Isolation of Two Isoforms of a Novel 15-kDa Protein from Rabbit Polymorphonuclear Leukocytes That Modulate the Antibacterial Actions of Other Leukocyte Proteins*

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We have recently reported the use of the highly selective and reversible binding of the potent bacterial/permeability-increasing protein (BPI) to target Gram-negative bacteria (Escherichia coli) for its isolation from crude extracts of human polymorphonuclear leukocytes (PMN). We now report the use of the same procedure for the purification from rabbit PMN of BPI and also of a novel 15-kDa species that consists of two nearly identical isoforms. These 15-kDa proteins have no demonstrable antibacterial activities by themselves. However, one isoform (p15A) potentiates strongly and the other (p15B) weakly the early antibacterial effects of both rabbit and human BPI. Both isoforms inhibit the late lethal action of BPI. Whereas the potentiating effect is specific for BPI the inhibitory effect is seen also with another antibacterial protein of PMN granules, azurocidin. Thus, we have identified in rabbit BPI a previously unrecognized 15-kDa protein species in the crude extract was identified, in addition to BPI, which displayed high affinity for surface sites on E. coli.

We report here the isolation of this protein species and the initial characterization of its molecular and functional properties. The results show that this 15-kDa protein exists as two isoforms, both of which manifest no antibacterial action by themselves. However, one of the isoforms strongly potentiates the early, reversible antibacterial effects on E. coli by BPI, while the other closely similar isoform does not. In addition, both 15-kDa isoforms inhibit the late bactericidal effects of BPI.

EXPERIMENTAL PROCEDURES

Materials

E. coli J5, a "rough" UDP-4-galactose-epimerase-less mutant was the gift of Dr. Loretta Leive (National Institutes of Health, Bethesda, MD). Human defensins were the gift of David Campanelli (Division of Hematology-Oncology, Cornell University Medical College). Normal human serum was prepared from peripheral blood of healthy donors. Polymyxin B was purchased from Roerig (Pfizer, Inc.), New York, NY.

Polymorphonuclear Leukocytes

Rabbit leukocytes (>90% granulocytes) were obtained from overnight glycogen-induced peritoneal exudates, pelleted by centrifugation, and stored at -70 °C for up to 6 months (9).

Acid Extraction of Protein from PMN

PMN were homogenized in distilled water and extracted with 0.16 N sulfuric acid at 4 °C as described previously (9). The extract was neutralized by prolonged dialysis against 1 mM Tris/HCl, pH 7.5, and precipitated were removed by centrifugation for 20 min at 23,000 g. The resulting supernatant fluid is referred to as "crude acid extract" and contains 10-20% of total leukocyte protein at 8 mg/ml.

Purification of Human BPI and Its 25-kDa NH2-terminal Fragment

Holo-human BPI and its 25-kDa NH2-terminal fragment were purified as described before (13, 14).

Bacterial Cultures

Bacteria were grown overnight and then in subculture at 37 °C in triethanolamine-buffered medium (8). Bacterial subcultures were harvested at mid-logarithmic phase and resuspended to the desired concentration in sterile physiological saline. "Smooth" E. coli with long chain lipopolysaccharides were obtained by growing E. coli J5 in triethanolamine-buffered medium supplemented with 1 mM galactose (11).
Binding of Proteins in Crude Extracts of Rabbit PMN to E. coli

A modification of the procedure described by Mannion et al. (13) was used. PMN crude extract was incubated with E. coli (5 × 10⁷/ml) for 15 min at 37 °C in 10 mM sodium acetate buffer, pH 4.0. After centrifugation at 3000 x g for 10 min to remove unbound material, the bacterial pellets were washed twice with half volumes of the incubation medium. Bound proteins were eluted from E. coli suspensions (5 × 10⁷/ml) during incubation in 200 mM MgCl₂ buffered with sodium acetate/acetic acid, pH 4.0, for 15 min at 37 °C. After removal of the bacteria by centrifugation, the supernatant was dialyzed against 50 mM sodium acetate buffer, pH 4.0, and referred to as the "Mg⁺-eluate."

