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Evaluation of the expression of the cationic peptide gene in various types of leukocytes

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To understand the regulation of the production of antimicrobial cationic peptide (CP) in leukocytes, expression of the CP gene was evaluated in various types of leukocytes using guinea pig neutrophils, monocytes, macrophages, eosinophils, lymphocytes and bone marrow cells. Acid-urea PAGE and SDS-PAGE/immunoblot analyses showed that CP was present in neutrophils and bone marrow cells, but not in other leukocytes. Northern blot and transcription run-off analyses revealed that only bone marrow cells expressed CP mRNA and transcribed the CP gene. Interestingly, in situ hybridization analysis using bone marrow cells demonstrated that CP mRNA was expressed in the neutrophil precursor cells, such as promyelocytes and myelocytes, but was not detected in the mature neutrophils and other bone marrow cells. Moreover, immunocytochemical study indicated that CP was present in the neutrophil precursor cells and the mature neutrophils in the bone marrow. Thus, the CP gene appears to be expressed during a limited period of neutrophil maturation, and CP is likely synthesized by the neutrophil precursor cells in the bone marrow.

Cationic peptide; Antimicrobial peptide; Gene expression; In situ hybridization; Leukocyte; Ginea pig

1. INTRODUCTION

Leukocytes play an important role in protecting hosts against microbial infection. Both oxidative and non-oxidative mechanisms are used by leukocytes to restrict infection [1–3]. Molecules such as H_2O_2, O_2^- and HOCl are examples of microbicidal agents that are derived from oxidative metabolism [1,3]. On the other hand, leukocytes contain antimicrobial components in the granules, which contribute to the oxygen-independent host defense mechanism [2,3]. Representatives of these components are highly basic proteins and peptides, such as lysozyme, bactericidal/permeability-increasing protein (BPI), cationic peptide (CP), major basic protein and eosinophil cationic protein [2–9].

Lysozyme is distributed in the granules of neutrophils and mononuclear phagocytes (monocytes and macrophages), and is bactericidal for Gram-positive bacteria, but lacks activity against Gram-negative bacteria [2,3,5]. BPI can be isolated from neutrophils, and displays bactericidal activity against Gram-negative bacteria but lacks activity against Gram-positive bacteria and fungi [2–5]. Major basic protein and eosinophil cationic protein are localized in the eosinophil granules, and are cytotoxic toward a range of parasites but exhibit minimal antibacterial activity [2,8,9]. In contrast, CP exhibits a broad antimicrobial spectrum for Gram-positive and Gram-negative bacteria, fungi and viruses [2–7]. In addition, CP expresses cytotoxic activity toward tumor cells [2,3,5,6]. CP has been isolated from human, rabbit, rat and guinea pig neutrophils [7,10]. However, it is not known whether leukocytes other than neutrophils are capable of producing CP. In this study, therefore, to understand the regulation of CP production in leukocytes, we have evaluated the CP gene expression in various types of leukocytes using guinea pig neutrophils, monocytes, macrophages, eosinophils, lymphocytes and bone marrow cells.

Abbreviations: CP, cationic peptide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kDa, kilodaltons; kb kilobases.

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2. MATERIALS AND METHODS

2.1. *Preparation of cells*

Guinea pig neutrophils, lymphocytes, monocytes, peritoneal macrophages, alveolar macrophages and eosinophils were prepared as described previously [11,12]. The purity of the cells examined by Wright-Giemsa stain was >90%. Bone marrow cells were obtained from guinea pig femoral bones.

2.2. *Polyacrylamide gel electrophoresis and immunoblot analysis*

The cells were suspended in 0.34 M sucrose, and distrupted in ice by sonication (four 10 s bursts at 25 W). The sonication was centrifuged at 100 g for 10 min to sediment the nuclei, and the supernatant

(the post-nuclear fraction) was analyzed by acid-urea PAGE (7.5% polyacrylamide, 6.25 M urea) [13,14] and SDS-PAGE with a 7.5–15% linear gradient of polyacrylamide [15]. The gel was silver-stained [16] using a commercially available reagent kit (Daiichi Pure Chemicals). In some experiments, after SDS-PAGE the proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was incubated with Block Ace (Dainippon Pharmaceutical Co.) and reacted with a 1:1,000 dilution of a rabbit anti-CP serum which had been prepared against purified guinea pig neutrophil CP [10] conjugated to ovalbumin [17]. The proteins were detected by incubation with a 1:2,000 dilution of horseradish peroxidase-labeled goat anti-rabbit IgG (Organan Teknika) followed by development with diformazan using the POD Immunostain Set (Wako Pure Chemical Industries). Protein concentrations were measured by the method of Lowry et al. [18].

