EOSINOPHILIC DIFFERENTIATION OF THE HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE, HL-60

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By the addition of specific chemical agents to the culture medium, the HL-60 leukemia cell line, derived from a patient with acute promyelocytic leukemia (1), can be induced to mature to cells with many of the functional, morphological, and biochemical features of either neutrophils or macrophages (2, 3). Two recent studies strongly suggested that HL-60 cells also differentiated to eosinophils when cultured in soft agar (4, 5); the colonies from soft agar stained with Luxol-fast-blue, a stain specific (among hematopoietic cells) for eosinophilic granules (6). Unfortunately, few cells differentiated to eosinophils, and it was technically difficult to harvest cells from semi-solid medium. These factors limit the usefulness of that system as a model for eosinophilopoiesis. Furthermore, the two reports disagreed on the role of added colony-stimulating factors. The recent description of increased numbers of morphologically abnormal eosinophils in the bone marrow of patients with FAB type M4 leukemia (AMMoL)[1] who have abnormalities of chromosome 16, del(16)(q22) (7) and inv(16)(p13q22) (8), has further focused attention on the phenomenon of eosinophilic differentiation of leukemia cells. We observed that HL-60 cells cultured in slightly alkaline liquid medium develop granules typical in appearance to those found in eosinophils and eosinophilic precursors. Our higher induction efficiency and ease in harvesting cells from liquid culture permitted a more complete characterization of the differentiated cells. To determine whether HL-60 cells might serve as a model system for eosinophilopoiesis in general, or eosinophilic differentiation of leukemia cells in particular, the HL-60–derived eosinophils were characterized morphologically, histochemically, and cytogenetically.

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[1] *Abbreviations used in this paper:* AMMoL, Acute Myelomonocytic Leukemia; Bicine, *NN*-bis(2-Hydroxyethyl)glycine; CLC, Charcot-Leyden crystal; dmin, double minute chromosome; DMSO, dimethylsulfoxide; FAB, French-American-British Classification; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; MBP, eosinophil major basic protein; PAS, periodic acid-Schiff reaction; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane.

Materials and Methods

Cell Culture. Experiments were done with HL-60 cells between passage levels 10 and 30, except as noted. Control cultures were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA, Lot 29101556 or Sterile Systems, Logan, UT, Lot 100273) and penicillin and streptomycin (50 IU/ml and 50 μg/ml, respectively; Gibco). These were kept in a humidified atmosphere of 5% carbon dioxide at 37°C. Cells were subcultured once weekly to a density of ~5×10^5 cells/ml and fed with an equal volume of medium at midweek. As added to the cells, this medium was at pH 7.4 and contained no additional organic buffers. Unless otherwise stated, control cells were taken from these cultures during the logarithmic growth phase.

Except as noted, eosinophil inductions were performed by resuspending HL-60 cells in plateau growth phase, at 5×10^5 cells/ml in 10 ml of fresh medium, buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (HEPPS) and titrated to pH 7.8, in T-25 flasks (Corning Glass Works, Corning, NY) at 37°C for 7 d. The flask caps were tightened because this tended to delay the fall in medium pH that occurs with time. Organic buffers (Sigma Chemical Co., St. Louis, MO) were kept as concentrated stock solutions in sterile normal saline. They were added to medium at the specified concentration and adjusted to the desired pH by addition of 1 M NaOH or HCl solutions. The pH was measured at 37°C using a model MI-410 combination pH electrode (Microelectrodes Inc, Londonderry, MA) and a pHI 70 pH meter (Beckman Instruments, Irvine, CA). Induction of neutrophilic differentiation was performed by culturing 5×10^5 cells/ml in medium, without added organic buffers, with 176 mM dimethyl sulfoxide (DMSO) (Aldrich, Gold Label, Milwaukee, WI) for 7 d at 37°C. Viable cell counts were performed in a hemocytometer by the exclusion of trypan blue.

Light Microscopic Evaluation. Cytocentrifuge preparations were made with a Cytospin Model 1 (Shandon Southern Instruments, Sewickley, PA). For routine evaluation, they were stained with Wright-Giemsa. The following special stains were also used (all reagents from Sigma): Luxol-fast-blue (6), toluidine blue (9), peroxidase and eosinophil peroxidase (cells fixed in formalin vapor [10]), acid phosphatase (9), arylsulfatase (11), chloroacetate esterase (9), α-napththyl acetate esterase (9), Feulgen reaction (12), Sudan Black (9), periodic acid-Schiff (PAS) (9).

Electron Microscopic Evaluation. Cells were fixed in a phosphate-buffered fixative containing 1% glutaraldehyde and 4% formaldehyde. Pellets were sectioned, stained, and examined on a JEOL 100 CX electron microscope (JEOL, Tokyo, Japan).

Immunofluorescence Techniques. Cytocentrifuge preparations were stained with antibodies specific for human eosinophil granule major basic protein and for Charcot-Leyden Crystal (CLC) protein (lysophospholipase), as described previously (13, 14). Briefly, slides were fixed in absolute methanol, washed in Dulbecco's phosphate-buffered saline (PBS), and incubated overnight in 10% heat-inactivated normal goat serum and PBS at 4°C. Slides were washed again and placed in a humidified chamber. To stain for eosinophil major basic protein (MBP), slides were overlaid with either protein A–purified normal rabbit IgG as a negative control or affinity chromatography–purified rabbit antibody to human MBP at equal IgG concentrations. To stain for CLC protein, slides were overlaid with the preimmunization serum from the CLC-immunized rabbit as negative control or the postimmunization anti-CLC antiserum. In both instances, after incubation overnight at 4°C slides were washed with PBS, stained with a 1% solution of chromotrope 2R, and washed again with PBS. Slides were then overlaid with fluorescein-conjugated IgG fraction of goat anti-rabbit IgG, incubated, washed with PBS and mounted with 10% PBS/90% glycerol solution containing *p*-phenylenediamine, coverslipped, and sealed with clear nail polish. Slides were examined with a Zeiss standard microscope equipped with vertical illumination for epi-fluorescence and fluorescein filter system. Addition of *p*-phenylenediamine to the mounting medium significantly reduced fluorescent quenching allowing for prolonged examination and for multiple fluorescence photomicrographs of individual fields. In some cases fluorescent cells were photographed, the coverslips removed, the slides counterstained with Wright's stain, and the identical fields rephotographed under

transmitted light. Our specificity controls for the immunofluorescence staining have been presented previously (13).

