Extracellular Accumulation of Potently Microbicidal Bactericidal/ Permeability-increasing Protein and p15s in an Evolving Sterile Rabbit Peritoneal Inflammatory Exudate

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Abstract

To what extent the host defense role of granule-associated antibacterial proteins and peptides of PMN includes extracellular action has not been established. To address this question, we have analyzed the antibacterial activity of cell-free (ascitic) fluid (AF) obtained from glycogen-induced sterile inflammatory rabbit peritoneal exudates in which > 95% of the accumulating cells are PMN. AF, but not plasma collected in parallel, exhibits potent activity toward serum-resistant Gram-negative and Gram-positive bacteria. Total and specific antibacterial activity of AF increases during the first 12 h after injection of glycogen in parallel with the influx of PMN. At maximum, > 99% of 10^7 encapsulated *Escherichia coli* and *Staphylococcus aureus* are killed in 30 min/ml of AF. Neutralizing antibodies against the bactericidal/permeability-increasing protein (BPI) of PMN abolishes activity of AF toward encapsulated *E. coli* but has no effect on activity vs staphylococci. However, BPI alone (~ 1 μg/ml in AF) can only account for ≤ 20% of AF activity toward *E. coli.* AF also contains 15 kD PMN proteins (p15s) that act in synergy with BPI. Purified BPI and p15s, in amounts present in AF, reconstitute the growth-inhibitory activity of AF toward encapsulated *E. coli.* These findings show for the first time an extracellular function of endogenous BPI, providing, together with the p15s, a potent microbicidal system toward Gram-negative bacteria resistant to plasma-derived proteins and phagocytes in inflammatory exudates. (*J. Clin. Invest.* 1995. 95:1916–1924.) Key words: polymorphonuclear leukocyte • inflammation • bacteria • ascites • synergy

Introduction

The passage of PMN from the circulation into inflammatory sites is a complex process that is initiated by interactions between PMN and endothelial cells and that is accompanied by heightened functional activity of the PMN (1–4). Among numerous responses to signals that prompt PMN to translocate, the cells increase surface expression of various receptors (1, 3) and may release portions of the contents of their storage granules (2, 5). The extracellular release by PMN in an inflammatory exudate of granule constituents is viewed as secretion by a viable cell rather than by a disintegrating PMN (2). What biological role the released material serves in inflammation is not clear. The extracellular release by PMN of proteinases such as elastase and collagenase may facilitate transendothelial migration of PMN but is also considered a major factor in the genesis of joint damage in inflammatory joint disease (2, 6, 7). Other secreted PMN products exhibit monocyte-specific chemotactic activity in vitro (8–10) and may promote influx of these cells later in the inflammatory response. Since the PMN granules contain an array of antimicrobial agents (10–13), we have considered the possibility that their release into the extracellular environment might add to the antimicrobial potential of an inflammatory exudate. To this end we took advantage of a long-standing animal model of inflammation (14), namely, a sterile peritoneal exudate induced in rabbits by intraperitoneal injection of glycogen in physiological saline. We found that, during the course of the development of the exudate, the cell-free (ascitic) fluid (AF)[1] gains potent antibacterial activity directed against both Gram-negative and Gram-positive bacteria. The activity toward a Gram-negative serum-resistant encapsulated *Escherichia coli* strain is largely attributable to two PMN-derived antimicrobial proteins, the bactericidal/permeability-increasing protein (BPI) and a 15-kD protein (p15). This report concerns the identification, quantitation, and antimicrobial activities of these proteins in ascitic fluid.

Methods

Bacteria. Bacteria used in this study included *E. coli* J5, a rough UDP-galactose-4-epimeraseless mutant of the smooth strain O111:B4 (15), *E. coli* K1/r and O7K1 (ATCC 23503; American Type Culture Collection, Rockville, MD), K1-encapsulated strains producing LPSs of rough and smooth chemotype, respectively, obtained from Dr. Alan Cross (Department of Bacterial Diseases, Walter Reed Army Medical Center, Washington, DC) (16) and the American Type Culture Collection, *Staphylococcus aureus* 52A (collection of Department of Microbiology, New York University Medical School, New York), and *Staphylococcus epidermidis* (14990; American Type Culture Collection). Bacteria were grown either in nutrient broth containing 0.9% (wt/vol) saline (*E. coli* J5) or in trypticase soy broth (other bacteria). Stationary phase over-

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1. *Abbreviations used in this paper:* AF, ascitic fluid; BPI, bactericidal/permeability-increasing protein; LBP, LPS-binding protein

night 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. 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.

Collection of AF and PMN from inflammatory peritoneal exudates. Sterile inflammatory peritoneal exudates were elicited in New Zealand white rabbits (2–3 kg) by intraperitoneal injection of 250–300 ml of sterile physiological saline supplemented with oyster glycogen (2.5 mg/ml). At various times (4–24 h) after injection, the inflammatory exudate was collected from the peritoneal cavity via a 16-gauge needle. The cell concentration of the exudate was measured by counting an aliquot in a hemocytometer. The cells were collected by sedimentation (100–200 *g* for 5 min), and the cell differential was determined after staining a smear of the cells with a leukostat stain kit (Fisher Diagnostics, Pittsburgh, PA). Cell pellets were stored at −20°C before use. The inflammatory AF was collected by sedimentation of the cells in the exudate followed by centrifugation of the recovered supernatant at 20,000 *g* for 10 min to remove particulate material before storage at 4°C. To normalize protein concentrations, AFs collected at earlier times were concentrated up to 10-fold by ultrafiltration (Centricon-10 concentrator; Amicon Corp., Danvers, MA) without loss of antibacterial activity.

