Killing of Gram-Negative Bacteria by Polymorphonuclear Leukocytes

ROLE OF AN O₂-INDEPENDENT BACTERICIDAL SYSTEM

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ABSTRACT Previous studies have suggested that a cationic bactericidal/permeability-increasing protein (BPI) present in both rabbit and human polymorphonuclear leukocytes is the principal O₂-independent bactericidal agent of these cells toward several strains of *Escherichia coli* and *Salmonella typhimurium* (1978. *J. Biol. Chem.* 253: 2664–2672; 1979. *J. Biol. Chem.* 254: 11000–11009). To further evaluate the possible role of this protein in the killing of gram-negative bacteria by polymorphonuclear leukocytes, we have measured the bactericidal activity of intact rabbit peritoneal exudate leukocytes under aerobic or anaerobic conditions and of intact human leukocytes from a patient with chronic granulomatous disease. Anaerobic conditions were created by flushing the cells under a nitrogen stream. Effective removal of oxygen was demonstrated by the inability of nitrogen-flushed leukocytes to mount a respiratory burst (measured as increased conversion of 1-[¹⁴C]glucose → ¹⁴CO₂ or by superoxide production) during bacterial ingestion. At a bacteria/leukocyte ratio of 10:1, killing of gram-positive, BPI-resistant, *Staphylococcus epidermidis* is markedly impaired in the absence of oxygen (76.4±3.3% killing in room air, 29.2±8.2% killing in nitrogen). Essentially all increased bacterial survival is intracellular. In contrast, both a nonopsonized rough strain (MR-10) and an opsonized smooth strain (MS) of *S. typhimurium* 395 are killed equally well in room air and nitrogen. A maximum of 70–80 MR-10 and 30–40 MS are killed per leukocyte either in the presence or absence of oxygen. There is no intracellular

bacterial survival in either condition indicating that intracellular O₂-independent bactericidal system(s) of rabbit polymorphonuclear leukocytes can at least match the leukocyte's ingestive capacity. Whole homogenates and crude acid extracts manifest similar bactericidal capacity toward *S. typhimurium* 395. This activity can be accounted for by the BPI content of these cell fractions and is virtually eliminated by immune (anti-BPI), but not by preimmune goat IgG-rich fractions. Opsonization of smooth MS, required for bacterial killing by intact leukocytes, does not alter bacterial sensitivity to BPI in crude or purified form. Leukocytes of a patient with chronic granulomatous disease killed ingested *S. typhimurium* 395 MS nearly as well as did normal leukocytes.

The bactericidal activity toward *E. coli* (J5) of crude acid extracts of the CGD and normal human leukocytes was virtually the same and was nearly completely inhibited by anti-BPI IgG-rich fractions, but not by preimmune IgG-rich fractions. These findings suggest that the killing of gram-negative bacteria such as *S. typhimurium* by intact polymorphonuclear leukocytes may also be attributed to the action of BPI.

INTRODUCTION

The multiple microbicidal activities of polymorphonuclear leukocytes (PMN)¹ can be broadly divided into two categories: (a) oxygen-dependent microbicidal systems, generated during the respiratory burst that accompanies (or may precede) phagocytosis (1); and

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¹ *Abbreviations used in this paper:* BPI, bactericidal/permeability-increasing protein; CGD, chronic granulomatous disease; HBSS, Hanks' balanced salt solution; PMN, polymorphonuclear leukocyte(s).

(b) oxygen-independent systems consisting primarily of proteins synthesized during cell differentiation (2). The latter group includes a membrane-active cationic protein with potent bactericidal activity toward several species of gram-negative bacteria. This bactericidal/permeability-increasing protein (BPI) has been recently isolated from both rabbit and human PMN (3, 4). Rabbit and human BPI are closely similar in several molecular and biological properties including molecular size ($M_r = 50\text{--}60,000$), net charge ($pI > 9.5$), pH optimum (neutral), bactericidal potency (10 nM BPI vs. 10^7 sensitive bacteria) and antimicrobial specificity (a range of gram-negative bacterial species [3–5]). All available evidence suggests that in crude leukocyte fractions BPI is the principal oxygen-independent bactericidal agent toward several strains of *Escherichia coli* and *Salmonella typhimurium* (3–6).

In this study we have sought to determine the importance of BPI in the overall bactericidal capabilities of the intact PMN toward gram-negative bacteria, comparing killing of rough and smooth *S. typhimurium* by rabbit PMN under aerobic and anaerobic conditions. We find that optimal killing of both bacterial strains by intact PMN does not require oxygen. Killing of smooth *S. typhimurium* by human leukocytes from a healthy individual and from a patient with CGD is also nearly the same. Our findings suggest that the O_2 -independent bactericidal activity of intact PMN as well as of PMN fractions toward these gram-negative bacteria is mainly attributable to BPI.

METHODS

PMN. PMN were obtained from overnight, sterile peritoneal exudates elicited in New Zealand White rabbits by injection of glycogen-saturated physiological saline (7). Differential counts showed that >95% of the cells were PMN. The cells were sedimented at 50 g for 10 min and resuspended at a concentration of 5×10^7 /ml in 90% Hanks' balanced salt (HBSS, without phenol red, Microbiological Associates, Inc., Bethesda, MD) and 10% 0.4 M Tris-HCl, pH 7.5. Whole homogenates of PMN, crude 0.16 N H_2SO_4 extracts (Sup II) and BPI were prepared as recently described (3, 6).

