Enhancement of Human Neutrophil Bactericidal Activity by Chemotactic Factors

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Neutrophils are important effector cells in the defense against microorganisms. They migrate into infected sites and then phagocytose and kill bacteria. Chemotactic factors may be important for initiating neutrophil migration. We investigated whether chemotactic factors might also influence an event subsequent to chemotaxis, namely bacterial killing. It was found that preincubation (20 min at 37°C) of human leukocytes with chemotactic substances such as zymosan-activated serum, a C5a-containing fraction of zymosan-activated serum, N-formyl methionyl phenylalanine or N-formyl methionyl-leucine-phenylalanine, enhanced leukocyte killing of Staphylococcus aureus, Escherichia coli, and Streptococcus faecalis in a dose-dependent fashion. The concentration of chemotactic factor required to enhance killing was similar to that required to induce neutrophil chemotaxis. In addition, zymosan-activated serum, C5a fraction, and the two N-formyl methionyl peptides increased the hexose monophosphate shunt activity of resting and phagocytosing neutrophils by two- to threefold. In contrast, bacterial killing by sodium azide-treated neutrophils and neutrophils from a patient with chronic granulomatous disease was not increased by any chemotactic factor. These findings suggest that chemotactic factors stimulate neutrophil oxygen-dependent microbicidal pathways. These observations illustrate another important contribution of biologically active molecules to effector cell function and host defense.

Chemotaxis of neutrophils into inflammatory sites is central to host defense against microorganisms. Neutrophils may migrate in response to a variety of chemotactic factors. The mechanism(s) by which these chemoattractants initiate neutrophil movement is not known. In addition to inducing neutrophil migration, chemotactic factors may modulate other neutrophil functions which could also be important in inflammation. For example, chemotactic factors stimulate extracellular lysosomal enzyme release (3, 10, 17). Furthermore, neutrophil metabolism, for example, hexose monophosphate shunt (HMPS) activity (9), superoxide production (11), and cellular chemiluminescence (2, 12), may be stimulated by chemotactic factors.

When bacteria invade the host, neutrophils probably first interact with chemotactic factors and then migrate to the site of invasion. Upon arrival, neutrophils proceed to phagocytose and kill bacteria. Thus, neutrophils may often interact with chemotactic factors before interacting with bacteria. The importance of chemotactic factors for cell migration is clear. However, their role in modulating phagocytosis and bacterial killing is less well understood. For this reason, and because of the known effects of chemotactic factors on neutrophil metabolism, we investigated their effect on the bactericidal capacity of human neutrophils. We report here that zymosan-activated serum (ZAS), partially purified C5a, and two N-formyl-methionyl chemotactic peptides enhance bacterial killing by neutrophils.

MATERIALS AND METHODS

Preparation of chemotactic factors. Normal human serum was incubated with zymosan (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 5 mg/ml for 60 min at 37°C to activate complement. Zymosan was removed by centrifugation at 2,000 × g for 15 min. This will be referred to as ZAS.

Partially purified C5a was prepared by gel filtration of 5 ml of ZAS on a column (2.5 by 100 cm) of Sephadex G-75 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and eluted with 0.15 M phosphate-buffered saline, pH 7.0, at 30 ml/h as previously described (8). This column was calibrated with ovalbumin (molecular weight [MW], 45,000), chymotrypsinogen A (MW, 25,000) and ribonuclease A (MW, 13,700) calibration standards (Pharmacia). Fractions were pooled into 20-ml portions and concentrated to 5 ml by ultrafiltration using a UM-2 filter (Amicon Corp., Lexington, Mass.). The fractions were then tested for...
chemotactic activity and for their effect on neutrophil bactericidal capacity. This method actually yields a crude preparation of C5a dist arg, since the anaphylotoxin inactivator was not inhibited (5). However, we will refer to this preparation as partially purified C5a. The active fractions were pooled, and the protein concentration was determined (7). The protein concentration was used only to standardize the concentration of the different fractions and of different preparative runs. Of course, most of the protein measured was contaminant protein because of the crude preparation and because C5a has a very small tyrosine content (6). The active pool was divided into small volumes and stored at −70°C.

The N-formyl methionyl-phenylalanine (FMP), L-methionyl-phenylalanine (LMP) (Sigma), and N-formyl methionyl-leucine-phenylalanine (FMLP) (Peninsula Chemical Laboratories, San Carlos, Calif.) were dissolved in Hanks balanced solution (HBSS) (Microbiological Associates, Walkerville, Md.) and frozen in portions at −70°C. A new portion was thawed for each experiment.