Collection of plasma and PMN from blood. Rabbit peripheral blood was collected from the marginal ear vein both just before and 16 h after intraperitoneal inoculation of glycogen/saline. Blood was collected into a tube containing buffered citrate as anticoagulant. Plasma was isolated by sedimentation of blood cells by centrifugation at 2,500 *g* for 10 min. To control for the possible effect of citrate on antibacterial activity, AF was assayed without added buffer or with citrate or phosphate buffer, pH 7.4. PMN were enriched by sedimentation of erythrocytes in citrated blood in 1% (wt/vol) dextran T-500 at 1 *g* and by centrifugation of the leukocyte-rich upper phase. Residual erythrocytes were lysed by resuspension of the cells in isotonic ammonium chloride as described in reference 17. Cell concentration and the proportion of PMN (≥ 70% PMN) were determined as described above.

Fractionation of AF or plasma by CM-Sephadex chromatography. AF or plasma was incubated with rotation for 1 h at room temperature with CM-Sephadex (100 μl resin/ml of AF or plasma) (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Before use, the resin was prepared according to the directions of the manufacturer, equilibrated in 0.9% (wt/vol) saline buffered with 2.5 mM Tris-HCl, pH 7.5, and spun to remove excess solution. Unbound proteins were recovered in the supernatant after sedimentation of the resin by centrifugation at 1,000 *g* for 30 s. The resin was washed twice with gel equilibration buffer (each wash equaled the original volume of AF or plasma). Bound proteins were eluted by washing the resin twice with 0.1 vol of 1.5 M NaCl containing 20 mM sodium acetate/acetic acid buffer, pH 4.0 ("high salt eluate"), or by washing twice with 0.05 vol of 2× SDS sample buffer ("SDS eluate"). The recovered fractions were stored at 4°C. For the purpose of Western blot analysis of the high salt eluate, this fraction was mixed with 0.1 vol of 100% ice-cold TCA, incubated on ice for 30 min, and spun at 10,000 *g* for 15 min at 4°C. The recovered pellet was washed with ice-cold 5% TCA, rinsed with ether, dried in air, and resuspended in 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreiotol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol). Recovery of BPI present in AF or plasma in the SDS eluate was ≥ 75% as assessed by spiking AF and plasma with radiolabeled BPI (< 0.1 μg/ml) or unlabeled BPI (1 μg/ml; plasma only) and monitoring the SDS eluate by autoradiography of immunoblots (see below). BPI could not be directly measured in AF or plasma by immunoblot analysis because of the high concentrations of closely migrating albumin in these biological fluids.

Assay of bacterial growth–inhibitory activity. An effect of various protein fractions on bacterial viability was determined by measuring bacterial colony–forming ability after incubation of the bacteria with or without the protein fraction at 37°C. Typical incubation mixtures contained 10^5 bacteria in a total volume of 100 μl of nutrient broth/0.9% physiological saline buffered with 20 mM sodium phosphate, pH 7.4, and the protein fraction, as indicated. Incubations were for 15 min at 37°C. In the experiments shown in Fig. 4, incubation mixtures contained 90% (vol/vol) buffered (buffered citrate or 10 mM sodium phosphate, pH 7.4) AF or plasma and 10% (vol/vol) physiological saline containing a final concentration of 10^6 bacteria/ml, and the incubations were carried out for up to 60 min at 37°C. At the end of the incubation, aliquots of the suspensions were taken and serially diluted in sterile physiological saline. A 25-μl aliquot of the diluted sample was transferred to 5 ml of molten (48°C) 1.3% (wt/vol) 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 staphylococci). 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.

Neutralization of antibacterial activity of AF or plasma by goat anti-BPI serum. Buffered AF and plasma, either undiluted or diluted in the typical incubation medium, were preincubated with 3% (vol/vol) goat serum (normal or anti-BPI) for 15 min at room temperature before addition of bacteria (in 10 μl) and continuation of incubation for up to 60 min at 37°C. Bacterial viability was then measured as described above. Neutralizing effects of this antiserum on BPI-mediated antibacterial activity were previously described (16).

Preparation of PMN lysates for Western blots. Cell pellets were resuspended in lysis buffer (1% Triton X-100, 1% deoxycholate, and 1 mM PMSF) at 10^8 PMN/ml and sonicated for 2 min in a water bath sonicator (40 W; 4°C). After incubation on ice for 1 h, an equal volume of 2× SDS sample buffer was added, and the samples were boiled until no turbidity was visible. Samples representing 2.5 and 5 × 10^4 PMN equivalents were applied to SDS polyacrylamide gels.

Immunodetection of BPI. Protein samples were resolved by SDS-PAGE in 10% polyacrylamide gels and transferred to 0.2 μm nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) at 200 mA for 16 h (18). The blots were blocked by incubation for 1 h at room temperature with 0.3% BSA buffered with TBS (10 mM Tris-HCl, pH 7.4, in 0.9% NaCl) containing 0.1% sodium azide, washed with TBS for 5 min, and then exposed to 0.1% (vol/vol) goat anti-BPI serum in 0.3% BSA/TBS/0.1% sodium azide/0.5% NP-40/5mM EDTA, pH 8.0 (incubation buffer), at room temperature for 3 h. Blots were washed six times with TBS and once with incubation buffer (5 min each). Bound antibody was detected by incubation for 3 h in incubation buffer supplemented with 0.2% (vol/vol) ^{125}I-protein G (~ 4 × 10^5 cpm/ml; Amersham Corp., Arlington Heights, IL). After six 5-min washes with TBS, blots were exposed for autoradiography on XAR film (Eastman Kodak Co., Rochester, NY). To distinguish between BPI and a structurally related plasma-derived LPS-binding protein (LBP; 19), duplicate samples were resolved in gradient Phast Gels (10–15% polyacrylamide; Pharmacia LKB Biotechnology, Inc.) and reacted after transfer with either 0.1% (vol/vol) goat anti-BPI serum or anti-LBP serum.

