Bactenecins, Defense Polypeptides of Bovine Neutrophils, Are Generated from Precursor Molecules Stored in the Large Granules

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Abstract. Bactenecins are highly cationic polypeptides of bovine neutrophil granules and exert in vitro a potent antimicrobial activity. We have previously purified two bactenecins, designated in an abbreviated form Bac7 and Bac5 from their approximate molecular masses of 7 and 5 kD (Gennaro, R., B. Skerlavaj, and D. Romeo. 1989. *Infect. Immun.* 57:3142–3146). Here we have studied the biosynthesis, processing, and localization of precursors of Bac7 and Bac5 in bovine bone marrow cells of the myeloid lineage. In vitro translation directed by mRNA isolated from these cells has shown that the primary translation products are preprobactenecins of 23.5 and 21 kD, and are processed to polypeptides of 20 and 15.8 kD, respectively. The 20-kD polypeptide is the granule storage form of Bac7, or proBac7, as also demonstrated by Western blot analysis of lysates of peripheral neutrophils. Between 15 and 50 min from the beginning of its biosynthesis the 15.8-kD polypeptide is converted into the 15-kD granule storage form of Bac5, or proBac5. As shown by immunogold EM, proBac7 and proBac5 are sorted and targeted to the matrix of the so called large granules, which are the predominant organelles in the cytoplasm of bovine neutrophils and are the exclusive store of the nonoxidative antimicrobial system of these cells. Solubilization of granules with Triton X-100 with concomitant unmasking of proteases leads to cleavage of the proforms to Bac7 and Bac5. Experiments performed with protease inhibitors suggest that the proteolytic cleavage is catalyzed in detergent-solubilized neutrophils by neutral serine protease(s), very likely derived from the azurophil granules.

T HE vacuolar apparatus of neutrophils of most animal species, including man, essentially consists of two granule populations, the specifics, and the lysosome-like, peroxidase-positive azurophils (4). Tightly packaged in these granules are different sets of proteins that are designed for microbial inactivation and degradation. Upon ingestion of microorganisms by the neutrophil, granules of both populations fuse with newly formed phagocytic vacuoles, thus exposing the ingested material to the action of these proteins (15). Apart from myeloperoxidase (17), the contribution to the overall antimicrobial activity given by the discharged enzyme proteins, such as the acid hydrolases and neutral proteases of azurophils, or lysozyme of azurophils and specifics, has not in general been clearly established. In contrast, some of the noncatalytic proteins have been identified as potent microbicidal factors (8). Mixing of these factors and enzymes in the phagosomes may lead to important cooperative effects, thus providing the neutrophil with very efficient defense mechanisms.

The neutrophils of cow, goat, and sheep also contain a third population of granules, called large granules, which outnumber the azurophils and the specifics (3, 10). Analysis of bone marrow specimen has shown that the formation of the large granules is a major event in the maturation process of the neutrophil, and occurs at the myelocyte stage (3).

In comparison with the azurophils and specifics, the large granules contain no serine or metalloproteases, acid hydrolases, or peroxidase (10). In contrast, they contain a set of strongly cationic polypeptides, which are thought to be responsible for the nonoxidative antimicrobial activity, associated with these granules (10).

The large granules of neutrophils may thus play an important function in the defense of ruminants against invading microbes, very likely involving in a cooperative effort proteins belonging to the azurophils and the specifics.

The study of the biosynthesis of the defense proteins of the large granules and of their maturation to cidal factors may reveal the interplay of these proteins with components of other granules playing supportive roles as well as help define their function in vivo.

We have addressed this point by studying the processing of precursors of two cationic defense polypeptides of 7 and
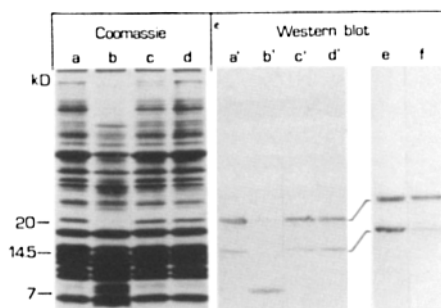
Figure 1. Immunodetection of Bac7 precursors. The IgG fraction of rabbit antiserum against Bac7 was used to screen for the presence of precursor molecules in bovine neutrophils or isolated granules. Proteins (40–60 μg/lane) were fractionated by SDS-PAGE, electroblotted onto nitrocellulose paper, and immunostained, as detailed in Materials and Methods. Lanes *a–d*, Coomassie blue stain; lanes *a′–d′*, *e*, and *f*, Western blot. Before SDS-PAGE, bovine neutrophils were either acid-precipitated with 10% TCA (lanes *a* and *a′*) or solubilized with 1% Triton X-100 in either PBS (lanes *b* and *b′*) or 0.2 M Na-acetate buffer, pH 4 (lanes *c* and *c′*). Alternatively, before the solubilization in Triton X-100, neutrophils were treated with DFP (lanes *d* and *d′*). Isolated granules were TCA-precipitated (lane *e*). Secretion of the recognized products upon cell stimulation with a secretory stimulus is analyzed in lane *f*; neutrophils were incubated for 30 min at 37°C with 30 nM PMA, proteins were TCA precipitated from the cell-free media, and then subjected to electrophoresis and blotting.

5 kD that we have previously purified from granules of bovine neutrophils (11). These peptides have been called bactenecins, from the Latin words bacterium and necare (to kill), and are designated Bac7 and Bac5 in an abbreviated form. The basic tools in our studies have been specific antibodies raised against Bac7 and Bac5, which both contain >45% proline and 24% arginine (11) although with a totally different amino acid sequence (Frank et al., 1990).

In vitro Bac7 and Bac5 efficiently kill *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae*, and arrest the growth of *Enterobacter cloacae*, at concentrations of 10–50 μg/ml. Their mechanism of action involves binding to the outer membrane of Gram-negative bacteria, followed by a rapid translocation to the inner membrane, where they impair the electron transport chain as well as some membrane activities dependent on the production of energy (Skerlavaj, B., D. Romeo, and R. Gennaro, manuscript submitted for publication). In addition, Bac7 also inactivates human herpes simplex virus types 1 and 2 (32).

Here we describe the biosynthesis of proforms of Bac7 and Bac5, which are targeted to the large granules of the neutrophils. To our knowledge, these are the first antigens identified as specific components of the large granules. We have also found that preprobactenecins are synthesized in immature bone marrow cells, where their processing is carried out to the storage forms of 20 and 15 kD (proBac7 and proBac5, respectively). The stored precursors can be cleaved to the mature antimicrobial peptides by neutral serine protease(s), which very likely derive from the azurophils. We conclude that this event occurs when the contents of both large granules and azurophils are discharged into the phagosome during the performance of the defense function by the neutrophil.