Cytogenetic Analysis. Chromosome studies were performed on logarithmically growing cells at passage 12. After a 10-min exposure to colcemid (0.05 μg/ml), the cells were treated with hypotonic KCl (0.075 M) for 20 min at 37°C, and then fixed with ethanol-acetic acid (3:1). Chromosome preparations were made by the air-drying method. Some of these preparations were stained with quinacrine mustard (50 μg/ml) to obtain Q-bands, and others were stained conventionally with Giemsa to better assess the incidence of cells with double minute chromosomes (dmin). Karyotypes were expressed according to the International System for Human Cytogenetic Nomenclature (1978), (1981) (15, 16).

Results

Light Microscopic Morphology. After culture in alkaline medium, cells with the appearance of eosinophils and eosinophilic precursors were observed with the Wright-Giemsa stain (Fig. 1). The least mature cells, which were recognizable as eosinophilic precursors by the presence of new granules, appeared to be eosinophilic promyelocytes (Fig. 1 *a*) by virtue of their high nucleo-cytoplasmic ratio, multiple prominent nucleoli, and blue cytoplasm. These cells contained large spherical, dark blue granules, frequently in vacuoles. In contrast, the most mature cells had segmented nuclei with condensed chromatin, indistinct nucleoli, a lower nucleo-cytoplasmic ratio, and pale cytoplasm (Fig. 1 *b*). These cells typically had smaller granules that stained red and were not usually vacuole associated. In general, <20% of cells identifiable as eosinophils were of this latter, most mature type. All intermediate stages of nuclear maturity could also be identified. While the large blue granules tended to be in the less mature cells and the smaller red granules in the more mature ones, occasionally the blue granules persisted in mature cells, and both granule types were often found in the same cell. The two types of granules will be referred to, for convenience, as Type I (large blue) and Type II (small red) without the implication that they are identical to primary and secondary granules of normal eosinophils.

Special Stains. Various special stains were performed on control, DMSO-induced, and alkaline medium–induced HL-60 cell populations. The results are summarized in Table I and presented in detail below. In HL-60–derived eosinophils, both Type I and Type II granules stained with Luxol-fast-blue; however, only rare cells were stained in control or DMSO-induced neutrophil populations. Toluidine blue, which is usually used to identify basophils by virtue of metachromatic staining of the granules (9), metachromatically stained the promyelocytic granules of control HL-60 cells, but not the new granules in alkaline medium or DMSO-induced cells. Control HL-60 cells uniformly demonstrated peroxidase activity in the promyelocytic granules; the DMSO-induced cells showed no peroxidase activity. Both Type I and Type II eosinophilic granules were peroxidase-positive, although they stained less intensely than the promyelocytic granules. In the control cells, 1×10^{-2} M KCN was required to completely inhibit the peroxidase staining reaction. When HL-60–derived eosinophils were stained in the presence of 10^{-2} M KCN, peroxidase positivity was seen in both Type I and Type II granules, signifying the presence of eosinophil peroxidase, which is relatively resistant to inhibition by cyanide, compared with myeloperoxidase (10). Prominent Sudan Black staining, which is dependent on the presence of peroxi-

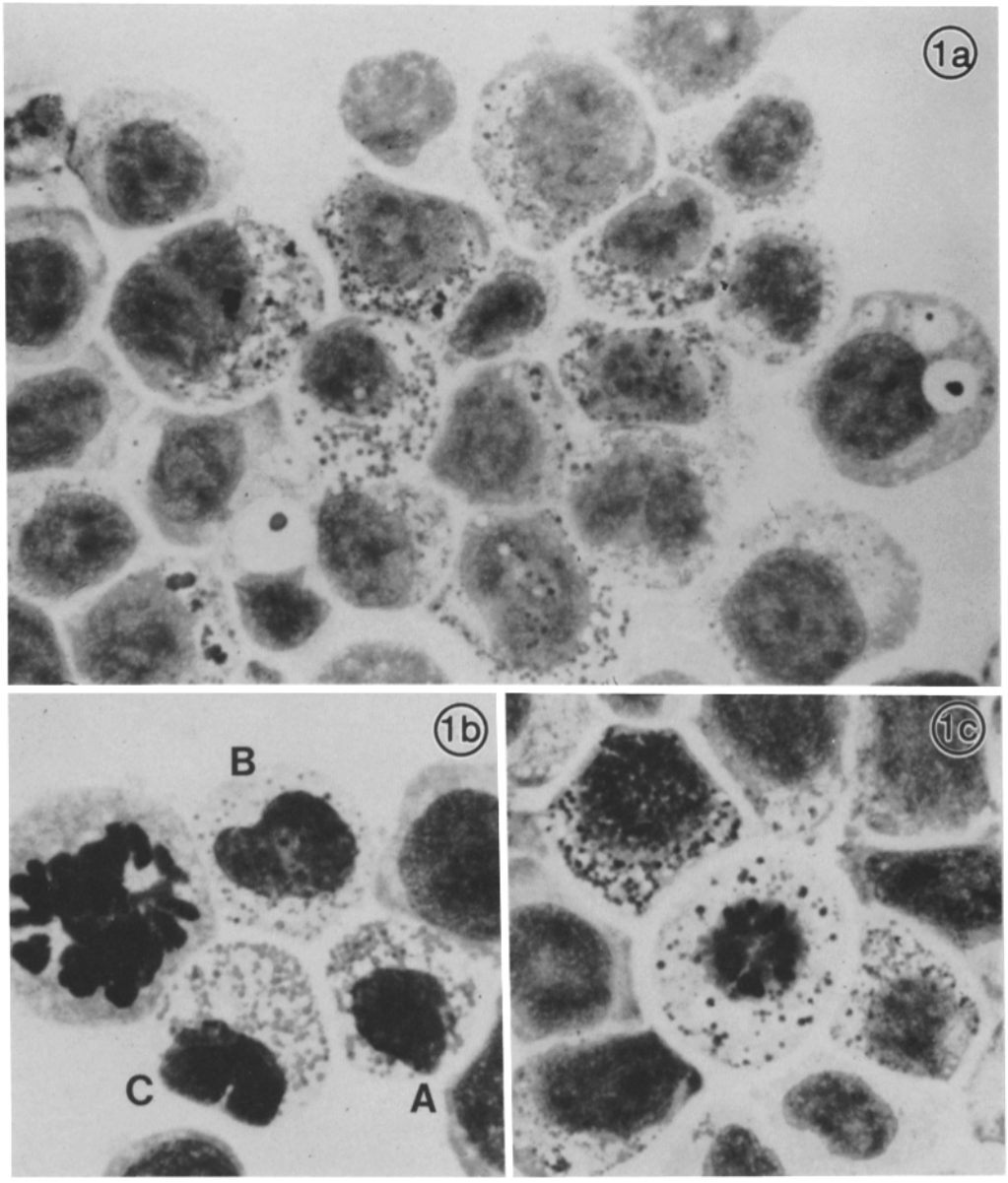

FIGURE 1. HL-60 cells were cultured for 7 d in medium buffered to pH 7.8 containing 25 mM HEPPS. Cytocentrifuge preparations were stained with Wright-Giemsa and photographed at final magnification ×1,280. (*a*) HL-60–derived eosinophils with immature nuclei. (*b*) More mature HL-60 derived eosinophils with nuclei in myelocyte (*A*), metamyelocyte (*B*), and band (*C*) stages of maturity. (*c*) HL-60 eosinophil undergoing mitosis.

