The mechanisms by which hematopoietic progenitor cells become lineage-committed remain poorly understood. A cloned subline of the AML14 cell line (AML14.3D10) that spontaneously differentiates to eosinophilic myelocytes in the absence of cytokine stimulation was obtained by limiting dilution. This subline exhibits augmented expression of interleukin-5 (IL-5) receptor α subunit mRNA and synthesizes all major eosinophil granule proteins. Exposure of this cell line to all-trans retinoic acid (ATRA) causes loss of eosinophilic granules and fast green staining within 48 hours, without cell death. In addition, mRNA for the IL-5 receptor α subunit becomes undetectable by 48 hours and the cells lose responsiveness to IL-5. Major basic protein, measured as a marker of eosinophilic granule content, decreases from more than 16 pg/cell to undetectable levels by 5 days after ATRA. Concomitant with the loss of major basic protein and fast green staining, surface expression of CD16 becomes detectable and is maximum by 10 days after ATRA. mRNA for the granulocyte colony-stimulating factor (G-CSF) receptor becomes detectable by day 5, and the cells become responsive to G-CSF. At this time, the cells appear morphologically as mature neutrophils and can reduce nitroblue tetrazolium.

With continued culture, the neutrophilic cells die and the culture becomes repopulated with eosinophilic myelocytes. These findings show that it is possible to change the differentiation program of hematopoietic cells even after they show evidence of advanced lineage commitment. The AML14.3D10 subclone of AML14 will be a valuable model for study of the transcriptional regulation of the eosinophil and neutrophil differentiation programs and lineage-specific gene expression.

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MATERIALS AND METHODS

Establishment of the AML14.3D10 Eosinophilic Subline

The establishment and properties of the eosinophil-inducible parent AML14 leukemic cell line have been previously described. After serial passages in culture with supplementation by interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5, a subline was observed to emerge in one flask that did not require cytokine supplementation for eosinophilic differentiation. Cells in this flask spontaneously differentiated to at least the eosinophilic myelocyte stage and retained vigorous proliferative ability with a doubling time of approximately 48 hours. Single cells from this flask were cloned in 96-well plates by limiting dilution in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 5 × 10⁻⁵ mol/L 2-mercaptoethanol (complete medium). No cytokine supplementation was administered. Several clones were isolated and expanded, showing various degrees of proliferative and spontaneous eosinophilic differentiative ability. The most vigorous and eosinophilic of these, designated AML14.3D10, was selected preferentially for further study. This clone has been stable in culture for more than 20 months. More than 55% of AML14.3D10 cells consistently show eosinophilic granules on Wright or fast green staining. Using light microscopy, the majority of the cells appear as eosinophilic promyelocytes or myelocytes, with some cells achieving more advanced stages of differentiation, including nuclear segmentation. These cells strongly express CD33 and CD13, are weakly positive for CD11b, and do not show detectable CD16.

ATRA Treatment of AML14.3D10 Cells

Cells were washed and resuspended at a concentration of 1 × 10⁶/mL in complete RPMI medium containing 1 × 10⁻⁴ mol/L ATRA (ICN, Costa Mesa, CA). Cultures were maintained in tissue culture flasks from which aliquots were periodically removed for viability determination, cytospin preparations, mRNA isolation, flow cytometric analysis, and cytokine measurements. After 5 days of culture with ATRA, the culture supernatant was collected and analyzed for IL-5, interferon-γ, and GM-CSF by enzyme-linked immunosorbent assay. The establishment and properties of the eosinophil-inducible parent AML14 leukemic cell line have been previously described. After serial passages in culture with supplementation by interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5, a subline was observed to emerge in one flask that did not require cytokine supplementation for eosinophilic differentiation. Cells in this flask spontaneously differentiated to at least the eosinophilic myelocyte stage and retained vigorous proliferative ability with a doubling time of approximately 48 hours. Single cells from this flask were cloned in 96-well plates by limiting dilution in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 5 × 10⁻⁵ mol/L 2-mercaptoethanol (complete medium). No cytokine supplementation was administered. Several clones were isolated and expanded, showing various degrees of proliferative and spontaneous eosinophilic differentiative ability. The most vigorous and eosinophilic of these, designated AML14.3D10, was selected preferentially for further study. This clone has been stable in culture for more than 20 months. More than 55% of AML14.3D10 cells consistently show eosinophilic granules on Wright or fast green staining. Using light microscopy, the majority of the cells appear as eosinophilic promyelocytes or myelocytes, with some cells achieving more advanced stages of differentiation, including nuclear segmentation. These cells strongly express CD33 and CD13, are weakly positive for CD11b, and do not show detectable CD16.

