EXPRESSION OF THE NEUTROPHIL ELASTASE
GENE DURING HUMAN BONE MARROW
CELL DIFFERENTIATION

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Neutrophil elastase (NE;[1] E.C.3.4.21.37) is a 220 residue,[2] single chain glycoprotein that functions as a potent serine protease capable of destroying a broad spectrum of substrates in the extracellular milieu (1–5). In addition to being one of the few mammalian proteases capable of functioning at a neutral pH to cleave mature, crosslinked elastin, NE can also attack the major forms of collagen, the protein components of proteoglycans, fibronectin, laminin, components of the complement and coagulation cascades, and *Escherichia coli* cell walls (1, 2, 4, 6). As such, NE is considered to be useful because of its likely role in normal tissue turnover and host defense, yet dangerous in its ability to destroy normal tissues. The latter concept is most dramatically illustrated by the hereditary disorder α1-antitrypsin (α1AT) deficiency, in which a deficiency of the major inhibitor of NE allows NE to attack alveolar walls in an unfettered fashion, resulting in the clinical disorder, emphysema (6, 7).

In the context of its broad and powerful spectrum of action, it is reasonable to assume that the expression of the NE gene is tightly controlled. In this regard, as the name suggests, NE is found in blood neutrophils, where it is stored in azurophilic (primary) cytoplasmic granules (8–11). When the neutrophil is activated by surface stimuli, or lysed, NE is released into the extracellular milieu along with the other contents of these granules (12). Consistent with its presence in mature neutrophils, NE can be detected in the granulocytic lineage of bone marrow cells as early as the promyelocyte stage (13). Interestingly, despite the fact the neutrophils contain large amounts of NE, they do not have NE mRNA transcripts, i.e., they are incapable of producing the enzyme (14). However, NE mRNA transcripts are found in bone marrow cells and in the human bone marrow–derived tumor cell lines U937 and HL60 (14). Furthermore, when the myelomonocytic-like HL60 cell line is stimu-

lated with DMSO to differentiate toward the granulocytic lineage, NE mRNA levels are increased, but when these cells are induced with phorbol esters to differentiate toward the monocytic lineage, NE transcripts disappear (14). Together, these observations are consistent with the hypothesis that the expression of the NE gene is limited to a very short period in leukocyte differentiation, likely to the promyelocyte and myelocyte stages.

To directly evaluate this hypothesis, the technique of in situ hybridization has been used to examine normal human bone marrow cells for the presence of NE mRNA transcripts. For comparison, we have also evaluated the same cells for the presence of transcripts of the genes for myeloperoxidase (MPO; a protein also stored in the azurophilic granules of neutrophils) (11), lactoferrin (LF; a protein stored in the secondary granules of neutrophils) (11), and β-globin (a protein expressed in the erythrocyte series of cells) (15).

Materials and Methods

Study Population.   Bone marrow cells were obtained from seven normal volunteers. "Normalcy" of the marrow was confirmed by conventional cytologic analysis. After dilution with an equal volume of DME, the marrow precursor cell population was enriched by Ficoll-Hypaque density centrifugation (16). Diploid human fetal lung fibroblasts (HFL1, ATCC CCL153) were used as controls (17).

RNA Extraction and Northern Analysis.   RNA was extracted and evaluated by Northern analysis using standard methods (18-20). The filters were evaluated with a ^{32}P-labeled DNA probe produced by nick-translation of the neutrophil elastase cDNA pPB15 (14), myeloperoxidase cDNA pMPO62 (provided by G. Rovera, Wistar Institute, Philadelphia, PA [21]), lactoferrin cDNA pHL41 (provided by E. Benz, Yale University, New Haven, CT [22]), and β-globin cDNA 18-6hβ (provided by S. Karlsson, NHLBI; initially cloned by B. Forget, Yale University).

RNA Probes for In Situ Hybridization.   To generate high specific activity ^{35}S-labeled single-stranded cRNA probes for in situ studies to detect NE, MPO, and LF mRNA transcripts, cDNAs were subloned into the transcription vector pGEM-3Z (Promega Biotec, Madison, WI), permitting in vitro transcription of sense and antisense mRNA from the SP6 and T7 promoters after appropriate linearization (23). Transcription conditions were those of the manufacturer (24) using [^{35}S]UTP (800 Ci/mmol; Amersham Corp., Arlington Heights, IL), yielding 3.5-4.5 × 10^8 dpm/μg template DNA.

