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Effects of Adhesins from Mannose-Resistant *Escherichia coli* on Mediator Release from Human Lymphocytes, Monocytes, and Basophils and from Polymorphonuclear Granulocytes

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We investigated the role of *Escherichia coli* expressing mannose-resistant hemagglutination and adhesins with regard to the induction of leukotrienes from a suspension of human lymphocytes, monocytes, and basophils (LMBs) compared with human polymorphonuclear granulocytes (PMNs). Genetically cloned *E. coli* strains expressing various types of mannose-resistant hemagglutination (MRH^+) were phagocytosed to a higher degree by monocytes than the nonadherent *E. coli* strain. The various strains differed in their capacity to induce a chemiluminescence response, which showed the same pattern for LMBs and PMNs. Stimulation of LMBs with bacteria alone, unlike granulocytes, did not activate the cells for the release of leukotrienes. However, preincubation of LMBs with bacteria decreased subsequent leukotriene formation when the cells were stimulated with calcium ionophore. The inhibitory effect was dependent on the concentration of bacteria used for preincubation as well as on the preincubation temperature. The various bacterial strains differed in inhibitory potency for mediator release. Preincubation of LMBs with zymosan, opsonized zymosan, the bacterial peptide FMLP, and peptidoglycan had no inhibitory effect or even increased subsequent leukotriene formation. Opsonized bacteria were far less inhibitory than nonopsonized bacteria. In contrast to human LMBs, preincubation of human PMNs with mannose-resistant bacteria led to increased leukotriene B_4 generation and reduced w-oxidation of leukotriene B_4. Our data suggest that phagocytes (neutrophils, monocytes) respond in a different way for leukotriene formation after interaction with mannose-resistant *E. coli*.

Bacterial adherence has been recognized as a prerequisite for various infectious disease processes (27). In this regard, the role of *Escherichia coli* adhesins for sepsis, urinary tract infections, and meningitis was analyzed. *E. coli* adhesins can be subdivided according to whether D-mannosides inhibit hemagglutination (2). The mannose-resistant (MR) hemagglutination phenotype is mediated by cell-bound adhesins or by specific protein fimbriae. Among the *E. coli* strains with MR hemagglutination, the P-adhesins recognize the sequence α-D-Gal-(1-4)-β-D-Gal on the target cell receptors (21). P-adhesin-expressing *E. coli* are frequently involved in human urinary tract infections. S-adhesins are widespread among *E. coli* isolates which cause sepsis or meningitis. These adhesins recognize a structure containing neuraminyl acid derivatives. In many strains these appear in the form of neuraminyl-α-(2-3)-galactoside (29).

It is well established that various kinds of human cells, e.g., erythrocytes and epithelial cells of the urinary tract, carry structures on their cell surface which serve as receptors for bacterial adhesins (26, 33, 41).

In recent years we presented evidence that mediators of inflammation such as leukotrienes, intracellular enzymes, and histamine are released from granulocytes and mast cells on interaction with *E. coli* expressing mannose-resistant hemagglutination (23, 24, 37). Leukotrienes are released from various cells on stimulation, e.g., from neutrophils, eosinophils, and monocytes via a 5-lipoxygenase (4, 15, 34). Activation of platelets leads via 12-lipoxygenase to 12-hydroxyeicosatetraenoic acid (12-HETE) formation (31); 12-HETE is transformed via a 5-lipoxygenase from lymphocytes, monocytes, and basophils (LMBs) to 5(S) 12(S)-DiHETE. 12-HETE is chemotactic for neutrophils, while 5(S)12(S)-DiHETE is biologically inactive (40).

Leukotriene B_4 (LTB_4) has been shown to be chemotactic for human neutrophils and eosinophils (4). LTB_4 is metabolized by omega oxidation into 20-OH- and 20-COOH-LTB_4, which are biologically inactive (8). Nonenzymatic hydrolysis of leukotriene A_4 (LTA_4) leads to the formation of 5(S)12(R)-6-*trans*-LTB_4 and 5(S)12(S)-6-*trans*-LTB_4, two LTB_4 isomers with minor biological activities. Leukotrienes C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4) have been identified as the slow-reacting substance of anaphylaxis due to the effect on smooth muscle, mucus production, and edema formation as well as vascular permeability. Lipoxygenase products and leukotrienes have been considered important mediators in bacterial infection, shock, and septicemia.

In the past it has been shown by us that human granulocytes respond differently to bacteria with defined surface characteristics (23). Appropriate stimulation, e.g., with the calcium ionophore and opsonized zymosan, also induces leukotriene formation from monocytes but to a lesser extent compared with granulocytes (31, 46). Lymphocytes by themselves do not release leukotrienes after stimulation but may influence the release by various cytokines (32). It was our purpose to study the effect of bacteria with mannose-resistant hemagglutinating properties with regard to the induction and modulation of leukotriene release from human monocytes.

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MATERIALS AND METHODS

Reagents. Reagents used were obtained from the following sources. Ficoll 400 was from Pharmacia, Uppsala, Sweden. Macrodex 6% (wt/vol) was from Knoll, Ludwigshafen, Federal Republic of Germany (FRG). Sodium metrizoate solution 75% (wt/vol) was from Nyegaard, Oslo, Norway. Peptidoglycan (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) was from Sigma Chemical Co., Munich, FRG. Lipopolysaccharide (LPS; *E. coli* O111:B4) was from Difco Laboratories, Detroit, Mich. *Salmonella minnesota* R345 LPS was a gift from C. Galanos, Max-Planck-Institut für Immunobiologie, Freiburg, FRG (16). Dilutions of LPS were performed with phosphate-buffered saline (PBS). Synthetic leukotrienes were a generous gift from J. Rokach (Merck-Frosst, Pointe-Claire, Quebec, Canada). The antibody against prostaglandin E_2 (PGE_2) was a gift from B. A. Peskar, Institut für Pharmakologie, Ruhr Universität Bochum, Bochum, FRG. [^{3}H]PGE_2 was supplied by New England Nuclear Corp. (Dreieich, FRG). Brain-heart infusion (BHI) was obtained from Oxoid, Wesel, FRG. The other chemicals were from Sigma and from Riedel-de-Häen, Seelze, FRG.

