

# The Effect of Dexamethasone on Functional Properties of HL60 Cells During All-*trans* Retinoic Acid Induced Differentiation. Are There Implications for the Retinoic Acid Syndrome?

Submitted on 06/04/96; revised 07/15/96

(communicated by Marshall A. Lichtman, M.D., 07/19/96)

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**ABSTRACT:** Differentiation therapy for acute promyelocytic leukemia (APL) using all-*trans*-retinoic acid (ATRA) has improved the prognosis of the disease. ATRA therapy also causes a newly recognized clinical syndrome, the “retinoic acid syndrome” (RAS), which can be successfully managed with dexamethasone. Because aberrant function of maturing leukemic granulocytes may cause this syndrome, and because dexamethasone is useful clinically, we studied functional properties of maturing HL60 cells cultured in the presence and absence of dexamethasone. HL60 cells were cultured for 4 days with ATRA and studied daily to determine acquisition of mature neutrophil-like properties including phagocytosis, NBT reduction, actin polymerization, chemotaxis and adhesion molecule expression. Undifferentiated HL60 cells could not polymerize actin or reduce NBT, and exhibited only a minimal ability to undergo chemotaxis or ingest latex beads. Following 4 days of maturation with ATRA, the cells would increase F-actin content in response to interleukin-8, ingest latex beads, migrate in a chemotaxis chamber, reduce NBT, and express CD11b. When dexamethasone was added to the cells in culture, there was no major enhancement or suppression of these properties. We also studied the effect of dexamethasone on functional properties of normal neutrophils and found minimal if any effect on their function. Overall, these studies suggest that *in vitro*, dexamethasone has little effect on the function of leukemic and normal granulocytes. To further investigate the pathophysiology of the retinoic acid syndrome, future studies may need to use endothelial cells, cytokines, or granulocytes obtained from APL patients.

**Keywords:** Acute promyelocytic leukemia, retinoic acid, phagocytosis, actin, chemotaxis, interleukin-8, glucocorticoids

## INTRODUCTION

The successful treatment of patients with acute promyelocytic leukemia (APL) using all-*trans* retinoic acid (ATRA) has been demonstrated in clinical trials and has led to the elucidation of the molecular basis of the disease (1-4). Our group and others have studied some of the functional characteristics of maturing APL cells and have studied leukemia cell lines which share many features with patient samples (5-8).

Differentiation of APL cells and HL60 cells with ATRA is associated with the acquisition of functional properties that are well characterized in neutrophils including chemotaxis, superoxide production, and actin polymerization. However, mature granulocytes from APL patients differ somewhat from normal neutrophils, and it has been postulated that some aberrant function may contribute to the “retinoic acid syndrome”(RAS) (9).

RAS has only been observed in patients

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The Blood Cells Foundation, La Jolla, California USA  
ISSN No. 1079-9796

Published by the Blood Cells Foundation  
Established by Springer-Verlag, Inc. in 1975

receiving ATRA for APL, thereby supporting the contention that maturing granulocytes play a role in this syndrome. Dexamethasone successfully ameliorates RAS by an unknown mechanism; possible explanations include alteration of leukocyte activation, cytokine production or endothelial changes. Some effects of glucocorticoids on characteristics of leukemia cell lines have been studied (10-13), however, the effect on functional properties of differentiating granulocytes has not been studied. Because RAS may be secondary to aberrant function of maturing granulocytes, we studied the effect of dexamethasone on functional properties of HL60 cells undergoing differentiation with ATRA. We also studied the effects of glucocorticoids on neutrophil properties because prior studies have shown variable and inconsistent results (14-19).

## MATERIALS AND METHODS

### *Cell Culture and Preparation of Neutrophils*

HL60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37° C in a humidified environment with 5% CO<sub>2</sub> in air. A final concentration 1µM ATRA (Sigma Chemical Co., St. Louis, MO) was added to the cultures, and samples were removed for study at desired time points. Cell morphology was assessed using Wright's stain.

Neutrophils were isolated from citrated blood obtained from normal volunteers using dextran sedimentation followed by Ficoll-Hypaque centrifugation. Saline lysis was used to remove erythrocytes. Neutrophils were resuspended in phosphate buffered saline (PBS) at a concentration of 1x10<sup>6</sup>/ml for use.

