Human Bactericidal/Permeability-increasing Protein and a Recombinant NH_2-Terminal Fragment Cause Killing of Serum-resistant Gram-negative Bacteria in Whole Blood and Inhibit Tumor Necrosis Factor Release Induced by the Bacteria

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Abstract

The bactericidal/permeability-increasing protein (BPI) of neutrophils and BPI fragments neutralize the effects of isolated Gram-negative bacterial lipopolysaccharides both in vitro and in vivo. Since endotoxin most commonly enters the host as constituents of invading Gram-negative bacteria, we raised the question: Can BPI and its bioactive fragments also protect against whole bacteria? To determine whether the bactericidal and endotoxin-neutralizing activities of BPI/fragments are expressed when Gram-negative bacteria are introduced to the complex environment of whole blood we examined the effects of added BPI and proteolytically prepared and recombinant NH_2-terminal fragments on: (*a*) the fate of serum-resistant encapsulated *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* that survive the antibacterial actions of whole blood and (*b*) the ability of these bacteria to trigger cytokine release. Added BPI in nanomolar concentrations killed each of three encapsulated strains of *E. coli* and in closely parallel fashion inhibited tumor necrosis factor (TNF) release. Holo-BPI and its NH_2-terminal fragment were equipotent toward a rough LPS chemotype K1-encapsulated strain, but the fragment was substantially more potent than holo-BPI toward two encapsulated smooth LPS chemotype strains. TNF release induced by *K. pneumoniae* and *P. aeruginosa* was also inhibited by both holo-BPI and fragment but, at the protein concentrations tested, *P. aeruginosa* was killed only by the fragment and *K. pneumoniae* was not killed by either protein. The bactericidal action of BPI/fragment toward *E. coli* is inhibited by C7-depleted serum, but accelerated by normal serum, indicating that BPI, acting in synergy with late complement components, enhances extracellular killing of serum-resistant bacteria. Thus, BPI and an even more potent NH_2-terminal fragment may protect against Gram-negative bacteria in the host by blocking bacterial proliferation as well as endotoxin-mediated effects, not only as components of the intracellular antibacterial arsenal of the neutrophil, but also as potentially therapeutic extracellular agents. (*J. Clin. Invest.* 1992. 90:1122–1130.)
Key words: complement • synergism • Gram-positive bacteria • lipopolysaccharides • neutrophils

Introduction

The continued high mortality and morbidity attributable to Gram-negative bacteremia and endotoxemia have prompted an intense search for therapeutic agents capable of counteracting the potentially devastating effects of circulating bacterial LPS. Recently it has been shown that administration to septic patients of monoclonal antibodies directed against lipid A, the toxic portion of the LPS molecule, may reduce mortality (1, 2). Since the systemic toxicity of endotoxin is thought to be mediated at least in part by excessive production of cytokines, such as tumor necrosis factor (TNF)α,[1] and various interleukins (1–9), protection by antibodies directed at these host mediators or by antagonists of their receptors is now also being investigated.

The bactericidal/permeability-increasing protein (BPI), a product of PMN of man and animals that is stored in the azurophilic granules, is cytotoxic for Gram-negative bacteria only (10–12). This remarkable target-cell specificity of BPI is attributable to its strong attraction to the LPS in the outer membrane of the Gram-negative bacterial envelope (13–15). This feature of BPI has led to the recognition that BPI is a member of a family of LPS-binding proteins (16) and is capable, when added extracellularly, of inhibiting effects of purified LPS in several in vitro settings (17–19) as well as in animal experiments (20). It has been suggested that under these conditions the LPS-neutralizing activity of BPI, directed at cell-free LPS, represents its main biological role, outweighing its importance as an antibacterial agent (8, 19). However, the most common presentation of LPS to the host is as an envelope component of invading bacteria. Therefore, the ability of added BPI and its bioactive fragments to attach to LPS in the bacterial envelope and to cause bacterial damage may be an important determinant of its protective action in vivo.

We now show that the addition of nanomolar concentrations of the 55-kD holo-BPI or of the ~ 23–25-kD NH_2-termi-

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1. *Abbreviations used in this paper:* BPI, bactericidal/permeability-increasing protein; CFU, colony-forming units; LBP, LPS-binding protein; TNF, tumor necrosis factor.

nal bioactive portion of the molecule to whole blood inoculated with live encapsulated Gram-negative bacteria can result in both killing of bacteria that survive the cellular and extracellular antibacterial systems present in whole blood and inhibition of the release of TNF prompted by the bacteria.

Methods

Bacteria. Bacteria used in this study included *Escherichia coli* J5, a rough UDP-galactose-4-epimeraseless mutant of the smooth strain 0111:B4 (13), a K1-encapsulated rough LPS chemotype strain of *E. coli* ("K1/r") kindly provided by Dr. Alan Cross (Department of Bacterial Diseases, Walter Reed Army Medical Center, Washington, DC) that was part of a collection of bacteremic isolates previously characterized by phage typing for K1 capsule (21), *E. coli* O7:K1 and *E. coli* O10:K5, two encapsulated smooth LPS chemotype strains (ATCC 23503 and 23506, respectively, American Type Culture Collection, Rockville, MD), one strain each of *Pseudomonas aeruginosa* (ATCC 19960) and *Klebsiella pneumoniae* (ATCC 29011), and *Staphylococcus aureus* (Cowan strain) that was obtained from the New York University Medical School Department of Microbiology bacteria collection. Bacteria were grown either in triethanolamine-buffered minimal salts medium (only *E. coli* J5; 22) or trypticase soy broth (all bacteria). Stationary phase overnight cultures were transferred to fresh medium (diluted 1:100) and grown to late logarithmic phase (~ 4 h) at 37°C. Bacterial concentrations were determined by measuring the OD_{550} in a spectrophotometer (Junior model; Coleman Instruments, Inc., Maywood, IL). Subcultures were harvested by sedimentation of bacteria at 3,000 *g* for 12 min. Bacteria were resuspended in sterile physiological saline to the desired concentration.