Bacteria. The smooth, mouse-virulent strain of *S. typhimurium* 395, MS, and the rough mutant derived from it, MR-10, have been described previously (8). *Staphylococcus epidermidis* 160K was kindly provided by Dr. Jeannette Winter (Department of Microbiology, New York University School of Medicine, New York). *E. coli* J5, a rough mutant that lacks UDP-galactose-4-epimerase activity, was obtained from Dr. Loretta Leive (Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolic and Digestive Diseases, National Institutes of Health, Bethesda, MD).

Growth and labeling of bacteria. Both strains of *S. typhimurium* 395 and *E. coli* J5 were grown in a triethanolamine-buffered (pH 7.75–7.9) minimal salts medium (9). *S. epidermidis* was grown in brain heart infusion broth (Difco Laboratories, Detroit, MI). All bacteria, after growth over-

night to stationary phase, were transferred to fresh medium (diluted 1:10), and the subcultures were grown to mid-late logarithmic phase. Where indicated, *E. coli* J5 was grown in media supplemented with 5 mM D-galactose to enable this bacterial strain to synthesize complete, long-chain lipopolysaccharide and to express a smooth phenotype (10). Bacterial concentrations were determined by measuring absorbance at 550 nm with a Coleman junior spectrophotometer (Coleman Instruments, Maywood, IL). The bacteria were sedimented by centrifugation at 6,000 g for 10 min and resuspended in sterile physiological saline at the desired concentration.

Bacterial RNA was labeled during growth in subcultures supplemented with 0.2 $\mu\text{Ci}/\text{ml}$ 2-[^{14}C]uracil (New England Nuclear, Boston, MA; 40–60 mCi/mmol). After incubation for 3 h the bacteria were washed once, resuspended in fresh growth medium, and reincubated for 30 min to permit incorporation of the remaining unincorporated radioactive precursor. After sedimentation and resuspension in isotonic saline, suspensions of 10^7 bacteria contained $\sim 3,000$ cpm, >98% of which were precipitable in cold 5% trichloroacetic acid.

Normal and hyperimmune rabbit serum and IgG. Normal serum was obtained from blood collected from New Zealand White rabbits (11). Rabbit hyperimmune serum against *S. typhimurium* 395 MS and anti-MS IgG were obtained as previously described (12, 13).

Opsonization. Opsonization of *S. typhimurium* 395 MS was accomplished by incubation of the bacteria at 37°C for 20 min in a mixture consisting of 100 μl HBSS, 100 μl 0.4 M Tris-HCl, pH 7.5, 20 μl of 5% casamino acids (Difco Laboratories), 10^9 *S. typhimurium* 395 MS, anti-MS serum or IgG (2%, unless indicated otherwise), brought up to a final volume of 1.0 ml with sterile saline. The opsonized bacteria were then sedimented by centrifugation at 6,000 g for 10 min and resuspended to the desired concentration in sterile isotonic saline.

Incubation procedures with intact PMN: Aerobic or anaerobic conditions. To create aerobic or anaerobic incubation conditions, PMN were equilibrated at room temperature in room air or nitrogen, respectively. Individual samples contained 10^7 PMN in 200 μl of resuspension medium in 10 ml siliconized Vacutainer tubes (no additive; Becton, Dickinson & Co., Rutherford, NJ). Nitrogen (pre-purified; Ohio Medical Products, Madison, WI) flushing was accomplished via 21- (inlet) and 18- (outlet) gauge needles inserted through the rubber stopper that seals these tubes. Flushing was carried out for 15 min before addition of bacteria. Longer nitrogen pretreatment of PMN (up to 60 min) did not alter the results. During pretreatment (in room air or nitrogen), the PMN suspensions were periodically hand-shaken to reduce cell clumping.

The bacteria were added in a volume of 50–150 μl of isotonic saline via the 18-gauge needle. Sterile saline was then added to rinse the needle and to bring the final volume of the suspension to 0.4 ml. The anaerobic samples were briefly flushed with nitrogen (<5 min) before removing the needles and resealing tubes with parafilm. Incubations were carried out for 30 min in a 37°C water bath with moderate shaking. At the end of incubation, the tubes were placed in an ice bath to prevent further phagocytosis and killing of bacteria.

Hexose monophosphate shunt activity. For measurement of hexose monophosphate shunt activity of PMN under aerobic or anaerobic conditions, the same pretreatment of PMN (2×10^7 cells/sample) in room air or nitrogen was carried out except that 10-ml erlenmeyer flasks capped with

rubber stoppers were used. After pretreatment, 0.03 μCi of 1- ^{14}C glucose (45 mCi/mol; New England Nuclear) in 100 μl of HBSS, 4×10^8 bacteria, and saline, to a final volume of 0.8 ml, were added through the 18-gauge needle as described above. Incubations were carried out for 30 min in a 37°C shaking water bath. Hexose monophosphate shunt activity was measured as conversion of 1- ^{14}C glucose $\rightarrow$ $^{14}\text{CO}_2$ (14). $^{14}\text{CO}_2$ formed in the absence of PMN was <5% of the amount formed in the presence of PMN. Evolved $^{14}\text{CO}_2$ was collected in polyethylene cups suspended from the rubber stoppers. At the end of incubation, the reaction was stopped by injecting 0.2 ml of 10 N H_2SO_4 through the rubber stopper into the flask. After 15 min, hydroxide of hyamine (0.4 ml) was added to the collection cups, and incubation at 37°C was continued for 1 h. At this time the cups were removed, placed in counting vials, and vigorously shaken with 8 ml of toluene-BBOT (2,5-tert-butylbenzoxazoly)-thiophene, Packard Instrument Co., Inc., Downers Grove, IL) scintillation mixture. Counting was performed in a LS 100C liquid scintillation counter (Beckman Instruments Inc., Irvine, CA).

