Inflammatory Lipid Mediator Generation Elicited by Viable Hemolysin-forming Escherichia coli in Lung Vasculature

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Summary

Escherichia coli hemolysin, a transmembrane pore-forming exotoxin, is considered an important virulence factor for E. coli-related extraintestinal infections and sepsis. The possible significance of hemolysin liberation for induction of inflammatory lipid mediators was investigated in isolated rabbit lungs infused with viable bacteria (concentration range, 10^4-10^8/ml). Hemolysin-secreting E. coli (E. coli-Hly+), but not an E. coli strain that releases an inactive form of the exotoxin, induced marked lung leukotriene (LT) generation with predominance of cysteinyl L Ts. Eicosanoid synthesis was not inhibited in the presence of plasma with toxin-neutralizing capacity. Pre-application of 2 x 10^8 human granulocytes, which sequestered in the lung microvasculature, caused a severalfold increase in leukotriene generation in response to E. coli-Hly+ challenge both in the absence and presence of plasma. Data are presented indicating neutrophil-endothelial cell cooperation in arachidonic acid lipoxygenase metabolism as an underlying mechanism. We conclude that liberation of hemolysin from viable E. coli induces marked lipid mediator generation in lung vasculature, which is potentiated in the presence of neutrophil sequestration and may contribute to microcirculatory disturbances during the course of severe infections.

Approximately 50% of Escherichia coli isolates causing extraintestinal infections in man elaborate a proteinaceous hemolysin, which thus represents one of the most prevalent bacterial exotoxins encountered in humans (1–5). The relevance of this hemolysin as a determinant of bacterial pathogenicity has been established in animal models with the use of genetically engineered, isogenic bacterial strains (6, 7). An analogous role in human infection has been inferred from the high association of hemolysin production with disease, including pyelonephritis and septicemia (2, 3). The hemolysin was characterized as a novel prototype of a pore-forming protein that may insert as a monomer into target lipid bilayers to generate a functional transmembrane lesion of 1-2 nm effective diameter (8–10). Structurally related toxins are also produced by other Gram-negative rods including Morganella, Proteus, and Pasteurella haemolytica. These agents constitute a family of protein toxins with similar functional and immunological properties (11–13).

Granulocytes have been identified as preferred target cells for E. coli hemolysin in human blood, and the exotoxin turned out as most potent leucocidin known to date (14). In addition, intravascular application of the hemolysin in blood-free perfused rabbit lungs revealed high sensitivity of the pulmonary microvasculature to this bacterial agent (15). Key events of acute respiratory failure were evoked, hallmarkmed by thromboxane-mediated pulmonary hypertension and sustained vascular leakage with progressive lung edema formation. These biophysical alterations were preceded by a burst of inflammatory lipid mediator generation, in particular, release of leukotrienes (16). These findings may be of clinical relevance as bacterial sepsis is the most consistent factor associated with the development of acute respiratory failure in adults, with Gram-negative rods, including E. coli, currently representing the predominant infectious agents (17, 18).

In physiological environments, including plasma constituents and granulocytes, the capacity of hemolysin-secreting E. coli to evoke lung microvascular disturbances may possibly be limited due to the presence of hemolysin-inactivating proteins (e.g., naturally occurring antibodies, lipoproteins [14]) and rapid bacterial phagocytosis. Based on this reasoning, we conducted a study on the interaction of hemolysin-secreting viable E. coli with the vasculature of isolated rabbit lungs, perfused both in the absence or presence of plasma and granulocytes. Acute pulmonary hypertension and protracted lung vascular leakage were again noted, corresponding to the effects of the isolated hemolysin (W. Seeger et al., manuscript in preparation). In the present communication, we report on marked lipid mediator generation elicited by the exotoxin-
Materials and Methods

Reagents. The leukotrienes (LTs) \( \text{LTC}_4 \), \( \text{LTD}_4 \), \( \text{LTE}_4 \), and LT\( \beta \_4 \), 20-OH- and 20-COOH-LT\( \beta \_4 \), and the synthetic LT\( \alpha \)-methyl ester were a generous gift from Dr. J. Rokach, Merck Frosst, Canada. 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE), as well as 5S,12S-diHETE, 5,15-diHETE, and 12-HHT, were obtained from Paezel AG, Frankfurt, FRG. All LTs were checked for purity and quantified spectrophotometrically before use as described (19). Tritiated LTs, used as internal standards, were obtained from New England Nuclear, Dreieich, FRG. Chromatographic supplies included HPLC-grade solvents, distilled in glass (Fluka KG, Heidelberg, FRG), octadecylsilyle 5-\( \mu \)m (Hypersil), and silica gel 5-\( \mu \)m column packing (Machery-Nagel, Duren, FRG), and C-18 Sep-pack cartridges (Waters Associates, Milford, MA). Anti-LTB\_4 , antiserum was received from Dr. J. Salmon (Wellcome Research Laboratories, Beckenham, UK), and anti-LTC\_4 , antiserum was from New England Nuclear. d,L-Lysin-monoacetylsalicilic acid/glucin (9:1) was obtained from Bayer AG (Leverkusen, FRG), and BSA (96% purity, reduced in FFA to <5 mg/g) was from Paezel AG (Frankfurt, FRG). All other biochemicals were obtained from Merck AG (Darmstadt, FRG) in pro-analysis quality.