dase (17), was observed in control cell granules but disappeared in DMSO-induced cells; small, less intensely stained granules were visible in the HL-60–derived eosinophils. Arylsulfatase activity could not be detected in undifferentiated cells, but lead sulfide deposits were associated with some granules in the

TABLE I
Summary of Staining Properties of Promyelocytic and Eosinophilic Granules

Stain	Promyelocytic granules	Eosinophilic granules (Types I and II)
Luxol-fast-blue	−	+
Toluidine blue metachromasia	+	−
Sudan black	+	+
Peroxidase	+	+
Peroxidase + 10^{-2} M KCN	−	+/−
Arylsulfatase	−	+/−
Acid phosphatase	−	+/−
Chloroacetate esterase	+	+
α-Naphthyl acetate esterase	−	−
PAS	−	+
Feulgen	−	−

Special stains were performed on control, DMSO-induced, and alkaline medium–induced HL-60 cells. Granules were not seen in DMSO-induced cells with any of the above stains, so no results are shown. While differences in staining intensity were seen between Type I and II eosinophilic granules with several of the special stains, the results were qualitatively similar and are presented together. The results are given as (+) or (−) if virtually all granules stain positively or negatively, respectively; (+/−) indicates heterogeneity of staining.

HL-60–derived eosinophils, signifying the presence of arylsulfatase activity. Acid phosphatase staining was usually confined to the region of the Golgi apparatus in undifferentiated cells; in both DMSO-induced cells and HL-60–derived eosinophils, virtually the entire cytoplasm was strongly positive. A variable proportion of Type I and Type II eosinophilic granules were positive as well. The great majority of control HL-60 cells were strongly positive for chloroacetate esterase activity, whereas only a rare cell showed α-naphthyl acetate esterase staining. Similarly, both Type I and Type II granules of HL-60–derived eosinophils were chloroacetate esterase–positive and were rarely positive for α-naphthyl acetate esterase. The PAS stain was either negative or faintly positive in control cells, but became strongly positive in HL-60–derived eosinophil cytoplasm. Type I granules were faintly stained but the Type II granules were often brightly positive. Both types of granules were Feulgen-negative and thus did not contain DNA.

Electron Microscopic Morphology. The HL-60 cells were examined after 7 d of culture in HEPPS-buffered medium at pH 7.8. The cells that contained eosinophilic granules were characterized by the following features (Fig. 2*a*). The mitochondria were relatively sparse and the Golgi apparatus was not prominent. The rough endoplasmic reticulum was dilated and contained flocculent material. A fraction of the cells had one or more vacuoles, some of which contained granules. The granules themselves were of two basic types. The more abundant type was spherical (0.4–0.7 μm in diameter), homogeneously electron dense, often membrane bound, and sometimes localized within vacuoles. These were

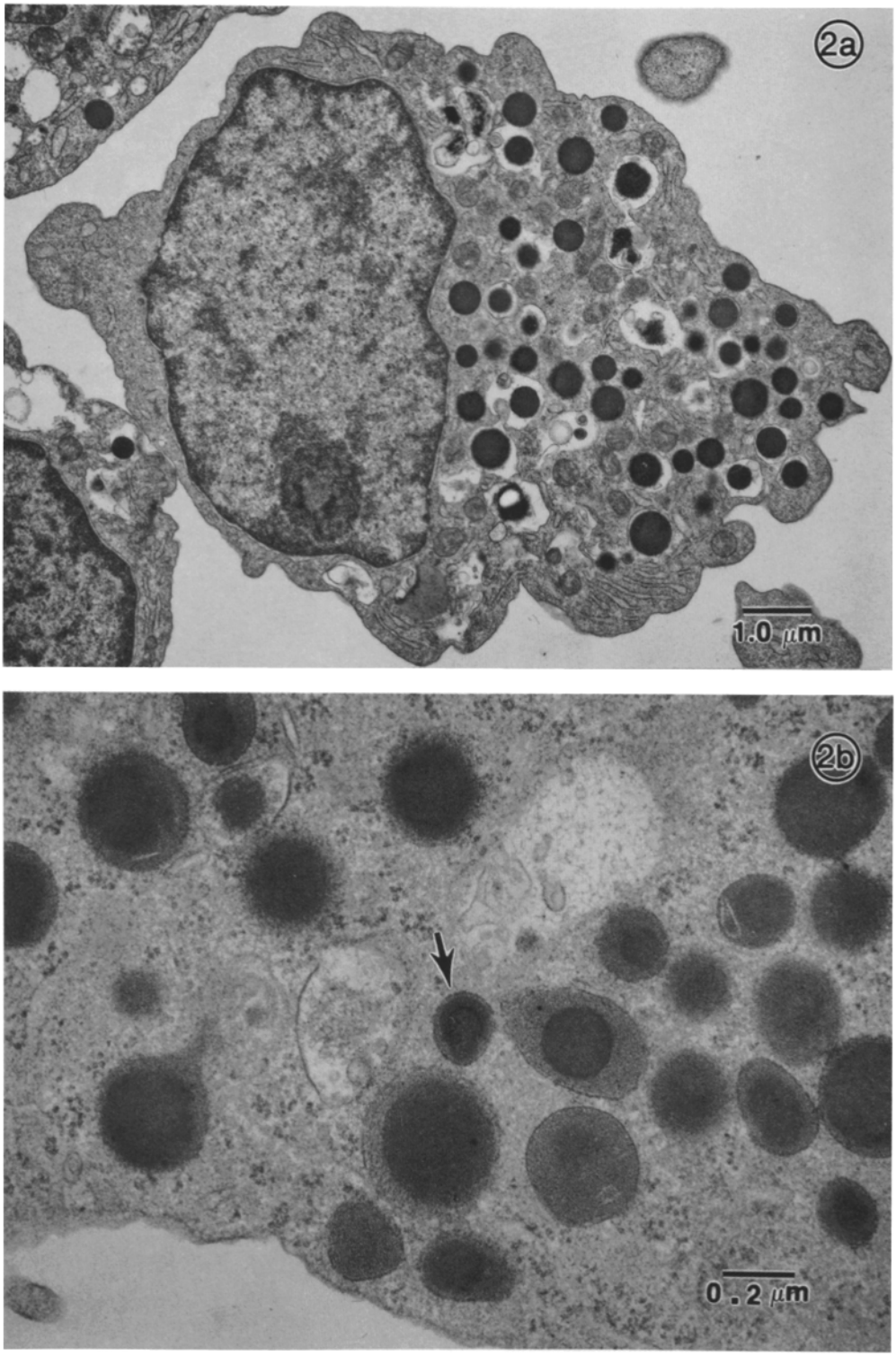

FIGURE 2. Electron micrographs of HL-60 cells cultured for 7 d in medium buffered to pH 7.8 containing 25 mM HEPPS. (*a*) Immature HL-60 eosinophil. (*b*) High magnification micrograph of granules in more mature HL-60 eosinophil. Note multiple granules containing electron dense cores, a rare finding. Arrow points to granule containing a multilamellar inclusion.

