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Chemotactic and protease-inhibiting activities of antibiotic peptide precursors

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We have recently shown that two antimicrobial peptides (Bac5 and Bac7) and/or their immature forms (proBac5 and proBac7) can be released extracellularly from activated neutrophils. In the present study we have investigated the biological activities of the immature forms, which do not exhibit antimicrobial effects. We show that proBac7 is a monocyte-selective chemoattractant, potentially contributing to the recruitment of these cells to infection sites, whereas proBac5 efficiently inhibits the in vitro activity of cathepsin L, a cysteine proteinase thought to contribute to tissue injury in inflammation.

Bactenecin; Probactenecin; Monocyte chemotaxis; Cathepsin L inhibition

1. INTRODUCTION

Neutrophils are the primary effector cells in acute inflammation. They are rapidly recruited from the blood stream by adhesion to, and migration through, the vascular endothelium, and chemotaxis to injured or infected tissue. The various stages of this complex process are accompanied by the release of specific factors, which are instrumental to transendothelial migration, amplification of the inflammatory and immune responses, and defense reactions [1–5].

In recent years we have characterized a variety of antimicrobial peptides of bovine neutrophils [6–11]. These include two proline- and arginine-rich peptides, bactenecins or Bac5 and Bac7, which are stored in the large granules as precursors [9]. Activation of neutrophils causes the release of bactenecins and/or their proforms [10]. Since proBac5 and proBac7 are devoid of any antimicrobial activity [9,11], we thought they might perform other bioactivities unrelated to pathogen inactivation. In particular, since the initial wave of neutrophils, typical of the inflammatory response, is rapidly followed by a second wave of monocytes, we checked whether they might become engaged in monocyte recruitment. Second, since the pro-region of Bac5 has a high identity with a specific inhibitor of cathepsin L [12], proBac5 was tested for its potential inhibitory effect on such a cysteine proteinase.

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Abbreviations: TFA, trifluoroacetic acid; PBS, phosphate buffered saline; BSA, bovine serum albumin; Z, benzyloxycarbonyl-; BANA, Na-benzoyl-DL-Arg-β-naphtylamide; AMC, 7-amido-4-methylcoumarin; PMA, phorbol 12-mirystate 13-acetate.

2. MATERIALS AND METHODS

2.1. *Purification of Bac5 and Bac7 and their precursors*

Bac5 and Bac7 were purified from granule extracts (200 mM sodium acetate, pH 5) of bovine blood neutrophils as previously described [7]. To purify their precursors, the granular extracts were applied to a CM-cellulose column (CM-52, Whatman Inc., Maidstone, Kent, UK) equilibrated with the extraction buffer. The column was eluted with 0.1, 0.2, 0.3 and 1 M NaCl in the same buffer, and the fractions collected were dialysed against 0.1% TFA. Those containing proBac5 or proBac7, as identified by Western blot analysis, were vacuum concentrated, re-dissolved in 0.1% TFA, and then loaded on a Pep RPC 10/10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Elution was performed with a 0–100% acetonitrile gradient in 0.1% TFA, and fractions corresponding to proBac5 or proBac7 were pooled, lyophilised, and either diluted in PBS (pH 7.4)/ 0.5 mM Ca^{2+}/ 1% BSA for assays of chemotactic activity, or dissolved in distilled water for protease inhibition studies.

2.2. *Analytical assays*

Protein concentration was measured with bicinchoninic acid (Pierce Chemical Co., Rockford, IL) using BSA as the standard. SDS-PAGE was carried out under reducing conditions in 14% polyacrylamide gel, and Western blots were performed as in [9].

2.3. *Monocyte preparation and chemotaxis assay*

Monocytes were prepared from the interface buffy coat, generated after differential centrifugation (2 000 × *g*, 20 min) of bovine blood. After lysing the erythrocytes, the mononuclear cells were centrifuged at 700 × *g* for 20 min at 4°C through a Ficoll-Paque gradient (Pharmacia LKB Biotechnology). The final preparations contained 60–70% monocytes and 30–40% lymphocytes, as determined by non-specific esterase assay [13]. Migration of monocytes was evaluated in a multiwell chamber (Neuro Probe Inc., Cabin John, MD) [14]. A 5-μm pore size polycarbonate filter (Nucleopore Corp., Pleasanton, CA) was used to separate the upper wells containing 0.75×10^5 monocytes in 50 ml of PBS/0.5 mM Ca^{2+}/1% BSA from the lower wells, which

contained the putative chemotactic factors in 50 μl of the same buffer. Neutrophil chemotaxis was performed with 8 μm polyvinylpirrolidone-free polycarbonate filters Nucleopore). Negative- and positive controls were run with dilution buffer and 5% zymosan-activated bovine serum, respectively. Experiments were carried out in quadruplicate with 10^{-7}–10^{-11} M proBac7, Bac7, proBac5 or Bac5. After 90 min at 37°C in air with 5% CO_2, the filters were carefully removed and stained with Diff-Quick (Harleco, Gibbstown, NJ). Five oil immersion fields were counted, and the activity was expressed as percentage of cells migrated over negative controls.