Incubation procedure with crude and purified PMN fractions. Typical incubation mixtures (unless indicated otherwise) contained 2×10^7 bacteria in a total volume of 0.4 ml of sterile physiological saline that also contained 25 μl 0.4 M Tris-HCl buffer (pH 7.5), 25 μl of HBSS, 250 μg of vitamin-free casamino acids and the indicated amount of PMN fraction.

Bactericidal activity. The bactericidal activity of intact PMN or PMN fractions was determined by measuring the effect of PMN (fraction) on bacterial viability, i.e. colony-forming ability. From bacterial suspensions incubated with or without PMN (fraction), 20- μl samples were taken, serially diluted in sterile saline, and plated on either nutrient agar (*S. typhimurium*) or brain heart infusion agar (*S. epidermidis*). After incubation of bacteria with or without intact PMN, the bacterial suspensions were placed in an ice bath and brought to a volume of 1.0 ml by addition of ice-cold sterile saline. Samples for serial dilution and plating were taken before and after sonication (40–50 W; 15 s; twice at 0–4°C; Sonifier Cell Disrupter, Heat Systems-Ultrasonics, Inc., Plainview, NY) of the suspensions to determine extracellular and total (extra- and intracellular) bacterial viability, respectively. Sonication under the indicated conditions does not affect bacterial viability, but breaks up PMN, releasing intracellular bacteria. The number of colony-forming units on the plates was determined after incubation at 37°C overnight.

Hydrophobic interaction chromatography. Chromatography was carried out on a phenyl-Sepharose CL 4B column (0.8 $\times$ 2.8 cm) at room temperature ($\sim$ 20°C). The gel was washed with 10 vol of each eluant before use and equilibrated in isotonic saline containing 5% HBSS and 20 mM Tris-HCl, pH 7.5. [^{14}C]Uracil labeled bacteria (2×10^7 bacteria in 0.4 ml of standard incubation mixture) were applied to the column and elution was carried out in the above gel buffer at an initially slow flow rate of 2–4 ml/h (maintained by hydrostatic pressure) to maximize bacterial interaction with the gel. After collection of one column volume (1.2 ml) of eluate, the flow rate was increased to 15–20 ml/h. Five fractions of 1.2 ml each were collected and bacterial elution was determined by measuring the radioactivity present in duplicate aliquots of each fraction. Parallel viability determinations showed a close correlation between eluted radioactivity and viable units. In all experiments, >80% of the bacteria eluted in the gel buffer, were recovered in the first two fractions. Subsequent elution conditions are noted in the text.

Anti-BPI antiserum. Antibodies against purified rabbit or human BPI (3, 4) were generated by subcutaneous injections of 0.3 mg protein emulsified in a 1:1 mixture of distilled water/Freund's complete adjuvant into goats. Generation of antibody was determined by double immunodiffusion of antiserum in 1% agarose gels according to Ouchterlony (15). The same goat was bled prior to immunization to provide preimmune serum. IgG-rich fractions were obtained from sera by QAE-Sephadex chromatography (16). Total protein and IgG contents of immune and preimmune IgG fractions are closely similar as judged by agarose gel electrophoresis and immunodiffusion (using rabbit [anti-goat IgG] IgG).

Miscellaneous. Protein concentration was determined by the method of Lowry et al. (17). Superoxide production was measured as described by Cohen and Chovanec (18).

RESULTS

Effect of nitrogen flushing on bactericidal and respiratory burst activities of PMN. Incubation of intact PMN in room air with ingestible bacteria such as rough, gram-negative *S. typhimurium* 395 MR-10 or gram-positive *Staph. epidermidis*-160 provokes a respiratory burst by the phagocyte that includes a 5–10-fold stimulation of glucose oxidation via the hexose monophosphate shunt (Table I). During similar incubations with nitrogen-flushed PMN the respiratory burst, measured either by hexose monophosphate shunt activity or by O_2^- production, is almost completely eliminated.

Nitrogen flushing does not reduce the bactericidal activity of PMN towards *S. typhimurium* MR-10, regardless of whether residual respiratory burst activity was still detectable at up to 5% of control values (room air) or was undetectable. During 30 min incubation either under room air or nitrogen only 5% of the bacteria added remain viable outside the PMN (Table I). To determine whether any ingested bacteria survive intracellularly, aliquots of the cell suspensions were briefly sonicated to lyse the PMN, thereby permitting colony formation by viable intracellular as well as extracellular bacteria. No greater bacterial viability is measured in the sonicated samples (Table I). Hence, there is no intracellular survival of *S. typhimurium* MR-10 either in the presence or absence of oxygen. In contrast, killing of *Staph. epidermidis* by PMN is markedly impaired under nitrogen. Greater bacterial survival under nitrogen is evident only after sonication (Table I). Thus, ingestion of *Staph. epidermidis* is normal but intracellular killing is reduced when oxygen is absent. When both *S. typhimurium* MR-10 and *Staph. epidermidis* are added together to the PMN the same results are obtained. *S. typhimurium* MR-10 is killed equally well in room air and nitrogen. In contrast, without oxygen most of the intracellular *Staph. epidermidis* survived (Table I). These results may be juxtaposed to those with purified BPI, which is potently active toward *S. typhimurium*, but inactive toward

TABLE I
N₂ Flushing of Rabbit PMN: Effect on Bactericidal and Respiratory Burst Activity