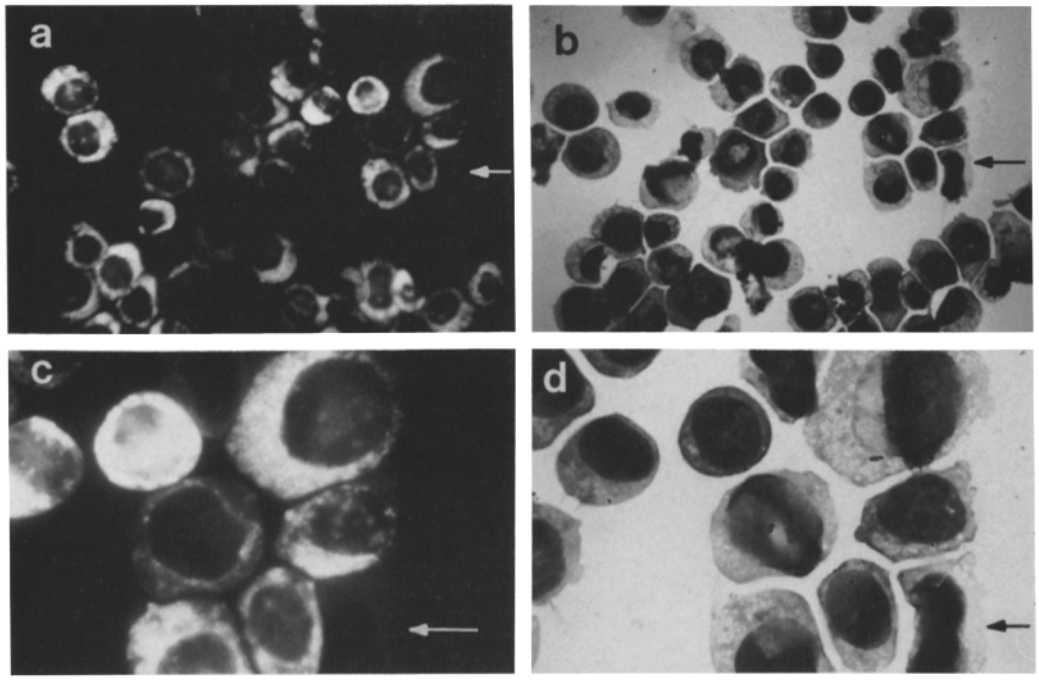

FIGURE 3. Immunofluorescent staining for MBP of HL-60 cells cultured at pH 7.8 for 7 d. In *a* and *c* (original magnifications ×400 and ×1,000), cells were stained with rabbit anti-MBP and show a variety of staining patterns: bright cytoplasmic staining, granular cytoplasmic staining, perinuclear staining, and cells that do not stain (arrow). *b* and *d* show fields identical to *a* and *c*, respectively, counterstained with Wright's stain.

similar in appearance to the primary granules of normal eosinophilic precursors, although the electron microscopic appearance alone is not sufficiently unique to distinguish them from early granules of other myeloid lineages (i.e., basophils). A few had a "swiss-cheese" appearance analogous to the fenestrated granules described in immature eosinophils (18). The second type of granule was smaller (0.15–0.8 μm on the long axis), ovoid, often membrane bound, and not typically found in vacuoles. Multilamellar inclusions with a periodicity of 10 nm, similar to those reported in eosinophilic leukemia (19) were occasionally observed. However, the rhomboid-shaped crystalloid typical of normal, mature eosinophils was not seen. Rarely, circular intragranular inclusions, which may represent atypical crystalloids, have been seen (Fig. 2*b*). The small granule described by Parmley and Spicer (20) has not been identified to date in these cells.

Immunofluorescent Labeling. HL-60 cells were cultured for 7 d in HEPPS-buffered medium at pH 7.8 and cytocentrifuge preparations made. Control HL-60 slides were prepared from control cultures in the midlogarithmic growth phase. These were incubated with affinity-purified antibodies against the MBP or the CLC protein (13, 14) followed by a fluorescein-conjugated second antibody and examined under a fluorescence microscope as described. The results shown in Fig. 3 and Table II indicate that a high percentage of control HL-60 cells show positive staining for MBP. Fig. 3 shows the patterns of staining for MBP;

TABLE II
Fluorescent Staining of HL-60 Cells

Experiment	Initial culture pH	Culture additives & length	Fluorescence pattern: Granulated cells	Stain	Cells counted	Negative	Perinuclear	Faint cytoplasmic	Coarsely granular	Bright cytoplasmic
		d	%			%	%	%	%	%
I	7.4	None (3)	4.0	aHMBP	149	17	9	15	26	34
	7.8	HEPPS (7)	52.5	aHMBP	183	25	6	11	16	42
	7.4	DMSO (7)	0.5	aHMBP	202	65	25	2.5	6	1.5
	7.4	None (3)	4.0	aCLC	352	94	2	3	0	1
	7.8	HEPPS (7)	52.5	aCLC	204	79	0	15	0	6
	7.4	DMSO (7)	0.5	aCLC	350	93	0	6	0	1
II	7.2	HEPPS (7)	16.5	aHMBP	201	27	9	13	50	1
	7.4	HEPPS (7)	27.5	aHMBP	200	40	5.5	15.5	38	1
	7.6	HEPPS (7)	44.0	aHMBP	201	29	6	24	38	3
	7.8	HEPPS (7)	51.0	aHMBP	201	35	5.5	18	34	7.5
	8.0	HEPPS (7)	53.0	aHMBP	300	31.3	3.3	29	31	5.3