For NE mRNA, three different probes were initially evaluated, corresponding to the following regions of the NE gene (all derived from the NE cDNA clone pPB15 [14]): (*a*) a 280-bp Eco RI-Pst I fragment corresponding to exon III and the 5' portion of exon IV; (*b*) a 370-bp Pst I-Eco RI fragment corresponding to the 3' portion of exon IV, the whole of exon V, including a portion of untranslated region; and (*c*) a 550-bp Eco RI-Ava I fragment (containing sequences of exons III, IV, and the 5' portion of exon V) (25). Preliminary studies using the 370-bp and 550-bp NE antisense probes demonstrated results identical to those obtained with the 280-bp probe. Thereafter, the 280-bp probe was used for all subsequent experiments. The equivalent ^{35}S-labeled NE cRNA sense probe was used, as a control, as a measure of nonspecific hybridization.

For MPO mRNA, the transcription vector included a 460-bp Kpn I-Kpn I fragment of plasmid pMPO62 (21). For LF mRNA, the transcription vector included a 650-bp Eco RI-Pst I fragment of plasmid pHL41 (22). To evaluate β-globin mRNA transcripts, antisense and sense RNA probes covering exon III and a portion of the 5' and 3' flanking introns were generated by inserting the 622-bp Dra I fragment of the human β-globin gene into the transcription vectors pTZ18R and pTZ19R (provided by S. Karlsson, NHLBI [26]). The two resulting transcription vectors were identical, except that the β-globin DNA fragment was inserted in the reverse orientation to generate the antisense and sense RNA probes.

In Situ Hybridization.   To prepare samples for in situ hybridization, cytocentrifuge preparations from bone marrow cells (1.5 × 10^5 cells per slide; 500 rpm, 7 min; Cytospin II,

Shandon Southern Instruments, Inc., Sewickley, PA) were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 3 min and kept in 70% ethanol at 4°C until used. Post-fixation in Karnovsky's fixative (27) was carried out before the in situ procedure. Hybridization was performed as described by Harper et al. (28) with minor modifications as previously described (29). For autoradiography, slides were dipped in Kodak NTB-2 emulsion diluted 1:1 with distilled water at 40–42°C, dried, exposed for 5 d at 4°C, developed in Kodak D-19 developer, and fixed in Kodak rapid fixer.

In initial experiments after the development of the autoradiograms, the slides were stained with Giemsa, Wright-Giemsa, or May-Grunwald-Giemsa. However, the cytoplasmic detail with these stains was not optimal for cell identification, presumably because the in situ hybridization procedures modified the staining characteristics. In contrast, with hematoxylin-eosin, the staining characteristics were excellent, permitting accurate identification of the cells at various stages of marrow differentiation. For comparative purposes, air dried cytospin slides were stained with Giemsa or with hematoxylin-eosin, and the different cell stages were determined in parallel, with the late promyelocyte stage identified as described by Ackerman (30). The results were consistent with the standard values for differential cell counts of Giemsa-stained smears from normal individuals (31) with the majority of cells being of the neutrophil lineage (62.5%), 27.3% erythroid lineage and 1.7% blasts, 2.4% promyelocytes, and 1.8% late promyelocytes. Using this approach, the hybridized preparations were evaluated using a Zeiss microscope at a magnification of ×1,000. For each preparation a minimum of 450 cells were counted and a minimum of 100 positive cells were identified.

Statistical Evaluation.   Data are presented as mean ± SEM. Comparisons between groups were made using the two-tailed Student's *t*-test or Mann-Whitney U test where appropriate.

Results

mRNA Transcripts in RNA Isolated from Bone Marrow Cells.   Using the NE cDNA probes, Northern analysis of RNA isolated from bone marrow cells revealed 1.3-kb NE mRNA transcripts, 3.5-kb MPO mRNA transcripts, 2.7-kb LF mRNA transcripts, and 0.7-kb β-globin mRNA transcripts. In contrast to these observations, RNA extracted from the HFL-1 cell line revealed no transcripts with the same NE, MPO, LF, and β-globin cDNA probes (Fig. 1), consistent with the knowledge that human diploid fetal lung fibroblasts do not produce the proteins coded for by these genes.

Identification of Bone Marrow Cells Containing Specific mRNA Transcripts.   Bone marrow cells hybridized with the ^{35}S-labeled antisense NE, MPO, LF, and β-globin cRNA probes were considered positive if they contained greater than six grains per cell. This was based on results with all four sense probes demonstrating 99% of the cells
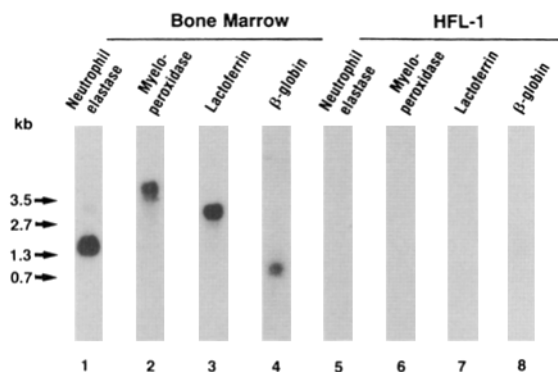