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analysis of differentiation related antigens, and other studies, as described below.

Assessment of Lineage-Specific Gene Expression

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from cells using standard methodology as previously described. The RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT), following the manufacturer’s instructions. Optimal primers and annealing conditions were computer selected using the Oligo program (National Biosciences, Plymouth, MN). To control for augmented signals caused by contaminating genomic DNA, the following precautions were taken. Primers selected for RT-PCR of mRNA for major basic protein (MBP), eosinophil peroxidase (EPO), and Charcot-Leyden crystal protein (CLC) were chosen to frame sequences that cross introns, based on the published genomic DNA
sequences for these genes. The primers used for amplification of granulocyte colony-stimulating factor (G-CSF)-receptor mRNA also span introns. For studies of eosinophil-derived neurotoxin (EDN) and the IL-5 receptor \(\alpha\) subunit (IL-5 Re), duplicate amplifications were performed, including all primers and reagents but omitting reverse transcriptase. Amplified products were assumed to be derived from RNA, rather than contaminating DNA, if no amplified product was detected in the duplicate reactions from which reverse transcriptase was omitted. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to ensure the quality of the RNA and reagents used in the reactions and as the standard for semiquantitative comparisons. Reverse-transcribed cDNA was subjected to 28 cycles of amplification. Primers used were as follows: IL-5 receptor \(\alpha\) subunit: sense, 5′GCCAAGAATACAGCAAAG-ACA3′, antisense, 5′TGAGAAACCCACATAAATAGG3′, which were derived from the published sequence of the IL-5 receptor \(\alpha\) subunit cDNA, beginning at position 545 and 982 of the coding region, respectively. Primers used for amplification of MBP, EPO, CLC, and EDN mRNA have been described elsewhere. The above oligonucleotides were synthesized on an Applied Biosystems model 300 oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). Primers for amplification of mRNA for the G-CSF receptor were sense, 5′AAGAGCCCCCTTACCCACTACCATTT3′, and antisense, 5′TGCTGTGAGCTGGGTCTGGGACACT3′, derived from the published sequence of the G-CSF receptor cDNA, beginning at position 1835 and 2174, respectively. These oligonucleotides were obtained from Clontech Laboratories, Inc (Palo Alto, CA).

Radioimmunoassay (RIA) of MBP. Production of a rabbit anti-MBP antibody and the double-antibody RIA method used to measure the MBP content of AML14.3D10 cells before and after exposure to ATRA. Production of antibody against MBP was previously described.11 Aliquots of 1 × 10^6 cells were placed in microcentrifuge tubes and washed in phosphate-buffered saline (PBS), pH 7.2. Granule proteins were extracted from cells by the addition of 500 \(\mu\)L of 0.5% NP-40 in 0.01 mol/L HCl, pH 2.0. After 30 minutes of incubation, supernatants were harvested and frozen at −80°C for later analysis.

