

Early commitment to the eosinophil lineage by cultured human peripheral blood CD34⁺ cells: Messenger RNA analysis

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Early hematopoietic progenitors expressing the CD34⁺ phenotype can be harvested from the peripheral blood of normal individuals. We have optimized the liquid culture of human CD34⁺ peripheral blood progenitors (PBPs) to achieve differentiation into a population of cells consisting almost entirely of eosinophil progenitors and maturing eosinophils. Growth of CD34⁺ PBPs for 28 days in the presence of the combination of IL-3, granulocyte-macrophage colony-stimulating factor, and IL-5 resulted in an almost 250-fold increase in cell number, yielding a population that contained 83% maturing eosinophils. The residual population consisted of basophils and mast cells (3% by acidic toluidine blue staining, 15.2% by flow cytometric assay for binding to high-affinity IgE receptor) and immature cells. This provides an opportunity to examine the kinetics of the acquisition of specialized mature eosinophil characteristics during eosinophil differentiation. Several host-defense and bioactive proteins are found almost exclusively in eosinophil granules. In addition, stimulated eosinophils, like neutrophils, produce copious amounts of toxic oxygen radicals. We used our culture system and the sensitive technique of reverse-transcriptase polymerase chain reaction to analyze the kinetics of production of messenger RNA transcripts encoding several eosinophil proteins, including five eosinophil granule proteins and four subunit peptides of the superoxide-generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in small numbers of differentiating eosinophils from peripheral blood CD34⁺ cells. Freshly isolated CD34⁺ PBPs contained transcripts for the ubiquitously present housekeeping protein phosphoglucokinase but contained no eosinophil granule protein transcripts and barely detectable amounts of some oxidase protein transcripts. On day 3 of culture, no cells recognizable by histochemical staining as eosinophils could be detected, but transcripts for all five eosinophil granule proteins were present. These transcripts increased several fold during the entire culture period. Similar kinetics were seen for all but one of the NADPH oxidase protein transcripts. However, transcripts for the p67^{phox} NADPH oxidase protein were not detected until day 7, and functional oxidase activity did not appear until day 12. From that point, oxidase activity increased dramatically over the culture period. These studies demonstrate that commitment of CD34⁺ PBPs to the eosinophil lineage occurs very early, by day 3, but that further events in differentiation must take place before the appearance of histologically staining eosinophil granules and acquisition of functional oxidase capacity. (J Allergy Clin Immunol 1996;98:344-54.)

Key words: Eosinophils, peripheral blood CD34⁺ cells, mRNA, growth factors

The peripheral blood of normal individuals contains small numbers of cells with the primitive

CD34⁺ progenitor phenotype.¹ We and others have demonstrated that cells of the myeloid lineage can be grown from human CD34⁺ peripheral blood progenitors (PBPs).^{2,3} These PBPs are easily accessible and may serve as a useful source for studying the maturation process of hematopoietic cells.

Several groups have demonstrated production of eosinophils and other myeloid cells in liquid cultures from mononuclear cells derived from human bone marrow or cord blood.⁴⁻⁸ We have found that

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Abbreviations used

CLC:	Charcot-Leyden crystal protein
ECP:	Eosinophil cationic protein
EDN:	Eosinophil-derived neurotoxin
EPO:	Eosinophil peroxidase
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
MBP:	Major basic protein
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
PBPs:	Peripheral blood progenitors
PGK:	Phosphoglucokinase
RT-PCR:	Reverse-transcriptase polymerase chain reaction

eosinophils can be grown from the primitive CD34⁺ cells circulating in the peripheral blood of normal individuals, and we have defined optimal culture conditions for production of eosinophils from these progenitors.⁹ In this study by using the combination of growth factors—IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5—we explore further the genetic regulatory events that occur during differentiation of eosinophils from primitive precursors.

Eosinophils have been shown to play an important role in certain inflammatory conditions and to act as effector cells in immunity to parasitic infestations. These effects of eosinophils are mediated by granule proteins and by oxygen radicals generated by the activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex.^{10, 11} Eosinophil granules contain a variety of unique cationic proteins including eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin (EDN), and Charcot-Leyden crystal protein (CLC).

Activation of the superoxide-generating NADPH oxidase in stimulated phagocytes involves interaction of several cytosolic proteins with a membrane-associated flavocytochrome.¹² Two of the cytosolic oxidase proteins, p47^{phox} and p67^{phox}, and the two subunits of the flavocytochrome b₅₅₈, gp91^{phox} and p22^{phox}, are found uniquely in cells of the host defense system. Most of the work defining this system and its development in myeloid differentiation has been done with neutrophils, monocytes, and myeloid leukemic cell lines.¹³ Not only are these proteins found in mature eosinophils,^{14, 15} but stimulated eosinophils actually produce more superoxide per cell than neutrophils.¹⁵

No studies have been done to define the development of this system during eosinophil differentiation.

In this report we document the appearance of messenger RNA transcripts for five eosinophil granule proteins and components of NADPH oxidase during differentiation of eosinophils from CD34⁺ PBPs. In the case of the oxidase proteins, we correlate the appearance of transcripts with the kinetics of the development of functional oxidase activity in eosinophils. These studies demonstrate that commitment of CD34⁺ PBPs to the eosinophil lineage occurs very early, by day 3, but that the appearance of histologically staining eosinophil granules and functional oxidase activity is manifested only later.