### *Phagocytosis Measurements*

Cells were suspended in RPMI 1640 medium plus 20% human plasma at a concentration of 2.5x10<sup>6</sup> cells per ml. Aliquots of 0.5 ml of cells were incubated with a suspension of 1.75 µm diameter microspheres (Fluoresbrite carboxylate,

Polysciences, Inc., Warrington, PA) at a final concentration of 50 microspheres per cell. The cells were incubated at 37° C without agitation, washed with phosphate buffered saline (PBS) containing 5mM ethylenediamine tetraacetic acid (EDTA), fixed in glutaraldehyde (1% v/v in PBS), and stored at 4° C for future analysis by flow cytometry (20).

Uptake of fluorescent microspheres was measured on an EPICS Profile flow cytometer (Coulter Corp., Hialeah, FL). A 15 mw laser was utilized for fluorescence excitation at 488 nm, and cell size and fluorescence distribution was measured at 525 nm. At least 5 distinct peaks could be resolved, corresponding to cells that had ingested 1, 2, 3, 4 or 5 microspheres. Percent phagocytosis was based on the ratio of cells containing one or more microspheres to the total number of cells.

### *Actin Polymerization Measurements*

FMLP was stored in DMSO at -20° C and was diluted in RPMI/0.1% BSA prior to use. IL-8 was refrigerated in distilled water and used directly from the stock solution. All experiments were done at 37° C. For each experiment, aliquots of the cell suspension containing 5 x10<sup>5</sup> cells/200 µl were removed at appropriate timepoints and placed into ice cold 3.2% paraformaldehyde and refrigerated for 48 hours to permeabilize the cells for flow cytometry (21,22). Cells were washed with PBS/0.1% BSA and incubated with 7-nitrobenz-2-oxa-diazole (NBD) phalloidin (0.6µM) (Molecular Probes Inc., Eugene, OR) for 45 minutes. The F-actin content was measured on an EPICS Profile flow cytometer(Coulter Corp. Hialeah, FL) using a fluorescence excitation at 488 nm and green fluorescence was measured at 525 nm as was forward angle light scatter. The log green fluorescence was converted to a relative linear scale and plotted against time.

### *Chemotaxis*

Chemotaxis was assayed as previously

described (23) using a 48 well chemotaxis chamber (Neuro Probe, Cabin John, MD) with 8  $\mu\text{m}$  pore size Sartorius cellulose nitrate filters (Neuro Probe). The lower portions of the wells were filled with 12 nM IL-8 (Upstate Biotechnology, Inc., Lake Placid, NY), 10 nM fMLP (Calbiochem, La Jolla, CA), or buffer. The upper compartments of the wells were filled with 50  $\mu\text{l}$  of a cell suspension at  $5 \times 10^6$  cells/ml. The assembled chamber was incubated for 2 hours at 37° C in humidified air, the filter was fixed in 70% ethanol, stained with hematoxylin solution, and dehydrated in a series of alcohols. The filter was cleared with Hemo-De clearing agent (Fisher Scientific, Pittsburgh, PA), mounted onto glass slides and coverslips were placed. Distance to the leading front was determined under high power using the calibrated fine focus adjustment knob on a standard light microscope. A minimum of 6 fields were observed for each experimental condition, and all assays were for 2 hours.

#### *NBT Reduction Determinations*

Samples were centrifuged and resuspended in RPMI with 0.1% nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO) in PBS. 1  $\mu\text{g/ml}$  12-o-tetradecanoyl phorbol-13-acetate (TPA) (Sigma Chemical Co.) was added and the mixture was incubated at 37° C for 20 min. Cells were fixed with 2% paraformaldehyde and analyzed on the EPICS Profile flow cytometer (Coulter Corp., Hialeah, FL). Measurement of forward and 90° light scatter allowed distinction between cells that were positive and negative for NBT reduction. NBT negative cells had an increased forward and decreased 90° light scatter, while NBT positive cells had a decreased forward and increased 90° light scatter (24). Samples were also examined for blue insoluble formazan granules using light microscopy.

#### *Staining for Cell Surface Adhesion Molecules*

Cells were incubated in PBS/ 20% human AB serum with 5 $\mu\text{g/ml}$  anti-CD11b or 0.625 $\mu\text{g/ml}$  anti-CD18 (Becton Dickinson, San Jose, CA) for

30 min at 4° C. The cells were washed with PBS/0.1% BSA, incubated with 25 $\mu\text{g/ml}$  FITC conjugated anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gathersburg, MD), fixed in PBS containing 1% paraformaldehyde and analyzed on an Epics PROFILE flow cytometer. Isotypic controls (IgG<sub>2a</sub> for anti-CD11b and IgG<sub>1</sub> for anti-CD18) were used to correct for non specific staining. Stained cells were also viewed with fluorescence microscopy.