FIGURE 1.   Identification of neutrophil elastase, myeloperoxidase, lactoferrin, and β-globin mRNA in bone marrow cells. Shown are Northern analyses of total cellular RNA (10 μg/lane) isolated from bone marrow cells from normal volunteers, using the following ^{32}P-labeled cDNA probes. (Lane *1*) neutrophil elastase; (lane *2*) myeloperoxidase; (lane *3*) lactoferrin; (lane *4*) β-globin. As negative controls, lanes *5–8* are identical to lanes *1–4*, but the extracted RNA came from HFL-1 fibroblasts. The sizes of the mRNA transcripts were determined using conventional markers.

containing less than five grains per cell. On the average, 5.0 ± 0.7% of the marrow cells were found to be positive for NE mRNA transcripts, with a range of 7 to 122 grains per cell. All of the positive cells belonged to early marrow precursors and to cells in the early stages of neutrophil differentiation (Fig. 2). In this regard, blasts, promyelocytes, late promyelocytes, and neutrophil myelocytes were observed to have NE transcripts, while more mature cells (neutrophil metamyelocytes, bands, and neutrophils) did not demonstrate hybridization with the antisense probe. No cells of the eosinophil, or erythroid lineages showed hybridization. Because of the difficulty in identifying monocyte precursors, it was not possible to definitively determine whether transcripts were or were not present in this population.

Bone marrow cells hybridized with the ^{35}S-labeled antisense MPO probe demonstrated that, on average, 5.4 ± 1.4% of bone marrow cells were positive for MPO mRNA transcripts, with a range of 8 to 83 grains per cell. Like NE, MPO$^+$ cells were confined to early precursors and cells in the early stages of neutrophil differentiation (Fig. 3, A and B).

For the LF cRNA antisense probe, on the average, 30.2 ± 5.2% of the bone marrow cells were positive for LF mRNA transcripts, with a range of 7 to 58 grains per cell. Quite different from NE$^+$ or MPO$^+$ cells, the LF$^+$ cells were found only in the late stages of neutrophil differentiation (Fig. 3, C and D).

Cells of the erythroid lineage were uniformly negative after hybridization with the NE, MPO, and LF cRNA probes. When evaluated with the ^{35}S-labeled β-globin antisense probe, of the total population of bone marrow cells, 10.5 ± 3.3% were positive for β-globin mRNA transcripts; all were of the erythroid lineage (Fig. 3, E and F). A range of 7 to 35 grains per positive cell was observed.