Immunodetection of p15s. Analyses were carried out in the same way as described above for BPI except that samples were resolved in 14% polyacrylamide gels, and 0.3% (vol/vol) guinea pig anti-p15 serum was used.

For quantitation of BPI and p15s in experimental samples, all blots also contained known amounts of purified BPI or p15 (25–400 ng). Measurement of protein bands in autoradiograms was carried out using the Optical Imaging System (Ambis Systems Inc., San Diego, CA). The standard curves obtained were highly reproducible and readily dis-

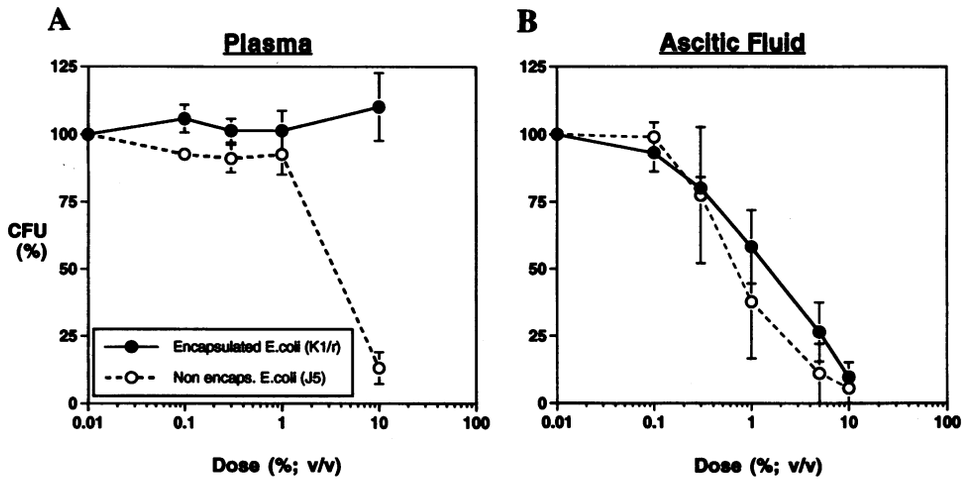

Figure 1. Comparison of antibacterial activity of rabbit plasma and inflammatory AF toward *E. coli.* The effect of increasing amounts of plasma (*A*) or AF (*B*) on viability of *E. coli* J5 (*open circles*) and K1/r (*closed circles*) was determined as described in Methods. All AFs tested were from inflammatory exudates collected 16 h after injection of glycogen/saline. The number of viable bacteria (colony-forming units, *CFU*) is expressed as the percent viability of bacteria incubated alone. The data shown represent the mean±SEM of five or more determinations.

tinguished twofold differences in BPI and p15 content (see Figs. 6 and 8).

Miscellaneous. Protein concentrations were determined by $A_{280/260}$ and by protein assay (Bio-Rad Laboratories, Richmond, CA) as described by the manufacturer, using serum albumin as standard.

Results

Potent antibacterial activity of rabbit AF toward both serum-sensitive and -resistant E. coli. Cell-free AF collected 16 h after intraperitoneal injection into rabbits of 250 ml of sterile physiological saline supplemented with oyster glycogen (2.5 mg/ml) possesses potent antibacterial activity against both serum-sensitive *E. coli* (J5) and serum-resistant encapsulated *E. coli* (K1/r) (Fig. 1). The activity of AF toward *E. coli* J5 was greater than or equal to that of plasma (or serum, not shown). The activity of AF toward *E. coli* K1/r far exceeded that of plasma (or of serum; not shown) collected either before or 16 h after inoculation (Fig. 1). Thus, this activity in AF is not simply a result of exudation of plasma constituent(s) but rather appears to be due to component(s) released locally into AF.

The antibacterial activity of AF was highly consistent, not only in samples collected from a single rabbit over a period of several months but also in samples obtained from different rabbits (Fig. 2). There was no reduction in activity during storage of AF at 4°C for up to 6 mo.

The total (Fig. 3 *A*) and specific antibacterial activity/ml of AF (toward *E. coli* K1/r) (Fig. 3 *B*) increased with time up to 24 and 12 h, respectively, after injection of the glycogen solution, further indicating that the antibacterial activity is attributable to component(s) of AF that accumulate at a rate different from that of the bulk protein (mainly plasma albumin). However, the increase in antibacterial activity did parallel the influx of PMN into the peritoneal exudate, suggesting that products released from PMN may be the source of antibacterial activity in AF.

Effect of anti-BPI serum on antibacterial activity of AF toward encapsulated E. coli. The most potent major protein product of PMN known to be toxic toward encapsulated *E. coli* is BPI (11, 16, 20). We therefore tested the effect of neutralizing BPI antibodies on the antibacterial activity of AF. Fig. 4 *A* shows that anti-BPI serum virtually abolished the antibacterial activity of AF toward *E. coli* K1/r, whereas addition of similar amounts of normal serum had no effect. These effects were seen over the entire dose range of AF tested, suggesting that all antibacterial activity of AF toward *E. coli* K1/r was dependent on BPI. The same results were obtained with each of several AFs tested. At high concentrations (90% vol/vol), plasma had a modest bactericidal effect during longer incubations (≥ 30 min) that was also (partially) inhibited by anti-BPI serum (Fig. 4 *B*).