Before assay, cell lysates (100 \(\mu\)L) were first reduced and alkylated in 300 \(\mu\)L of 0.35 mol/L Tris-HCl, pH 8.0, 0.12 mol/L NaCl, and 0.01 mol/L EDTA containing 7.5 mmol/L dithiothreitol for 1 hour, followed by the addition of 30 \(\mu\)L of 0.2 mol/L iodoacetamide and incubation for 15 minutes in the dark at room temperature. Reduced and alkylated purified MBP standards or reduced and alkylated cell lysates were diluted in PPF buffer (0.1 mol/L phosphate buffer [pH 7.5], 0.1% proteamine sulfate, 0.5% fetal bovine serum [FBS], and 0.1% NaNO₃). The samples were mixed with 100 \(\mu\)L of 1:5,000 rabbit anti-MBP in PPF and 125I-MBP (2.0 ng/tube). After an overnight incubation at 4°C, 1 mL of ice-cold goat-antirabbit IgG, 1:80 dilution in PPF, and 100 \(\mu\)L 1:20 normal rabbit serum in PPF were added. The tubes were mixed and incubated at 4°C for 3 hours.
Assessment of Cytokine mRNA by AML14.3D10 Cells

To screen AML14.3D10 cells for production of cytokines active on eosinophils or neutrophils, RT-PCR was performed on RNA isolated from cells before and 10 days after ATRA induction. Oligonucleotide primers for the detection of mRNA for IL-3, GM-CSF, IL-5, and G-CSF were obtained from Clontech Laboratories, Inc. All primer sets were known to span introns based on published genomic sequences. Conditions for reverse transcription and amplification were as described above.

Nitroblue Tetrazolium (NBT) Reduction

NBT reduction was assayed as described. Cells were incubated at a concentration of 2 x 10^5/mL for 25 minutes at 37°C with an equal volume of 0.2% NBT dissolved in Dulbecco's PBS containing 200 ng of freshly diluted tetradeacynophorol acetate per milliliter. Reaction tubes were grossly examined for colored precipitate, and cytopreparations were made to assess the cellular content of blue-black formazan deposits.

Assessment of Proliferative Response to Cytokines

AML14.3D10 cells were cultured in complete medium for 72 hours with and without ATRA. After this, cells from both groups were placed in 96-well plates (1 x 10^5 cells/well) in a total volume of 200 µL complete medium alone or with either recombinant human IL-5 (rhIL-5; a gift of Michael Grace, Schering Plough Corp; Kenilworth, NJ) or rhG-CSF (Genzyme, Cambridge, MA) in quadruplicate for 48 hours before the addition of 1 µCi [3H]-thymidine and culture for an additional 12 hours. Cells were then harvested onto glass fiber filters for liquid scintillation counting.

RESULTS

ATRA Treatment of AML14.3D10 Cells

Light microscopic study of AML14.3D10 cells cultured with ATRA showed striking changes over a 3-week period (Fig 1). Within 24 hours, the cells had lost most of their eosinophilic granules as assessed by both Wright and fast green staining. This continued until 7 days, by which time no eosinophilic granules were visible and no fast green staining was detectable. Also during this time period, nuclear chromatin became more condensed and many cells developed nuclear indentations or segmentation and showed fine neutral-staining cytoplasmic granulation. The appearance of the cells at this stage strongly resembled that of maturing neutrophils. Although cell proliferation slowed during this period, it did not completely stop and there was no significant cell death in culture as assessed by serial inverted microscopic examination of cultures and assessment of viability by trypan blue staining. The cultures attained maximum morphologic maturity by day 10 to 14. After this, cell viability steadily declined until after day 21, when the cultures began to be repopulated by vigorously growing cells that now clearly evidenced eosinophilic differentiation by Wright and fast green staining. Identical results were obtained in eight separate experiments.

Assessment of Lineage-Specific Gene Expression

Analysis of mRNA expression of differentiation-related genes. AML14.3D10 cells show augmented expression of mRNA for the IL-5 Rα subunit in comparison to parental AML14 cells or TF-1 cells. Treatment of AML14.3D10 cells with ATRA caused a rapid loss of detectable mRNA for the IL-5 Rα subunit that was evident by 3 hours and complete by 48 hours. RNA remained undetectable for at least 10 days (Fig 2).

