

SUBCELLULAR LOCATION AND PROPERTIES OF BACTERICIDAL FACTORS FROM HUMAN NEUTROPHILS

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At least two antimicrobial systems exist in polymorphonuclear leukocytes (PMN),¹ one that depends on the production of reactive oxygen intermediates and another that is independent of the respiratory burst (1–3). Neutrophil-derived proteins have been implicated as components of the respiratory burst-independent microbicidal pathway (4–6). However, their nature, subcellular localization, mode of action, and actual contribution to killing *in vivo* are still a matter of debate. We have examined the location of bactericidal factors (BF) in human neutrophils, taking advantage of a new method for the efficient subcellular fractionation of these cells: disruption by nitrogen cavitation and centrifugation of the postnuclear supernatant on a discontinuous Percoll density gradient (7). In contrast to previous work, we have looked at the distribution of BF at each step of the fractionation procedure and in all the fractions, from whole cell lysates to isolated cellular compartments. On the basis of our localization results, we have used a highly purified organelle population, azurophilic granules, as our starting material for the isolation of azurophil-derived bactericidal factors (ADBF).

Materials and Methods

Isolation of Neutrophils. Blood was obtained from healthy donors who gave informed consent. The blood was anticoagulated with 25 mM sodium citrate and mixed with an equal volume of 6% dextran in 0.9% NaCl to enhance the sedimentation of erythrocytes. After 60 min at room temperature, the leukocyte-rich supernatant was collected and centrifuged at 200 *g* for 10 min. The cell pellets were resuspended in 0.9% NaCl. PMN were separated from mononuclear cells by centrifugation through Ficoll-Hypaque and contaminating erythrocytes removed by two successive cycles of hypotonic lysis, as described (7, 8). >98% of the cells were PMN, of which >95% were neutrophils and <3% were eosinophils. We will refer to this preparation as neutrophils. One unit of blood yielded 10⁹ neutrophils.

Subcellular Fractionation of Neutrophils. Isolated neutrophils in PBS (2 × 10⁷ cells/ml)

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¹ *Abbreviations used in this paper:* ADBF, azurophil-derived bactericidal factor(s); BF, bactericidal factor(s); KU, killing unit; PMN, polymorphonuclear leukocyte(s).

were treated with 5 mM DIFP for 15 min at 4°C. The DIFP-treated cells were centrifuged at 130 *g* for 10 min at 4°C, and the pellet was resuspended in ice-cold relaxation buffer containing 100 mM KCl, 3 mM NaCl, 1 mM ATP(Na)₂, 3.5 mM MgCl₂, 10 mM Pipes, pH 7.3. The cell suspension was disrupted by nitrogen cavitation for 20 min at 350 psi, in a bomb (Parr Instrument Co., Moline, IL) at 4°C and the cavitate was collected into EGTA, pH 7.4 at a final concentration of 1.5 mM. Nuclei and unbroken cells were pelleted (P₁) by centrifugation at 500 *g* for 10 min at 4°C. The postnuclear supernatant (S₁) was centrifuged for 15 min at 20,000 rpm (SS 34 rotor; Sorvall Instruments, Newtown, CT) on a discontinuous Percoll density gradient, as described by Borregaard et al. (7). Fractions of ~1 ml were collected at 4°C and assayed for specific markers of azurophilic granules (β -glucuronidase and myeloperoxidase), specific granules (vitamin B₁₂-binding protein), and plasma membrane (alkaline phosphatase) as described below. Percoll was removed from pooled fractions by centrifugation at 35,000 rpm (180,000 *g*) for 2 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA). The layer that sedimented above the packed Percoll was resuspended in relaxation buffer, and stored in aliquots at -70°C.

Assays for Specific Markers in Subcellular Fractions. To ensure complete solubilization, aliquots from each fraction were diluted 1:5 in Triton X-100 (0.05% wt/vol final concentration) before enzyme or protein assays. Alkaline phosphatase was assayed with 1 mg/ml *p*-nitrophenyl phosphate as substrate in a 0.3 mM MgCl₂, 50 mM sodium barbital buffer, pH 10.5. 50- μ l samples diluted in Triton X-100 were assayed. Samples were incubated for 80 min at 37°C in the assay mixture (1-ml vol) and the reaction was terminated by addition of 100 μ l of 1 N NaOH. The absorbance at 410 nm was read immediately. The enzyme activity was calculated as described (9).

β -Glucuronidase was assayed by liberation of phenolphthalein from 1 mM phenolphthalein β -monoglucuronic acid in 100 mM sodium acetate buffer, pH 4.4, at 37°C for 3 h. 25- μ l samples diluted in Triton X-100 were assayed in 550 μ l of assay mixture. The reaction was terminated by adding 200 μ l of 1 M glycine, 1 M NaCl, and 1 M NaOH, and the absorbance was read at 550 nm. The enzyme activity was calculated as described (7).

Vitamin B₁₂-binding protein was measured on 25-, 50-, and 100- μ l samples diluted in Triton X-100 essentially as described by Gottlieb et al. (10). [⁵⁷Co]vitamin B₁₂ was prepared by mixing 5 ng/ml vitamin B₁₂ (Sigma Chemical Co., St. Louis, MO) with 0.025 μ Ci/ml [⁵⁷Co]cyanocobalamin (Amersham Corp., Arlington Heights, IL; sp. act. 10⁵ cpm/ng). 750 μ l of saline was mixed with 350 μ l of [⁵⁷Co]vitamin B₁₂ and with 100 μ l of the final volume of the sample. 0.5 ml of albumin-coated charcoal was then added and the test tubes were centrifuged for 2 min at 10,000 *g* at room temperature. 1 ml of the supernatant was collected and counted in a Packard auto-gamma scintillation counter (Packard Instrument Co., Downers Grove, IL) to determine the amount of bound [⁵⁷Co]vitamin B₁₂ in each sample.

Protein was determined as described by Lowry et al. (11) using BSA as standard. To prevent Triton X-100 interference with the assay, 0.1% SDS was added to the alkaline copper solution (12). Percoll at the concentration present in the fractions did not affect the assay.