Quantitative Evaluation of Specific mRNA Transcripts at Different Cell Stages. Evaluation of cells positive for NE mRNA transcripts showed they were present during a very limited period of neutrophil differentiation. In this regard, positive cells were found predominantly in the promyelocyte (95.8 ± 2.6%) and late promyelocyte (75.0 ± 9.2%) stages with a much smaller percentage of positive myelocytes (Fig. 4). Additionally, a small percentage of blasts contained NE transcripts, but NE mRNA was not detected in the more mature cells of the neutrophil series (neutrophil metamyelocytes, bands, and neutrophils) or in cells from the eosinophil or erythroid lineages. To assess whether the distribution of NE mRNA transcripts among cells of early granulocytic lineage was specific for NE or was part of a generalized upregulation of mRNA transcripts for granule proteins, the percentages of cells positive for NE transcripts were contrasted with those for MPO and LF mRNA. Interestingly, the proportion of NE$^+$ cells among all bone marrow cells was quite similar to that of the MPO$^+$ cells (Fig. 4). In this context, the percentages of MPO$^+$ blasts, promyelocytes, and late promyelocytes were similar to the percentage of NE$^+$ blasts (p = 0.07), promyelocytes (p > 0.1), and late promyelocytes (p > 0.05). However, the proportions of neutrophil myelocytes containing NE were higher than those containing MPO transcripts (NE 7.8 ± 1.2%; MPO 0.8 ± 0.5%; p < 0.02), suggesting that NE transcripts persisted to a greater extent in granulocytic differentiation than did MPO mRNA. In marked contrast, evaluation with the LF probe revealed no transcripts until the neutrophil myelocyte stage. Furthermore, the LF mRNA was present in almost half the total of neutrophil metamyelocytes and band forms, a time in differentiation when NE and MPO transcripts were absent (Fig. 4). As a final
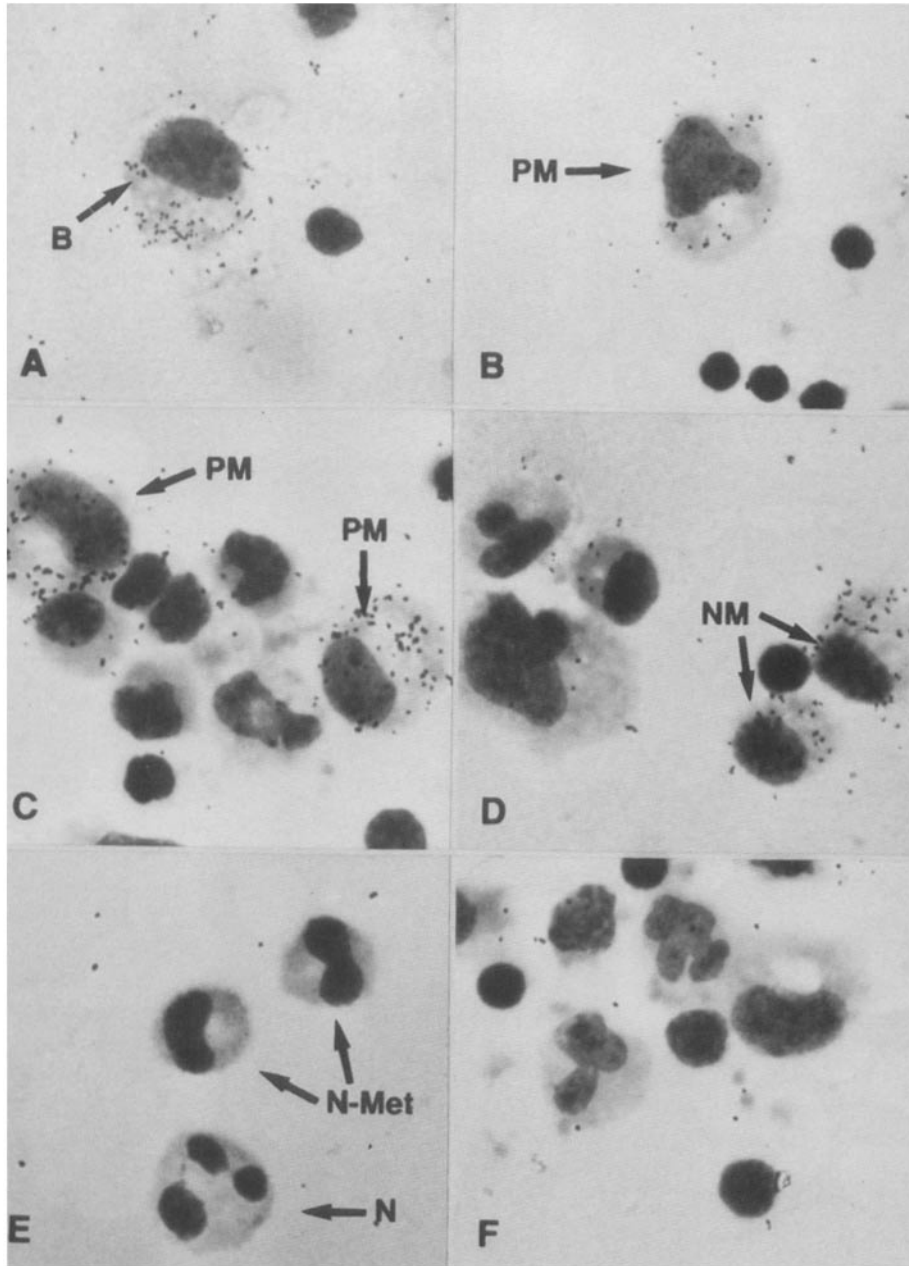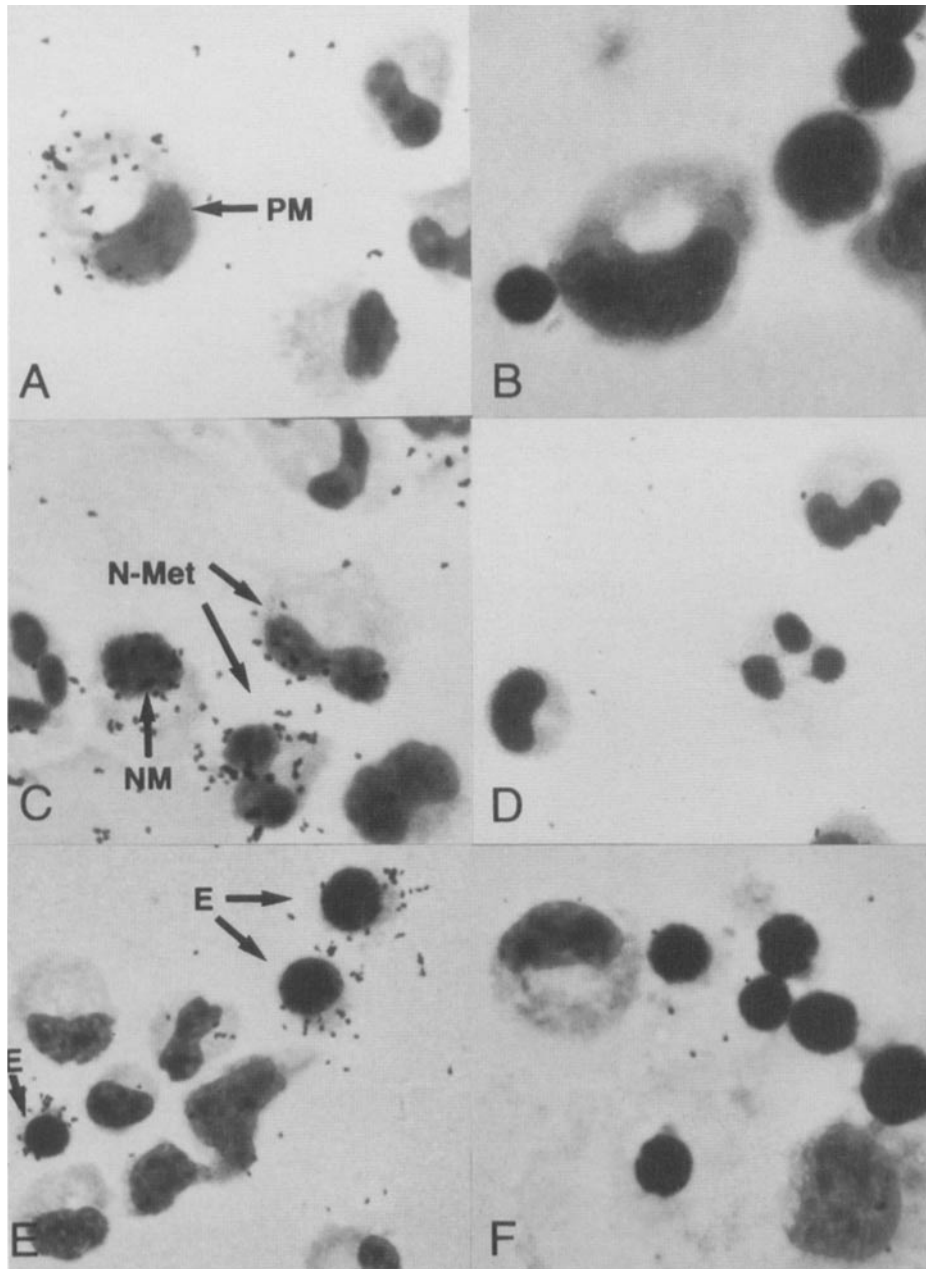
FIGURE 2. Examples of autoradiographs demonstrating in situ hybridization of ^{35}S-labeled neutrophil elastase (NE) cRNA probes to NE mRNA transcripts in cytocentrifuge preparations from bone marrow cells of normal individuals (hematoxylin-eosin stain, ×630). (A–E) Bone marrow cells hybridized with the ^{35}S-labeled NE antisense probe. (A–D) Examples of bone marrow cells exhibiting mRNA transcripts. (A) Blast (B). (B) Early promyelocyte (PM). (C) Two promyelocytes (PM). (D) Two neutrophil myelocytes (NM). (E) Metamyelocytes (N-Met) and a mature neutrophil (N) are negative. (F) Similar to panels A–E, but using the ^{35}S-labeled NE sense cRNA probe. Shown are a number of cells, all of which exhibit no hybridization signal.