2.3 *Evaluation of CP mRNA transcripts*

Expression of CP mRNA was evaluated by Northern blot analysis. Total cellular RNA isolated by the guanidinium thiocyanate extraction method [19] was electrophoresed on a formaldehyde-containing agarose gel. RNA was blotted onto a nylon membrane (Hybond-N, Amersham), and hybridized with CP cDNA probe [20] or γ-actin cDNA probe (pHFγA-1 [21]) (graciously provided by P. Gunning and L. Kedes, Stanford University) labeled with [^{32}P]dCTP (3,000 Ci/mmol, Amersham) by the random hexanucleotide primer method [22].

2.4. *Evaluation of CP gene transcription*

The transcription of the CP gene was evaluated by a transcription 'run-off' assay, as described previously [20]. Nuclei isolated from the cells were incubated with [^{32}P]UTP (800 Ci/mmol, Amersham) to label nuclear RNA. Transcription of the CP gene and, as a control, the γ-actin gene, was determined by hybridization (40 h, 42°C) of the ^{32}P-labeled nuclear RNA (10^7 cpm) to the filter-bound (nylon membrane, Amersham; dot-blot apparatus, Bio-Rad Laboratories), unlabeled cDNA targets (CP cDNA and γ-actin cDNA) in the presence of 50% formamide and 10% dextran sulfate.

2.5. *Evaluation of CP mRNA transcripts by in situ hybridization*

In situ hybridization was performed as described previously [23] with minor modifications [24]. Cytocentrifuge preparations (10^5 cells/slide) were prepared using a Cytospin 2 (340 rpm, 10 min, Shandon Instruments) and siliconized RNAse-free glass slides. The slides were fixed in 4% paraformaldehyde and then treated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) to reduce the non-specific binding of the probe. The slides were then treated with proteinase K and hybridized (14–16 h, 50°C) with the ^{35}S-labeled antisense CP cRNA probe (10^7 cpm/ml) in the presence of 50% formamide and 10% dextran sulfate. After RNAse treatment, the slides were dipped in Konica NR-M2 emulsion, dried, exposed for 3 days at 4°C, developed in a Konica-Konidor developer, and fixed in Fuji Fix. The cells were then stained with May-Grünwald-Giemsa to demonstrate nuclear morphology. To generate ^{35}S-labeled single-stranded cRNA probe, the CP cDNA was subcloned into the transcription vector Bluescript SK/− (Promega) [20], and the labeled CP antisense and sense cRNA probes were synthesized by transcription of the cDNA using [^{35}S]UTP (800 Ci/mmol, Amersham). When ^{35}S-labeled CP sense cRNA probe was used as a negative control no silver grain was observed in the cells examined (not shown).

2.6. *Immunocytochemical staining of CP*

The cytocentrifuge preparations were immunostained for CP, as described previously [25]. To inactivate the endogenous peroxidase activity, the slides were treated with 0.1 M periodic acid and 0.02% sodium borohydride. The slides were then washed, and incubated in a 1:1,000 dilution of the rabbit anti-CP serum (described above). The slides were extensively washed, and then incubated in a 1:2,000 dilution of peroxidase-labeled goat anti-rabbit IgG (Organon Teknika). After extensive washing, the slides were developed in 0.05% 3,3′-diaminobenzidine/0.01% hydrogen peroxide. The cells were then counterstained with May-Grünwald-Giemsa.

The two CPs with a difference of only one amino acid residue [10] and the corresponding cDNAs with >99% homology [20] have been already isolated by us. When the effects of the two antisera or two cDNAs (corresponding to each CP) were examined, no difference was observed between the two (not shown).

3. RESULTS AND DISCUSSION

3.1. *Evaluation of the cells containing CP and CP mRNA transcripts*

To evaluate the regulation of CP production in leukocytes, the presence of CP in various types of leukocytes was first examined by electrophoretic analysis. Acid-urea PAGE analysis showed the characteristic CP bands [10] in mature neutrophils and bone marrow cells; the band was not detected in lymphocytes, peritoneal macrophages and eosinophils (Fig. 1A). SDS-PAGE and immunoblot analysis also demonstrated that the 3.8-kDa bands corresponding to CP were detected in mature neutrophils and bone marrow cells, but not in other types of leukocytes (Fig. 1B and C). The CP band was not detected in monocytes and alveolar macrophages by acid-urea PAGE and SDS-PAGE/immunoblot analyses (not shown).