To assay myeloperoxidase, 200 μ l of each fraction was diluted fivefold in relaxation buffer containing 0.2% Triton X-100, and introduced into the sample compartment of a Perkin-Elmer 557 double-beam spectrophotometer (Coleman Instruments Div., Perkin-Elmer Corp., Oak Brook, IL). Absorption spectra, from 400 to 600 nm, of oxidized fractions versus fractions reduced with dithionite were then measured (E_{472} = 75/mM·cm) (13).

Preparation of ADBF. Fractions from the Percoll gradients corresponding to azurophilic granules were pooled and Percoll was removed by centrifugation as described (7). The azurophilic granule preparation was resuspended in relaxation buffer and stored either on ice at 4°C or at -70°C. The azurophilic granules stored on ice at 4°C appeared to be intact in that no β -glucuronidase or myeloperoxidase release from the granules could be detected for 2 wk. Freezing of the azurophilic granules at -70°C resulted in some leakage (\leq 20%) of the β -glucuronidase but not of the myeloperoxidase. The isolated

azurophilic granules were extracted with 0.05 M glycine-HCl buffer, pH 2.0, for 40 min at 25°C. The acid extract was centrifuged at 10,000 *g* for 20 min and the supernatant used as a source of ADBF. The supernatant was either diluted in or dialyzed against the incubation medium prior to bactericidal assays. For the dialysis of ADBF extracts, a membrane tubing of 1,000 *M*, cutoff (Spectra/Por, Spectrum Medical, Los Angeles, CA) was used. Fractions from the Percoll density gradients were extracted by following the same procedure; Percoll had no effect on the extraction or activity of bactericidal factor(s).

Bactericidal Assays. Bactericidal activity was tested against *Escherichia coli* K12 (MC 4100) in routine assays and, where indicated, against *Salmonella typhimurium* LT2 (gifts of Dr. H. Shuman, Columbia University), *Pseudomonas aeruginosa* PAC and PAO (gift of Dr. A. Prince, Columbia University), *Listeria monocytogenes* (gift of Dr. R. Steinman, The Rockefeller University), *Staphylococcus aureus* (gift of Dr. S. Blander, The Rockefeller University), and *Streptococcus pneumoniae* type III, type II, and an unencapsulated variant of the *S. pneumoniae* type II strain (gifts of Dr. E. Tuomanen, The Rockefeller University). Trypticase soy broth and trypticase soy agar plates were used to cultivate most bacteria. In the case of *S. pneumoniae*, Cy medium and 5% defibrinated sheep blood agar plates were used (14).

Organisms from a single colony on agar plates were inoculated into liquid medium and cultured overnight at 37°C. Aliquots of the overnight culture were inoculated into fresh nutrient broth and grown to the midexponential phase. Bacterial cultures were then diluted into the test medium to the appropriate concentration. Most experiments were performed in 0.05 M citrate buffer, pH 5.5. Control experiments showed that this buffer did not affect the viability of any of the bacteria tested except *P. aeruginosa* and *S. pneumoniae* type II, for which 0.05 M phosphate buffer, pH 6.0, was used. Other buffers such as acetate, phosphate, or citrate-phosphate at a concentration of 0.01 or 0.05 M were used in some bactericidal assays, as specified in the text.

Bacteria (4×10^4 CFU in a final volume of 200 μ l) were incubated for 30 min at 37°C with indicated amounts of azurophilic granule extract diluted in the incubation medium. Samples were then diluted 1:100 in M63 minimal medium (15), and spread onto agar plates. CFU were counted after incubation at 37°C for 16 h. Bactericidal activity was expressed as the percentage of bacteria killed after exposure to ADBF compared with control. Alternatively, we defined 1 unit of killing activity (killing units, KU) as the reciprocal of the number of micrograms per milliliter of protein necessary to kill 10^5 bacteria/ml in 30 min at 37°C (LD₅₀).

Isolation of Azurophil Granule Membranes. Isolated azurophilic granules in relaxation buffer (pH 7.3) were disrupted by seven cycles of freezing in an acetone/dry ice bath and thawing at 37°C followed each time by immersion for 30 s in a sonicating water bath (Branson B3, Heat Systems-Ultrasonics Inc., Plainview, NY). A pelleted material was then obtained by centrifugation of the lysed granules at 10,000 *g* for 60 min or at 135,000 *g* for 6 min at 4°C. We refer to this pelleted material as azurophilic granule membrane. Isolated granule membranes and soluble granule contents were assayed for ADBF activity after incubation with 0.05 M glycine, pH 2.0, as previously described.

Results

Subcellular Distribution of BF. To determine the subcellular location of BF, we fractionated the neutrophils, using the method of Borregaard et al. (7). Neutrophils were treated with 5 mM DIFP before fractionation because DIFP, a potent serine protease inhibitor, has been shown to inhibit proteolysis very effectively in PMN extracts (16, 17). 10^9 DIFP-treated cells were disrupted by nitrogen cavitation, and the postnuclear supernatant was centrifuged on a discontinuous Percoll density gradient. Each fraction of the gradient was assayed for specific markers of azurophilic granules (myeloperoxidase), specific granules (vitamin B₁₂-binding protein) and plasma membrane (alkaline phosphatase). As shown in Fig. 1, the method resulted in efficient separation of these three