Isolated Lung Model. The model has been previously described (15, 20-22). Briefly, rabbits of either sex (body weight, 2.2-2.6 kg) were deeply anesthetized and anticoagulated with 1,000 U of heparin per kilogram bodyweight. Blood was centrifuged in a discontinuous Percoll gradient (23, 24) to yield a PMN fraction of ~97% purity. The granulocytes were kept in RPMI 1640 with 20% calf serum and suspended in KHAB. Rabbit blood was obtained from anesthetized animals by femoral artery catheterization after anticoagulation with heparin. Blood was centrifuged for 10 min at 3,000 g, and plasma was stored at -70°C until experimental use.

Preparation of E. coli. A hemolysin-producing E. coli strain (LE2001; Hly\(^{+}\)) and an E. coli strain secreting an inactive form of the toxin (MC4100; Hly\(^{-}\)) (25) were used. Both strains were grown to late logarithmic phase, washed three times in KHAB (3 min, 690 g), and quantified by absorbance measurement at 578 nm. A calibration curve established for each E. coli strain was used. Based on this approximate quantification, aliquots of the E. coli suspension were mixed with the lung buffer medium directly after the final washing procedure to achieve the required bacterial concentrations. Aliquots were plated on agar, and CFU were counted for definitive quantification in each experiment. Data evaluation was undertaken only when the approximate quantification differed from counts by <30%.

Analytical Procedures. LTs and HETEs were extracted from lung perfusion fluid by octadecylsilyle solid phase extraction columns as described (19, 26). Conversion into methylsters was performed by addition of freshly prepared diazomethane in ice-cold diethyl ether. Reversed-phase (RP)-HPLC of nonmethylated compounds was carried out on octadecylsilyle columns (Hypersil; 5-\( \mu \)m particles) with a mobile phase of methanol/water/acetic acid (72:28:0.16, pH 4.9) (19). In addition to the conventional UV detection at 270 nm (LTs) and 237 nm (HETEs), a photodiode array detector (990; Waters Associates) was used that provided full UV spectra (190-600 nm) of eluting compounds and allowed checking for peak purity and substraction of possible coeluting material. For further verification, samples were collected in 15-s fractions and subjected to post-HPLC-RIA with anti-LTC\_4 and anti-LTB\_4 as described (19, 26). RP-HPLC of methylated compounds was performed isocratically (72:28:0.16, pH 4.9) for 5 min, followed by a linear gradient to 90:10:0.16 over 10 min (gradient former model 250; Gynkothek, Munich, FRG). Single-phase (SP)-HPLC was carried out using a modification of the method of Powell (27). Isocratic elution with hexane/isopropanol/acetate (97:3:0.1; 12 min) was followed by a linear gradient to 86:14:0.1 over 10 min. All data obtained by the different analytical procedures were corrected for the respective recoveries of the overall analytical procedure and are given in nanomoles per total perfusate volume. Recovery was determined by separate recovery experiments using different quantities of the individual compounds in the appropriate concentration range. Factors for recovery were further confirmed by addition of 40-80 \( \mu \)Ci \( [\text{H}] \) LT\( \alpha \), \( [\text{H}] \) LT\( \beta \), and \( [\text{H}] \) 5,15-HETE to the perfusate as internal standards in selected samples. Detection limits of the different compounds are indicated in Table 2.

E. coli hemolysin concentrations in the buffer fluid were determined by an ELISA that uses a monoclonal anti-hemolysin antibody to capture the antigen, and a second, polyclonal antibody for development, as described (28).

Experimental Protocol. After a steady-state period of 40 min, the recirculating perfusate was exchanged by fresh buffer medium, which contained 500 \( \mu \)M acetylsalicilic acid (ASA) in all experiments. This cyclooxygenase inhibitor was used to suppress hemolysin-evoked severe pulmonary hypertension, known to be mediated predominantly by lung thromboxane generation (15). In particular experiments, the KHB perfusate was supplemented with 15% (vol/vol) rabbit plasma. Human plasma cannot be used in perfused rabbit lung studies, as its complement system is known to be activated spontaneously at rabbit cell surfaces via the alternate pathway (29, 30). Higher percentages of rabbit plasma were not used because of analytical limitations of the chromatographic techniques arising...
under these conditions. 10 min after perfusate exchange, 2 × 10⁸ PMN in 2 ml KHAB or the same volume of cell-free buffer were slowly injected into the pulmonary artery. Random cell counts in the recirculating medium 3 and 10 min after PMN application documented a nearly quantitative sticking of these cells in the lung vasculature (<3% circulating PMN). Microscopical examinations revealed that the vast majority of the PMN were sequestered in the pulmonary capillaries. This is in line with previous investigations, in which >90% of freshly prepared PMN were noted to marginate in alveolar microvessels after bolus application in isolated perfused lungs (31-33). 10 min after PMN or sham application, live E. coli bacteriae were admixed to the recirculating buffer volume to give final concentrations of 10⁴, 10⁵, 10⁶, or 10⁷ CFU/ml; and time was set as zero. 10-ml perfusate samples for determination of arachidonic acid (AA) lipoxygenase products were taken at -2, 5, 10, 20, 30, 60, and 90 min (end of experiment). Samples were immediately centrifuged at 0°C (5,000 g, 5 min) and kept on ice until transfer to the extraction columns.

