Bactericidal activity of human eosinophilic granulocytes against Escherichia coli

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Eosinophilic granulocytes are believed to participate in the body’s defense against infestation with parasites, especially helminths (such as nematodes and trematodes) in the larval stage (35). They are also typically present in the allergic inflammatory response seen in conditions such as allergy and asthma, where they may contribute to tissue damage and connective tissue remodeling (35). However, the roles of eosinophils in host defense against bacterial invasion are possible. In a system for bacterial viable counts, the bactericidal activity of eosinophils and the contribution of different cellular antibacterial systems against *Escherichia coli* were investigated. Eosinophils showed a rapid and efficient killing of *E. coli* under aerobic conditions, whereas under anaerobic conditions bacterial killing decreased dramatically. In addition, diphenylene iodonium chloride (DPI), an inhibitor of the NADPH oxidase and thereby of superoxide production, also significantly inhibited bacterial killing. The inhibitor of nitric oxide (NO) (L-arginine) production L-5(1-iminoethyl)-ornithine dihydrochloride (L-NIO) did not affect the killing efficiency, suggesting that NO or derivatives thereof are of minor importance under the experimental conditions used. To investigate the involvement of superoxide and eosinophil peroxidase (EPO) in bacterial killing, EPO was blocked by azide. The rate of *E. coli* killing decreased significantly in the presence of azide, whereas addition of DPI did not further decrease the killing, suggesting that superoxide acts in conjunction with EPO. Bactericidal activity was seen in eosinophil extracts containing granule proteins, indicating that oxygen-independent killing may be of importance as well. The findings suggest that eosinophils can participate in host defense against gram-negative bacterial infection and that oxygen-dependent killing, i.e., superoxide acting in conjunction with EPO, may be the most important bactericidal effector function of these cells.

**Materials and Methods**

Eosinophilic granulocytes were isolated from the bone marrow of healthy volunteers by using dextran sedimentation. The cells were plated in 96-well plates and incubated for 3 days at 37°C in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were then washed and used for the experiment. The bacterial strain used in the experiment was *Escherichia coli* O55:B5, which was obtained from Difco, Detroit, Mich. Boron dipyrromethan (BODIPY)-labeled neutrophils were obtained from Molecular Probes Europe BV, Leiden, The Netherlands.

**Results**

The bactericidal activity of eosinophils against *Escherichia coli* was investigated. In the presence of azide, whereas addition of DPI did not further decrease the killing, suggesting that superoxide acts in conjunction with EPO. Bactericidal activity was seen in eosinophil extracts containing granule proteins, indicating that oxygen-independent killing may be of importance as well. The findings suggest that eosinophils can participate in host defense against gram-negative bacterial infection and that oxygen-dependent killing, i.e., superoxide acting in conjunction with EPO, may be the most important bactericidal effector function of these cells.
Acid extraction of proteins from eosinophils. Pellets of frozen eosinophils were resuspended in ice-cold water and subjected to intermittent sonication in a water bath in the presence of ice for 10 s at 40 W until no cells could be seen settling to the bottom of the tube. The lysate was extracted with 0.16 M H2SO4 and kept on ice for 30 min with intermittent vortexing. After centrifugation at 23,000 × g for 20 min to sediment insoluble material, the recovered supernatant was dialyzed against 1 mM Tris-HCl (pH 7.4) until the pH of the dialysate was ≥7.0. Thereafter, the extracted protein was dialyzed against 1 mM Tris-HCl-150 mM NaCl at pH 7.4 in dialysis cassettes (Slide-A-Lyzer; Pierce, Rockford, Ill.) and stored at 4°C until use. The protein content was determined using a colorimetric assay based on bicinchoninic acid (BCA protein assay reagent; Pierce).

Antibacterial assay. The bactericidal activity of eosinophils was determined by measuring the effect on bacterial colony-forming activity as described previously (28). From bacterial suspensions incubated with or without cells, aliquots were taken at the indicated time intervals and serially diluted in sterile ice-cold PBS. A 30-μl aliquot of the diluted sample was transferred to 5 ml of 1.3% (wt/vol) mohr’s salt at 48°C with 0.8% (wt/vol) nutrient broth and 0.5% (wt/vol) NaCl and poured into a petri dish. The temperature of the agar causes disruption of the granulocytes and release of intracellular bacteria. The agar was allowed to solidify, and the number of CFU was determined on the plates after overnight incubation at 35°C.

In some experiments, the agar was supplemented with 1 mg of bovine serum albumin/ml to allow sublethally injured bacteria to grow (29).