Buffers and media. The buffer used for washing the peripheral blood leukocytes and for mediator release was a PBS buffer, pH 7.4. It consisted of 0.137 M NaCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 15 mM KH_2PO_4, and 27 mM KCl. Calcium chloride ($CaCl_2$; 0.6 mM for ionophore stimulation and 2 mM for zymosan stimulation) and magnesium chloride ($MgCl_2$; 1.0 mM in all experiments) were added shortly before stimulation to minimize the spontaneous release of mediators. Bacteria were grown in BHI broth (Oxoid). EDTA-lysozyme-PBS for the phagocytic assay consisted of 40 mM EDTA and 100 μg of lysozyme per ml in PBS, pH 7.4.

Preparation of human peripheral leukocytes. Human blood leukocytes were obtained from heparinized (15 U/ml) blood of healthy donors, which was separated on a Ficoll-metrizoate gradient followed by dextran sedimentation (3). The LMB fraction, containing 84.6 ± 4.6% lymphocytes, 14.2 ± 4.1% monocytes, and 1.2 ± 0.5% basophilic granulocytes was isolated by Ficoll-metrizoate sedimentation and washed twice with PBS at 300 × *g*. The proportion of monocytes was determined by the esterase assay of Tucker et al. (42). The amount of contaminating platelets was about 5×10^7 to 10×10^7 per 10^7 LMBs. The polymorphonuclear granulocytes (PMNs) were isolated by dextran sedimentation and washed two times at 300 × *g* to remove the platelets. This method led to more than 97% pure PMNs (6).

Counterflow elutriation. In a series of experiments, human LMBs were further purified by an elutriation technique, modified according to De Boer and Ross (13) and Schönfeld et al. (38). Elutriation was performed with a Beckman centrifugation system (J2-21 centrifuge; Beckman Instruments, Palo Alto, Calif.) with a JE-6 Mark elutriation rotor. PBS buffer containing 1.5% EDTA and bovine serum albumin (5 mg/ml) was the eluent. A temperature of 4°C was used throughout the separation steps. To separate the different cell populations, the rotation was kept constant at 2,000 rpm. The chamber (standard Beckman chamber) was loaded with 10^8 cells per ml. Fraction I, which was eluted with a flow of 7 ml/min, contained the remainder of the platelets. Fraction II was obtained with a flow of 12 ml/min and contained most of the lymphocytes (monocyte content was <0.5%). The third fraction was collected at 18 ml/min (more than 70% monocytes), and the fourth fraction (monocytes >70%, ≈4% basophils) was collected at 20 ml/min. The fraction volume was 100 ml. After the separation procedure, the cells were washed twice in PBS to remove EDTA and bovine serum albumin. Cell identification was performed by light microscopy. Cell viability was determined by the dye exclusion test with trypan blue (Merck, Darmstadt, FRG). Cell suspensions with a viability of more than 95% were used for the experiments. The cells of each fraction were adjusted to 2×10^7/ml and stimulated for leukotriene formation with the Ca ionophore.

TABLE 1. Strains used

E. coli strain	Relevant characteristics
536/21	Hly$^-$ MSH$^-$ MS-Fim$^-$ MRH$^-$ MR-Fim$^-$
536/21(pANN801-1)	Hly$^-$ MSH$^-$ MS-Fim$^+$ S-MRH$^+$ S-Fim$^-$
536/21(pANN801-4)	Hly$^-$ MSH$^-$ MS-Fim$^-$ S-MRH$^+$ S-Fim$^+$
536/21(pANN921)	Hly$^-$ MSH$^-$ MS-Fim$^-$ P-MRH$^+$ P-Fim$^+$

Bacterial strains. *E. coli* 536 (O6:K15:H31) was isolated from a patient with a urinary tract infection. This strain exhibits mannose-resistant hemagglutination (MRH$^+$). Furthermore, it is serum resistant, expresses fimbriae, and produces hemolysin. The hemolysin-negative strain 536/21 is a spontaneous mutant of strain 536 that has lost the ability to produce fimbriae (Fim$^-$) and exhibit hemagglutination (MRH$^-$). *E. coli* 536/21 was transformed with recombinant plasmids which coded for different types of adherence (17, 18). Transformation with the plasmid pANN801-1 produced the strain 536/21(pANN801-1), expressing S-adherence but no fimbriae (S-MRH$^+$ S-Fim$^-$). The variant *E. coli* 536/21(pANN801-4) is S-hemagglutinin positive and possesses fimbriae (S-MRH$^+$ S-Fim$^+$) (17). Introduction of the plasmid pANN921 into *E. coli* 536/21 led to the expression of P-hemagglutination and P-fimbriae (P-MRH$^+$ P-Fim$^+$) (18). Cloning and functional characterization of the bacterial strains were performed at the Institut für Genetik und Mikrobiologie, Universität Würzburg, Würzburg, FRG. The strains studied are listed in Table 1.

Bacterial growth. Strain 536/21 was grown in BHI medium without antibiotics. The plasmid-carrying strains were grown in BHI with 10 μg of tetracycline per ml. BHI (10 ml) was inoculated with 100 μl of an overnight culture and cultivated for 3 to 3.5 h at 37°C on a shaker (150 rpm). Bacteria in the late logarithmic growth phase were used. Bacterial cultures were centrifuged at 3,291 × *g* (Heraeus Christ Minifuge T; Heraeus, Osterode, FRG) for 20 min at 4°C and washed in PBS. The bacterial titer used was adjusted to 10^{10} bacteria per ml of PBS.

Heat inactivation of bacteria. For heat inactivation, 2×10^{10} bacteria from a 3.5-h culture were washed twice in RPMI 1640 medium and suspended in 10 ml of the medium. The bacterial suspension was incubated at 65°C for 1 h. After centrifugation, the bacterial pellet was suspended in 2 ml of sterile RPMI 1640 medium. Samples of these suspensions were used for the preincubation of human peripheral leukocytes.

