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Comparison of Intracellular Bactericidal Activities of Human Neutrophils and Eosinophils

By Lawrence R. DeChatelet, Regina A. Migler, Pamela S. Shirley, Hyman B. Muss, Pamela Szejda, and David A. Bass

Eosinophils from a patient with hypereosinophilia were observed to phagocytize radiolabeled *E. coli* or *S. aureus* as effectively as neutrophils from a normal control. This was observed at a number of bacteria/cell ratios and at various time intervals following initial challenge and was confirmed by direct microscopic examination. In spite of comparable rates of phagocytosis, the eosinophils were consistently less capable of killing the bacteria. This correlates with an inability of eosinophil peroxidase to catalyze the peroxidase-H_2O_2-Cl^-–mediated decarboxylation of amino acids; in contrast, both eosinophil and neutrophil peroxidases showed similar capabilities to iodinate protein in vitro. These data are compatible with the importance of the chloride-mediated mechanism in the bactericidal activity of intact phagocytes.

BOTH EOSINOPHIL AND NEUTROPHIL leukocytes are capable of ingesting and killing microorganisms in vitro.[1,2] During these events there is a marked increase in cellular metabolism, a respiratory burst, that results in the production of potential bactericidal agents such as hydrogen peroxide and superoxide anion.[3,4] This respiratory burst is necessary for the killing of many bacteria; however, the mechanism(s) by which the oxidative metabolic burst results in bacterial killing remains controversial. Part of the bactericidal capability may reside in direct toxic effects of oxidative substances such as superoxide and hydrogen peroxide.[3] Alternatively, greater bactericidal activity may be shown by the combination of leukocyte peroxidase with hydrogen peroxide and a halide. Peroxidase obtained from neutrophil leukocytes can form such a bactericidal system with either chloride or iodide as the cofactor. In the presence of iodide, protein iodination results;[5] with the chloride, amino acid decarboxylation and aldehyde formation occur.[6,7] In contrast, peroxidase obtained from eosinophil leukocytes can serve in the peroxidase-H_2O_2-iodide reaction, resulting in protein iodination and bacterial killing, but such peroxidase cannot decarboxylate amino acids efficiently in the presence of chloride and similarly has no bactericidal effect in the presence of chloride.[8]

Eosinophil leukocytes have been observed to be less efficient in killing bacteria than neutrophil leukocytes, and this reduced bactericidal ability has been ascribed to the lower phagocytic potential of the eosinophils obtained for these studies in the past.[1,2] In the present study we examined the phagocytic potential of eosinophil leukocytes obtained from a patient with eosinophilia of unknown etiology. These eosinophils were observed to have a phagocytic rate equal to that

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From the Departments of Biochemistry and Medicine, The Bowman Gray School of Medicine, Winston-Salem, N.C.

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Address for reprint requests: Dr. Lawrence R. DeChatelet, Dept. of Biochemistry, The Bowman Gray School of Medicine, Winston-Salem, N.C. 27103.

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of normal neutrophils; this provided an opportunity to reexamine the basis of the bactericidal defect of eosinophil leukocytes. Since the only known defect in the eosinophil oxidative system resides in the inability to partake in the peroxidase-H_2O_2-chloride reaction, the presence of a bactericidal defect in such cells could provide evidence for the importance of this mechanism in the bactericidal activity of phagocytes.

MATERIALS AND METHODS

Preparation of leukocytes. Leukocytes were isolated from 90 ml heparinized venous blood by sedimentation of the red blood cells with plasmagel (HTI, Buffalo, N.Y.) as previously described.[9] Isolated cells were washed in 5 ml Dulbecco's phosphate-buffered saline (PBS) pH 7.4, and contaminating erythrocytes were removed by hypotonic lysis for 6 sec with 6 ml cold deionized water. Then 2 ml of 3.5% NaCl was quickly added to restore isotonicity. The cells were collected by centrifugation and resuspended in PBS. Total counts were performed in a hemocytometer under phase microscopy; differential counts were performed on Wright-stained smears on each sample. All experiments involved simultaneous study of eosinophil and neutrophil preparations. The isolated eosinophil preparations contained 82% 94% eosinophils (a typical differential count by Wright stain showed 86% eosinophils, 8% neutrophils, and 6% lymphocytes; the percentage neutrophils in the different samples varied from 4% to 12%); control samples, obtained from healthy volunteers, always contained less than 6% eosinophils in these experiments. The eosinophils used were obtained from a single patient (J.C.) with hypereosinophilia of long duration and unexplained etiology; a description of the clinical course of the patient has been previously published.[4] The experiments were performed over a period of 1 yr; different normal control samples were employed in each experiment.