METHODS

Enrichment of human PBPs

Human PBPs were obtained from leukapheresis collection of 2 to 4 × 10⁹ light-density leukocytes from healthy adult donors after informed consent was obtained. After lysis of erythrocytes with ammonium chloride, cells were incubated with biotin-anti-CD34 monoclonal antibody and subjected to positive selection by using a commercially available kit with an avidin affinity column (Cellpro, Bothell, Wash.). These cells (2 to 4 × 10⁶) represented about a 1000-fold enrichment for CD34⁺ cells (purity of 60% to 80%) from the leukapheresis collection. The enrichment and CD34⁺ purity were calculated by flow cytometry, as described in detail previously.² The CD34⁺-enriched cells were centrifuged at 500 g and resuspended in 5 ml of Iscove's modified Dulbecco's medium (Gibco, Grand Island, N.Y.) containing 10% fetal calf serum (ICN, Costa Mesa, Calif.), 4 mmol/L glutamine (Gibco), and 10 μm mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.) (enriched medium). The partially purified progenitors were further subjected to adherence in a plastic tissue culture flask for 2 hours to deplete the few contaminating monocytes.

Liquid cultures of CD34⁺ PBPs

CD34⁺ PBPs (0.3 to 0.5 × 10⁶/ml) at about 75% purity were grown in 25 cm² tissue culture flasks (Becton Dickinson, Lincoln Park, N.J.) in enriched medium with growth factors at 37° C in a humidified incubator with 7% CO₂. Eosinophil differentiation was induced by addition of optimal concentrations (as previously determined by us) of IL-3 (25 ng/ml), GM-CSF (10 ng/ml), and IL-5 (10 ng/ml) (R&D Systems, Minneapolis, Minn.). Half of the culture medium was replaced weekly with freshly prepared medium containing the same concentrations of growth factors. The cultured CD34⁺ PBPs were enumerated on day 0 (day of isolation of cells) and subsequently on days 3, 7, 14, 21, and 28 of culture. The percent of cells of eosinophil lineage was determined in cytopsin preparations and was based on

counts of 300 cells stained with Luxol fast blue (Sigma Chemical Co.). The percent of cells of the basophil and mast cell lineage under these culture conditions was determined by acidic toluidine blue staining (300 cells counted) and flow cytometric assay for binding to the high-affinity IgE receptor, as previously described.^{16,17} As a control for basophil and mast cell lineage, we used the same culture conditions for peripheral blood CD34⁺ cells, but with IL-3 as the only growth factor.

Analysis of eosinophil mRNA transcripts by reverse-transcriptase polymerase chain reaction (RT-PCR) in the cultured PBPs

Cultured cells (1 to 2×10^6) were taken on days indicated previously, starting from day 0, washed twice with phosphate-buffered saline, placed directly into 2 ml of RNAzol B (Tel Test, Friendswood, Texas), and stored at -70°C until further processing was done to isolate RNA. RNA isolation was performed in a single-step method as described by Chomczynski and Sacchi.¹⁸ Total cellular (2 μg) RNA was reverse-transcribed in a 50 μl reaction volume with dithiothreitol 4 mmol/L (Promega, Madison, Wis.), 12 units of RNA Guard (Pharmacia, Madison, Wis.), 0.8 mmol/L deoxyribonucleoside triphosphate mix (Boehringer Mannheim, Indianapolis, Ind.), 50 ng of random hexamer oligonucleotides (Promega), and 54 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, Petersburg, Fla.). Incubation was carried out for 40 minutes at 42°C , and the reverse-transcriptase enzyme was inactivated by heating the mixture to 94°C for 5 minutes. The complementary DNA was then diluted 1:1 with water, and 5 μl of the diluted product was used for specific amplification of the various eosinophil proteins, with 2.5 units of Taq DNA polymerase (Boehringer Mannheim) in a PCR Thermocycler (Perkin Elmer, Norwalk, Conn.). PCR products were separated on 1% agarose gel, transferred to a Hybond N⁺ membrane (Amersham, Arlington Heights, Ill.) with standard blotting techniques, and probed with internal oligonucleotides specific for each of the cDNA products. Bands were visualized by using the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham) as previously described.¹⁹ Autoradiographs were scanned with a 600 ZS scanner (Molecular Dynamics, Torrance, Calif.) and quantitated with image analysis software (Image, National Institutes of Health, Bethesda, Md.). PCR reaction conditions were defined such that a log linear relationship was obtained between the specific cDNA and the signal density of the probed PCR product in the detection system. This log linear relationship occurred at 28 cycles for a wide range of conditions. To control for the relative amount of the total mRNA transcribed in each reverse-transcriptase reaction, the reverse-transcriptase product used for analysis of each specific mRNA was also analyzed by identical PCR and measurement technique for the constitutively expressed housekeeping gene phosphoglucokinase (PGK).²⁰ The

data are expressed as the ratio of density of the specific cDNA band in arbitrary units divided by the density of the PGK cDNA band for that sample. This ratio is unique for each product of the PCR reaction.

Oligonucleotide sequences

PCR primer pairs were designed to span at least one intron, when this information was available. Nucleotide sequences for primers and probes, respectively, were as follows (sense 5' primer, antisense 3' primer, and probe for PCR product):

- PGK: GGTCGTTATGAGAGTCGACTTCAA, TAGGACCCGCTCGATTTCACCGT, and GCTAAGCAGATTGTGTGGAATGGTCCCTGTG
- ECP: GCACATCAGTCTGAACCCCCCTCG, TAGAACCTCCTTCCTGGTCTGTCT, and CCAAGTATACGCTGCCCTCATAACAGAACTCTC
- MBP: TCTATCTCAGTGCCAGATATGGTG, TCAGTAGGAACAGATGAAAGGAAG, and ACTTGCCGGAGGTGCTACAGGGGCAACCTG
- EDN: GCACATCAATATGACCTCCAGCA, TAGAACATGTTTGCTGGTGTCTGC, and CCAAGATATGACCTGTCTAGTAACAAAACCTCGGC
- CLC: CTACTGTGACAATCAAAGGGCGAC, AGCCTCAGGCTTGATTCTATGGTC, and CAAGTGTGCTTTGGTCTGCTGTGGTTCATG
- EPO: ATGCATCTGCTCCCAGCCCTGGCA, GAGTGGGCGCAGGTCCTCTCACA, and GAGG-GCACTGACCCAGCCTCCCCCT
- p47^{phox}: AGCCAGCACTATGTGTACATGTTCC, GACGTATGGCTCACCTGCATAGTTG, and GAGTACTGCAGCACGCTCATGAGCCTGCC
- p67^{phox}: CTCCGCAGTCCGACATCCCAGCTC, TGAAGAGTGCCCTCCACTTGGTGC, and CAAGTACACGGTAGTCATGAAGACTCAGCC
- gp91^{phox}: TGGAATGGTGTGTGTGTGAATGCCGAG, AGCTTGGACACCTTTGGGCACCTG, and GGCAGGCATCACTGGAGTTGTCTATCACGCT
- p22^{phox}: GCCAACGAGCAGGCGCTGGCGTCC, GGCTGCTTGATGGTGCCTCCGATC, and GACAGAAGCACATGACCCCGCTGGTGAAGC