Incubation conditions		Bacterial survival		[1- ¹⁴ C]Glucose→ ¹⁴ CO ₂	O ₂ [°] formed
Bacteria	N ₂	-Sonication	+Sonication	% Stimulation	nmol [°]
<i>S. typhimurium</i> MR10 (20/1)	—	4.9±1.9	3.4±1.6	1,410±240	3.9
	+	6.4±4.1	6.2±4.3	49±24	0.2
<i>Staph. epidermidis</i> (10/1)	—	17.8±4.3	23.6±3.3	480	
	+	18.8±5.0	70.8±8.2	10	
<i>S. typhimurium</i> MR10 (20/1)	—	3.7±2.0	3.6±1.8		
	+	0.3±0.2	0.4±0.1		
<i>Staph. epidermidis</i> (10/1)	—	13.2±1.1	29.2±9.5		
	+	19.3±6.8	85.7±6.7		

After preincubation of 10⁷ rabbit PMN in room air or under a nitrogen atmosphere (see Methods), *S. typhimurium* MR10 and/or *Staph. epidermidis* (16OK) were added. The numbers in parentheses indicate the bacteria/PMN ratio. Following incubation at 37° for 30 min, bacterial survival or hexose monophosphate shunt activity of PMN was measured as described in Methods. Bacterial survival is expressed as percentage of viability of bacteria incubated alone.

[°] Oxidation of [1-¹⁴C]glucose → ¹⁴CO₂ by PMN incubated with bacteria is expressed as the percent stimulation over the amount of glucose oxidized by PMN incubated alone. [°]Superoxide (O₂[•]) production was measured as superoxide dismutase-inhibitable cytochrome *c* reduction. The results shown represent the increase in O₂[•] formation by phagocytosing (vs. resting) PMN and are expressed as nanomoles O₂[•] produced by 10⁷ PMN per 30 min. These values represent the mean of two experiments. All other results shown represent the mean and, where indicated, standard error of the mean of three or more experiments.

Staph. epidermidis (3, 4, unpublished observation). At lower *Staphylococcus*/PMN ratios (1:1), ingested *Staph. epidermidis* are effectively killed in the absence of oxygen (not shown) suggesting the operation of a non-BPI oxygen-independent system at low bacterial doses.

O₂-independent killing of smooth *S. typhimurium* 395 MS. To determine the effectiveness of oxygen-independent bactericidal activities of PMN toward a more virulent strain of gram-negative bacteria, we examined the fate of smooth *S. typhimurium* 395 MS incubated with rabbit PMN under room air or nitrogen. Unlike the rough MR-10, smooth MS resist ingestion and killing by PMN unless first opsonized with immune serum (Table II) or partially purified IgG fractions (not shown). Normal serum, even at concentrations as high as 50%, is ineffective. The effect of immune serum is dose-dependent. When opsonized with 2% immune serum, ≤20% of the bacteria added remain viable during 30 min incubation with PMN either under room air or under nitrogen. Bacterial viability measured in unsonicated and sonicated cell suspensions is essentially the same indicating that all surviving bacteria are extracellular (Table II) and that optimal killing of both nonopsonized rough and opsonized smooth *S. typhimurium* by rabbit PMN does

not require O₂. This conclusion is supported by the results shown in Fig. 1. In these experiments, 10⁷ PMN were incubated, in room air or nitrogen, with increasing numbers of rough or (opsonized) smooth *S. typhimurium* 395 to determine the maximal bactericidal capacity of PMN toward these bacteria in the presence and absence of oxygen. It is apparent that, toward either strain, the bactericidal capacity of PMN is not reduced by the exclusion of oxygen-dependent antimicrobial systems. A maximum of 70–80 MR-10 or 30–40 MS are killed per PMN, with or without oxygen. Again, comparison of bacterial viability in unsonicated and sonicated cell suspensions reveals that essentially all surviving bacteria are extracellular (not shown). Thus, the number of bacteria killed is determined by the number of bacteria ingested. Toward both rough and smooth *S. typhimurium* 395, the oxygen-independent bactericidal capacity of rabbit PMN at least equals the phagocytic capacity of the PMN.

Surface hydrophobicity of gram-negative bacteria: Affinity for phenyl-Sepharose and effect of opsonization. The effectiveness of oxygen-independent activities toward ingested smooth *S. typhimurium* is particularly striking in view of the relatively greater potency of crude and purified PMN fractions against

TABLE II
*O₂-Independent Killing of Smooth S. typhimurium 395 MS by Rabbit PMN:
Immune Serum Requirement*

Immune serum	Bacterial survival (% control)			
	Room air		N ₂	
	- Sonication	+ Sonication	- Sonication	+ Sonication
%				
0	104±11.2	107		
0.5	74.5	66.4	49.7	38.8
1.0	34.5±8.3	34.5±7.2	29.3±5.8	29.5±6.6
2.0	16.9±4.6	19.6±5.4	11.9±4.2	14.1±5.3
50 (Normal)	76			

S. typhimurium 395 MS was preincubated with the indicated concentration of immune (or normal) serum as described in Methods. The bacteria were then washed and resuspended in physiological saline; 2×10^8 bacteria were added to 10^7 rabbit PMN in room air or nitrogen. After incubation at 37°C for 30 min, bacterial survival was measured as described in Methods and expressed as percentage of viability of bacteria incubated alone. Viability of serum pretreated bacteria ranged from 88 to 101% of control values. The values shown represent the mean and standard error of the mean of three or more experiments (or the mean of two separate determinations).

rough gram-negative bacteria than against smooth strains (5, 19, 20). Thus, differences in sensitivity of rough and smooth bacteria to the action of PMN relate to surface properties (such as exposure of negative charges and hydrophobic groups) that determine the relative ease of PMN (protein)-bacterium interaction. Following opsonization, the surface of smooth bacteria becomes more hydrophobic, hence more similar to that of rough bacteria (21, 22). This can be shown by hy-

drophobic-interaction chromatography of the ¹⁴C-labeled bacteria on phenyl-Sepharose (Table III). Rough bacteria, like *E. coli* J5, and *S. typhimurium* 395 MR-10, interact strongly with this hydrophobic matrix and few bacteria are recovered in the effluent. In contrast, the smooth phenotype of this *E. coli* strain (obtained