Next, the expression of CP mRNA or the transcription of the CP gene was examined. Northern blot analysis revealed that bone marrow cells expressed the 0.6-kb CP mRNA transcript, whereas no CP mRNA transcript was observed in mature neutrophils, lymphocytes, peritoneal macrophages and eosinophils (Fig. 2A). In contrast, when the same RNA samples were analyzed with a γ-actin cDNA probe, the 2.2-kb γ-actin mRNA transcripts were observed in these cells. Consistent with the results of Northern blot analysis, the transcription run-off assay demonstrated that the CP gene was transcribed in bone marrow cells but not in other types of leukocytes, although the γ-actin gene was transcribed in these cells (Fig. 2B). The expression of CP mRNA and the transcription of the CP gene was not observed in monocytes and alveolar macrophages (not shown). Thus, among various types of leukocytes examined, mature neutrophils and bone marrow cells contained CP, whereas only bone marrow cells transcribed the CP gene and expressed CP mRNA transcripts.

3.2. *Identification of bone marrow cells expressing CP mRNA*

Bone marrow cells are composed of various type of cells. Then, the bone marrow cells expressing CP mRNA transcripts were identified with in situ hybridization using ^{35}S-labeled antisense CP cRNA probe. All the cells expressing CP mRNA belonged to the neutrophil precursor cells (Fig. 3A–D). In this regard, meylobiasts, promyelocytes, myelocytes and metamyelocytes were observed to express CP mRNA transcripts, while mature neutrophils did not express CP mRNA transcripts. CP mRNA was not detected in the cells of eosinophil, lymphocyte, monocyte and erythroid lineages

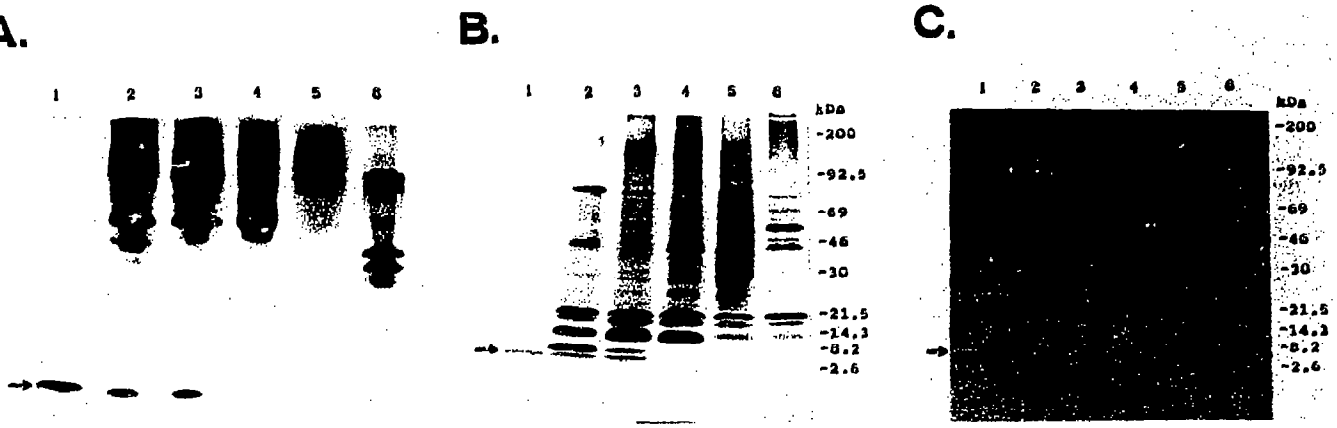

Fig. 1. Acid-urea PAGE and SDS-PAGE/immunoblot analyses of the cell extracts. The postnuclear fractions of various types of leukocytes were subjected to (A) acid-urea PAGE or (B) SDS-PAGE followed by (C) immunoblot analysis. Lane 1, purified neutrophil CP; lane 2, neutrophils; lane 3, bone marrow cells; lane 4, lymphocytes; lane 5, peritoneal macrophages; lane 6, eosinophils. 3 μg of purified CP and 15–25 μg of the post-nuclear fractions were analyzed by acid-urea PAGE, whereas 500 ng of purified CP and 5–8 μg of the post-nuclear fractions were analyzed by SDS-PAGE/immunoblot analysis. Molecular weight standards: myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa), myoglobin I (8.2 kDa) and myoglobin III (2.6 kDa). The arrows indicate the bands corresponding to CP.