Chemiluminescence assay for oxygen radicals by NADPH oxidase in differentiating eosinophils

Aliquots of 1×10^5 cultured PBPs were taken every 2 to 3 days and washed twice in Hanks' balanced salt solution (Biowhittaker, Walkersville, Md.). Cells were then resuspended in 0.1 ml Hanks' balanced salt solution and placed in duplicate in a 96-well flat-bottom plate (Dynatek Microlite 3; Dynatek Laboratories Inc., Chantilly, Va.). Diogenes luminol enhancer solution (0.1 ml) (National Diagnostics, Atlanta, Ga.), containing 1 $\mu\text{g}/\text{ml}$ phorbol myristate acetate (Sigma Chemical Co.) was added to each well, and chemiluminescence of each well was immediately measured for 0.5 seconds, every minute for 15 minutes in a single-channel microplate

Luminoscope luminometer (Labsystems, Raleigh, N.C.). The maximum rate of chemiluminescence (oxidase activity) is reported as the relative peak light emitted (height of the curve in arbitrary units).

Purification of eosinophils from peripheral blood

Mature eosinophils were isolated from peripheral blood of mildly eosinophilic atopic volunteers (4% to 8% eosinophils) by using immunomagnetic negative selection as previously described.²¹ The purity of the eosinophil preparation was always greater than 95% as determined by histochemical staining, with the remainder of the cells being lymphocytes. The detection of mRNA transcripts for the various eosinophil proteins and chemiluminescence of mature eosinophils were performed exactly as described for the human PBPs.

RESULTS

Differentiation of CD34⁺ cells to eosinophils

Purified CD34⁺ PBPs were cultured with optimal concentrations of IL-3 (25 ng/ml), GM-CSF (10 ng/ml), and IL-5 (10 ng/ml) for 28 days. The number of cells in cultures and the time course of appearance of cells belonging to the eosinophil lineage (Luxol stain-positive) were monitored from day 0 to day 28. As shown in Fig. 1, *A* the total number of cells in cultures at day 0 increased 5.7- ± 1.9-fold by day 7, 41.3- ± 18.2-fold by day 14, and 247- ± 74.5-fold by day 28 of culture. In other studies (not shown), IL-5 alone at 10 ng/ml without the presence of other growth factors did not support any proliferation or differentiation of the purified preparations of CD34⁺ cells. This suggests that late committed eosinophil progenitors were absent from the CD34⁺ cell preparations.

Histochemical staining of cytopsin preparations of the cultured progenitors revealed no cells of the eosinophil lineage on days 0 and 3 of culture (Fig. 1, *B*). However, by day 7, 5.6% ± 2.6% of the cells in culture were Luxol-positive eosinophil precursors. The percentage of cells of eosinophil lineage increased to 52% ± 9.7% by day 14. On day 28 of culture, 83.3 ± 4.1 of all cells in the culture contained Luxol-positive granules. Almost 50% of these cells had nuclear features of mature (segmented) eosinophils, and the rest were myelocytic and metamyelocytic eosinophils. No cells of the maturing neutrophil lineage were seen (Fig. 1, *B*). The percent of basophil and mast cells on day 14 of culture (IL-3, GM-CSF, IL-5) was 3% as determined by acidic toluidine blue staining (not shown) and 15.2% as determined by a flow cytometric assay for binding to the high-affinity IgE receptor