TABLE III
Chromatography of Rough and Smooth Bacteria on Phenyl-Sepharose: Effect of Anti-MS IgG Treatment of Smooth S. typhimurium 395 MS

Bacteria	Anti-MS IgG added	Percent recovery in gel buffer wash
	μg/ml	
<i>E. coli</i> J5 (+ galactose) (S)	0	99
<i>E. coli</i> J5 (- galactose) (R)	0	5
<i>S. typhimurium</i> MS 395 (S)	0	86
<i>S. typhimurium</i> MS 395 (S)	2	78
<i>S. typhimurium</i> MS 395 (S)	20	69
<i>S. typhimurium</i> MS 395 (S)	200	16

FIGURE 1 *O₂-independent bactericidal capacity of rabbit PMN toward S. typhimurium 395 MR 10 and MS. After pretreatment of 10^7 PMN in room air (—) or nitrogen(---), various numbers of either MR-10 (●) or opsonized MS (○) were added. After incubation at 37°C for 30 min, bacterial killing was measured as described in Methods. The loss of bacterial viability measured before and after sonication of cell suspensions was the same; for purposes of clarity only the values obtained after sonication are shown. The values shown represent the mean of the results of two or more experiments.*

Before chromatography, the bacteria (2×10^7), prelabeled during growth with [2-¹⁴C]uracil, were incubated for 20 min at 37°C in the standard incubation mixture containing anti-MS IgG where indicated. Phenyl-Sepharose chromatography was carried out as described in Methods. The recovery of [2-¹⁴C]uracil-labeled bacteria in the gel buffer wash is expressed as the percentage of total bacterial radioactivity applied to the columns. The values shown represent the mean of the results of two similar experiments.

by growth in galactose-supplemented medium) and nonopsonized *S. typhimurium* MS are quantitatively recovered in the buffer wash. Pretreatment of *S. typhimurium* MS with increasing concentrations of anti-MS IgG (or immune serum; not shown) results in progressively greater retention of the bacteria by phenyl-Sepharose. Nearly all bacteria are retained following opsonization with 200 µg/ml anti-MS IgG (or 2% immune serum). Both rough *E. coli* J5 and IgG-treated *S. typhimurium* MS remain bound to phenyl-Sepharose during elution with distilled water or 1 M sodium chloride ($\pm 50\%$ ethylene glycol); $\geq 60\%$ of the bound bacteria can be eluted with 5% Triton X-100 in Tris-HCl buffer (not shown).

Sensitivity of *S. typhimurium* MS to crude and purified bactericidal fractions of PMN: Effect of opsonization. Judging by its affinity to phenyl-Sepharose, opsonized *S. typhimurium* MS exhibits "rough-like" surface hydrophobicity that could facilitate its intracellular killing as well as its ingestion by PMN. However, as shown in Table IV, opsonization does not increase the sensitivity of *S. typhimurium* MS to killing by either whole PMN homogenates or crude acid extracts of PMN (Sup. II). A maximum of 25–40 *S. typhimurium* MS are killed per cell equivalent of homogenate or Sup. II, whether or not the bacteria are opsonized. The activity of the solubilized bactericidal protein in Sup. II is highly consistent. Whole homogenates, however, do not always exhibit full activity, presumably because interaction between bacteria and the bactericidal protein fraction in particulate form (6) varies. The bactericidal capacity of Sup. II, expressed per cell equivalent, towards *S. typhimurium*

MS and *S. typhimurium* MR-10 (not shown) is closely similar to that of the intact PMN suggesting that preexisting acid-extractable activities are sufficient to account for the oxygen-independent bactericidal activity of the intact cell.

Evidence of BPI-action in the killing of S. typhimurium by PMN

Increased outer membrane permeability of ingested *S. typhimurium* MR-10. A cardinal feature of the antibacterial action of BPI is the remarkably discrete nature of the lesions produced by BPI (3–6). In bacteria rendered nonviable within 1 min of incubation, the bacterial inner membrane remains essentially intact both structurally and functionally, allowing the biosynthesis of various classes of bacterial macromolecules to continue at near normal rates (for at least 30–60 min). In contrast, alterations of the bacterial outer membrane, including breakdown of the normal permeability barrier to small hydrophobic molecules like actinomycin D, are produced almost immediately after binding of BPI to the bacterial surface.

As previously shown for *E. coli* (23), after ingestion and intracellular killing of *S. typhimurium* MR-10 by intact rabbit PMN, either in room air or under nitrogen, bacterial protein synthesis in the absence of actinomycin D is also nearly normal, but is markedly inhibited in the presence of the drug (Table V). Thus, even when the bacteria are exposed to the full antibacterial equipment of the intact PMN, the protein biosynthetic machinery of ingested (and killed) *S. typhimurium* remains largely intact for at least 15–30 min, while the same rapid alteration in outer membrane permeability is produced that renders these organisms sensitive to the normally impermeant antibiotic. These findings indicate that the same discrete lesions in the bacterial envelope accompany killing by intact PMN (under N₂ as well as in room air) as by isolated BPI.