FIGURE 3. Examples of autoradiographs demonstrating in situ hybridization of ^{35}S-labeled myeloperoxidase (MPO), lactoferrin (LF), and β-globin cRNA probes to mRNA transcripts within bone marrow cells of normal individuals (hematoxylin-eosin stain, ×630). (A and B) Hybridization with the ^{35}S-labeled MPO probes. (C and D) Hybridization with the ^{35}S-labeled LF probes. (E and F) Hybridization with the ^{35}S-labeled β-globin probes. (A) MPO antisense probe. Shown is a promyelocyte (PM) exhibiting MPO mRNA transcripts. (B) Similar to panel A but hybridized to the MPO sense probe. All cells are negative. (C) LF antisense probe. Shown are a neutrophil myelocyte (NM) and two metamyelocytes (N-Met) demonstrating positive hybridization. (D) Similar to A but with the LF sense probe. All cells are negative. (E) β globin antisense probe. Shown are a number of erythroid lineage cells (E) showing positive hybridization. (F) Same as E but with the β-globin sense probe. All cells are negative.
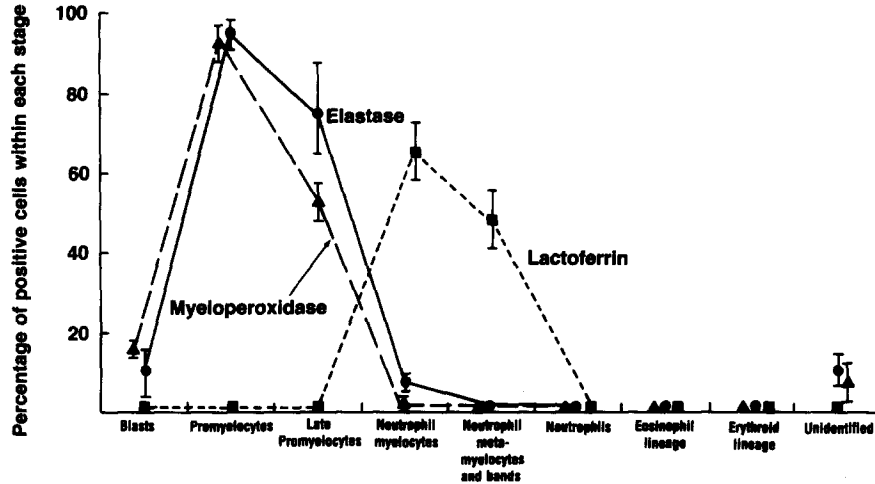
FIGURE 4.   Proportions of bone marrow cells within each cell stage expressing transcripts of the neutrophil elastase (NE; ●) gene, the myeloperoxidase (MPO; ▲) gene, and the lactoferrin (LF; ■) gene. After hybridization with the appropriate ^{35}S-labeled sense and antisense probes, the number of positive cells within each stage was evaluated for each gene and expressed as a percentage of the total number of cells in each stage. Each data point represents the mean of marrow samples from at least four normal individuals (blasts, promyelocytes, late promyelocytes, NE vs. MPO, NS; neutrophil myelocytes, NE vs. MPO, $p < 0.02$).