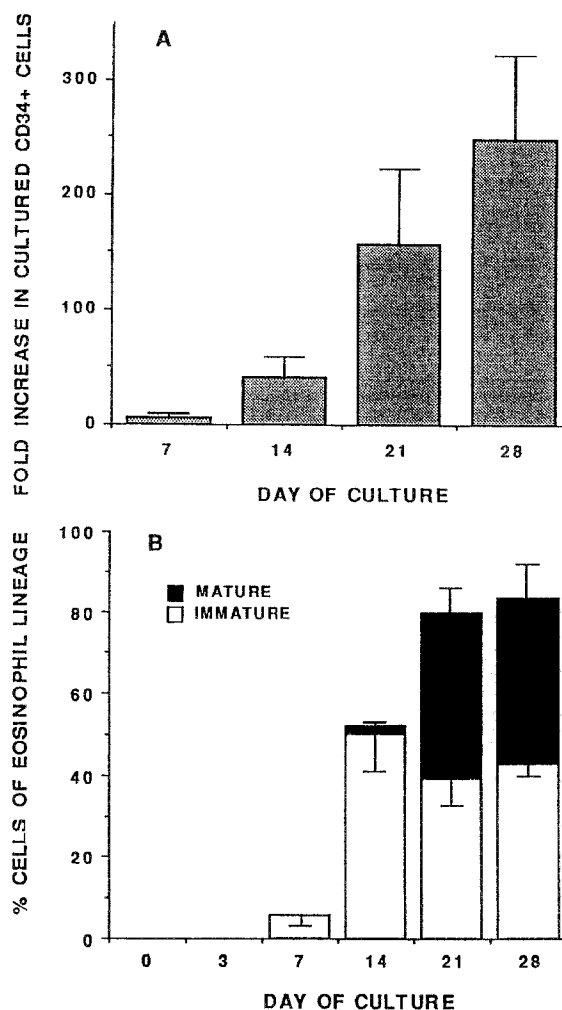


FIG. 1. Measures of proliferation (**A**) and differentiation toward the eosinophil phenotype (**B**) of purified normal human peripheral blood CD34⁺ cells cultured in enriched medium containing IL-3 (25 ng/ml), GM-CSF (10 ng/ml), and IL-5 (10 ng/ml). On indicated days, cells were counted, and results are expressed as fold increase (mean ± SD) in cell numbers compared with day 0 (**A**). Cytospin preparations were stained with Luxol fast blue to identify eosinophils and eosinophil precursors. Percentages of mature eosinophils with segmented nucleus (*filled bars*) and immature eosinophils with typical myelocytic and metamyelocytic structure (*open bars*) were determined on the basis of counts of 300 cells in each cytopsin preparation (**B**). On days 0 and 3, no Luxol stain-positive cells were detected. Data represent the mean ± SD of five to eight experiments.

(Fig. 2, *lower right panel*). However, when PBPs were cultured in IL-3 alone, the percent of basophils and mast cells was 16% as determined by to acidic toluidine blue staining (not shown) and 28.5% as determined by flow cytometric assay for binding to the high-affinity IgE receptor (Fig. 2, *upper right panel*).

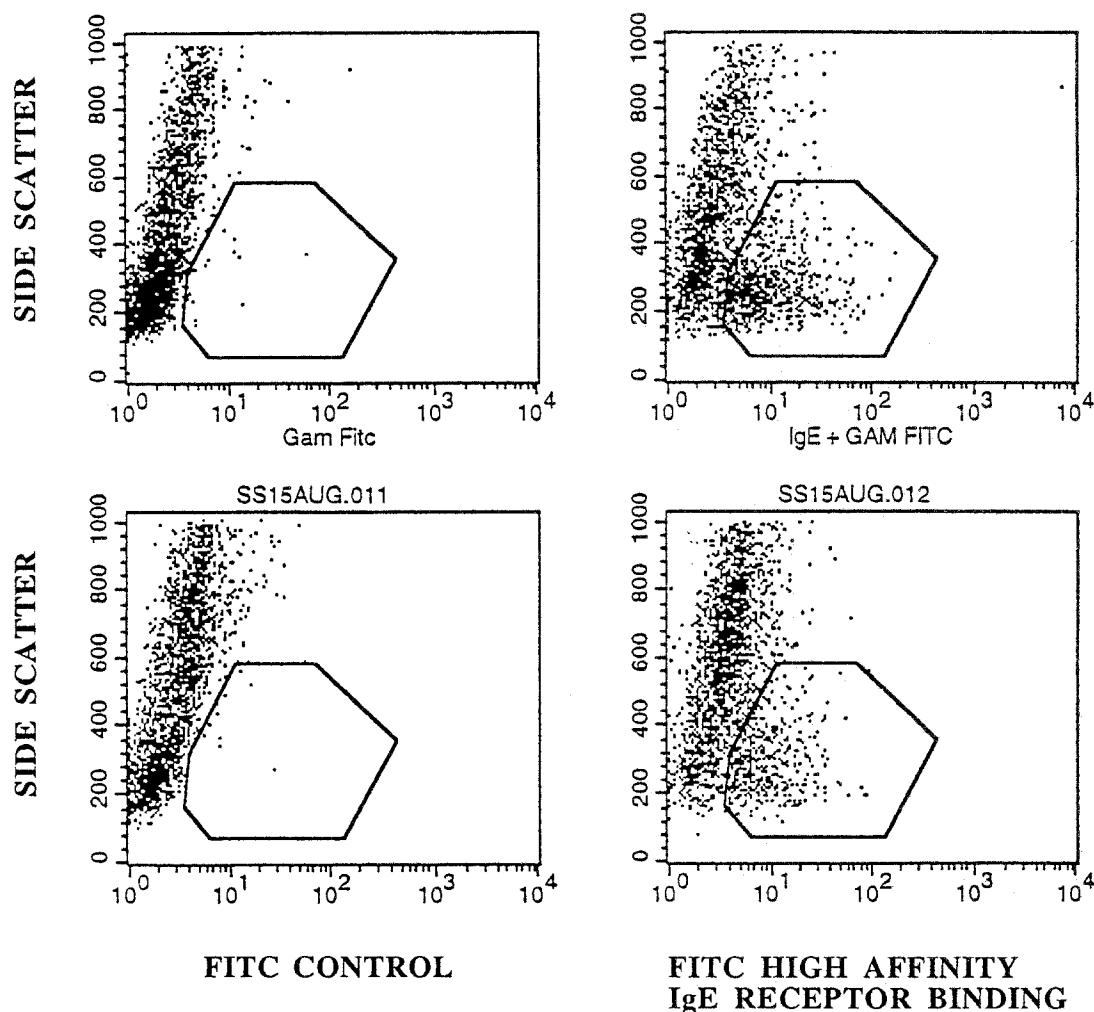


FIG. 2. Flow cytometric dot-blot analysis for binding to the α -subunit of the high-affinity IgE receptor. The two upper panels show PBP-derived cells on day 14 of culture in medium containing IL-3 alone, and the two lower panels show PBP-derived cells on day 14 of culture in a combination of IL-3, GM-CSF, and IL-5. The two panels on the left are controls in which cells have been stained with an irrelevant subclass antibody, followed by the secondary antibody goat-anti-mouse Ig-FITC, and cells in the right panels were incubated with the antibody to the α -subunit of the high-affinity IgE receptor and then stained with the secondary goat-anti-mouse Ig-FITC antibody.