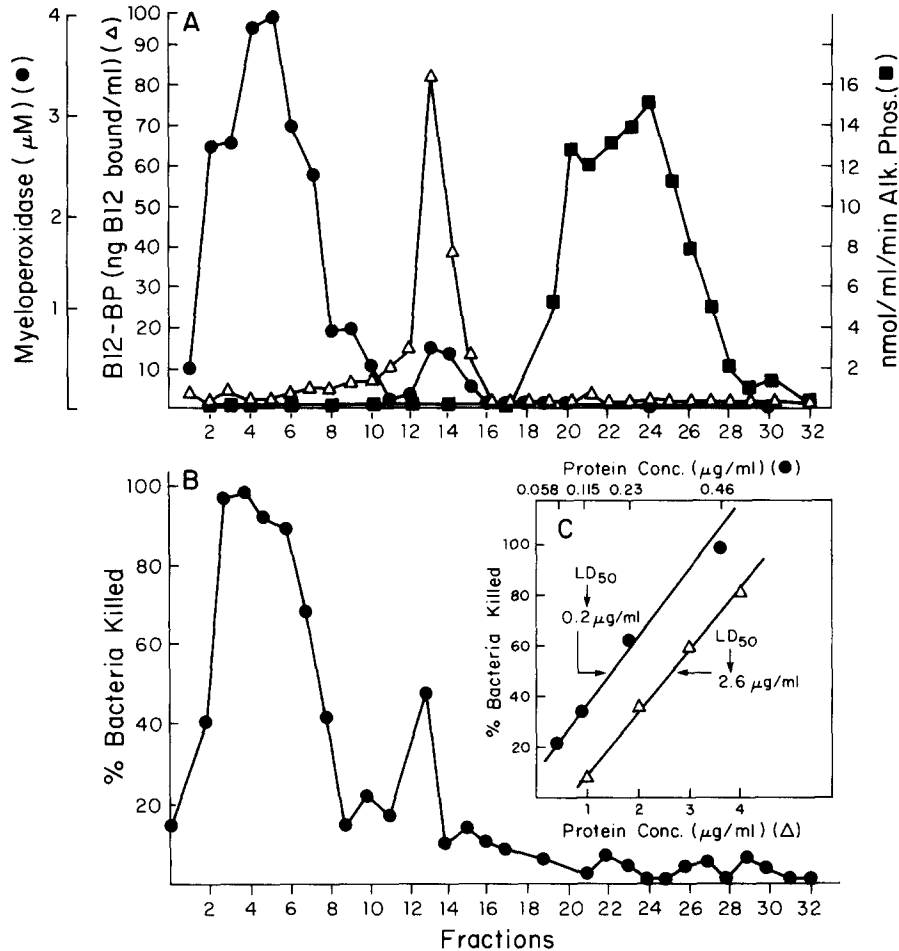


FIGURE 1. Subcellular distribution of bactericidal activity in human neutrophils. (A) Distribution of markers for (●) azurophilic granules (myeloperoxidase), (Δ) specific granules (vitamin B₁₂-binding protein [B12-BP]), and (■) plasma membrane (alkaline phosphatase). (B) Profile of bactericidal activity. Each fraction was extracted with 0.05 M glycine-HCl, pH 2.0, and centrifuged at 10,000 *g* for 20 min, and the supernatant was incubated with *E. coli* K12, at a protein concentration of 5 μg/ml. The percentage of bacteria killed after 30 min incubation at 37°C is presented here. Inset (C) shows the amount of protein from (●) purified azurophilic and (Δ) purified specific granules necessary to produce 50% reduction in bacterial CFU (LD₅₀).

compartments. Azurophilic granules showed no contamination by markers of specific granules or plasma membranes. Specific granules were not contaminated by plasma membranes but had some contamination by azurophilic granules, as indicated by the presence of 10% of the myeloperoxidase in this peak.

Bactericidal activity, obtained by extraction of cellular fractions at pH 2.0, distributed as shown in Table I. The majority of activity of the cavitate was present in the postnuclear supernatant (S₁). 6% of activity was associated with the nuclear fraction, perhaps because of adherence of a few granules to nuclei. The location of BF in the granule fraction was further indicated by a specific

TABLE I
Distribution of BF in Human Neutrophils

Purification step	Volume	Protein	KU	Sp act (KU/mg protein)	Yield
	<i>ml</i>	<i>mg/ml</i>			<i>%</i>
Cavitate	9.8	3.0	2.5	0.8	100
Pellet	2.0	3.0	0.75	0.25	6
S ₁ *	8.8	2.3	2.75	1.2	97
S ₂ ‡	8.0	1.3	0.025	0.02	0.8
γ (plasma membrane)	3.0	0.65	0.03	0.05	0.4
β (specific granules)	3.0	1.05	0.38	0.36	4.5
α (azurophilic granules)	3.0	1.1	7.0	6.4	86

* Postnuclear supernatant.

‡ Cytosol.

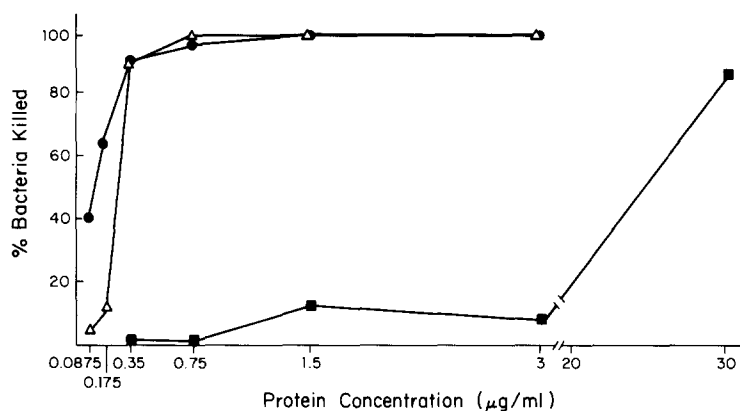


FIGURE 2. Membrane association of ADBF. Isolated azurophilic granules in relaxation buffer (pH 7.3) were disrupted by freeze-thaw/sonication and centrifuged. The supernatant (■), the pelleted material (●), and the total granules (Δ) were extracted at pH 2.0 and tested for bactericidal activity.

activity that was twice that of the unfractionated cavitate. As shown in Fig. 1, >90% of BF was present in the azurophilic granule fraction. The low level of activity present in the specific granule fraction could be attributed to the 10% contamination by azurophilic granules, as detected by myeloperoxidase assay (see Fig. 1). Indeed, ~10 times more protein from the specific than from the azurophilic granule fraction was required to kill 50% of the bacteria (*inset*, Fig. 1). These results strongly suggest that these BF are located exclusively in the azurophilic granule of human neutrophils.

