Monocyte-Chemotactic Activity of Defensins from Human Neutrophils

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Abstract

We investigated the monocyte-chemotactic activity of fractionated extracts of human neutrophil granules. Monocyte-chemotactic activity was found predominantly in the defensin-containing fraction of the neutrophil granules. Purified preparations of each of the three human defensins (HNP-1, HNP-2, HNP-3) were then tested. HNP-1 demonstrated significant chemotactic activity for monocytes: Peak activity was seen at HNP-1 concentrations of 5×10^{-9} M and was 49±20% (mean±SE, $n = 9$) of that elicited by 10^{-8} M FMLP. HNP-2 (peak activity at 5×10^{-9} M) was somewhat less active, yielding 19±10% ($n = 11$). HNP-3 failed to demonstrate chemotactic activity. Checkerboard analysis of monocyte response to HNP-1 and HNP-2 confirmed that their activity was chemotactic rather than chemokinetic. Neutrophils demonstrated a low level of response to defensins but this reaction was primarily chemokinetic. Defensins may play a role in the recruitment of monocytes by neutrophils into inflammatory sites.

Introduction

Neutrophils, major cellular components of the inflammatory response, are known to contribute to the regulatory control of the inflammatory cell population by producing low molecular weight chemotactic factors such as leukotriene B4, which can attract more neutrophils and monocytes into the inflammatory site (1). Since many of the neutrophil's biologically active components are contained within its granules and are released into the extracellular space in response to stimuli, we examined PMN granules for proteins with direct chemotactic activity for leukocytes.

In the initial screening we employed gel permeation chromatography to fractionate neutrophil granule extracts into four principal protein fractions, as described by Modrzakowski and colleagues (2). The composition of these factions has been partially determined: Fraction A contains myeloperoxidase and lactoferrin, fraction B contains proteases (including elastase and cathepsin G), fraction C contains lysozyme and fraction D in rich is defensins (2, 3), a family of peptides with a broad spectrum of microbicidal activity in vitro (4).

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Methods

Leukocytes for chemotactic studies were obtained from heparinized peripheral blood by Ficoll-Hypaque density separation to obtain mononuclear cells, followed by dextran sedimentation to obtain neutrophils (5). Cells were washed and resuspended at 10^6 monocytes or neutrophils/ml in HBSS containing 0.1% BSA (Calbiochem-Behring Corp., La Jolla, CA).

Granule-rich fractions were prepared from neutrophils from single donor leukophoresis packs (Hemacare, Van Nuys, CA) containing 1–3 $\times 10^{10}$ cells, of which > 90% were viable PMN. After suspension in HBSS (pH 7.4) with 2.5 mM $MgCl_2$, the cell suspension was sealed in a nitrogen "bomb" (Parr Instrument Co., Moline, IL) and pressurized to 750 psi for 20 min. The suspension was then released drop-wise into HBSS with 5 mM Na_2EDTA while stirring. Nuclei and cellular debris were removed by centrifugation at low speed (200 *g* for 10 min). The pellet was examined by phase-contrast microscopy to ensure > 90% cellular disruption. The granule-rich supernatant was sedimented at 27,000 *g* for 20 min, and the pellets were stored at −70°C until extracted.

Granule proteins were extracted and fractionated essentially as described by Modrzakowski et al. (2). Briefly, the granule pellets (~ 1 $\times 10^{10}$ cell equivalents total) were extracted three times in a volume of 0.2 M sodium acetate buffer with 0.01 M $CaCl_2$ (pH 4.0) at a final concentration of 2×10^8 cell equivalents/ml. Each extraction was done over 12 h at 4°C with gentle stirring, and the residue was separated by centrifugation at 27,000 *g* for 20 min. The pooled acetate extracts were concentrated by ultrafiltration (YC-05 filter; Amicon Corp., Danvers, MA) and placed on a Sephadex G-100 column (2.5 by 150 cm). ~ 70 mg of crude granule protein extract was eluted with 0.2 M sodium acetate buffer (pH 4.0) and collected in 10-ml fractions. The fractions were pooled into four fractions (A, B, C, and D) defined by their A_{280} pattern (Fig. 1), concentrated by ultrafiltration (Amicon YC-05 filter), and dialyzed against PBS in Spectrapore 3 tubing (3,500 mol-wt cutoff; Spectrum Medical Industries, Los Angeles, CA). ~ 50% of the protein in the original granule extract was recovered by this technique.