(not shown). Next, the cells containing CP mRNA were quantitated at different cell stages (Fig. 4). CP mRNA was detected to a limited extent in myeloblasts. Interestingly, the percentage of the CP mRNA-positive cells increased markedly in the promyelocyte and myelocyte stages (>90%). However, the levels of the positive cells remarkably decreased thereafter.

Furthermore, the bone marrow cells containing CP

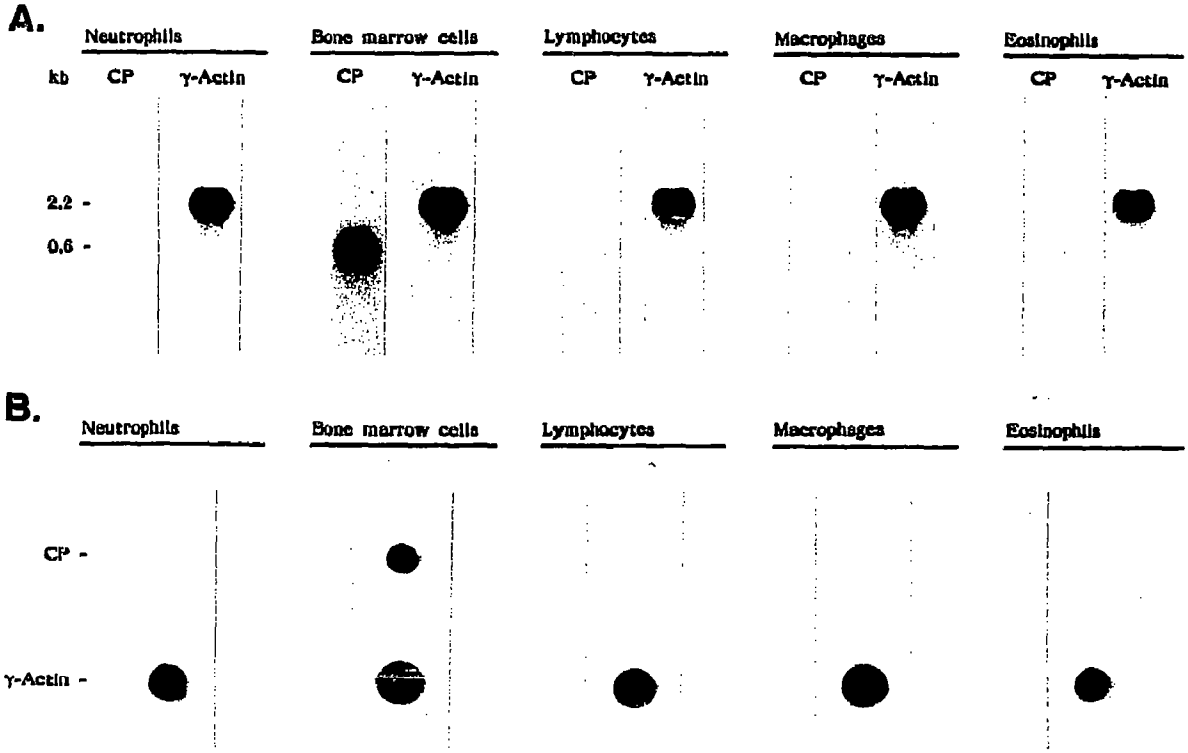

Fig. 2. Evaluation of the expression of CP mRNA and the transcription of CP gene. (A) Northern blot analysis of CP mRNA. Total cellular RNA (10 μg) of various types of leukocytes was electrophoresed on an agarose gel, and blotted onto a nylon membrane. The blot was hybridized to ^{32}P-labeled CP or γ-actin cDNA probe. The 0.6-kb CP mRNA and the 2.2-kb γ-actin mRNA transcripts are indicated. (B) Transcription run-off analysis of CP gene. Nuclei isolated from the cells were labeled with [^{32}P]UTP, and the labeled nuclear RNA was hybridized to filter-bound CP (top) and γ-actin (bottom) cDNA targets.

