Cyclic Nucleotide-induced Maturation of Human Promyelocytic Leukemia Cells

THOMAS J. CHAPLINSKI and JAMES E. NIEDEL, *Department of Medicine, Division of Hematology-Oncology, Duke University Medical Center, Durham, North Carolina 27710*

ABSTRACT Myeloid differentiation in vitro is characterized by the sequential appearance of morphological, functional, and biochemical markers of maturation. We examined the effect of agents that increased the intracellular concentration of adenosine 3'5'-cyclic monophosphate on the expression of these markers by human promyelocytic leukemia cells (HL60). Cells treated with 500 μM N^6,O^2-dibutyryl adenosine 3'5'-cyclic monophosphate expressed formyl peptide and complement receptors, reduced nitroblue tetrazolium, adhered to substrate, demonstrated chemotaxis and stimulated lysosomal enzyme release, rapidly ceased proliferation, and assumed the morphology of myelocytes and metamyelocytes. Prostaglandin E_2 (100 nM) and theophyllin (500 μM) induced similar functional changes but the cells did not mature beyond the myelocyte stage. Cholera toxin (1 or 50 nM) induced formyl-peptide receptor expression and adherence, but the cells did not reduce nitroblue tetrazolium, continued to proliferate, and were unchanged morphologically. Formyl-peptide receptor expression was the earliest marker of these modified programs of maturation. The receptor appeared within 2 h after treatment and increased linearly for 72 h. Receptor expression was dependent on new protein synthesis. At 48 h, Scatchard analysis demonstrated 2.4×10^5 receptors/cell with a K_D of 1.3 nM.

In contrast to induction of HL60 differentiation by dimethyl sulfoxide, retinoic acid, or phorbol myristate acetate, the developmental programs initiated by agents that raised intracellular adenosine 3'5'-cyclic monophosphate shared several unique features: (*a*) plasma membrane maturation was dissociated from morphological maturation; (*b*) no latent period was evident following induction—the earliest membrane

marker was expressed within 2 h; (*c*) commitment to terminal differentiation was delayed.

INTRODUCTION

The human leukemic cell line (HL60), derived from a patient with acute promyelocytic leukemia, has been maintained in continuous culture for >5 yr (1). These cells, which grow as myeloblasts and promyelocytes, can be induced to differentiate into mature myeloid forms with a variety of agents. For example, after 3–7 d of continuous exposure to dimethyl sulfoxide (DMSO),[1] HL60 cells exhibit a reduced nuclear-cytoplasmic ratio, loss of nucleoli, decreased azurophilic granules, and assume the morphologic characteristics of metamyelocytes (2). Biochemically, these cells reduce nitroblue tetrazolium (NBT), generate superoxide, and express membrane receptors for complement and chemotactic formyl peptide (3–5). Functionally, the differentiated cells are similar to neutrophils, as they demonstrate chemotaxis, chemokinesis, adherence, phagocytosis, and stimulated enzyme release (3–6).

The HL60 system, thus, provides a model for the study of plasma membrane development and receptor expression during myeloid differentiation. Unfortunately, DMSO-induced membrane maturation is often variable and incomplete (5). We therefore sought alternative inducers that would reproducibly cause

membrane maturation and chemotactic peptide receptor expression in all of the treated cells. In addition to defining a new class of inducers, use of such agents may help to define the molecular mechanisms of cell differentiation.

A number of agents that increase intracellular cyclic AMP (cAMP) by either stimulating adenylate cyclase, inhibiting cyclic nucleotide phosphodiesterase, or being a cell-permeant analogue of cAMP, inhibit proliferation of hematopoetic cells as assessed in a soft-agar cloning assay (7–10). Such treatment can also induce irreversible morphological differentiation in certain malignant cell lines (11, 12). In this study, we show that N^6,O^2 dibutyryl adenosine 3'5' cyclic monophosphate (dbcAMP) and other related agents induce a modified program of myeloid differentiation in the HL60 cell line. After exposure to dbcAMP, the chemotactic receptor is rapidly synthesized and the cells are able to reduce NBT, release lysosomal enzymes, demonstrate chemotaxis, and adhere to substrate. Agents that presumably increase intracellular cAMP induced HL60 cells to undergo a program of plasma membrane differentiation, without concomitant morphologic maturation.

METHODS

Materials. Antibiotics, RPMI 1640, HEPES buffer, Eagle's minimal essential medium (MEM), leucine-free, glutamine, and fetal bovine serum were obtained from Gibco Laboratories (Grand Island Biologial Co., Grand Island, NY). Butyrate, NBT, nitrophenyl-β-D-glucuronide, cycloheximide, cholera toxin, DMSO, theophyllin, isoproterenol, adenosine, dbcAMP and all cyclic nucleotide analogues were obtained from Sigma Chemical Co. (St. Louis, MO). Prostaglandin E_1 (PGE$_1$) and prostaglandin E_2 (PGE$_2$) were provided by Dr. John Pike (Upjohn Co., Kalamazoo, MI) and stock solutions were prepared at 5 mM in ethanol. These solutions were stored at −20°C and serially diluted in media before use. ^{125}I- and [^3H]leucine were obtained from New England Nuclear (Boston, MA). N-Formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosinyl-lysinyl (N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys) (formyl peptide) was synthesized by S. Wilkinson (Wellcome Research Laboratories, Beckenham, England) and was stored in stock solution at 0.5 mM in dimethyl formamide at −20°C. Triton X-100 and Permablend were obtained from Packard Instrument Co. (Downers Grove, IL). Phorbol myristate acetate (PMA) was obtained from Consolidated Midland Corp. (Brewster, NY) and NCS tissue solubilizer from Amersham Corp. (Arlington Heights, IL).

Cells. The HL60 cells obtained from R. C. Gallo, National Institutes of Health, had been stored in liquid nitrogen at passage 17 until 3 mo before use. They were maintained in continuous culture and determined to be free of Mycoplasma. The cells were grown in suspension culture in RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, penicillin (50 U/ml), streptomycin (50 μg/ml), and 15% heat-inactivated fetal bovine serum. Doubling time was 24–36 h. The cell cultures were divided every 2–3 d to maintain a density of 2.5×10^5 to 10×10^5 cells/ml. Mortality assessed by trypan-blue dye exclusion was <5%.

Radioligand and fluorescent binding assay. N-Formyl-Nle-Leu-Phe-Nle [^{125}I]Tyr-Lys (formyl-^{125}I-peptide) was prepared by the chloramine-T method (13). The specific activity of the iodinated peptide varied from 200 to 600 cpm/fmol. The tetramethyl-rhodamine-labeled formyl peptide (fluorescent formyl peptide) was prepared and used in fluorescent studies (14).

Cells were harvested by centrifugation and washed twice with assay buffer (15 mM sodium phosphate/123 mM sodium chloride/0.1% bovine albumin pH 6.75). The formyl ^{125}I-peptide binding assay was a modification of the method described elsewhere (5, 14). Briefly, 360,000 cells and formyl ^{125}I-peptide (500 fmol) in 200 μl assay buffer were incubated at 22°C for 30 min or 4°C for 3 h. Binding was terminated by addition of 2 ml of 4°C incubation buffer and rapid filtration through a Whatman GF/C filter (Whatman Inc., Chemical Separation Div., Clifton, NJ). The filters were washed with 10 ml of 4°C phosphate-buffered saline (PBS) and counted for ^{125}I. Nonsaturable binding was determined by an identical method except for the addition of 125 nM unlabeled formyl peptide during the binding incubation. Specific binding refers to total binding minus nonsaturable binding. Data points were measured in duplicate or triplicate with a standard error consistently < ±10%.