LPS. Purified LPS from *E. coli* J5 (Rc chemotype) and from *Salmonella minnesota* Re595 (Re chemotype) were obtained from List Biological Laboratories, Inc. (Campbell, CA). Solutions of LPS were prepared by sonication and serially diluted in HBSS (minus Ca^{2+} and Mg^{2+} [$HBSS^-$]; Gibco Laboratories, Grand Island, NY) as previously described (23).

BPI and BPI NH_2-terminal fragments. Human BPI (nBPI-55) was purified from crude extracts of PMN-rich populations using *E. coli* as an affinity matrix, as previously described (15). An ~ 25-kD NH_2-terminal fragment of human BPI (nBPI-25) was isolated after limited proteolysis of purified human BPI as described before (24). A recombinant NH_2-terminal fragment of BPI (rBPI-23) was expressed in Chinese hamster ovary cells after transfection with an expression vector containing human BPI cDNA (25) modified to contain a stop codon after amino acid 199. Details of the design of the expression vector containing BPI cDNA and the procedure for purification of the recombinant protein will be presented in a separate manuscript (Gazzano-Santoro, H., J. B. Parent, L. Grinna, A. Horwitz, T. Parsons, G. Theotan, P. Elsbach, J. Weiss, and P. J. Canlan, manuscript in preparation). The identity and purity of the recovered protein were confirmed by NH_2-terminal amino acid sequencing and SDS-PAGE. Protein mass was estimated by a protein assay kit (Bio-Rad Laboratories, Richmond, CA) and by absorbance at 280 nm, with BSA as the standard. Protein estimates were confirmed by Coomassie blue staining after SDS-PAGE. All protein samples were dialyzed against and stored in 20 mM sodium acetate/acetic acid buffer, pH 4.0.

Blood. Blood was collected from healthy human volunteers after informed consent into tubes containing citrate or heparin as an anticoagulant (Becton Dickinson, Mountain View, CA). Unless indicated otherwise, blood samples contained 275 μl of citrated blood, 5 μl of BPI (fragment) in acetate buffer (or buffer alone) and 20 μl of bacteria or LPS diluted in sterile physiological saline or $HBSS^-$ (or diluent alone), respectively. Bacteria or LPS was added last. Samples were incubated at 37°C for up to 5 h before measurement of bacterial viability and TNF in the extracellular medium.

Sera. Venous blood was collected from healthy volunteers. The serum was collected after clot formation, centrifuged at 10,000 *g* for 20 min to remove any debris, and stored at −70°C. C7-depleted human serum and purified human C7 were purchased from Quidel (San Diego, CA). The quality of these products was pretested by confirming the loss in C7-depleted serum of bactericidal activity toward a serum-sensitive strain of *E. coli* (J5) and the restoration of bactericidal activity to the level of normal serum by supplementing the depleted serum with purified C7 (final concentration of 60 μg/ml).

Measurement of bacterial viability. After the indicated incubations at 37°C, aliquots were taken and serially diluted in sterile physiological saline. A 25-μl aliquot of the diluted sample was transferred to 5 ml of 1.3% (wt/vol) molten (48°C) Bactoagar (Difco Laboratories Inc., Detroit, MI) containing either 0.8% (wt/vol) nutrient broth and 0.5% (wt/vol) NaCl (for plating of *E. coli*) or 3% (wt/vol) trypticase soy broth (for plating of *S. aureus*) and poured into a petri dish. Where indicated, the molten agar was supplemented with 1 mg/ml BSA (United States Biochemical Corp., Cleveland, OH). The agar was allowed to solidify at room temperature, and bacterial viability was measured as the number of colonies formed after incubation at 37°C for 18–24 h. Brief sonication of blood samples before serial dilutions, as previously described (26), did not increase the number of bacterial colonies formed.

Measurement of extracellular accumulation of TNF in whole blood. After incubation of blood samples for 5 h at 37°C, the samples were diluted four times with RPMI and spun at 500 *g* for 5 min to collect the extracellular medium. TNF in the recovered medium was measured by ELISA using the Biokine TNF test kit (T Cell Sciences, Inc., Cambridge, MA).

Results

Selection of a suitable experimental model. To provide an experimental setting in which both the bactericidal and the antiendotoxin activities of BPI and its NH_2-terminal fragment could be tested in whole blood, we initially examined the ability of added live *E. coli* to survive in blood and trigger extracellular accumulation of TNF. Among the test organisms selected first was a K1-encapsulated *E. coli* strain, containing short chain (rough chemotype) LPS ("K1/r"). This highly BPI-sensitive organism (21) because of the presence of (K1) capsule is likely to be resistant to the cellular and extracellular antibacterial systems in blood (21, 27, 28) and representative of strains that are prominent in clinical infections (29–31). We have shown before that (K1) capsule does not increase bacterial resistance to BPI (21). At low doses of bacteria added to whole blood, few bacteria survived during 5 h incubations (Fig. 1 *A*). However, with larger inocula, the surviving fraction increased and outgrowth of surviving bacteria was observed. In contrast, a rough strain of *E. coli* (J5; Fig. 1 *A*), as well as two clinical blood isolates of *E. coli* (data not shown), were rapidly and virtually completely killed in blood at all bacterial doses tested (up to 10^7/ml). Despite their differences in survival, both *E. coli* J5 and the K1-encapsulated strain were potent inducers of TNF release in blood (Fig. 1 *B*). The TNF-inducing activity of the bacteria was dose-dependent and was even more pronounced than that of purified LPS added in amounts corresponding to the LPS content of the whole bacteria (Fig. 1 *B*).