Chemotaxis assay. Human peripheral blood leukocytes were isolated and examined for chemotaxis under agarose as described previously by Cutler (4), Nelson et al. (16), and modified by us (14). Briefly, leukocytes were isolated by sedimentation, washed, and resuspended in medium 199 (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.). The chemotaxis assay consisted of establishing a concentration gradient of chemotactic factor, or appropriate control substance, in agarose and exposing the leukocyte suspension to these gradients. Under these conditions, the neutrophils migrated under the agarose on the surface of a tissue culture dish. After 2.5 h of incubation at 37°C, the cells were fixed to the dish with methanol and Formalin. The agarose was removed, the cells were stained with Giemsa, and neutrophil migration was measured by using a projecting microscope (×45 magnification). The linear distance that the cells moved towards the control substance was subtracted from the distance the cells moved towards the chemotactic factor. This difference is called the chemotactic differential (CD).

Bactericidal assay. The bactericidal capacity of human peripheral blood leukocytes was assayed as previously described (1) with the following modifications. Leukocytes were separated from heparinized venous blood with 6% dextran in saline (10 U of heparin per ml, 1 part of dextran-saline to 4 parts of blood). The leukocyte-rich plasma was removed after 45 to 60 min of sedimentation and centrifuged at 200 × g for 6 min, and the leukocytes were washed twice in HBSS. The leukocytes were resuspended to a concentration of 11.4 × 10⁶ neutrophils per ml of HBSS. The bactericidal capacity of neutrophils was assayed by using the following incubation mixtures added to duplicate tubes. To 0.35 ml of leukocyte suspension (4 × 10⁶ neutrophils), 0.06 ml of either HBSS (control) or a test substance (C5a, FMP, FMLP, or LMP) was added. This mixture was incubated for 20 min at 37°C, and then 0.2 ml of the bacterial suspension was added. The final concentration of bacteria was usually 10⁶ colony-forming units per ml. The bacteria were quantitated for each experiment by pour-plate colony counts. Lastly, fresh autologous serum was added to each tube to a final concentration of 10%. The tubes were then incubated at 37°C on a Lab Tech Tilter for 180 min. Bacterial control tubes were incubated in parallel. These were identical to the experimental tubes except that no leukocytes were added.

In experiments employing autologous ZAS as the test substance, fresh autologous serum was the control substance. Leukocytes were incubated with ZAS or autologous serum for 20 min, washed once with HBSS, and resuspended in 0.4 ml of HBSS. The leukocytes were washed so that the final concentration of serum in the bactericidal assay could be standardized at 10%. The bacteria and serum were then added to this washed leukocyte suspension as described above.

At different time intervals (20, 90, and 180 min), 0.1 ml of the incubation mixtures were diluted in distilled water to lyse the leukocytes. The total viable bacteria (intracellular and extracellular) were determined by pour-plate colony counts. To quantitate the number of viable intracellular bacteria, penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively) (GIBCO) were added to one of the duplicate tubes after 20 min of incubation as previously described (1). Only viable intracellular bacteria were quantitated after washing the leukocytes and then lysing them with distilled water. When sodium azide (Sigma)-treated leukocytes were examined, sodium azide was added to the leukocyte suspension at the start of the first incubation. Leukocytes from a patient with chronic granulomatous disease (CGD) were also examined. The patient was a male whose leukocytes had the characteristic metabolic and bactericidal abnormalities previously described for this disease (13).

Enhanced bacterial killing by leukocytes was calculated according to the formula: percent increased killing = viable bacteria with control leukocytes – viable bacteria with treated leukocytes/viable bacteria with control leukocytes × 100.

Results are expressed as a percent increase relative to control leukocytes examined in parallel. Although there was some day-to-day variation in the bactericidal capacity of control leukocytes, there was never any overlap between chemotactic factor-treated leukocytes and control leukocytes. Neutrophil viability was assessed by trypan blue dye (0.2%) (GIBCO) exclusion.

Bacteria. Staphylococcus aureus 502A, Escherichia coli, and Streptococcus faecalis were used. Bacteria were grown in nutrient broth for 16 h before use. When preopsonized S. aureus was required, the pellet of bacteria from a 10-mL broth tube (16-h culture) was resuspended in 2 ml of a 1:2 dilution of fresh serum and incubated for 60 min at 37°C. The opsonized bacteria were then washed with 10 volumes of HBSS.