Protein concentration was measured by the bicinchoninic acid protein assay system (Pierce Chemical Co., Rockford, IL) with chicken egg-white lysozyme as the standard, according to the manufacturer's instructions. Electrophoresis of 1–4 μg of crude granule extract, fractions A–D, and purified human lysozyme, neutrophil elastase, and defensins was performed on 12.5% acid–urea polyacrylamide gels to confirm the identification of each peak. These preparations were diluted to the indicated concentrations in HBSS containing 0.01% BSA and tested for chemotactic activity. The approximate molar concentrations of the fractions were estimated using a molecular weight of 100,000 for fraction A (myeloperoxidase and lactoferrin), 25,000 for fraction B (elastase and cathepsin G), 12,000 for fraction C (lysozyme), and 4,000 for fraction D (defensins).

The human defensins HNP-1, HNP-2, and HNP-3 were purified from neutrophil granule preparations. The granules were extracted with 10% acetic acid, and the extract was concentrated under vacuum and chromatographed on Biogel P-10 to yield a crude mixture of the three defensins. These were separated from each other and further

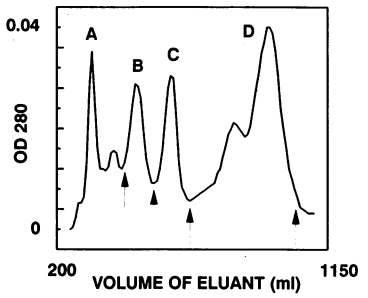

Figure 1. A representative chromatogram of neutrophil granule extract on a Sephadex G-100 column. The pattern of four major peaks was highly reproducible between preparations, with only minor variations in the relative size of the absorbance peaks. Arrows designate the cutoff points for fraction pools A, B, C, and D.

purified by ion-exchange and reverse phase HPLC as described previously (6). The purified proteins were dissolved in 0.01% acetic acid and diluted to the desired concentrations in HBSS containing 0.01% BSA (HBSS-BSA). In some assays, 5% autologous human serum (heat inactivated at 56°C for 30 min) was substituted for BSA in the dilution buffer (HBSS-serum).

Chemotaxis was determined in triplicate samples in multiwell chambers (Neuro Probe, Inc., Cabin John, MD) (7) using a 5-μm pore size filter (Nucleopore Corp., Pleasanton, CA) that separated the upper well which contained the leukocytes from the lower well which contained the putative chemotactic factors. Negative controls using the appropriate dilution buffer were run simultaneously. After 1 h incubation at 37°C, the filters were removed and stained with Giemsa. With a microgrid, we determined the number of cells that had migrated to the lower aspect of the filter (cells/hpf) toward the putative chemoattractant, and subtracted from it the number of cells that migrated toward the diluent control. This activity was then compared with that of 10^{-8} M FMLP and the results were expressed as percentage of FMLP activity.

Checkerboard analysis (8) was performed to distinguish chemotactic from chemokinetic activity. Serial dilutions of the tested factor were placed in the lower well of the chamber, and similar concentrations of the factor were used to dilute the leukocytes used in the upper well of the chamber. Directional movement of the cells in response to a concentration gradient indicates chemotaxis, whereas increased random locomotion of the cells induced by the factor unrelated to the concentration gradient indicates chemokinesis. Results are expressed as cells/hpf.

Results

Monocyte-chemotactic activity of fractions A–D from four different neutrophil donors is summarized in Fig. 2 *A*. Only fraction D contained statistically significant chemotactic activity, which was found at concentrations 10^{-7}–10^{-9} M (10 of 12 determinations showed activity > 0%, $P = 0.019$, exact test). The highest levels of monocyte chemotactic activity, 75±41%, occurred at 10^{-8} M (40 ng/ml) of fraction D. None of the fractions displayed chemotactic activity for neutrophils (Fig. 2 *B*).

Since > 90% of fraction D consists of the three defensins HNP-1, HNP-2, and HNP-3, we investigated the chemotactic activity of the individually purified defensins. In HBSS-BSA, HNP-1 demonstrated statistically significant chemotactic activity for monocytes, in the range of concentrations 5×10^{-8}–1×10^{-10} M (43 of 50 triplicate experiments had activity > 0%, $P = 8.9 \times 10^{-8}$). Peak activity was seen at HNP-1 concentrations of 5×10^{-9} M (20 ng/ml), where the response was 49±20% ($n = 9$) of FMLP activity.