Two experiments were done to determine the effect of culture conditions on the immunofluorescence patterns of HL-60 cells stained with anti-human eosinophil major basic protein (aHMBP) or anti-Charcot-Leyden Crystal protein (aCLC). In all experiments, HL-60 cells were suspended at 5×10^5 cells/ml in medium at the stated pH containing either no additive, 25 mM HEPPS, or 176 mM DMSO. Cells were harvested at the time points shown and cytocentrifuge preparations stained with aHMBP, aCLC, or the Wright stain as described in Materials and Methods. The percentage of cells containing eosinophilic granules in each culture as determined by the Wright stain (200 cells counted) is given above as *Granulated cells*. The corresponding immunofluorescence patterns are shown for the same cultures, expressed as the percentages of cells demonstrating one of five general distributions of fluorescence: none (*Negative*); fluorescence confined to perinuclear area (*Perinuclear*); diffuse, faint cytoplasmic fluorescence (*Faint cytoplasmic*); fluorescence predominately in cytoplasmic granules (*Coarsely granular*); or diffuse, bright cytoplasmic fluorescence (*Bright cytoplasmic*).
Experiment I compares control HL-60 cells cultured at pH 7.4 for 3 d (harvested during mid-logarithmic growth) to cells cultured for 7 d with 25 mM HEPPS initially at pH 7.8 (to induce eosinophilic differentiation), to cells cultured 7 d at pH 7.4 with DMSO (to induce neutrophilic differentiation).
Experiment II compares HL-60 cells cultured 7 d in medium containing 25 mM HEPPS at 5 different initial pHs. These cells had been previously routinely passaged at pH 7.4.

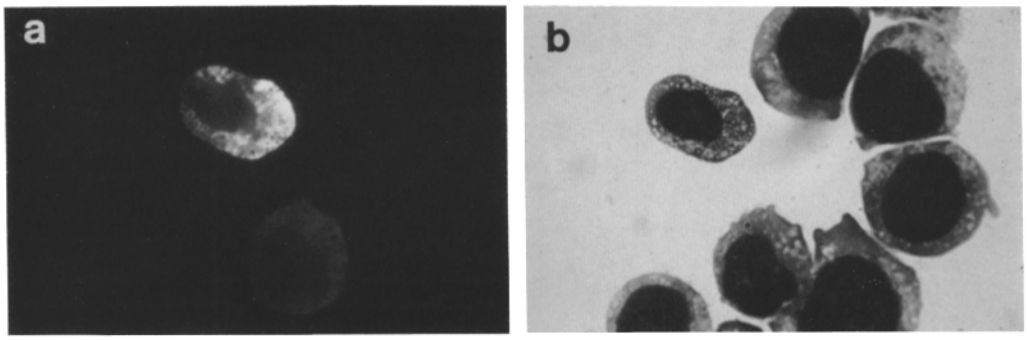

FIGURE 4. Immunofluorescent staining for CLC protein of HL-60 cells. Culture conditions identical to those in Fig. 3.

this was graded as presented in Table II. Exposure of these cells to pH 7.8 does not markedly alter the percentage of MBP-positive cells, whereas incubation with DMSO causes a striking reduction in the percentage of cells staining for MBP. The results for CLC (Fig. 4) show that only a small percentage of cells stained and, in general, there was not a marked difference between control HL-60 cells and those exposed to pH 7.8 or to DMSO.

Induction Conditions. The percentage of cells developing recognizable eosinophil granules and the proportion of cells with eosinophilic granules that had mature nuclei depended on several factors: time, pH of medium, and feeding

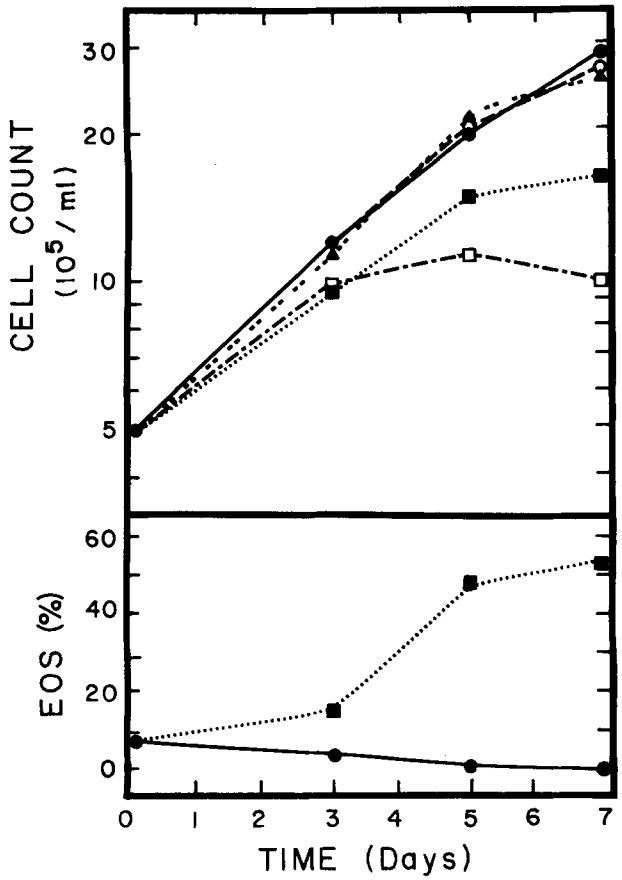

FIGURE 5. HL-60 cells were cultured in 10 ml of RPMI-1640 with 10% fetal calf serum and 25 mM HEPPS in T-25 flasks with tight caps. The initial cell concentration was 5×10^5 cells/ml and initial medium pH was 7.2 (●——●), 7.4 (▲- - - -▲), 7.6 (○– – –○), 7.8 (■· · · · ·■), or 8.0 (□· - — - — ·□). Cell counts and cytocentrifuge preparations were obtained on days 0, 3, 5, and 7 from identical triplicate flasks at each pH. Upper panel shows the means of the cell counts. The means of the percentages of cells grown at pH 7.2 and 7.8 forming eosinophilic granules are shown in the lower panel.

schedule. The presence or absence of fetal calf serum was not a factor as eosinophils could be observed in a serum-free medium (21). The passage level of the cells was also unimportant as eosinophils formed at passage levels ranging from 11 to over 100. Similarly, HL-60 cells obtained from other laboratories could differentiate to eosinophils. The time course of growth and eosinophilic differentiation of the HL-60 cells cultured at several different pHs without refeeding is shown in Fig. 5. This figure shows a steady increase in cells with eosinophilic granules during a 7-d incubation when cultured at an initial medium pH of 7.8. In addition, there was an increase in the proportion of cells with both eosinophilic granules and mature nuclei. There was a decrease in viability of cells after 7 d without medium replenishment so cells were harvested at day 7 for the tests described in this study.