Opsonization of bacteria. Serum-resistant bacteria (10^{10}) obtained from the 3.5-h culture were suspended in 50% fresh human serum (5×10^9/ml) and incubated for 30 min at 37°C. Subsequently, the bacteria were washed twice in PBS, and the bacterial titer was adjusted to 10^{10} bacteria per ml in PBS. Serum resistance was confirmed by replating the bacteria on BHI medium.

Preparation of opsonized zymosan. Zymosan A (2 mg per tube) was suspended in 5 ml of PBS and boiled for 15 min. Resuspended zymosan A was then washed twice in PBS for

10 min at 4°C at 1,900 × *g*. The pellet was suspended in fresh human serum (10 mg/ml) and incubated for a further 30 min at 37°C. After centrifugation, the pellet was washed twice with PBS and resuspended to a final concentration of 40 mg of opsonized zymosan per ml of PBS.

Adherence and uptake. Two experimental procedures were performed to evaluate bacterial adherence and uptake. [^{3}H]thymidine (555 kBq) 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 PBS (15 min, 4,000 × *g*, 4°C) and suspended at a concentration of 10^{10}/ml in PBS. The bacteria incorporated 13.2 to 17.0% of the added radioactivity. LMBs (2×10^7) in 500 μl of PBS with 0.6 mM calcium and 1.0 mM magnesium were stimulated with 50 μl of the bacterial suspension and incubated at 37°C for various times. Two similar portions were used for the parallel detection of uptake and adherence. Subsequently, the reaction mixture was placed on ice, and the cells were separated by centrifugation at 300 × *g* for 15 min. Adherent bacteria were removed from the cells by incubating them on ice with 500 μl of PBS-EDTA-lysozyme for 30 min. By repeated washing in PBS and subsequent lysis in distilled water, the percentage of uptake was determined. To calculate the total amount of uptake and adherence, lysozyme treatment was omitted. The percentage of adherence was calculated as the difference between the lysozyme-treated and untreated cell suspensions (36, 43).

Scintigel (9 ml; Roth, Karlsruhe, FRG) was added to the cell lysates of both series, and each sample was counted for 1 min in a β-counter (Tricarb Liquid Scintillator Spectrometer 3255; Packard). Labeled bacterial suspension (50 μl) served as a control.

Adherence and uptake were also determined by counting CFU (43). *E. coli* bacteria (10^8) were incubated with LMBs (10^7) for 15 and 30 min at 37°C. Subsequently, the LMBs were washed in PBS and lysed in distilled water. A portion of the suspension was plated on BHI agar plates. The CFU were counted after 20 h of incubation at 37°C. In parallel, samples were analyzed in which adherent bacteria were removed from the cells by lysozyme treatment on ice for 30 min. Bacterial lysis was assayed by incubating pure bacteria in the lysozyme solution. No CFU were detected after this procedure. The calculation of uptake and adherence was performed as described above. A similar procedure was carried out when PMNs were used as target cells.

Chemiluminescence. LMBs or PMNs (2×10^6) in 0.25 ml of PBS with 0.6 mM calcium and 1.0 mM magnesium and luminol (20 μl of a 0.25 mM solution) were incubated for about 15 min at 37°C until a stable signal was obtained. Subsequently, 50 μl of the bacterial suspension (5×10^9 cells per ml) was added. Chemiluminescence was monitored in a Lumacounter M 2080 (Lumac) at different times for 10 s. The results are expressed as counts per minute.

Leukotriene release from human peripheral blood cells. Human PMNs (10^7) and LMBs (10^7) in 500 μl of PBS were incubated with 50 μl of bacterial suspension (10^{10} bacteria per ml) in the presence of calcium and magnesium for 15 min or for various times at 37°C as indicated in the figure legends. Portions of the supernatants were assayed for leukotriene release. After preincubation, the stimulus (e.g., Ca ionophore A23187) or the same volume of PBS was added to the cell suspension, and incubation proceeded for 15 min. The reaction mixture was then placed on ice to stop the reaction; the cells were centrifuged at 300 × *g* for 15 min at 4°C. The supernatant was collected and analyzed by reversed-phase high-pressure liquid chromatography (RP-HPLC); PGE_2 was analyzed by radioimmunoassay.

TABLE 2. Phagocytosis and adherence[a]

E. coli strain	% of total radioactivity		
	Uptake	Adherence	Total radioactivity associated with LMBs
536/21 (MRH$^-$ Fim$^-$)	6.8 ± 2.8	7.5 ± 2.0	14.3 ± 4.1
536/21(pANN801-4) (S-MRH$^+$ S-Fim$^+$)	10.9 ± 4.0	14.9 ± 3.2	25.8 ± 6.6
536/21(pANN801-1) (S-MRH$^+$ S-Fim$^-$)	23.1 ± 3.5	2.9 ± 1.5	26.0 ± 3.8
536/21(pANN921) (P-MRH$^+$ P-Fim$^+$)	19.4 ± 4.3	7.5 ± 2.7	26.9 ± 4.0

[a] Human LMBs (2×10^7, with 14.2 ± 4.1% monocytes) were stimulated with 5×10^8 washed bacteria at 37°C. Values are expressed as percentages of total radioactivity (100% = 33,541 to 43,279 cpm/5×10^8 bacteria). Data represent mean values of six independent measurements.

In control experiments, human LMBs (10^7) were preincubated with zymosan (2 mg), opsonized zymosan (2 mg), opsonized bacteria (5×10^8), the bacterial peptide FMLP (10^{-7} M), peptidoglycan (1 to 50 μg/ml), or LPS (1 to 100 μg/ml) from *E. coli* O111:B4 or *S. minnesota* R345. Subsequently, the cells were stimulated with the Ca ionophore as indicated above.

Analysis of leukotrienes. The supernatant of the stimulated cells was deproteinized by the addition of 2 ml of acetonitrile–methanol (1:1, vol/vol), covered with nitrogen, and stored overnight at −70°C. After centrifugation at 3,000 × *g*, the supernatants were evaporated to dryness in a freeze dryer and suspended in 600 μl of methanol-water (30:70, vol/vol). RP-HPLC analysis of leukotrienes was performed as described previously (22).

