Induction of Differentiation of Human Myeloid Cell Lines by Tumor Necrosis Factor in Cooperation with 1α,25-Dihydroxyvitamin D₃

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ABSTRACT

We analyzed the combined effect of tumor necrosis factor and 1α,25-dihydroxyvitamin D₃ on the differentiation of human myeloid cell lines HL-60, ML3, and U937. The two compounds synergize in inducing terminal differentiation of myelomonocytic cells. Dimethylsulfoxide (4), retinoic acid (5), and other substances induce HL-60 cells to differentiate along the myeloid pathway, whereas phorbol diesters (6), 1,25(OH)₂D₃ (7), and leukocyte products contained in medium conditioned from PHA-CM (8) induce them to differentiate into cells with characteristics of monocytic/macrophage lineage. HL-60 and U937 cells bear specific receptor molecules for 1,25(OH)₂D₃ (9, 10); an HL-60 blast cell line (9), which does not differentiate in response to 1,25(OH)₂D₃, expresses only about 8% of the number of 1,25(OH)₂D₃ receptors as the parental responsive cell line. HL-60 cells treated with 1,25(OH)₂D₃ have been reported (7) to differentiate to cells with the differentiated morphology of metamyelocytes and monocytes, capable of phagocytosis and NBT reduction. These cells have been identified as monocytic/macrophage cells because they adhere to glass, contain nonspecific esterase, and express Fc receptors for IgG.

Partial monocytic differentiation of human myeloid cell lines (12–17) is induced by IFN-γ, a potent inducer of the high-affinity Fc receptor (13, 18) and of class II major histocompatibility antigens (19). IFN-γ is present in PHA-CM but in concentrations insufficient to account for its differentiation-inducing activity, and other differentiation-inducing factors have been detected in PHA-CM-depleted PHA-CM that synergize with IFN-γ in inducing differentiation (16). IFN-γ was shown to synergize with 1,25(OH)₂D₃ in inducing differentiation of human myeloid cell lines (20, 21). Also, a synergism between IFN-γ and 1,25(OH)₂D₃ was observed in the induction of class II major histocompatibility antigens on murine WEHI-3 myelomonocytic cells (22). Interestingly, IFN-γ has been shown to stimulate synthesis of 1,25(OH)₂D₃ by normal human macrophages (23). This interplay between IFN-γ and 1,25(OH)₂D₃ may suggest a role for 1,25(OH)₂D₃ in the regulation of hematopoiesis by IFN-γ-producing lymphocyte subsets, i.e., T and natural killer cells.

TNF and LT are two partially homologous factors originally described on the basis of their cytostatic effects on tumor cell lines, TNF as a tumor cytotoxic factor present in the serum of animals injected with endotoxin (24), and LT as a class of cytotoxic/cytostatic substances released by lymphocytes upon antigenic or mitogenic stimulation (25, 26). The cytotoxic/cytostatic effects of both cytokines are potentiated by IFN-γ (27, 28). Recently, purification and cloning of the genes for TNF and LT have allowed more detailed studies of the biology of these factors (29–33). The two cytokinins also appear to mediate regulatory effects on various cell types, e.g., they act as growth factors for fibroblasts (34, 35) and regulate proliferation, differentiation, and functions of myelomonocytic cells at all stages of differentiation (36). In particular, we (36, 37) showed that TNF and LT, at concentrations of the order of 10⁻¹⁵ M, induce monocytic differentiation of human myeloid cell lines. After 5 days of culture in the presence of TNF or LT, a significant proportion of the HL-60 myeloid cells express monocytic differentiation antigens and nonspecific esterase activity and become able to reduce NBT and to mediate low levels of Ab-CMC against tumor target cells. These markers of differentiation, however, are maximally induced when IFN-γ is present simultaneously with the cytotoxic, and the two classes of cytokines act synergistically to induce terminal differentiation. The appearance of monocytic antigens is accompanied by acquisition of the morphologic and functional properties of mature monocytic cells, such as chemiluminescence and phagocytosis, and by expression of Fc receptors for monomeric IgG. A decrease in cell proliferation accompanies induced differentiation.

The differentiation induced by TNF on myeloid cell lines closely resembles that induced by 1,25(OH)₂D₃. We report here that TNF and 1,25(OH)₂D₃ in combination induce differentiation of the HL-60, ML3, and U937 human cell lines. We show that the interaction of the two differentiation-inducing compounds is synergistic, especially when the compounds are used at suboptimal concentrations.