The dependence of eosinophilic differentiation on medium pH is shown in

TABLE III
Effect of Medium pH on Eosinophilic Differentiation of HL-60 Cells

Initial pH	Buffer	Final pH	Cell count (mean ± SD)	Cells containing eosinophilic granules (mean ± SD)	Nuclear maturity
			$\times 10^{-5}/ml$	%	%
7.2	HEPES	6.9	19.4 ± 2.5	12.1 ± 4.0	4.1
7.4	HEPES	7.0	20.1 ± 1.5	22.1 ± 6.2	4.1
.... 7.6	HEPES	7.1	20.6 ± 3.5	25.1 ± 3.5	13.1
7.6	HEPPS	7.1	18.3 ± 5.3	27.0 ± 4.0	17.6
7.8	HEPPS	7.1	14.0 ± 3.4	26.6 ± 6.5	12.8
8.0	HEPPS	7.3	9.6 ± 2.0	30.1 ± 6.6	17.2

HL-60 cells were seeded at an initial density of 5×10^5 cells/ml in 10 ml of medium buffered to various pHs with 25 mM concentrations of the buffers listed in T-25 flasks. These flasks were incubated for 7 d with tightened caps at 37°C without medium replenishment. At this point the final medium pH, the cell count, and the percentage of cells containing eosinophilic granules by Wright-Giemsa staining of cytocentrifuge preparations were determined. The above data represents the average and standard deviation of six cultures. 200 cells were scored on cytocentrifuge preparations. Nuclear maturity refers to the percentage of granule-containing cells with nuclei in the metamyelocyte, band, or segmented stage of development.

Table III. HL-60 cells were cultured for 7 d without medium replenishment in HEPES or HEPPS-buffered medium adjusted initially to various pHs. At pH 7.2 relatively few cells contained granules; the granules tended to be Type I, and the cells were immature by nuclear morphologic criteria. When the starting medium pH was raised to pH 7.8, cell growth slowed, although not at the expense of cytotoxicity, as viabilities exceeded 85% by trypan blue exclusion even at the highest pH throughout the incubation period. In addition, at an initial medium pH of 7.8, the percentage of cells containing eosinophilic granules increased, as did the proportion of cells with Type II granules and mature nuclei. Because of the slowing of growth at the highest pHs, the optimal yield of eosinophils was obtained at an initial pH range of 7.6–7.8.

There was little additional effect of raising the pH above 7.6 to 7.8 or 8.0. Several other buffers were tested (Table IV). There were no differences in cell growth or induction efficiency among any of the buffers. We chose 25 mM HEPPS as it was not cytotoxic and maintained the pH most effectively in the face of metabolic production of acidic wastes by the cells. This is probably due to the closeness of its pKa at 37°C to the initial optimal inducing pH range. While it was possible to maintain a more constant alkaline pH over the 7-d incubation by adding alkaline medium during the induction, the percentage of cells with eosinophilic granules was lower than when the cells were cultured without refeeding. When cells were cultured for 4 d in HEPPS-buffered medium, initially at pH 7.8, and then resuspended in similar fresh medium for 3 additional days, the percentage of cells containing granules did not exceed 7.5% (6 experiments). This suggests that medium depletion plays a role in the induction process,

TABLE IV
Effect of Organic Buffer on Eosinophilic Differentiation

Initial pH	Buffer	Final pH	Cell count (mean ± SD)	Cells containing eosinophilic granules (mean ± SD)
			$\times 10^{-5}$/ml	
7.8	HEPPS	7.6	20.6 ± 1.3	63 ± 11
7.8	HEPES	7.5	23.7 ± 1.3	57 ± 24
7.8	Bicine	7.3	21.1 ± 1.9	60 ± 7
7.8	Tricine	7.4	18.7 ± 2.2	59 ± 12
7.8	Tris	7.2	20.3 ± 3.4	67 ± 6

HL-60 cells were seeded at an initial density of 5×10^5 cells/ml in 10 ml of medium buffered to various pHs with 25 mM concentrations of the buffers listed in T-25 flasks. These flasks were incubated for 7 d with tightened caps at 37°C without medium replenishment. At this point the final medium pH, the cell count, and the percentage of cells containing eosinophilic granules by Wright-Giemsa staining of cytocentrifuge preparations were determined. The above data represents the average and standard deviation of six cultures. 200 cells were scored on cytocentrifuge preparations.

either directly or indirectly through taking cells out of the logarithmic growth phase.

Cytogenetic Analysis. 25 Q-banded cells were analyzed in detail. The modal chromosome number was 45, and a representative karyotype designated 45,X,−X,−5,−9,−10,−16,−16,−17,+?18,+der(5)t(5;17)(q13;q12),-+der(9)t(9;?)(q34.3;?),+der(10)t(10;13)(p11.2;q12.1),+der(16)t(16;?)(q22;?),-+der(16)t(16;?)(q23;?),del(9)(p13) is shown in Fig. 6. Double minute chromosomes were observed in 4 of these 25 cells. In addition, 100 consecutive mitotic cells were examined under the microscope for counts of dmin. 8 of the 100 mitotic cells had 1 to 12 dmin with an average of 3 per cell.

The karyotype at passage 12 appeared to be essentially the same as that at passage 35 reported by Gallagher et al. (1), in which three marker chromosomes (M1, M2, M3) were described. We have tentatively identified these three chromosomes as der(10)t(10;13)(p11.2;q12.1), del(9)(p13), and der(5)t(5;17)(q13;q12), respectively. Although no abnormality of chromosome 16 was described in the original report on the HL-60 cell line, additional bands on the terminal end of the long arm of both 16 homologues were recognized in our cells. Since the karyotypes published by Gallagher et al. (1) were prepared from mitoses with quite contracted chromosomes, it is extremely difficult to discern whether abnormalities of chromosome 16 were actually present in those cells.

These two der(16) chromosomes differ from each other. One is somewhat longer than the other, and the break points of these two derivative chromosomes are at band 16q23 in the former and at 16q22 in the latter (Fig. 6). These same abnormalities of chromosome 16 were also found in HL-60 cells obtained from other laboratories (Testa and Misawa, unpublished data). Despite heterogeneity in various aspects of the karyotypes of HL-60 cells, the abnormalities of chro-