Added BPI inhibits survival of E. coli and accumulation of extracellular TNF in whole blood. Based on the preceding experiments, we chose the K1-encapsulated strain (*E. coli* K1/r) to test the antibacterial activities of BPI/fragments added to whole blood. Fig. 2 shows that holo-BPI (nBPI-55) potently inhibited both bacterial survival (Fig. 2, *A–C*) and release of TNF (Fig. 2 *D*) in whole blood. Both effects of BPI were dose dependent and apparent over a broad range of bacterial concentrations and were observed in blood anticoagulated either with citrate or with heparin (10 U/ml). At the higher dose (2.2

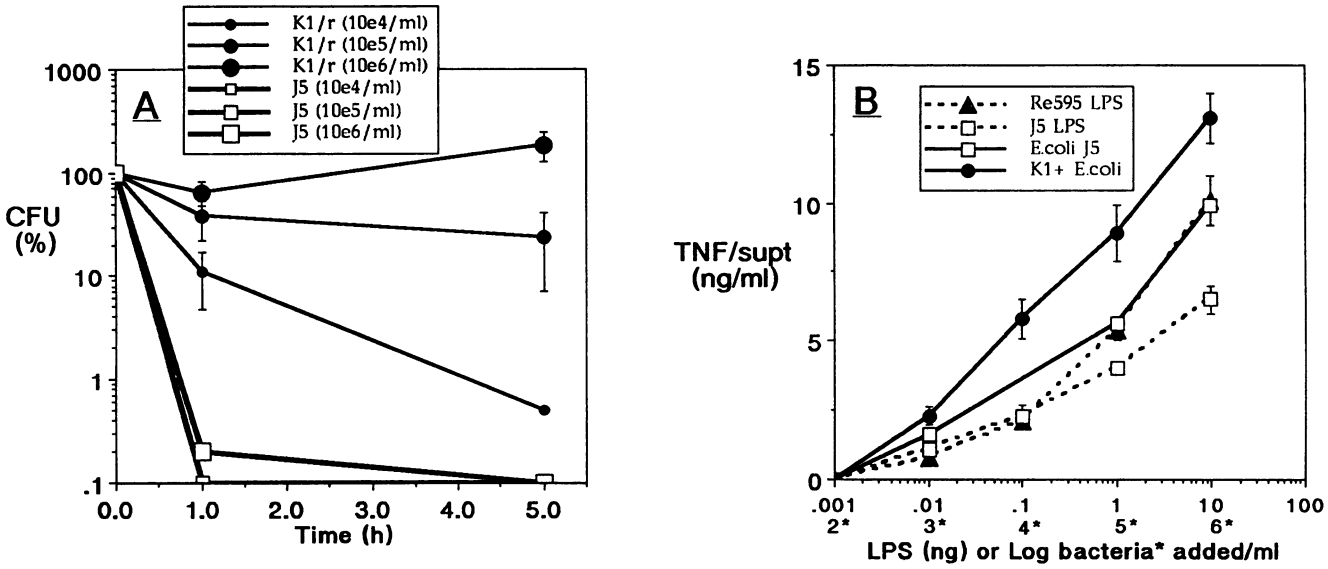

Figure 1. Incubation of *E. coli* in whole blood. Effect on survival (*A*) and accumulation of TNF (*B*). Increasing amounts of *E. coli* or purified LPS were incubated at 37°C in blood and, at the indicated times, bacterial viability (*A*) and extracellular TNF (*B*) were measured as described in Methods. The scale on the abscissa is arranged so that the amount added either as isolated LPS or as LPS associated with intact *E. coli* added/ml is the same. *E. coli* are estimated to contain ~ 1 ng of LPS/10^5 bacteria (50). The number of viable *E. coli* recovered after incubation in blood is expressed as the percentage of the number of viable *E. coli* initially added to the blood. The results shown represent the mean±SEM of at least three independent experiments.

μg/ml; ~ 40 nM) tested, BPI reduced bacterial survival by > 100-fold and bacterial TNF-inducing activity by ~ 100-fold (i.e., caused a 100-fold increase in the bacterial dose necessary for induction of TNF).

Effects of NH_2-terminal fragment(s) of BPI. An ~ 25-kD NH_2-terminal fragment of BPI, generated during limited proteolysis, exhibits all the known biological activities of holo-BPI (18, 24). Fig. 3 shows that this fragment and a corresponding recombinant product are as potent as holo-BPI in reducing survival of *E. coli* K1/r (Fig. 3 *A*) and bacterial induction of TNF accumulation (Fig. 3, *B–E*) in whole blood. The activities exhibited by the recombinant BPI fragment confirm our prediction that all of the molecular determinants for the antibacterial activities of BPI toward Gram-negative bacteria reside within the NH_2-terminal region encompassed by residues 1–199 (18).

Late components of complement act in synergy with BPI to accelerate killing of serum-resistant E. coli. The antibacterial action of BPI occurs in two stages: (*a*) almost immediate sublethal alterations, including growth inhibition, that are reversible; and (*b*) later, irreversible effects, that include the lethal lesion(s) (32). Mannion et al. (32) have shown that serum albumin (≥ 0.5 mg/ml) specifically inhibits the progression of BPI action from sublethal to lethal injury and that during prolonged incubation (≥ 2 h) in albumin-supplemented media BPI-treated bacteria that have suffered only sublethal damage regain their normal ability to grow (e.g., ability to form colonies in nutrient agar). In whole blood the albumin effect is not apparent. Fig. 2, *A–C,* shows that the growth-inhibitory effect of added BPI on *E. coli* K1/r is sustained during long incubations (up to 5 h) in whole blood (and not reversed when samples from blood are plated on nutrient agar supplemented with albumin) therefore indicating that in whole blood added BPI causes irreversible inhibition of bacterial growth (i.e., bacterial killing) despite the presence of high concentrations of albumin. To determine whether other elements in the extracellular environment of blood can override the inhibitory effect of albumin on BPI action, we compared the antibacterial action of BPI on *E. coli* K1/r in buffered mixtures of nutrient broth and physiological saline either without or with added purified albumin (12.5 mg/ml) or with whole serum containing the same concentration of albumin. In each medium, BPI promptly arrested colony formation in nutrient agar by > 98% of the bacteria (Fig. 4 *B, broken lines*). However, as shown before (32), after 15 min incubation, most of the bacteria formed colonies when plated in nutrient agar supplemented with albumin (Fig. 4 *B, solid lines*) indicating that damage to these bacteria at this time was still sublethal and hence reversible during incubation in albumin-supplemented medium. After longer incubation in nutrient broth without added albumin, progressively fewer BPI-treated bacteria could form colonies in albumin-supplemented nutrient agar (Fig. 4 *B, open squares, solid line*) demonstrating the slow progression of BPI action to the irreversible lethal stage (32). This progression was blocked when albumin was included in the incubation medium (Fig. 4 *B, open circles, solid line*). In contrast, a nonlethal dose of serum (Fig. 4 *A*) overcame the albumin block and actually markedly accelerated the rate of killing of BPI-treated bacteria (Fig. 4 *B, closed circles, solid line*). Serum depleted of C7 acted like purified albumin and inhibited killing of BPI-treated bacteria (Fig. 4 *B, open triangles, solid line*). Purified C7 alone had no effect on BPI action but together with C7-depleted serum reproduced the effect of normal serum (Fig. 4 *B, closed triangles, solid line*) showing that the enhancement of bacterial killing by normal serum plus BPI is dependent on late components of the complement system acting synergistically with BPI.