Statistical analysis. Data from different experiments with different donor cells were combined and reported as the mean ± standard deviation (SD). Student's *t* test for independent means was used to provide statistical analysis ($P > 0.05$ was considered not significant).

If not otherwise stated, the indicated *P* values refer to a comparison of each value with the other values.

RESULTS

Adherence, uptake, and chemiluminescence response. Genetically cloned *E. coli* bacteria with different adhesive properties were studied with regard to adherence and uptake. As target cells we used an LMB cell suspension (4×10^7/ml), which was incubated for 10 min with radiolabeled washed bacteria (5×10^8). In kinetic studies over various time periods up to 20 min, it was shown that maximal uptake and adherence were obtained after 10 min of incubation.

Table 2 shows the data obtained after 10 min of incubation with radiolabeled bacteria for the various strains. The adherence rates of the various strains were significantly different [$P < 0.05$ for the comparison of *E. coli* 536/21(pANN801-4) with *E. coli* 536/21 and *E. coli* 536/21(pANN921); $P < 0.01$ for all other pairwise comparisons]. It is apparent that *E. coli* 536/21 without adhesins showed an intermediate rate of uptake (6.8 ± 2.8%). Although the combined amounts of uptake and adherence for the strains with MR properties were nearly equal (≈25.8 to 26.9%), there were differences. *E. coli* 536/21(pANN801-1) (S-MRH$^+$ S-Fim$^-$) showed the lowest rate of adherence (2.9 ± 1.5%) but the highest rate of

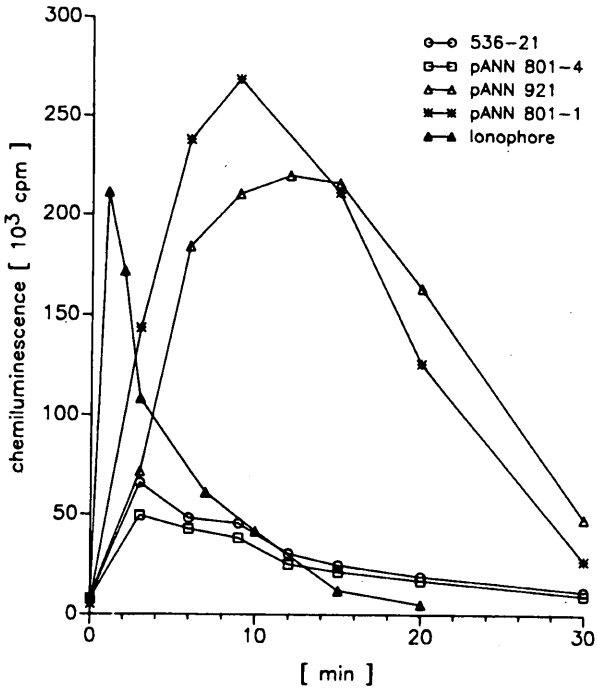

FIG. 1. Induction of chemiluminescence response by *E. coli* strains with various adhesive properties. Human LMBs (2×10^6) were stimulated with washed bacteria (2.5×10^8) at 37°C. The chemiluminescence response was monitored at different times for 10 s. The results are expressed as counts per minute. The experiment was performed four times, and the mean values are shown.

uptake (23.1 ± 3.5%). In comparison, *E. coli* 536/21(pANN801-4) (S-MRH$^+$ S-Fim$^+$) with S-fimbriae showed a high adherence rate and an intermediate rate of uptake. A less pronounced adherence than with the former strain was obtained with *E. coli* 536/21(pANN921), while this strain expressed rather high uptake (19.4 ± 4.3%). In comparison, the combined amounts of uptake and adherence for *E. coli* 536/21 compared with *E. coli* 536/21 with adhesins were significantly less ($P < 0.01$).

Experiments were also carried out by counting CFU. The results were similar to those obtained by radiolabeling experiments. For the MR *E. coli* with adhesins, the correlation coefficients for the two procedures were: uptake, $R = 0.993$; adherence, $R = 0.989$. In both experimental procedures, the total binding of MR *E. coli* bacteria was higher than that of the nonadherent strain ($P < 0.01$).

E. coli 536/21(pANN921) and *E. coli* 536/21(pANN801-1) showed the highest uptake (44.2 and 69.3%, respectively); the most pronounced adherence rate was observed for *E. coli* 536/21(pANN801-4) (73.8%), and low adherence was obtained with *E. coli* 536/21(pANN801-1 (<1%).

A similar pattern for adherence and uptake was obtained when human PMNs were used as target cells (data not shown).

These results emphasize that the interaction of bacteria with the target cells is dependent on distinct surface properties.

The chemiluminescence response was then analyzed with the various strains (Fig. 1). As a positive control, we used the activation of the cells with the calcium ionophore A23187 (4.9 μM). Human LMBs (2×10^6) were stimulated with the various washed bacterial cells (2.5×10^8) at 37°C. Kinetic studies were performed over 30 min. There were pronounced differences in induction of the chemiluminescence response. The most potent stimuli were *E. coli* 536/21(pANN801-1) and *E. coli* 536/21(pANN921). These strains showed an optimum after 5, 10, and 15 min of activation, with a decline at later times. A four- to fivefold-lower chemiluminescence response was obtained with *E. coli* 536/21 and *E. coli* 536/21(pANN801-4) ($P < 0.01$). In comparison, the calcium ionophore as stimulus induced a steep and early increase in the chemiluminescence response, with a rapid decline. With human PMNs as the target cells, the various strains were similarly active in inducing a chemiluminescence response (25).

TABLE 3. Leukotriene release from PMNs by adherent *E. coli* strains[a]

E. coli strain	Leukotrienes released (ng) ± SD	
	LTB_4	LTC_4
536/21(MRH$^-$ Fim$^-$)		
536/21(pANN801-4) (S-MRH$^+$ S-Fim$^+$)	16.0 ± 5.0	14.0 ± 4.0
536/21(pANN801-1) (S-MRH$^+$ S-Fim$^-$)	8.0 ± 2.0	10.0 ± 3.0
536/21(pANN921) (P-MRH$^+$ P-Fim$^+$)	4.0 ± 2.0	8.0 ± 4.0

[a] The experiments were carried out three times. Incubation proceeded for 15 min at 37°C.