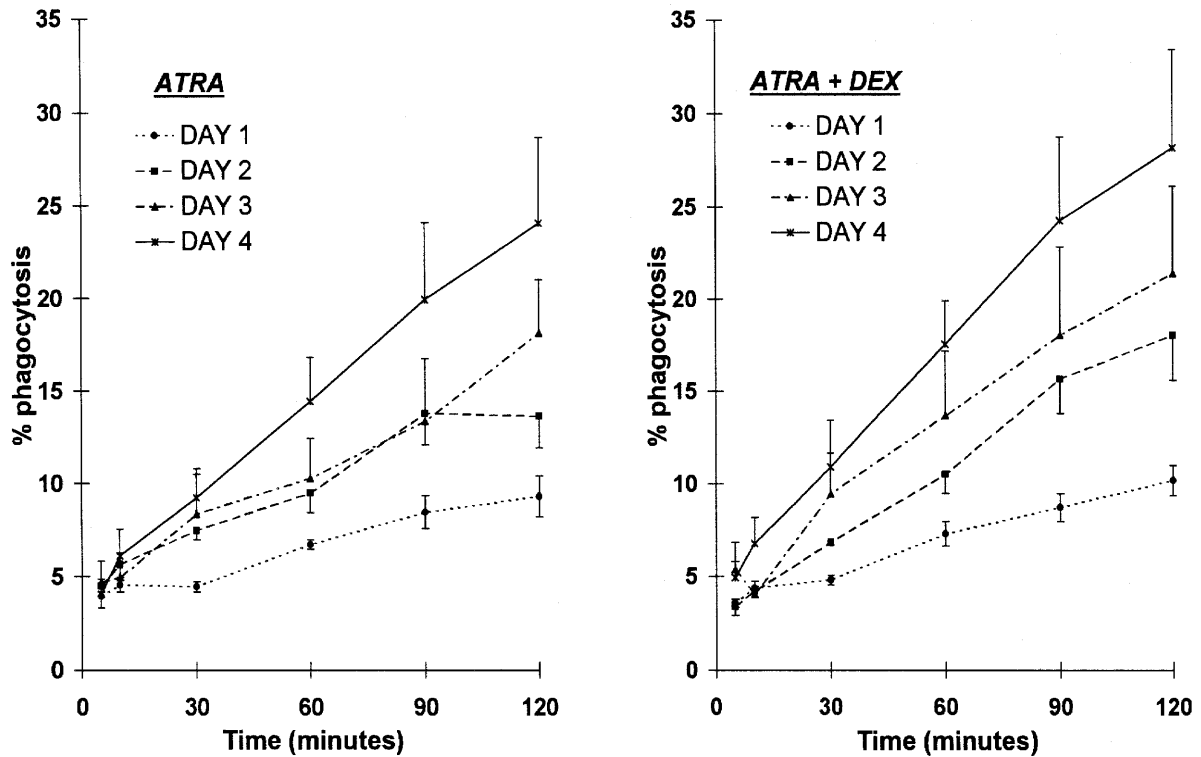
## RESULTS

### *Phagocytosis: Determination with Flow Cytometry and the Effect of Dexamthasone on Phagocytosis in HL60 Cell Matured with ATRA*