RT-PCR analysis of mRNA encoding for eosinophil granule proteins

Using the sensitive RT-PCR technique, we monitored the appearance of mRNA transcripts of eosinophil granule proteins. Each granule protein mRNA PCR product was normalized to the PCR product of the mRNA of PGK, and a ratio was established. As shown in Figs. 3 and 4, on day 0 of culture, no mRNA transcripts of eosinophil granule proteins could be detected. Although no eosinophil lineage cells could be identified by Luxol staining on day 3 of culture (Fig. 1, *B*), a small amount of specific mRNA transcripts of EPO, ECP, MBP, EDN, and CLC could be demon-

strated. A further increase in the amount of these mRNA transcripts was seen by day 7, with the levels rising slightly or remaining stable from day 14 to day 28 of culture. When similar mRNA transcripts were sought in mature human peripheral blood eosinophils, no mRNA transcripts of EPO and MBP were detected. By contrast, the transcript PCR ratio (granule protein PCR signal / PGK PCR signal) for ECP, EDN, and CLC detected in mature eosinophils (1.26 ± 0.36 , 1.92 ± 0.73 , and 3.77 ± 2.0 , respectively) was comparable to the ratio in progenitors differentiated to eosinophils in culture for 28 days (1.43 ± 0.31 , 1.22 ± 0.36 , and 4.41 ± 0.96 , respectively).

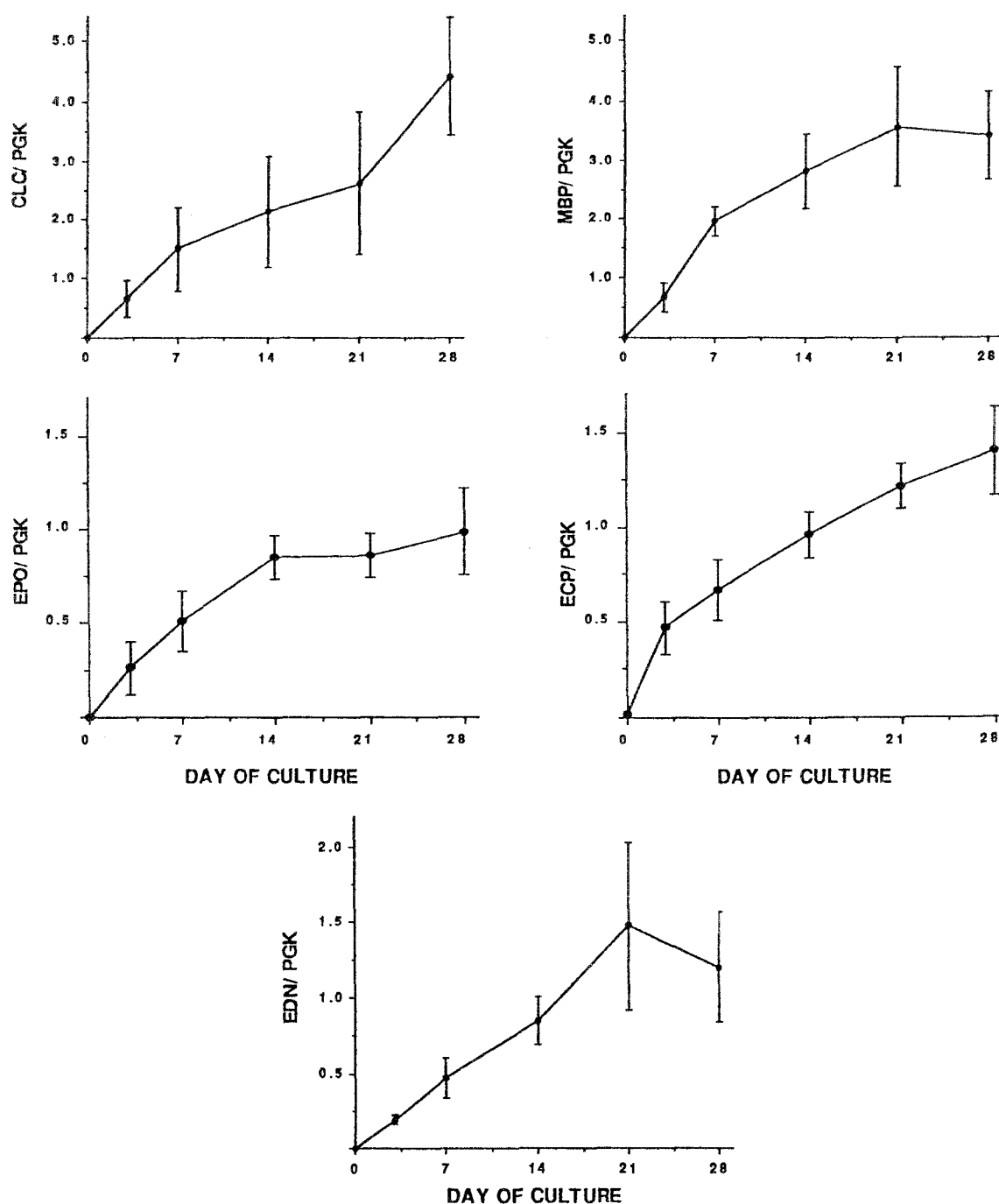


FIG. 3. Relative levels of mRNA transcripts for the eosinophil granule proteins—CLC, EDN, EPO, MBP, and ECP—produced by the normal human peripheral blood CD34⁺ cells in the cultures described in the legend to Fig. 1. Transcripts were detected by RT-PCR as described in Methods, and results are expressed on the vertical axis of each graph as the ratio of granule protein RT-PCR densitometry signal to the PGK RT-PCR densitometry signal. On the x axis is the day of culture on which the analysis was performed. Data represent the mean $\pm$ SD of three to five experiments.