Effects of BPI/fragment on survival and TNF-inducing activity of other bacteria in whole blood. Because the presence of long polysaccharide chains (O-antigen) in LPS reduces the affinity of BPI for the envelope of Gram-negative bacteria (13,

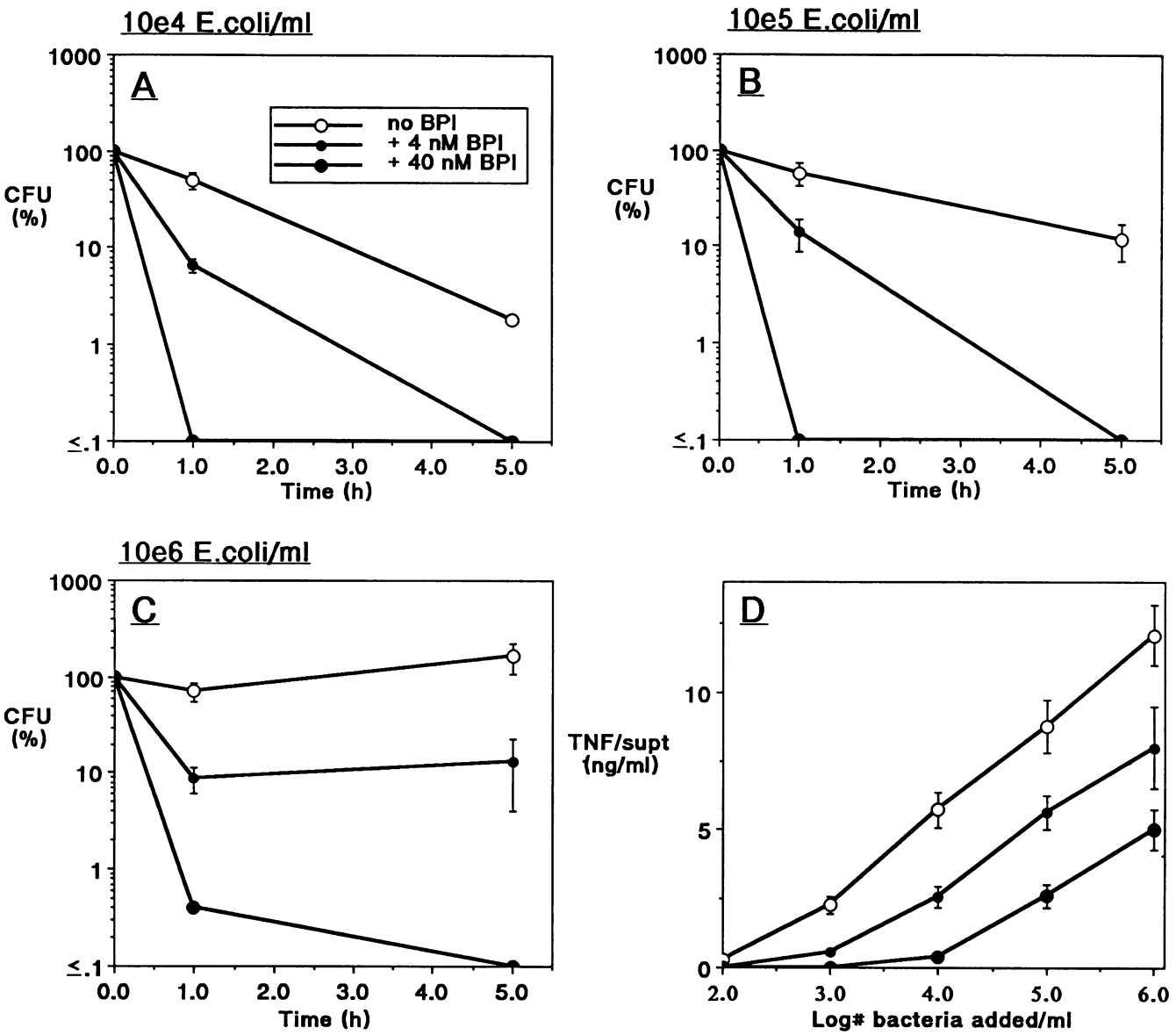

Figure 2. BPI potently inhibits both survival of encapsulated *E. coli* and bacterial induction of TNF in whole blood. Increasing concentrations of encapsulated *E. coli* were incubated in citrated blood without further addition or with 4 or 40 nM holo-BPI. Bacterial viability (*A–C*) and accumulation of extracellular TNF (*D*) were measured as described in Methods. The results shown represent the mean±SEM of at least four independent experiments.