Strains of *E. coli* producing long-chain (smooth chemotype) LPS are more resistant to BPI (11, 15) and also to AF (data not shown). However, at high concentrations (90% vol/vol of 16- and 24-h exudates), AF but not plasma or serum produced complete inhibition of the viability of up to 10^6 *E. coli* O7K1/ml (Fig. 4, *C* and *D*). The antibacterial activity of AF toward

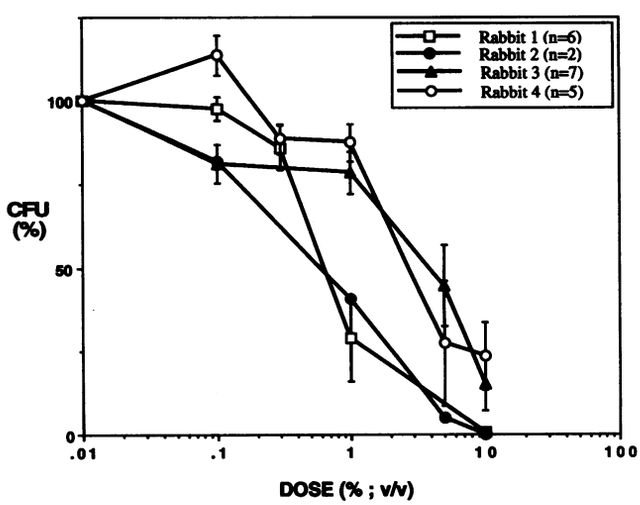

Figure 2. Comparison of antibacterial activity toward serum-resistant *E. coli* K1/r of AF from different rabbits. Experiments were carried out and data were presented as described in the legend to Fig. 1. The number in parentheses represents the number of independent collections of AF (from 16-h exudates) from each rabbit.

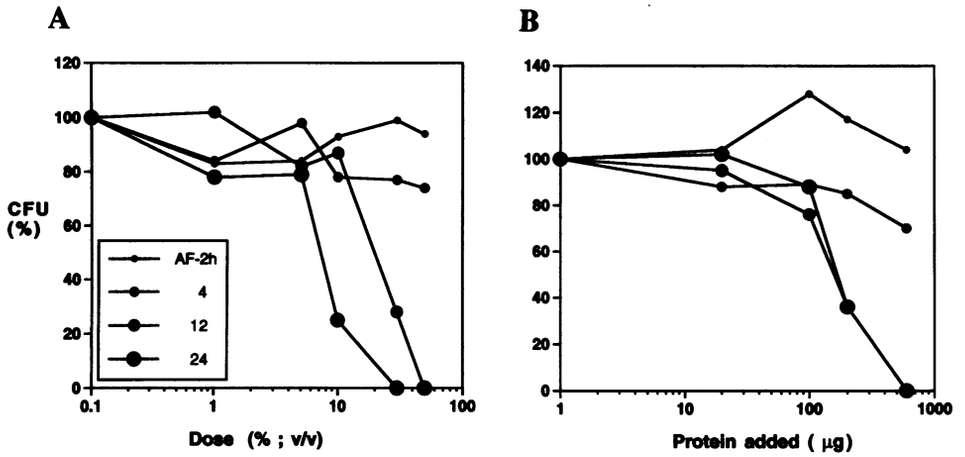

Figure 3. Comparison of antibacterial activity toward *E. coli* K1/r of AF collected from a single animal at various times after intraperitoneal injection. The total protein concentration in AF collected at 2, 4, 12, and 24 h after injection was ~ 3, 4, 8, and 22 mg/ml, respectively. AFs collected at earlier times were concentrated by ultrafiltration (see Methods) to permit testing of equal amounts of protein (*B*). The data shown represent the mean of two or more similar experiments.

E. coli O7K1 was also completely inhibited by anti-BPI serum (Fig. 4 *C*).

BPI-independent antibacterial activity of AF toward staphylococci. AF (but not plasma) also caused killing of Gram-positive bacteria, including *S. aureus* (Fig. 4, *E* and *F*) and *S. epidermidis* (data not shown). BPI alone is nontoxic to Gram-positive bacteria (11, 21). Killing of the staphylococci by AF was not inhibited by anti-BPI serum, confirming that these effects of AF were not BPI-dependent. Thus, in contrast to plasma (or serum), AF contains potent broad-spectrum antimicrobial activity, some of which appears to be dependent on BPI.

Partial purification by ion-exchange chromatography of antibacterial activity of AF toward encapsulated E. coli. To further document and characterize the contribution of BPI to the antibacterial activity of AF toward *E. coli* K1/r, proteins in AF were fractionated by chromatography on CM-Sephadex, a cation exchange resin that, under the conditions used, quantitatively adsorbs BPI and other PMN cationic proteins but not the bulk proteins in AF derived from plasma (21–23; see Methods). As expected, ~ 99% of the protein in AF but no antibacterial activity toward *E. coli* K1/r was recovered in the unbound (buffered physiological saline wash, pH 7.4) fraction (Fig. 5 *A*). In contrast, the fraction adsorbed to CM-Sephadex and eluted with acetate (pH 4.0)–buffered 1.5 M NaCl ("high salt eluate") contained ≤ 0.5% of the total AF protein but ~ 50% of the total antibacterial activity. The activity of this bound protein fraction was unaffected by recombining with the unbound proteins recovered from AF or from plasma (Fig. 5 *A*). The distribution of antibacterial activity in AF from different animals and collected at 4, 12, and 24 h after inoculation was essentially the same. Antibacterial activity toward *E. coli* K1/r was not unmasked by fractionation of plasma (Fig. 5 *B*), sug-