RT-PCR analysis of mRNA encoding for eosinophil NADPH oxidase subunit proteins

Unlike the eosinophil granule proteins for which no RT-PCR product was detectable in freshly

isolated CD34⁺ PBPs on day 0, there was some detectable RT-PCR product for the oxidase subunits p47^{phox}, gp91^{phox}, and p22^{phox} on day 0 (Figs. 4 and 5). The amount of RT-PCR product for

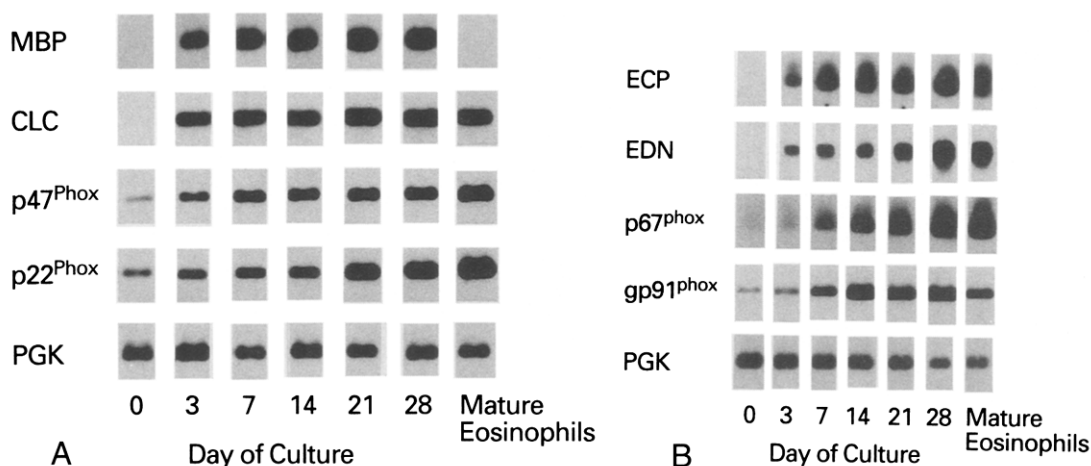


FIG. 4. Luminescence bands detected in each of two representative experiments of RT-PCR analysis of mRNA transcripts in normal human peripheral blood CD34⁺ cells differentiating in culture into eosinophils as described in the legend to Fig. 1. **A**, An experiment in which a culture was analyzed for expression of transcripts encoding granule proteins MBP and CLC, as well as the oxidase proteins p47^{phox} and p22^{phox}. The bottom panel shows analysis of transcripts for the housekeeping protein PGK, which serves as a control for the amounts of template in the different samples. **B**, A different experiment in which a culture was analyzed for expression of transcripts encoding granule proteins ECP and EDN, as well as the oxidase proteins p67^{phox} and gp91^{phox}. The bottom panel again shows analysis of transcripts for the housekeeping protein PGK as a control. Analysis for EPO was not performed as part of either of the representative experiments shown in **A** or **B** but was similar to that seen with ECP.

these three transcripts increased two to three times by day 3 of culture. However, only a trace of RT-PCR product for p67^{phox} transcript was detected on either day 0 or day 3, but it was easily detected by day 7. From day 7 onward, the RT-PCR product ratios for these oxidase proteins continued to increase over the rest of the culture period. By day 28, the RT-PCR ratios of the NADPH oxidase subunit protein transcripts from cultured PBPs and purified peripheral blood eosinophils were similar (data for mature eosinophils not shown).

Chemiluminescence assay for NADPH oxidase function in differentiating eosinophils in CD34⁺ PBP cultures

To evaluate the NADPH oxidase activity of progenitors grown in culture, a chemiluminescence assay was performed every 2 to 3 days, as shown in Fig. 6. Oxidase activity was undetectable in cultured PBPs during the first 10 days of culture with the three growth factors, IL-3, GM-CSF, and IL-5. On day 12 oxidase activity was first detected in the population of differentiating progenitors and was equivalent to 4.6% of the amount produced by an equal number of mature eosinophils (539 ± 169 luminescence units, $n = 4$). Oxidase activity increased linearly, reaching a plateau on day 18, at

which point the activity of the culture population was 20% of that produced by an equal number of mature eosinophils.

DISCUSSION

We have shown that CD34⁺ PBPs differentiate and proliferate predominantly toward the eosinophil lineage under the influence of the hematopoietic growth factors IL-3, GM-CSF, and IL-5.⁹ In this report we used this combination of growth factors to induce maturation of peripheral blood CD34⁺ cells into eosinophils in liquid cultures in order to study the kinetics of expression of genes encoding granule and NADPH oxidase proteins found in mature eosinophils. Under our culture conditions PBPs proliferated sixfold by day 7 of liquid culture, with a dramatic increase in growth seen by days 14 to 28 (41- to 247-fold). Only at the end of the first week of culture could a few Luxol-staining cells of eosinophil lineage be detected. The percent of Luxol-positive cells increased significantly by days 14 through 28 (50% to 80% of total cells in culture). Only a small fraction of cells of the eosinophil lineage was morphologically mature by day 14, but half of them were mature by day 21.