24), we also examined the antibacterial actions of BPI/fragment against two encapsulated strains of *E. coli* (O7:K1 and O10:K5) containing long chain (smooth chemotype) LPS. At inocula of 10^5 or 10^6 bacteria/ml of either strain, bacterial viability was not reduced after 1 h in blood alone (Fig. 5). Addition of either holo-BPI or its NH_2-terminal fragment produced a dose-dependent inhibition of bacterial viability and, in closely parallel fashion, inhibition of TNF release. However, in contrast to the equal potency of holo-BPI and the fragment toward *E. coli* K1/r, the fragment (at fivefold higher doses than required for killing of *E. coli* K1/r) was from 5–10-fold more potent than the holo-protein toward both *E. coli* O7:K1 and *E. coli* O10:K5. The fragment was also substantially more potent than holo-BPI toward one strain of *P. aeruginosa.* However, in contrast to the parallel bactericidal and TNF-inhibitory effects of both proteins on *E. coli,* these effects were not similar in the case of *P. aeruginosa* and a mucoid strain of *K. pneumoniae.* Both holo-BPI and the fragment potently inhibited TNF release induced by these bacteria, but holo-BPI had no significant effect on survival of either organism (at the doses tested) and the fragment killed only the *Pseudomonas* strain at a 5–10-fold higher concentration than needed for its TNF-inhibitory effect (Fig. 5). Thus, the induction of TNF by various Gram-negative bacteria was inhibited by the two proteins whether or not the bacteria were killed.

In contrast to the effects of BPI/fragment on each of the Gram-negative bacteria tested neither BPI nor the fragment affected survival or extracellular TNF accumulation when the Gram-positive bacterium *Staphylococcus aureus* was introduced to whole blood (Fig. 5).