HMPS activity. HMPS activity of leukocytes at rest and during phagocytosis was determined by measuring the quantity of ^14C CO₂ generated from [1-^14C]-glucose as previously described (15). Leukocytes were preincubated for 20 min at 37°C with a test substance (C5a, ZAS, FMP, FMLP, or LMP) or appropriate control substance (HBSS or autologous serum). After this, the HMPS activity was determined during a second 20-min incubation for resting and phagocytos-
ing leukocytes. Phagocytosing leukocytes were challenged with opsonized S. aureus at bacteria-to-neutrophil ratios of 10:1 and 100:1.

RESULTS

Effect of ZAS on leukocyte bactericidal activity. Figure 1 shows the results of a representative experiment designed to test the effect of ZAS on the phagocytosis and killing of bacteria by leukocytes. Leukocytes preincubated with 4.5% ZAS killed more S. aureus at 20, 90, and 180 min than control leukocytes preincubated with normal autologous serum (Fig. 1, top). When intracellular bacteria were quantitated in the tubes containing penicillin and streptomycin, leukocytes treated with ZAS contained fewer viable intracellular bacteria than control leukocytes. These findings indicate that the increased killing of S. aureus by ZAS-treated leukocytes can not be explained by enhanced phagocytosis alone. In the bottom of Fig. 1, the results of six experiments are shown. The S. aureus-to-neutrophil ratio in these experiments ranged from 1:5 to 10:1. After 20 min, killing by ZAS-treated leukocytes was increased by 40%. At 90 and 180 min, killing was increased by 72 and 68%, respectively.

Maximum enhancement of bacterial killing was observed when leukocytes were incubated with 4.5% ZAS. By comparison, 4.5% ZAS induced approximately 30% of the maximum chemotactic response (CD = 2.5) while 15% ZAS (CD = 7.5) induced a maximum response.

Fractionation of ZAS. ZAS was fractionated by Sephadex G-75 column chromatography to isolate the bactericidal enhancing activity and to determine its elution relative to the chemotactic activity. These results are summarized in Fig. 2. In the experiment shown, the neutrophil bactericidal-enhancing activity eluted in the same fractions as the neutrophil chemotactic activity. Both activities eluted between the ribonuclease and chymotrypsinogen A standards with an apparent MW of 14,000 to 18,000. Previous reports have identified this chemotactic activity as C5a (5, 6, 8). This partially purified C5a was used in subsequent experiments.

The effect of increasing concentrations of partially purified C5a on the bactericidal capacity of leukocytes was examined. For these experiments, the leukocytes were preincubated for 20 min at 37°C but they were not washed, since separate experiments with C5a (not shown) indicated that washing did not influence the results. The bactericidal activity measured at 180 min was enhanced by the C5a fraction in a dose-dependent fashion (Fig. 3). Enhancement of bactericidal activity was maximal at a concentration of 1.2 µg of protein per ml. At this concentration, bacterial killing at 180 min was enhanced by 63 ± 5% (standard error of the mean [SEM]). In experiments not shown, this same concentration enhanced bacterial killing at 20 and 90 min by 45 ± 8% and 67 ± 5%, respectively. For these experiments the bacteria-to-neutrophil ratio ranged from 1:5 to 10:1.

In the absence of leukocytes, C5a had no effect on bacterial growth. In addition, when bacteria were preincubated with C5a for 20 min, washed, and then added to control leukocytes with serum at a final concentration of 10%, bacterial killing was not affected.

When increasing concentrations of the C5a fraction were tested for chemotactic activity, a concentration of 1.2 µg/ml elicited 25% (CD = 1.8) of the maximum response (CD = 7.2), which
was elicited by 4 μg/ml.

Effect of synthetic peptides on leukocyte bactericidal activity. The effect of the two chemotactic peptides, FMP and FMLP, on the bactericidal activity of leukocytes was examined. Figure 4 shows that the bactericidal activity of leukocytes was enhanced by FMP and FMLP in a dose-dependent fashion. Enhancement of bacterial killing was maximal in the presence of 10^{-5} to 10^{-6} M FMP and 10^{-9} M FMLP. By comparison, FMP was chemotactic for human neutrophils at concentrations between 10^{-4} and 10^{-5} M, while FMLP was chemotactic between 10^{-7}.
and $10^{-8}$ M.