Marker studies. Cytocentrifuged samples were evaluated with Wright's stain, and morphologic maturity was determined by multiple factors including decrease of azurophilic granules, decreased nuclear-cytoplasmic ratio, loss of nucleoli, increased nuclear density, and identations of nucleus. α-Naphthol AS-D acetate (nonspecific) esterase was assayed as described elsewhere (15). Adherence was determined on a hemocytometer glass cover slip after incubation at 37°C for 15 min.

Formyl peptide-stimulated β-glucuronidase release was determined by a modification of established methods (16, 17). Briefly, 3×10^6 cells were suspended for 10 min in 250 μl buffer (PBS containing 0.5 mM MgCl$_2$, 0.5 mM CaCl$_2$, 0.2% bovine serum albumin, and cytochalasin B 10 μg/ml). Each concentration of formyl peptide was added, and after 5 min at 37°C, cells were centrifuged at 500 g for 15 min. Control cells were lysed with Triton X-100 and teflon-glass homogenized. Cells and lysates were centrifuged at 39,000 g for 20 min at 4°C. 100 μl of supernatant of the formyl peptide-stimulated and control lysed cells were incubated with 900 μl buffer (50 mM sodium acetate, 0.1% Triton X-100, 1 mM p-nitrophenyl-β-D-glucuronide, pH 5.00) at 37°C for 12 h. The reaction was terminated by the addition of 2.0 ml of 0.1 M sodium hydroxide. The β-glucuronidase assay measured the formation of p-nitrophenol from p-nitrophenyl β-D-glucuronide at 410 nM and was expressed as percent release of total cellular enzyme.

For NBT reduction studies, 2×10^6 cells in 1 ml RPMI 1640 with 15% fetal bovine serum were incubated at 37°C for 20 min with 1 ml 0.2% NBT solution in the presence of 200 ng of freshly diluted PMA or 50 nM N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys. The percentage of cells containing membrane deposits of reduced black formazan on a cytospin preparation was determined for 200 cells and repeated in triplicate for each score. The chemotactic response was estimated by the method of Boyden (5) as reported previously using 5-μm filters.

Complement receptors on HL60 cells were determined by rosetting of complement-coated erythrocytes (EAC). Accordingly, 0.5 ml of a 2% human erythrocyte suspension was sensitized with 0.5 ml of a 1:100 dilution of high titer cold agglutinin serum and 0.5 ml of fresh plasma. The erythrocytes, serum, and plasma were incubated for 10 min on ice,

then 10 min at 37°C, and washed three times. The sensitized erythrocytes were then incubated with sample HL60 cells for 1 h (erythrocytes/HL60, 10:1). An HL60 cell rosetted by at least three erythrocytes was determined as positive and 200 HL60 cells were scored for each sample.

Cycloheximide inhibition. For each concentration of cycloheximide studied, 5×10^6 HL60 cells were suspended in 5 ml of MEM Eagle's leucine-free medium with 10% heat-inactivated fetal bovine serum. The concentration in each flask was 10 μCi/ml of [U^{3}H]leucine (50 Ci/mmol sp act) and 500 μM dbcAMP. The cells were cultured for 24 h with the indicated concentration of cycloheximide. At 24 h, a 1-ml aliquot was removed, the cells washed and formyl-^{125}I-peptide binding assessed under standard assay conditions. A second 1-ml aliquot was removed to determine leucine incorporation into total cellular protein. The cell pellet, obtained by centrifugation after three washes in MEM Eagle's medium, was dissolved in 0.2 ml 1 M NaOH at 37°C for 20 min. 0.1 ml of 5% bovine serum albumin and 2.0 ml of 10% trichloracetic acid were added and the mixture was incubated at 4°C for 1 h. The pellet obtained by centrifugation was dissolved in 1.0 ml of NCS tissue solubilizer and added to 10 ml of scintillation fluid (20.9 g Permablend/1.0 liter scintillation grade toluene). Samples were counted in a Beckman LS9000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA) with an efficiency of 45%.

RESULTS

Characteristics of dbcAMP-treated HL60 cells. Cells of the HL60 line, in response to specific chemical inducers will differentiate into either granulocytes or macrophages. These cells, grown under the influence of dbcAMP, develop morphologic characteristics of granulocyte differentiation, but do not progress beyond the metamyelocyte stage (Fig. 1). Maturing cells remain peroxidase positive and nonspecific esterase negative. An increase in saturable binding of formyl ^{125}I-peptide was demonstrated within the first 24 h of exposure to dbcAMP; at this time >95% of the cells were receptor positive (Table I). Glass adherence and oxygen-free radical generation demonstrated by NBT dye reduction occurred after 3 d of treatment, similar to the time course in DMSO- or butyrate-treated cells. Complement receptors were not present after 24 h of exposure to dbcAMP, but appeared on the majority of cells by the 4th d of treatment. Cell mortality was increased (20%) by the 5th d of treatment and therefore experiments were generally terminated at or before this time.

Exponential growth of HL60 cells in suspension culture was inhibited at dbcAMP concentrations >100 μM. At the higher concentrations of dbcAMP tested, the cell density approximately doubled over the first 24 h of treatment and then remained stable over the next 4 d. With other agents that induced granulocyte differentiation (DMSO, butyrate, retinoids, for example), >48 h of exposure was necessary before inhibition of exponential growth in suspension culture was evident (2, 18, 19).

Expression of formyl-peptide receptor induced by dbcAMP. Dibutyryl cAMP induced a program of differentiation similar in many respects to that induced by DMSO or retinoic acid (2–4, 18, 19). On striking difference, however, was the early appearance of the formyl-peptide receptor. In response to 500 μM dbcAMP, an increase in formyl ^{125}I-peptide binding was seen within 2 h and continued to increase linearly for 72 h, finally reaching a plateau at >300 fmol formyl peptide bound per 10^6 cells. With DMSO induction, receptor expression was not seen until day 3 and plateaued at ~100 fmol/10^6 cells (Fig. 2). The extent of formyl-peptide receptor expression showed a reproducible dose-dependent relationship with increasing concentrations of dbcAMP (Fig. 3). Below 100 μM dbcAMP, neither an increase in chemotactic receptor nor morphologic changes were evident. The maximum receptor expression occurred in response to 500 μM–1 mM dbcAMP.

By 24 h of exposure of 500 μM dbcAMP, >95% of the HL60 cells demonstrated saturable receptor-mediated internalization of the fluorescent formyl peptide (data not shown). This cellular homogeneity was in contrast to DMSO-treated cells, which showed fluorescent binding and internalization in a maximum of 30% of the cells even after prolonged exposure to DMSO (5). Fluorescent peptide uptake by peripheral neutrophils was qualitatively similar, but less intense than the uptake by dbcAMP-treated cells (14). The rates of internalization of the fluorescent peptide by dbcAMP-treated cells, peripheral neutrophils, and the subpopulation of untreated HL60 cells, which spontaneously differentiated (5), appeared identical.

The standard receptor assay at 22°C measures both membrane surface binding and internalization of the radioligand (13, 14). Cellular uptake of formyl ^{125}I-peptide was rapid at 22°C with a maximum value at 30 min. At 4°C, bound formyl ^{125}I-peptide remained on the cell surface of dbcAMP-treated cells and equilibrium was not achieved until 3 h of exposure. At both temperatures, binding to dbcAMP-treated cells was saturable with a plateau above 2.5 nM formyl peptide. After 48 h of HL60 exposure to 500 μM dbcAMP, Scatchard analysis of binding at 4°C revealed a receptor density of 240,000 receptors/cell with a K_d of 1.3 nM. This binding affinity was similar to that of peripheral neutrophils, but the receptor density was approximately fourfold greater in the induced HL60 cells, although the cells remained morphologically immature at 48 h (Fig. 1). Scatchard analysis of binding to uninduced cells at 4°C was not possible because binding was too low to be reliably measured.