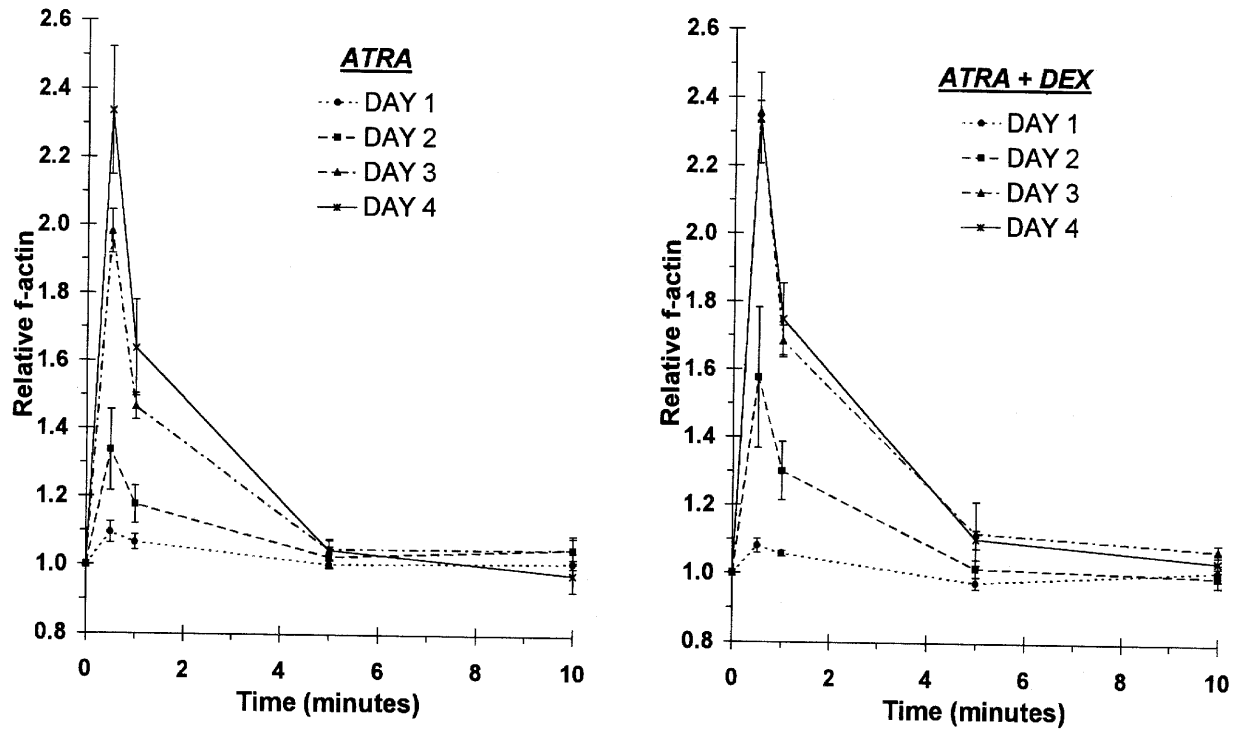
Neutrophils isolated from healthy donors were initially used for these experiments. Cells were prepared as described above and 70-80% of the cells ingested latex beads. Fluorescence microscopy was used to verify the flow cytometry results. Experiments were then carried out in the presence of 1 $\mu\text{M}$  and 10 $\mu\text{M}$  dexamethasone and showed that phagocytosis was neither enhanced nor inhibited by this agent (data not shown).

HL60 cells cultured for 4 days in ATRA showed progressive morphologic evidence of nuclear and cytoplasmic maturation. They were sampled daily to assess their ability to ingest latex microspheres at varying stages of maturation, and there was a progressive increase in the percentage of cells capable of phagocytosis as shown in Figure 1A. Most cells ingested only 1 or 2 beads, although an occasional cell ingested 10 or more beads.

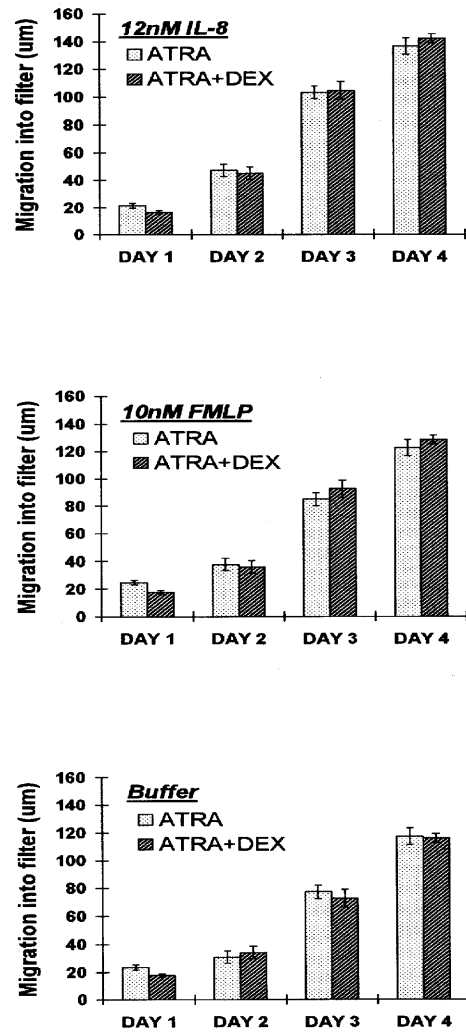
Dexamethasone was added to the cell culture media to study any possible effect on HL60 cell phagocytosis during ATRA induced maturation. There was a progressive increase in the percentage of cells capable of phagocytosis as shown in Figure 1b, however, there was no significant difference when comparing cells matured with ATRA alone versus those treated with ATRA and dexamethasone.