In parallel with each lung experiment in which granulocytes were applied, six aliquots of 4 × 10⁸ PMN (corresponding preparation) were suspended in a total volume of 5 ml buffer fluid, composed in analogy to the lung perfusate (KHAB or KHAB plus rabbit plasma). According to the time schema of the lung experiments, the incubation was terminated after 5, 10, 20, 30, 60, and 90 min, respectively, by centrifugation at 0°C (5,000 g, 5 min). The total 5-ml supernatant was subjected to eicosanoid analysis.

Control experiments, following an identical time schedule but performed in the absence of E. coli, included lung perfusions with buffer, buffer plus rabbit plasma, buffer plus PMN, or buffer plus rabbit plasma plus PMN (for each, n = 2). Corresponding in vitro incubations of PMN in the absence of E. coli were performed. In additional controls, 10⁶/ml nonhemolytic E. coli (E. coli-Hly-) were applied using the same protocols.

Control experiments for determination of the disappearance rate (metabolism/uptake) of individual LTs and 5-HETE in the isolated lung preparation were performed by use of radiolabeled standards (Table 1). Concentrations used were those corresponding to the range of LTs and 5-HETE appearing in the model of pulmonary leukostasis.

**Results**

**Lung Perfusion with E. coli-Hly⁺ in the Absence of PMN.** Under baseline conditions, AA lipoxygenase products in lung perfusates ranged below the detection limits of the respective analytical procedures. Application of viable E. coli-Hly⁺ elicited a dose- and time-dependent release of eicosanoids into the recirculating buffer medium (Figs. 1-6), commencing within 5–10 min after bacterial challenge, with cysteinyl LTs representing the quantitatively dominating compounds. The time course of LTC₄ liberation (rapid onset, followed by subsequent decline) vs. that of LTE₄ appearance (delayed onset, plateauing after 30-60 min) suggested metabolism of LTC₄ via LTD₄ to LTE₄. These metabolic steps have been demonstrated for perfused lungs of different species, including the rabbit (19, 21, 34). The very rapid conversion of LTD₄ to LTE₄ is responsible for low perfusate levels of the former, ranging below or just above the respective detection limit (LTD₄ data not given in detail). In addition to the cysteinyl LTs, LTB₄ appeared in the buffer medium of E. coli-Hly⁺-challenged lungs. This compound displayed similar time course (plateau after 30-60 min) but lower total amount as compared with the cysteinyl LTs. No ω oxidation products of LTB₄ appeared in these experiments performed in the absence of infused granulocytes. This lack of LTB₄-ω hydroxylation has previously been noted (19, 21). It is explained by the relatively low quantities of marginated PMN in this blood-free perfused lung model, and by the virtual absence of ω oxidation capacity of rabbit (F. Grimminger et al., unpublished data) and porcine (35) PMN, a finding that contrasts with that of human neutrophils (36).

| Injected ³H standard | Recovery from the perfusate at: | 5 min | 30 min | 55 min | 95 min |
|----------------------|--------------------------------|-------|--------|--------|--------|
| LTC₄ + metabolites    | LTC₄ and LTE₄                  | 84.2 (2.9) | 74.9 (3.2) | 62.1 (4.3) | 50.9 (5.3) |
| LTB₄⁺                |                                | 77.4 (5.4) | 54.6 (4.4) | 43.5 (3.8) | 32.2 (4.9) |
| 5-HETE               |                                | 80.8 (8.4) | 44.9 (4.1) | 21.3 (2.4) | 11.7 (0.9) |
| 6-trans-LTB₄         |                                | 85.2 (2.9) | 70.4 (5.2) | 58.4 (4.2) | 48.5 (6.1) |
| 6-trans-epi-LTB₄     |                                | 82.8 (2.1) | 64.9 (3.8) | 54.0 (2.8) | 44.5 (4.8) |

Radiolabeled standards (15 nmol LTC₄ and LTBr; 5 nmol 5-HETE, 6-trans-LTB₄, and 6-trans-epi-LTB₄) were bolus injected into the pulmonary artery of KHAB-perfused rabbit lungs under baseline conditions. The recoveries from the perfusate at the different times after application are indicated (mean [SE] of five independent experiments).

* In blood-free perfused rabbit lungs without pre-application of human PMN, LTB₄ is not ω oxidized (24). Metabolites of LTB₄ do thus not need to be considered for calculation of recovery under these conditions.

