. **58**:2127–2132.
14. **Colditz, I. G., R. D. Zwahlen, and M. Baggiolini.** 1990. Neutrophil accumulation and plasma leakage induced in vivo by neutrophil-activating peptide-1. J. Leukocyte Biol. **48**:129–137.
15. **Dinarello, C. A.** 1991. Interleukin-1, p. 48–82. *In* A. Thomson (ed.), The cytokine handbook. Academic Press, New York.
16. **Goebel, W., and J. Hedgpeth.** 1982. Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. J. Bacteriol. **151**:1290–1298.
17. **Goetzl, J., M. Woods, and R. R. Gorman.** 1977. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid. J. Clin. Invest. **59**:179–186.
18. **Grimminger, F. U., U. Sibelius, S. Bhakdi, N. Suttorp, and W. Seeger.** 1991. *Escherichia coli* hemolysin is a potent inductor of phosphoinositide hydrolysis and related metabolic responses in human neutrophils. J. Clin. Invest. **88**:1531–1539.
19. **Hacker, J., H. Hof, L. Ernicky, and W. Goebel.** 1986. Influence of cloned *Escherichia coli* hemolysin genes, fimbriae and serum resistance on pathogenicity in different animal models. Microb. Pathog. **1**:533–548.
20. **Hirano, T., S. Akira, T. Taga, and T. Kishimoto.** 1990. Biological and clinical aspects of interleukin 6. Immunol. Today **11**:443–447.
21. **Hughes, C., J. Hacker, A. Roberts, and W. Goebel.** 1983. Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by *Escherichia coli*. Infect. Immun. **39**:546–551.
22. **Jorgensen, S. E., P. F. Mulcahy, and C. F. Louis.** 1986. Effect of *Escherichia coli* hemolysin on permeability of erythrocyte membranes to calcium. Toxicon **24**:559–563.
23. **König, B., and W. König.** 1991. Roles of human peripheral blood leukocyte protein kinase C and G proteins in inflammatory mediator release by isogenic *Escherichia coli* strains. Infect. Immun. **59**:3801–3810.
24. **König, B., and W. König.** 1993. The role of the phosphatidylinositol turnover in 12-hydroxyeicosatetraenoic acid (12-HETE) generation from human platelets by the *E. coli* alpha-hemolysin, thrombin, and fluoride. Immunology **80**:633–639.
25. **König, B., and W. König.** 1993. Induction and suppression of cytokine release (TNF-alpha; IL-6; IL-1β) by *E. coli* pathogenicity factors (adhesins, alpha-hemolysin). Immunology **78**:526–533.
26. **König, B., W. König, J. Scheffer, J. Hacker, and W. Goebel.** 1986. Role of *Escherichia coli* alpha-hemolysin and bacterial adherence in infection: requirement for release of inflammatory mediators from granulocytes and mast cells. Infect. Immun. **54**:886–892.
27. **König, W., W. Schönfeld, M. Raulf, M. Köller, J. Knöller, J. Scheffer, and J. Brom.** 1990. The neutrophil and leukotrienes—role in health and disease. Eicosanoids **3**:1–22.
28. **König, B., W. Schönfeld, J. Scheffer, and W. König.** 1990. Signal transduction in human platelets and inflammatory mediator release induced by genetically cloned hemolysin-positive and -negative *Escherichia coli* strains. Infect. Immun. **58**:1591–1599.
29. **Kraig, E., T. Dailey, and D. Kolodrubetz.** 1990. Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to the alpha-hemolysin/leukotoxin gene family. Infect. Immun. **58**:920–929.
30. **Ludwig, A., T. Jarchau, R. Benz, and W. Goebel.** 1988. The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. Mol. Gen. Genet. **214**:553–561.
31. **Ludwig, A., A. Schmid, R. Benz, and W. Goebel.** 1991. Mutations affecting pore formation by haemolysin from *Escherichia coli*. Mol. Gen. Genet. **226**:198–208.
32. **Magenus, P. W., D. B. Wilson, T. M. Connolly, T. B. Bross, and G. J. Neufeld.** 1985. Phosphoinositide turnover provides a link in stimulus-response coupling. Trends Biochem. Sci. **10**:168–170.
33. **Marre, R., J. Hacker, W. Henkel, and W. Goebel.** 1986. Contribution of cloned virulence factors from uropathogenic *Escherichia coli* strains to nephropathogenicity in an experimental rat pyelonephritis model. Infect. Immun. **54**:761–767.
34. **Scheffer, J., K. Vosbeck, and W. König.** 1986. Induction of inflammatory mediators from human polymorphonuclear granulocytes and rat mast cells by hemolysin-positive and -negative *E. coli* strains with different adhesins. Immunology **59**:541–544.
35. **Shalaby, M. R., B. B. Aggarwal, E. Rinderknecht, L. P. Svedensky, B. S. Finkle, and M. A. Palladino, Jr.** 1985. Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factor. J. Immunol. **135**:269–272.
36. **Spector, A. A., J. A. Gordon, and S. A. Moore.** 1988. Hydroxyeicosatetraenoic acids (HETES). Prog. Lipid Res. **27**:271–302.
37. **Tracey, K., J. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami.** 1987. Anticachectin/TNF-alpha antibodies prevent septic shock during lethal bacteremia. Nature (London) **330**:662–664.
38. **Tucker, S. B., R. V. Pierre, and R. E. Jordon.** 1977. Rapid identification of monocytes in a mixed mononuclear cell preparation. J. Immunol. Methods **14**:267–268.
39. **Van Zee, K. J., L. E. DeForge, and E. Fischer.** 1991. IL-8 in septic shock, endotoxemia, and after IL-1 administration. J. Immunol. **146**:3478–3481.
40. **Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Esperik.** 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock: association between interleukin-6, interleukin-1, and fatal outcome. J. Exp. Med. **169**:333–338.
41. **Waage, A., A. Halstensen, and T. Espevik.** 1987. Association between tumor necrosis factor in serum and fatal outcome in patients with meningococcal disease. Lancet **i**:355.
42. **Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow.** 1981. Hemolysin contributes to virulence of extra-intestinal *E. coli* infections. Nature (London) **294**:665–667.
43. **Willems, J., M. Joniau, S. Cinque, and J. van Damme.** 1991. Human granulocyte chemotactic peptide (IL-8) as a specific neutrophil degranulator: comparison with other cytokines. Immunology **67**:540–542.