Reversed-phase HPLC

High performance liquid chromatography was carried out on a C₄ column (Vydac) using a linear gradient of acetonitrile (J. T. Baker Co.), as described previously (14). BP1 eluted at ~70% acetonitrile and was immediately dialyzed versus 10 mM sodium acetate buffer, pH 4.0. The 15-kDa proteins that eluted at lower acetonitrile concentrations were dried down in a Speed-Vac microconcentrator (Savant) and resuspended in 10 mM sodium acetate buffer, pH 4.0, with no apparent loss of biological activity.

Ion-exchange FPLC

Cation-exchange chromatography was performed on a Pharmacia LKB Biotechnology Inc. FPLC system using a Mono-S column. The buffer system used was 50 mM sodium phosphate and 1 mM sodium acetate buffer, pH 4.0. After removal of the bacterial pellets were washed twice with half volumes of the incubation medium. Bound proteins were eluted with a linear gradient of sodium chloride. Elution of protein was monitored by measuring absorbance at 214 nm.

SDS-PAGE

SDS-PAGE of crude PMN extracts after incubation with E. coli at pH 4.0 for 15 min at 37 °C was analyzed by SDS-PAGE. Comparison of the starting extract with the supernatant of the bacterial suspensions shows selective depletion of two protein species from the PMN extract; one with an apparent molecular mass of 50 kDa co-migrating with purified rabbit BP1, and one a 15-kDa species. (Fig. 1, lanes a-g). The seletivity of depletion of these components of the extract was most pronounced at higher ratios of extract:bacteria. Under these conditions, "E. coli 55 at subsequent purification of 50-ko- and 15-kDa proteins. The procedure was carried out as described under "Experimental Procedures" and "Results." Lane a, 2.5 μg of crude PMN extract; lanes b-g, unbound material representing 2.5 μg of crude extract, after incubation, respectively, of 50, 100, 200, 500, 1250, and 2500 μg of crude extract with 5 × 10⁷ E. coli 55; lanes h and i, 2.5 × 10⁷ E. coli 55, respectively, before and after incubation with crude extract (conditions of lane j); lane j, 1 μg of protein eluted from E. coli (conditions of lane i) with 200 mM MgCl₂; lanes k-m, protein fractions from reversed-phase HPLC, 0.5 μg, respectively, of BP1, p15A, and p15B.

RESULTS

Preferential Binding to E. coli of 50-kDa and 15-kDa Proteins Present in Crude Acid Extracts of Rabbit PMN

Human BP1 in crude acid extracts of peripheral blood PMN or spleen of patients with chronic myelocytic leukemia can be purified to near homogeneity in one step by taking advantage of the preferential and reversible binding of BP1 to target E. coli (13).

To determine whether the same procedure could be used for the purification of rabbit BP1, crude acid extracts of rabbit PMN were incubated at pH 4.0 for 15 min with E. coli J5 at ratios of extract:E. coli varied over a 50-fold range. After separation of bound and unbound protein by centrifugation of the bacterial suspension, both the supernatant and the bacterial pellets were analyzed by SDS-PAGE. Comparison of the starting extract with the supernatants of the bacterial suspensions shows selective depletion of two protein species from the PMN extract; one with an apparent molecular mass of 50 kDa co-migrating with purified rabbit BP1, and one a 15-kDa species. (Fig. 1, lanes a-g). The selectivity of depletion of these components of the extract was most pronounced at higher ratios of extract:bacteria. Under these conditions, Coo-

Hydrolysis of bacterial phospholipids was measured as release of radiolabel from bacteria that had been pre-labeled during growth with [1-¹⁴C]oleic acid (Du Pont-New England Nuclear) (9). Incubation mixtures were buffered at pH 7.4 with 20 mM sodium phosphate and supplemented with 0.25% BSA to capture labeled phospholipid breakdown products (14). After incubation for 60 min, incubation mixtures were centrifuged in an Eppendorf centrifuge at 14,000 rpm for 3 min to separate unhydrolyzed phospholipids retained in the pellet and breakdown products complexed to the albumin in the supernatant. Hydrolysis was quantitated by counting radioactivity in the supernatant in an LS 5000 TD (Beckman), and was expressed as percent of the total radioactivity present in the incubation mixture.