Table 1. Disappearance Rate of Radiolabeled AA Lipoxygenase Products from the Pulmonary Circulation
Figure 1. Time-dependent liberation of eicosanoids into the buffer medium of lungs and/or PMN challenged with 10⁶/ml E. coli-Hly⁺. Lungs (Lu) were perfused with 200 ml KHAB in the absence or presence of rabbit plasma (Pla; 15% [vol/vol]) and human PMN (2 × 10⁸). Total amounts of the different AA lipoxygenase products in the recirculating perfusate volume are given. In accompanying in vitro experiments, 5 × 10⁶ PMN were incubated with 10⁹/ml E. coli-Hly⁺ in a buffer volume of 5 ml; total amounts of eicosanoids measured in these studies were corrected (factor 40) to correspond to the buffer volume in the perfused lung experiments. 20-OH-LTB₄ and 20-COOH-LTB₄ are comprised as ω oxidation products (ω-ox) of LTB₄. The LTA₄ hydrolysis products include the 5,6-diastereomeric pair of LTB₄, both nonenzymatically formed 5,6-diHETEs. Means of four independent experiments are given for each group and each time point. SEM data are included in Table 2.

All eicosanoids formed in the E. coli-Hly⁺-challenged lungs (Table 2), as well as the total sum of AA lipoxygenase products (Fig. 6), displayed corresponding dose dependency. Lipid mediator formation was not detectable at 10⁴ bacteria/ml, commenced at 10⁵/ml, and plateaued at 10⁶-10⁷/ml. In contrast to the leukotrienes, no 5-, 8-, 9-, 11-, 12-, or 15-HETEs, di-HETEs (5S,12S- or 5,15-), or nonenzymatic degradation products of LTA₄ were detected. Due to the presence of ASA in all experiments, liberation of thromboxane A₂ and prostaglandin I₂ ranged below detection limits (random measurements with RIA in lung perfusate). The presence of rabbit plasma did not affect profile, time course,
and amount of AA lipoxygenase product formation (Lu + Pla in Figs. 1-6). A dose-dependent liberation of hemolysin into the buffer medium was documented by ELISA (Table 3).

**E. coli-Hly** Challenge in the Presence of PMN. Incubation of PMN with *E. coli-Hly* in vitro induced rapid generation of LTB4, peaking after 20 min. It was accompanied and followed by a more delayed appearance of 20-OH- and 20-COOH-LTB4, combined as ω oxidation products in Fig. 2 and Table 2, in accordance with the known ω oxidation capacity of human PMN (36). In parallel with LTB4 generation, substantial amounts of 6-trans- and 6-trans-epi-LTB4, as well as 5,6-diHETEs, were detected in the incubation medium. These products indicate secretion of intact LTA4 into the extracellular space; they are comprised as nonenzymatic LTA4 hydrolysis products in Fig. 3 and Table 2. All AA lipoxygenase products displayed corresponding dose dependency. Release reaction commenced at 10^5 bacteria/ml and plateaued at 10^6–10^7 ml. 5-HETE ranged just above the detection limit in single experiments performed with the higher bacterial load. Cysteinyl LTs were not measured upon PMN challenge in vitro, indicating absence of significant amounts of eosinophils in the granulocyte preparation. 12-HETE, 5S,12S-DiHETE, and 15-HETE all ranged below detection limits, indicating absence of contaminating platelets and lymphocytes. The admixture of rabbit plasma to PMN incubated with *E. coli-Hly* in vitro did not alter profile and time course of AA lipoxygenase product formation. However, all LTA4-derived enzymatic and nonenzymatic products ranged at two- to threefold higher values in the presence of opsonin.

Infusion of *E. coli-Hly* in rabbit lungs, in which 2 × 10^8 human PMN were sequestered, again elicited predominant cysteinyl LT generation, with profile and time course (early LTC4 release, protracted LTE4 accumulation) corre-
Table 2. Generation of Eicosanoids in E. Coli-HLY* -challenged Perfused Lungs and PMN In Vitro

| E. coli/ml Protocol | LTB₄ | 20-ox-LTB₄ | LTA₄ hydrolysis | 5-HETE | LTC₄ | LTE₄ |
|--------------------|------|------------|-----------------|--------|------|------|
|                    | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min |
| 10⁴                 |       |         |       |         |       |         |       |         |       |         |       |         |       |         |
| Lu                 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 |
| Lu + Pla           | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 |
| Lu + PMN           | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 |
| Lu + PMN + Pla     | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 |
| PMN                | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 |
| PMN + Pla          | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 | <0.2 ± 0.2 |