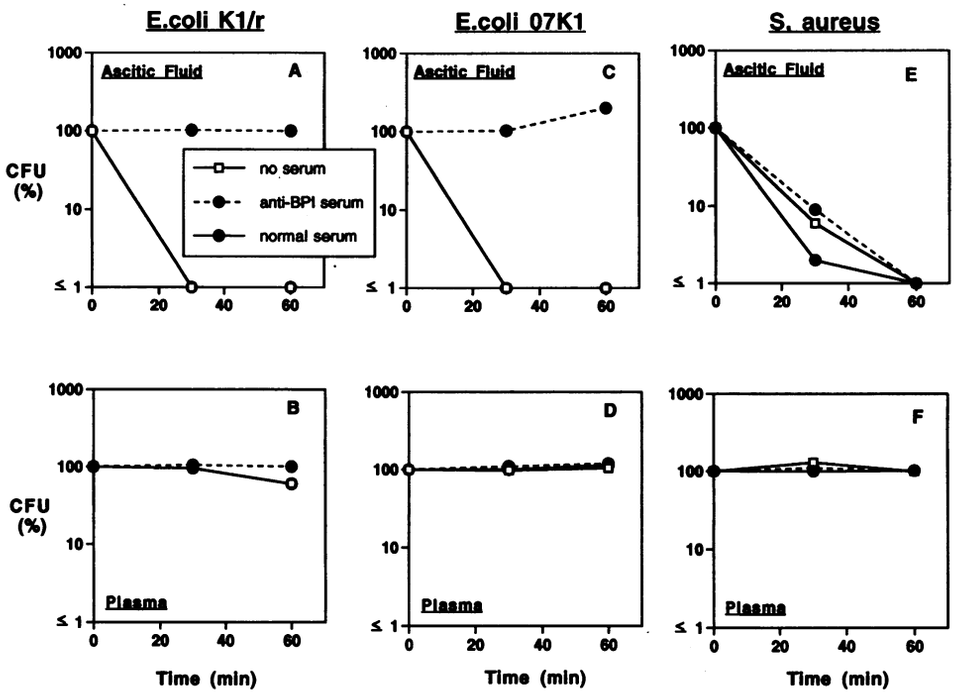

Figure 4. Anti-BPI serum blocks antibacterial activity of AF toward *E. coli* but not toward staphylococci. Effects of nearly undiluted AF (*A, C,* and *E*) or plasma (*B, D,* and *E*) incubated alone or with 3.6% (vol/vol) goat anti-BPI serum or normal serum on viability of *E. coli* K1/r (*A* and *B*), *E. coli* O7K1 (*C* and *D*) and *S. aureus* (*E* and *F*) were measured as described in Methods. Incubations were carried out for 30 and 60 min at 37°C. The results shown represent the mean of three or more closely similar experiments. Virtually identical results were obtained with citrate- or phosphate-buffered AF. Note that in A–C, effects on CFU in absence of serum or in presence of normal serum are identical.

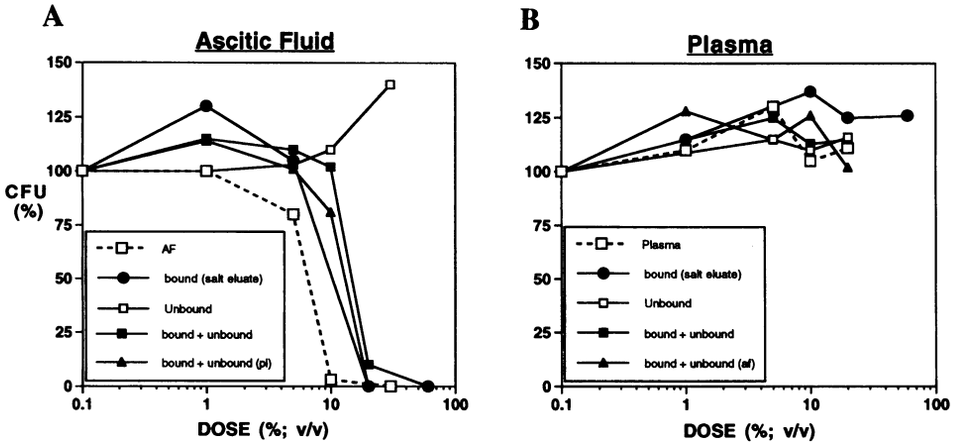

Figure 5. Antibacterial activity of AF toward *E. coli* K1/r is recovered in the high salt eluate after CM-Sephadex chromatography. AF (*A*) and plasma (*B*) were fractionated by CM-Sephadex, and unbound and/or bound (high salt eluate) protein fractions were tested for effects on viability of *E. coli* K1/r as described in Methods and in the legend to Fig. 1. The data shown represent one experiment representative of three independent and closely similar fractionations.

gesting that the difference in activity between AF and plasma is due to the presence of antibacterial factor(s) in AF and not inhibitor(s) of activity in plasma.

Immunoblots of the high salt eluate of AF recovered from CM-Sephadex using anti-BPI serum demonstrated immunoreactive species comigrating with BPI (Fig. 6 *A*). Acetate-buffered 1.5 M NaCl was only about half as effective as SDS (see Methods) in eluting these species from CM-Sephadex (Fig. 6 *A*, lanes *6* and *7*), therefore accounting for the ~ 50% recovery of antibacterial activity in the high salt eluate (Fig. 6 *A*). To insure that the immunoreactive species was BPI and not the related plasma-derived LBP, SDS eluates of AF and plasma proteins bound to CM-Sephadex were further analyzed by immunoblotting using both anti-BPI and anti-LBP sera. Fig. 6 *B* shows that rabbit BPI (lane *4*) migrates more rapidly during SDS-PAGE than LBP (lane *3*). In AF, species reactive with BPI antibodies comigrated with BPI (lane *5*) and did not react with LBP antibodies, establishing the presence and identity of BPI in AF. A species reactive with anti-BPI was faintly detected in plasma (lane *6*). In contrast, LBP was more abundant in plasma than in AF (Fig. 6 *B*, lanes *1* and *2*).