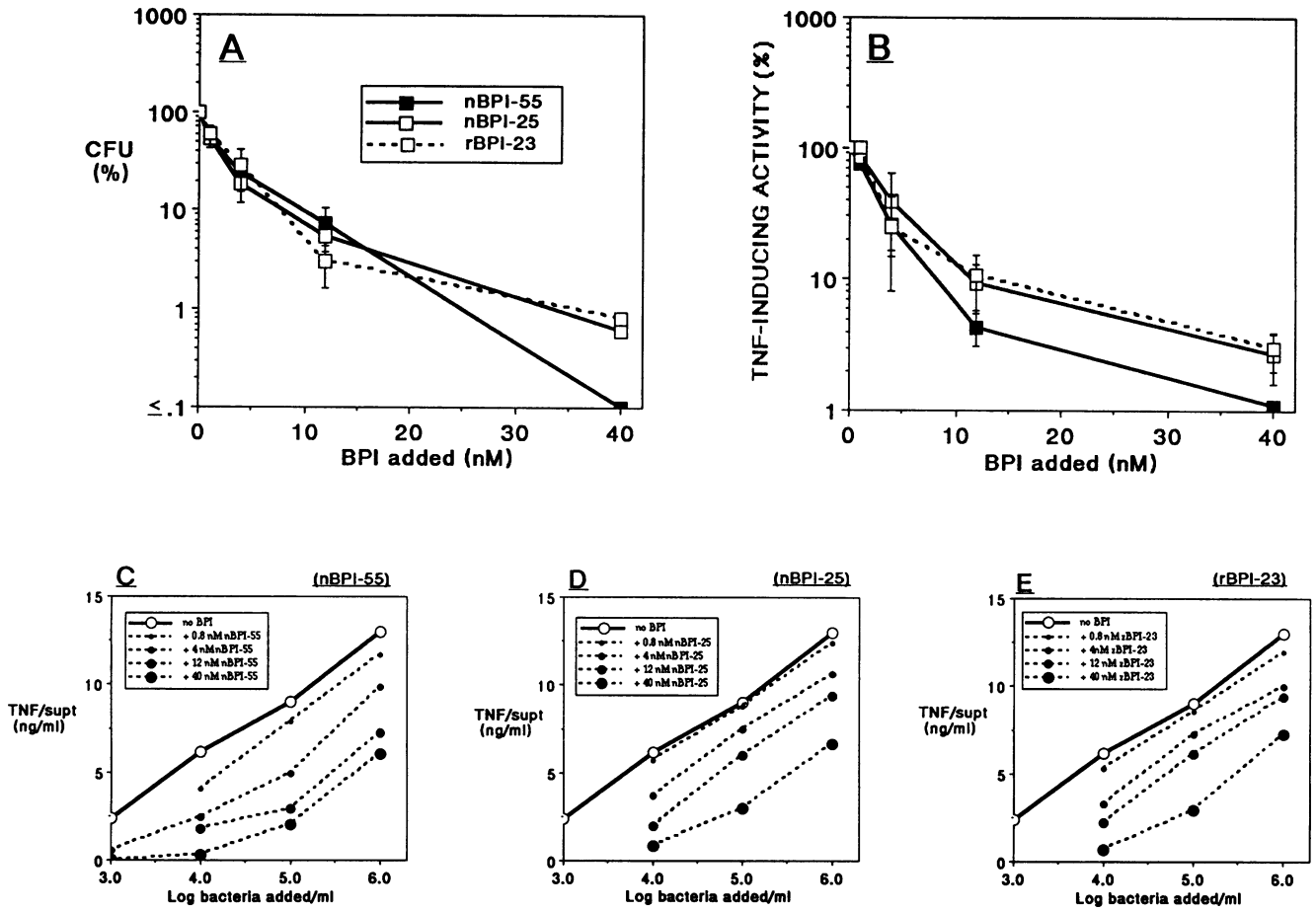

Figure 3. Comparison of antibacterial activities of holo-BPI and its NH_2-terminal (23–25 kD) fragments in whole blood. Bacterial survival (*A*) and extracellular accumulation of TNF (*C–E*) were measured as described in Methods after incubation of K1-encapsulated *E. coli* (10^3–10^6/ml) in blood supplemented with increasing concentrations of native holo-BPI (nBPI-55) or an NH_2-terminal fragment of BPI obtained either by limited proteolysis (nBPI-25) or by expression of a truncated form of human BPI cDNA (rBPI-23). Bacterial colony-forming units (CFU) were measured after 1 h incubation of 10^6 *E. coli*/ml and are expressed as the percentage of CFU of bacteria incubated in blood alone (representing 75±15% of the CFU of the initial inoculum). The effect of added holo-BPI and BPI fragments on the ability of the bacteria to induce extracellular TNF accumulation (*TNF-inducing activity; B*) was calculated by comparing the bacterial dose requirements for triggering TNF accumulation in the absence and presence of BPI (fragment) (*C–E*). For example, an increase in bacterial dose requirement of 2-, 10-, and 100- fold corresponds, respectively, to a reduction of bacterial TNF-inducing activity to 50, 10, and 1% of control (untreated bacteria). For a given amount of BPI/fragment added, a similar shift in the dose curve was observed at each bacterial dose tested. Therefore, each dose point was used to calculate the effects of added BPI (*B*). This effect is expressed as percent TNF-inducing activity of the added bacteria, using a bacterial standard curve as shown in Fig. 2 *D*. Each value shown represents the mean±SEM of at least four independent determinations (*A and B*) or of at least two determinations (*C–E*).

Discussion

BPI is located in the primary granules of PMN and is the most potent antibacterial agent yet identified in the antimicrobial arsenal of this phagocyte, directed specifically at a broad range of Gram-negative bacterial species (10, 33). We have demonstrated before that the remarkable target cell specificity of BPI reflects the strong attraction of this protein for the lipopolysaccharides that are the dominant surface molecules of the outer membrane of the Gram-negative bacterial envelope (13–15, 21). It is to this interaction that we attribute the prompt growth inhibitory effects and surface alterations that isolated BPI exhibits upon binding to *E. coli* and other Gram-negative bacteria (10, 32). The fate of bacteria ingested by PMN in vitro, both under aerobic and anaerobic conditions, closely mimics that of bacteria exposed to purified BPI, lending credence to the conclusion that BPI also is primarily responsible for growth arrest within the phagocyte (10, 26, 34).

In light of the strong evidence implicating the essential role of the recognition by BPI of LPS in the bacterial envelope in the selective action of BPI on Gram-negative bacteria, it is not surprising that recent studies have revealed that BPI is a member of a family of LPS-binding proteins (16). One of these, LPS-binding protein or LBP, has been shown to mediate and amplify host responses to LPS (35–37). In contrast, BPI mutes such responses by inhibiting the actions of isolated LPS in vitro as well as in whole animal experiments (17–20), raising expectations that BPI and bioactive fragments of BPI may be used as therapeutic agents directed against the clinical consequences of endotoxemia (8, 17–20).

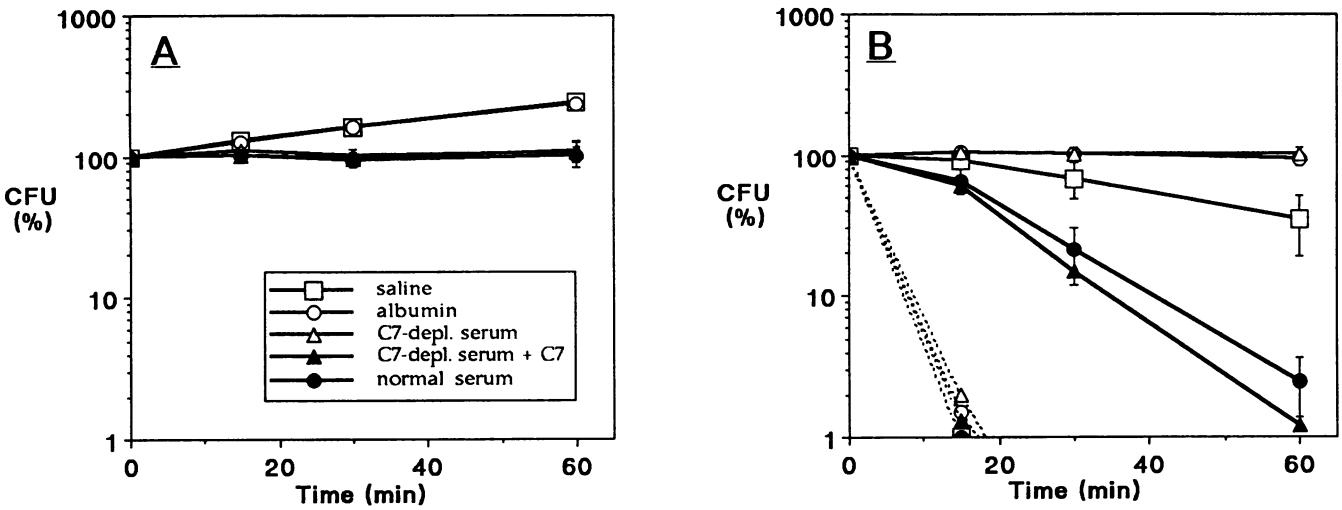

Figure 4. Synergistic bactericidal action of BPI and complement against serum-resistant K1-encapsulated *E. coli*. *E. coli* (2×10^6/ml) were incubated in the absence (*A*) and presence (*B*) of holo-BPI (2 μg/ml) in buffered (20 mM sodium phosphate, pH 7.4) nutrient broth supplemented with physiological (0.9%) saline or saline containing purified albumin (12.5 mg/ml), 25% normal serum (vol/vol), 25% C7-depleted serum, or 25% C7-depleted serum plus purified C7 (50 μg/ml). After incubation at 37°C for the indicated times, aliquots were taken to measure bacterial viability in unsupplemented nutrient agar (*broken lines*) and in nutrient agar supplemented with 1 mg of albumin/ml (*solid lines*). In the absence of BPI, there was no difference in the number of CFU apparent in nutrient agar±albumin. The CFU in a given sample are expressed as the percentage of the CFU of the added bacteria at time zero. The data shown represent the mean±SEM of at least three independent experiments.