Membrane Association of BF. To determine the location of BF within azurophilic granules, we lysed the intact purified granules at neutral pH by repeated freeze-thaw and sonication, centrifuged the disrupted granules, and separated soluble granule contents from pelleted granule membranes. Under these conditions, >90% of β-glucuronidase and myeloperoxidase were found in the supernatant fraction (data not shown). In contrast, 98% of ADBF was associated with azurophil membrane, as shown in Fig. 2.

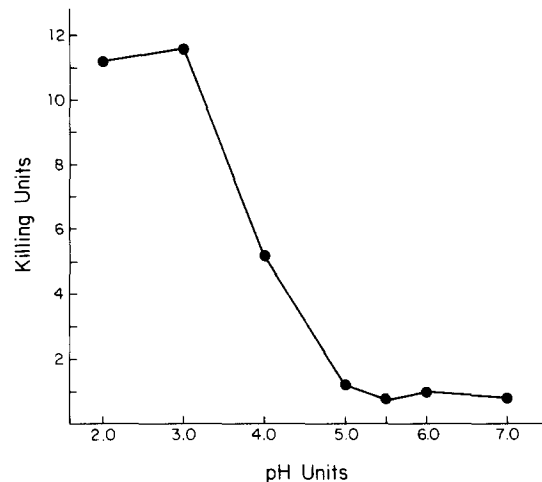


FIGURE 3. Effect of pH on the extraction of BF from azurophilic granule membranes. Aliquots of azurophil membranes were added to various buffer systems (0.05 M): glycine, pH 2.0–3.0; citrate, pH 4.0–6.0; phosphate, pH 7.0. After incubation at 25°C for 40 min, the suspensions were centrifuged at 10,000 *g* for 20 min and the supernatants assayed for protein content and bactericidal activity. Killing units (KU) correspond to the reciprocal of the number of microgram per milliliter of protein necessary to kill 10^5 bacteria in 30 min at 37°C. In each case, the bactericidal assay was conducted in 0.05 M citrate buffer, pH 5.5.

We next examined the ability of a number of agents to release ADBF from azurophil membranes. ADBF activity was assayed in the supernatant and pellet obtained after centrifugation of azurophil membranes treated with buffers of varying pH. Fig. 3 shows that <10% of ADBF activity was released from the membrane at pH 5.0–7.0, 50% at pH 4.0, and all at pH 2.0–3.0. The extraction of BF from intact azurophilic granules followed the same pH curve (data not shown).

Other agents commonly used to solubilize peripheral and integral membrane proteins were then tested (Table II). Treatment of the azurophil membranes with 1% Triton X-100 released ADBF activity as effectively as acid. In contrast, neither 2 M NaCl, 6 M urea, nor 0.1 M sodium bicarbonate, pH 11, released ADBF from the granule membrane.

Triton X-114 has been used on isolated membranes or whole cells to separate integral membrane proteins from hydrophilic proteins; hydrophilic proteins are recovered in the aqueous phase, whereas amphiphilic integral membrane proteins are found in the detergent phase after the phase separation of this detergent at $\geq 20^\circ\text{C}$ (18, 19). When azurophil membranes were extracted with Triton X-114, 87% of ADBF activity partitioned with the detergent phase (Table II).

ADBF Activity In Vitro Depends on Release from the Azurophil Membrane. We sought to determine if ADBF is active when associated with the membrane or if activity requires its release from the membrane. Azurophil membranes were treated at pH 2.0, which solubilizes ADBF, or at pH 5.5, which represents intralysosomal pH (20–22) but does not release ADBF from the membrane (Fig. 3). Bactericidal activity was then assayed at pH 5.5 in total membranes, and in the supernatant and pellet fractions obtained after centrifugation of the mem-

TABLE II
Effect of pH, Ionic Strength, and Surface-active Agents on the Release of ADBF from the Azurophilic Membrane

Azurophil membranes treated with:		Bactericidal activity of material released into supernatant	Bactericidal activity of material remaining in membrane pellet
Buffer	Additional agents		
<i>KU</i>			
0.05 M glycine, pH 2.0	—	14.0	<0.1
0.05 M citrate, pH 5.5	—	0.2	11.6
0.1 M sodium bicarbonate, pH 11.0	—	<0.1	10.8
0.05 M phosphate, pH 7.0	2 M NaCl	<0.1	8.0
None	6 M urea	<0.1	12.8
0.05 M phosphate, pH 7.0	1% Triton X-100	12.8	<0.1
0.01 M Tris, pH 7.4	0.15 M NaCl	1.7*	NA [§]
	0.5% Triton X-114	11.3 [‡]	NA

50 μ l of azurophil membranes (0.5 mg protein/ml) were incubated at 25°C for 40 min in 200 μ l of the various agents listed above. After centrifugation at 10,000 *g* for 20 min (4°C), the supernatants were collected, dialyzed against 0.05 M citrate, pH 5.5, and tested for protein content and bactericidal activity. Pellets were washed three times with ice-cold 0.05 M phosphate, pH 7.0, incubated at 25°C for 40 min with 0.05 M glycine, pH 2.0, and centrifuged, and the supernatants were assayed for protein and killing activity. For Triton X-114 treatment of azurophil membranes, we followed the procedure described by Bordier (18).

* Aqueous phase.

[‡] Detergent phase.

[§] Not applicable.

branes treated at both pH's. ADBF from membranes treated at pH 5.5 (membrane-bound ADBF, 1 KU) was 10 times less active than ADBF from membranes treated at pH 2.0 (soluble ADBF, 11.6 KU). Bactericidal activity could be recovered almost completely from membranes treated at pH 5.5 by reextraction at pH 2.0 (Table II).

Effect of Dose, Time, Bacterial Growth Status, and Buffer. ADBF activity was linear with respect to protein concentration over the range of 0.3–30 μ g/ml (Fig. 1 and further data not shown). The effect of bacterial concentration is shown in Fig. 4. Up to 10⁷ bacteria/ml could be killed by 30 μ g/ml of ADBF-containing extract in 30 min at 37°C. Killing was rapid; 50% of the cells were killed within 5 min at 37°C by the azurophilic granule extract containing 1.4 μ g of protein/ml (Fig. 5). The physiologic state of the bacteria incubated in the test medium did not affect their susceptibility to ADBF. Thus, bacteria in exponential growth or in a stationary phase were equally sensitive. The addition of glucose (20 mM) to the incubation medium did not affect ADBF activity. The killing activity of ADBF was approximately the same when citrate, acetate or phosphate salts were used as a buffer (data not shown).