HNP-1 was also chemotactic in the presence of serum. Chemotactic assays performed in HBSS-serum resulted in a

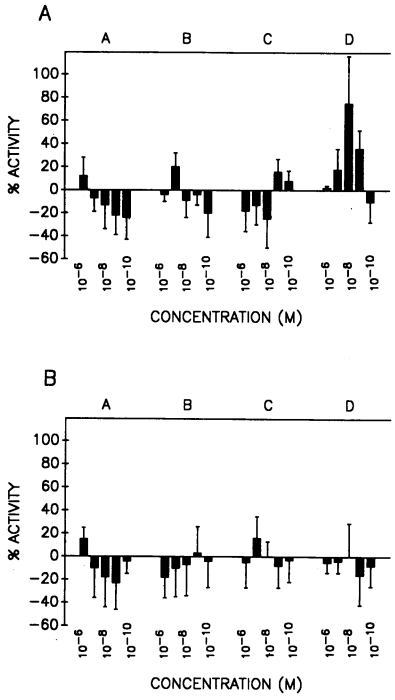

Figure 2. Chemotactic activity of neutrophil granule fractions A–D for monocytes (*A*) and for neutrophils (*B*). Each fraction was tested at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M. Data represent mean±SE of four different granule preparations, each tested in triplicate.

higher baseline (control) activity of the cells tested (28±7 monocytes/hpf in 5% heat-inactivated autologous serum vs. 6±4 monocytes/hpf in BSA, $n = 3$), and a lower incremental activity (level above the baseline) for FMLP (28±13 monocytes/hpf in serum vs. 37±17 monocytes/hpf in BSA, $n = 3$). In HBSS-serum, HNP-1 showed peak activity at concentrations of 5×10^{-9} M HNP-1, giving 50%±24% ($n = 3$) of that elicited by FMLP.

In HBSS-BSA, HNP-2 demonstrated some chemotactic activity at 5×10^{-8} to 1×10^{-10} M (42 of 64 experiments showed activity > 0%, $P = 0.006$) with peak at 5×10^{-9} M, that was 19±10% ($n = 11$) of FMLP activity. HNP-3, which differs from HNP-1 and HNP-2 only in the single amino terminal residue, failed to demonstrate chemotactic activity (Fig. 3).

Checkerboard analyses of HNP-1– and HNP-2–induced monocyte migration showed that the observed activity was chemotactic rather than chemokinetic (Fig. 4). Neutrophils demonstrated low levels of migration toward the three defensins HNP-1, HNP-2, and HNP-3 (Fig. 5), but this activity was found to be primarily chemokinetic rather than chemotactic (data not shown).

Discussion

The influx of phagocytes into sites of inflammation is controlled by multiple signals generated by invading pathogens,

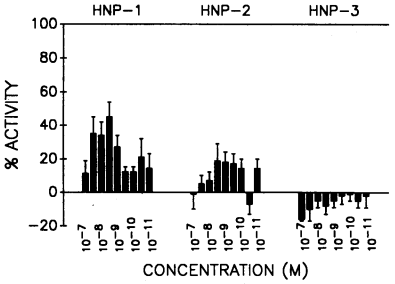

Figure 3. Monocyte chemotactic activity of the three human defensins (HNP-1, HNP-2, and HNP-3) tested at concentrations 10^{-7}, 5×10^{-8}, 10^{-8}, 5×10^{-9}–10^{-11} M. Data represent mean±SE ($n = 6 - 11$).

HNP-1

LOWER WELL (CONC.) \ UPPER WELL (CONC.)	0%	5%	10%	50%	100%
0%	0	1	1	1	1
5%	1	2	0	1	1
10%	2	3	0	1	1
50%	6	3	1	1	2
100%	18	7	2	2	1

HNP-2

LOWER WELL (CONC.) \ UPPER WELL (CONC.)	0%	5%	10%	50%	100%
0%	0	0	0	0	0
5%	2	0	0	0	0
10%	3	1	0	0	0
50%	5	0	0	0	0
100%	11	3	1	0	0

Figure 4. Checkerboard analysis of HNP-1 (*top*) and HNP-2 (*bottom*) for monocytes. Various dilutions of HNP were placed in the lower well of the chamber as well as in the monocyte-containing upper well of the chamber. 100% concentration was at 10^{-9} M for HNP-1 and 5×10^{-9} M for HNP-2.

serum activation, injured cells, and phagocytes already present at the site. It has been suggested that neutrophils contribute to the recruitment of inflammatory cells by releasing lipid derivatives such as leukotriene B_4 and platelet-activating factor (9, 10), and a complement-activating factor contained in specific granules (11). Our experiments demonstrate that the PMN azurophil granule proteins, defensins, can also exert chemotactic activity and may be an additional mechanism whereby neutrophils influence recruitment.