Formyl peptide-stimulated β-glucuronidase release, chemotaxis, and NBT reduction were used as measures of the physiologic function of the newly expressed for-
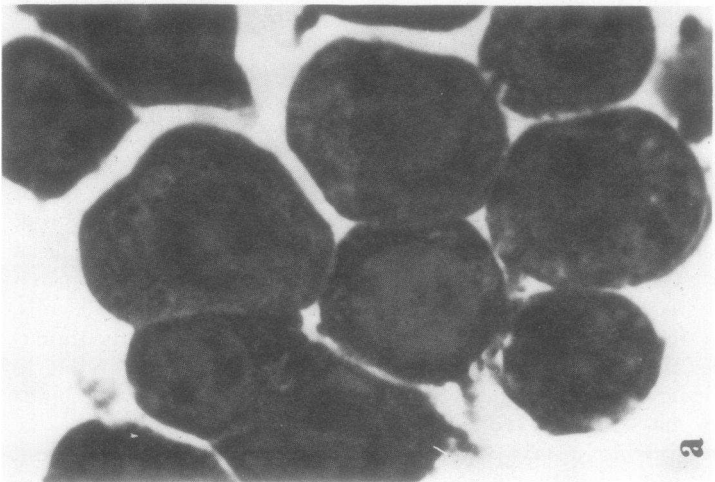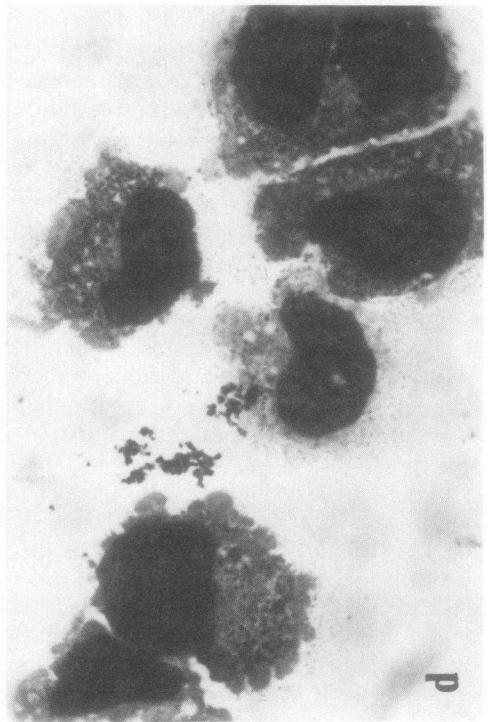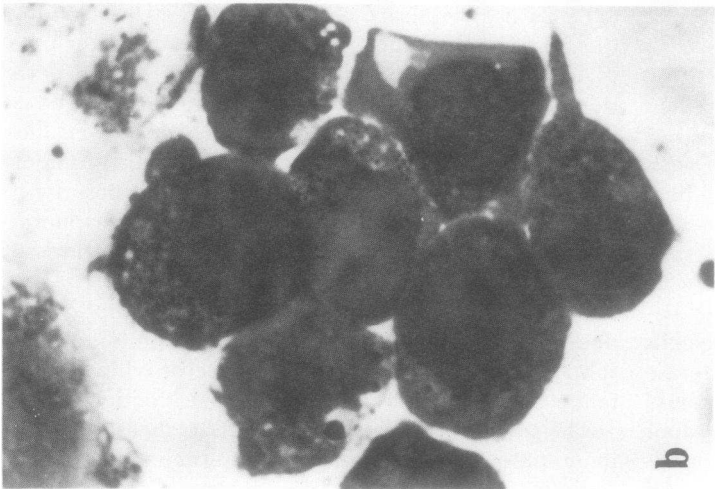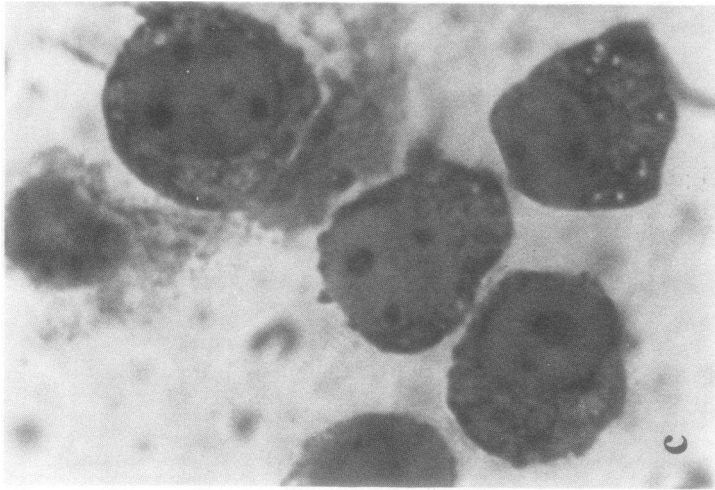
TABLE I
Assessment of Maturation of HL60 Cells Exposed
to 500 μM dbcAMP

Day	Maturity*	Chemotactic receptor‡	NBT reduction§	Glass adherence‖	Complement receptor¶
0	5	10 (5)	5	5	0
1	5	125 (>95)	10	5	0
2	9	225 (>95)	60	5	ND
3	22	300 (>95)	88	56	ND
4	45	313 (>95)	90	76	60
5	70	325 (>95)	99	78	ND

* Percentage of HL60 cells showing morphological characteristics beyond the promyelocyte. 200 cell differential counts in triplicate of Wright-stained cytofuge preparations.

‡ Formyl-^{125}I-peptide bound (fmol/10^6 cells) under standard assay conditions (Methods). In parentheses are shown the percentage of receptor-positive cells as demonstrated with the rhodamine-labeled peptide (Methods).

§ Percentage of HL60 cells containing reduced NBT. 200 cells were scored in triplicate with SEM < 6%. Assayed as described in Methods.

‖ Percentage of HL60 cells that spread on a glass cover slip after incubation for 15 min at 37°C, 100 cells counted.

¶ Percentage of HL60 cells to which greater than three complement-sensitized erythrocytes had rosetted. 200 HL60 cells were scored.

myl-peptide receptors. Uninduced HL60 cells did not demonstrate any of these functions in response to the peptide. Cells treated for 24 h with 500 μM dbcAMP released a maximum of 17.8% of total cellular β-glucuronidase (uninduced cells released 0.9%). An ED$_{50}$ of 4 nM N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys was determined for release, in close agreement with the measured K_D and similar to the ED$_{50}$ for peripheral neutrophils.

After 4 d of dbcAMP treatment, 85% of the cells were able to reduce NBT in response to peptide stimulation. Chemotaxis was also evident at this time as 75 cells per high power field migrated in response to a 0.5 nM peptide concentration as compared to <3 cells per high power field for controls. This chemotactic response is ~30% of the response seen in our laboratory with peripheral neutrophils under similar assay conditions.

Inhibition of dbcAMP-induced chemotactic receptor expression by cycloheximide. Induction with

dbcAMP resulted in a 10-fold stimulation in the rate of [^{3}H]leucine incorporation into cellular protein. HL60 cells exposed for 24 h to the protein synthesis inhibitor cycloheximide at concentrations <3 μg/ml, showed no change in cellular mortality or morphology. The dose-response curve for cycloheximide inhibition of [^{3}H]leucine incorporation into protein of dbcAMP-induced cells correlated with the inhibition of expression of the formyl-peptide receptor (Fig. 4).