10⁵

| Lu                  | 0.59 ± 0.13 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | 0.24 ± 0.2 | 0.61 ± 1.35 | 0.2 ± 0.2 | ± 0.09 ± 0.22 ± 0.32 |
| Lu + Pla            | <0.61 ± 0.21 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | <0.2 ± 0.2 | 1.02 ± 1.64 | ± 0.2 ± 0.31 ± 0.50 |
| Lu + PMN            | 0.69 ± 0.12 | 0.66 ± 0.21 | 2.13 ± 0.41 | <0.1 ± 0.1 | <0.15 ± 0.15 | 0.48 ± 0.2 | ± 0.12 ± 0.24 ± 0.41 |
| Lu + PMN + Pla      | 0.88 ± 0.31 | 0.56 ± 0.22 | 3.02 ± 0.55 | <0.1 ± 0.1 | <0.15 ± 0.15 | 0.99 ± 0.2 | ± 0.28 ± 0.44 ± 0.68 |
| PMN                 | <0.1 ± 0.13 | <0.1 ± 0.08 | <0.2 ± 0.21 | 0.29 ± 0.46 | <0.2 ± 0.15 | <0.2 ± 0.2 | ± 0.14 ± 0.05 ± 0.10 |
| PMN + Pla           | 0.59 ± 0.31 | 1.13 ± 0.13 | 1.94 ± 0.21 | 0.29 ± 0.31 | <0.15 ± 0.31 | <0.2 ± 0.2 | <0.2 ± 0.2 | ± 0.06 ± 0.05 ± 0.10 |

10⁶

| Lu                  | 1.21 ± 0.32 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | 0.46 ± 0.28 | 1.22 ± 2.53 | ± 0.16 ± 0.31 ± 0.41 |
| Lu + Pla            | 0.96 ± 0.24 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.1 ± 0.1 | <0.15 ± 0.15 | 0.31 ± 0.2 | 1.35 ± 2.41 | ± 0.10 ± 0.26 ± 0.62 |
| Lu + PMN            | 1.44 ± 0.41 | 2.31 ± 0.38 | 3.78 ± 0.51 | <0.1 ± 0.1 | <0.15 ± 0.15 | 1.05 ± 0.34 | 2.48 ± 5.13 | ± 0.31 ± 0.51 ± 0.92 |

Control Studies. In the absence of plasma or PMN, the E. coli strain producing inactive hemolysin (Hly⁻; used at 10⁴/ml) did not induce any measurable liberation of AA lipoxigenase products both in the perfused lung experiments and in the isolated PMN studies. In the absence of both plasma and PMN, eicosanoid liberation occurred with profiles corresponding to those evoked by E. coli-Hly⁺ (Table 4). The total amounts of the different lipoxigenase products were, however, only ~30% of those evoked by challenge with the corresponding concentration of E. coli-Hly⁺.

In all control studies using plasma, PMN, or both in the absence of E. coli, no measurable lipoxigenase product formation was noted in the isolated lungs and upon PMN incubation in vitro.

Discussion

In a preceding study in isolated rabbit lungs, bolus application of purified E. coli hemolysin (250 ng/ml perfusate) was
Table 2. (continued)

| E. coli/ml | Protocol | LTB₄ | 20-ox-LTB₄ | LTA₄ hydrolysis | 5-HETE | LTC₄ | LTE₄ |
|-----------|----------|------|------------|-----------------|-------|------|------|
|           |          | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min | 30 min | 90 min |
| Lu + PMN + Pla | 2.82 | 1.58 | 2.41 | 6.82 | <0.1 | <0.1 | <0.15 | <0.15 | 2.53 | 1.32 | 3.98 | 7.66 |
| ± 0.46 | ± 0.32 | ± 0.61 | ± 1.04 | ± 0.69 | ± 0.44 | ± 0.92 | ± 1.21 |
| PMN | 0.41 | 0.15 | 9.22 | 1.62 | <0.1 | <0.1 | <0.15 | <0.15 | <0.2 | <0.2 | <0.2 | <0.2 |
| ± 0.16 | ± 0.06 | ± 0.24 | ± 0.37 | ± 0.18 | ± 0.11 | ± 0.39 | ± 0.51 |
| PMN + Pla | 1.98 | 0.49 | 2.13 | 3.92 | 0.69 | 0.48 | 0.68 | <0.15 | <0.2 | <0.2 | <0.2 | <0.2 |
| ± 0.36 | ± 0.21 | ± 0.44 | ± 0.56 | ± 0.28 | ± 0.16 | ± 0.10 |