Decarboxylation of L-alanine-1-^{14}C was determined by a modification of a previously described method.[6] Each flask contained in a volume of 3.0 ml: 2.20×10^{-4} *M* H_2O_2 (prepared in 0.02 *M* acetate buffer pH 5.5), 1.67×10^{-3} *M* L-alanine (Sigma Chemical, St. Louis, Mo.); 0.833 μCi L-alanine-1-^{14}C (New England Nuclear, Boston, Mass.), and 0.10 *M* KCl. Eosinophil or neutrophil sonicates were added to initiate the reaction, which was performed at 37°C with constant shaking. The reaction was terminated after 60 min by the addition of 1.0 ml 5% trichloroacetic acid (TCA). $^{14}CO_2$ released during the course of the incubation was collected in 0.5 ml hyamine hydroxide (New England Nuclear) and counted in a liquid scintillation spectrometer. Blanks contained PBS in place of the leukocyte sonicate.

Cell-free iodination. The ability of leukocyte sonicates to iodinate zymosan was studied in parallel with the decarboxylation studies described above. Each flask contained in a total volume of 1.0 ml: 1×10^{-4} *M* KI, ^{125}I (New England Nuclear) as NaI to yield 200,000 cpm, 1×10^{-5} *M* H_2O_2, and calcium-free PBS to give the indicated final volume after all additions. Assays were performed in the presence and absence of 0.20 ml zymosan (1.7×10^8 particles) and were initiated by the addition of either neutrophil or eosinophil sonicate corresponding to 5×10^5 intact cells. The reaction was terminated after 60 min with 2 ml cold 5% TCA and the precipitate collected by centrifugation at 1000 *g* for 5 min. It was washed four times by repeated suspension and centrifugation in 2 ml of 5% TCA and counted in a gamma well counter. Experimental values were corrected for a blank that contained all the reagents except leukocyte sonicate.

Phagocytosis assays. Phagocytosis of bacteria was quantitated by measuring leukocyte uptake of radiolabeled organisms.[10] *Staphylococcus aureus* 502-A or *Escherichia coli* were grown overnight in 6 ml penassay broth (Difco, Detroit, Mich.) containing 0.20 μCi uniformly ^{14}C-labeled L-amino acids (New England Nuclear). The labeled bacteria were collected by centrifugation, washed twice in 0.1% gelatin solution, and suspended to an absorbance of 0.60 at 620 nm in a Coleman Junior Spectrophotometer. This routinely yielded 5 10×10^9 colony-forming units (CFU)/ml.

The assay was performed in 25-ml flasks in a reciprocating shaker bath containing 5×10^6 leukocytes (either neutrophils or eosinophils), 10% fresh human serum, various amounts of viable labeled bacteria as indicated in the individual experiments, and Hanks' balanced salt solution (HBSS) to provide a total volume of 1.0 ml. The reactions were stopped at various times by the addition of 4.0 ml cold 0.04 *M* NaF in PBS containing 10% calf serum. The cells were collected by centrifugation at 800 *g* for 10 min and washed free of noningested bacteria by repeated suspensions in 4 ml of the buffer used to stop the reaction. After three such washes, the pellet was dried overnight at 60°C and digested with 0.50 ml of 0.2 *N* NaOH for 2 hr at 60°C. The solution was neutralized with 0.20 ml of 3% glacial acetic