Because the systemic entry of LPS into the host is usually secondary to invasion by Gram-negative organisms we addressed the questions: Can BPI and its bioactive fragments perform their antibacterial functions in the complex environment of whole blood and prevent in this setting the effects of the bacteria on cellular responses typical of Gram-negative bacteremia?

Mannion et al. (32) have shown before that when albumin is added as an isolated protein to an artificial incubation medium, the initial (almost immediate) actions of BPI, including binding, outer membrane alterations, and (reversible) growth inhibition are unaffected, but the later stage(s) of BPI action, involving cytoplasmic membrane damage and concomitant irreversible growth inhibition (cell death), is (are) blocked. These observations have prompted some to speculate that any extracellular function of BPI in vivo would be limited to neutralization of LPS (8, 19). However, we have now shown that added (extracellular) BPI and its bioactive fragments not only inhibit the accumulation of TNF in whole blood in response to the introduction of live *E. coli*, but also increase killing of *E. coli* in blood. These effects are evident at the same nanomolar concentrations that are effective in simple laboratory media, indicating that in the far more complex environment of whole blood neither cellular nor extracellular elements prevent BPI and its NH_2-terminal fragments from finding their natural targets. In fact, when encapsulated *E. coli* is exposed to BPI in the presence of nonlethal concentrations of serum, bacterial killing is markedly accelerated reflecting synergistic effects of BPI/fragment and late components of the complement system on *E. coli* (see Fig. 4). We have previously observed similar potentiation by late components of complement on intracellular killing of *E. coli* by PMN (26), suggesting that the functions of BPI and the complement system are joined to serve a major role in host defenses against Gram-negative bacteria. Because BPI causes prompt bacteriostasis and discrete surface alterations, even in the presence of albumin as sole additive (Fig. 3 *B*) (32), it is possible that BPI renders the serum-resistant K1-encapsulated *E. coli* more susceptible to complement in a manner similar to what has been reported for a nonbactericidal derivative of polymyxin B (38, 39). However, the late effects of BPI (32) and the bactericidal action of the membrane attack complex (40–42) cannot be distinguished so that the role of each, and possibly of additional antimicrobial blood elements, in the sequence of events in whole blood leading to bacterial death cannot yet be determined. Whatever the nature of the synergistic action of BPI and late complement components, it is the addition of BPI or the NH_2-terminal fragment that results in killing of surviving *E. coli*. These findings raise the possibility that BPI and its NH_2-terminal fragment, administered to whole animals and man, may serve a dual beneficial role, i.e., by enhancing the destruction of extracellular Gram-negative bacteria and by inhibiting their induction of potentially harmful cytokine (TNF) release.

The spectrum of bactericidal activity of BPI against the organisms tested in blood mirrored the action of BPI in simpler (serum-free) media (data not shown), suggesting that the inherent ability of BPI to act on a given organism primarily determines that organism's fate in blood when BPI is added. Because encapsulated bacteria (*E. coli*) are as sensitive to BPI as nonencapsulated strains (10, 21), bacteria that are highly resistant to both the cellular and extracellular elements of normal blood can still be effectively eliminated upon addition of nanomolar concentrations of BPI. The presence of long polysaccharide chains in outer membrane LPS, however, does increase bacterial resistance to holo-BPI (10, 13, 21, 43) but much less so to the NH_2-terminal fragment (24; Fig. 5), indicating that the fragment is substantially more potent toward this clinically important group of organisms. Many other species and strains