INTRODUCTION

Leukemia-derived human myeloid cell lines, such as the promyelocytic HL-60 (1) and ML3 (2) and the histiocytic U937 (3), can be induced to differentiate in vitro by several synthetic and natural inducers and are often used as models to study terminal differentiation of myelomonocytic cells. Dimethylsulfoxide (4), retinoic acid (5), and other substances induce HL-60 cells to differentiate along the myeloid pathway, whereas phorbol diesters (6), 1,25(OH)₂D₃ (7), and leukocyte products contained in medium conditioned from PHA-CM (8) induce them to differentiate into cells with characteristics of monocytic/macrophage lineage. HL-60 and U937 cells bear specific receptor molecules for 1,25(OH)₂D₃ (9, 10); an HL-60 blast cell line (9), which does not differentiate in response to 1,25(OH)₂D₃, expresses only about 8% of the number of 1,25(OH)₂D₃ receptors as the parental responsive cell line. HL-60 cells treated with 1,25(OH)₂D₃ have been reported (7) to differentiate to cells with the differentiated morphology of metamyelocytes and monocytes, capable of phagocytosis and NBT reduction. These cells have been identified as monocytic/macrophages because they adhere to glass, contain nonspecific esterase, and express monocyte-specific enzymes (9, 11).

Partial monocytic differentiation of human myeloid cell lines (12–17) is induced by IFN-γ, a potent inducer of the high-affinity Fc receptor for monomeric IgG (13, 18) and of class II major histocompatibility antigens (19). IFN-γ is present in PHA-CM but in concentrations insufficient to account for its differentiation-inducing activity, and other differentiation-inducing factors have been detected in PHA-CM-depleted PHA-CM that synergize with IFN-γ in inducing differentiation (16). IFN-γ was shown to synergize with 1,25(OH)₂D₃ in inducing differentiation of human myeloid cell lines (20, 21). Also, a synergism between IFN-γ and 1,25(OH)₂D₃ was observed in the induction of class II major histocompatibility antigens on murine WEHI-3 myelomonocytic cells (22). Interestingly, IFN-γ has been shown to stimulate synthesis of 1,25(OH)₂D₃ by normal human macrophages (23). This interplay between IFN-γ and 1,25(OH)₂D₃ may suggest a role for 1,25(OH)₂D₃ in the regulation of hematopoiesis by IFN-γ-producing lymphocyte subsets, i.e., T and natural killer cells.

TNF and LT are two partially homologous factors originally described on the basis of their cytostatic effects on tumor cell lines, TNF as a tumor cytotoxic factor present in the serum of animals injected with endotoxin (24), and LT as a class of cytotoxic/cytostatic substances released by lymphocytes upon antigenic or mitogenic stimulation (25, 26). The cytotoxic/cytostatic effects of both cytokines are potentiated by IFN-γ (27, 28). Recently, purification and cloning of the genes for TNF and LT have allowed more detailed studies of the biology of these factors (29–33). The two cytokinins also appear to mediate regulatory effects on various cell types, e.g., they act as growth factors for fibroblasts (34, 35) and regulate proliferation, differentiation, and functions of myelomonocytic cells at all stages of differentiation (36). In particular, we (36, 37) showed that TNF and LT, at concentrations of the order of 10⁻¹⁵ M, induce monocytic differentiation of human myeloid cell lines. After 5 days of culture in the presence of TNF or LT, a significant proportion of the HL-60 myeloid cells express monocytic differentiation antigens and nonspecific esterase activity and become able to reduce NBT and to mediate low levels of Ab-CMC against tumor target cells. These markers of differentiation, however, are maximally induced when IFN-γ is present simultaneously with the cytotoxic, and the two classes of cytokines act synergistically to induce terminal differentiation. The appearance of monocytic antigens is accompanied by acquisition of the morphologic and functional properties of mature monocytic cells, such as chemiluminescence and phagocytosis, and by expression of Fc receptors for monomeric IgG. A decrease in cell proliferation accompanies induced differentiation.

The differentiation induced by TNF on myeloid cell lines closely resembles that induced by 1,25(OH)₂D₃. We report here that TNF and 1,25(OH)₂D₃ in combination induce differentiation of the HL-60, ML3, and U937 human cell lines. We show that the interaction of the two differentiation-inducing compounds is synergistic, especially when the compounds are used at suboptimal concentrations.

MATERIALS AND METHODS

Cell Lines. All cell lines were grown in RPMI 1640 medium (Flow Laboratories, Rockville, MD). The human promyelocytic cell line HL-60 was maintained in medium supplemented with 15% FBS (GIBCO, Grand Island, NY), the human promyelocytic ML3, the histiocytic U937, the B-lymphoblastoid RPMI 8866, the bladder carcinoma 5637, and the mouse mastocytoma line P815 were maintained in medium
supplemented with 10% FBS. All cell lines used in this study were mycoplasma-free.