Recently, a few studies have demonstrated production of eosinophils in liquid cultures by using

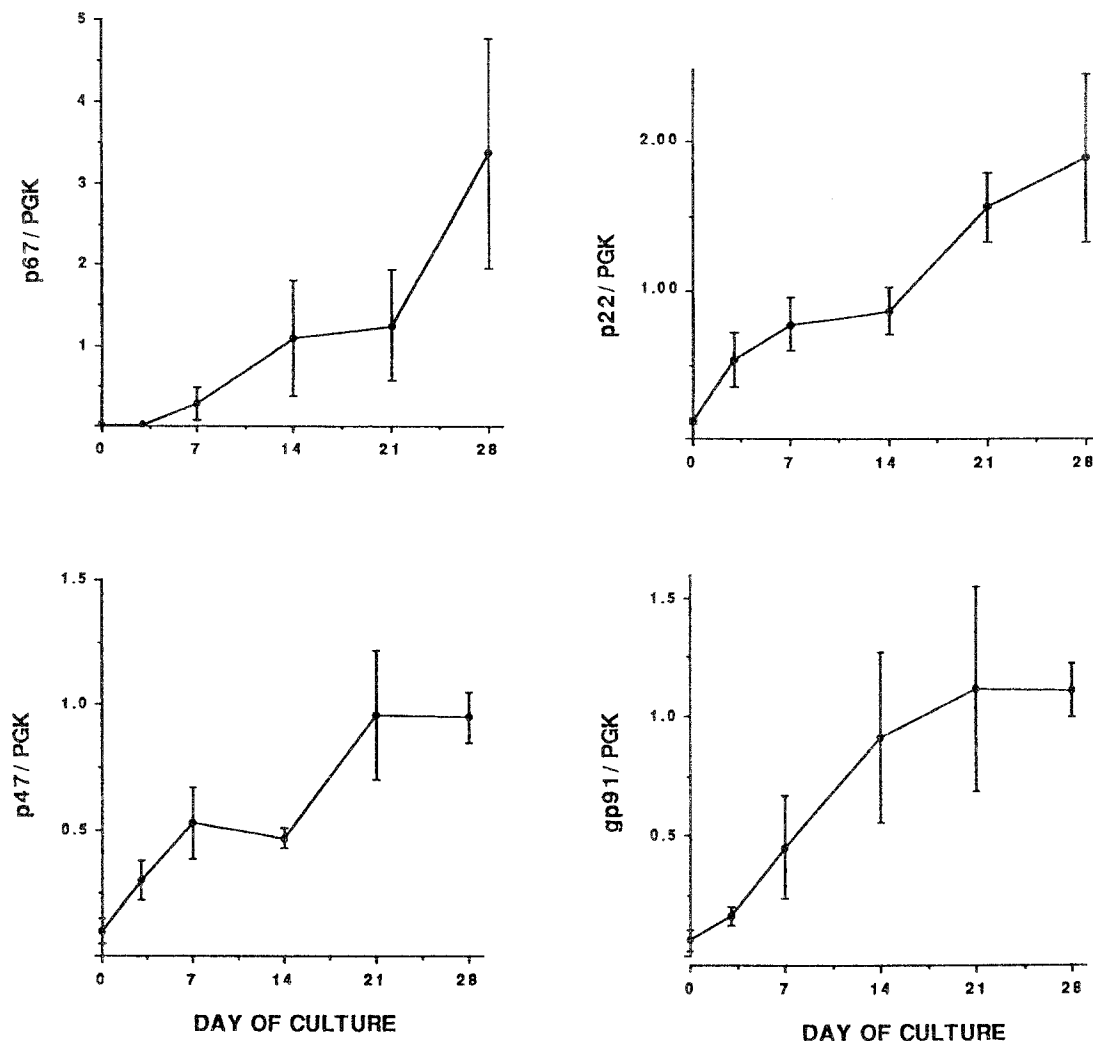


FIG. 5. Relative levels of mRNA transcripts for four NADPH oxidase peptide subunits—p67^{phox}, p22^{phox}, p47^{phox}, and gp91^{phox}—produced by normal human peripheral blood CD34⁺ cells in cultures described in the legend to Fig. 1. Transcripts were detected by RT-PCR as described in Methods, and results are expressed on the vertical axis of each graph as the ratio of granule protein RT-PCR densitometry signal to the PGK RT-PCR densitometry signal. On the x axis is the day of culture on which the analysis was performed. Data represent the mean $\pm$ SD of three to five experiments.

nonselected mononuclear cells derived from either bone marrow or cord blood.⁴⁻⁶ In apparent contradiction to our results, these studies demonstrated a much higher percentage of eosinophils by day 7 of culture and also showed cell proliferation with IL-5 alone in the medium. This is most likely due to the presence of late progenitors and committed cells in the mononuclear cell populations used. From our data, it appears that the CD34⁺ PBPs used in our system provide a cell population that is more uniformly primitive as a starting point for evaluation of events during differentiation of eosinophils. At the same time, our conditions for differentiation yield a high percent of mature eosinophils that

are free of contaminating neutrophils by day 21. However, our culture conditions with IL-3, GM-CSF, and IL-5 did induce differentiation of a small percentage (3% by acidic toluidine blue staining and 15.2% by flow cytometric assay for binding to the high-affinity IgE receptor) of cells toward the basophil or mast cell lineage. Previous reports indicate that use of IL-3 alone to differentiate CD34⁺ cells results in appearance of significant numbers of cells with basophil or mast cell characteristics.²² In our studies, culture of CD34⁺ cells in IL-3 alone generated 16% to 28.5% of the cells with basophil or mast cell characteristics. The addition of GM-CSF and IL-5 to IL-3 in the

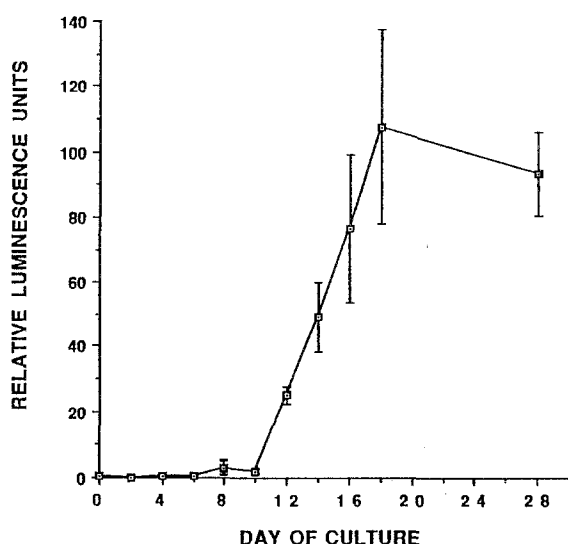


FIG. 6. Phorbol myristate acetate-stimulated oxygen radical-induced chemiluminescence by differentiating eosinophils in cultures of normal human peripheral blood CD34⁺ cells, described in the legend to Fig. 1. Assays were performed as described in Methods. The vertical axis shows oxidase activity in relative luminescence units, and the horizontal axis indicates the day of culture on which analysis was performed. Data represent the mean $\pm$ SD of five experiments.