| 10⁷ | Lu | 1.34 | 0.51 | <0.1 | <0.1 | <0.1 | <0.15 | <0.15 | 0.31 | <0.2 | 0.99 | 1.68 |
| ± 0.42 | ± 0.18 | ± 0.14 | ± 0.24 | ± 0.42 |
| Lu + Pla | 1.84 | 0.26 | <0.1 | <0.1 | <0.1 | <0.15 | <0.15 | <0.5 | <0.2 | 1.62 | 2.39 |
| ± 0.31 | ± 0.11 | ± 0.17 | ± 0.39 | ± 0.51 |
| Lu + PMN | 1.80 | 0.52 | 2.59 | 4.10 | <0.1 | <0.1 | <0.15 | <0.15 | 0.62 | 0.25 | 2.08 | 3.96 |
| ± 0.44 | ± 0.21 | ± 0.43 | ± 0.56 | ± 0.24 | ± 0.1 | ± 0.41 | ± 0.49 |
| Lu + PMN + Pla | 2.72 | 0.66 | 3.96 | 6.68 | <0.1 | <0.1 | <0.15 | <0.15 | 2.08 | 0.66 | 4.31 | 6.49 |
| ± 0.54 | ± 0.16 | ± 0.81 | ± 1.23 | ± 0.51 | ± 0.22 | ± 0.67 | ± 0.83 |
| PMN | 0.52 | 0.22 | 0.83 | 1.98 | <0.1 | <0.1 | <0.15 | <0.15 | <0.2 | <0.2 | <0.2 | <0.2 |
| ± 0.13 | ± 0.11 | ± 0.24 | ± 0.41 |
| PMN + Pla | 2.43 | 0.68 | 2.12 | 4.61 | 0.81 | 0.62 | 0.81 | <0.15 | <0.2 | <0.2 | <0.2 | <0.2 |
| ± 0.41 | ± 0.21 | ± 0.62 | ± 0.71 | ± 0.21 | ± 0.11 | ± 0.33 |

Lungs (Lu) were perfused with 200 ml KHAB in the absence or presence of rabbit plasma (Pla; 15% [vol/vol]) and human PMN (2 x 10⁸). They were challenged with different concentrations of E. coli-Hly⁺ in the buffer medium. Total amounts of the various AA lipoxygenase products in the perfusate medium 30 and 90 min after application of the bacteria are given in nanomoles. In accompanying in vitro experiments, 5 x 10⁶ PMN were incubated with 10⁶/ml E. coli-Hly⁺ in a buffer volume of 5 ml; total amounts of eicosanoids measured in these studies were corrected (factor 40) to correspond to the buffer volume in the perfused lung experiments. 20-OH-LTB₄ and 20-COOH-LTB₄ are comprised as oxidation products of LTB₄. The LTA₄ hydrolysis products include the 5,6-diastereomeric pair of LTB₄, both nonenzymatically formed 5,6-diHETEs. Each point represents mean ± SEM of four independent experiments.

Table 3. Hemolysin Concentration in the Recirculating Buffer Fluid of Perfused Lungs

| E. coli/ml | Protocol | Concentration after E. coli (Hly⁺) application interval of: | 30 min | 60 min | 90 min |
|-----------|----------|-------------------------------------------------------------|-------|-------|-------|
|           |          |                                                              |       |       |       |
| 10⁴ (n = 4) | Lu      | <20                                                          | <20   | <20   | <20   |
|           | Lu + PMN | <20                                                          | <20   | <20   | <20   |
| 10⁵ (n = 4) | Lu      | <20                                                          | <20   | <20   | <20   |
|           | Lu + PMN | <20                                                          | <20   | <20   | <20   |
| 10⁶ (n = 4) | Lu      | 32.5 ± 15.6                                                  | 59.0 ± 21.3 | 63.2 ± 20.2 | 48 ± 14 |
|           | Lu + PMN | 15.0 ± 5.5                                                   | 35.0 ± 7.8 | 134.4 ± 29.9 | 48 ± 14 |
| 10⁷ (n = 4) | Lu      | 79.6 ± 41.3                                                  | 110.6 ± 38.6 | 117.0 ± 19.6 | 48 ± 14 |
|           | Lu + PMN | 66.0 ± 24.4                                                  | 90.4 ± 20.2 | 117.0 ± 19.6 | 48 ± 14 |

Lungs (Lu) were perfused with 100 ml KHAB in the absence or presence of human PMN (2 x 10⁶). They were challenged with different concentrations of E. coli-Hly⁺ in the buffer medium. Hemolysin was determined in aliquots of the perfusate taken at the indicated intervals after application of the bacteria. Measurements in the presence of plasma could not be performed due to nonspecific disturbances in the ELISA. Each point represents mean ± SEM of four independent experiments.
Lungs (Lu) were perfused with 200 ml KHAB in the absence or presence of rabbit plasma (Pla; 15% [vol/vol]) and human PMN (2 × 10⁸) and challenged with 10⁶/ml E. coli-Hly- in the buffer medium. Total amounts of the various AA lipoxygenase products in the perfusate at the different time intervals after application of the bacteria are given in manomoles. In accompanying in vitro experiments, 5 x 10⁶ PMN were incubated with 10⁶/ml E. coli-Hly- in a buffer volume of 5 ml; total amounts of eicosanoids measured in these studies were corrected (factor 40) to correspond to the buffer volume in the perfused lung experiments. No eicosanoid liberation was detected in E. coli-Hly+-challenged lungs perfused with KHAB, KHAB plus plasma, or KHAB plus PMN; these constellations of buffer fluid have thus been omitted from the table. Similarly, in vitro incubation of PMN elicited AA lipoxygenase product generation only in the presence of plasma. 20-OH-LTB₄ and 20-COOH-LTB₄ are comprised as oxidation products of LTB₄. The LTA₄ hydrolysis products include the 5,6-diastereomeric pair of LTB₄, both nonenzymatically formed 5,6-diHETEs. Each point represents mean ± SEM of four independent experiments.