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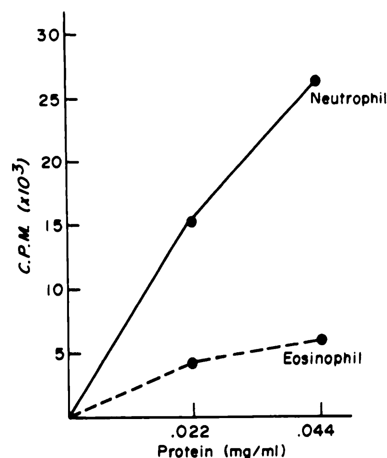

Fig. 1. Decarboxylation of L-alanine-1-^{14}C by sonic extracts of human eosinophils and neutrophils. Assay conditions described in Materials and Methods. Each point, mean of closely agreeing triplicate determinations.

acid, 0.50 ml of deionized water was added, and 1.0-ml volumes were counted in 10 ml Aquasol (New England Nuclear) in a liquid scintillation spectrometer. As a standard, 0.10 ml of the original labeled bacteria (5×10^7 bacteria) was similarly counted in 10 ml Aquasol.

In one experiment, phagocytosis of *S. aureus* was also determined by direct microscopic examination. Samples were removed from each flask at various times after addition of bacteria. Smears were made of each sample and stained and 100 cells (neutrophils or eosinophils) were examined for the presence of intracellular bacteria. Results were expressed as average number of bacteria ingested per cell.

Bactericidal assay. Bactericidal assays were run by a modification of the method of Maaløe[11] in parallel with the phagocytic assays previously described. The same suspension of bacteria, grown on radioactive amino acids, was used in both cases. The assay mixture consisted of 5×10^6 leukocytes, 10% fresh human serum, 2×10^7 labeled bacteria, and HBSS to a final volume of 1.0 ml in 16×125 mm plastic tubes. The tubes were rotated end-over-end at 10 rpm in a Fisher Roto Rack at 37°C for 60 or 120 min. For determination of viable bacteria, the cells were lysed by the addition of 9.0 ml of 0.1% gelatin and held at room temperature for 5 min. The samples were serially diluted, and pour plates were prepared. Viable bacteria were enumerated by colony count after overnight incubation at 37°C. Controls run in the absence of leukocytes contained bacteria alone or bacteria and serum. No killing was observed with the controls over the 120-min incubation period; frequently some growth of bacteria occurred.

RESULTS

The decarboxylation of L-alanine-1-^{14}C by sonicates of neutrophil and eosinophil leukocytes plus hydrogen peroxide and chloride are compared in Fig. 1. Eosinophil sonicate showed a markedly reduced activity in this reaction. In contrast, eosinophil and neutrophil sonicates, in the presence of hydrogen peroxide and io-

Table 1. Iodination by Neutrophil and Eosinophil Sonicates (cpm)

Neutrophil		Eosinophil	
Without Zymosan	With Zymosan	Without Zymosan	With Zymosan
76	41,396	20	39,094

The standard reaction mixture consisted of 5×10^6 sonicated leukocytes, 0.1 ml of ^{125}I (200,000 cpm), ±0.2 ml zymosan, 10^{-7} mol KI, 10^{-8} mol H_2O_2, and calcium-free PBS pH 7.4 to a total volume of 1 ml. Values obtained from blanks, consisting of all reagents without leukocytes, were subtracted from experimental values. Each value represents the average of triplicate determinations.

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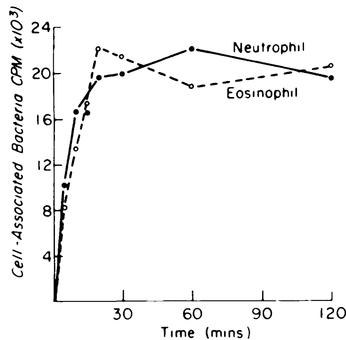

Fig. 2. Phagocytosis of radiolabeled *E. coli* by human eosinophils and neutrophils. Each point, mean of triplicate determinations; all points corrected for control stopped at zero time. Zero-time values were 5947 and 4828 cpm for eosinophils and neutrophils, respectively. Total number of bacteria present in each incubation was equivalent to 42,000 cpm. Data from a single experiment representative of two separate experiments. Ratio of bacteria to cells was approximately 4:1.

dide, showed comparable activity in the iodination of protein (Table 1). Virtually no iodination was detectable in the absence of zymosan as an acceptor molecule with either sonicate.