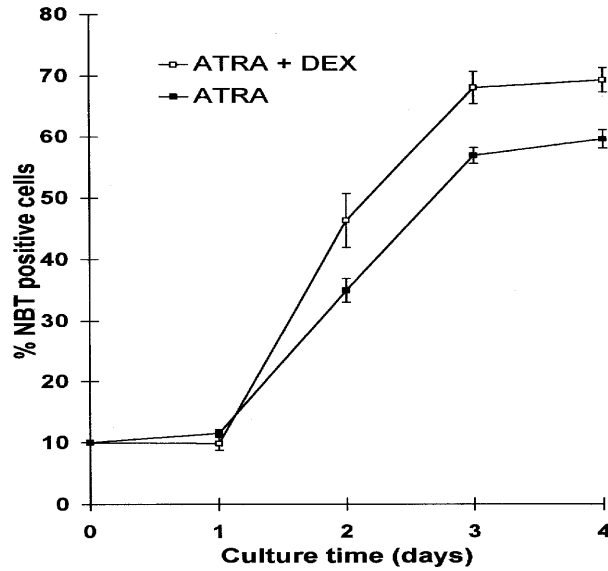
**Figure 1.** The effect of dexamethasone on phagocytosis in HL60 cells matured with ATRA. Cells were cultured with 1  $\mu$ M ATRA and phagocytosis of latex beads was studied in the presence and absence of 1 $\mu$ M dexamethasone. Throughout maturation, there was a progressive increase in the percentage of cells capable of phagocytosis with 8% of the undifferentiated cells ingesting beads compared with 24% of the matured cells. (A) The results were not significantly different when the cells were cultured in the presence of 1 $\mu$ M dexamethasone; 9% of the undifferentiated cells were capable of phagocytosis and 28% of the matured cells. (B) Results shown are the mean of 5 experiments  $\pm$  S.E.



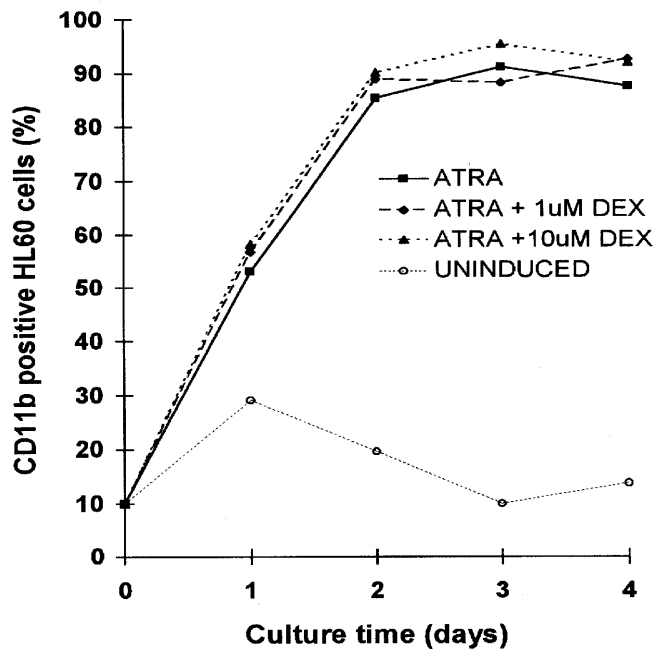
**Figure 2.** The effect of dexamethasone on actin polymerization in HL60 cells matured with ATRA. Cells were cultured with  $1\mu\text{M}$  ATRA and samples were assayed daily for changes in F-actin content in response to  $12\text{ nM}$  IL-8. The relative F-actin content was determined by flow cytometry and is plotted against time. The individual curves represent sequential days of maturation as noted. (A) Cells matured with ATRA developed the ability to respond to IL-8; day 4 cells showed a 2.35 fold increase in F-actin content at 0.5 min. (B) Identical experiments were carried out in the presence of  $1\mu\text{M}$  dexamethasone. The student t-test showed a statistically significant difference in the actin responses at two timepoints, 0.5 and 1 min timepoint of the day 3 cells ( $p < 0.05$ ), where the dexamethasone treated cells had a greater actin response. Results shown are the mean of 3 experiments  $\pm$  S.E.