Materials and Methods

Extraction of Bovine Neutrophil Granules and Purification of Bactenecins

Granules of peripheral bovine neutrophils were extracted in 200 mM sodium acetate, 5 mM EDTA, pH 4, as previously reported (19). Bac7 and Bac5 were purified from the granule extracts as previously described (11).

Antibodies

Rabbit antisera to Bac7 or Bac5 were obtained after repeated immunizations of rabbits with 100 μg of Bac7 or Bac5 in complete Freund adjuvant. The Ig fraction of these antisera was precipitated with 40% sodium sulfate, followed by affinity purification of the IgG fraction on Protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Uppsala, Sweden).

When required, monospecific anti-Bac7 IgG were further purified from total IgG by immunoaffinity chromatography on purified Bac7, coupled to Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA). The Ig fraction of anti-human myeloperoxidase antiserum was purchased from Dakopatts (Glostrup, Denmark), and shown to cross-react with bovine myeloperoxidase by Western blot analysis.

Preparation of Peripheral Leukocytes and of Bone Marrow Cells

Polymorphonuclear leukocytes were purified from fresh bovine blood by differential centrifugation followed by hypotonic lysis of contaminating erythrocytes (21). This procedure led to leukocyte preparations containing >93% neutrophils.

Eosinophils were separated from neutrophils by centrifugation through a gradient of colloidal silica (Percoll; Pharmacia LKB Biotechnology) (21).

The monocyte/lymphocyte fraction was prepared from the interface buffy coat, generated after differential centrifugation of bovine blood, by lysing the erythrocytes with isotonic NH₄Cl. Less than 1% granulocytes was present in this fraction.

Human polymorphonuclear leukocytes, containing >95% neutrophils, were prepared from peripheral blood by the standard technique of dextran sedimentation and Ficoll-Hypaque gradient centrifugation.

Bone marrow was collected from the first and the second rib of cattle soon after slaughtering. Ribs were cut open with a cast cutter. The marrow was then quickly transferred with a spatula into a tube containing 1 mM EDTA in PBS prewarmed to 37°C. Coarse debris and clots were sedimented and lipid aspirated with a Pasteur pipette. The cell suspension was then passed through a screen of filter cloth to remove small clumps and quickly diluted in PBS containing 10 mM glucose. Erythrocytes and erythroid cells were lysed with isotonic NH₄Cl and mature granulocytes were removed by Ficoll-Hypaque gradient centrifugation at 800 *g* for 40 min (30). The layer at the Ficoll-Hypaque/PBS interface, enriched in immature cells of the myeloid lineage, was drawn off and used for subsequent experiments after washing twice in PBS.

Solubilization of Cells and Protease Inhibitors

Before Western blot analysis, neutrophils (5 × 10⁷/ml) were either treated with 10% TCA or solubilized with 1% Triton X-100 in PBS in the presence or the absence of a protease inhibitor. The inhibitors used were 1 mM PMSF (Sigma Chemical Co., St. Louis, MO), 50 μg/ml leupeptin (Sigma Chemical Co.), 50 μg/ml cystatin (a gift of V. Turk, J. Stefan Institute, Ljubljana, Yugoslavia), 1 mM iodoacetic acid, or 5 mM EDTA. The effects of diisopropyl fluorophosphate (DFP,[1] Fluka Chemie AG, Buchs, Switzerland) were studied by incubating neutrophils with the inhibitor (0.1 mM) for 10 min in an ice bath (1), and washing once in PBS before solubilizing the cells with 1% Triton X-100. Solubilization in acidic medium was performed with 1% Triton X-100 in 0.2 M sodium acetate, pH 4.

Metabolic Labeling of Cells and Immunoprecipitation

Bone marrow cells or neutrophils were suspended at 37°C in leucine-free RPMI 1640 medium (4 × 10⁷ cells/ml), supplemented with 20 mM glutamine, 5% FCS, and 20 mM Hepes (pH 7.4), and then incubated for various

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1. *Abbreviations used in this paper*: BPI, bactericidal/permeability increasing protein; DFP, diisopropylfluorophosphate.
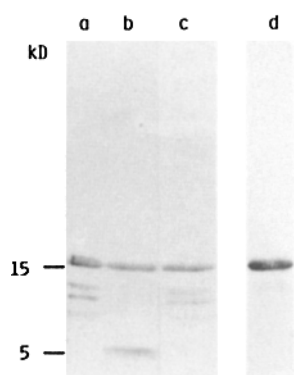
Figure 2. Immunodetection of Bac5 precursors. The IgG fraction of rabbit antiserum against Bac5 was used for Western blot analysis. Lane *a*, TCA-precipitated neutrophils; lanes *b* and *c*, total granules solubilized with 1% Triton X-100 in either PBS or 0.2 M Na-acetate buffer, pH 4, respectively; lane *d*, immunorecognized products released into the extracellular medium, after incubation of the cells with 30 nM PMA for 30 min at 37°C.

periods of time with L-[4,5³H]leucine (120–190 Ci/mmol; 2.5 mCi added per 1 × 10⁸ cells; Amersham International, Amersham, UK). At the end of the incubation, cells were washed twice with PBS by centrifugation for 10 s in an Eppendorf microfuge. For pulse–chase experiments, cell suspensions were incubated with [³H]leucine (3.5 mCi/ml) for 10 min, and then diluted with prewarmed medium containing 2 mM unlabeled leucine. At fixed time intervals, aliquots of 5 × 10⁶ cells were removed, diluted with ice-cold PBS and sedimented. Cell pellets were solubilized in lysis buffer (4 × 10⁷ cells/ml of 20 mM Tris-HCl, pH 8.2, containing 1.5% SDS), boiled for 4 min, and centrifuged for 5 min in an Eppendorf microfuge. The SDS-extracts were used immediately or stored at −70°C.

Cell extracts containing 3–6 × 10⁶ cell equivalents were six-fold diluted in 1.5% NP-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8.2, supplemented with 2 mg/ml BSA, 1 mM EDTA, and 2 mM PMSF. Samples were first preadsorbed for 60 min with 60 µl of a 50% protein A-Sepharose CL-4B slurry (Pharmacia LKB Biotechnology). After sedimenting the beads, normal rabbit serum (15 µl) was added to the samples for 2 h. 60 µl of 50% protein A-Sepharose were then added and after 60 min the beads were pelleted. This preclearance procedure was repeated twice.

Finally, the NaCl concentration in the sample was brought to 500 mM, and 110 µg of total IgG were added. After standing overnight, the immunocomplexes were precipitated by incubation with 100 µl of 50% protein A-Sepharose for 60 min. Otherwise, the antigen was immunoprecipitated by overnight incubation of the sample with IgG previously cross-linked to protein A-Sepharose (25). The whole procedure was carried out at 4°C. The beads were washed twice in 1 ml of 500 mM NaCl, 0.5% NP-40, 20 mM Tris-HCl, pH 8.2, boiled for 4 min in SDS-sample buffer, and the supernatants analyzed by SDS-PAGE and fluorography.