The function of the intact cells was then examined. There was no significant difference in either the rate or extent of uptake of radioactive *E. coli* between the two cell types (Fig. 2). In contrast, the eosinophil was not nearly as efficient in destroying the bacteria (Fig. 3). These data are from a single experiment representative of two separate experiments performed with a bacteria/cell ratio of 4:1.

In order to determine that this was not an artifact of the procedure, the experiment was repeated with a 12-fold lesser concentration of bacteria. As shown in Fig. 4, uptake of the labeled bacteria by the two cell types was comparable. The low number of cpm in Fig. 4 reflects simply the very low bacterial challenge (there was approximately one bacterium for every six cells). Again, the bacteria were killed substantially better by the neutrophils than by the eosinophils (Fig. 5).

These observations were also obtained with *S. aureus* as the test organism (Figs. 6 and 7). These data are from a single experiment representative of three

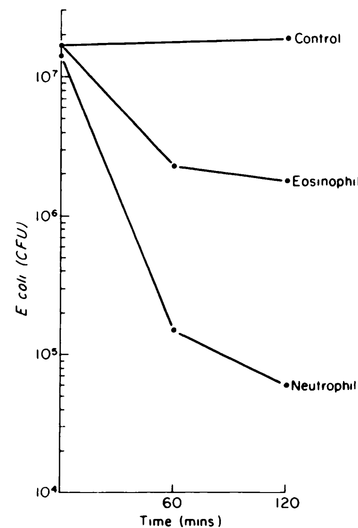

Fig. 3. Killing of *E. coli* by intact human eosinophils and neutrophils. Representative of two separate experiments run in parallel (i.e., with same cells and at same time) as those described in Fig. 2.

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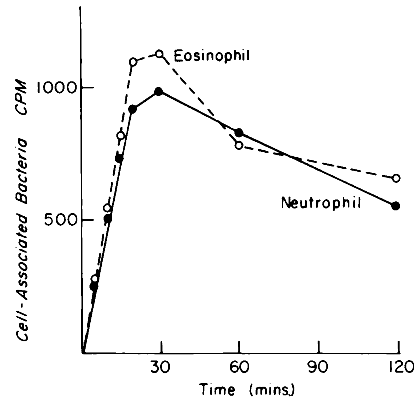

Fig. 4. Phagocytosis of radiolabeled *E. coli* by human eosinophils and neutrophils at a low ratio of bacteria to cells (1:6). Total bacterial challenge represented approximately 1800 cpm. Each point, mean of triplicate determinations; all values corrected for control stopped at zero time. Zero-time values were 129 and 97 cpm for eosinophils and neutrophils, respectively.

separate experiments performed at bacteria/cell ratios of from 1:1 to 2:1. If anything, bacterial uptake by the eosinophil was slightly greater than that by the neutrophil. In spite of this, the eosinophil preparations were consistently less effective in killing the bacteria than the corresponding neutrophil preparations.

In order to confirm these observations, we evaluated phagocytosis by direct microscopic examination in one experiment, employing a bacteria/cell ratio of approximately 12:1. This relatively high ratio was employed to facilitate visualization of intracellular bacteria. The results (Table 2) were in substantial agreement with previous experiments employing radiolabeled bacteria. Phagocytosis occurred very rapidly and was virtually complete by 10 min. As before, there was no significant difference in the phagocytic index with the two cell types. Virtually all cells examined contained one or more bacteria, and the average numbers of bacteria/cell for the neutrophils and eosinophils were similar.