Table 4. Eicosanoid Formation Evoked by Inactive Hemolysin-forming E. coli in the Presence of Plasma and PMN

| Variable     | Protocol                  | Amount after interval of: |
|--------------|---------------------------|---------------------------|
|              |                           | 10 min | 20 min | 30 min | 60 min | 90 min |
| LTB₄         | PMN + Pla                 | 0.36 ± 0.09 | 0.93 ± 0.24 | 1.12 ± 0.26 | 0.44 ± 0.11 | 0.23 ± 0.06 |
|              | Lu + PMN + Pla            | <0.1 | 0.54 ± 0.18 | 1.22 ± 0.41 | 0.64 ± 0.19 | 0.33 ± 0.09 |
| 20-ox-LTB₄   | PMN + Pla                 | <0.1 | 0.41 ± 0.07 | 0.89 ± 0.25 | 1.12 ± 0.34 | 0.98 ± 0.29 |
|              | Lu + PMN + Pla            | <0.1 | 0.39 ± 0.13 | 0.95 ± 0.34 | 1.85 ± 0.49 | 2.23 ± 0.80 |
| LTA₄ hydrolysis | PMN + Pla                 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
|              | Lu + PMN + Pla            | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| 5-HETE       | PMN + Pla                 | <0.15 | 0.24 ± 0.10 | <0.15 | <0.15 | <0.15 |
|              | Lu + PMN + Pla            | <0.15 | <0.15 | <0.15 | <0.15 | <0.15 |
| LTC₄         | PMN + Pla                 | <0.2 | <0.2 | <0.2 | <0.2 | <0.2 |
|              | Lu + PMN + Pla            | 0.31 ± 0.13 | 0.91 ± 0.34 | <0.2 | 0.35 ± 0.09 | <0.2 |
| LTE₄         | PMN + Pla                 | <0.2 | <0.2 | <0.2 | <0.2 | <0.2 |
|              | Lu + PMN + Pla            | 0.38 ± 0.11 | 0.99 ± 0.34 | 1.61 ± 0.43 | 1.58 ± 0.54 | 2.59 ± 0.71 |

Lungs (Lu) were perfused with 200 ml KHAB in the absence or presence of rabbit plasma (Pla; 15% [vol/vol]) and human PMN (2 × 10⁸). They were challenged with 10⁶/ml E. coli-Hly- in the buffer medium. Total amounts of the various AA lipoxygenase products in the perfusate at the different time intervals after application of the bacteria are given in manomoles. In accompanying in vitro experiments, 5 x 10⁶ PMN were incubated with 10⁶/ml E. coli-Hly- in a buffer volume of 5 ml; total amounts of eicosanoids measured in these studies were corrected (factor 40) to correspond to the buffer volume in the perfused lung experiments. No eicosanoid liberation was detected in E. coli-Hly+-challenged lungs perfused with KHAB, KHAB plus plasma, or KHAB plus PMN; these constellations of buffer fluid have thus been omitted from the table. Similarly, in vitro incubation of PMN elicited AA lipoxygenase product generation only in the presence of plasma. 20-OH-LTB₄ and 20-COOH-LTB₄ are comprised as oxidation products of LTB₄. The LTA₄ hydrolysis products include the 5,6-diastereomeric pair of LTB₄, both nonenzymatically formed 5,6-diHETEs. Each point represents mean ± SEM of four independent experiments.

Noted to induce rapid leukotriene generation (16). The total amount of AA lipoxygenase products was ~8-10 nmol in those experiments, and the profile of metabolites corresponded well to that evoked by infusion of viable hemolysin-forming E. coli in the present investigation. A more protracted time course (plateau after 30-60 min) and lower total amounts of products (~3 nmol), noted in the current study, are readily explained by the retarded liberation of the exotoxin from the bacteria and by the lower maximum hemolysin concentrations achieved (~135 ng/ml at the highest E. coli-Hly⁺ contents used). The complete absence of LT release in lungs challenged with isogenic E. coli—secreting functionally inactive hemolysin clearly indicates that the bacterial exotoxin represents the causative agent of inflammatory lipid mediator induction and excludes a predominant role of bacterial LPS under the present conditions. This is in line with the preceding study, in which purified hemolysin with a far-reaching reduction in endotoxin content was used. In the presence of plasma, no diminution of E. coli-Hly⁺-evoked eicosanoid generation was noted. This finding is noteworthy in view of the hemolysin-inactivating capacity of naturally occurring antibodies and lipoproteins (14). It supports the notion that rapid toxin binding to target cells competes with toxin neutralization.

Furthermore, it is conceivable that direct transfer of the exotoxin from cell-adherent bacteria occurs. In such a "hidden microenvironment," plasma inactivators might be virtually ineffective.