The analog LMP was not chemotactic at concentrations between $10^{-3}$ and $10^{-1}$ M. This peptide had a minimal effect on leukocyte bactericidal activity at one concentration only ($10^{-4}$ M, $n = 3$) (Fig. 4).

In the absence of leukocytes, FMP, FMLP, and LMP had no effect on bacterial growth. Furthermore, when bacteria were preincubated with these peptides and washed, the bacteria were killed normally by leukocytes.

The ability of chemotactic factors to increase leukocyte killing of different bacteria was examined. The C5a fraction at 1.2 µg of protein per ml enhanced leukocyte killing of *E. coli* by 63 ± 7% and *S. faecalis* by 45 ± 12% at 180 min. Similarly, FMP ($10^{-5}$ M) enhanced leukocyte killing of *E. coli* by 45 ± 5% and *S. faecalis* by 43 ± 12% at 180 min.

**Effect of chemotactic factors on leukocyte HMPS activity.** The effect of ZAS, C5a, FMP, FMLP, and LMP on the HMPS activity of leukocytes was investigated next. Leukocytes were preincubated for 20 min at 37°C with each factor as detailed for the bactericidal assay. C5a (1.2 µg of protein per ml), FMP ($10^{-5}$ M), and FMLP ($10^{-9}$ M), at concentrations which enhanced bacterial killing, also increased the HMPS activity of resting leukocytes by 2.7- to 3.2-fold relative to control leukocytes preincubated with HBSS (Fig. 5). Similarly, ZAS (4.5%) increased the HMPS activity by 2.2-fold relative to control leukocytes treated with normal serum.

When chemotactic factor-treated leukocytes were challenged with opsonized *S. aureus* (10 bacteria to 1 neutrophil), the HMPS activity was increased 1.6- to 2.4-fold over cells challenged with bacteria but not exposed to a chemotactic factor (Fig. 5). At a bacteria-to-neutrophil ratio (100:1), which caused maximal stimulation of HMPS, no further increase in HMPS was observed when leukocytes were treated with a chemotactic factor. LMP ($10^{-9}$ to $10^{-3}$ M) had no significant effect on the HMPS activity of resting leukocytes or leukocytes challenged with bacteria.

To determine whether chemotactic factors enhanced bactericidal pathways dependent or independent of oxidative metabolism, sodium azide-treated normal leukocytes and leukocytes from a patient with CGD were examined. Sodium azide (1 mM) markedly inhibited bacterial killing by normal leukocytes, although cell viability as judged by trypan blue dye exclusion, was not impaired (Fig. 6). When sodium azide-treated leukocytes were exposed to C5a, no increase in bacterial killing was observed. Similar results were observed with FMP (not shown). In addition, the abnormal killing of *S. aureus* by leukocytes from a patient with CGD was not influenced by C5a at several different concentrations (0.4, 1.2, and 3.6 µg of protein per ml) and on three separate occasions.

On two occasions CGD leukocytes killed *S. faecalis* normally, but killing of this bacterium could also not be enhanced by C5a or FMP (not shown). In contrast, C5a and FMP did enhance the killing of *S. faecalis* by normal leukocytes (45 ± 12% and 43 ± 10%, respectively, at 180 min).

Finally, neither C5a nor FMP stimulated the HMPS of CGD leukocytes (CGD - control, 180 cpm/2.5 × 10⁶ PMN per 20 min; C5a treated, 165 cpm/2.5 × 10⁶; FMP treated, 200 cpm/2.5 × 10⁶). In contrast, CGD neutrophils migrated normally towards the two chemotactic factors (C5a-CGD, CD = 6; control = 6.0 ± 0.5; FMP-CGD, CD = 8; control CD = 9 ± 0.6 SEM).

**DISCUSSION**

Our initial experiments examined the effect on neutrophil bactericidal activity of serum in
which chemotactic factors were generated by zymosan. Our findings demonstrated that ZAS, at a concentration (4.5%) which was chemotactic, enhanced the killing of *S. aureus* by neutrophils (Fig. 1). This enhancing effect was observed at several bacteria-to-neutrophil ratios. Fractionation of ZAS by gel filtration (Fig. 2) demonstrated that the chemotactic and bactericidal-enhancing activities of ZAS were present in the same elution volume and therefore appeared to be of similar molecular weight (14,000 to 18,000). The chemotactic activity of these fractions is reported to be C5a (8, 5, 6).