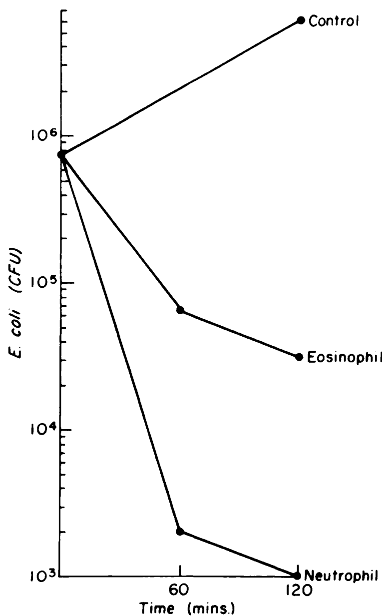

Fig. 5. Killing of *E. coli* by intact cells. Experiment run in parallel with that illustrated in Fig. 4. Each point, mean of triplicate determinations.

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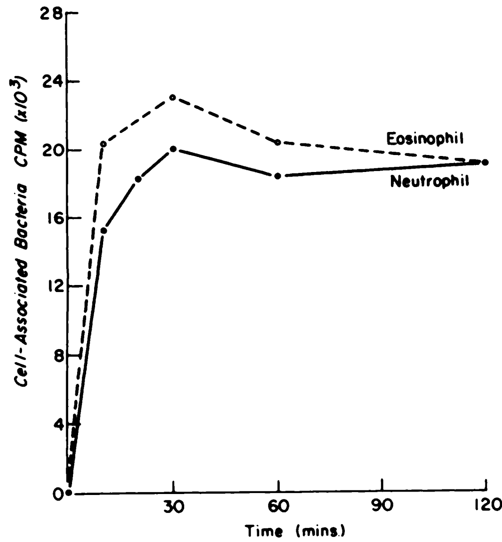

Fig. 6. Phagocytosis of radiolabeled *S. aureus* by human leukocytes. Each point, mean of triplicate determinations. This experiment employed total challenge of approximately 47,000 cpm and bacteria to cell ratio of 1:1. Zero-time values were 5347 and 7352 cpm for eosinophils and neutrophils, respectively. Representative of three separate experiments performed with bacteria to cell ratios from 1:1 to 2:1.

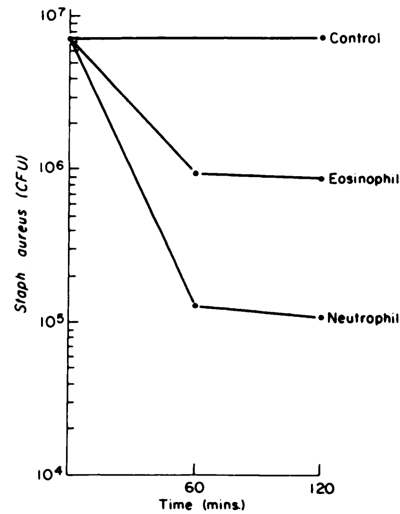

Fig. 7. Killing of *S. aureus* by intact cells. This experiment was run in parallel with that illustrated in Fig. 6 and is representative of three separate experiments employing bacteria to cell ratios from 1:1 to 2:1.

Table 2. Phagocytosis of *S. Aureus*: Microscopic Evaluation

Time (min)	Average No. Bacteria/Cell	
	Eosinophils	Neutrophils
5	2.16	2.52
10	5.70	4.88
15	4.27	4.10
20	5.97	6.18
25	6.36	5.72
30	5.80	5.96

Smears were made at the end of the specified incubation time and stained and 100 cells (eosinophils or neutrophils) were examined for intracellular bacteria. Results are expressed as the average number of bacteria/cell at each time interval.

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DISCUSSION

The present findings that eosinophils and neutrophils had comparable rates of phagocytosis were quite surprising to us and certainly conflict with most reports in the literature, which generally agree that human eosinophils are less avid phagocytes than human neutrophils.[1,2,12,13] Because of these previous reports, we went to some length to document the similar degree of phagocytosis observed in these experiments. In spite of numerous experimental manipulations, including the use of different bacteria, varying the bacteria/cell ratio, and evaluating ingestion by direct microscopic examination, the results were always the same. In every experiment both the rate and extent of phagocytosis were comparable for the neutrophils and eosinophils. This observation is not without precedent, in that a previous report documented a similar degree of phagocytosis of *Candida albicans* by human eosinophils and neutrophils.[11] Recently, Dr. Stephanie Pincus compared the phagocytic rate and capacity of guinea pig peritoneal eosinophils and neutrophils by measuring the uptake of oil red-O paraffin oil emulsions coated with *E. coli* lipopolysaccharide. The rate of ingestion by the eosinophils was, if anything, somewhat greater than that with neutrophils, while the capacity of the two cell types was equal after 30 min incubation (personal communication).