Effect of cyclic nucleotides and prostaglandins on chemotactic receptor expression. Dibutyryl cAMP may have induced chemotactic receptor synthesis, inhibited cellular proliferation, and induced morphologic maturation by mechanisms unrelated to cyclic nucleotides. To explore these alternative possibilities, other nucleotide analogues, prostaglandins, and inhibitors of cyclic nucleotide phosphodiesterase were tested and compared with the results of dbcAMP treatment (Table II). Those agents believed to raise intracellular levels of cAMP consistently induced early receptor expression, early inhibition of proliferation, but minimal morphological maturation.

Prostaglandins increase cAMP by stimulating adenylate cyclase. With 100 nM PGE$_1$ or PGE$_2$, early chemotactic receptor expression occurred within the first 24 h of treatment, similar to the standard dbcAMP treatment. These agents inhibited cellular proliferation, increased membrane deposits of NBT, and stimulated glass adherence. The treatment did not induce obvious granulocytic maturation, but the majority of cells were not morphologically identical to untreated promyelocytes. The most obvious change was loss of azurophilic granules and apparent increase in cytoplasmic volume (Fig. 1). Theophyllin (500 μM) alone did not induce any of these cellular changes. However, early chemotactic receptor expression in response to suboptimal doses of PGE$_2$ (10 nM) was increased by simultaneous exposure to theophyllin (500 μM). In other experiments, theophyllin acted synergistically with DMSO; the two agents together induced early receptor expression. Cholera toxin, another agent that stimulates adenylate cyclase, induced an early increase in receptor binding but failed to decrease proliferation or induce morphologic change. Isoproterenol, a potent β-agonist, neither influenced morphologic appearance nor chemotactic receptor expression.

Unmodified cyclic AMP, which does not traverse the cell membrane, was ineffective in inducing any cel-

FIGURE 1 Morphology of HL60 cells after culture with dbcAMP, PGE$_2$, and theophyllin or cholera toxin. Cytospin cell preparations were stained with Wright's stain and photographed through a ×100 oil-immersion objective. (a) Control cells demonstrating prominent primary granules and nucleoli and large nucleus with immature chromatin. (b) Cells cultured for 48 h with 500 μM dbcAMP, unchanged from control cells. (c) Cells cultured with 1 nM cholera toxin for 5 d, unchanged from control cells. (d) Cells cultured for 5 d with 500 nM PGE$_2$ and 1 mM theophyllin retain prominent specific granules and nucleoli but have reduced nuclear/cytoplasmic ratio and altered nuclear shape. (e) Cells cultured for 5 d with 500 μM dbcAMP appear as myelocytes and metamyelocytes.
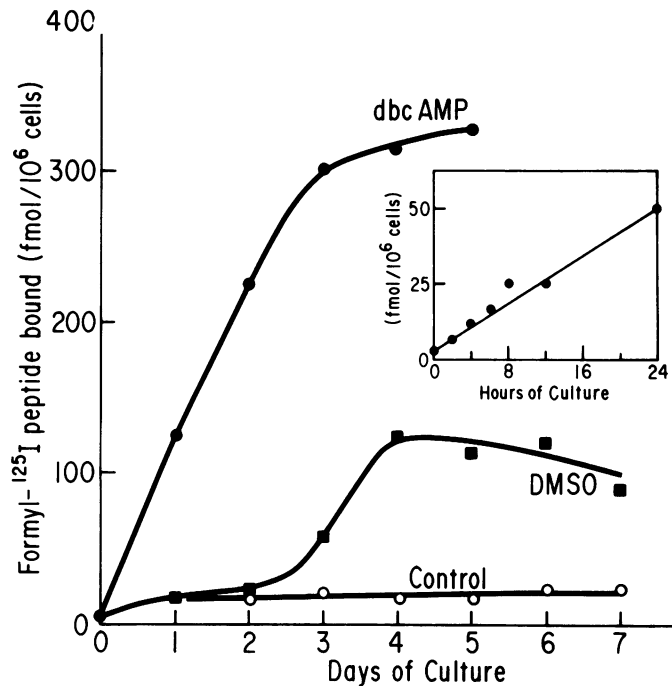
FIGURE 2 Effect of inducers on chemotactic receptor expression. HL60 cells, 2.5 × 10⁵/ml, were cultured with either 500 μM dbcAMP (●) or 160 mM DMSO (■). At each interval, specific binding of formyl ^{125}I-peptide was determined by the standard assay as described in Methods. Insert depicts a separate experiment in which HL60 cells at an initial cell density of 1 × 10⁶ cells/ml were cultured with 500 μM dbcAMP. Receptor expression was evaluated at frequent intervals over the first 24 h.
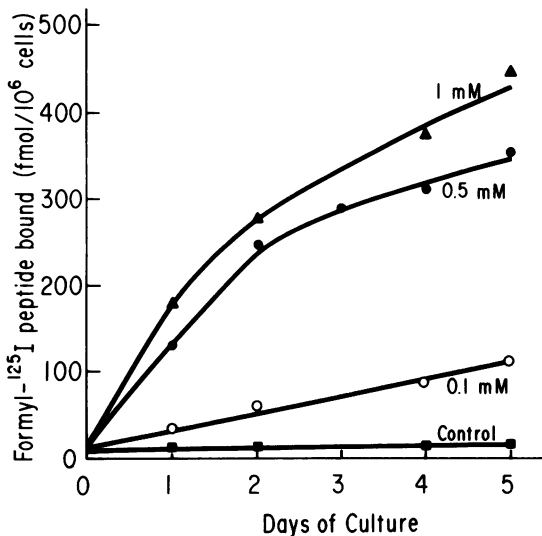

FIGURE 3 Effect of various concentrations of dbcAMP on chemotactic receptor expression. HL60 cells, 1 × 10⁶/ml, were cultured in the continuous presence of 1 mM (▲), 0.5 mM (●), 0.1 mM (○), or 0.0 mM (■) dbcAMP as indicated. Specific binding of formyl ^{125}I-peptide was determined by the standard assay as described in Methods.

lular changes. The cell-permeant cyclic nucleotide 8Br cAMP induced only a fourfold increase of the chemotactic receptor with little morphologic change. An interesting finding was the delayed increase of formyl ^{125}I-peptide binding that was evident with both 500 μM 5'AMP and 500 μM 8Br 5'AMP. The 5'AMP might induce this effect by conversion to the cell-permeant nucleoside adenosine. Chemotactic receptor expression, however, did not increase when 500 μM adenosine was used as an inducing agent.

Butyrate is known to induce HL60 cells to differentiate (2). In the current studies, 500 μM butyrate induced a delayed appearance of receptor at a time when cells morphologically resembled metamyelocytes. Toxicity was significant at concentrations >750 μM butyrate. The addition of dbcGMP did not induce early receptor expression. However, by day 5, both morphologic maturation and a fivefold increase in receptor were evident. Intracellular butyrate, produced by hydrolysis of dbcAMP or N^6,O^2-dibutyryl guanosine 3'5'-cyclic monophosphate (dbcGMP), may have resulted in a portion of the late receptor expression and morphologic maturation seen with butyrate derivatives of cyclic nucleotides.
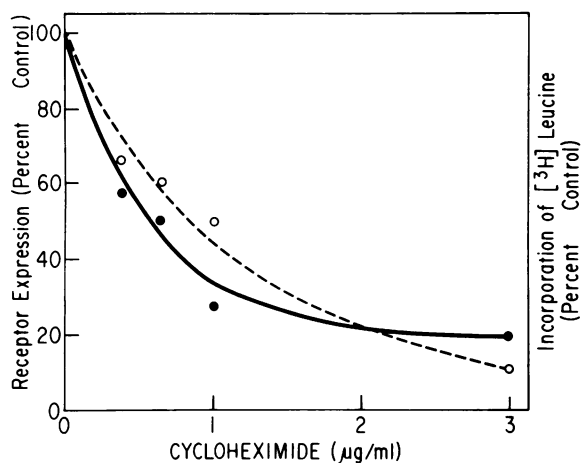
FIGURE 4 Effect of inhibition of protein synthesis on receptor expression. HL60 cells, 5 × 10⁶/ml, were cultured in leucine-free Eagle's MEM with [³H]leucine, 500 µM dbcAMP, and the indicated concentration of cycloheximide (Methods). After 24 h of culture, aliquots of cells were removed from control and cycloheximide cultures and assayed for formyl ^{125}I-peptide binding. Receptor expression by the cycloheximide-treated cells is expressed as a percentage of the receptor expression exhibited by control cells cultured without cycloheximide (●). Duplicate aliquots were assayed for incorporation of [³H]leucine into trichloroacetic acid-precipitable material (○).