Treatment with Endoglycosidase H

Digestion with endoglycosidase H was performed as recommended by the manufacturer (Boehringer, Mannheim, FRG).

Preparation of mRNA

Ficoll-enriched bovine bone marrow cells were washed once in cold PBS and then homogenized for 3 min with a Polytron homogenizer in 6.6 M urea/3 M LiCl (1 × 10⁸ cells/ml). The homogenate was left overnight at 4°C and then centrifuged for 20 min at 10,000 g in a rotor (model SS34; Sorvall Instruments Div., Newton, CT). The pellet was digested for 20 min at 40°C with proteinase K (100 µg/ml; Merck, Darmstadt, FRG) in 10 mM Tris-HCl, pH 7.4, containing 0.5% SDS and 1 mM EDTA, and then extracted twice with phenol-chloroform (1:1, vol/vol). The final aqueous layer was made 0.2 M in ammonium acetate (pH 5) and 70% in ethanol, and left overnight at −20°C. RNA was collected by centrifugation for 15 min in an Eppendorf microfuge, rinsed with ethanol by centrifugation, and then dissolved in deionized water.

Poly(A)⁺ RNA was separated from total RNA by using Hybond poly(U) paper (Amersham International), according to the manufacturer's instructions.

In Vitro Translation

mRNA from bovine bone marrow cells (300–700 ng) and [³H]leucine (50 µCi) were included in 25 µl of the translation mixture containing a rabbit reticulocyte lysate (Amersham International). When the initial steps of

preprobactenecin processing were investigated, a canine pancreatic microsomal membrane fraction (Amersham International) was also present in the translation reaction mixture. After 60 min at 37°C, the reaction was stopped by adding 1 vol of 4% SDS in 20 mM Tris-HCl, pH 8.2, containing 10 mM EDTA, and immediately boiled for 3 min. 50 µl of the SDS-lysate were diluted with 1 vol of deionized water and then with 5 vol of 20 mM Tris-HCl, pH 8.2, containing 2% NP-40, 5 mM EDTA, 150 mM NaCl, and 1 mM PMSF. The preclearance and immunoprecipitation steps were carried out as above, except that BSA (4 mg) was added when the NaCl concentration was brought to 500 mM.

Neutrophil Stimulation

Neutrophils (5 × 10⁷) in 1 ml PBS with 10 mM glucose were exposed to 20–50 nM PMA for 10–30 min, and the extracellular fluid was then separated from the cells by a 10-s centrifugation in an Eppendorf microfuge. Proteins in 600 µl of the supernatant were precipitated by adding 200 µl of 40% TCA and the pellet solubilized in SDS-sample buffer. The remaining portion of the supernatant was used for enzyme assays.

Analytical Assays

Protein, lactate dehydrogenase, N-acetyl-β-glucosaminidase and vitamin B₁₂-binding protein were assayed according to established methods (10). Lactate dehydrogenase in the extracellular medium was used as a means of evaluation of integrity of cells stimulated with PMA.

SDS-PAGE was performed using the Laemmli system (18). Samples were run under reducing conditions. For protein separation and blotting, a 14% polyacrylamide gel (8 × 9 cm) was used. Protein transfer to nitrocellulose (0.2 µm; Schleicher and Schuell, Dassel, FRG) was performed with a 30-min blot (28) in a semidry electrophoretic transfer cell (Trans-Blot SD; Bio-Rad Laboratories). Proteins were then fixed to the nitrocellulose paper by incubating for 45 min with 0.2% glutaraldehyde in PBS followed by a 15-min quench with NH₄Cl. After a 2-h exposure to rabbit IgG, blots were incubated for 90 min with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham International) and the color reaction developed with 4-chloronaphthol (Bio-Rad).

Autoradiography

Immunoprecipitates were electrophoresed on a 8–20% gradient slab polyacrylamide gel (18 × 17 cm). For the detection of labeled immunoprecipitates, gels were soaked in Enhance (Dupont Corp., Boston, MA), dried, and subjected to autoradiography using a Kodak X-OMAT AR5 x-ray film and a Cronex Lightning-plus intensifying screen (Dupont Corp.). Exposure times at −70°C ranged from 24 h to 21 d.

Immunocytochemistry

Cell pellets were fixed for several hours in 8% paraformaldehyde in 200 mM Hepes, pH 7.4. For cryosectioning, small pieces of the fixed pellets were infused with 2.3 M sucrose, mounted on specimen holders, cooled in liquid nitrogen, and cryosectioned. The sections were labeled with affinity-purified IgG (14–70 µg/ml) against either Bac7 or Bac5, or with the Ig fraction of anti-human myeloperoxidase antiserum, and visualized using 6 and 9 nm protein A-gold particles (14). The sections were stained with uranyl acetate and embedded in methyl cellulose.