Effect of pH and Divalent Cations. Because it has been shown that phagosomes rapidly reach and maintain a pH value of 5.5 during intracellular killing of bacteria *in vivo* (20–22), we examined the effect of pH on the bactericidal activity of ADBF *in vitro*. ADBF was effective over a broad range of pH (5.0–8.0) (Fig.

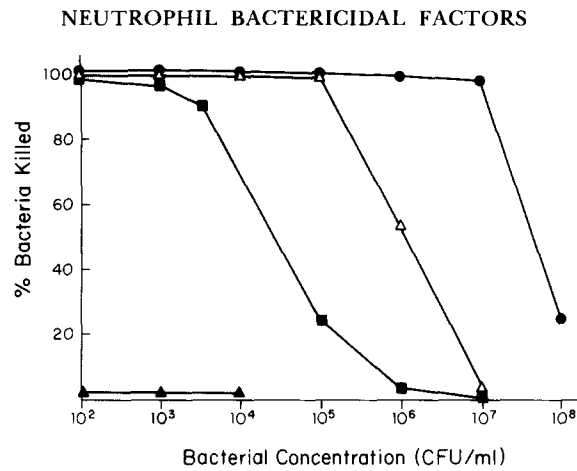


FIGURE 4. Bactericidal activity of azurophilic granule extract on increasing concentrations of bacteria. Granule extract was prepared as described in Materials and Methods. 30 µg/ml (●), 3 µg/ml (Δ), 0.3 µg/ml (■), or 0.03 µg/ml (▲) of the azurophil extract was added for each bacterial concentration tested.

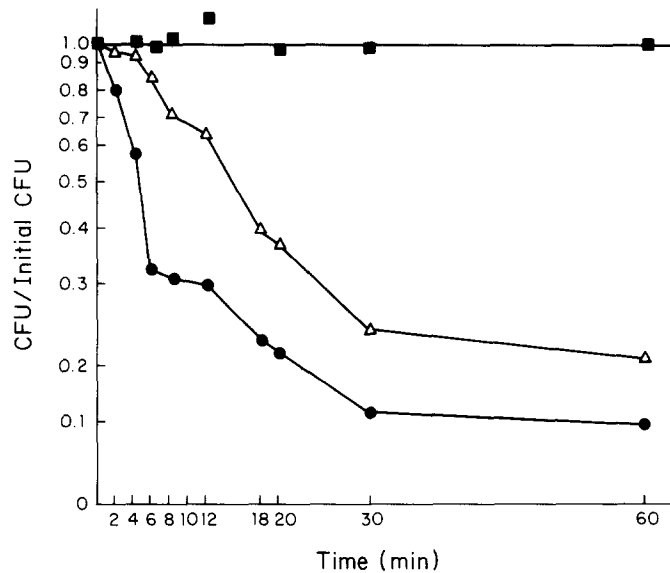


FIGURE 5. Kinetics of bactericidal activity of ADBF. *E. coli* K12 (2×10^5 CFU/ml) was incubated with 1.4 µg/ml (●) or 0.7 µg/ml (Δ) of azurophilic granule extract in 0.05 M citrate buffer, pH 5.5, and with citrate buffer alone (■).

6), with an optimum at pH 5.5. Media more acid than pH 5.0, which are bactericidal per se, could not be used to test ADBF killing.

Because ions such as Mg^{2+} and Ca^{2+} play a critical role in phagocytic processes (23) and also affect the surface properties of Gram-negative bacteria (24), we examined the effect of these ions on ADBF bactericidal activity. Mg^{2+} ions antagonized but did not completely block ADBF activity. The effect of Mg^{2+} ions was maximal at 1 mM, with a 25% reduction in bactericidal activity (data not shown). In contrast, Ca^{2+} ions inhibited all ADBF activity at a concentration

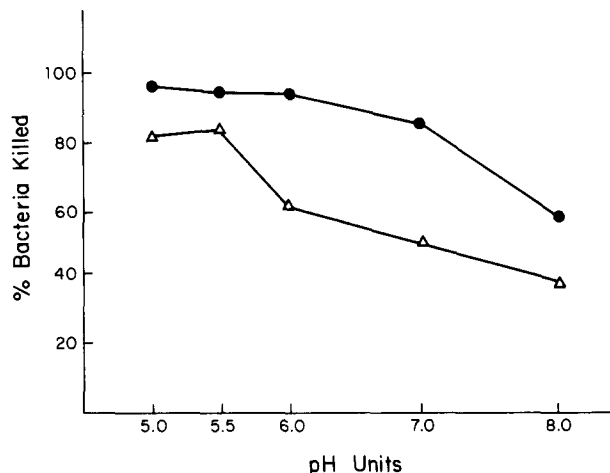


FIGURE 6. Effect of pH on bactericidal activity of ADBF. *E. coli* (2.5×10^5 CFU/ml) was incubated for 30 min at 37°C with 2.8 µg/ml (●) or 0.7 µg/ml (Δ) of azurophilic granule extract in citrate buffer, pH 5 and 5.5, and sodium phosphate or sodium phosphate-citrate buffer, pH 6.0–8.0.