TABLE II
Formyl-peptide Receptor Expression and Morphologic Change in Response to Treatment with Various Agents

	Chemotactic receptor*			Morphology‡
	Day 1	Day 2	Day 5	Day 5
Control	10	10	10	5
Early inducers				
Dibutyryl cAMP, 500 µM	125	225	325	70
PGE₁, 100 nM	61	98	96	5
PGE₂, 10 nM	28	38	43	5
PGE₂ 10 nM + theophyllin 500 µM	86	428	281	10§
PGE₂, 100 nM	76	89	144	8§
Cholera toxin, 10 nM	66	73	116	7
Late inducers				
DMSO, 160 mM	10	12	110	55
Butyrate, 500 µM	12	15	60	58
Theophyllin, 500 µM	6	20	63	5
5' AMP, 500 µM	10	18	120	46
DbcGMP, 500 µM	18	16	110	23
8Br cAMP, 500 µM	14	8	60	8
8Br 5'AMP, 500 µM	12	8	300	52
Noninducers				
Adenosine, 500 µM	23	31	24	5
Isoproterenol, 20 µM	12	12	17	5
cAMP, 500 µM	10	10	23	5

* Femtomoles formyl ^{125}I-peptide bound per 10⁶ HL60 cells under standard assay conditions (Methods).

‡ Percentage of cells showing morphological characteristics beyond the promyelocyte stage.

§ Majority of cells in this group did not show obvious morphologic granulocytic maturation but did display loss of azurophilic granules and increase in cytoplasmic volume.

Commitment of differentiation and chemotactic receptor expression. Chemotactic receptor expression was determined for HL60 cells treated with inducers for various intervals. In these experiments, cells were exposed to either dbcAMP, PGE₂, or cholera toxin for either 4 or 24 h, then washed and resuspended in new media without inducer. These cells were compared with cells continuously exposed to the inducers. Continuous exposure to dbcAMP was required for the linear increase in formyl ^{125}I-peptide binding (Fig. 5a) and for the development of morphologic maturation and NBT reduction (Fig. 5b). When dbcAMP was removed after 4 h, there was no increase in receptor expression nor morphologic change. Following washout of dbcAMP at 24 h, formyl-^{125}I-peptide binding decreased toward base line (Fig. 5a) and the cells gradually resumed proliferation (Fig. 5b). These cells did not reduce NBT or show evidence of morphologic maturation (Fig. 5b). The increased expression of chemotactic receptor at 24 h was quite homogeneous as 95% of cells bound and internalized the fluorescent formyl peptide. However the majority of these cells must not have been committed to receptor expression because following removal of dbcAMP at 24 h, only 16% remained receptor positive at 72 h. Cell density increased from 1.7 × 10⁶ cells/ml to 2.3 × 10⁶ cells/ml over the interval. In a separate experiment, cells were washed free of dbcAMP at 48 h; receptor expression slowly increased between 48 and 72 h and then remained stable from day 3 to day 5. 50% of the cells were NBT positive and morphologically mature and 95% were receptor positive at day 5, indicating commitment after at least 48 h of continuous dbcAMP treatment. If cells were washed at 72 h and resuspended in fresh medium containing dbcAMP, no significant change in the linear rate of receptor expression was seen indicating stability of the nucleotide derivative in culture.

A similar experiment with continuous exposure to 500 nM PGE₂ and 1 mM theophyllin for 5 d showed a rapid rise in formyl ^{125}I-peptide binding as 95% of cells bound the fluorescent-labeled peptide at each time point tested (Fig. 6a). This treatment did not lead
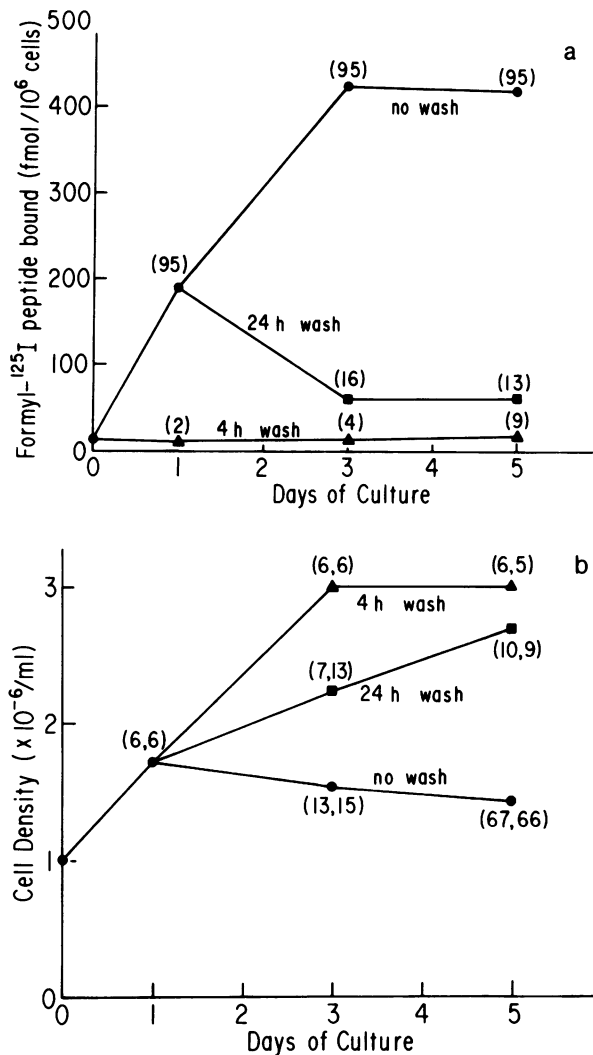
FIGURE 5 Commitment to differentiation and cessation of proliferation induced by dbcAMP. Cells, 1×10^6/ml, were cultured with 500 μM dbcAMP. After 4 and 24 h of culture, aliquots of cells were removed, washed, resuspended in fresh medium without dbcAMP and cultured for a total of 5 d. (a) After 1, 3, and 5 d of culture, specific formyl ^{125}I-peptide binding by an aliquot of cells was determined for each of the culture conditions; no wash (●), 24-h wash (■), and 4-h wash (▲). At each time point, the percentage of receptor-positive cells was determined by fluorescent peptide uptake and is shown in parentheses. (b) Cell density is plotted for the three culture conditions. At each time point, the percentage of cells matured beyond the promyelocyte is shown as the first number in parenthes. The second number in the parentheses represents the percentage of cells that reduced NBT.