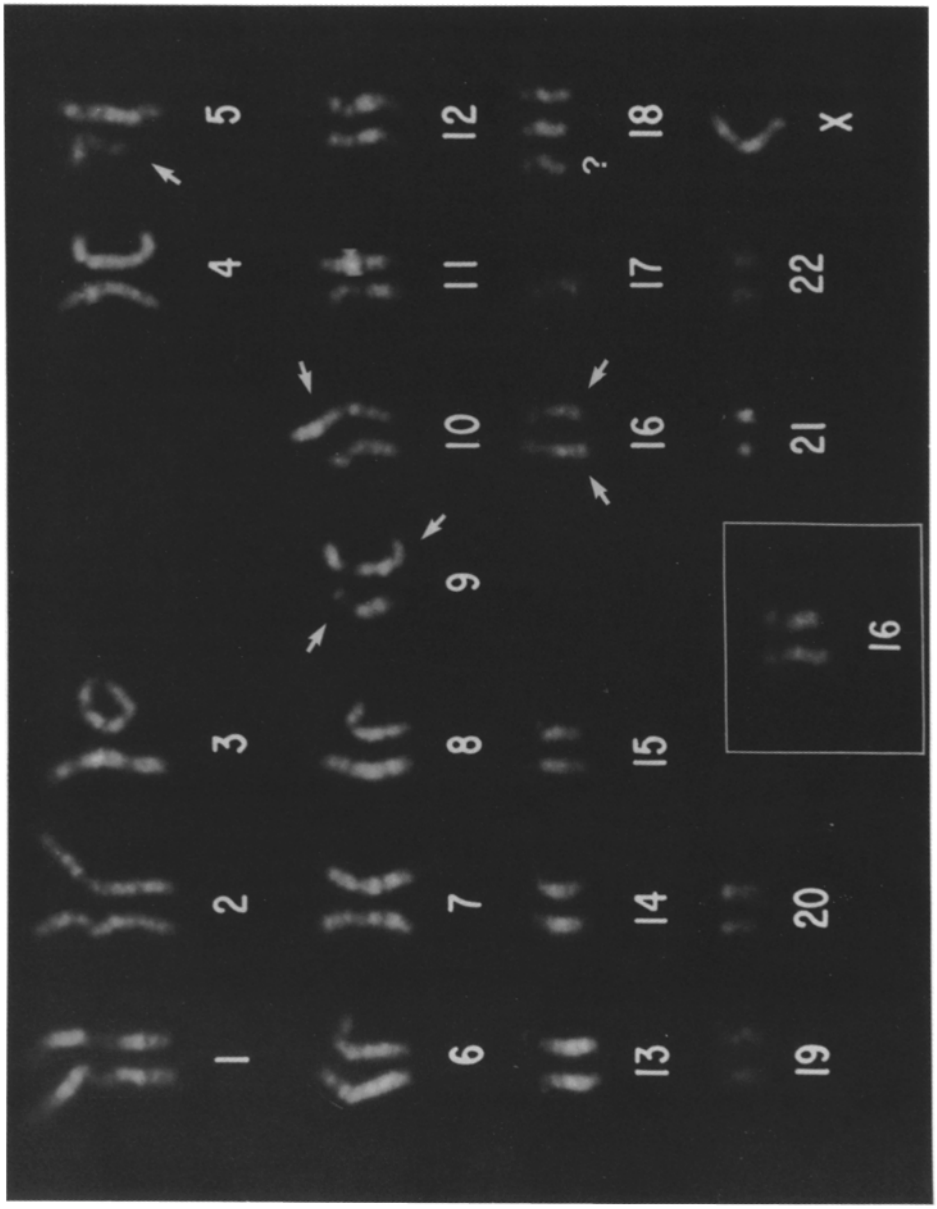

FIGURE 6. A karyotype of a Q-banded H1-60 mitotic cell at passage 12. 16 from another cell. In each cell, both No. 16 chromosomes show additional Rearranged chromosomes are indicated by arrows. *Inset* Chromosome pair bands at the terminal end of the long arm.

mosome pair 16 were found in every metaphase examined, regardless of passage level, source of the cells, or presence of other specific chromosomal abnormalities.

Discussion

These data demonstrate that HL-60 cells can differentiate to eosinophils and eosinophilic precursors when cultured in mildly alkaline medium as determined by morphological and biochemical criteria. Morphologically, the cells contain new granules which, when stained with Wright-Giemsa, are typical in size and color for eosinophilic granules, both in the mature and less mature cells. These granules stain with Luxol-fast-blue, which, among blood cells, is specific for eosinophils, but they are not metachromatic when stained with toluidine blue, as would be basophilic granules. Furthermore, using immunofluorescent and histochemical techniques, five granule proteins characteristic of the eosinophil can be demonstrated in these cells: MBP, CLC protein (lysophospholipase), eosinophil peroxidase, arylsulfatase, and acid phosphatase. Except for eosinophil peroxidase, each of these proteins has also been described in other myeloid cell types (13, 22), but the combination of the presence of all these proteins, plus the light morphologic appearance and staining characteristics of the granules permits rigorous identification of these cells as eosinophils.

The detection of MBP in the undifferentiated HL-60 cells was unexpected, as this suggests that this protein is constitutively produced in this cell line. The granular appearance of many of the cells after immunofluorescent staining for this protein implies that it is localized to the promyelocytic granules in the control cells. There is, however, no significant change in the quantity or distribution of MBP (as judged by immunofluorescent appearance) as the cells mature to eosinophils, despite the easily visible acquisition of new granules (Table II). On the other hand, DMSO-induced neutrophilic differentiation appears to be associated with the cessation of MBP production and the loss of preformed MBP. The following hypothesis is proposed to explain these observations. Actively growing, immature HL-60 cells express MBP, a marker of the eosinophilic lineage, and may be naturally preprogrammed to undergo terminal eosinophilic differentiation given favorable culture conditions. Under these favorable alkaline culture conditions, the cells gradually lose their proliferative potential, and undergo nuclear and cytoplasmic maturation to eosinophils. Cytoplasmic maturation consists of the synthesis of new eosinophil-associated proteins and the assembly of these new and preexisting proteins into granules, among other changes. Whether the MBP in the new granules is derived from that in the promyelocytic granules or is synthesized de novo is currently unknown, but the immunofluorescence pattern of MBP is unaltered, as the new granules are in the same general cellular location as the old promyelocytic granules. DMSO-induced neutrophilic differentiation may involve a switch to a different differentiation pathway such that eosinophil-associated genes are no longer expressed and other neutrophil-specific genes are expressed as the cells mature to neutrophil-like forms.

The HL-60–derived eosinophils resemble morphologically the abnormal eosinophils seen in certain malignant states. Notably, they share certain peculiarities of their granules with the bone marrow eosinophils in patients with AMMoL

whose blasts have the inv16(p13q22) chromosomal abnormality (8). Specifically, both red and blue granules can be seen in the HL-60 eosinophils stained with Wright-Giemsa, the granules are chloroacetate esterase and PAS positive (eosinophilic granules from normal persons are negative for both), and crystalloids are rare (8). The persistence of chloroacetate esterase activity has previously been described in eosinophilic leukemia (23) and the presence of multilamellar inclusions and the paucity of crystalloids in secondary granules as seen through the electron microscope has also been described in eosinophils from lymphoma patients (19). Additionally, vacuolization of the cytoplasm has been described in circulating eosinophils in various pathologic states although normal eosinophilic precursors may vacuolate in the bone marrow (24), as may circulating normal eosinophils upon appropriate stimulation (25). Cytotoxicity may also manifest as vacuolization, but this is unlikely to be the explanation in our studies of HL-60 cells as there were no other findings of cellular degeneration; also cell viabilities were high in cultures carried up to 7 d, as measured by trypan blue exclusion.