2.4. *Enzyme assays*

Human kidney cathepsin L (Calbiochem, CO), human liver cathepsin B (Novabiochem, CO) were assayed with Z-Phe-Arg-AMC, and Z-Arg-Arg-AMC, respectively [15]. The fluorescence intensity (λ_{ex} 370 nm, λ_{em} 460 nm) of the released aminomethylcoumarin was recorded continuously for 15–20 min. Papain (Sigma, St. Louis, MO) was assayed with BANA (Sigma) as substrate [16]. For the determination of the inhibition constant, 0.1 nM cathepsin L was incubated 30 min at 37°C with 20–500 nM proBac 5 before adding 5, 10, 20 μM Z-Phe-Arg-AMC (final concentrations). The reaction was stopped after 10 min with 2 ml of 1 mM iodoacetic acid. Fluorescence intensity was measured as above, and the K_i was calculated by the Eisenthal–Cornish Bowden and the Bieth and Dixon plots.

3. RESULTS AND DISCUSSION

3.1. *Chemotactic activity*

Previous experiments [10] showed that over 50% of the proBac5 and proBac7 content of neutrophil granules can be released into the extracellular medium upon cell exposure to PMA, commonly used as a secretagogue for neutrophils. This finding thus indicates that neutrophils may respond to some stimuli by delivering proBac5 and proBac7 to extracellular targets. Based on the observation that neutrophil recruitment to inflammation sites precedes a large mobilization of monocytes, we examined whether the released antibiotic precursors may contribute to the generation of chemotactic signals for monocytes .

The chemotactic activity was tested using the modified Boyden chamber technique, with various concentrations of the putative chemoattractants. Fig. 1 shows that proBac7 displays a chemoattractant activity which peaks at 10^{-9} M. At this concentration the activity of proBac7 is about one third of that exerted by activated serum (624 ± 53). The chemoattractant activity of the antibiotic peptide Bac7, generated by proteolytic maturation of proBac7, is much lower (Fig. 1), and does not follow the bell-shaped curve typical of chemotactic factors. ProBac5 and Bac5 in the same concentration range do not cause monocyte chemotaxis (not shown). Finally, at the concentrations tested, all these peptides practically do not induce any activation of random or oriented neutrophil migration (not shown).

The monocyte-selective chemotactic activity exhibited by proBac7 suggests that this peptide may be one of the factors released by bovine neutrophils to amplify the inflammatory response. Although the observed chemoattraction is not as marked as that exhibited by complement fragments present in zymosan-activated serum,

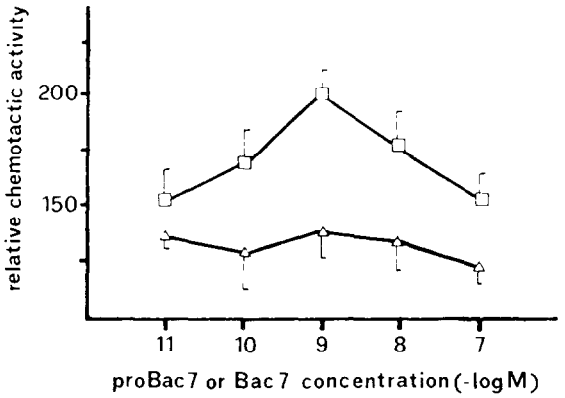

Fig. 1. Chemotactic response of bovine monocytes to proBac7 (□) and Bac7 (△). Migration of monocytes was evaluated by using a multiwell microchamber technique. Relative chemotacic activities are means ± S.E.M. of 5–12 cell preparations, and were calculated as a percentage of migrated cells over negative controls

the contribution of proBac7 to the generation of chemotactic signals is nevertheless likely to be significant. In fact, a gradual release of this polypeptide from neutrophils in the inflammatory site, coupled to its relative stability [10], would secure cumulative and persistent effects in monocyte chemoattraction.

The chemotactic activity of proBac7 appears to be confined to the pro-region of the molecule, since its maturation product Bac7, endowed with antibiotic activity, has little or no effect on the migration of monocytes. Conversely, other neutrophil antimicrobial peptides, such as human defensins and CAP37, are good monocyte-selective chemoattractants [17–18].

3.2. *Inhibition of cathepsin L by proBac5*

Molecular cloning of Bac5 has shown that the region upstream of the antibiotic peptide (pro-sequence) shares about 70% identity with cathelin [12], an inhibitor of the lysosomal cysteine proteinase, cathepsin L, isolated from pig leukocytes [19]. Cathepsin L is present in inflammatory cells [20], and may contribute substantially to tissue injury at inflammatory lesions, due to its potent elastinolytic and collagenolytic activities [21,22].

ProBac5 purified from bovine neutrophils was therefore tested for its potential inhibitory activity on this enzyme as well as on other cysteine proteinases.