Results

Storage Forms of Bactenecins and their Localization in the Large Granules

Neutrophils or granules were precipitated with TCA, to prevent proteolysis, and then subjected to SDS-PAGE. The corresponding Western blots were incubated with the IgG fraction of antisera against either Bac7 or Bac5. As shown in Fig. 1, antibodies against Bac7 recognize a main molecular species of 20 kD and a minor species of 14.5 kD, both in neutrophils (lane *a'*) and in isolated granules (lane *e*). This result suggests that the granules, wherefrom the bactenecin is purified, contain a probactenecin (proBac7) of 20 kD. Likewise,
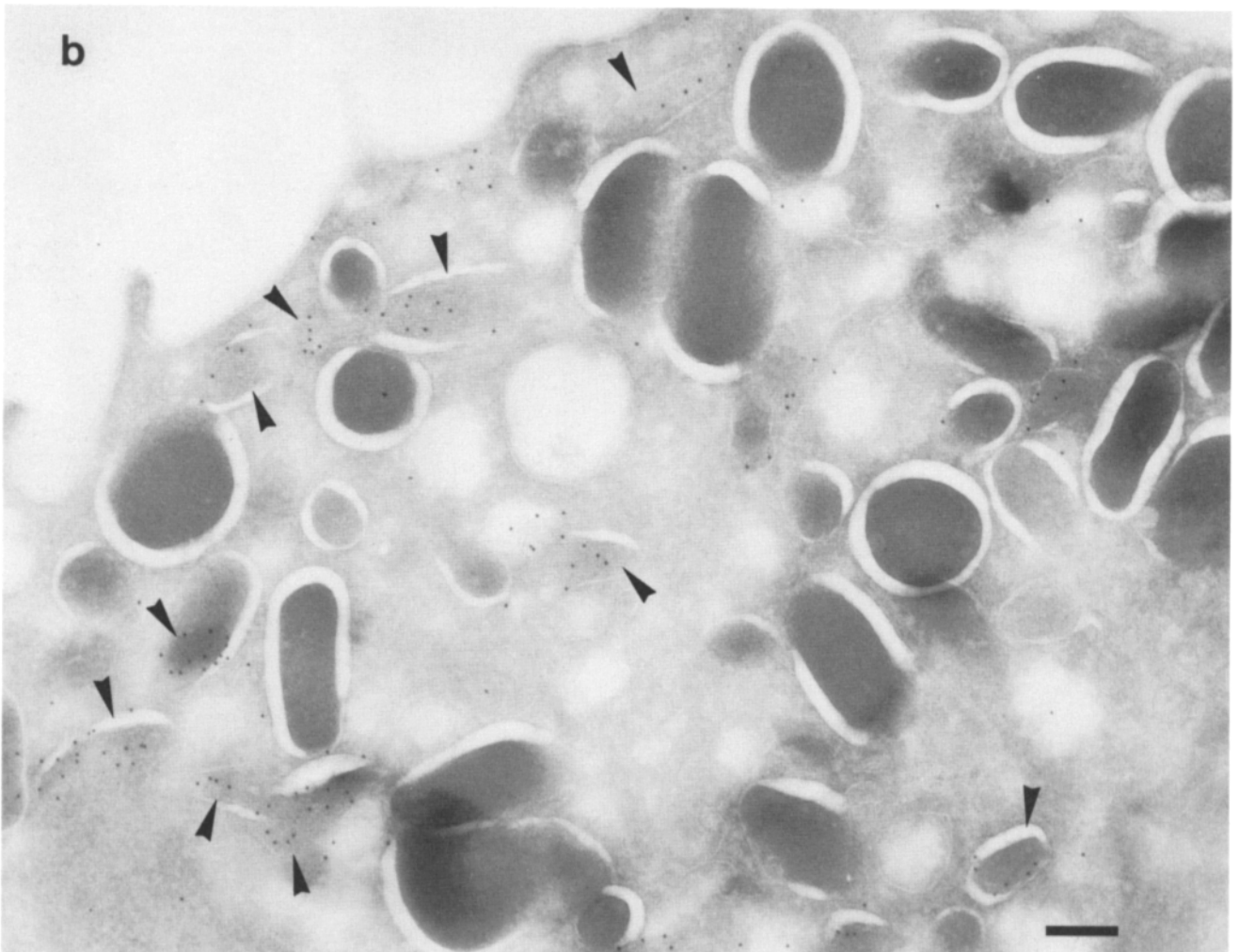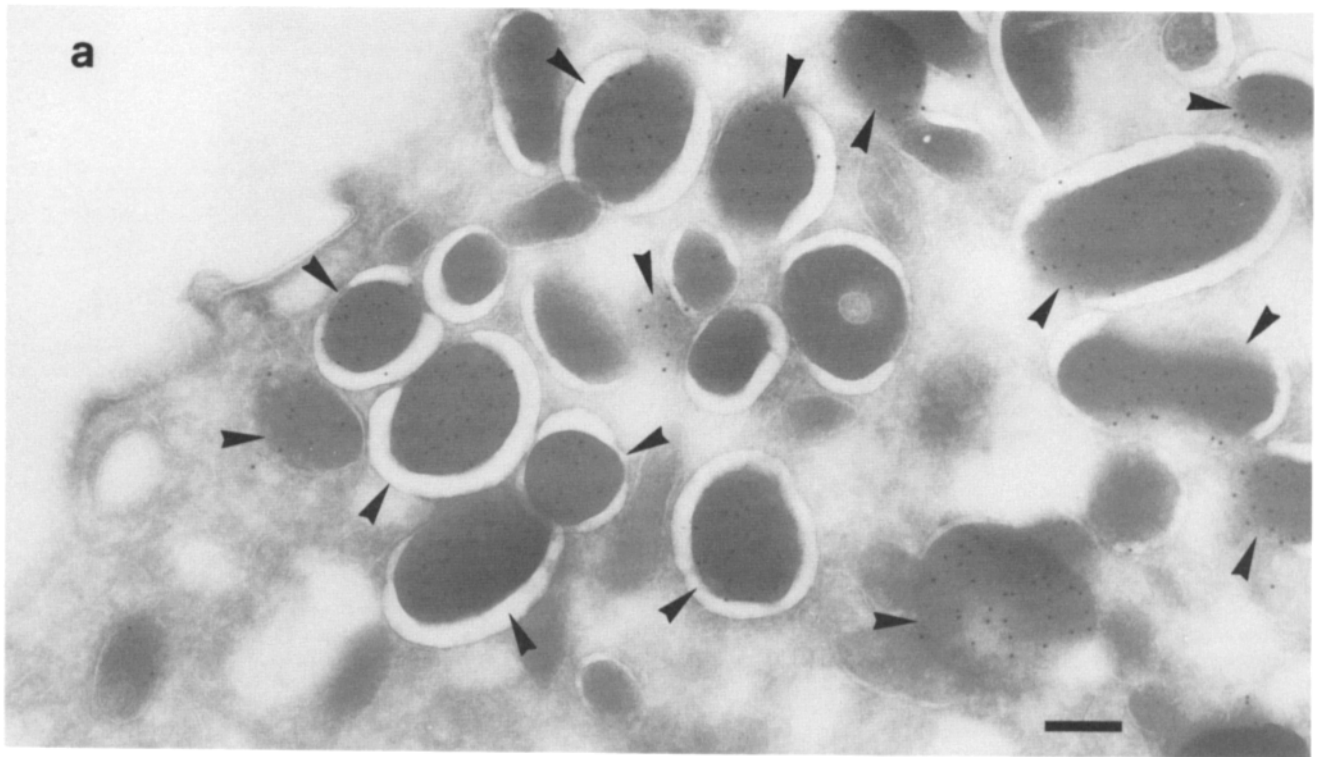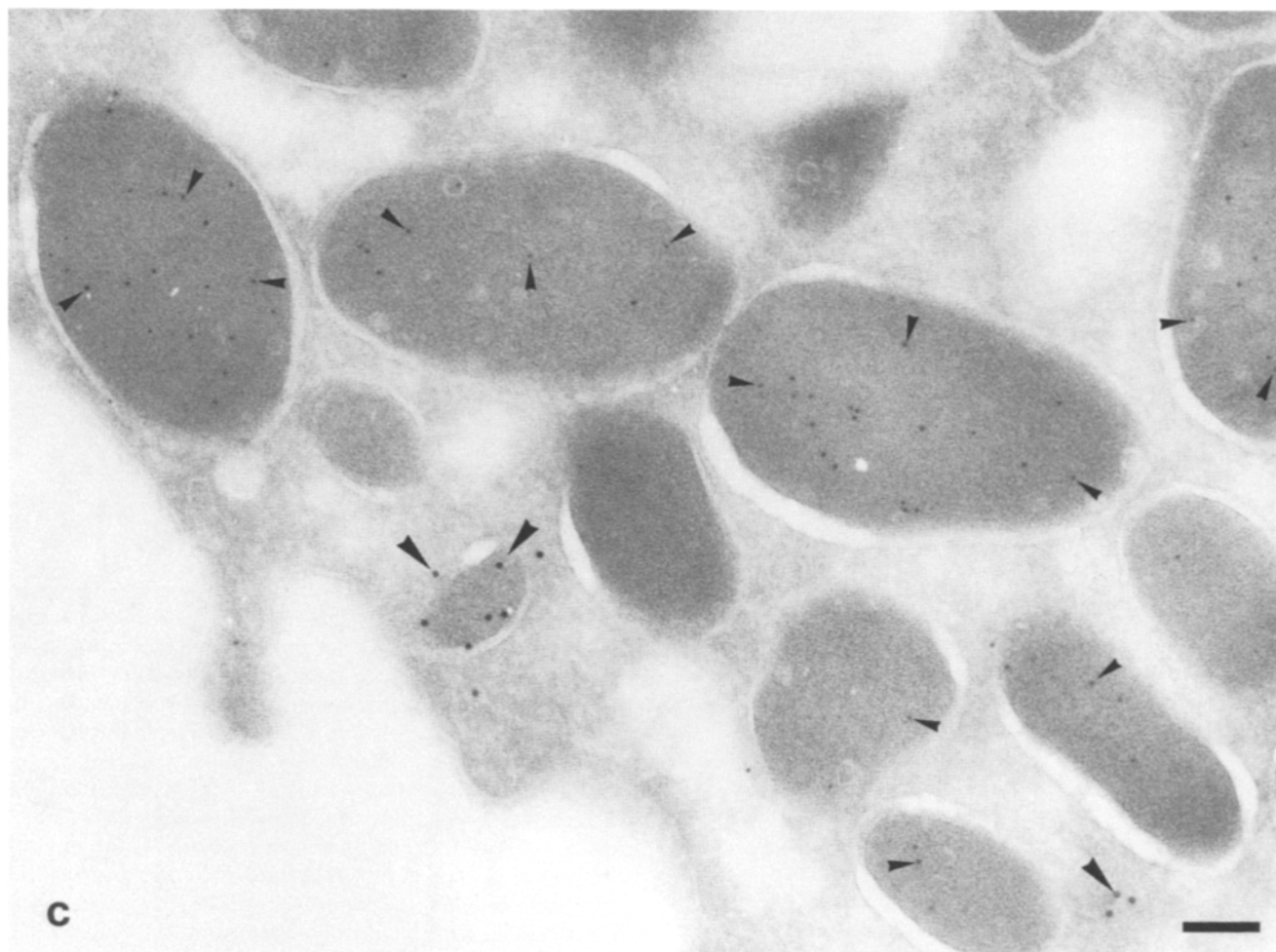
Figure 3. Immunogold electron microscopy of neutrophils, using anti-Bac7 IgG (*a* and *c*) and antimyeloperoxidase Ig (*b* and *c*). In *a*, the arrowheads indicate the presence of probactenecin in the large granules, labeled with 9 nm gold particles. In *b*, the arrowheads point to myeloperoxidase of the azurophils, also labeled with 9 nm gold particles. In *c*, the smaller gold particles (dots of 6 nm) denote the presence of bactenecin and the larger gold particles (dots of 9 nm) the presence of myeloperoxidase. Bar: (*a* and *b*) 0.2 μm; (*c*) 0.1 μm.