Amino Acid Analysis

Amino acid composition was determined using a Waters Pico-Tag amino acid analyzer. Samples were pre-treated in vacuo for 2 h at 110 °C with 5.7 mM HCl containing 0.05% phenol.

NH₂-terminal Amino Acid Sequencing

The NH₂-terminal sequence analyses were performed by sequential Edman degradation on an Applied Biosystems 470A or 477.

Determination of Protein Mass

Protein mass was measured either by the Bio-Rad protein assay kit or by the Lowry method (17), using bovine serum albumin as a standard.

- Fig. 1. SDS-PAGE of crude rabbit PMN extracts after incubation with E. coli J5 and subsequent purification of 50-kDa and 15-kDa proteins. The procedure was carried out as described under "Experimental Procedures" and "Results." Lane a, 2.5 μg of crude PMN extract; lanes b-g, unbound material representing 2.5 μg of crude extract, after incubation, respectively, of 50, 100, 200, 500, 1250, and 2500 μg of crude extract with 5 × 10⁷ E. coli J5; lanes h and i, 2.5 × 10⁷ E. coli J5, respectively, before and after incubation with crude extract (conditions of lane j); lane j, 1 μg of protein eluted from E. coli (conditions of lane i) with 200 mM MgCl₂; lanes k-m, protein fractions from reversed-phase HPLC, 0.5 μg, respectively, of BP1, p15A, and p15B.
massie Blue-stained protein profiles of E. coli that had been incubated with or without PMN extracts confirmed that only the 50- and 15-kDa proteins bound appreciably to E. coli (Fig. 1, lanes h and i). At the highest ratio of extract:E. coli, the depletion of the 15-kDa protein became less complete than that of the 50-kDa protein (Fig. 1, lane g), with corresponding enrichment of the 50-kDa protein in the bacterial pellet (not shown), implying that this protein binds more avidly to E. coli. Thus, by choosing the optimal concentration of rabbit PMN extract for a given number of E. coli, selective adsorption of the 50-kDa and the 15-kDa proteins can be achieved, setting the stage for purification of both proteins.

Purification of the 50-kDa and 15-kDa Proteins after Adsorption to E. coli

The adsorbed 50-kDa and the 15-kDa rabbit proteins could be eluted nearly quantitatively when the bacteria were resuspended in 200 mM MgCl₂ (Fig. 1, lane j). This procedure results in no detectable elution of bacterial proteins (13). Fig. 2 shows that the 50-kDa and the 15-kDa protein species in the Mg²⁺ eluate could be separated completely by reversed-phase HPLC using a shallower acetonitrile gradient of 0–70% acetonitrile over 60 min (not shown). Both species migrated with an apparent molecular mass of 15 kDa (Fig. 1, lanes l and m), and are designated p15A (earlier peak) and p15B (later peak).

A rough estimate of the BPI and 15-kDa protein content in PMN extracts based on Coomassie Blue staining after SDS-PAGE indicated that the two protein species were present in approximately the same amounts of ~1 mg/10 ml extract (1.8 × 10⁸ PMN equivalents). About 400 µg of each protein was obtained after the purification, resulting in a recovery of ~40%. Subsequent separation of p15A and p15B revealed an apparent mass ratio of ~1:2, respectively.

Evidence that p15A and p15B Are Structurally Similar Isoforms

Differences between p15A and p15B were also observed during cation-exchange chromatography on a Mono-S column (Fig. 3). Elution of p15A required a higher NaCl concentration than elution of p15B (1.5 vs. 1.1 M NaCl, respectively), suggesting that the former is more basic than the latter. The amino acid compositions of p15A and p15B are indistinguishable, each with a high content of arginine, glutamine/glutamic acid, and proline residues (Table Ia). In addition, the NH₂-terminal amino acid sequences of the two proteins are identical in at least 19 of 20 residues (Table Ib). A search in the Bionet data bank revealed no significant similarity with any known protein sequences, including that of human BPI. Thus p15A and p15B are closely similar but distinct proteins.