Uninduced AML14.3D10 cells did not contain detectable mRNA for the G-CSF receptor. After exposure to ATRA, G-CSF receptor mRNA was clearly evident by 5 days and appeared to be maximally expressed by days 7 to 10 (Fig 2). Identical reciprocal modulation of IL-5 Rα subunit and G-CSF expression was observed for both the human TF-1 cells and the parental AML14.3D10 cells.
Fig 5. Flow cytometric analysis of CD16 and CD33 expression on AML14.3D10 cells before and at sequential times after ATRA exposure. Y axis, cell number; X axis, relative fluorescence intensity. The control histograms represent background fluorescence of cells incubated with a fluorochrome-conjugated isotype-matched MoAb with no human tissue specificity. The CD16 histogram is directly superimposed on the control histogram before addition of ATRA and at 3 days afterward.

Fig 6. Ethidium-stained gel of RT-PCR products of mRNA for IL-3, GM-CSF, IL-5, and G-CSF from AML14.3D10 cells before (0) and 7 days after (+) ATRA exposure. Products were of expected mass for the primers used. GAPDH was used to verify the integrity of RNA and reagents.
cells or 16 pg/cell. Five days after ATRA exposure, MBP was undetectable in cell lysates by RIA (Fig 4).

Flow cytometric analysis of differentiation-related antigen expression. The CD16 antigen is expressed on neutrophils but not on eosinophils, a fact that has been found useful in purifying eosinophils by negative selection with CD16-coated magnetic beads. After ATRA treatment, CD16 became detectable on AML14.3D10 cells by 6 days and increased in intensity until 8 to 10 days (Fig 5), after which expression decreased until it was no longer detectable by 17 to 21 days (data not shown). Over the time course studied, expression of CD33 remained essentially constant. These results were reproducible in four separate experiments.

Production of Cytokine mRNA by AML14.3D10 Cells
mRNA for GM-CSF was easily detectable in AML14.3D10 cells. No mRNA for IL-3, IL-5, or G-CSF was detected. After ATRA-induced neutrophilic differentiation, mRNA for GM-CSF was not detected (Fig 6).

Proliferative Response of AML14.3D10 to IL-5 or G-CSF
AML14.3D10 cells showed a proliferative response to IL-5, but no response to G-CSF (Fig 7A). Seven days after exposure to ATRA, the cells no longer proliferated in response to IL-5, but now could be shown to be growth inhibited in response to G-CSF (Fig 7B).

NBT Reduction
Little or no NBT was reduced by unstimulated AML14.3D10 cells. However, cells that were treated with ATRA for 7 days rapidly reduced NBT. The reduced blue precipitate was easily visible in the reaction tubes (Fig 8). Examination of cytopreparations showed formazan precipitates in the majority of ATRA stimulated cells, but not in cells that had not received ATRA.

DISCUSSION
These studies show that a clonal cell line that is committed to eosinophilic differentiation can change its differentiation program to the neutrophilic lineage after exposure to ATRA. A possible mechanism for this could be the induction of an alternative differentiation program in a small subpopulation of uncommitted stem cells present in phenotypically eosinophilic AML14.3D10 cultures. This is the mechanism that is apparently operative in other leukemic cell lines in which differentiation along multiple lineages can be induced. The HL-60 cell line shows spontaneous differentiation to the promyelocyte stage and can be induced to neutrophilic, monocytic, eosinophilic, or basophilic lineages with a variety of agents. Sublines of HL-60 have been obtained that will differentiate predominantly to eosinophils when exposed to butyric acid or IL-5. These events show the induction of an immature myeloid precursor to a more differentiated phenotype.