The C5a fraction was found to enhance bacterial killing in a dose-dependent fashion (Fig. 3). Furthermore, the concentration required to enhance neutrophil bactericidal activity was similar to the concentration required to induce neutrophil chemotaxis. This suggests, but does not prove, that the C5a chemotactic factor did enhance bacterial killing.

To test the hypothesis that chemotactic factors can enhance the bactericidal activity of neutrophils, we studied the effect of the two chemotactic peptides, FMP and FMLP. These peptides are chemotactic at concentrations between $10^{-4}$ and $10^{-5}$ M for FMP and between $10^{-7}$ and $10^{-8}$ M for FMLP. In addition, both peptides enhanced neutrophil killing of *S. aureus* (Fig. 4) to the same extent as the C5a fraction and like C5a, at a concentration which was similar to that required to induce chemotaxis in the agarose assay. The finding that a 3- to 10-times higher concentration of factors was required to induce optimal chemotaxis than to optimally enhance bacterial killing may have been due to dilution of the chemotactic factors during diffusion in agarose. These observations suggest that the same molecule which induced neutrophil chemotaxis also enhanced bacterial killing. This conclusion is further supported by the finding that LMP, a peptide analog of FMP, which lacked chemotactic activity, had a negligible effect on the bactericidal activity of neutrophils (Fig. 4).

The enhancement of neutrophil bacterial killing by chemotactic factors may have involved several mechanisms. First, these factors may have increased the number of bacteria phagocytosed. However, this is unlikely because at the bacteria-to-neutrophil ratios of 1:5 and 1:1, greater than 95% of the bacteria are ingested within 30 min even by control leukocytes (personal observations). Furthermore, Verhoef et al. (18) found that even at bacteria-to-neutrophil ratios of 10:1, 80% of the *S. aureus* was ingested within 20 min by control neutrophils. Second, the chemotactic factors may have stimulated the initial rate of phagocytosis. Our results do not exclude this possibility. However, it is unlikely that a stimulatory effect limited to this early event would result in enhanced bacterial killing which persisted for 3 h. Furthermore, if the chemotactic factors primarily acted to increase the rate or extent of phagocytosis, then the number of viable intracellular bacteria would have been greater in chemotactic factor-treated cells than in control cells. On the contrary, the number of viable bacteria inside chemotactic factor-treated leukocytes as well as the number viable in the total inoculum was less at each time point than with control leukocytes (Fig. 1; similar results with C5a, FMP, and FMLP are not shown). Although these observations do not absolutely rule out a minor effect of the chemotactic factors on phagocytosis, it must be con-
cluded that the predominant action of these factors was to stimulate neutrophil intracellular bactericidal mechanisms.

The chemotactic factors may have stimulated oxygen-dependent or oxygen-independent bactericidal pathways. The requirements for oxygen-dependent bactericidal pathways was explored by using sodium azide-treated leukocytes, and leukocytes from a patient with CGD. Sodium azide is known to inhibit bacterial killing, probably by inhibiting myeloperoxidase (15) and perhaps other oxygen-dependent pathways. CGD neutrophils are defective in killing catalase-positive bacteria because of a primary defect in oxygen-dependent bactericidal pathways. In contrast, CGD neutrophils kill catalase-negative bacteria such as S. faecalis normally. C5a and FMP did not enhance the killing of S. aureus by azide-treated normal leukocytes or by CGD leukocytes (Fig. 6). Furthermore, these agents did not enhance the killing of S. faecalis by CGD leukocytes (not shown), although they did enhance killing by normal neutrophils (see above). These findings suggest that chemotactic factors can enhance bacterial killing primarily by stimulating oxygen-dependent bactericidal pathways.

Further support for this hypothesis is gained from the finding that chemotactants stimulate the HMPS activity of resting and phagocytosing neutrophils (Fig. 5) as was reported previously (4). Furthermore, C5a enhances superoxide production (11) and FMP (10^{-6}M) and FMLP (10^{-8}M) stimulate neutrophil chemiluminescence (2, 12), which was likely due to superoxide generation (12). All of these reactions are associated with neutrophil killing of bacteria. Our finding that chemotactic substances enhance the bactericidal capacity of neutrophils may be an important link to understanding the interaction of biologically active molecules with effector cells in host defense.

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LITERATURE CITED