Biological Properties of the 15-kDa Isoforms

p15A Potentiates Early Growth Inhibition by BPI—The avid binding of the two 15-kDa isoforms to E. coli raised the possibility that these previously unrecognized proteins contribute to the antimicrobial action of rabbit PMN. When tested against E. coli J5, neither p15A nor p15B, alone or in combination, caused any loss of viability even when added at molar concentrations that were 100-fold higher than a minimal growth inhibitory dose of BPI (Fig. 4). However, addition of p15A in combination with BPI markedly reduced the dose of BPI required for inhibition of bacterial growth (Fig. 4a). The magnitude of this potentiating effect depended on the dose of both BPI and p15A. At a very low non-growth inhibitory BPI dose (e.g. 10 ng/10⁸ bacteria), addition of ≥1 µg of p15A caused 50–60% reduction in colony forming units in nutrient agar. More complete (>95%) growth inhibition was produced at higher BPI doses, with the amount of p15A required declining as the BPI dose was increased (Fig. 4a).

Under optimal conditions, p15A reduced by 6–8-fold the inhibitory BPI dose (e.g. 10 ng/10⁸ bacteria), addition of ≥1 µg of p15A caused 50–60% reduction in colony forming units in nutrient agar. More complete (>95%) growth inhibition was produced at higher BPI doses, with the amount of p15A required declining as the BPI dose was increased (Fig. 4a).
Analyses of amino acid composition (a) and partial NH₂-terminal sequence (b) of the 15-kDa isoforms

The analyses were carried out as described under "Experimental Procedures."

<table>
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<th>Amino acid</th>
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<th>p15B (mol %)</th>
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<td>Glx</td>
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<td>Leu</td>
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<tr>
<td>Lys</td>
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(b)

p15A: IlePro( )ArgArgLeuArgTyrGluValValAlaGlnAlaLeuGlnPheGluAsn
p15B: IleProHisArgArgLeuArgTyrGluValValAlaGlnAlaLeuGlnPheGluAsn

* Not determined.

FIG. 4. Dose-dependent effect of p15A and/or p15B on inhibition by BPI of growth of E. coli J5.
Inhibition of colony formation was measured after addition of p15A (0-5 µg) (a), p15B (0-5 µg) (b), or p15A + p15B (mass ratio ~1:2) (0-2 µg) (c) to 10⁶ bacteria (~5 min at room temperature), followed by rabbit BPI (0-0.08 µg), under the incubation conditions described under "Experimental Procedures." The data are expressed as percent of colony forming units in samples of bacteria incubated alone. The results shown are of one of at least three similar experiments.

The absence of a potentiating effect at high p15B doses is also illustrated in Fig. 5b.
When both p15A and p15B (p15A:p15B, ~1:2) were present together with sub-inhibitory doses of BPI, significant potentiation of growth inhibition was observed at low (≤1 µg) p15A/p15B doses with little or no effect at higher doses (≥1 µg), reflecting the influence of both isoforms under the former conditions, and the dominance of the p15B effect in the latter case (Fig. 4c).

The potentiating effect of the 15-kDa proteins on bacterial growth inhibition by BPI was also seen with more BPI-resistant smooth E. coli (results not shown).

Both p15A and p15B Inhibit the Late Bactericidal Action of BPI—Bacterial growth arrest in nutrient agar triggered by lethal amounts of BPI can initially be overcome by adding serum albumin to the growth medium (Fig. 6, a and b; Ref. 16). After longer incubations with BPI, an increasing fraction of the bacteria can no longer be rescued by albumin (Fig. 6b), coincident with progression of injury from the outer bacterial
Novel Protein Modulators of PMN Cytotoxins