Bactericidal activity of crude PMN fractions: Specific inhibition by anti-BPI antibody. We have recently provided evidence suggesting that BPI is the principal oxygen-independent bactericidal agent of PMN toward several rough gram-negative bacteria (3, 4). The very similar bactericidal activities of Sup. II and highly purified BPI vs. *S. typhimurium* MS (Fig. 2), now further suggest that BPI also plays a prominent role in the killing of these smooth bacteria. The units on the double scale of the abscissa in Fig. 2 are chosen to indicate the same amounts of BPI, whether added in crude or purified form. (BPI represents $5 \pm 1.2\%$ ($n = 8$) of the total protein in Sup. II (4). The identical dose-dependent killing of *S. typhimurium* MS by Sup.

TABLE IV

Killing of S. typhimurium 395 MS by Whole Homogenates and Crude Acid Extracts of Rabbit PMN: Effect of Opsonization

PMN Fraction	Number of bacteria added	Number of bacteria killed	
		Nonopsonized	Opsonized
Whole homogenate	2.0×10^7	2.0×10^7	1.9×10^7
	10×10^7	8.1×10^7	8.3×10^7
	75×10^7	33×10^7	27×10^7
Sup II	2.0×10^7	1.9×10^7	1.8×10^7
	10×10^7	7.1×10^7	7.0×10^7
	75×10^7	29×10^7	25×10^7

Whole homogenates or crude acid extracts (Sup II) of PMN, representing 10^7 PMN equivalents, were incubated with increasing numbers of nonopsonized or opsonized *S. typhimurium* 395 MS for 30 min at 37°C in the standard incubation mixture. Bacterial killing was measured as described in Methods. The results shown represent the mean of two experiments with whole homogenate and three closely similar experiments with Sup II.

TABLE V
Effect of Phagocytosis by Rabbit PMN on Viability and Susceptibility
to Actinomycin D. of *S. typhimurium* MR-10
under Aerobic and Anaerobic Conditions

	¹⁴ C-AA incorp. → TCA ppt		Bacterial viability	
	-ActD	+ActD	- Sonication	+ Sonication
Room air				
-PMN	100 (3)	86.1±17.0	100 (2)	110
+PMN	122±20 (3)	17.0±5.5	23±5 (3)	20±8
N ₂				
-PMN	100 (4)	96.0±30.0	100 (4)	87±4
+PMN	72.6±8.0 (4)	23.0±12.0	22 (2)	25

S. typhimurium MR-10 (3.75×10^8 bacteria/ml) were incubated under room air or a nitrogen atmosphere in a 1:1 mixture of isotonic saline/buffered HBSS containing rabbit PMN (3.75×10^7 /ml) and/or actinomycin D (ActD; 100 µg/ml) where indicated. After 10 min at 37°, an aliquot was taken to measure extra- and intracellular bacterial viability of samples not containing ActD. At this time an equal volume of saline/buffered HBSS containing ¹⁴C-amino acids (0.1 µCi/ml; 12.5 µCi/µmol) and cycloheximide (0.5 mM, which fully inhibits protein biosynthesis by PMN) was added. After an additional 15 min incubation at 37°, 3 ml of ice-cold 10% trichloroacetic acid were added. Collection and counting of acid-precipitable radioactivity were carried out as previously described (23). The values shown represent the mean and standard error of the mean of the indicated number (in parentheses) of determinations. When only two values were obtained their mean is shown. All results are expressed as percentage of values obtained for bacteria incubated alone.

The difference in protein biosynthetic activity of ingested bacteria in room air vs. nitrogen (as percentage of control) is not statistically significant ($P > 0.05$).

FIGURE 2 Sensitivity of *S. typhimurium* 395 MS to crude and purified bactericidal rabbit PMN fractions, the effect of anti-MS IgG pretreatment. After preincubation with (solid symbols) or without (open symbols) anti-MS IgG, as described in Methods, *S. typhimurium* 395 MS (2×10^7) were incubated for 30 min at 37°C with increasing amounts of Sup II (squares) or purified BPI (circles) in the standard incubation mixture. At the end of incubation bacterial viability was determined as described in Methods. The values shown represent the mean of the results of three or more similar experiments.

II and purified BPI indicates that all acid-extractable bactericidal activity of PMN toward *S. typhimurium* MS can be attributed to BPI. Opsonization of the bacteria with anti-MS IgG (or immune serum) does not alter the sensitivity of *S. typhimurium* MS to Sup. II or purified BPI as judged by either the dose curve (Fig. 2) or the time course (not shown) of bacterial killing.

Further evidence of the prominent role of BPI among the O₂-independent bactericidal systems of the PMN in the killing of *S. typhimurium* is provided by the blocking of the bactericidal effects of whole homogenate and Sup II by immune IgG-rich fractions (Table VI). Similar concentrations of preimmune IgG fractions (from the same goat) have little or no inhibitory effect.

Killing of S. typhimurium by leukocytes of a patient with chronic granulomatous disease (CGD). Table VII shows the results of two separate experiments carried out with leukocytes from an asymptomatic patient with CGD and from a normal individual, incubated with opsonized *S. typhimurium* 395 MS at ~1.4 or 4 bacteria/PMN. The CGD leukocytes ingested and killed the bacteria nearly as well

TABLE VI
Inhibition of Anti-BPI Serum of Bactericidal Activity of Whole Homogenates and Crude Extracts of Rabbit PMN toward *S. typhimurium* MR-10

	Viability (percent bacteria alone)					
	No IgG		+ Immune IgG		+ Preimmune IgG	
			0.25	0.8	2	0.25 0.8 2 mg*
Bacteria alone (1.5×10^7)	100				100	100
+ Whole homogenate; $10^6 \dagger$	<1	9	108	108	<1	<1 18
2×10^6	<1	<1	46	106	<1	<1 3
+ Neutralized acid extract; 10^6	<1	6	72	94	<1	<1 27
2×10^6	<1	<1	25	96	<1	<1 6

Incubation mixtures (see methods) contained 1.5×10^7 *S. typhimurium* MR-10 and either 1 or 2×10^6 cell equivalents ($\dagger$) of whole homogenate or neutralized crude acid extracts of rabbit PMN. The indicated amounts (milligrams protein*) of partially purified immune or preimmune serum were added just before the PMN-fractions. Incubations at 37°C were carried out for 30 min, after which samples were taken for determination of bacterial viability.

as did the leukocytes of a normal individual. Whether the somewhat greater intracellular bacterial survival in the CGD cells reflects the defect in respiratory burst (24), or merely biological variation (resuspension of the CGD cells was more difficult because of clumping

than of the normal leukocytes) cannot be determined from this small number of observations. It is evident, however, that the absence of the respiratory burst does not markedly impair the bactericidal efficiency of the PMN of this patient with CGD (24).