Induction and modulation of leukotriene formation. Human PMNs (10^7) were incubated with the various bacterial strains for 15 min at 37°C. The S-MRH$^+$ S-Fim$^+$ strain [*E. coli* 536/21(pANN801-4)] induced up to 16 ± 5 ng of LTB_4 and 14 ± 4 ng of LTC_4 (Table 3). In comparison, Ca ionophore-stimulated PMNs led to 68.8 ± 18.0 ng of LTB_4 and 52.3 ± 10.7 ng of LTC_4.

Human LMBs (10^7) stimulated with the calcium ionophore (4.9 μM) for 15 min at 37°C produced 22.5 ± 12.1 ng of LTB_4, 17.0 ± 8.8 ng of LTC_4, and 9.6 ± 5.1 ng of LTB_4 isomers ($n = 11$). With opsonized zymosan, 8.3 ± 3.3 ng of LTB_4, 3.2 ± 2.0 ng of LTC_4, and 1.7 ± 1.2 ng of LTB_4 isomers were generated.

Stimulation of the LMB suspension with the calcium ionophore also led to 851.7 ± 227.8 ng of 12-HETE, which is a product of the contaminating platelets. 5(S)12(S)-DiHETE (40.6 ± 11.3 ng) was also detected by RP-HPLC analysis. This product is produced from 12-HETE by metabolization via a 5-lipoxygenase. Incubation of human LMBs with the MR *E. coli* strains did not induce leukotriene formation; leukotriene formation required an additional stimulus (e.g., the Ca ionophore).

Dose-response studies were carried out with the various bacteria with from 1.25×10^8 to 10×10^8 cells (Fig. 2). Human LMBs (10^7) were incubated for 15 min at 37°C with bacteria or with buffer as a control and subsequently stimulated with the calcium ionophore A23187. It is apparent that *E. coli* 536/21 in increasing concentrations inhibited leukotriene formation from the cells. The overall leukotriene release was significantly decreased compared with the PBS control after treatment with 5×10^8 bacteria ($P < 0.01$) but not with 2.5×10^8 or 1.25×10^8 bacteria ($P > 0.05$). Prestimulation of LMBs at a concentration of 10×10^8 bacteria inhibited leukotriene formation completely.

Among the various strains, significant differences in deactivation for leukotriene release were obtained after pretreatment of LMBs with 5×10^8 bacteria. At a concentration of 5×10^8, the strain *E. coli* 536/21 without adhesins showed an

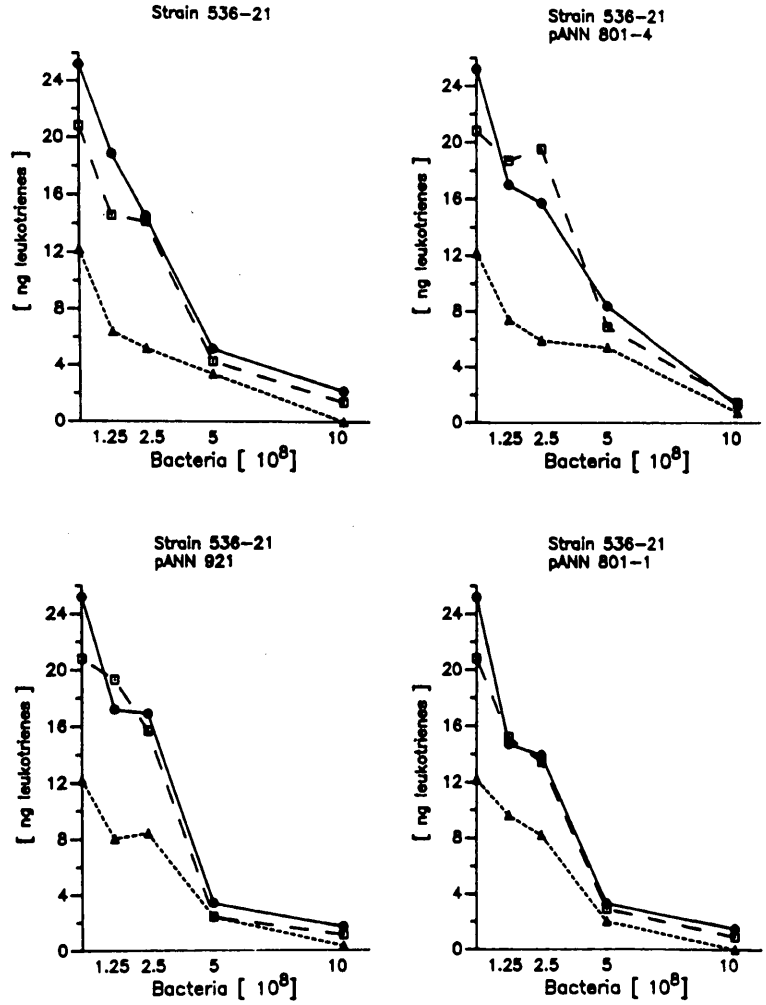

FIG. 2. Inhibition of ionophore-induced leukotriene release by various concentrations of bacteria used for preincubation. Human LMBs (10^7) were incubated with various concentrations of bacteria (1.25×10^8 to 10×10^8) at 37°C for 15 min. Ca ionophore (4.9 μM) was added, and the cells were incubated for an additional 15 min. Data are mean values of different experiments ($n = 4$ for 10×10^8, 2.5×10^8, and 1.25×10^8 bacteria, $n = 11$ for 5×10^8 bacteria). Symbols: ●, LTB_4; □, LTC_4; △, LTB_4 isomers.

intermediate rate of deactivation. *E. coli* 536/21(pANN921) as well as 536/21(pANN801-1) showed a steep decline for subsequent ionophore-induced LTB_4 (79.4 to 86.9% inhibitory efficacy) and LTC_4 formation (79.3 to 88.5%) ($P < 0.01$ compared with the two other strains). *E. coli* 536/21(pANN801-4) was poorly active in deactivation. A less pronounced inhibition of LTB_4 (66.7%, $P < 0.01$) and LTC_4 (66.8%, $P < 0.05$) was obtained when this strain was analyzed compared with the remaining adherent strains.