**FIG. 6.** Inhibition by the 15-kDa proteins of BPI-mediated irreversible growth inhibition of *E. coli* J5. Experiments were carried out with both rabbit and human BPI and yielded similar results. The results were therefore pooled and are presented as means ± S.E. Data are from at least five experiments. **a**, growth arrest in liquid media. Bacterial growth was monitored by measuring the absorbance at 550 nm as described under "Experimental Procedures." Growth was followed in the absence (control) or presence of lethal (0.4-1.5 μg/10^7 bacteria) and sub-lethal (0.2-0.5 μg/10^7 bacteria) doses of BPI, and in the presence of sub-lethal BPI doses supplemented with p15A (2.5-5 μg/10^7 bacteria). The data are from at least five experiments. **b**, time-dependent bacterial growth inhibition in albumin-supplemented nutrient agar. Bacterial viability in bovine serum albumin-supplemented nutrient agar was measured after incubation with lethal BPI doses, or sub-lethal BPI doses with or without p15A and/or p15B for 10-120 min, as described under "Experimental Procedures." Results are expressed as percent of colony forming units of bacteria at time 0. The effects on irreversible growth inhibition by BPI of p15A or p15B alone were the same as of the two isoforms in combination, and were therefore pooled.

**FIG. 7.** Effect of the 15-kDa isoforms on the action of BPI on the bacterial outer envelope. The increase in permeability of the outer membrane of *E. coli* J5 to actinomycin D and the activation of bacterial phospholipolysis were measured as described under "Experimental Procedures." In all assays the 15-kDa proteins were added and incubated with the bacteria at room temperature for 5 min before adding BPI and further incubation at 37°C. **a** and **b**, permeability-increasing activity. *E. coli* (10^7) were incubated with p15A (a) or p15B (b) (0-5 μg) and actinomycin D, followed by rabbit BPI (0-0.18 μg). After addition of BPI incubation was continued at 37°C for 10 min. Loss of colony formation on BSA-supplemented nutrient agar after exposure to actinomycin D reflects permeabilization of the bacteria to this drug. The data are expressed as percent of bacteria incubated alone. **c**, activation of bacterial phospholipolysis. *E. coli* (10^7) prelabeled with [3H]oleic acid were incubated with 2 μg of either p15A or p15B, followed by rabbit BPI (0-3 μg). Results are presented as percent of total labeled bacterial phospholipid that was hydrolyzed (measured as described under "Experimental Procedures").

envelope to the cytoplasmic membrane (i.e. from sublethal to apparently lethal) (16). Addition of p15A to sublethal doses of BPI triggered similar growth inhibition (Fig. 6a) that remained reversible by albumin throughout prolonged incubation (Fig. 6b), indicating that p15A potentiates the initial (reversible) but not the late (irreversible) effects. Addition of either p15A or p15B with normally lethal doses of BPI actually inhibited progression to late bactericidal events (Fig. 6b).

**p15A Potentiates the Outer Membrane Alterations of BPI**—The initial growth inhibitory effect of BPI is typically accompanied by an increase in bacterial outer membrane permeability (e.g. to the normally impermeant drug actinomycin D) and activation of bacterial phospholipolysis (8, 9). Fig. 7, a
and c. shows that p15A when added alone did not affect bacterial outer membrane permeability or phospholipolysis, but reduced by 10-20-fold the amount of BPI required to produce these effects. The amount of p15A required for maximal potentiation of these effects (-2 μg) was similar to that required for potentiation of bacterial growth inhibition. Again, in contrast to p15A, p15B caused little or no potentiation of these envelope effects of BPI (Fig. 7, b and c).

Effect of the 15-kDa Proteins on the Action of Other Antibacterial Agents—Table II shows that the 15-kDa proteins potentiated similarly the growth inhibitory effects of rabbit and human BPI, and the bioactive 25-kDa NH₂-terminal fragment of human BPI (14). In contrast, the action of two other human PMN antibacterial proteins, the defensins (18, 19) and azurocidin (10, 20), were, respectively, unaffected and inhibited by the 15 kDa proteins. Serum complement- and polymyxin B-mediated killing of E. coli were also unaffected by these 15-kDa proteins (Table II).