upon incubation of TCA-treated granules with anti-Bac5 IgG, a 15-kD proBac5 is recognized, as well as some minor polypeptides with molecular masses intermediate between 15 and 5 kD (Fig. 2, lane *a*).

The 14.5-kD protein recognized by anti-Bac7 IgG migrates quite close to the 15-kD molecule recognized by anti-Bac5 IgG (see Figs. 1 and 2). Apart from the small but clearly detectable difference in molecular weight these proteins are also immunologically unrelated. In fact, after loading with granule lysates an immunoaffinity column set up by coupling anti-Bac7 IgG to Affi-gel 10, it was found by SDS-PAGE that the 15-kD molecule is present in the mixture of unbound proteins, whereas the 14.5-kD polypeptide is recovered from the retained material (not shown). Western blot analysis confirms that only the retained 14.5-kD band reacts with anti-Bac7 IgG, whereas anti-Bac5 IgG specifically recognize the unretained 15-kD band (not shown).

The specificity of the immune recognition of the proforms of bactenecins was tested by probing with anti-Bac7 or anti-Bac5 IgG Western blots of bovine eosinophils or monocytes and lymphocytes, of a bovine leukemic cell line, of human neutrophils, of the promyelocytic cell line HL60 or of un-

related cell lines such as the murine NIH-3T3 or the hamster CHO, which are all totally negative (not shown).

In an attempt to evaluate which protease(s) might carry out the processing of probactenecins to mature antimicrobial Bac7 and Bac5, we dissolved neutrophils, or isolated neutrophil granules, with the nondenaturing detergent Triton X-100, thus abolishing the latency of the granule proteases. A similar condition, in which proteins from different granules are forced together, is met with phagocytosis, when different granule populations release their contents into the microenvironment of the phagosome. No difference was found in these experiments, whether whole cells, or isolated total cell granules were solubilized with Triton X-100.

The Western blot of neutrophils treated with Triton X-100 at pH 7.4 reveals a significant generation of Bac7 (Fig. 1, lane *b'*). Under these conditions, the disappearance of the 20-kD proBac7, recognized by anti-Bac7 IgG, can also be easily noticed in Coomassie blue-stained gels (Fig. 1, lane *b* vs. lane *a*). Incubations with Triton X-100 for various time lengths have shown that the proteolytic cleavage is completed within 5 min (not shown).

Generation of Bac7 in the presence of Triton X-100 is vir-

tually prevented when neutrophils are solubilized at pH 4. In fact, the resulting Western blot is comparable with the TCA-treated cells (Fig. 1, lane c' vs. lane a') or granules (Fig. 1, lane c' vs. lane e).

The conversion of proBac7 is also totally inhibited when neutrophils are pretreated with the serine protease inhibitor DFP, which can penetrate intact cells and granules and neutralize their serine proteases (1), and then lysed with Triton X-100 at pH 7.4 (Fig. 1, lanes d and d'). In addition, this conversion can partially be prevented by the serine–protease inhibitor PMSF, whereas it is not affected by leupeptin and other inhibitors of cysteine proteases, such as cystatin and iodoacetic acid, or by EDTA, an inhibitor of metalloproteases (not shown).

Similarly, the 15-kD proBac5 is converted into Bac5, following solubilization of isolated neutrophil granules with Triton X-100 at pH 7.4 (Fig. 2, lane b). This conversion is also prevented by DFP (not shown), or by Triton-solubilization of granules at pH 4 (Fig. 2, lane c).

These results thus strongly suggest the involvement of neutral serine protease(s) in the generation of the bactenecins from their proforms. The serine proteases so far identified in the bovine neutrophils are components of the azurophil granules (10).