In contrast to studies of the differentiation of HL-60 and other cell lines, our observations suggest that the lineage switch in our system occurs at least in part by a change in the differentiation program of cells that already show advanced evidence of eosinophilic differentiation. The addition of ATRA to AML14.3D10 cultures does not result in cell death. Despite this, mRNA for the IL-5 Rα subunit is markedly diminished within hours and is undetectable by 48 hours. Serial light microscopic examination of cytopreparations of cultures after ATRA induction clearly shows a gradual loss of eosinophil-specific fast green staining in individual cells, with concomitant gradual acquisition of neutrophil morphology. During the first few days, mRNA for eosinophil granule proteins becomes undetectable, and granule protein levels also decrease, as reflected by measurement of MBP. By 5 days after ATRA, mRNA for the G-CSF receptor is detectable; by 6 days, CD16 is expressed. Modulation of cytokine receptor mRNA content is reflected in the biologic behavior of the cells, which are responsive to IL-5 and unresponsive to G-CSF before ATRA induction, but are unresponsive to IL-5 and responsive to G-CSF after ATRA. Because cell death does not occur during the first 10 to 14 days after ATRA treatment, the fact that the cultures do not contain both phenotypically eosinophilic and neutrophilic cells during this interval strongly suggests that individual eosinophilic cells have suppressed the eosinophil differentiation program and activated the neutrophil program. We speculate
that this phenomenon is observable in the AML14.3D10 cell line because of its apparent arrest at the eosinophilic myelocyte stage. Cells at this stage, although showing clear lineage commitment, are still capable of division and presumably still responsive to other influences regulating gene expression. After ATRA induction, cell death does occur after the peak expression of the neutrophilic phenotype at 10 to 14 days. This is likely due to a more complete or terminal differentiation along this lineage in response to ATRA. A small subpopulation of cells resistant to ATRA must exist in this system, because phenotypically eosinophilic cells invariably repopulate cultures after about 21 days. After this repopulation, it is possible to repeat the whole sequence of eosinophil to neutrophil transformation in the same culture by again adding ATRA.

It is possible that endogenous production of GM-CSF plays a role in the maintenance of proliferation and eosinophilic differentiation of the AML14.3D10 cell line. It is noteworthy that the phenotypically undifferentiated AML14 cell line, from which AML14.3D10 was subcloned, does not produce GM-CSF (data not shown). After ATRA exposure, GM-CSF production is greatly reduced, suggesting the possibility that suppression of GM-CSF production could be permissive for the induction of the neutrophil differentiation program. Future experiments will address these issues.

The addition of ATRA to purified human hematopoietic progenitor cells cultured in the presence of IL-3 and GM-CSF has not been found to affect the eosinophilic differentiation induced by these cytokines. However, ATRA has been shown to cause a decrease in erythroid differentiation and an increase in granulocytic differentiation in progenitor cultures supplemented with low doses of IL-3 and GM-CSF and saturating amounts of erythropoietin. These findings suggest a possible role for retinoids in the process of lineage commitment of normal progenitors.

Conventional models of hematopoiesis begin with multipotent stem cells that, in addition to self-renewal, either stochastically commit to a single lineage or produce progeny having progressively restricted lineage potential, resulting ultimately in progenitors that are capable of differentiation along only a single lineage. In either model, once lineage commitment has occurred, the process has been thought to be irreversible. Although performed on an admittedly abnormal leukemic cell line, our studies suggest that lineage commitment may not be final. This concept is unconventional in regard to hematopoietic cells, but evidence has been obtained that differentiation shifts may occur in other tissues. Lung cancer cells, for example, have been shown to be switchable from neuroendocrine (small cell) to epithelial (adenocarcinoma).

Cell differentiation is thought to be regulated largely at the level of gene transcription. There has been intense interest in recent years in the transcription factors that regulate expression of lineage-specific genes. Although a number of these have been described and functionally characterized in hematopoietic cells, the hypothetical master switches that activate entire differentiation programs have remained elusive. Systems such as the one that we have described may provide valuable opportunities for the isolation of signals initiating or suppressing entire programs of differentiation.

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Changing the differentiation program of hematopoietic cells: retinoic acid-induced shift of eosinophil-committed cells to neutrophils

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