BPI does not account for all antibacterial activity of AF toward E. coli *K1/r: role of p15s.* Although the antibacterial activity of AF toward *E. coli* K1/r was completely inhibited by anti-BPI serum, at equivalent amounts of BPI added, AF was 5–10 times more active than purified BPI (Fig. 7), suggesting that other agent(s) in AF contribute to antibacterial activity in a BPI-dependent fashion. We have previously described 15-kD cationic proteins in rabbit PMN granules that act synergistically with BPI against *E. coli* (24–26). Immunoblots of AF and the high salt eluate of AF using anti-p15 serum revealed the presence of substantial amounts of p15 (up to 20 times more p15 than BPI; Table II). At this concentration, purified p15s alone did not inhibit bacterial viability (data not shown). However, addition of both purified BPI and p15s in amounts corresponding to their concentrations in AF produced the same dose-dependent growth inhibition as the AF (Fig. 7). These findings strongly suggest that the antibacterial activity of AF reflects the combined actions of BPI and the p15s.

Unlike BPI, the p15 content in AF could be measured directly by immunoblots of whole AF (Fig. 8 *B*). This permitted verification of the quantitative binding of the p15s to CM-Sephadex (i.e., no p15 is detected in the unbound protein fraction; Fig. 8 *B*, lane *7*) and nearly quantitative recovery from CM-Sephadex by elution with 2× SDS sample buffer (Fig. 8 *B*, lanes *5* and *8*). Plasma collected either before or 16 h after intraperitoneal injection of glycogen/saline did not contain detectable p15 (Fig. 8 *A*, lanes *5* and *6*). Purified p15 added to plasma could be fully recovered in immunoblots of whole plasma (Fig. 8 *A*, lane *7*) and in SDS eluates of bound proteins (Fig. 8 *A*, lane *10*), further demonstrating that p15s in complex fluids including AF and plasma can be quantitatively monitored.

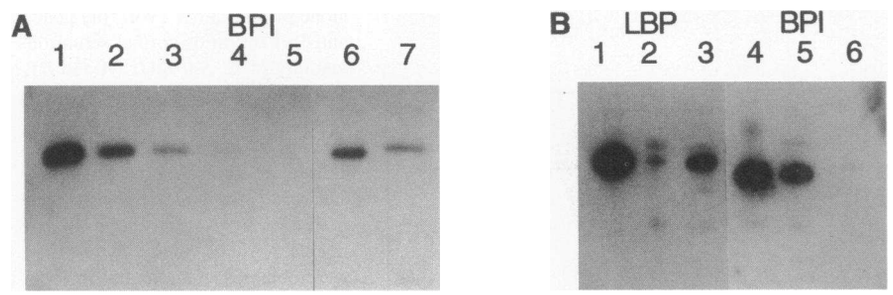

Figure 6. Immunological detection of BPI and LBP in AF and plasma. (*A*) The BPI content of AF and plasma from rabbit #3 was determined by immunoblot analysis after fractionation by CM-Sephadex chromatography as described in Methods. Lanes *1–4* contain 200, 100, 50, and 25 ng of purified rabbit BPI (24), respectively; lane *5*, SDS eluate of 200 μl of plasma bound to CM-Sephadex; lanes *6* and *7*, SDS and high salt eluates, respectively, of 200 μl of AF bound to CM-Sephadex. (*B*) The same samples shown in lanes *5* and *6* of the left panel were also resolved in gradient gels (see Methods) and reacted with anti-LBP (lanes *1–3*) and anti-BPI (lanes *4–6*) sera. Lanes *1* and *6:* SDS eluate of 80 μl of plasma; lanes *2* and *5:* SDS eluate of 80 μl of AF; lane *3:* 50 ng of purified LBP; lane *4:* 100 ng of purified BPI.

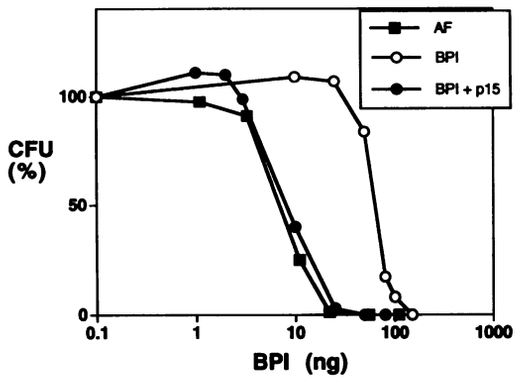

Figure 7. Antibacterial activity toward *E. coli* K1/r of AF is reconstituted by purified BPI and p15s. The BPI and p15 content of AF (rabbit #1) was measured as described in Methods. BPI and p15s were purified from PMN of the same rabbit (24) and added in amounts corresponding to that present in AF. Effects on viability of *E. coli* K1/r were measured as described in Methods and in the legend to Fig. 1. The results shown represent the mean of two closely similar experiments.

Time course of accumulation of BPI and the p15s in AF. The cellular and extracellular (plasma and AF) contents of BPI and p15s in peripheral blood and peritoneal exudates were measured using the same quantitative immunodetection methods. The BPI and p15 content of AF increased with time of collection (Table I) in parallel with the increase in antibacterial activity toward *E. coli* K1/r (see Fig. 3). In contrast, the amount of BPI and p15 in PMN, derived either from blood or from the peritoneal exudate, was essentially the same at all times collected. At maximum, ~ 10% of the total p15 content in the inflammatory exudate (cells + fluid) and < 2% of BPI was recovered in the AF.