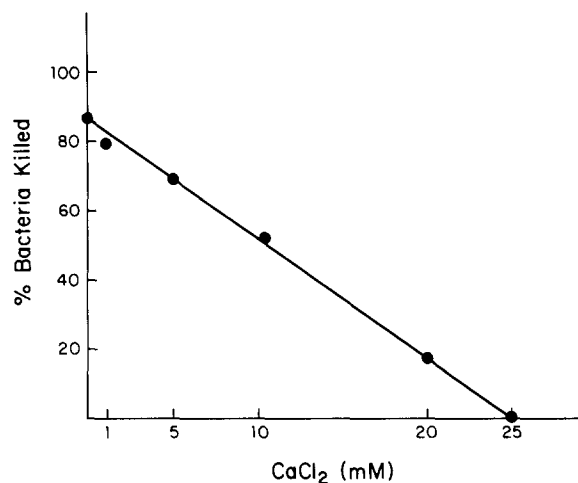


FIGURE 7. Effect of Ca²⁺ ions on ADBF activity. *E. coli* K12 (2.5×10^5 CFU/ml) was incubated for 30 min at 37°C with 2.8 µg/ml of azurophilic granule extract in 0.05 M citrate buffer, pH 5.5, supplemented with CaCl₂ as shown.

of 25 mM (Fig. 7). The decrease of bactericidal activity was linear with respect to calcium concentration over the range of 1–25 mM. In that the medium used for these tests contains citrate, which chelates divalent cations, the concentration of free cations in solution is lower than the nominal concentration. However, citrate does not bind significant amounts of Mg²⁺ and Ca²⁺ at low pH (25). The addition of EDTA (1–25 mM) to the incubation medium, to chelate cations, did not affect ADBF activity (data not shown). NaCl inhibited at concentrations of ≥ 0.3 M (Fig. 8). Physiologic concentrations of NaCl or KCl did not inhibit ADBF activity, when the latter was tested at a concentration ≥ 2.8 µg/ml.

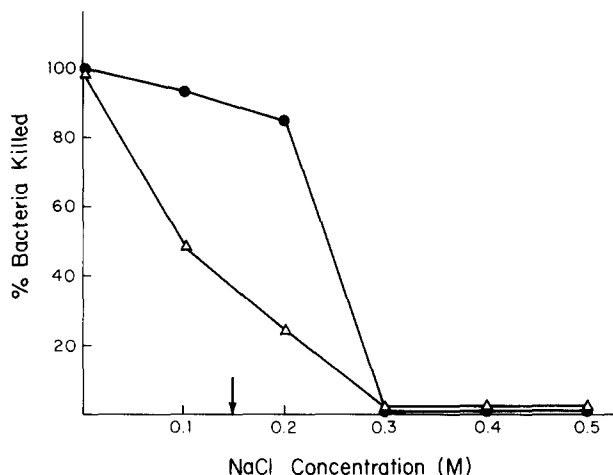


FIGURE 8. Effect of NaCl concentration on bactericidal activity of ADBF. *E. coli* K12 (2.5×10^5 CFU/ml) was incubated for 30 min at 37°C with 2.8 µg/ml (●) or 1.4 µg/ml (Δ) of azurophilic granule extract in 0.05 M citrate buffer, pH 5.5, supplemented with NaCl as shown. Arrow indicates NaCl concentration of plasma.

TABLE III
Antibacterial Spectrum of ADBF

Organism	Strain or type	ADBF activity*
<i>Staphylococcus aureus</i>	S27	+
<i>Staphylococcus aureus</i>	450	+
<i>Staphylococcus aureus</i>	TSS-1†	+
<i>Staphylococcus aureus</i>	TSS-2†	+
<i>Streptococcus pneumoniae</i>	Type III	-
<i>Streptococcus pneumoniae</i>	Type II	+
<i>Streptococcus pneumoniae</i>	R6	+
<i>Listeria monocytogenes</i>	450	(+)
<i>Pseudomonas aeruginosa</i>	PAC	+
<i>Pseudomonas aeruginosa</i>	PAO 103-0	+
<i>Salmonella typhimurium</i>	LT2	+
<i>Escherichia coli</i> K12	MC 4100	+

* ADBF activity is scored according to the micrograms per milliliter of protein in azurophil extract necessary to kill 10^5 bacteria in 30 min at 37°C: +, 0.1–0.3 µg/ml; (+), 1–2.5 µg/ml; -, >20 µg/ml.

† Clinical isolates from two patients with toxic shock syndrome.

Bacterial Spectrum of ADBF Killing. ADBF kills both Gram-positive and Gram-negative bacteria (Table III). The Gram-positive bacteria susceptible to ADBF killing include different strains of *Staphylococcus aureus* (two isolated from patients with toxic shock syndrome), β-hemolytic streptococci (with the exception of the capsulated streptococcus type III) and to some extent *Listeria monocytogenes*. All the Gram-negative bacteria tested were killed as efficiently as *E. coli*.

Discussion

Since the early studies by Hirsch (26–28), various bactericidal proteins have been described and in some cases isolated from neutrophils (6, 17, 29–33). The

subcellular location of these or other human neutrophil-derived bactericidal proteins has not been established. In this report, we treated human neutrophils with DIFP to prevent proteolysis and fractionated them using a scheme that results in clear separation of cytosol, plasma membrane, specific granules, and azurophilic granules, with minimal proteolysis or alteration of granule integrity and density. We then determined the optimum conditions for the extraction and assay of bactericidal factors; BF were maximally extracted at pH 2.0–3.0 and assayed at pH 5.5 (phagolysosomal pH). Under these conditions, we screened all cellular compartments and found that most of the bactericidal activity (97%) was associated with the postnuclear supernatant (S_1) and ~90% of the activity comigrated with the azurophilic granule population, after centrifugation of S_1 on Percoll density gradients. Neither the cytosol nor the plasma membrane fractions had significant activity. The low level of activity (5–10%) in the specific granule fraction could be accounted for by the 5–10% contamination of this fraction by myeloperoxidase-positive azurophil granules. Our data showed a single location for human neutrophil BF, in the azurophilic granule.

In support of these results, a single location for BF was found in bovine neutrophils, where bactericidal activity was exclusively localized to a large myeloperoxidase-negative granule population (34). In addition, a recent preliminary report (35) indicated that one of the neutrophil-derived BFs (bactericidal permeability-increasing protein) can be recovered (90%) in the cellular fractions containing azurophilic granules. In contrast, Rest et al. (36) found BF in both azurophilic and specific granules of human neutrophils. Because they used a different technique of fractionation (homogenization in sucrose and isopycnic centrifugation on sucrose density gradients), as well as different methods of extraction and assay, their results are not directly comparable with ours.