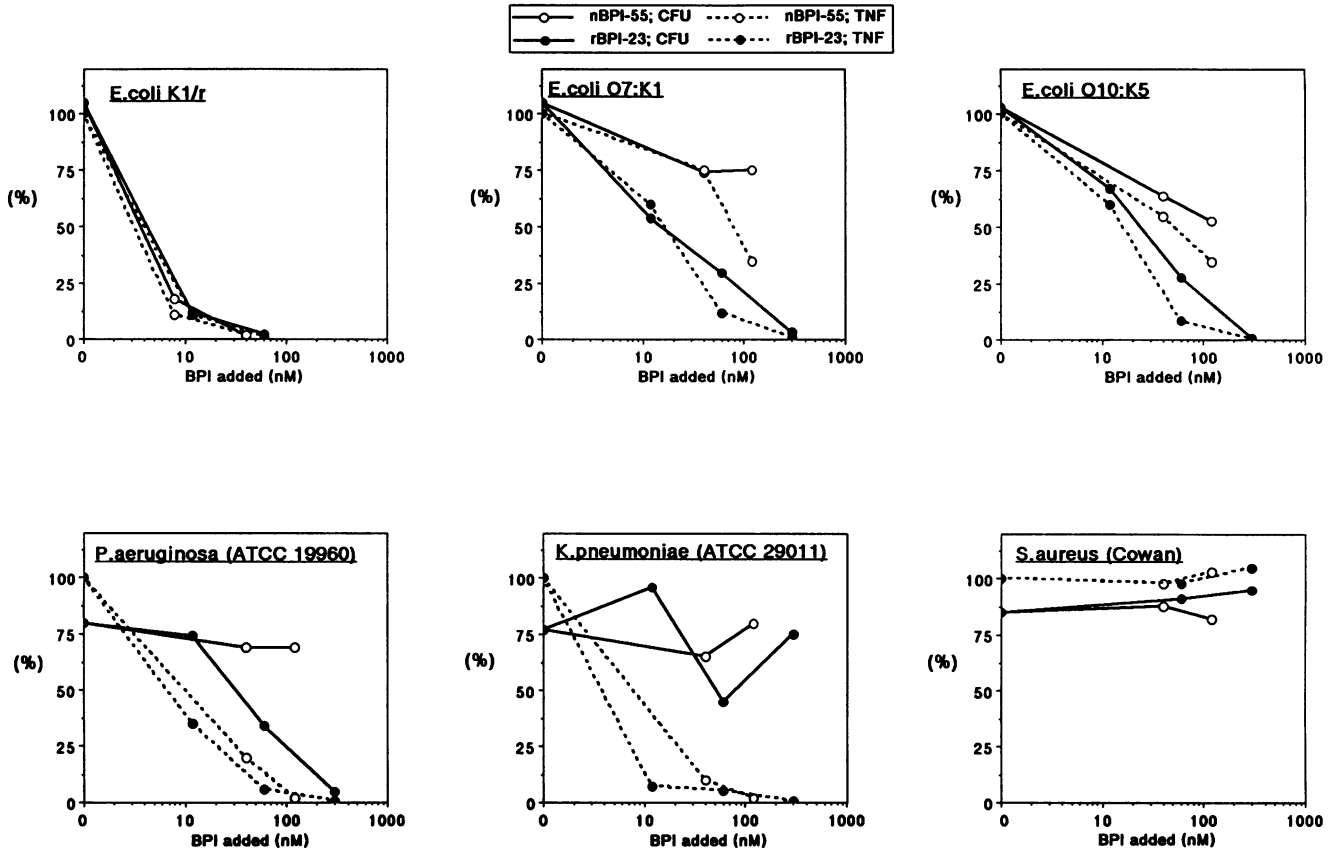

Figure 5. Comparison of antibacterial activities of holo-BPI (nBPI-55; *open circles*) and its NH_2-terminal fragment (rBPI-23; *closed circles*) in whole blood. Bacterial survival (*solid lines*) and extracellular accumulation of TNF (*broken lines*) were measured as described in Methods after incubation of various bacteria (10^4–10^6/ml) in blood supplemented with increasing concentrations of BPI/fragment. Bacterial CFU were measured after 1 h incubation of 10^5 or 10^6 bacteria/ml and are expressed as the percentage of CFU of bacteria added to blood. The effect of added holo-BPI and BPI fragments on bacterial TNF-inducing activity was calculated by comparing TNF accumulation triggered by increasing concentrations of bacteria in the absence and presence of BPI (fragment) as described in the legend to Fig. 3. Each value shown represents the mean of from two to six independent determinations.

of Gram-negative bacteria, including *P. aeruginosa* and *K. pneumoniae,* are sensitive to BPI (10, 44, 45; unpublished observations). The unexpected resistance of the strains of these organisms used in this study most likely reflects our selection of phenotypes that survive in normal blood. Both *P. aeruginosa* and *K. pneumoniae* are opportunistic pathogens that often cause disease when host defense is compromised.

The potency of all the Gram-negative bacteria tested as inducers of TNF accumulation in whole blood is remarkable. Addition of as little as 1 bacterium per μl of blood (containing ca. 7000 leukocytes) suffices to trigger the release of detectable amounts of extracellular TNF (Fig. 1 *B*). The TNF-inducing activity of these whole Gram-negative bacteria is at least as great as of amounts of isolated LPS that equal the total bacterial LPS content (Fig. 1 *B*). Thus, if LPS released by the bacteria is to account for the TNF-inducing activity of *E. coli* (46, 47), such release must be nearly quantitative. This seems unlikely in the case of organisms that remain viable in blood. Alternatively, the TNF-inducing potency of LPS released by the bacteria is far greater than of equivalent amounts of extracted and purified LPS. It is also possible that LPS within the bacterial envelope, perhaps in combination with other envelope constituents, further contributes to cytokine production and release. Whatever the nature of the inciting bacterial agent(s), we show that holo-BPI and the NH_2-terminal fragment are potent inhibitors of TNF release induced not only by isolated LPS (17–19) but also by added bacteria, whether dead or alive. Presentation to blood of equal numbers of bacteria that either survive or are promptly killed in blood alone (Fig. 1 *B*) triggers comparable TNF responses. Thus, the detection in blood cultures from septic patients of viable organisms is not an accurate index of the bacterial challenge to host responses. In the uncompromised host with Gram-negative bacteremia many species of Gram-negative bacteria are effectively killed by the intrinsic antimicrobial systems, but without protection against the actions of the cytokines released in response to circulating dead bacteria or their products. However, sepsis is particularly prevalent among patients with impaired antibacterial defenses in whom the bacteremia includes viable and proliferating organisms.

Earlier work in this laboratory has shown that BPI is less readily released from the PMN upon stimulation with degranulating agents than are other granule proteins (48), consistent with some of BPI's structural properties (25) and a primary intracellular (phagosomal) function. Indeed, BPI has been shown to coat ingested, but not extracellular, *E. coli* during

incubation with PMN (49). However, these observations do not exclude the possibility that under certain in vivo conditions BPI is released in biologically active form and concentrations. Thus far there is no evidence to support the claim that BPI is released in appreciable amounts by PMN under physiologic conditions. The fact that minute amounts of LPS added to whole blood ex vivo elicit cytokine production and release and that added BPI or BPI fragments in nanomolar concentrations inhibit this response implies that if BPI is present in the extracellular environment of normal blood its concentration does not exceed 10 nM (Fig. 3).

The results of this study, in combination with very recent whole animal experiments, showing protection against administered LPS by recombinant holo-BPI (20), as well as by recombinant NH_2-terminal fragment (unpublished observations) support the possibility that BPI and particularly the bioactive NH_2-terminal portion of the molecule are important additions to a group of potential therapeutic proteins and agents that may serve in the treatment of Gram-negative bacteremia and endotoxemia. BPI stands out among these agents in two ways: (*a*) as a natural component of the antimicrobial systems of the host, and (*b*) as the only agent so far identified with both bactericidal and endotoxin-neutralizing activities. This report further shows that the NH_2-terminal recombinant fragment as an administered extracellular agent may possess greater antibacterial range and potencies than the holo-protein.

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