The production of 5(S)12(S)-DiHETE decreased after bacterial pretreatment of LMBs to the same amount as the other metabolites (data not shown). These results suggest that bacterial pretreatment may reduce the 5-lipoxygenase activity of monocytes.

Under the experimental conditions used, no significant difference was obtained for PGE_2 formation ($P > 0.05$) (data not shown).

For the MR-expressing strains [*E. coli* 536/21 carrying pANN801-1, pANN801-4, or pANN921), the following correlations were obtained: uptake-adherence, $R = -0.996$; uptake-inhibition of leukotriene release, $R = +0.960$; uptake-chemiluminescence, $R = +0.999$; adherence-inhibition of leukotriene release, $R = -0.931$; adherence-chemiluminescence, $R = -0.992$; chemiluminescence-inhibition of leukotriene release, $R = +0.969$. For *E. coli* 536/21 without adhesins, there was no correlation of the parameters under study.

In a series of experiments, the human LMB population was separated by elutriation. The purified cells, either lymphocytes or monocytes, were then analyzed for leukotriene formation after stimulation with the Ca ionophore. When monocytes were purified from LMBs (14.9% monocytes) by elutriation and centrifugation to 75.2%, the ionophore-stimulated leukotriene release of 10^7 cells was about four- to fivefold higher than from the original LMB fraction.

These studies provided evidence that the monocyte is the major leukotriene-producing cell type within the LMB fraction. Lymphocytes were not able to generate leukotrienes.

Prestimulation of the monocytes with the MRH^+ *E. coli* strains showed the same pattern of deactivation as was

TABLE 4. Influence of pretreatment with bacteria on the Ca ionophore-induced release of leukotrienes from PMNs[a]

Stimulus	Leukotrienes released (ng)				
	LTB_4	20-OH- + 20-COOH-LTB_4	20-OH- + 20-COOH-LTB_4 + LTB_4	LTC_4	LTB_4 isomers
PBS (control)	68.8 ± 18.0	379.8 ± 130.5	448.6 ± 23.5	52.3 ± 10.7	87.0 ± 32.0
536/21 (MRH^- Fim^-)	139.8 ± 41.4**	147.3 ± 121.5**	287.1 ± 90.7**	51.1 ± 35.1	62.2 ± 31.9
536/21(pANN 801-4) (S-MRH^+ Fim^+)	146.6 ± 53.3**	121.7 ± 60.2**	268.4 ± 79.7**	56.9 ± 25.9	70.6 ± 57.2
536/21(pANN801-1) (S-MRH^+ Fim^-)	141.4 ± 32.7**	123.5 ± 77.9**	264.9 ± 90.1**	49.2 ± 31.8	62.5 ± 40.9
536/21(pANN921) (p-MRH^+ Fim^+)	151.9 ± 52.6**	146.5 ± 97.0**	298.3 ± 90.7**	77.2 ± 46.9	64.4 ± 32.3

[a] Human PMNs (10^7 per 0.5 ml) were incubated with bacteria (5×10^8) for 15 min at 37°C. Subsequently, Ca ionophore (4.9 μM) was added, and the stimulation proceeded for an additional 15 min. The experiment was performed five times. Data are mean values ± SD. Symbols: **, $P < 0.01$ versus PBS control.

obtained with the unseparated LMBs. However, 12-HETE and 5(S)12(S)-DiHETE were not detectable in the platelet-free cell suspension.

We then compared leukotriene formation from 10^7 human PMNs which were incubated with washed bacteria for 15 min and subsequently stimulated with the Ca ionophore for an additional 15 min (Table 4). Granulocytes incubated with buffer and then stimulated with the Ca ionophore served as the control. Preincubation of granulocytes with bacteria increased LTB_4 formation by two- to threefold ($P < 0.01$) on account of the omega-oxidation products of LTB_4 (20-OH-LTB_4 and 20-COOH-LTB_4), which were reduced by about threefold. The formation of LTC_4 as well as LTB_4 isomers was not affected ($P > 0.05$). A calculation of the combined amounts of LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4 showed an overall decrease in LTB_4 formation in human PMNs. There were no statistically significant differences between the various strains ($P > 0.05$).

Characteristics of deactivation. The inhibitory effect of the various *E. coli* strains required a certain time of preincubation. Human LMBs (10^7) were preincubated with the washed bacterial strains (5×10^8) for 5, 15, and 30 min; subsequently, the cells were stimulated with the Ca ionophore (Fig. 3). No significant effects compared with the PBS control were observed after 5 min of pretreatment. After 15 min of preincubation, significant differences ($P < 0.01$) between the cells incubated with and without bacteria were detected. Among the various *E. coli* strains, the results differed significantly when the cells were pretreated with 5×10^8 bacteria ($P < 0.01$, 0.05). As shown, the subsequent

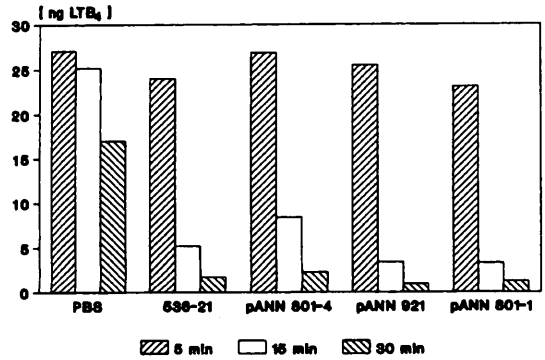

FIG. 3. Kinetics of bacterially induced inhibition of leukotriene release by Ca ionophore. LMBs (10^7) were incubated with 5×10^8 washed bacteria or PBS for the time indicated. Ca ionophore (4.9 μM) was added, and incubation proceeded for a further 15 min. Data are mean values of three experiments each for 5 and 30 min and 11 experiments for 15 min of preincubation.