**DISCUSSION**

The preferential binding to E. coli of BPI in crude acid extracts of human PMN provides a remarkably easy and efficient means of purification of this protein (13). Application of the same procedure to crude extracts of rabbit PMN has now led to the identification and ultimate isolation of two closely similar 15-kDa proteins that, in addition to BPI, bind avidly to E. coli. The preferential binding to E. coli of BPI and the 15-kDa proteins in the complex mixture of antibacterial cationic proteins present in the crude extracts is apparently a function of both the relative affinities of the various proteins to these bacteria, and of the number of bacterial binding (surface) sites available. At relatively low doses of PMN extract added, many proteins in the extract bind to E. coli (Fig. 1). However, as the amount of extract added is increased, the spectrum of proteins depleted from the extract by binding to the bacteria becomes more limited, indicating an unequal competition among the various proteins of the PMN extract for a limited number of bacterial surface sites. Our findings suggest that a hierarchy is evident under a broad range of experimental conditions including pH (4.0-7.5) and salt concentration (10-150 mM NaCl) (results not shown). The purified 15-kDa proteins also bind to E. coli in the absence of BPI showing that BPI-induced surface changes are not needed for their binding.²

The two 15-kDa proteins cannot be distinguished by analysis of their amino acid compositions and the NH₂-terminal sequences of the first 20 residues (Table I), indicating that these two proteins are structurally nearly identical isoforms. Whether the different behavior of the two proteins during reversed-phase HPLC and ion-exchange chromatography is attributable to primary structural differences or to post-translational modifications remains to be established. Functionally, p15A and p15B are also very similar in many ways. The binding properties of the two proteins to E. coli, either in the presence or absence of BPI, are thus far indistinguishable, and neither protein by itself or together with the other isoform produces any detectable antibacterial effects (Fig. 4). Both isoforms can alter the antibacterial activity of BPI but it is here that p15A and p15B show significant functional differences.

The effects of the 15-kDa proteins on BPI action are apparently complex, mirroring the complexity of BPI action on target bacteria. We have recently shown that BPI produces its antibacterial effects in two distinct stages: 1) early and reversible growth arrest and envelope alterations that are restricted to the outer membrane, and 2) later, apparently irreversible, growth inhibition that is accompanied by cytoplasmic membrane damage affecting the bacterial metabolic machinery (16). The 15-kDa proteins have opposite effects on these two stages of BPI action, potentiating the early (Figs. 4, 5, and 7), but inhibiting the late effects (Fig. 6). It is in the potentiating effect that the functional differences between p15A and p15B are manifest. The isoform p15A strongly potentiates the early reversible action of BPI, reducing the amount of BPI needed for initial growth inhibition and envelope alterations by 5-20-fold. In contrast, p15B only modestly potentiates these early effects of BPI at low concentrations and has no appreciable effect at higher concentrations.

The basis of the synergistic action of p15A and BPI is not known. It is possible that sublethal concentrations of BPI unmask an otherwise cryptic antibacterial effect of p15A, but we believe it is more likely that p15A potentiates the action of BPI. All aspects of the combined action of p15A and BPI closely simulate the early antibacterial effects produced when higher concentrations of BPI are added alone. This includes the manifestations of outer envelope injury and continued metabolic integrity of the growth-inhibited bacteria (not shown) and the ability of these bacteria to be rescued by BSA, all hallmarks of BPI-treated E. coli (8, 9, 16). Moreover, no synergism was observed when p15A was combined with sublethal amounts of several other membrane-disruptive antibacterial agents (Table II).

We have postulated before that only a fraction of the bound BPI is needed for growth-inhibition because removal of 80-90% of bound BPI by high concentrations of Mg²⁺ or Ca²⁺ does not reverse the growth inhibition (21). The fact that only 10-20% of the normal growth-inhibitory dose of BPI is needed when p15A is present supports this hypothesis and shows further that initial "full occupancy" by BPI is not necessary to arrest bacterial growth. BPI is also more potent at pH 6.0 (versus pH 7.4) despite equal binding (16, 21) further dem-