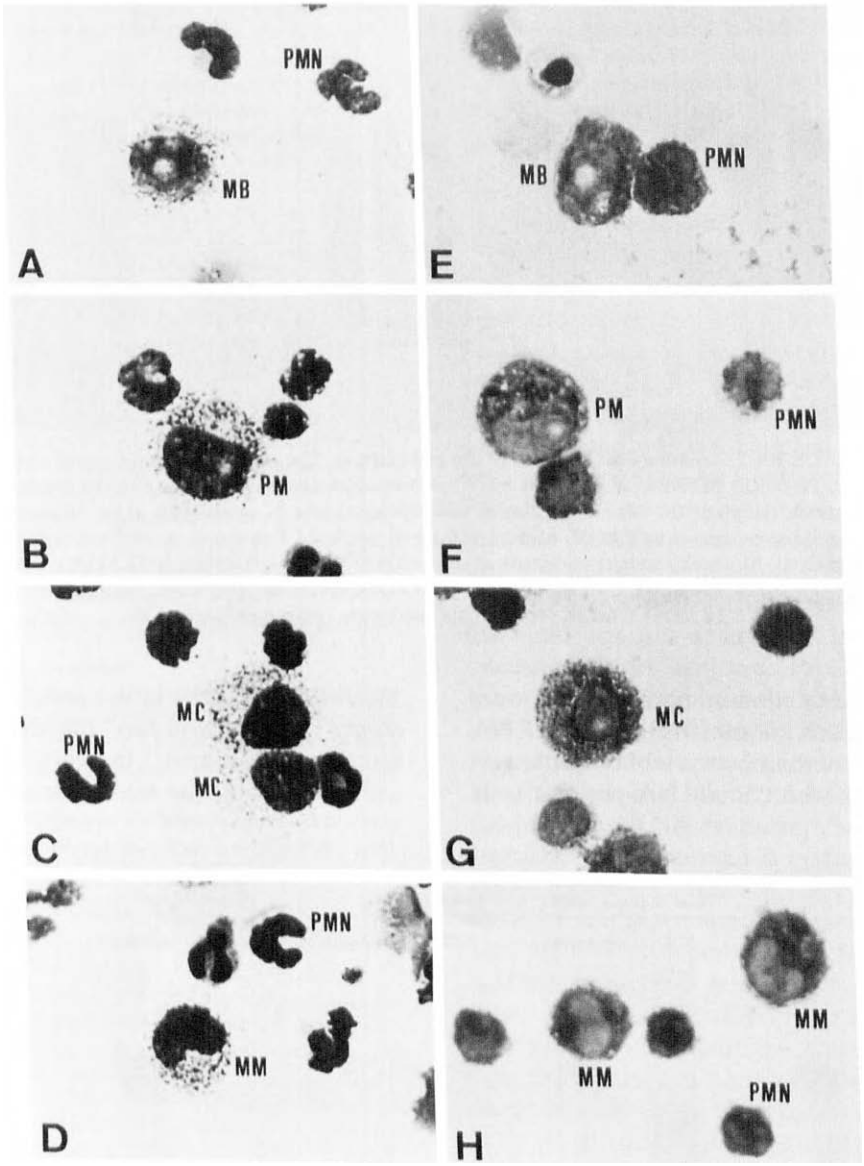

Fig. 3. Evaluation of CP mRNA by in situ hybridization and immunocytochemical staining of CP. (A–D) Examples of bone marrow cells exhibiting CP mRNA transcripts. Bone marrow cells were hybridized with ^{35}S-labeled CP antisense cRNA probe, and CP mRNA transcripts were detected. Myeloblast (MB), promyelocyte (PM), myelocytes (MC) and metamyelocyte (MM) show positive hybridization exhibiting silver grains. Mature neutrophils (PMN) are negative. (E–H) Examples of bone marrow cells containing CP. CP of the bone marrow cells was immunocytochemically evaluated using the rabbit anti-CP serum and peroxidase-labeled anti-rabbit antibody. MB, PM, MC, MM and PMN are stained and positive. Magnification ×500.

were immunocytochemically evaluated using the anti-CP serum. CP was detected in the cells of neutrophil lineage such as myeloblasts, promyelocytes, myelocytes, metamyelocytes and mature neutrophils (Fig. 3E–H). On the other hand, CP was not detected in the cells of other cell lineages (not shown). When the CP-positive cells were quantified at different cell stages, a small percentage of myeloblasts was observed to possess CP (Fig. 4). Interestingly, the percentage of the CP-positive cells increased markedly at the promyelocyte and myelocyte stages (>90%) in parallel with the increase of CP mRNA. The increased levels of the positive cells remained almost constant after the myelocyte stage, although the expression of CP mRNA was remarkably reduced.