To check whether probactenecins are members of the secretory protein pool of neutrophil granules, we then exposed neutrophils to PMA. Phorbol esters are known secretagogues for specific and large granules, but not for azurophils (10, 12), as confirmed by the presence among the secreted proteins of the vitamin B_{12}-binding protein, a component of the specific granules, and the absence of the azurophil-associated N-acetyl-β-glucosaminidase (not shown). Under these conditions both proBac7 and proBac5 are secreted in the extracellular medium (Fig. 1, lane f and Fig. 2, lane d).

Mature bactenecins are not detected among the secreted proteins, indicating that essentially no conversion of probactenecins occurs upon exposure of neutrophils to PMA. This is a further evidence that the protease(s) responsible for the conversion of probactenecins are not components of the granule populations discharged by effect of PMA, i.e., specific and large granules.

PMA-stimulated secretion of probactenecins also suggests that they are part of the granule matrix and are not bound to the granule membrane. This conclusion is consistent with the results obtained with immunogold EM, showing that the antigens reacting with anti-Bac7 IgG (or anti-Bac5, not shown) are dispersed in the matrix of the large granules (Fig. 3, a and c). Double labeling of neutrophils with antibodies to Bac7 and to myeloperoxidase (Fig. 3 c) indicates that the proforms of the antimicrobial peptides are not localized in the azurophils, which in addition to myeloperoxidase (Fig. 3 b) contain neutral proteases (4, 10).

Bactenecin Precursors Are Synthesized in Bone Marrow Cells But Not in Peripheral Neutrophils

First, peripheral neutrophils were tested for their ability to synthesize probactenecins. After incubation with [³H]leucine, although a vast array of proteins are labeled in neutrophils (not shown), none of the labeled proteins is immunoprecipitated by IgG to Bac7 (Fig. 4, lane a'). Only an

unlabeled product of 20 kD, corresponding to the proBac7 previously shown in Western blots, is immunoprecipitated from these lysates (Fig. 4, lane a).

In contrast, a 20-kD newly synthesized product is immunoprecipitated from lysates of [³H]leucine-labeled bone marrow cells of the myeloid lineage (Fig. 4, lane b'). Generation of this immunoprecipitate is inhibited by an excess of Bac7 (Fig. 4, lane c'), thereby confirming that the 20-kD protein has common antigenic determinants with Bac7.

Likewise, IgG to Bac5 can immunoprecipitate labeled 15-kD proBac5 from lysates of [³H]leucine-labeled bone marrow cells (Fig. 5, lane b'), but not from lysates of labeled neutrophils (Fig. 5, lane a'). The immunoprecipitation of proBac5 is prevented by an excess of Bac5 (Fig. 5, lane c').

The above results thus indicate that probactenecins are synthesized in immature myeloid cells and are present in peripheral neutrophils as prepackaged, granule-associated proteins.

Primary Translation Products of Bactenecins

To investigate the initial steps in the biosynthesis of probactenecins, poly(A)⁺ RNA from immature bone marrow cells was used to direct the in vitro protein synthesis in the presence of rabbit reticulocyte lysates. The translation products were then analyzed for the presence of bactenecin precursors by immunoprecipitation with IgG purified from the antisera against either Bac7 or Bac5.

As shown in Fig. 6 (lanes c and f), the primary translation products corresponding to proBac7 and proBac5 have molecular masses of 23.5 and 21 kD, respectively. If the in vitro translation is carried out in the presence of a microsomal membrane fraction, the preprobactenecins are cotranslationally modified to products of 20 and 15.8 kD, respectively (Fig. 6, lanes b and e). These molecules comigrate with proteins immunoprecipitated from bone marrow cells, pulsed with [³H]leucine for 10 min (Fig. 6, lanes a and d).
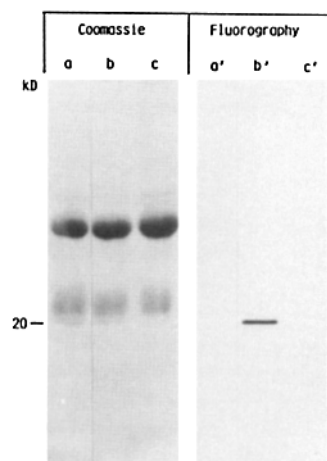
Figure 4. Immunorecognition of [³H]leucine-labeled proBac7. Coomassie blue stain (lanes a–c) and fluorography (lanes a'–c') of polypeptides immunoprecipitated with anti-Bac7 IgG from metabolically labeled neutrophils (lanes a and a') or bone marrow cells (lanes b, b', c, and c'). 3–5 × 10⁶ neutrophils or bone marrow cells were metabolically labeled with [³H]leucine for 2–5 h, detergent-solubilized, immunoprecipitated with the IgG fraction of rabbit antiserum against Bac7, as described in Materials and Methods, and analyzed in a 8–20% gradient acrylamide gel. The immunoprecipitation from bone marrow cells was carried out either in the absence (lanes b and b') or presence (lanes c and c') of purified Bac7, which was added in an ∼10-fold molar excess over proBac7. A rough estimation of the concentration of proBac7 in bone marrow cell lysates was carried out by a comparative evaluation of the stain intensity of electrophoretic bands, after exhaustive immunoprecipitation of the Bac7 precursor from the lysates.
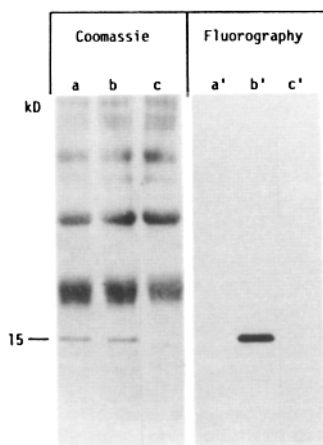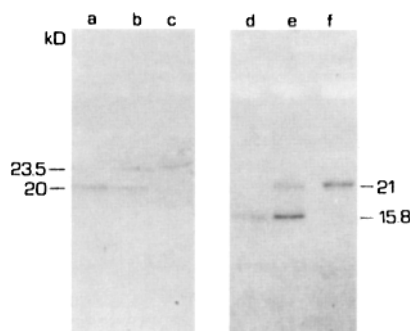
Figure 5. Immunorecognition of [³H]leucine-labeled pro-Bac5. Experimental conditions and lettering as in Fig. 4.