control, using the β-globin RNA probe, positive cells were found only within the erythroid lineage.

The same distinctions in the distribution of cells containing NE, MPO, and LF mRNA transcripts were observed when the data were expressed in terms of the distribution of positive cells among the different cell types in the total population of marrow cells expressing each specific gene (Fig. 5). In this context, the distribution of the cells found to be positive for NE transcripts was quite similar to the distribution of cells expressing MPO transcripts, although a somewhat higher proportion of promyelocytes expressed MPO than NE transcripts ($p < 0.03$) and a smaller proportion of neutrophil myelocytes expressed MPO than NE ($p < 0.03$). In contrast, the distribution of LF⁺ cells was completely different, with 22.3 ± 3.4% of the cells that expressed the gene being neutrophil myelocytes and 77.7 ± 3.4% being neutrophil metamyelocytes and band forms.

Evaluation of the Relative Expression of NE Gene Transcripts in the Neutrophil Lineage.   To assess the relative differences in the number of mRNA transcripts among each of the different transcripts, in cells at different stages of development, for each probe, the average number of grains present over each of one hundred positive cells was evaluated. For NE, the average number of grains per positive cell differed significantly among the different cell types (Fig. 6). The average number of grains per positive cell was significantly higher in promyelocytes compared with blasts ($p < 0.03$), late promyelocytes ($p < 0.01$), and neutrophil myelocytes ($p < 0.005$), but similar numbers of grains were observed in blasts, late promyelocytes, and neutrophil myelocytes ($p > 0.05$).