Incubation of bacteria with eosinophils under aerobic and anaerobic conditions. Cell suspensions were preincubated for 30 min at 37°C in an anaerobe box containing 10% H2, 10% CO2, and 80% N2. In parallel, cells were incubated with bacteria in a normal atmospheric environment at the same temperature.

To investigate the function of the NADPH oxidase, cells were incubated with DCF (5 μM) under anaerobic or aerobic conditions and also moved from anaerobic to aerobic conditions or incubated with DPI followed by stimulation with PMA (100 ng/ml). The cells were then put on ice and fixed with formaldehyde to a final concentration of 1% (vol/vol) before measurement of the mean fluorescence intensity by flow cytometry using the fluorescein isothiocyanate channel.

DCF is a cell-permeable nonfluorescent probe that is de-esterified intracellularly and converts into highly fluorescent DCF upon oxidation (36). In short, a stem solution of DCF (5 mM) was prepared fresh every day by dissolving the substance in 95% (vol/vol) ethanol. This solution was kept in the dark at room temperature. Eosinophils at 106/ml PBS; final concentration of 10% H2, 10% CO2, and 80% N2. In parallel, cells were incubated with bacteria in a normal atmospheric environment at the same temperature.

Eosinophils at 106/ml were incubated with DCF (5 μM) for 15 min at 37°C before use in the experiments.

NO production. L-NIO was used to inhibit NO synthase (37). L-NIO (10 mM in PBS; final concentration of 100 μM) was added to the cells suspended in HBSS supplemented with 0.6 mM L-arginine. Bacterial incubations and viable counts were performed under aerobic conditions at 37°C. L-NIO incubated with bacteria alone did not affect the viability of the bacteria.

Eosinophils were incubated in DMEM (2 × 106 cells/ml) at 37°C for 24 h in the absence or presence of IL-5 (1 nM) to investigate their NO production. The supernatants were recovered, and proteins were precipitated with ZnSO4 (30% [wt/vol]) before measurement of NO, as determined by the Griess reaction, described below. The viability of eosinophils after 24 h of incubation was determined by trypan blue exclusion and was >98% in medium alone and >98% in the presence of IL-5.

As a positive control of NO production and its inhibition by L-NIO, rat aorta was used. After institutional approval was obtained, male Sprague-Dawley rats (200 to 250 g of body weight) were anesthetized with halothane and exsanguinated. The descending thoracic aorta was removed and cut into circular segments weighing 2.5 to 4 g. The segments were each incubated in 1 ml of DMEM. LPS was added to a final concentration of 100 ng/ml. Two segments were incubated with LPS and served as controls. L-NIO (100 μM) was added to one of the segments incubated with LPS. The segments were incubated with gentle agitation for 24 h at 37°C in an atmosphere containing 8% carbon dioxide in air. After incubation, the segments were removed, blotted on filter paper, and weighed. The DMEM was transferred to clean vials and centrifuged for 5 min at 11,000 × g at 4°C to remove cellular debris and stored at −20°C until assayed for nitrate and nitrite.

Nitrate and nitrite levels, reflecting NO production, were determined by the Griess reaction. In short, 100 μl of supernatant from duplicate incubations was transferred to the wells of a transparent microplate. Nitrate reductase and NADH were added, and the plate was incubated at room temperature for 2 h. Then, 100 μl of Griess reagent (40 mg/ml) was added, and absorbance was measured at 562 nm and compared with standards prepared by diluting sodium nitrate in DMEM. The detection limit for this assay of nitrate in DMEM was 750 nM.

Determination of phagocytosis. Eosinophils (106/ml) were incubated with serum-opsonized BODIPY-labeled E. coli at a ratio of 1:100. The serum opsonization was performed as described above. Incubations were performed under both aerobic and anaerobic conditions at 37°C in medium alone or after addition of IL-5 (1 nM) or PAF (1 μM). Aliquots were taken after 15, 30, and 60 min, and eosinophil binding of bacteria was determined using an Eipix XL-MCL flow cytometry system (Beckman Coulter Inc., Fullerton, Calif.). Eosinophils were gated using their characteristics in side and forward scatter, excluding unbound bacteria. Fluorescence from extracellular bacteria was quenched by the addition of trypan blue. The percent intracellular bacteria was calculated by using the following formula: [mean fluorescence intensity after quenching(initial mean fluorescence intensity − eosinophil autofluorescence)].

RESULTS

Killing of E. coli. The killing of E. coli by eosinophils was measured by viable count technique (Fig. 1). The killing of the bacteria reached a maximum after 1 h of incubation. Addition of the eosinophil-activating agents PAF (1 μM) or IL-5 (1 nM) (4, 26) did not further increase the killing efficiency (data not shown). The vast majority of bacteria were killed and not sublethally injured (data not shown).