Comparison of BPI and p15 contents of 16-h AFs collected from different animals. Table II shows that the higher antibacterial activity of AF collected from rabbit No. 1, in comparison to AF from rabbits Nos. 3 and 4 (see Fig. 2), also paralleled the higher BPI and p15 content of the AF of this animal. In contrast, the total protein concentration in AF from rabbit No. 1, the PMN concentration in the exudates from this animal, and the BPI and p15 content in the PMN of rabbit No. 1 were not significantly different from those of the other animals.

Discussion

This study demonstrates potent and increasing antibacterial activity toward serum-resistant encapsulated *E. coli* in the AF of an evolving sterile inflammatory exudate induced by injection of glycogen into the peritoneal cavity of the rabbit. The accumulation of antibacterial activity parallels the increase in the concentration of PMN in the exudate and is of comparable magnitude from rabbit to rabbit and on repeated challenge. As early as 4 h after injection of glycogen, antibacterial activity of the AF is readily detectable and continues to increase during the next 8–20 h. In contrast, the plasma does not gain antimicrobial activity after the local inflammatory challenge. Antibacterial activity is stable during storage of AF for months at 4°C, indicating that this activity is largely attributable to biologically stable agents. The fact that neutralizing antibodies against BPI can abolish virtually all antibacterial effects of AF toward *E. coli* K1/r and O7K1 implies that BPI is an essential element in the antimicrobial activity of AF against these organisms. However, by itself, the BPI concentration in AF accounts for ≤ 20% of the observed antibacterial activity (Fig. 7). We have recently shown that other PMN granule proteins, the p15s, and the most potent rabbit defensins (NP-1, 2) can act synergistically with BPI, reducing the dose of BPI required for growth inhibition as much as 50-fold when subinhibitory doses of the p15s and/or NP-1(2) are also present (24–26). The AF contains substantial amounts of the p15s (up to 2 μM; Table II). These concentrations of the p15s alone do not affect growth of *E. coli* K1/r but, together with BPI (10–20 nM), in the amounts present in AF, display essentially the same growth-inhibitory potency toward *E. coli* K1/r as the whole AF. Thus, BPI + p15s can account for nearly all the growth-inhibitory activity of the AF toward *E. coli* K1/r. NP-1 and -2 are also present in the AF (Weinrauch, Y., and J. Weiss, unpublished observations). However, in contrast to BPI and the p15s, the bulk of the defensins are not recovered in native form but as slow migrating species during acid-urea PAGE, probably reflecting high molecular weight complexes with α_2-macroglobulin or C1 (27, 28). These complexes are apparently devoid of antimicrobial activity.

At the concentrations of albumin present in AF, the antibacterial effects of purified BPI±p15 do not rapidly progress from reversible, sublethal alterations to actual killing (24, 26, 29). In contrast, the whole AF in most instances produces rapidly lethal effects (Weinrauch, Y., and J. Weiss, unpublished obser-

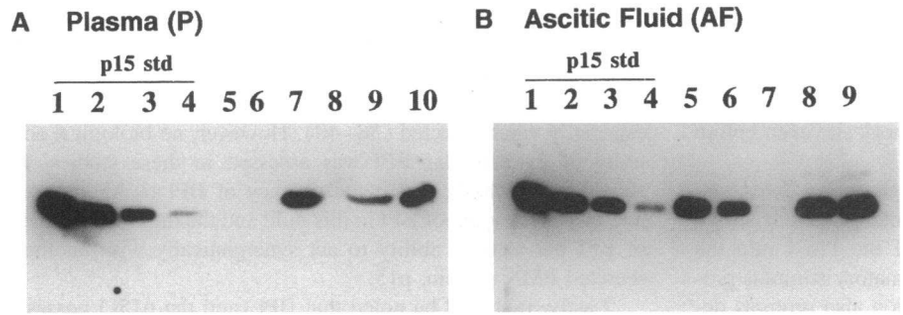

Figure 8. Immunological detection of p15s in plasma (*A*) and AF (*B*). (*A*) Lanes *5* and *6* contain 20 μl of plasma collected, respectively, before and 16 h after injection of glycogen/saline; lanes *7–10,* plasma spiked with purified p15 (10 μg/ml): 10 μl equivalent, unfractionated (lane *7*), 20 μl equivalent, CM-Sephadex unbound protein fraction (lane *8*), 5 and 10 μl, respectively, of CM-Sephadex bound fraction (SDS eluate, lanes *9* and *10*). (*B*) 20 and 10 μl, respectively, of unfractionated AF (lanes *5* and *6*), and 20 μl equivalent of AF, CM-Sephadex unbound protein fraction (lane *7*) and SDS (lane *8*), or high salt (lane *9*) eluates of bound protein fractions. Lanes *1–4* in both *A* and *B* contain 200, 100, 50, and 25 ng, respectively, of purified p15s.

Table I. Cellular and Extracellular Content of p15 and BPI in Blood and Inflammatory Exudate Collected at Different Times

	Time of collection	PMN	p15		BPI	
			Fluid	PMN	Fluid	PMN
	h	*cells/ml*	*μg/ml*	*μg/10⁷*	*μg/ml*	*μg/10⁷*
Blood	0	5×10^6	<0.2	30±9.0	<0.05	19±2.8
Exudate	2	1×10^6	0.3±0.04	ND*	0.1±0.01	ND*
	4	6×10^6	1.0±0.1	33±5.0	0.1±0.01	16±2.0
	12	3×10^7	2.1±0.3	38±5.0	0.4±0.03	16±2.5
	24	4×10^7	3.3±0.9	28±3.4	1.1±0.01	19±1.0

Measurements of p15 and BPI content in cells and extracellular fluid were carried out as described in Methods. For quantitation of p15 and BPI in experimental samples, two doses of each sample were analyzed on immunoblots and compared to a standard curve of purified p15 and BPI, present in the same blot (see Figs. 6 and 8). The values shown represent the mean±SEM from three or more independent determinations. * ND, not determined.