Figure 7. Pulse–chase analysis of [³H]leucine-labeled proteins recognized by rabbit IgG against Bac5. Bone marrow cells were pulse-labeled for 10 min with [³H]leucine (lane *a*) and chased in the presence of an excess unlabeled leucine for 50 min (lane *b*). Cells were detergent-solubilized, and immunoprecipitation was carried out with IgG against Bac5. Immunoprecipitates were analyzed by electrophoresis on a 8–20% gradient SDS-PAGE and visualized by fluorography.
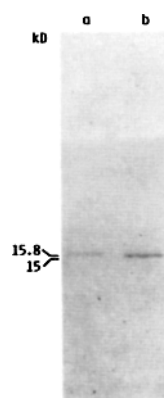
The cotranslational processing of preproBac7 thus generates a polypeptide with a mol wt corresponding to that of the granule-associated proBac7. Conversely, the polypeptide derived from preproBac5 migrates with a slightly slower mobility than the mature storage form, suggesting a higher molecular weight. To gain insight into the final maturation step of preproBac5, we pulsed bone marrow cells with [³H]leucine for 10 min and then chased for various times, up to 240 min. The conversion of the 15.8-kD polypeptide into the 15-kD mature form begins between 15 and 25 min of chase time. Between 25 and 50 min it is possible to discern the two products in different proportions (not shown), whereas after a 50-min chase only the mature, labeled form is detected (Fig. 7).

Discussion

Highly cationic antimicrobial polypeptides with mol wt ranging from 1,600 to 8,000 have been extracted from granules of bovine neutrophils (11, 19, 23), which constitute an efficient cell-dependent defense system against bacteria (31), viruses (24, 32) and parasites (29). By Western blot analysis

Figure 6. In vitro translation. Newly synthesized proteins were immunoprecipitated with rabbit IgG against Bac7 (lanes *a*, *b*, and *c*) or against Bac5 (lanes *d*, *e*, and *f*). mRNA from bovine bone marrow cells were used to direct the cell-free translation in a reticulocyte lysate translation system in the presence of [³H]leucine. The translation was carried out in the absence (lanes *c* and *f*) or presence (lanes *b* and *e*) of dog pancreas microsomal membranes. Lanes *a* and *d*, probactenecins immunoprecipitated from bone marrow cells labeled with [³H]leucine for 10 min. The immunoprecipitation products were analyzed by SDS-PAGE and fluorography.

performed with specific antibodies against two of these polypeptides, the 7- and 5-kD bactenecins or Bac7 and Bac5, we have shown here that they are present in bovine neutrophil granules as higher mol wt precursors. The prevailing precursor molecules in Western blots are probactenecins of 20 and 15 kD, respectively.

ProBac7 and proBac5 are synthesized in immature bone marrow cells of the myeloid lineage as preprobactenecins of 23.5 and 21 kD, which are then processed and targeted to the so called large granules, as shown by immunoelectron microscopy. Mature peripheral neutrophils are unable to synthesize the bactenecin precursors. The biosynthesis of preprobactenecins presumably occurs at the myelocyte stage of cell maturation, when the large granules are assembled (3), and is switched off with the progress of myeloid differentiation.

Bovine neutrophils are characterized by the presence of three populations of granules, which arise at different stages of cell maturation (3). The large granules (0.4–0.5 μm in diameter) appear when the production of peroxidase-containing granules (azurophils) ceases, whereas the specifics (0.15–0.3 μm in diameter) are formed when most of the large granules are already mature. It is not known at present how the genes for the components of the three types of granules are sequentially activated, whereas it can be reasonably assumed that the shutdown of their biosynthesis coupled to the progress of the myeloid differentiation is carried out at the level of transcription (7, 16).

Preprobactenecins appear to be processed in a simple and rather fast manner. PreproBac7 generates proBac7 in a single step. In the case of Bac5, after removal of the signal peptide, the complete maturation of the molecule requires an additional step from a 15.8-kD polypeptide to the final 15-kD storage form. The 15.8-kD intermediate is not affected by treatment with endoglycosidase H (not shown), thereby indicating that addition of N-linked oligosaccharides is not required for the appropriate targeting of these molecules to the granules. Pulse–chase experiments have shown that the conversion of the 15.8-kD intermediate to the 15-kD mature molecule becomes evident after 15 min of chase, suggesting that it is carried out in a post-ER compartment, and is completed within 50 min.

Solubilization of neutrophils, or of isolated total granules with a nondenaturing detergent causes the conversion of the storage forms of bactenecins into Bac7 and Bac5. Western blot analysis has shown that this process does not occur when

the solubilization is carried out under acidic conditions or in the presence of DFP, thereby suggesting that it is catalyzed by a neutral serine protease. The significance of this proteolytic cleavage can be discussed in relation to the features of other cytotoxic polypeptides of neutrophils and eosinophils.

The proportion of the precursors of these proteins or their domains not directly involved in the toxic effects have been suggested to play different roles. In the 60-kD bactericidal/permeability-increasing protein (BPI), the C-terminal domain linked to the NH_2-terminal 25-kD portion which carries all the antibacterial activity (22), may anchor BPI to the granule membrane and prevent its secretion (13). A function of this type for the proportion of probactenecins can be excluded, since immunogold EM has shown that proBac7 and proBac5 are embedded in the matrix of the large granules, wherefrom they can be easily released upon neutrophil stimulation with PMA.

An appealing possibility would be that the proportion of probactenecins contains sequences important for their sorting out and appropriate intracellular transport to the granules. Such a function has been implied for the proregion of the precursors of defensins (7), low molecular weight antimicrobial polypeptides purified from neutrophils of several animal species (9). Unlike bactenecins, however, the proregion of defensins, whose amino acid sequence has been inferred from analysis of cDNA clones, is cleaved before, or soon after, the polypeptides are stored in the azurophil granules. In fact, no evidence for prodefensins has been found in mature neutrophils (7).

The proportion of probactenecins may possess toxicity-neutralizing properties. Actually, analysis of cDNA clones of the major basic protein (MBP) of eosinophils, a toxic factor for several parasites as well as for various mammalian cells, has indicated that the acidic amino acid sequence of the proportion of proMBP may interact with the basic mature MBP and minimize autotoxic effects in the cell (5, 20).

Preliminary observations provide support to this hypothesis. In fact, purified proBac7 and proBac5 have been found to be inactive on Bac7- and Bac5-susceptible bacteria (unpublished). Bac7 and Bac5, which cause a significant perturbation of biomembranes (Skerlavaj, B., D. Romeo, and R. Gennaro, manuscript submitted for publication), thus appear to be stored in the large granules as harmless species, which require a cleavage by neutral serine protease(s) to generate antimicrobial molecules. Interestingly, neutral serine proteases such as cathepsin G and elastase are constituents of the azurophils (2). In the stimulated neutrophil the activation of probactenecins may thus occur only after their discharge into the phagocytic vacuoles, whose pH remains slightly alkaline for a few minutes after sealing of the boundary membrane (27). Here they would meet neutral protease(s), which are known to be also released from the azurophils upon phagocytosis (6, 26). Such a potential interplay between azurophils and large granules in the activation of probactenecins is indirectly supported by the observation that in the supernatant of PMA-treated neutrophils, in which there is no discharge of the content of azurophils (10, 12), proBac7 and proBac5 are found as uncleaved molecules.