**Figure 3.** The effect of dexamethasone on chemotaxis of HL60 cells matured with ATRA. HL60 cells cultured with 1  $\mu$ M ATRA were studied in the presence and absence of 1  $\mu$ M dexamethasone and exposed to either buffer, fMLP or IL-8 in a chemotaxis chamber. The graphs display data from days 1-4 and are grouped according to the chemoattractant used. Uninduced HL60 cells had essentially no ability to migrate; the cells migrated between 10 and 20  $\mu$ m in all conditions tested. When cultured with ATRA, they showed a progressive increase in their ability to migrate during 4 days of maturation. This result was seen with IL-8, fMLP, and buffer. The addition of 1  $\mu$ M dexamethasone did not alter the chemotactic responses. Results shown are the mean of 2 experiments  $\pm$ S.E.



**Figure 4.** The effect of dexamethasone on NBT reduction in ATRA induced HL60 cells. HL60 cells cultured in  $1\mu\text{M}$  ATRA had NBT reduction measured daily over 4 days of maturation. 10% of the uninduced cells reduced NBT and this increased to 60% by the fourth day of ATRA treatment. Parallel experiments done with ATRA and  $1\mu\text{M}$  dexamethasone showed slightly greater responses on days 3 and 4 that were statistically significant ( $p \leq 0.05$ ). Results shown are the mean of 3 experiments  $\pm$  S.E.



**Figure 5.** The effect of dexamethasone on adhesion molecule expression in HL60 cells matured with ATRA. HL60 cells were cultured for 4 days in  $1\mu\text{M}$  ATRA and CD18 and CD11b were measured using immunofluorescent labeled monoclonal antibodies and flow cytometry. 97% of undifferentiated HL60 cells express CD18 (data not shown), however, only 10% of the cells express CD11b. Throughout the 4 days of maturation, there was no change in CD18 expression, but there was a progressive increase in the percentage of cells expressing CD11b (Fig 5). This pattern of expression was not altered by the addition of  $1\mu\text{M}$  dexamethasone.

### *The Effect of Dexamethasone on Actin Polymerization in HL60 Cells Matured with ATRA*

HL60 cells cultured with ATRA were sampled daily for determination of relative F-actin content before and after the addition of IL-8 to assess early cytoskeletal changes. The potential to polymerize actin in response to IL-8 increased throughout maturation as demonstrated graphically (Fig 2A).

To study the possible effect of dexamethasone on actin polymerization in HL60 cells during ATRA induced maturation, 1 $\mu$ M dexamethasone was added to the cell culture media and experiments were performed as above. The overall pattern of response was quite similar to the cells without dexamethasone, however, 2 timepoints showed small but statistically significant difference in actin responses with values slightly greater than those studied without dexamethasone (Fig 2B).

Normal neutrophils were also used to evaluate any effect of dexamethasone on actin polymerization. Dexamethasone doses of 1 $\mu$ M and 10 $\mu$ M were used and had no effect on IL-8 induced actin polymerization in neutrophils (data not shown).

### *The Effect of Dexamethasone on Chemotaxis of HL60 Cells Matured with ATRA*

Chemotaxis of maturing HL60 cells was assessed in the presence and absence of 1 $\mu$ M dexamethasone using the chemoattractants IL-8 and fMLP, or buffer. Cells cultured with ATRA were assessed daily and showed a progressive increase in the ability of the cells to migrate with successive days of maturation. The presence of dexamethasone had no significant effect on chemotaxis (Fig 3).

Normal neutrophils were studied in a similar manner. 1 $\mu$ M dexamethasone did not enhance or inhibit chemotaxis in these experiments. 10 $\mu$ M dexamethasone had a slight but statistically significant inhibitory effect on chemotaxis when fMLP was used as the chemoattractant (P=0.027)

(data not shown).

### *Effect of Dexamethasone on NBT Reduction in ATRA Induced HL60 Cells*

HL60 cells were cultured in ATRA and NBT reduction was measured daily using flow cytometry. The cells exhibited a progressive and marked increase in their ability to reduce NBT as shown in Figure 4. HL60 cells treated with ATRA and dexamethasone had slightly greater responses on days 3 and 4 as shown (Fig. 4).