TABLE VII
Comparison of Ingestion and Killing of Opsonized *S. typhimurium* 395 MS by PMN of a Patient with CGD and of a Normal Individual

Bacteria added:	Expt.	Normal		CGD	
		Bacteria killed	Intracellular survival	Bacteria killed	Intracellular survival
1×10^7	1	8.5×10^6	$<5 \times 10^4$	7.3×10^6	$<5 \times 10^4$
	2	8.5×10^6	$<5 \times 10^4$	6.9×10^6	1.1×10^6
3×10^7	1	2.4×10^7	$<5 \times 10^4$	1.9×10^7	$<5 \times 10^4$
	2	2.3×10^7	$<5 \times 10^4$	1.6×10^7	4.8×10^6

Incubation mixtures (see Methods) contained 1×10^7 leukocytes (65–70% PMN) and the indicated number of opsonized *S. typhimurium* 395 MS. After incubation at 37°C for 60 min, the tubes were placed in ice and samples were taken for serial dilution and plating. The remaining mixtures were sonicated (see Methods) and samples were taken again for plating, for determination of intraleukocyte bacterial survival.

The results are shown of two separate experiments, each in duplicate, carried out with leukocytes from the same donation of each individual. Processing of the heparinized blood from the patient with CGD and from the normal individual was performed in parallel. The PMN-rich fractions were obtained by dextran (6% Macrodex [Pharmacia Fine Chemicals, Uppsala, Sweden]; final concentration 1%) sedimentation and washed twice in HBSS before resuspension in HBSS at a concentration of 1×10^7 leukocytes/200 μ l.

The difference in intracellular bacterial survival in normal vs. CGD cells is not statistically significant ($P > 0.20$).

The bactericidal activity of crude acid extracts of the PMN of these two individuals toward *E. coli* J5 and *S. typhimurium* MR-10 (not shown) was the same. Anti-BPI IgG-rich fractions (but not preimmune IgG-rich fractions) nearly completely inhibited this bactericidal activity of both extracts (Table VIII). Thus, BPI seems primarily responsible for the killing of gram-negative bacteria such as *E. coli* and *S. typhimurium* by the PMN of this CGD patient.

DISCUSSION

Of the multiple microbicidal activities present in PMN, those that are oxygen-dependent have received the greatest attention. This interest has been stimulated by the discovery of a clinical disorder of PMN antimicrobial function (chronic granulomatous disease) that seems linked to the inability of the PMN to generate a respiratory burst and, consequently, oxygen-dependent microbicidal activities (1). In vitro these cells exhibit a profound defect in microbicidal activity toward several microorganisms, several of which produce frequent infections in these patients. The importance of O₂-requiring bactericidal systems of the PMN in host defense against infection is therefore firmly established. However, it has become evident in recent years how variable the clinical course is of patients with CGD. Several patients with well-documented CGD, i.e., whose leukocytes cannot mount a respiratory burst, have been reported to live reasonably normal lives with relatively few bacterial infections (24, 25). The varying consequences of a presumably uniform absence of the respiratory burst suggest that abnormalities in addition to those connected to

oxidative metabolism may contribute to impaired bactericidal activity of CGD cells. For example, O₂-independent bactericidal mechanisms may be more or less capable, in different individuals with CGD, of making up for the defect in O₂-dependent killing.

Significant O₂-independent microbicidal activity is evident in PMN toward many microbial species. A number of such activities have been demonstrated in crude cell fractions and extracts of PMN in which the oxygen-dependent systems are not functional (2, 26–28). Furthermore, under anaerobic conditions intact PMN are capable of substantial oxygen-independent killing of several microbial species (29).

In this study we have provided further evidence of the effectiveness of oxygen-independent bactericidal systems in the killing of rough and smooth *S. typhimurium* by intact PMN in two ways. First, we have shown that intracellular killing of these gram-negative bacteria by rabbit PMN under aerobic or anaerobic conditions is virtually identical, even when ingestion is maximal. The possibility that traces of residual respiratory burst activity after nitrogen flushing are actually sufficient to kill ingested *Salmonella* is unlikely, not only because killing was complete even in experiments in which no stimulation of hexose monophosphate shunt activity was detectable, but particularly because the *Salmonella* strains used in these experiments produce both superoxide dismutase and catalase (30), which should protect against small increments in oxygen metabolism. Second, leukocytes from a patient with CGD killed a smooth strain of *S. typhimurium* almost as effectively as did leukocytes from a normal individual.