to typical mature granulocytic features, but 70% of the cells were able to reduce NBT by day 5 (Fig. 6b). 4 h of exposure to PGE$_2$ did not induce receptor expression nor decrease cellular proliferation. Cells washed
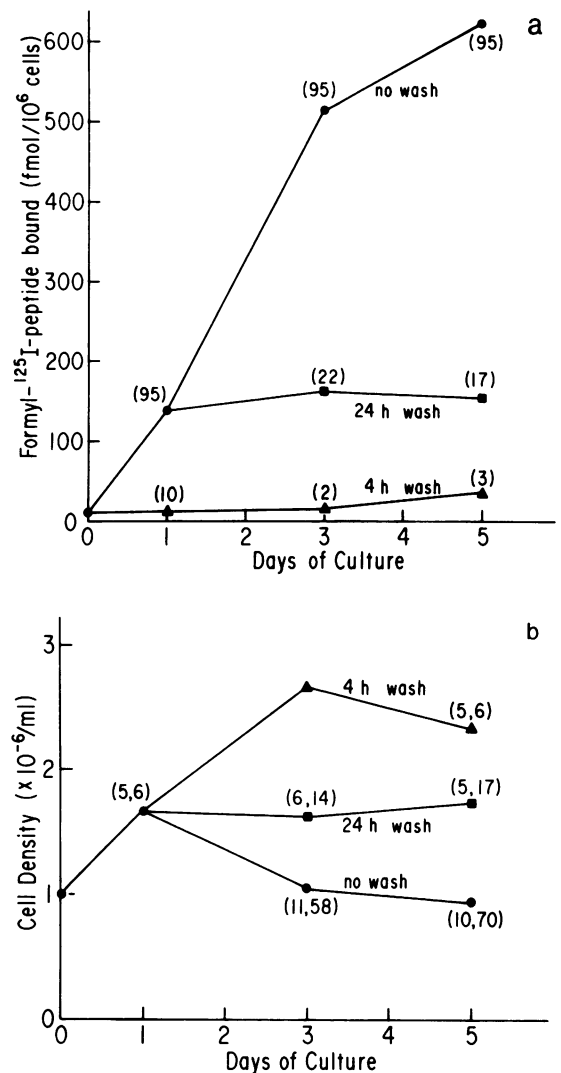
FIGURE 6 Commitment to differentiation and cessation of proliferation induced by PGE$_2$ and theophyllin. Cells, 1×10^6/ml, were cultured with 500 nM PGE$_2$ and 1 mM theophyllin. After 4 and 24 h of culture, aliquots of cells were removed, washed, resuspended in fresh medium without PGE$_2$ or theophyllin and cultured for a toal of 5 d. (a) Receptor expression and (b) cell density, NBT reduction, and maturity as detailed in the legend to Fig. 5.

free of PGE$_2$ after 24 h exhibited a stable plateau in peptide binding. Over this interval, a decrease in the proportion of cells positive for fluorescent-peptide binding was evident (Fig. 6a), although cell density did not change (Fig. 6b). When PGE$_2$ and theophyllin were removed after 48 h of treatment, receptor expression and NBT reduction reached a level ~70% of maximum. Commitment to chemotactic receptor expression or to the ability to generate superoxide required exposure to PGE$_2$ and dbcAMP for >24 h.

Cholera toxin is known to irreversibly activate adenylate cyclase and lead to a prolonged increase in intracellular cAMP (20). Incubation of HL60 cells with 1 nM cholera toxin induced an increase in binding of both formyl ^{125}I-peptide and fluorescent peptide (Fig. 7). A similar increase in receptor expression was seen when cells were treated for only 4 or 24 h, consistent with the irreversible nature of adenylate cyclase activation by cholera toxin. Binding of formyl ^{125}I-peptide slowly increased during the 5 d of culture, but the proportion of cells positive for the fluorescent peptide gradually decreased. At 1 or 50 nM cholera toxin, neither changes in the rate of cell proliferation, NBT reduction, nor morphology could be detected.

DISCUSSION

The study of myeloid differentiation has become experimentally accessible since the establishment of the HL60 cell line by Collins et al. (1). Many of the agents, including DMSO, dimethyl formamide, butyric acid, and acetamide derivatives, which induce differentiation of other malignant cell lines, also induce granulocytic maturation of the HL60 cell line (18). Retinoids induce granulocytic maturation at concentrations ~5 logs lower than the concentrations required for other inducers (19). All these agents induce a similar developmental program of differentiation as an orderly sequence of myeloid markers appears. The phorbol diesters uniquely induce differentiation of HL60 cells into macrophages, without production of granulocyte elements (21).

Although a unifying mechanism underlying induction of differentiation has not been established, evidence from other cell lines suggests that cyclic nucleotides may be involved (11, 12, 22, 23). Synchronized mouse erythroleukemia cells demonstrate a five- to sixfold increase in intracellular cAMP during mid-S phase after exposure to chemically diverse inducers (24). Elevation of intracellular cAMP in mouse and human neuroblastoma lines is one of the presumed mechanisms for inducing morphologic differentiation to mature neurons (25–28) and increasing neurotransmitter-related enzyme synthesis (29). The effects of dbcAMP in this line are reversible during the first 24 h; longer exposure commits the cells to a program of differentiation.

Our current studies demonstrate that agents which increase intracellular cAMP, such as cell-permeant cAMP analogues, prostaglandins of the E series, and cholera toxin are inducers of a modified program of differentiation in HL60 cells. The program initiated by these agents is unique in the following respects: (a) there is no latent period following treatment; the appearance of a differentiation-related membrane re-
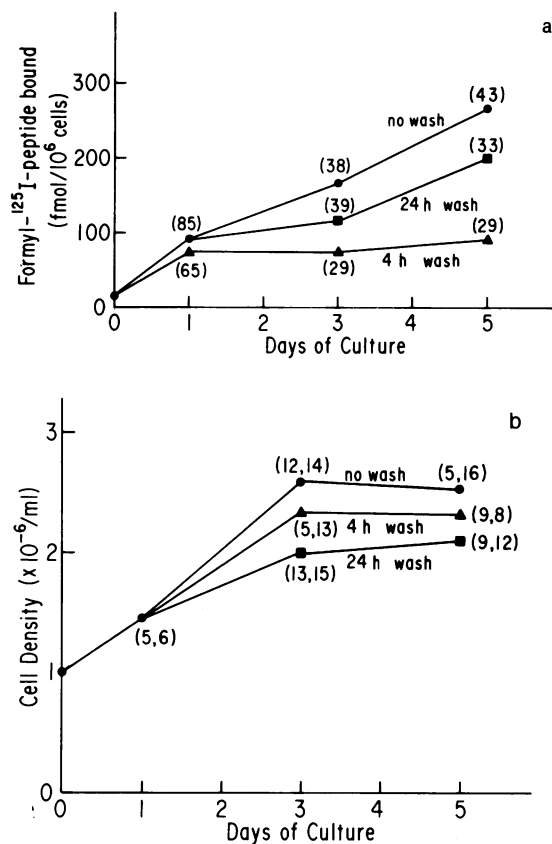

FIGURE 7 Commitment to differentiation and cessation of proliferation induced by cholera toxin. Cells, 1×10^6/ml, were cultured with 1.0 nM cholera toxin. After 4 and 24 h of culture, aliquots of cells were removed, washed, resuspended in fresh medium without cholera toxin and cultured for a total of 5 d. (a) Receptor expression and (b) cell density, NBT reduction, and maturity as detailed in the legend to Fig. 5.

ceptor is detectable within 2 h; (b) a dissociation occurs between differentiation of the plasma membrane and morphologic maturation; (c) a prompt cessation of cellular proliferation accompanies early membrane changes; and (d) commitment to terminal differentiation has not occurred in a majority of the cells at a time when >95% of the cells have expressed a differentiation marker, the formyl-peptide receptor. Cyclic AMP analogues and prostaglandins had been tested previously in leukemic lines, using a clonogenic soft-agar assay system, but these compounds had not led to obvious morphologic change and were not studied further (10). Our study confirms that terminal myeloid differentiation is not evident after treatment with such agents. However, several markers of plasma membrane maturation, including the chemotactic and complement receptors, NBT dye reduction, chemotaxis, and cell adherence to glass, are expressed after treat-

ment, at a time when the cells remain morphologically immature.