Because it is impossible to obtain sufficient quantities of pure eosinophils from normal peripheral blood, all such studies (including the present one) have utilized cells from patients with eosinophilia. Such cells have necessarily been exposed to prior stimuli and might be either activated or exhausted when isolated for study in vitro. Thus it is impossible to define the phagocytic activity of "normal" unstimulated eosinophils. Whether our patients' eosinophils have normal phagocytic capability (and most previous studies were using partially exhausted cells) or increased capability (and the other studies were using more normal cells) cannot be ascertained. Nevertheless, the availability of eosinophils with a phagocytic rate identical to normal neutrophils provides an excellent opportunity to examine the intracellular bactericidal activities of these two types of leukocytes.

The organisms used in the present study (*E. coli* and *S. aureus*) were selected because their intraleukocytic killing depends to a large extent on the oxidative metabolism of the cell. For example, cells from patients with chronic granulomatous disease are profoundly defective in their ability to destroy these bacteria.[15] Several publications have reported that the respiratory burst following phagocytosis by eosinophils is in many ways more potent than that produced by neutrophils.[1,2,4] Such observations were made with cells obtained from the same patient utilized in the present study.[1] Thus these experiments employed eosinophils with a phagocytic rate equal to the neutrophils and an oxidative burst equal to (and in many ways greater than) that shown by neutrophils. Yet in experiments run in parallel the neutrophils consistently killed both *E. coli* and *S. aureus* to a greater extent than did the eosinophils.

In the neutrophil, several mechanisms of oxidative bactericidal activity have been suggested that involve the interaction of peroxidase, H_2O_2, and a halide. In the presence of iodide, this results in iodination of the bacteria,[5] while in the presence of chloride this results in the decarboxylation and deamination of amino acids with the generation of bactericidal aldehydes[6] and possibly in the cleavage of peptide bonds.[7] Both the iodide and chloride systems have been shown to be bac-

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tericidal in vitro,[16] but the relative importance in vivo of the two systems is unknown.

The eosinophil contains large quantities of a peroxidase which is immunologically and biochemically distinct from the myeloperoxidase of the neutrophil.[17,18] We recently reported that the eosinophilic peroxidase was not efficient in the chloride-dependent decarboxylation of amino acids.[8] This appeared to be a general property of the eosinophilic peroxidase in that cells from three patients with eosinophilia were defective; the magnitude of the defect correlated well with the ratio of neutrophils to eosinophils in the isolated preparation.[8] This observation is verified in Fig. 1, where relatively little activity was seen with the eosinophilic sample compared to the neutrophilic preparation. In contrast, both preparations were equally efficient in the iodination reaction, which was performed under similar conditions (Table 1). Furthermore, eosinophil peroxidase will not show bactericidal activity in a cell-free system using H_2O_2 and chloride, whereas neutrophil peroxidase is very bactericidal under the same conditions.[8] Both peroxidases are equally effective in killing bacteria when iodide is utilized as the halide.[8]

The present study extends these earlier observations to the intact cell. These data suggest that a major difference between eosinophil and neutrophil systems lies in the inability of eosinophil peroxidase to catalyze the peroxidase-H_2O_2-Cl^- reaction(s). This could explain the relative bactericidal defect of eosinophils and suggests evidence for the importance of this bactericidal mechanism in neutrophil leukocytes. However, there is no certain proof that the relative bactericidal defect in the intact eosinophil is due to the lack of the peroxidase-H_2O_2-Cl^- reaction; the two phenomena have not been shown to be causally related. Furthermore, the fact that eosinophils did kill a substantial proportion of ingested bacteria indicates that other bactericidal mechanisms are certainly operative.

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