Experiments were also performed with normal neutrophils. Dexamethasone did not alter NBT reduction in these experiments (data not shown).

### *Effect of Dexamethasone on Adhesion Molecule Expression in HL60 Cells Matured with ATRA*

The presence of CD18 and CD11b was measured daily using HL60 cells cultured in ATRA. Most undifferentiated HL60 cells expressed CD18 whereas very few expressed CD11b. Throughout the 4 days of maturation, there was no change in CD18 expression, but there was a progressive increase in the percentage of cells expressing CD11b (Fig 5). This pattern of expression was not altered by dexamethasone.

## DISCUSSION

Differentiation of leukemic cells had been studied *in vitro* for greater than a decade prior to the successful clinical application of differentiating agents (25-28). Patients with APL who are treated with ATRA have *in vivo* differentiation of leukemic granulocytes and an improved event-free survival. RAS occurs only in patients with APL treated with ATRA, and has not been described in other patients receiving reinoids, or patients with APL treated with chemotherapy alone. It is postulated that RAS is secondary to aberrant interaction(s) between maturing leukemic granulocytes and host tissues. Dexamethasone is successful in treating RAS, yet the mechanism of its effect remains unknown. This study was undertaken to determine if dexamethasone alters

functional properties of leukemic granulocytes during differentiation with ATRA, and because there is conflicting data on the effects of glucocorticoids on neutrophil function, normal neutrophils were also studied.

HL60 cells treated with ATRA acquire the ability to ingest latex microspheres; this property was neither inhibited nor enhanced by dexamethasone treatment. When compared with normal neutrophils, however, mature HL60 cells have a relatively limited ability to ingest latex beads which may account for the inability to detect any effect of dexamethasone. Petroni *et al* (14) found a small but significant inhibitory effect of 200nM dexamethasone on  $\gamma$ -interferon induced phagocytosis in neutrophils. Our experiments did not use cytokine priming and did not demonstrate an inhibitor effect of dexamethasone on normal neutrophil phagocytosis.

The actin cytoskeleton of HL60 cells undergoes many changes with maturation. Actin content increases, chemotactic responses develop, and the cells acquire the ability to polymerize actin in response to chemotactic factors following differentiation (29-33). In our HL60 cell experiments, there were 2 timepoints in the actin studies where dexamethasone resulted in slight increases in response compared with untreated cells, however, the overall effect of dexamethasone was minimal. In neutrophils, actin polymerization responses were not altered by dexamethasone.

The effect of glucocorticoids on neutrophil chemotaxis has been studied previously and minimal effects have been reported (15,16). Our neutrophil studies concur with other investigators; dexamethasone caused a minor inhibitory effect on chemotaxis in the presence of fMLP and no effect in the other experimental conditions. In HL60 cells differentiated with ATRA, dexamethasone did not alter chemotactic responses

Neutrophil degranulation and superoxide generation contribute to tissue damage in inflammatory states. Our studies with normal neutrophils did not show any inhibitor effect of dexamethasone on NBT reduction, suggesting that

the beneficial action of glucocorticoids in inflammatory states is not mediated via this mechanism. HL60 cells treated with ATRA acquire the ability to reduce NBT throughout maturation, although the response are lower than in normal neutrophils. The slightly enhanced responses seen on days 3 and 4 were statistically significant, however, overall the increase was small and unlikely to reflect a clinically significant *in vivo* effect.

Lastly, our studies on surface adhesion molecule expression in HL60 cells treated with ATRA showed that the expression of these molecules was not altered by co-culture with dexamethasone. The absence of an observed effect of dexamethasone on isolated cells does not exclude that possibility that aberrant adhesive interactions occur *in vivo* or in other experimental conditions. Future studies utilizing endothelial cells may be helpful in addressing this issue.

In summary, we have used ATRA treated HL60 cells as a model system for studying the function of maturing leukemic granulocytes in an effort to learn more about the possible effects of glucocorticoids in RAS. These studies show that dexamethasone has no major effect on functional properties of normal neutrophils or HL60 cells undergoing differentiation with ATRA. Although dexamethasone is clinically useful in the treatment of RAS, its mechanism of action is unclear. It is possible that glucocorticoid effects are cytokine mediated, dependent on cell:cell interactions, or that APL cells and HL60 cells differ in their behavior and sensitivity to glucocorticoids.

#### ACKNOWLEDGMENTS

This work was supported by the Department of Medicine, Rochester General Hospital.

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