In contrast to NE, assessment of the amount of message present at different stages
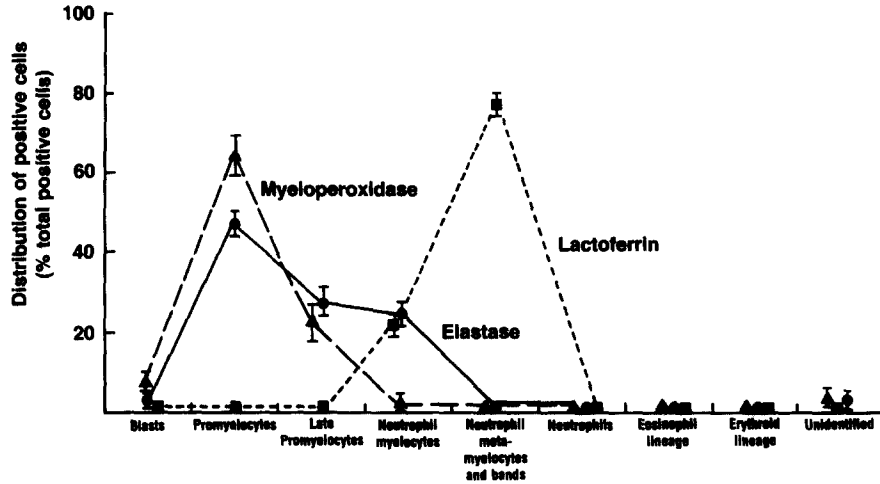
FIGURE 5.   Distribution of bone marrow cells expressing mRNA transcripts for the neutrophil elastase (NE; ●) myeloperoxidase (MPO; ▲), and lactoferrin (LF; ■) genes. After hybridization with each probe, the number of positive cells in each stage was determined and expressed as a percentage of the total number of cells positive for each gene. Each data point represents the mean of marrow samples from at least four normal individuals (promyelocytes, NE vs. MPO, $p < 0.03$; neutrophil myelocytes, NE vs. MPO $p < 0.03$).
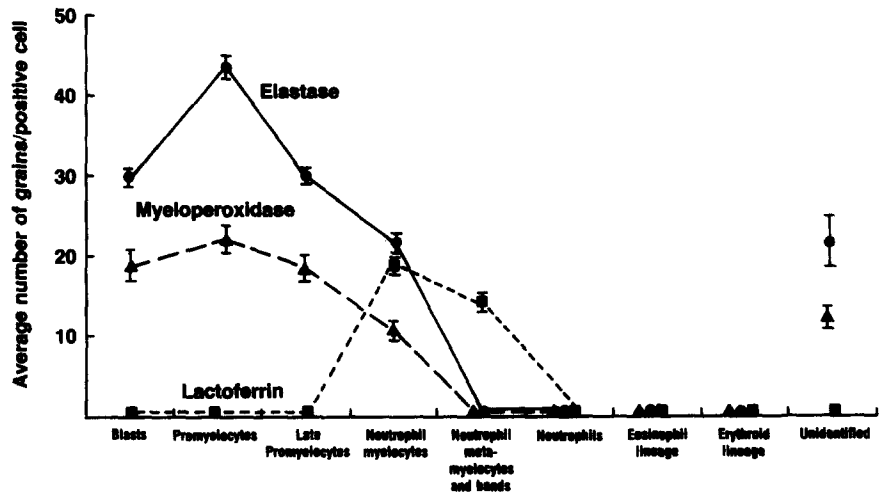

FIGURE 6.   Estimate of the relative number of mRNA transcripts in different bone marrow cells for the neutrophil elastase (NE; ●), myeloperoxidase (MPO; ▲), and lactoferrin (LF; ■) genes. The data are expressed as the average number of grains per positive cell for each gene at each cell stage. Each data point represents the mean of marrow samples from at least four normal individuals (NE; promyelocytes vs. blasts, $p < 0.03$; promyelocytes vs. late promyelocytes, $p < 0.01$; promyelocytes vs. neutrophil myelocytes, $p < 0.005$; other comparisons NS. MPO; promyelocytes vs. neutrophil myelocytes, $p < 0.02$).

of development for the MPO gene showed that the average number of MPO transcripts was comparable in blasts, promyelocytes, and late promyelocytes ($p > 0.1$, all comparisons), but that promyelocytes contained a larger average number of grains per cell than did neutrophil myelocytes ($p < 0.02$).

For lactoferrin, the two cell types that contained LF transcripts had equivalent amounts of mRNA transcripts per cell (neutrophil myelocytes, 19 ± 1 average number of grains per cell, neutrophil metamyelocytes and band forms, 18 ± 1, $p > 0.05$). However, the total number of grains in the two cell populations differed significantly (neutrophil myelocytes 427 ± 42, neutrophil metamyelocytes and band forms, 1,378 ± 136; $p < 0.04$), due to the higher number of neutrophil metamyelocytes and band forms present in 100 positive cells.

Discussion

Using in situ hybridization, this study demonstrates that there are specific stages in bone marrow differentiation during which the neutrophil elastase gene is expressed at the level of NE mRNA. Although first detected within blast cells, maximum NE mRNA levels are present during the promyelocyte stage. Thereafter, NE mRNA levels decline such that they are undetectable by the stage of the neutrophil metamyelocyte and thereafter. Furthermore, NE mRNA is not detectable in any other cell lineage in bone marrow. These observations are consistent with the observations that: (a) using Northern analysis, NE mRNA transcripts are not present in mature neutrophils or monocytes, but are found in RNA extracted from bone marrow cells (14); (b) in the HL60 myelomonocytic cell line, NE mRNA transcripts increase when the cells are induced to differentiate toward the granulocytic lineage by DMSO, but decrease when the cells are committed to the monocyte lineage by PMA (14); and (c) at the protein level, NE is first detected by immunocytochemical and cytoenzymatic methods at the promyelocyte stage (13). Interestingly, NE mRNA transcripts appear in bone marrow differentiation in a relatively similar fashion as transcripts for MPO, another neutrophil azurophilic granule component, although minor differences in the relative levels of NE and MPO transcripts during differentiation suggests the two genes are not coordinately controlled at the mRNA level. In marked contrast to the appearance of NE mRNA transcripts, transcripts for LF, a neutrophil secondary granule component, appear much later in neutrophil myeloid differentiation. Thus, the expression of the gene for NE appears to be very tightly controlled, with the majority of expression at the promyelocyte stage of myeloid cell differentiation, and the control of the NE gene at the level of mRNA transcripts appears to be independent of that of other proteins in neutrophil granules.