culture media reduced the percent of cells differentiating toward the basophil or mast cell lineage to 3% to 15%. The mechanism for the change in the percentages cannot be delineated from this observation because it is not possible to discern whether there is actual suppression of differentiation toward the basophil or mast cell lineage or simply enhancement of growth of cells differentiating toward the eosinophil lineage. Most of the previous studies examining the differentiation of these lineages have used unpurified bone marrow mononuclear cells, and not purified CD34⁺ progenitors, as in our study.²³

Relatively little information is available on the expression of eosinophil granule protein transcripts during differentiation of normal human progenitors. The study in which normal progenitors were used was done with cord blood-mixed mononuclear cell populations,⁶ whereas other studies have been done with leukemic cell lines, which may not reproduce the normal pattern of differentiation.²⁴ The specific growth of eosinophils from peripheral blood CD34⁺ cells enabled us to focus on the appearance of mRNA transcripts encoding for both the granule and NADPH oxidase proteins in differentiating eosinophils. The message for the five granule proteins studied

(MBP, EPO, ECP, EDN, and CLC) could not be demonstrated in the CD34⁺ progenitors by using a sensitive RT-PCR assay. This indicates that these cells, at the time of initiation of liquid culture, represent an early progenitor population without any characteristics of the eosinophil lineage. The mRNA for all five granule proteins studied was detected by day 3 of culture, though eosinophil-specific histochemical staining with Luxol did not reveal any cells with eosinophil structure at this time. The amounts of mRNA increased by day 7 of culture and stabilized by days 14 to 28. The kinetics of the increase in the mRNA transcripts encoding the five different granule proteins studied was similar.

Using Northern blot analysis, Gruart et al.⁶ observed the presence of mRNA transcripts encoding four granule proteins—MBP, EDN, ECP, and EPO—in the cultured cells derived from cord blood mononuclear cells. As in our studies, they did not observe transcripts on day 0. They detected transcripts for these four proteins at their next sampling point on day 7. As in our study, they did not find any message for MBP in mature eosinophils from patients with high eosinophilia from different etiologies. However, transcripts for EPO in peripheral blood eosinophils were barely detectable in a quarter of the patients they examined. Our data, obtained by using the more sensitive RT-PCR technique on purified eosinophils from mildly atopic individuals, indicated that no transcripts for either EPO or MBP could be detected. It is possible that with some of the patients they examined, immature eosinophil precursors were included in the peripheral blood samples, accounting for the small amount of EPO transcript detected. We found that the levels of EDN, ECP, and CLC mRNA transcripts in mature eosinophils were similar to the levels of these transcripts in PBP-derived eosinophils on days 14 to 28 of culture. The importance of this dissociation between the mRNA levels of the various granule proteins in mature eosinophils is not clear. However, any explanation of these differences in regulation of transcription of granule protein mRNAs must account for the presence of amounts of transcripts for EDN, ECP, and CLC in mature peripheral blood eosinophils, which are equivalent to amounts present during differentiation and presumably active granule formation.

The NADPH oxidase enzyme complex is an essential component of the host defense system of all phagocytic cells. We used our culture system for differentiation of eosinophils from CD34⁺ PBPs as

a model for studying the kinetics of expression of the four oxidase-specific subunit peptides of the NADPH oxidase. In addition, the acquisition of oxidase function by the differentiating eosinophils was monitored. Very small amounts of mRNA transcripts of the oxidase proteins were detectable at the initiation of culture. Though we cannot eliminate the possibility that this signal derived from a subset of CD34⁺ cells, most likely this derived from the small percentage of myelomonocytic lineage-positive cells contaminating the starting culture. An increase in the message for p47^{phox}, gp91^{phox}, and p22^{phox} was observed by day 3, with further gradual increase thereafter. However, the detection of any signal indicating p67^{phox} mRNA transcripts was delayed until day 7. We previously studied the appearance of NADPH oxidase proteins and activity during induced differentiation of the promyelocytic leukemia cell line HL-60 toward the neutrophil lineage. Particularly when retinoic acid was used as the inducing agent, we found that p47^{phox}, gp91^{phox}, and p22^{phox} proteins appeared early, whereas p67^{phox} protein production was delayed.¹³ Moreover, the appearance of functional oxidase activity followed the kinetics of the late appearance of the p67^{phox} protein, suggesting that this was the limiting factor in this HL60 cell model. It was not possible to determine whether this late appearance of p67^{phox} was a result of the specific inducer, retinoic acid, and/or the leukemic nature of the cell line or whether it correlated with any situation in normal differentiation of the oxidase system. This study supports the notion, at least in the eosinophil model tested here, that the late appearance of p67^{phox} relative to the other factors is a feature of normal differentiation, and may be the limiting factor for the relatively late appearance of functional oxidase activity.

Our report defines a system useful for the study of early events in eosinophil differentiation and should serve as a useful model for delineation of early gene regulation in normal hematopoietic progenitors. PBPs, unlike marrow cells, can easily be obtained from patients, including pediatric patients, on multiple occasions. Although we used apheresis-derived cells for these studies, we have recently found that almost 10⁵ CD34⁺ cells can be purified from as little as 50 ml of whole blood and that they proliferate well in our culture conditions. By using RT-PCR as we have outlined, many of the assays in this study can be performed on samples of cells of that size. It will thus be possible to define events during maturation of CD34⁺ PBPs from

different patient groups with inherited or acquired abnormalities of hematopoietic cells.

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