ADBF was associated with the azurophil membrane. Upon lysis of azurophilic granules at neutral pH, which solubilizes 90% of the myeloperoxidase, 98% of ADBF remained in the membrane-containing pellet. All of the membrane-associated ADBF could be extracted at pH 2.0 and released in soluble form after centrifugation of the acid-treated membrane. Possible explanations for the solubilizing effect of acid are: (a) acid induces a proteolytic event, (b) acid induces conformational changes that disrupt the association of ADBF with the granule membrane, or (c) acid displaces a charge interaction between ADBF and the membrane. Low pH was not strictly required for solubilization of ADBF. Triton X-100 at neutral pH could also release ADBF. Agents commonly used to solubilize peripheral membrane proteins, such as 2 M NaCl, 6 M urea, or 0.1 M sodium bicarbonate, pH 11, were unable to dissociate ADBF from the azurophil membrane. This indicates that ADBF is either tightly associated with or an integral constituent of the azurophil membrane. Indeed, upon treatment of azurophil membrane with Triton X-114, ADBF behaved similarly to an integral membrane constituent, in that it partitioned into the detergent phase.

We attempted to address the question of whether the release of BF from the membrane is necessary for bactericidal activity. Membrane-bound ADBF was 10 times less active than soluble ADBF, suggesting a coupling *in vitro* between ADBF solubilization and activity. The molecular mechanism of ADBF activation *in vivo* is yet to be understood.

On the basis of our localization results, we used intact, purified azurophil granules as a source of BF and studied their properties. This contrasts to previous work in which bactericidal proteins were obtained from an heterogeneous granule-enriched fraction by extraction with acid over long periods of time (6, 17, 30), in the absence of protease inhibitor (6, 30). ADBF resembles some of the bactericidal proteins previously described in being acid-extractable and granule-associated. In particular, ADBF shares many of the characteristics of the rabbit neutrophil-derived phagocytin (27, 28, 37): (a) Changes in the test medium such as a variation in the buffer, the addition of glucose, and inclusion of a metal binding agent had little effect on the bactericidal activity of ADBF. (b) Divalent cations (Mg^{2+} , Ca^{2+}) were not required for its lethal action. At high concentrations, these ions antagonized or, in the case of Ca^{2+} , inhibited completely the bactericidal effect (ID_{50} for Ca^{2+} , 10 mM). (c) Like phagocytin, ADBF was more active at a low pH (5.0–5.5), the estimated phagolysosomal pH during intracellular killing of bacteria.

ADBF differs from other reported neutrophil-derived antimicrobial proteins in a number of ways. ADBF is active over a wide range of pH, with optimum activity at pH 5.5, and is relatively insensitive to high ionic strength. This contrasts with bactericidal permeability-increasing factor, which is optimally active at pH 7.0, or defensins, which strictly require pH 7.0–8.0 and low ionic strength conditions for bactericidal activity (6, 30, 33, 34). By its antimicrobial spectrum, ADBF differs from purified factors such as bactericidal permeability-increasing factor or cationic antimicrobial proteins, which are only active on Gram-negative bacteria (17, 30, 33).

ADBF is extremely active; 0.1–0.3 $\mu g/ml$ can kill 10^5 bacteria per ml, using a wide range of test organisms—Gram-positive and Gram-negative bacteria. Purified BF specific for Gram-negative bacteria, such as bactericidal permeability-increasing factor (30) and the 57 kD cationic antimicrobial protein (17) have comparable activity, whereas others, such as defensins, seem to be active only at higher concentrations (>50 $\mu g/ml$) on both Gram-positive and Gram-negative bacteria (6, 33). In addition, ADBF kills bacteria rapidly; >50% killing is achieved within 5 min.

Because ADBF is not a homogeneous preparation, its activity in the azurophil membrane extract may be due to a single protein species or to the combined activity of several proteins that act synergistically. ADBF killing does not appear to involve H_2O_2 -dependent systems, since catalase (500 U/ml) does not significantly reduce its activity (data not shown) and since >90% myeloperoxidase is released in the soluble fraction upon isolation of membrane ADBF. The azurophil membrane is composed of a limited number of protein species, including two major polypeptides of 29 and 6 kD (38). Further characterization and purification of ADBF is needed to determine if it comprises a novel bactericidal protein or proteins or if it includes some of the bactericidal proteins previously described.

Summary

We examined the subcellular location of bactericidal factors (BF) in human neutrophils, using an efficient fractionation scheme. Nitrogen bomb cavities of

DIFP-treated PMN were centrifuged through discontinuous Percoll gradients, each fraction extracted with 0.05 M glycine, pH 2.0, and tested for the killing of *Escherichia coli*. >90% of BF coisolated with the azurophil granules. After lysis of azurophils, 98% of azurophil-derived BF (ADBF) sedimented with the membrane. ADBF activity was solubilized from azurophil membrane with either acid or nonionic detergent (Triton X-100, Triton X-114). Bactericidal activity was linear with respect to protein concentration over the range 0.3–30 $\mu\text{g/ml}$. 0.1–0.3 $\mu\text{g/ml}$ ADBF killed 10^5 *E. coli* within 30 min at 37°C. At 1.4 $\mu\text{g/ml}$, 50% of 2×10^5 bacteria were killed within 5 min. ADBF was effective between pH 5–8, with peak activity at pH 5.5. Glucose (20 mM), EDTA (1–25 mM), and physiologic concentrations of NaCl or KCl had little or no inhibitory effect on ADBF. ADBF killed both Gram-positive and Gram-negative virulent clinical isolates, including listeria, staphylococci, β -hemolytic streptococci, and *Pseudomonas aeruginosa*. Thus, under these conditions of cell disruption, fractionation, extraction, and assay, almost all BF in human PMN appeared to be localized to the membrane of azurophilic granules as a highly potent, broad-spectrum, rapidly acting protein(s) effective in physiologic medium. Some of these properties appear to distinguish ADBF from previously described PMN bactericidal proteins.