LTB_4 formation of LMBs was reduced by 80% after 15 min of preincubation. A similar inhibitory pattern was obtained for LTC_4 and LTB_4 isomers.

From additional experiments it became apparent that pretreatment of LMBs at 4°C and subsequent stimulation with the calcium ionophore A23187 did not induce deactivation (data not shown).

In order to analyze the calcium requirement, human LMBs (10^7) were incubated at 37°C with bacteria in the absence of cations (calcium and magnesium) for 15 min (Fig. 4). Subsequently, the cells were stimulated with the ionophore in the presence of calcium and magnesium. The cells pretreated with bacteria at 37°C in the absence of calcium showed a pronounced inhibition ($P < 0.01$) of leukotriene release compared with PBS-treated cells.

As was described previously (Fig. 2), differences in deactivation among the various *E. coli* strains became apparent at 37°C, which indicated that *E. coli* 536/21(pANN801-4) was less active than the other adhesin-carrying strains or *E. coli* 536/21.

These results clearly demonstrate that deactivation is temperature dependent and independent of exogenous calcium. The results suggest that deactivation apparently results from membrane biochemical changes.

Apparently, the receptor interaction by which the monocyte is triggered decides whether deactivation will occur.

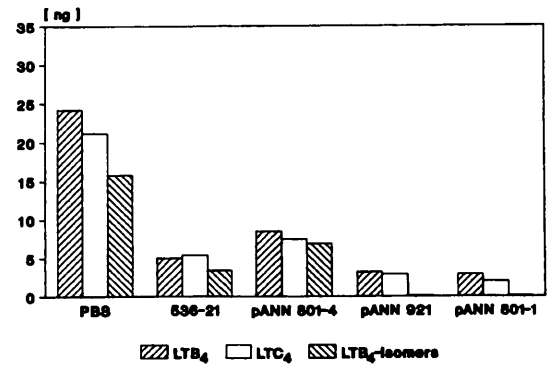

FIG. 4. Influence of the Ca and Mg concentration on the inhibition effect. Samples of human LMBs (10^7) were incubated without calcium and magnesium ions for 15 min at 37°C. At the end of the preincubation period, 4.9 μM Ca ionophore was added, and all samples were incubated at 37°C with calcium and magnesium for an additional 15 min. The amounts in control samples (preincubation at 37°C with calcium and magnesium) were: LTB_4, 29.8 ± 4.8 ng; LTC_4, 25.5 ± 8.4 ng; LTB_4 isomers, 15.6 ± 3.1 ng ($n = 4$). Data are mean values of independent experiments with cells from different donors.

TABLE 5. Leukotriene release from LMBs after treatment with different stimuli[a]

First stimulus	Second stimulus	Leukotriene release (% of control)		
		LTB_4	LTC_4	LTB_4 isomers
Zymosan	Ionophore	94.8 ± 9.7	98.1 ± 7.3	92.1 ± 10.2
	PBS	12.5 ± 2.8	—[b]	—
Opsonized zymosan	Ionophore	115.9 ± 8.8	136.6 ± 12.4	123.4 ± 8.4
	PBS	12.1 ± 4.0	—	—
E. coli 536/21 (5×10^8)	Ionophore	22.4 ± 10.1	15.6 ± 7.8	26.6 ± 17.8
	PBS	—	—	—
Opsonized *E. coli* 536/21 (5×10^8)	Ionophore	63.1 ± 18.9	68.2 ± 17.1	71.3 ± 15.3
	PBS	9.1 ± 4.3	—	—
FMLP (10^{-7} M)	Ionophore	107.7 ± 31.0	101.1 ± 2.6	115.3 ± 39.5
Peptidoglycan (10 μg/ml)	Ionophore	96.0 ± 36.2	104.9 ± 1.3	126.6 ± 49.8
E. coli O111:B4 LPS (10 μg/ml)	Ionophore	96.2 ± 10.3	102.5 ± 8.3	110.7 ± 19.8

[a] Human LMB's (10^7) were incubated with the stimuli indicated for 15 min at 37°C. Ca ionophore (4.9 μM) or 50 μl of PBS was added, and the samples were incubated for an additional 15 min at 37°C. Cells which were incubated in the presence of PBS for 15 min at 37°C and then stimulated with the Ca ionophore in the way described above served as the control. Data are expressed as a percentage of the PBS control. The results are mean values of three experiments (± SD).
[b] —, Not detectable.

When LMBs were treated with zymosan or opsonized zymosan and subsequently stimulated with the Ca ionophore, no deactivation was obtained (Table 5). With opsonized zymosan, enhanced formation of leukotrienes was observed. No deactivation was shown when the cells were treated with FMLP or peptidoglycan. Preincubation of human LMBs with opsonized bacteria was three- to fourfold less active in inhibition than nonopsonized bacteria.

Furthermore, under the same experimental conditions, living bacteria were more active in deactivating LMBs than sonicated or heat-inactivated microorganisms, which were three- to fivefold less active in inhibition (data not shown). When various preparations of LPS were analyzed, the following results were obtained. LPS from *S. minnesota* R345 at a concentration range of 5 to 50 μg/ml induced 25 to 75% reduction of ionophore-induced leukotriene release from LMBs, while the LPS from *E. coli* O111:B4 at a concentration from 1 to 100 μg/ml did not lead to deactivation.

DISCUSSION

Our results demonstrate that genetically cloned *E. coli* strains expressing defined MR hemagglutinating properties induce different effects on an LMB cell suspension with regard to chemiluminescence induction, adherence, and phagocytosis as well as mediator release. In comparison to the activation of human PMNs and rat mast cells (23, 25) the strains do not induce leukotriene formation from monocytes as well as histamine release from basophils by themselves. However, subsequent stimulation with the Ca ionophore revealed that preincubation of the LMBs with the various bacteria led to a potent deactivation of subsequent leukotriene formation as well as histamine release (data not shown). This deactivation was obtained with the adhesin- as well as the non-adhesin-expressing strains of *E. coli* 536/21. However, among the various MR strains, differences with regard to the efficacy of deactivation were obtained. For the MR *E. coli* strains, the inhibition of leukotriene release correlated well with the induction of the chemiluminescence response, bacterial adherence, and uptake.