Although selective influx of mononuclear phagocytes into inflammatory lesions is commonly observed, the monocyte-specific chemotactic signals that mediate this influx have not been extensively characterized. Degradation products of matrix proteins (collagen, elastin, and fibronectin) have been shown to recruit monocytes selectively (12). In addition, certain neutrophil products have also been shown to have this activity. In 1968, Ward (13) demonstrated that a low molecular weight cationic protein fraction from lysates of rabbit neutrophils had chemotactic activity for rabbit mononuclear cells (but not neutrophils). In retrospect, it is possible that Ward observed the activity of one or more of the rabbit neutrophil defensins, which make up most of the low molecular weight cationic protein fraction of rabbit neutrophil lysates. Such rabbit defensins are homologues of the human defensins employed in our experiments (6).

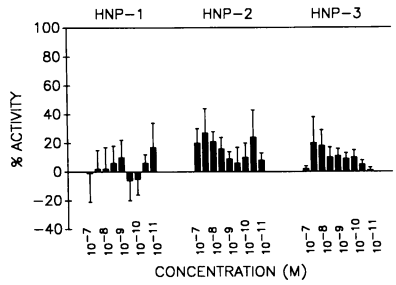

Figure 5. Neutrophil chemotactic activity of the three human defensins (HNP-1, HNP-2, and HNP-3) tested at concentrations 10^{-7}, 5×10^{-8}, 10^{-8}, 5×10^{-9}–10^{-11} M. Data represent mean±SE ($n = 5 - 12$).

Wright and Gallin described a complement activating factor contained in specific granules of neutrophils (11). Because our experiments detected chemotactic activity in the absence of serum, it is unlikely that complement activation or other serum factors are involved in the activity we observed. Whereas specific granule components are readily released by various soluble stimuli (reviewed in reference 14), the tissue concentrations of azurophil granule components may primarily reflect phagocytosis of particles by PMN or lysis of PMN at the inflammatory locus (reviewed in reference 15). Chemotactic activity of azurophil granule proteins could contribute to the generation of a chemotactic signal whose intensity is proportional to the phagocytic load and/or the number of senescent or destroyed PMN at the site. The activity of HNP-1 persists in the presence of serum, suggesting that HNP-1 may contribute to monocyte influx into inflammatory lesions in vivo.

The skin window response has been used as an in vivo test of recruitment and migration of cells in the inflammatory response. Typically, neutrophils migrate into the skin window early (6–12 h) in the response with a change to a monocyte infiltration at 24–48 h. Although monocyte migration into skin windows does occur in patients with significant neutropenia (16), neutrophils are felt to be major mediators of monocyte influx (17). A genetic disorder of neutrophils has been described in which neutrophils have abnormal or absent specific (secondary) granules. These patients with specific granule deficiency (SGD)[1] have a depressed inflammatory response and recurrent severe bacterial infections. Neutrophils from patients with SGD not only lack specific granule markers (such as lactoferrin and vitamin B12–binding proteins) (1, 18), but are also profoundly deficient in defensins (19), which are predominantly localized in azurophilic granules (6, 20). Patients with SGD have been noted to have an abnormal skin window response. Despite appropriate neutrophil recruitment in the early response period, they manifest failure of monocyte recruitment into the skin window in the late phase (18). In vitro, the monocytes of SGD patients migrate normally to standard chemotactic stimuli. It is noteworthy that unfractionated lysates of neutrophils from a patient with SGD are deficient in an unidentified monocyte-selective chemotactic factor that was present in normal neutrophils (18). Since our findings indicate that defensins can serve as a monocyte-selective chemotactic factor in neutrophil granules, we suggest that the in vitro and in vivo abnormalities of monocyte chemotaxis in SGD may result from defensin deficiency of SGD neutrophils. Because defensins comprise ~ 25% of granule protein (20) and 5–7% of cellular protein (21) of normal human neutrophils, defensins may be a major physiologic mediator of the monocyte recruitment by neutrophils in the inflammatory response.

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1. *Abbreviation used in this paper:* SGD, specific granule deficiency.

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