There seems to be a relationship between the pH of the culture medium and the appearance of eosinophilic granules in the HL-60 cells (Fig. 5). Medium alkalinity has been shown to be mitogenic to quiescent mammalian cells (26), and to alter their growth rate (27) and expression of protein products (28). Furthermore, high pH of the culture medium can accelerate the spontaneous differentiation of a cultured melanoma cell line, B16 (29). However, eosinophilic differentiation was less effectively induced when we tried to maintain an alkaline environment through refeeding of cultures with aliquots of fresh alkaline medium. It therefore appears that nutrient depletion may also have a role in the induction process. Precedents exist in numerous other inducible cell lines, where serum deprivation or medium exhaustion leads to terminal differentiation (30). Similarly, melanogenesis was promoted by alkaline stimulation in the B16 cell line only when in the stationary growth phase (29). On the other hand, because it was common to observe eosinophilic granules in mitotic cells (Fig. 1 *c*), it is unlikely that nutrient depletion acts solely via nonspecific suppression of cell growth.

The data in Table III shows a striking increase in the percentage of cells with eosinophilic granules and mature nuclei as the initial pH of the culture medium is increased from 7.2 to 7.6, but little increase thereafter. While the initial pH range of 7.6 to 8.0 is higher than that used in most laboratories for tissue culture medium (27), over the course of the 7 d of culture, the HL-60 cells are ultimately exposed to pHs similar to those of normal plasma (pH 7.35–7.45) due to acidification of the medium by metabolic wastes. This is in contrast to cells cultured initially at pH 7.2 where the pH falls below 7.0. Thus, an alternative explanation for the effect of pH on the HL-60 cells is that acidic conditions suppress spontaneous eosinophilic maturation of the cells that might occur at a more physiologic pH. Unrecognized variability in medium pH may have accounted for the reported discrepancies in the role of colony-stimulating factors in eosinophilic differentiation of HL-60 cells (4, 5). There was often significant variability in the percentages of cell developing eosinophilic granules in experiments set up on different days, despite apparently identical initial culture conditions (Tables II, III, IV and Fig. 5). This is likely to be due, at least in part, to

variability in the rate at which the pH of the culture medium falls during the incubation and the rate at which the cells multiply.

The possibility of a potentiating effect of the organic buffers on eosinophilic differentiation cannot be ruled out from the present data. The culture medium pH is unstable in the absence of organic buffers if the flask caps are tightened, precluding a precise comparison, under identical conditions, of inductions with and without these buffers; eosinophils can be seen, however, in the absence of these additives. There is not likely to be a major effect of these additives, as they do not appear to have significant effects on cell growth and protein synthesis (27), including growth and DMSO-induced neutrophilic differentiation of HL-60 cells (21).

The finding of abnormalities on the long arm of chromosome 16 in a leukemic cell line that can differentiate to eosinophils further strengthens the association between this cytogenetic finding and the clinical observation of abnormal eosinophils in the bone marrow of patients with acute myelomonocytic leukemia (AMMoL; references 7, 8, 31). However, HL-60 was derived from a patient with acute promyelocytic leukemia (FAB type M3), and there are not yet any reports of comparable abnormalities of chromosome 16 in patients with this type of leukemia. Although the specific types of rearrangement of chromosome 16 in HL-60 cells, described above, differ from those reported in patients with AMMoL and abnormal marrow eosinophils, the affected break points involve the same region on 16q. Moreover, there is recent evidence that AMMoL with dysplastic eosinophils may be associated with several related structural rearrangements (e.g., deletion, inversion, or translocation), all of which affect 16q22(31). Furthermore, the occurrence of rearrangements of the long arm of both chromosome 16 homologues, while not common, has been described in a subgroup of AMMoL patients with abnormal marrow eosinophils (8). The relationship between the chromosomal abnormalities and the abnormal granule cytochemistry and morphology is unclear. The paucity of crystalloids and persistence of chloroacetate esterase positivity in the granules of the HL-60–derived eosinophils has similarly been noted in the abnormal eosinophils of the AMMoL patients with the abnormal chromosome 16 (8). In addition, several reports in the past, before the availability of techniques to obtain extended, finely banded chromosomes, describe AMMoL patients with abundant bone marrow eosinophils that also have granules lacking crystalloids and with chloroacetate esterase positivity (32–34). These data provide further evidence that genes regulating eosinophilic differentiation are located in the chromosomal region 16q22-123.

This model system may prove valuable for future studies because of the following characteristics. First, it is the only human cell line inducible to form eosinophils. Second, the cells contain a large number of characteristic proteins for which reasonably facile assays exist. Third, as the pH of the culture medium appears to be an inducing stimulus, it may be a suitable model for the role of ion fluxes in the control of gene expression and differentiation. Fourthly, the presence of cytogenetic abnormalities in a region implicated by clinical studies with eosinophilic differentiation may facilitate future molecular studies of the control mechanisms of eosinophilic differentiation. Finally, as the HL-60–derived eosinophils have several of the ultrastructural abnormalities observed in eosino-

phils from patients with certain malignancies, this may be an appropriate model to study the abnormal granule morphogenesis seen in those diseases.

Summary

HL-60 promyelocytic leukemia cells differentiated to eosinophils and eosinophilic precursors when cultured under mildly alkaline conditions (pH 7.6–7.8) for 7 d without refeeding. New cytoplasmic granules appeared blue in the least mature cells and red in the most mature cells when stained with Wright-Giemsa. The granules also stained with Luxol-fast-blue, a characteristic of eosinophil granules. Furthermore, most cells contained the eosinophil major basic protein (MBP); the Charcot-Leyden Crystal (CLC) protein (lysophospholipase), eosinophil peroxidase, acid phosphatase, and arylsulfatase were also detected in a portion of these cells. The eosinophil major basic protein was found in a high proportion of undifferentiated cells, and thus may be constituitively produced. By examining finely banded chromosomes, translocation break points were demonstrated at q22 on one chromosome 16 and at q23 on the other homologue; abnormalities in this region of the long arm of 16 are a characteristic finding in the recently described syndrome of acute myelomonocytic leukemia (AMMoL) with abnormal bone marrow eosinophils. In common with the bone marrow eosinophils in these patients, the HL-60 eosinophil granules contained chloroacetate esterase and periodic-acid Schiff (PAS) reactive material; crystalloid inclusions were rare. Therefore, the HL-60 cell line appears to be an in vitro model for eosinophilopoiesis and may be specially suited for the study of the abnormal eosinophils seen in certain malignant conditions.

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