References

1. Amrein, P. C., and T. P. Stossel. 1980. Prevention of degradation of human polymorphonuclear leukocyte proteins by diisopropylfluorophosphate. Blood. 56:442-447.
2. Baggiolini, M., U. Bretz, and B. Dewald. 1978. Subcellular localization of granulocyte enzymes. In Neutral Proteases in Human Polymorphonuclear Leukocytes. K. Havemann and A. Janoff, editors. Urban & Schwarzenberg, Inc., Munich, FRG. 3-17.
3. Baggiolini, M., U. Horisberger, R. Gennaro, and B. Dewald. 1985. Identification of three types of granules in neutrophils of ruminants. Lab. Invest. 52:151-158.
4. Bainton, D. F. 1988. Phagocytic cells: developmental biology of neutrophils and eosinophils. In Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 265-280.
5. Barker, R. L., G. J. Gleich, and L. R. Pease. 1988. Acidic precursor revealed in human eosinophil granule major basic protein cDNA. J. Exp. Med. 168:1493-1498.
6. Cramer, E. M., J. E. Beesley, K. A. F. Pulford, J. Breton-Gorius, and D. Y. Mason. 1989. Colocalization of elastase and myeloperoxidase in human blood and bone marrow neutrophils using a monoclonal antibody and immunogold. Am. J. Pathol. 134:1275-1284.
7. Daher, K. A., R. I. Lehrer, T. Ganz, and M. Kronenberg. 1989. Isolation and characterization of human defensin cDNA clones. Proc. Natl. Acad. Sci. USA. 85:7327-7331.
8. Elsbach, P., and J. Weiss. 1988. Phagocytic cells: oxygen-independent antimicrobial systems. In Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 445-470.
9. Ganz, T., M. E. Selsted, D. Szklarek, S. S. L. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer. 1985. Natural peptide antibiotics of human neutrophils. J. Clin. Invest. 76:1427-1435.
10. Gennaro, R., B. Dewald, U. Horisberger, H. U. Gubler, and M. Baggiolini, 1983. A novel type of cytoplasmic granule in bovine neutrophils. J. Cell Biol. 96:1651-1661.
11. Gennaro, R., B. Skerlavaj, and D. Romeo. 1989. Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils. Infect. Immun. 57:3142-3146.
12. Goldstein, I. M., S. T. Hoffstein, and G. Weissmann. 1975. Mechanism of lysosomal enzyme release from human polymorphonuclear leukocytes. J. Cell Biol. 6:647-652.
13. Gray, P. W., G. Flaggs, S. R. Leong, R. J. Gumina, J. Weiss, C. E. Ooi, and P. Elsbach. 1989. Cloning of the cDNA of a human neutrophil bactericidal protein. J. Biol. Chem. 264:9505-9509.
14. Griffiths, G., R. Brands, B. Burke, D. Louvard, and G. Warren. 1982. Viral membrane proteins acquire galactose in trans-Golgi cisternae during intracellular transport. J. Cell Biol. 95:781-792.
15. Hirsch, J. G. 1962. Cinemicrophotographic observations on granule lysis in polymorphonuclear leukocytes during phagocytosis. J. Exp. Med. 116:827-833.
16. Johnson, K. R., W. M. Nauseef, A. Care, M. J. Wheelock, S. Shane, S. Hudson, H. P. Koeffler, M. Selsted, C. Miller, and G. Rovera. 1987. Characterization of cDNA clones for human myeloperoxidase: predicted amino acid sequence and evidence for multiple mRNA species. Nucleic Acids Res. 15:2013-2028.
17. Klebanoff, S. J. 1988. Phagocytic cells: developmental biology of neutrophils and eosinophils. In Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 391-444.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.
19. Marzari, R., B. Scaggiante, B. Skerlavaj, M. Bittolo, R. Gennaro, and D. Romeo. 1988. Small, antibacterial and large, inactive peptides of neutrophil granules share immunoreactivity to a monoclonal antibody. Infect. Immun. 56:2193-2197.
20. McGrogan, M., C. Simonsen, R. Scott, J. Griffith, N. Ellis, J. Kennedy, D. Campanelli, C. Nathan, and J. Gabay. 1988. Isolation of a complementary DNA clone encoding a precursor to human eosinophil major basic protein. J. Exp. Med. 168:2295-2308.
21. Mottola, C., R. Gennaro, A. Marzullo, and D. Romeo. 1980. Isolation and partial characterization of the plasma membrane of purified bovine neutrophils. Eur. J. Biochem. 111:341-346.
22. Ooi, C. E., J. Weiss, P. Elsbach, B. Frangione, and B. Mannion. 1987. A 25-kDa NH_2-terminal fragment carries all the antibacterial activities of the human neutrophil 60-kDa bactericidal/permeability-increasing protein. J. Biol. Chem. 262:14891-14894.
23. Romeo, D., B. Skerlavaj, M. Bolognesi, and R. Gennaro. 1988. Structure and bactericidal activity of an antibiotic dodecapeptide purified from bo-

vine neutrophils. *J. Biol. Chem.* 263:9573–9575.

24. Rouse, B. T., R. C. Wardley, L. A. Babiuk, and T. K. S. Mukkur. 1977. The role of neutrophils in antiviral defense. *In vitro* studies on the mechanism of antiviral inhibition. *J. Immunol.* 118:1957–1961.
25. Schneider, C., R. A. Newman, D. R. Sutherland, U. Asser, and M. F. Greaves. 1982. A one step purification of membrane proteins using a high efficiency immunomatrix. *J. Biol. Chem.* 257:10766–10769.
26. Segal, A. W., J. Dorling, and S. Coade. 1980. Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. *J. Cell Biol.* 85:42–59.
27. Segal, A. W., M. Geisow, R. Garcia, A. Harper, and R. Miller. 1981. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature (Lond.).* 290:406–409.
28. Szewczyk, B., and L. M. Kozloff. 1985. A method for the efficient blotting of strongly basic proteins from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose. *Anal. Biochem.* 150:403–407.
29. Townsend, J., and W. P. H. Duffus. 1982. *Trypanosoma theileri*: antibody-dependent killing by purified populations of bovine leucocytes. *Clin. Exp. Immunol.* 48:289–299.
30. Walker, J. M., V. E. O. Valli, and J. H. Lumsden. 1974. Colony formation in culture by bovine granulopoietic progenitor cells. *Can. J. Comp. Med.* 38:145–152.
31. Williams, M. R., and K. J. Bunch. 1981. Variation among cows in the ability of their blood polymorphonuclear leucocytes to kill *Escherichia coli* and *Staphylococcus aureus*. *Res. Vet. Sci.* 30:298–302.
32. Zerial, A., B. Skerlavaj, R. Gennaro, and D. Romeo. 1987. Inactivation of herpes simplex virus by protein components of bovine neutrophil granules. *Antiviral Res.* 7:341–352.