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References

1. Klebanoff, S. J., and R. A. Clark. 1978. The Neutrophil: Function and Clinical Disorders. North-Holland Publishing Company, Amsterdam. 1–810.
2. Kaplan, E. L., T. Laxdal, and P. G. Quie. 1968. Studies of polymorphonuclear leukocytes from patients with chronic granulomatous disease of childhood: bactericidal capacity for streptococci. *Pediatrics*. 41:591.
3. Mandell, G. L. 1974. Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. *Infect. Immun.* 9:337.
4. Elsbach, P., and J. Weiss. 1983. A reevaluation of the roles of the O_2 -dependent and O_2 -independent microbicidal systems of phagocytes. *Rev. Infect. Dis.* 5:843.
5. Spitznagel, J. K., and W. M. Shafer. 1985. Neutrophil killing of bacteria by oxygen-independent mechanisms: a historical summary. *Rev. Infect. Dis.* 7:398.
6. Ganz, T., M. E. Selsted, D. Szklarek, S. S. L. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer. 1985. Defensins, natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* 76:1427.
7. Borregaard, N., J. M. Heiple, E. R. Simons, and R. A. Clark. 1983. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J. Cell Biol.* 97:52.
8. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* 21 (Suppl.):77.
9. Lindhardt, K., and K. Walter. 1963. Phosphatases (phosphomonoesterases). *In* Methods of Enzymic Analysis. H.-U. Bergmeyer, editor. Academic Press, Inc., New York. 779.

10. Gottlieb, C., K.-S. Lau, L. R. Wasserman, and V. Herbert. 1965. Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B12 binding capacity, antibody to IF, and serum unsaturated B12 binding capacity. *Blood*. 25:875.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
12. Wang, C. S., and R. L. Smith. 1975. Lowry determination of protein in the presence of Triton X-100. *Anal. Biochem.* 63:414.
13. Bos, A., R. Wever, and D. Roos. 1978. Characterization and quantification of the peroxidase in human neutrophils. *Biochim. Biophys. Acta.* 525:37.
14. Tomasz, A. 1968. Biological consequences of the replacement of choline by ethanolamine in the cell wall of pneumococcus: chain formation, loss of transformability, and loss of autolysis. *Proc. Natl. Acad. Sci. USA.* 59:86.
15. Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 137-140.
16. Amrein, P. C., and T. P. Stossel. 1980. Prevention of degradation of human polymorphonuclear leukocyte proteins by diisopropylfluorophosphate. *Blood* 56:442.
17. Shafer, W. M., L. E. Martin, and J. K. Spitznagel. 1984. Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropylfluorophosphate. *Infect. Immun.* 49:29.
18. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604.
19. Lewis, V., S. A. Green, M. Marsh, P. Vihko, A. Helenius, and I. Mellman. 1985. Glycoproteins of the lysosomal membrane. *J. Cell Biol.* 100:1839.
20. Geisow, M. J., P. D'Arcy Hart, and M. R. Young. 1981. Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. *J. Cell Biol.* 89:645.
21. McNeil, P. L., L. Tanasugarn, J. B. Meigs, and D. L. Taylor. 1983. Acidification of phagosomes is initiated before lysosomal enzyme activity is detected. *J. Cell Biol.* 97:692.
22. Horwitz, M. A., and F. R. Maxfield. 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *J. Cell Biol.* 99:1936.
23. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:669.
24. Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* 49:1.
25. Chaberek, S., and E. E. Martell. 1959. *Organic Sequestering Agents*. John Wiley & Sons, editors. New York. 312-313.
26. Hirsch, J. G. 1956. Phagocytin: a bactericidal substance from polymorphonuclear leucocytes. *J. Exp. Med.* 103:589.
27. Hirsch, J. G. 1956. Studies of the bactericidal action of phagocytin. *J. Exp. Med.* 103:613.
28. Hirsch, J. G. 1960. Further studies on preparation and properties of phagocytin. *J. Exp. Med.* 111:323.
29. Zeya, H. I., and J. K. Spitznagel. 1963. Antibacterial and enzymic basic proteins from leukocyte lysosomes: separation and identification. *Science (Wash. DC)*. 142:1085.
30. Weiss, J., P. Elsbach, I. Olsson, and H. Odeberg. 1978. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. *J. Biol. Chem.* 253:2664.
31. Elsbach, P., J. Weiss, R. C. Franson, S. Beckerdite-Quagliata, A. Schneider, and L. Harris. 1979. Separation and purification of a potent bactericidal/permeability in-

- creasing protein and a closely associated phospholipase A₂ from rabbit polymorphonuclear leukocytes. *J. Biol. Chem.* 254:11000.
32. Lehrer, R. I., K. M. Ladra, and R. B. Hake. 1975. Nonoxidative fungicidal mechanisms of mammalian granulocytes: demonstration of components with candidacidal activity in human, rabbit, and guinea pig leukocytes. *Infect. Immun.* 11:1226.
 33. Selsted, M. E., D. Szklarek, and R. I. Lehrer. 1984. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. *Infect. Immun.* 45:150.
 34. Gennaro, R., B. Dewald, U. Horisberger, H.-U. Gubler, and M. Baggiolini. 1983. A novel type of cytoplasmic granule in bovine neutrophils. *J. Cell Biol.* 96:1651.
 35. Weiss, J., T. Goldberg-Klein, and I. Olsson. 1986. Cellular and subcellular localization of the neutrophil bactericidal/permeability-increasing protein. *Clin. Res.* 34:537A. (Abstr.)
 36. Rest, R. F., M. H. Cooney, and J. K. Spitznagel. 1978. Bactericidal activity of specific and azurophil granules from human neutrophils: studies with outer-membrane mutants of *Salmonella typhimurium* LT-2. *Infect. Immun.* 19:131.
 37. Cohn, Z. A., and J. G. Hirsch. 1960. The isolation and properties of the specific granules of rabbit polymorphonuclear leucocytes. *J. Exp. Med.* 112:983.
 38. Gabay, J. E., J. M. Heiple, and C. F. Nathan. 1986. Azurophil-derived bactericidal factor(s) from human neutrophils. *Clin. Res.* 34:676A. (Abstr.).