The principal agent in this oxygen-independent kill-

TABLE VIII
Bactericidal Activity of Crude Acid Extracts of CGD and Normal Leukocytes toward *E. coli* J5; Its Inhibition by an Anti-BPI IgG Fraction

	Viability (% bacteria alone)						
	No IgG	+ Immune IgG			+ Preimmune IgG		
		42	84	210	42	84	210 μ g*
Bacteria alone	100			99			113
+ Crude extract of:							
CGD leukocytes	<1	33	95	140	<1	2	4
Normal leukocytes	<1	36	34	120	2	7	3

Incubation mixtures contained 1×10^5 *E. coli* J5 in the absence and presence of crude acid extracts of CGD or normal leukocytes (50 μ g of protein). These human leukocyte extracts were prepared as previously described (3). The indicated amounts (micrograms protein*) of partially purified immune or preimmune serum were added just before the PMN fractions. Incubations were carried out for 30 min at 37°C after which samples were taken for determination of bacterial viability (colony forming units).

ing of gram-negative bacteria appears to be the BPI that has been recently isolated in our laboratory. BPI (rabbit or human) is at least 10–20 times more potent, toward these bacteria, than any non-BPI protein fraction obtained during purification (3, 4). Nearly all the bactericidal activity of crude PMN fractions toward several rough and smooth gram-negative bacteria is recovered in BPI-containing fractions. In fact, the rate and extent of killing of these bacteria by intact PMN, disrupted PMN, crude extracts or highly purified fractions, all containing comparable amounts of BPI, are remarkably similar (3, 4, 6, 23). Moreover, the bactericidal activity of crude fractions toward these gram-negative bacteria is virtually eliminated by anti-BPI-antibody (but not by preimmune IgG fractions).

BPI is tightly granule membrane-associated (3, 6, unpublished observations) and, therefore, intracellular sequestration of the bacteria is presumably a requisite for its action in the intact cell. Indeed, our findings are consistent with the view that ingestion is the limiting step in the killing of gram-negative bacteria by PMN. All ingested *S. typhimurium* (MR-10 or opsonized MS) are readily killed and bacterial survival is evident only when the phagocytic capacity of PMN has been exceeded. Nonopsonized and opsonized *S. typhimurium* MS are equally sensitive to BPI but non-opsonized bacteria resist ingestion and, hence, escape the bactericidal activity of intact PMN. Opsonization apparently promoted the intracellular killing of these smooth bacteria only by facilitating their delivery to this intracellular bactericidal system.

The sensitivity of gram-negative bacteria to BPI is largely determined by properties of the bacterial surface that mediate BPI-bacterium interaction (5). Rough strains, bearing surfaces with more anionic and hydrophobic character than the corresponding smooth strains, bind BPI more avidly and are more sensitive to the protein's antibacterial actions. The coating of smooth gram-negative bacteria with specific antibodies produces an apparent increase in the hydrophobicity of this bacterial surface (13, 21, 22; Table III). Since the activity of BPI against *S. typhimurium* MS is not enhanced by opsonization of the bacteria, an increase in surface hydrophobicity apparently is not sufficient to increase bacterial sensitivity to BPI. The greater affinity and sensitivity of rough strains for BPI, therefore, is most likely attributable to the greater accessibility of anionic groups on the surface of bacteria with short lipopolysaccharide-saccharide chains (31, 32). This is consistent with a model previously presented (5), suggesting that electrostatic attraction between BPI and the bacterial surface is the primary determinant of (gram-negative) bacterial sensitivity to this cationic bactericidal protein.

Considering the greater resistance of smooth strains to BPI, it may seem surprising that, once ingested, rough and smooth *S. typhimurium* appear equally susceptible to this oxygen-independent system. Smooth strains are effectively killed, however, at higher (3–10-fold) concentrations of BPI (3–5). Estimates of the amount of BPI in PMN granules (~5% of granule protein (3, 4) and of its specific activity indeed suggest that enough BPI is present to provide concentrations that are lethal to smooth gram-negative bacteria ingested by PMN. Further, preliminary findings suggest that the activity of purified and crude BPI toward smooth bacteria may be increased by carrying out the incubations in smaller volumes (i.e., higher protein and bacterial concentrations). The micro-environment of the phagocytic vacuole may provide such favorable conditions for the action of BPI, its activity matching any rate of ingestion, even of relatively resistant smooth strains.

Because oxygen-independent killing is so effective that no intracellular *S. typhimurium* survive in the absence of oxygen, any contribution to killing under aerobic conditions by oxygen-requiring systems cannot be determined. Although we can therefore not rule out the possibility that under physiological conditions oxygen-dependent systems actually do play a role in killing of these gram-negative bacteria by the PMN, several considerations favor a critical role for BPI. Toward several gram-negative bacterial species, BPI exhibits greater activity (on a molar basis) than powerful antibiotics such as polymixin B (33). This potent activity is remarkably specific in its target; only gram-negative bacteria seem susceptible (3, 4). Of interest, those gram-negative bacteria that are resistant to BPI (*Serratia marcescens*, *Proteus* species) (6, 23) may produce chronic infection in patients with chronic granulomatous disease, whereas bacteria sensitive to BPI (including *Pseudomonas aeruginosa*) usually do not (1, 34, 35). The antimicrobial spectrum and potency of rabbit and human BPI are closely similar and the two proteins display partial immunological cross-reactivity (3–5, unpublished observations). Such conservation during evolution of a protein with very specific and potent antimicrobial activity seems most consistent with an essential function. We therefore favor the idea that toward many gram-negative bacteria BPI is the principal bactericidal agent of PMN, whereas for many other microbial species the oxygen-dependent systems are most important. Such a specialization of bactericidal function in the PMN is, in fact, suggested by our experiments with *S. typhimurium* and *Staph. epidermidis* in which, under anaerobic conditions, one microbial species is killed and the other survives within the same cell, perhaps the same phagocytic vacuole.

More complete determination of the antibacterial spectrum of BPI and more direct evidence that its presence is essential for the antimicrobial function of PMN toward these bacteria await further study.

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