The formyl-peptide chemotactic receptor is an accepted marker of mature myeloid cells and is known to be expressed in HL60 cells after 3–5 d of DMSO or dimethylformamide treatment (5, 6). DMSO-induced receptor expression, however, is variable and occurs in only 20–40% of cells. Recently, Brandt et al. (30) described a twofold increase in this receptor when DMSO and dexamethasone were used concomitantly for induction, but whether this treatment recruited a higher percentage of receptor-positive cells is unknown. Table II demonstrates that treatment with various agents induced chemotactic receptor expression as either an early or late event. Cell-permeant cAMP analogues and stimulators of adenylate cyclase, with or without cyclic nucleotide phosphodiesterase inhibitors, all produced early expression of the chemotactic receptor. In contrast to other inducers, these agents reproducibly induced receptor expression in at least 95% of cells, to a level three to five times greater than that seen with other classes of inducers. The induced receptor displays binding properties similar to the receptor on mature neutrophils and is coupled to the mechanisms for lysosomal enzyme release, chemotaxis, NBT reduction, and peptide endocytosis. Receptor expression in response to these agents, therefore, provides an ideal in vitro system for the study of the formyl-peptide receptor.

Many experiments in our study make use of dbcAMP, a cell-permeant cyclic nucleotide. However, this is not an ideal model compound because intracellular hydrolysis of dbcAMP to butyrate and cAMP renders interpretation difficult. Elevated intracellular butyrate may also be responsible for the morphologic change and the increased receptor expression seen late in the culture with dbcGMP and dbcAMP treatment. Prostaglandins of the E series and cholera toxin increase intracellular cAMP without the complicating increase in butyrate. PGE_2 clearly dissociated receptor expression, NBT reduction, and adherence from morphologic maturation. Treatment with cholera toxin induced only receptor expression. The lack of significant receptor expression after treatment with 8 Br cAMP, another cell-permeant nucleotide, is not well understood. This derivative may not have the same effect on cAMP-dependent protein kinases as does unmodified cAMP or may not as readily enter the cells.

Variation in cAMP levels alone cannot completely explain the phenomenon of chemotactic receptor synthesis. Our studies showed an unexpected expression of receptor on the 5th d of treatment with both 5'AMP and 8 Br 5'AMP. 5'AMP has been reported to affect proliferation and differentiation in a number of cell lines (23, 25, 31). Unmodified nucleotide does not enter the cell but is readily dephosphorylated to adenosine,

which can freely enter the cell. Following intracellular rephosphorylation, the elevated concentration of 5'AMP is a potent inhibitor of phosphoribosyl-pyrophosphate synthetase, thereby blocking de novo pyrimidine synthesis and causing pyrimidine nucleoside starvation (32). Rescue of cells from this inhibitory effect of 5'AMP has been previously demonstrated by the addition of cytidine and uridine to the cultured cells (23). In our studies, the cell-permeant nucleoside, adenosine, neither stimulated differentiation nor induced receptor synthesis, rendering pyrimidine starvation an unlikely mechanism.

Previous studies with in vitro bone marrow culture have suggested that cAMP and prostaglandins may have a regulatory role in hematopoiesis (9). Colony-forming units of granulocyte precursors (CFU-GM) proliferation is under the dual control of macrophage-derived colony-stimulating factor (CSF) and prostaglandin of the E series (7–10, 33, 34). When soft-agar bone marrow culture was performed in the absence of adherent cells believed to produce CSF, cAMP and PGE were shown to be inhibitors of CFU-GM proliferation (9). HL60 proliferation may be influenced by autostimulatory growth factors similar to CSF (35). In preliminary studies, we have used acridine orange and the fluorescence-activated cell sorter to assess cellular DNA content. These studies demonstrate that the inhibition of proliferation observed in soft-agar culture is also evident in suspension culture within the first 24 h after treatment with agents that increase intracellular cAMP. This is in contrast to the 3–4-d delay in inhibition of exponential growth following treatment with DMSO and retinoids.

In addition to studying plasma membrane maturation as a function of increased intracellular cAMP, the present study was designed to study cAMP-mediated commitment to myeloid differentiation. Commitment is defined operationally as the capacity of cells that have been exposed to inducer to express differentiated characteristics in the absence of inducer. Exposure to DMSO for 12 h is the minimal requirement to promote a stochastic commitment to terminal myeloid differentiation in a fraction of HL60 cells (36). However, a latent period of 72 h occurs between induction by DMSO and the appearance of the earliest functions (2, 5, 36). In the mouse erythroleukemia system, a recent study has shown commitment to be a multistep process involving inducers and inhibitors of induction (37). Our studies demonstrate that increased intracellular cAMP abolished the latent period for the expression of an inducible membrane receptor in myeloid differentiation. However, commitment to membrane differentiation was delayed and incomplete. For instance, after 24 h of exposure to dbcAMP, >95% of the cells expressed the chemotactic receptor, but following wash-out of this inducer, 80% of these cells ceased

receptor expression (Fig. 5). Overgrowth of the small population of receptor-negative cells cannot explain this dramatic decrease in receptor-positive cells because cell density increased by only 35%. When washout of dbcAMP or PGE$_2$ was performed after 48 h of exposure, commitment to receptor expression, NBT reduction, and adherence was evident in >70% of cells, indicating that commitment had occured between 24 and 48 h. Cholera toxin, an irreversible activator of adenylate cyclase, induced the chemotactic receptor, but did not induce the membrane enzyme system responsible for NBT reduction. Receptor expression was slowed, but not abolished after washout of cholera toxin.

Our study implies a role for cAMP in the induction of plasma membrane maturation. Experiments of Gazitt et al., (24) in which cAMP levels were shown to be elevated in synchronized mouse erythroleukemia cells after treatment with various inducers, will serve as model for elucidating the role of cyclic nucleotides in HL60 differentiation. Increased intracellular cAMP may be a common mediator in response to chemically dissimilar inducers, as suggested by our demonstration of synergy in early receptor expression with DMSO and theophyllin. But even if a universal role for cAMP in HL60 differentiation cannot be defined, these agents will provide a model for the study of membrane maturation and may eventually have a role in the treatment of myeloid leukemia.

Myeloid leukemic cells accumulate in vivo because of their inability to mature to functional nonproliferative cells. Since maturation and cessation of proliferation appear to be linked events, leukemic cell accumulation may be diminished if cellular maturation were induced. Preliminary studies in our laboratory indicate that fresh explants of human acute myeloid leukemia cells in suspension culture also respond to treatment with dbcAMP and PGE$_2$. Cells from seven of nine patients with acute myeloid leukemia expressed the chemotactic receptor and became adherent after exposure to dbcAMP and PGE$_2$. Synergy between nontoxic agents such as retinoids, cyclic nucleotide phosphodiesterase inhibitors, dexamethasone, and prostaglandins may result in a potent inducer combination. In response to retinoic acid, human promyelocytic and acute myeloblastic leukemia cells in culture have recently been shown to both cease clonal proliferation and express morphologic maturity (38, 39). Subgroups of human myeloid leukemia may respond to in vivo modulation with such agents.