Aerobic versus anaerobic conditions. Anaerobic conditions and pharmacological uncoupling of the NADPH oxidase under aerobic conditions reduced eosinophil killing of E. coli significantly compared with incubation of eosinophils and bacteria under aerobic conditions (Fig. 2a and b). These findings suggest that superoxide formed by NADPH oxidase is important for bacterial killing in this system.
To determine the efficiency of anaerobic conditions and DPI in inhibiting the oxidase activity, cells were loaded with DCF, and the oxidase-dependent fluorescence was measured (13) (Fig. 2c). PMA was used to induce a respiratory burst in the cells. Anaerobic conditions and DPI both decreased the PMA-induced respiratory burst. When the cells were first incubated for 30 min under anaerobic conditions and then subjected to aerobic conditions for 30 min before stimulation with PMA, a respiratory burst almost of the order of that in stimulated cells held under aerobic conditions was seen. This shows that decreased killing during anaerobic conditions was not attributable to poor viability of the cells.

**Inhibition of NO synthesis.** No significant reduction in eosinophil killing of *E. coli* was seen in the presence of the NO synthesis inhibitor L-NIO (Fig. 3a). This suggests that the generation of NO does not contribute to the killing of bacteria in this system. No NO production could be detected from resting or IL-5-stimulated eosinophils after 24 h of incubation, as determined by the Griess reaction (five separate experiments; data not shown). However, both constitutive and LPS-stimulated NO production from segments of rat aorta was inhibited by the same concentration of L-NIO (Fig. 3b).

**Antibacterial activity of eosinophil protein extracts.** Protein extracts from purified eosinophils killed bacteria in a dose-dependent manner (Fig. 4). The 50% effective dose was approximately 3 μg/ml after 60 min of incubation with the bacteria.

**Phagocytosis versus bacteria bound to the cell surface.** To determine whether the bacteria were phagocytosed or bound to the surface of eosinophils, *E. coli* cells labeled with the fluorescent dye BODIPY were used. After 60 min of incubation under aerobic conditions, 11.6 ± 3.2% (mean ± standard error of the mean [SEM] for three separate experiments) of the bacteria were phagocytosed, as determined by quenching of extracellular BODIPY by trypan blue. There was no difference between the numbers of phagocytosed bacteria during aerobic and anaerobic incubation.

Treatment of eosinophils with cytochalasin B, an agent that disrupts the cytoskeleton and thereby inhibits phagocytosis, did not affect the bacterial killing efficiency of eosinophils (data not shown).

Taken together, these data suggest that the bacterial killing occurs mainly on the surface of the cells.

**DISCUSSION**

In the present study we show that eosinophils can kill *E. coli* and that the killing, to a large degree, is oxygen dependent under the conditions used. We also show that there is oxygen-independent bactericidal activity in eosinophil protein extracts.
Furthermore, our data suggest that the bactericidal activity of eosinophils is dependent on the interaction between superoxide generated by the NADPH oxidase and the granule-bound peroxidase EPO.

Several studies have shown that eosinophils are less capable of killing bacteria than neutrophils (2, 10, 32). However, many of these were performed at a time when it was difficult to obtain cells of high purity. In addition, some data regarding the bactericidal capacities of eosinophils are conflicting. Some investigators have reported that eosinophils are as efficient phagocytes as are neutrophils (11), especially after stimulation by certain cytokines (3). More recently, it has been shown that

**FIG. 3.** Killing of *E. coli* by eosinophils during inhibited NO production. (a) Eosinophils were incubated with bacteria in the absence or presence of the NO synthase inhibitor L-NIO (100 μM). Means ± SEMs from three independent experiments are shown. n. s., not significant. (b) The efficiency of L-NIO (100 μM) in inhibiting NO synthase and thereby NO production was determined by measurement of the NO metabolites NO$_2^-$ and NO$_3^-$ by the Griess reaction. Segments of rat thoracic aorta were incubated in the absence or presence of LPS (100 ng/ml) and in the presence of LPS plus L-NIO (100 μM). Results are means ± SEMs from five independent experiments. *, $P < 0.05$ (Student’s $t$ test for paired observations).

**FIG. 4.** Presence of bactericidal activity against *E. coli* in eosinophil crude protein extracts. Bacteria were incubated with extracts at the indicated concentrations for 60 min. Viable bacteria were assayed by viable counts.

**FIG. 5.** Superoxide acts in conjunction with peroxide in eosinophil killing of *E. coli*. Eosinophils were incubated with *E. coli* in medium alone (control), after addition of azide (5 mM), and in the presence of the combination of azide (5 mM) and DPI (5 μM). *, $P < 0.05$; n. s., not significant (Student’s $t$ test for paired observations).
they can, at least under certain circumstances, show bactericidal activities comparable to those of neutrophils (17, 38).