In this regard, *E. coli* 536/21(pANN801-4) (S-MRH$^+$ S-Fim$^+$) was poorly active. *E. coli* expressing S-Fim was nonstimulatory when bacterial uptake as well as the chemiluminescence response was studied. In fact, we cannot conclude that the S-fimbriae themselves induce this effect; the expression of fimbriae may cause a change in the properties of the bacterial cells. This could be a change in hydrophobicity of the outer membrane or an increase in the number of S-adhesins.

The pattern of results obtained by counting CFU to assess adherence and uptake was similar to that obtained from radiolabeling experiments.

It appears that *E. coli* bacteria expressing S-MRH$^+$ or P-adhesins are more potent in the deactivation for leukotriene release, in the induction of uptake, and in the chemiluminescence response than *E. coli* 536/21 and *E. coli* 536/21(pANN801-4) ($P < 0.01$). It has to be clarified in future studies whether purified adhesins express similar biological activities as bacterial expressed adhesins. The inhibitory effect is dependent on the concentration of bacteria used for preincubation as well as on the preincubation time and temperature (1). The presence of intracellular calcium was sufficient for deactivation after bacterial interaction with the LMBs (Fig. 4).

This raises the question of the mechanism of monocyte deactivation. Mediator release, such as leukotrienes from the LMB fraction, can be induced after incubation with zymosan, which requires a mannan receptor for cell activation (12, 20). Stimulation of the human LMB suspension with opsonized zymosan also induced leukotriene release, which was fivefold lower than that obtained with the ionophore. Preincubation of LMBs with opsonized zymosan and the subsequent activation of the cells with the ionophore enhanced leukotriene formation. Furthermore, pretreatment with zymosan did not induce a deactivation of the LMBs. In addition, opsonized bacteria were much less active in deactivation than nonopsonized bacteria. Preincubation of the LMBs with either FMLP, peptidoglycan, or crude bacterial suspension also did not deactivate the cells.

These results suggest that receptor-specific differences participate, which may also include signal-transducing events, e.g., protein kinase C (11, 30, 44), and inositol phosphate metabolism (39, 46).

We previously showed that LPS and lipid A deactivate human granulocytes for subsequent ionophore-induced leukotriene formation (6, 7). With monocytes as the target cells,

the LPSs from *S. minnesota* and *E. coli* expressed different effects. LPS from *S. minnesota* in a concentration range of 5 to 50 μg/ml was able to deactivate the monocyte from 25 to 75%, in contrast to the *E. coli* LPS, which showed no effect. The variable effects of LPS may be caused by differences between the O antigens of the two LPSs (19). The combined results therefore suggest that LPS alone is most likely not the component for deactivation (6, 28).

One may argue that cells other than monocytes, such as lymphocytes or basophils, contribute to the parameters studied (4, 9, 32). The interaction of the bacteria with lymphocytes may induce regulatory factors which may affect the cellular susceptibility for mediator release (4, 32).

Arachidonic acid metabolites such as PGE_2 from monocytes were not significantly affected by preincubation with the various strains. Experiments in which the LMB suspension was purified by the elutriation technique and treated with calcium ionophore and bacteria clearly demonstrated that the presence of lymphocytes and the low amounts of basophils (≈1%) did not influence the chemiluminescence response, deactivation for leukotriene release, or the rate of bacterial uptake; thus, within the suspension, the monocyte is responsible for the effects studied. Obviously, the reactivity of the monocyte obtained from a cell suspension is different from that obtained after adherence (14). In the latter case, the process of spreading in the culture already induces an activation of the cell which may affect the biological responses. Therefore, our major interest was to study the functional activity of the monocyte in suspension combined with lymphocytes as a potential source of cytokines.

The leukotriene data are in contrast to results obtained with PMNs. With these cells, a stimulation of leukotriene release was obtained (23, 36, 37). In preincubation experiments, human granulocytes behave differently than monocytes (Table 4). The amount of LTB_4 after preincubation with the bacterial strains was enhanced after stimulation with the calcium ionophore. However, preincubation with bacteria modulated the capacity of the granulocyte to metabolize the chemotactically active LTB_4 into the biologically inactive w-oxidation products. One may argue that this mechanism stabilizes the chemotactic activity in the case of a bacterial infection, facilitating the influx of granulocytes and subsequent phagocytosis.

Therefore, our data emphasize that, unlike human granulocytes, the monocyte expresses a completely different response pattern after interaction with MR *E. coli* bacteria (10, 25). In contrast, receptor-mediated events, which include the recognition of Fc and complement receptors by the monocyte, induced a low deactivation or even an enhanced generation of lipid mediators (35, 45). Apparently, this mechanism could play an important role during bacterial infection. The direct activation of human granulocytes with MR *E. coli* induces the release of mediators, resulting in an inflammatory response, which then provides the further influx of granulocytes. One may speculate that the monocyte deactivated for the release of low-molecular-weight mediators preferentially now operates as a phagocytosing and antigen-presenting cell. Obviously, the production of monokines, e.g., interleukins 1 and 6 and tumor necrosis factor, is then stimulated (9). Interestingly, Fc-coated particles, such as zymosan or bacteria, activate the monocyte directly for the release of low-molecular-weight mediators (15, 20). This may imply that once the antibody response has been established, the opsonized particle (bacteria) induces leukotriene formation also from monocytes and thus supports the influx of granulocytes, which may then support phagocytosis and the elimination of the bacteria.

Thus, our results suggest that after a stimulus, monocytes and granulocytes act differently with regard to leukotriene formation (10). Further studies are directed to elucidating the membrane biochemical mechanisms of deactivation, and the role of isolated adhesins, additional common bacterial factors, and cytokines for the regulation of the inflammatory response by MR *E. coli* strains.

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