ACKNOWLEDGMENTS

REFERENCES

1. Collins, S. J., R. C. Gallo, and R. E. Gallagher. 1977. Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature (Lond.). 260: 347–349.
2. Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA. 75: 2458–2462.
3. Newburger, P. E., M. E. Chovaniec, J. S. Greenberger, and H. J. Cohen. 1979. Functional changes in human leukemic cell line HL60. J. Cell Biol. 82: 315–322.
4. Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. 1979. Normal functional characteristics of cultured human promyelocytic leukemic cells (HL60) after induction of differentiation by dimethyl sulfoxide. J. Exp. Med. 149: 969–974.
5. Niedel, J. E., I. Kahane, L. Lachman, and P. Cuatrecasas. 1980. A subpopulation of cultured human promyelocytic leukemia cells (HL60) display the formyl peptide chemotactic receptor. Proc. Natl. Acad. Sci. USA. 77: 1000–1004.
6. Fontana, J. A., D. G. Wright, E. Schiffman, B. A. Corcoran, and A. B. Deisseroth. 1980. Development of chemotactic responsiveness in myeloid precursor cells: studies with a human leukemia cell line. Proc. Natl. Acad. Sci. USA. 77: 3664–3668.
7. Kurland, J. I., R. S. Bockman, H. E. Broxmeyer, and M. A. S. Moore. 1978. Limitation of excess myelopoiesis by the intrinsic modulation of macrophage-derived prostaglandin E. Science (Wash., DC). 199: 552–555.
8. Kurland, J. I., H. E. Broxmeyer, L. M. Pelus, R. S. Bockman, and M. A. S. Moore. 1978. Role for monocyte-macrophage-derived colony-stimulating factor and prostaglandin E in the positive and negative feedback control of myeloid stem cell proliferation. Blood. 52: 388–407.
9. Taetle, R., and A. Koessler. 1980. Effects of cyclic nucleotides and prostaglandins on normal and abnormal human myeloid progenitor proliferation. Cancer Res. 40: 1223–1229.
10. Koeffler, H. P., and D. W. Golde. 1980. Humoral modulation of human acute myelogenous leukemia cell growth in vitro. Cancer Res. 40: 1858–1862.
11. Friedman, D. L. 1976. Role of cyclic nucleotides in cell growth and differentiation. Physiol. Rev. 56: 652–708.
12. Tisdale, M. J. 1979. The significance of cyclic AMP and cyclic GMP in cancer treatment. Cancer Treat. Rev. 6: 1–15.
13. Niedel, J. E., S. Wilkinson, and P. Cuatrecasas. 1979. Receptor-mediated uptake and degradation of ^{125}I-chemotactic peptide by human neutrophils. J. Biol. Chem. 254: 10700–10706.
14. Niedel, J. E., I. Kahane, and P. Cuatrecasas. 1979. Receptor-mediated internalization of fluorescent chemotactic peptide by human neutrophils. Science (Wash., DC). 205: 1412–1414.
15. Li, C. Y., K. W. Lam, and C. T. Yam. 1973. Esterases in human leukocytes. J. Histochem. Cytochem. 21: 1–12.
16. Michell, R., M. J. Karnovsky, and M. L. Karnovsky. 1970. The distribution of some granule-associated enzymes in

guinea pig polymorphonuclear leukocytes. *Biochem. J.* **116**: 207–216.

17. Showell, H. J., R. J. Freer, S. H. Zigmond, E. Schiffman, S. Aswanikumar, B. Corcoran, and E. Becker. 1976. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretions for neutrophils. *J. Exp. Med.* **143**: 1154–1169.

18. Abraham, J., and G. Rovera. 1981. Inducers and inhibitors of leukemic cell differentiation in culture. *In* Tissue Growth Factors. R. Baserga, editor. Springer-Verlag, Berlin. 405–425.

19. Breitman, R. T., S. E. Selonick, and S. E. Collins. 1980. Induction of differentiation of the human promyelocytic leukemia cell line (HL60) by retinoic acid. *Proc. Natl. Acad. Sci. USA.* **77**: 2936–2940.

20. Moss, J., and M. Vaughn. 1979. Activation of adenylate cyclase by choleragen. *Annu. Rev. Biochem.* **48**: 581–600.

21. Rovera, G., D. Santoli, and C. Damsky. 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. *Proc. Natl. Acad. Sci. USA.* **76**: 2779–2783.

22. Tabuse, Y., M. Furusawa, H. Eisen, and K. Shibata. 1977. Prostaglandin E_1, an inducer of erythroid differentiation of Friend erythroleukemia cells. *Exp. Cell Res.* **108**: 41–45.

23. Rubin, C. S. 1978. Effects of cyclic AMP on the growth of differentiating and undifferentiated erythroleukemia cells. *J. Cell. Physiol.* **94**: 57–68.

24. Gazitt, Y., R. C. Reuben, A. D. Deitch, P. A. Marks, and R. A. Rifkind. 1978. Changes in cyclic AMP levels during induction of differentiation in murine erythroleukemia cells. *Cancer Res.* **38**: 3779–3783.

25. Furmanski, P., D. J. Silverman, and M. Lubin. 1971. Expression of differentiation-function in mouse neuroblastoma mediated by dibutyryl cyclic AMP. *Nature (Lond.).* **233**: 413–415.

26. Prasad, K. N., and A. Vernadakis. 1972. Morphological and biochemical study in dibutyryl cyclic AMP-induced differentiated neuroblastoma cells. *Exp. Cell Res.* **70**: 27–32.

27. Prasad, K. N. 1972. Morphological differentiation induced by prostaglandin in mouse neuroblastoma cells. *Nat. New Biol.* **236**: 49–52.

28. Prasad, K. N., S. K. Sahu, and P. K. Sinha. 1976. Cyclic nucleotides in the regulation of expression of differentiated functions in neuroblastoma cells. *J. Natl. Cancer Inst.* **57**: 619–629.

29. Waymine, J. C., N. Weiner, and K. N. Prasad. 1972. Regulation of tyrosine hydroxylase activity in cultured mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. USA.* **69**: 2241–2245.

30. Brandt, S. J., K. C. Bank, D. B. Glass, and J. M. Kinkade. 1981. Glucocorticoid-stimulated increase in chemotactic peptide receptor on differentiating human HL60 cells. *Cancer Res.* **41**: 4947–4951.

31. Masui, H., and L. D. Garner. 1971. Inhibition of replication in functional mouse adrenal tumor cells by adrenocorticotropic hormone mediated by cyclic AMP. *Proc. Natl. Acad. Sci. USA.* **68**: 3206–3210.

32. Fox, I. H., and W. N. Kelley. 1972. Human phosphoribosyl-pyrophosphate synthetase. *J. Biol. Chem.* **247**: 2126–2131.

33. Kurland, J. I., J. W. Hadden, and M. A. S. Moore. 1977. Role of cyclic nucleotides in the proliferation of committed granulocyte-macrophage progenitor cells. *Cancer Res.* **37**: 4534–4538.

34. Pelus, L. M., H. E. Broxmeyer, J. I. Kurland, and M. A. S. Moore. 1979. Regulation of macrophage and granulocyte proliferation. *J. Exp. Med.* **150**: 277–292.

35. Brenan, J. K., C. N. Abboud, J. F. Dipersio, G. H. Barlow, and M. A. Lichtman. 1981. Autostimulation of growth by human myelogenous leukemia cells (HL60). *Blood.* **58**: 803–812.

36. Tarella, C., D. Ferrera, E. Gallo, G. L. Pagliardi, and F. W. Ruscetti. 1982. Induction of differentiation of HL60 cells by DMSO: evidence for a stochastic model not linked to the cell division cycle. *Cancer Res.* **42**: 445–449.

37. Chen, Z. X., J. Banks, R. A. Rifkind, and P. A. Marks. 1982. Inducer-mediated commitment of murine erythroleukemia cells to differentiation: a multistep process. *Proc. Natl. Acad. Sci. USA.* **79**: 471–475.

38. Breitman, T. R., S. J. Collins, and B. R. Keene. 1981. Terminal differentiation of human promyelocytic leukemia cells in primary culture in response to retinoic acid. *Blood.* **57**: 1000–1004.

39. Douer, D., and H. P. Koeffler. 1982. Retinoic acid—inhibition of the clonal growth of human myeloid leukemia cells. *J. Clin. Invest.* **69**: 277–283.