The hydrolytic activity of cathepsin L on the synthetic substrate Z-Phe-Arg-AMC is inhibited by 80% within a few minutes after the addition of 10^{-6} M proBac5 (Fig. 2A). Parallel control experiments show that 10^{-7} M leupeptin exerts full inhibition of this proteinase. The inhibition constant for the interaction of proBac5 with cathepsin L is 6×10^{-8}M. Since the proteolytic fragment Bac5 has virtually no effect on the activity of cathepsin L (Fig. 2A), the inhibitory effect appears to be mediated by the pro-sequence of the precursor, as expected [12].

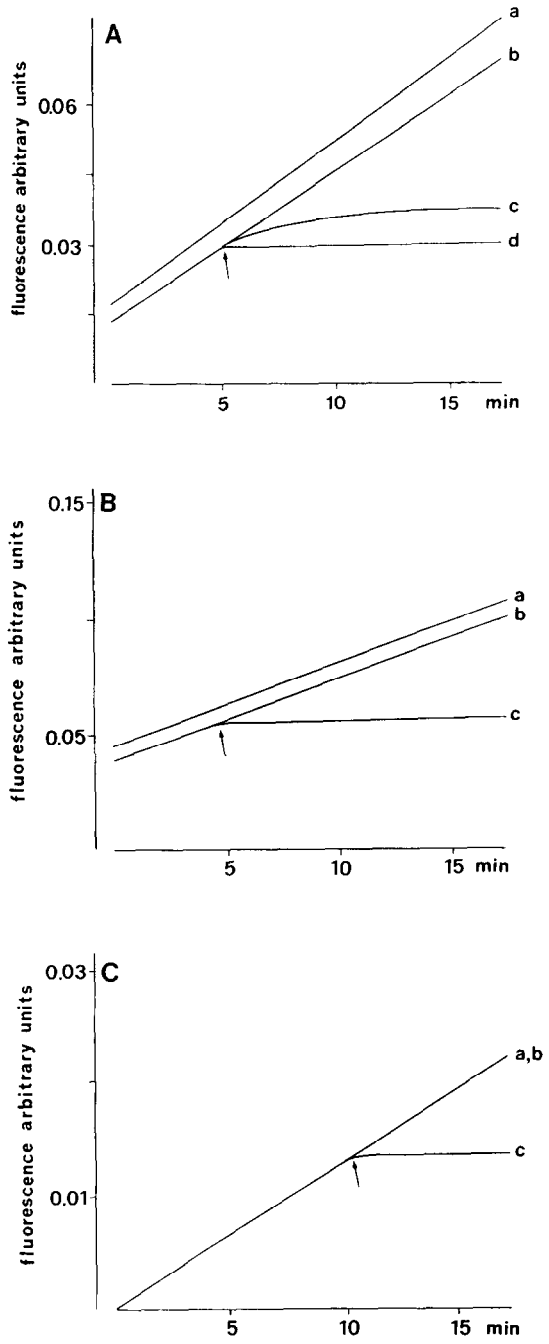

Fig. 2. Effect of proBac5 and Bac5 on the activity of cysteine proteinases. (A) Cleavage of the fluorogenic substrate Z-Phe-Arg-AMC by cathepsin L (2.5 ng) alone **(a)**, and in the presence of either 10^{-6} M Bac5 **(b)** or 10^{-6} M proBac5 **(c)** or 10^{-7} M leupeptin **(d)**. Fluorescence measurements were made at a concentration of cathepsin L corresponding to the hydrolysis of 33.75 pmol substrate/min. (B) Cleavage of the fluorogenic substrate Z-Arg-Arg-AMC by cathepsin B (20 ng) alone **(a)**, and in the presence of either 10^{-6} M proBac5 **(b)** or 10^{-7} M leupeptin **(c)**. Fluorescence measurements were made at a concentration of cathepsin B corresponding to the hydrolysis of 240 pmol substrate/min. (C) Cleavage of the fluorogenic substrate BANA by papain (10 mg) alone **(a)**, and in the presence of 10^{-6} M proBac5 **(b)** or 10^{-7} M leupeptin **(c)**. Fluorescence measurements were made at a concentration of papain corresponding to the hydrolysis of 207 nmol substrate/min. Arrows indicate the addition of proBac5 or Bac5 or leupeptin.

Cathepsin L appears to be a specific target for this inhibitor, since other cysteine proteinases, such as cathepsin B (Fig. 2B) and papain (Fig. 2C) are unaffected by proBac5, while completely inhibited by leupeptin.

Due to its elastinolytic and collagenolytic activities [21,22], cathepsin L is a major factor in tissue degradation. In particular, it can degrade glomerular basement membrane [23] and cause bone resorption [24]. Inhibition of this proteinase by a polypetide released in relatively large amounts from neutrophils at inflammatory sites might thus be of great significance in prevention of tissue damage.

Overall, our results emphasize the marked efficiency of cell-dependent innate immunity. Polypeptides secreted from neutrophils appear to be structured to respond to different functional requirements, including amplification of the response, host defense, and tissue protection from inflammatory damage, while in some cases combining more than one such activity within the same molecule.

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