Granulocytes are extremely susceptible to attack by E. coli hemolysin (14, 37-40), and exotoxin-induced leukotriene generation in these cells has been previously observed (41). We found that the toxin-provoked profile of lipoxygenase products corresponded to that of calcium ionophore-exposed neutrophils (24, 35, 36, 42). Via the 5-lipoxygenase pathway, a hydroperoxy intermediate (5-HPETE), and subsequently the 5,6-epoxide LTA₄, are formed, the latter being converted to LTB₄ as the major enzymatic secretion product. Reuptake of LTB₄ ultimately results in the appearance of its oxidation products (36, 42, 43). Diverging from this pathway, 5-HPETE is metabolized to 5-HETE, and "excessive" LTA₄ is directly released into the extracellular space (44). In aqueous medium, this unstable IT intermediate undergoes rapid nonenzymatic hydrolysis with a half-time of only a few seconds (45), resulting in the appearance of four nonenzymatic degradation products (diastereomeric pairs of 6-trans-LTB₄ and 5,6-DiHETE). In the absence of plasma, PMN eicosanoid generation was elicited only by the E. coli strain forming active...
hemolysin. Previous investigations, using different channel-forming bacterial protein toxins in subcytolytic concentrations, have suggested a central role of transmembrane calcium flux through toxin-created pores in triggering lipid mediator generation (20, 46–49). Surprisingly, the E. coli-Hly+-evoked AA lipoxygenase metabolism was even augmented in the presence of plasma. However, under these circumstances, moderate PMN eicosanoid generation was also elicited by the E. coli strain producing inactive toxin. These findings suggest an opsonizing function of plasma, causing enhanced bacterial uptake by the PMN; phagocytosis-related activation of AA metabolism is well known. Though phagocytosis was not directly measured in the present study, random microscopical examinations indeed revealed PMN uptake of both E. coli-Hly+ and E. coli-Hly− in the presence of plasma.

Under conditions of lung neutrophil sequestration, hemolysin attack on both microvascular endothelium and infused (adherent) granulocytes has to be expected. The profile of AA lipoxygenase products detected under these circumstances corresponded to that of non-PMN-charged lungs. LTB4 generation was, however, followed by rapid oxidation of this compound, reflecting metabolic activity of the infused granulocytes. The most striking finding was a severalfold increase in the total amounts of the different AA lipoxygenase products. Related to the data of non-PMN-charged lungs, the sum of lipoxygenase products was augmented approximately threefold in the presence of PMN and approximately sixfold after preceding application of both PMN and plasma (quantified 90 min after E. coli-Hly+-challenge). Related to the sum of lipoxygenase products evoked in E. coli-Hly+-exposed PMN in vitro, a three- to fourfold augmentation was calculated both in the absence and presence of plasma. Considering the disappearance rates of the different lipoxygenase products in the perfused lung model, which may surpass 50% after a 90-min recirculation (Table 1), this augmentation of product formation is even more apparent. Collectively, these data suggest PMN endothelial cooperativity in the model of pulmonary leukostasis in response to the E. coli-Hly+-challenge. PMN-secreted LTA4, whether evoked by hemolysin attack or, to a minor extent, by phagocytic activity, apparently completely escapes extracellular decay due to avid uptake and enzymatic conversion by lung vascular cells. Preceding in vitro studies have indeed demonstrated transcellular LTA4 shift between PMN and endothelial cells with subsequent conversion to LTC4 by the endothelial glutathione-S-transferase (50, 51). Bolus injection of synthetic LTA4 into the pulmonary artery has documented rapid uptake and nearly quantitative enzymatic conversion of this unstable LT precursor in the presently used lung model (19). Moreover, selective stimulation of neutrophils sequestered in rabbit lungs also elicited cysteinyl LT appearance at the expense of LTA4 degradation products, thus demonstrating intercellular LTA4 transfer (52). Modulation of PMN 5-lipoxygenase metabolisms with a shift in 5-HPETE metabolism from the peroxidase to the LTA4 synthetase pathway was additionally noted in these experiments. This notion corresponds to the present finding of 5-HETE generation in E. coli–challenged PMN in vitro, but not under conditions of microvascular adherence in situ. However, both phenomena, decrease in PMN 5-HETE generation in favor of LTA4 formation and PMN endothelial LTA4 transfer, can quantitatively not fully account for the severalfold increase in LTA4-derived products noted upon hemolysin challenge of the PMN-charged lungs. Similar potentiation of eicosanoid generation was noted upon in situ stimulation of microvascularly sequestered PMN (26, 52). “Feeding” of adherent granulocytes with endothelium-derived arachidonic acid, thus perpetuating PMN LTA4 generation and transcellular formation of LTA4-derived products, may be one underlying mechanism (53, F. Grimminger et al., unpublished results). Overall, the complex coordinative interactions between endothelial cells and adherent granulocytes are still poorly understood (54, 55).

In conclusion, the present findings support and extend the contention that exotoxin released in the immediate vicinity of target cells may escape the neutralizing capacity of plasma constituents. The described release of inflammatory mediators, amplified in the presence of endothelium-adherent granulocytes and suggestive for intercellular cooperativity, may be relevant for microcirculatory disturbances evoked by E. coli hemolysin and by bacteria secreting functionally related cytolysins.

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