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**TABLE II**

<table>
<thead>
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<th>Antibacterial agent</th>
<th>15-kDa protein*</th>
<th>Potentiation</th>
<th>Inhibition</th>
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<tr>
<td>Rabbit BPI</td>
<td>1.0-4.0</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Human BPI</td>
<td>1.2-4.0</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>25-kDa NH₂-terminal fragment of human BPI</td>
<td>1.2-4.0</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Azurocidin</td>
<td>0.2</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Human defensins</td>
<td>1.0-5.0</td>
<td>--</td>
<td>+++</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0.5-3.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>4.0</td>
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</table>

* The 15-kDa protein sample used was a mixture of the isoforms at a mass ratio of 1:3 of 15-A:15-B.

onstrating that there is no stringent relationship between total BPI binding and its initial growth-inhibitory effect. In fact, the combination of p15A and lower pH (6.0) reduces the BPI dose requirements for bacterial growth inhibition by nearly 20-fold.

Because removal of bound BPI by Mg\(^{2+}\) triggers repair of much of the outer envelope damage initially inflicted by BPI (21), we have concluded before that, in contrast to the effect on growth, the outer membrane alterations require sustained full occupancy of surface sites by BPI. However, the potentiation of p15A (and lower pH) of this aspect of BPI action strongly suggests that only a small fraction of the normal BPI dose also suffices for the permeability-increasing and phospholipase-activating effects. These findings imply that most of the bound BPI occupies sites that are not directly involved in early BPI action. Thus, a possible explanation for the potentiating effect of p15A is its occupancy of “nonessential” sites, thereby directing BPI to qualitatively different sites that must be occupied by BPI for expression of its early antibacterial actions.

It is unlikely that mere binding of p15A to E. coli accounts for this potentiation of BPI action. The other isoform p15B apparently binds to a similar extent with little or no potentiating effects. Furthermore, a lipopolysaccharide-binding protein isolated from serum (23) that also binds to E. coli actually inhibits the early BPI effects.

The inhibition by both 15-kDa proteins of the late BPI effects, while apparently contradictory to the potentiating effects, actually solidifies our evidence showing that the early and later actions of BPI on E. coli can be completely separated (16) and suggests that BPI produces different effects, depending on what anatomical or “functional” sites it occupies.

We have taken the preferential binding to E. coli of BPI in crude extracts of human PMN (13) as another argument that, within the phagosomal environment where many other antimicrobial proteins and peptides are present, BPI plays a primary role in the action of PMN toward Gram-negative bacteria. We have now shown that rabbit PMN contain yet another protein species (p15A and p15B) that binds preferentially to E. coli, and that each of the isoforms, either alone or in combination, although without apparent independent effects on the bacteria, can modulate the action of BPI and other PMN proteins (Fig. 4; Table II). The 15-kDa proteins may be delivered to ingested bacteria along with BPI, since granule-rich preparations of rabbit PMN are enriched with both BPI and the 15-kDa proteins. These observations underscore the potential for complex interactions among the antibacterial proteins of the PMN, not only as isolated proteins but also in the intact PMN. The 15-kDa proteins may optimize the utilization of BPI for initial growth arrest of large numbers of ingested bacteria or of more resistant strains when the amount of available BPI might be limiting. A contribution of the 15-kDa proteins to the fate of ingested E. coli is also suggested by our recent demonstration that these bacteria are actually killed much more slowly than when treated by BPI (24). In fact, this prolonged intracellular survival is remarkably similar to the effect of the 15-kDa proteins on the killing of BPI. The discovery of the 15-kDa isoforms that can modulate the actions of other PMN proteins further illustrates the need for caution in the assignment of definitive roles to individual members of the antimicrobial arsenal, particularly when viewed within the intact phagocyte.

Acknowledgments—We thank Drs. David Campanelli and Carl Nathan (Division of Hematology-Oncology, Cornell University Medical College) for carrying out the bioassays with azurocidin and for the generous gift of human defensins and Mike Jefferies for carrying out the protein sequence homology searches.

REFERENCES

Isolation of two isoforms of a novel 15-kDa protein from rabbit polymorphonuclear leukocytes that modulate the antibacterial actions of other leukocyte proteins.

C E Ooi, J Weiss, O Levy and P Elsbach


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