Table II. p15 and BPI Content of 16-h AF from Different Rabbits

Rabbit number	p15	BPI
	μg/ml	*μg/ml*
1	14.0±0.8	1.1±0.2
3	5.1±0.3	0.4±0.1
4	6.0±0.7	0.3±0.1

p15 and BPI content in AF were measured as described in Methods and the legend of Table I. The values shown represent the mean±SEM of three or more independent determinations.

vations). Thus, by this criterion, the antibacterial activity of AF toward *E. coli* K1/r (and O7K1) does not simply reflect the combined action of BPI and p15. We have shown before that exposure of *E. coli* to concentrations of serum (Complement) that alone produce no detectable damage markedly accelerates the slow transition from reversible to irreversible bacterial growth inhibition in BPI-treated bacteria and overrides the inhibitory effect of albumin on this progression (20, 30). In addition to the Complement system (31, 32), AF contains high concentrations of a 14-kD phospholipase A2 that is potently active against *E. coli* treated with BPI±p15 (23, 33) and may further enhance bacterial destruction in AF. The fact that AFs can differ in the rate and extent of killing of *E. coli* K1/r despite similar growth-inhibitory activity supports the view that, in addition to BPI and p15, other host defense elements, including some derived from plasma such as Complement and phospholipase A2, also contribute to the overall antibacterial activity of a given AF toward this test organism.

Although our experiments have focused mainly on the antibacterial activity of AF toward *E. coli,* it is apparent that this inflammatory fluid possesses potent, broad-spectrum antimicrobial activity, including antistaphylococcal activity (see Fig. 4). This BPI-independent activity is not present in plasma and therefore may also reflect the secretion of antimicrobial (poly)-peptides by PMN or other cells in the inflammatory environment. The presence of potent antibacterial activity against serum-sensitive and -resistant Gram-negative as well as Gram-positive bacteria suggests that inflammatory fluids may contribute substantially to host defense against both Gram-negative and Gram-positive bacteria at an inflammatory site even before the bacteria are sequestered intracellularly.

Among the alterations that PMN undergo during extravasation is extracellular release of granule proteins (2). It seems plausible, therefore, that the migration of the PMN into the peritoneal cavity in response to the inflammatory stimulus provided by the injection of a glycogen solution also prompts degranulation. Both BPI and p15 are mainly granule-associated proteins of PMN (18, 25, 34). Our measurements of the cellular and extracellular content of these proteins in the inflammatory exudate (Tables I and II) reveal that a much higher fraction of the p15s than of BPI accumulates in the AF (up to 20% vs < 3%, respectively). The precise granule localization of BPI and the p15s in rabbit PMN has not yet been determined. Therefore, it is possible that differences in accumulation of BPI and p15s in AF reflect residence of the two proteins in granules that degranulate to a different extent (5). Alternatively, exocytosis of granules containing both proteins may yield less released extracellular BPI because BPI is more tightly associated with PMN membranes (18, 35). In addition, other sources of the two proteins under these inflammatory conditions must also be considered, including other cells in the peritoneal cavity, even though so far the production of BPI and p15 can be traced only to myeloid cells (18, 25). Further, in light of growing evidence that mature PMN under certain conditions selectively synthesize proteins (36, 37), we have begun to explore the possibility that, in the inflammatory environment, biosynthesis of one or more antimicrobial proteins is stimulated. Preliminary studies show p15 mRNA in exudate cells (but not blood PMN) collected as early as 2–4 h after injection of glycogen, whereas BPI mRNA is not detected in exudate cells until at least 8–12 h after injection (Zarember, K., and J. Weiss, unpublished observations). The extent to which the mRNA detected in these cells is translated and contributes to the accumulation of PMN proteins in the AF is under study.

Whatever the mechanism(s) of the accumulation of such proteins in AF, this study demonstrates for the first time the extracellular mobilization of sufficient endogenous BPI to account for prominent extracellular BPI-like antibacterial activity. This is the first time that an antibacterial role in a natural environment has been documented for any of the PMN-derived oxygen-independent antimicrobial proteins or peptides. Recently, others have also observed elevated extracellular levels of BPI in plasma of septic patients and volunteers after LPS administration and, to an even greater extent (as in our study), in AF collected from patients on chronic peritoneal dialysis, especially when infected (38–40). However, no biological activity of extracellular BPI was assessed in these studies. It should be emphasized that the potency of BPI in AF reflects not only the amount of BPI in this fluid and the intrinsic potency of BPI but also its ability to act synergistically with another secreted PMN protein, p15.

Lastly, it should be noted that BPI (and the p15s) possess potent LPS (endotoxin)–neutralizing activity (20, 41–44). It has been speculated previously that release of BPI from PMN

at inflammatory sites could provide a feedback mechanism to turn off the inflammatory response provoked by invading Gram-negative bacteria (43, 45). It should be noted that levels of LBP, a homologue of BPI that is an important positive regulator of endotoxin activity (19), are far lower in inflammatory fluid than in plasma (Fig. 6 *B;* 40). Thus, in inflammatory fluids, extracellular BPI and p15s may not only exert their antibacterial (cytotoxic) action but may also downregulate further inflammatory reactions triggered by bacteria or their constituents (LPS). In contrast to analyses of occasional clinical samples, this highly reproducible rabbit model of (acute) inflammation should permit the detailed study of the evolution of the inflammatory response and its regulation by the mobilization and integration of cellular and extracellular host defenses.

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