The microbicidal systems of granulocytes can be divided into oxygen-dependent and -independent systems (15, 24). The production of superoxide by the membrane-bound NADPH oxidase, of eosinophils is three to six times greater than that of neutrophils (12). The product of the oxidase system, superoxide, seems to have limited cytotoxicity, and the hydrogen peroxide that is formed by dismutation of superoxide is bactericidal only at high concentrations (19). Eosinophils are exceptionally rich in peroxidase, e.g., EPO, which is present in the matrix of eosinophil granules at 15 μg/10⁷ eosinophils (9, 35). In systems using purified EPO, superoxide, and hydrogen peroxide, the generation of potent cytotoxic species such as the hydroxyl radical in eosinophils has been observed (31). When combined with H₂O₂ and halides (for example, chloride, iodide, or bromide), EPO catalyzes the formation of highly cytotoxic hypohalous acids (22). Recently, thiocyanate was suggested to be an important substrate in this reaction, generating hypoiodocyanate and cyanate (1). Our findings obtained by using azide to block the heme group of EPO and DPI to block the NADPH oxidase system suggest that the oxidase system acting in conjunction with EPO is important for the bactericidal activity of eosinophils. This finding is in agreement with the finding that myeloperoxidase and NADPH oxidase act in conjunction in neutrophils (18), but it is in contrast to an earlier report showing that EPO did not contribute to the bactericidal activity of intact eosinophils (7).

The bactericidal activity of EPO is pH dependent (22). During incubation, the pH of the medium decreased, but there was no difference between the pHs of the media under aerobic and anaerobic conditions (data not shown), arguing against the possibility that such an effect contributed to the difference in bacterial killing.

It has been suggested that eosinophils can produce NO through a constitutive expression of inducible NO synthase (14). NO can react with superoxide and form peroxynitrate, which possesses potent cytotoxic properties against, for example, E. coli (6). Therefore, eosinophil NO production together with superoxide production by the oxidase system may present a powerful source of cytotoxicity (33). In our experiments, however, we were not able to detect NO production by the eosinophils, nor did we see any effects of the inducible NO synthase inhibitor L-NIO on bacterial killing by eosinophils. The reason for this could be the source of cells or the means of detection used. These methodological problems have been reported in experiments with neutrophils (37).

We found potent bactericidal activity in protein extracts from eosinophils. The oxygen-independent antibacterial effects of eosinophils may be attributable to several granule-bound proteins that have, in purified forms, been shown to possess bactericidal effects (25). The characteristic proteins making up the bulk content of eosinophil granules are the highly cationic proteins eosinophil cationic protein (ECP), EPO, eosinophil-derived neurotoxin (EDN), and major basic protein. ECP and eosinophil-derived neurotoxin possess RNase activity, but the bactericidal activity of at least ECP seems to be independent of its RNase activity (34). The effect of ECP on bacteria may instead come from its pore formation (40). Interestingly, the cytotoxicity of ECP can be potentiated by the presence of a functioning oxidase system (39), but the mechanism behind this has not been characterized.

Other granule proteins possessing antibacterial activity have been reported to be present in eosinophil granules. Among these are the 14-kDa secretary phospholipase A₂ (5), the bactericidal/permeability-increasing protein (8), and the bacterial cell wall-degrading enzyme N-acetyl muramyl-l-alanine amidase (21). The contribution of each of these is difficult to determine, but together they could represent a powerful and, to a large degree, oxygen-independent bactericidal system. They may also be important for bactericidal activities after release to the extracellular environment.

Since IL-5 is important for eosinophil differentiation, increased susceptibility to infection in IL-5 knockout mice could be expected. Surprisingly, IL-5-deficient mice still produce basal levels of eosinophils that appear to be morphologically and functionally normal (30). In the case of host defense against parasitic infestation, the basal levels of eosinophils has been shown to compromise defense against several helminths. In addition, IL-5 deficient mice also appear to have functional deficiencies in B-1 B lymphocytes and in immunoglobulin A production (30). To our knowledge, the susceptibility to bacterial infection of IL-5 knockout mice has not been investigated. Furthermore, the regulation of the immune systems in mice is different at several levels from that in humans, and therefore firm conclusions with regard to the roles of eosinophils in mice and humans may be difficult to make.

Our study suggests that eosinophils can be efficient in host defense against invasion by gram-negative bacteria and that oxygen-dependent killing, e.g., superoxide acting in conjunction with EPO, may be the most important bactericidal effector function of these cells. This can be important at mucosal interfaces and in the mucosa of, for example, the large intestine, where the conditions are aerobic.

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