Together these observations suggest that the CP gene is expressed during a limited stage of neutrophil lineage maturation, and CP is likely synthesized by the neutrophil precursor cells in the bone marrow and stored in the cells after synthesis.

It is known that mature neutrophils do not express the mRNA transcripts for neutrophil elastase and myeloperoxidase, although they have these proteins in the granules [26–29]. A recent study using in situ hybridization has revealed that neutrophil elastase and myeloperoxidase mRNA transcripts are expressed only in the

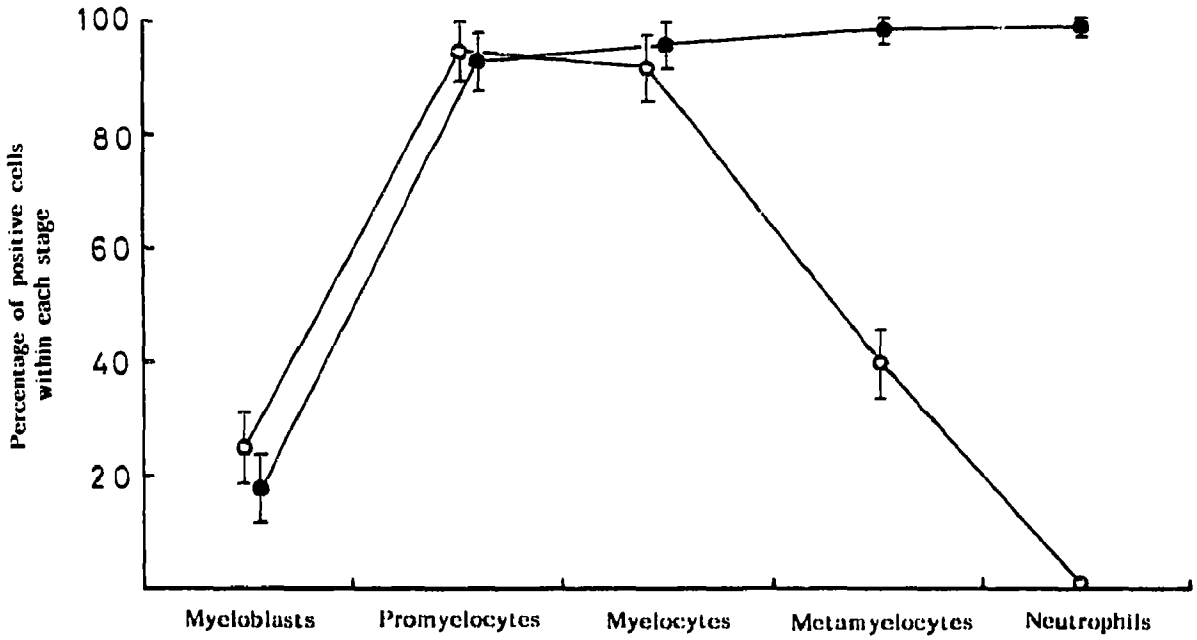

Fig. 4. Proportions of bone marrow cells within each cell stage exhibiting CP mRNA transcripts and CP. After the hybridization with ^{35}S-labeled antisense CP cRNA probe or the treatment with anti-CP serum, the number of positive cells for CP mRNA (○) and CP (●) within each stage was evaluated, and expressed as a percentage of the total number of cells in each stage. Each data point represents the mean ± S.D. of at least four experiments.

neutrophil precursor cells in the bone marrow [30], suggesting that the synthesis of these granule proteins occurs during a period of neutrophil maturation that takes place in the bone marrow. The present study has shown that CP mRNA is also expressed in the neutrophil precursor cells in the bone marrow, but not in the mature neutrophils. Thus, CP, one of the major antimicrobial components, also seems to be synthesized by the maturing neutrophils in the bone marrow, as are neutrophil elastase and myeloperoxidase.

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