Comparative Expression of NE, MPO, and LF Genes at the mRNA Level. In the mature neutrophil, the azurophilic granules contain abundant amounts of NE and MPO but no LF, while the secondary granules contain LF, but no NE or MPO (10, 11, 13, 32–38). During neutrophil lineage differentiation, the azurophilic granules appear at the neutrophil promyelocyte stage (32–34). The data at the mRNA level in the present study are entirely consistent with these observations, and suggest there are different "classes" of genes expressed at different times during granulocyte differentiation. In this regard, the NE and MPO genes appear to represent a class that is expressed early, initially at the blast stage, and then most intensely at the promyelocyte stage. In contrast, the LF gene appears to represent a class of genes that is ex-

pressed much later, initially at the neutrophil myelocyte stage, and most intensely at the neutrophil metamyelocyte and band stages. Thus, taken together, the data at the mRNA and protein levels suggest that different classes of granule genes are expressed in different phases of neutrophil myeloid differentiation.

The expression of the NE gene at the mRNA level likely represents the expression of a general class of neutrophil azurophilic granule genes; however, at least for the NE and MPO genes, there are minor differences at the mRNA level that suggest the two genes are not coordinately controlled in an exact fashion. Two lines of evidence support this concept. First, at the neutrophil myelocyte stage, NE mRNA transcripts are found in a higher proportion of the cells than are MPO transcripts. Second, while the relative numbers of NE transcripts among cells of different stages appear to increase between blasts and promyelocytes, the relative numbers of MPO transcripts seem to remain constant among cells at the blast and promyelocyte stages. Thus, the expression of these genes may have several levels of regulation, i.e., they appear to be switched on in a coordinate fashion, but the minor relative differences in mRNA levels of NE and MPO during myeloid differentiation suggest that there are additional levels of control. Consistent with this concept, when the HL60 cell line is induced to differentiate with DMSO into the granulocytic lineage, NE mRNA transcripts are upregulated while MPO transcripts are reduced (14, 39).

Implications for Possible Therapeutic Modulation of NE Gene Expression. While the neutrophil contains abundant amounts of neutrophil elastase, the fact that the mature neutrophil has no NE mRNA transcripts has important implications for how the levels of this potent destructive enzyme can be controlled. From the observations in the present study, NE clearly can be synthesized only at the early stages of myeloid differentiation, only in the bone marrow, i.e., there is no possibility for regulation at the DNA, RNA, or protein synthesis levels in the neutrophil itself. Thus, in contrast to other mediators of inflammation such as cytokines and growth factors, which can be modulated at the site of inflammation at the DNA, RNA, or protein synthesis levels, the control of NE gene expression occurs long before the neutrophil reaches the site of action of this mediator. In this regard, for human disorders in which a relative excess of NE may be playing a central role (e.g., emphysema) (4, 6, 7, 40), therapeutic strategies to modulate the amount of neutrophil elastase in the target organ will, by necessity, be directed at suppressing the numbers of neutrophils that reach the site of inflammation, suppressing the ability of the neutrophil to release NE, or suppressing the expression of the NE gene in the bone marrow in the early stages of myeloid differentiation.

Summary

Neutrophil elastase, a potent serine protease carried and released by activated neutrophils, is not synthesized by neutrophils, but by their bone marrow precursor cells. Using in situ hybridization with ^{35}S-labeled antisense and sense neutrophil elastase cRNA probes, the present study demonstrates that expression of the neutrophil elastase gene is tightly controlled in bone marrow precursors and occurs during a very limited stage of differentiation of the neutrophil myeloid series, almost entirely at the promyelocyte stage. Neutrophil elastase mRNA transcript levels are detectable to a limited extent in blasts, increase markedly in the promyelocyte stage, and then disappear as promyelocytes further differentiate. Control probes specific

for myeloperoxidase, lactoferrin, and β-globin mRNA transcripts, respectively, demonstrated contrasting gene expression. Myeloperoxidase mRNA transcripts were also found almost exclusively at the promyelocyte stage, but myeloperoxidase mRNA levels disappeared earlier than do neutrophil elastase mRNA levels, suggesting that expression of these genes may be differently controlled. In comparison, lactoferrin mRNA transcripts were detected late in the neutrophil lineage, while β-globin mRNA was detected only in cells of the erythroid lineage. Together these observations suggest that the expression of the neutrophil elastase gene is likely under very tight control, and is likely different than that for other constituents of the neutrophil granules.

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