Monoclonal Antibodies. Monoclonal antibodies B52.1, B33.1, and B147.2 were produced and characterized in our laboratory; OKM1, and 5E9 were produced from cell lines obtained from the American Tissue Culture Collection (Rockville, MD); 3G8 was kindly donated by Dr. J. Unkeless (Mount Sinai Hospital, New York) and Kufc79 was a gift of Dr. P. Gambel (Medical College of Virginia). Antibody OKM1 (IgG2b, 38) reacts with an epitope on the C3b receptor which is expressed on all differentiated neutrophilic granulocytes and monocytes and which, during differentiation, appears at the myelocytic and promyelocytic stages. Antibody B52.1 (IgM, 16, 39) reacts with all peripheral blood monocytes and all ANAE-positive cells in the bone marrow, and cross-compete for binding to monocytes with antibody Mo2 (40, 41). Antibody B33.1 (IgG2a, 42) reacts with class II HLA antigens. Antibody 5E9 (IgG1, 43) reacts with the transferrin receptor. Antibody 3G8 (IgG1, 44) reacts with a surface molecule with a molecular weight of 70,000, identified as the high-affinity FcR for monomeric IgG.

Induction of Myeloid Cell Lines. U937 were seeded at 1 x 10^5 and 0.75 x 10^5 cells/ml, respectively, in RPMI 1640 supplemented with 15% FBS. ML3 and U937 were seeded in 144-well, 150-µl round-bottom microtiter plates (10^4 cells/ml) for the indicated periods of time and pulsed for the last 6 h of culture with 1 µCi/well of ^3H]TdR (New England Nuclear); cells were collected on glass-fiber filters with an automated cell harvester (Skatron, Sterling, VA), and the cell-associated radioactivity was assayed by liquid scintillation.

RESULTS

Proliferation of Myeloid Cell Lines in the Presence of 1,25(OH)_(2)D_3 and TNF. ^3H]TdT uptake of HL-60 and ML3 cell lines on day 5 of culture, was inhibited by ≥10^(-9) M 1,25(OH)_(2)D_3 and by 100 U/ml TNF but not significantly by 100 U/ml rIFN-γ (Fig. 1). Maximal inhibition was obtained using 1,25(OH)_(2)D_3 and rTNF in combination. On day 5 of culture, in the presence of the various inducers, cell viability, as judged by vital dye exclusion, was always higher than 90% and the number of cells recovered was between 50 and 80% of that from control cultures in medium without inducers. The extent of inhibition of proliferation by 1,25(OH)_(2)D_3 and TNF was confirmed by autoradiography after ^3H]TdT pulse and by cell cycle analysis (flow cytometry) after propidium-iodide staining of DNA (not shown).

Surface Phenotype of Myeloid Cell Lines Induced by 1,25(OH)_(2)D_3 and rTNF. rTNF (100 U/ml) and 1,25(OH)_(2)D_3 (10^-8 or 10^-7 M) induced expression of the myelomonocytic differentiation antigen recognized by antibody OKM1 and of the monocye-specific antigen recognized by antibody B52.1 (Table 1). The proportion of HL-60 cells induced to express this antigen by the two compounds in combination was higher that induced by either compound alone. rIFN-γ, a poor inducer of the two differentiation antigens by itself, potentiates the induction by rTNF but not that by 1,25(OH)_(2)D_3. Two

![Fig. 1. Effect of different concentrations of 1,25(OH)_(2)D_3, rTNF, and/or rIFN-γ, on the ^3H]TdT uptake of HL-60 (A) and ML3 (B) cells. ^3H]TdT uptake was evaluated on day 5 from initiation of culture. The experiment is representative of three performed with analogous results and data are average of triplicate determinations. Cells were cultured in: culture medium (O); 100 U/ml rIFN-γ (•); 100 U/ml rTNF (O); 100 U/ml rIFN-γ and 100 U/ml rTNF (□).](https://cancerres.aacjournals.org)
Table 1: Effect of 1,25(OH)2D3, rTNF, and rIFN-γ on surface antigen expression by HL-60 cells.

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<th>Inducers</th>
<th>Antibody*</th>
<th>1,25(OH)2D3 (m)</th>
<th>rIFN-γ (U/ml)</th>
<th>rTNF (U/ml)</th>
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<th>SE9</th>
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<td>57.9 (173)</td>
<td>53.9 (137)</td>
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* Percentage of positive cells.

Fig. 2. Effect of 1,25(OH)2D3, rTNF, and rIFN-γ at various concentrations on expression by HL-60 cells of the antigens recognized by antibody OKM1 (A) and B52.1 (B), of the ability to mediate Ab-CMC (C), and of ANAE activity (D). Surface antigen expression was determined by indirect immunofluorescence, Ab-CMC in a 3-h 51Cr-release assay (effector-to-target cell ratio, 25:1), and ANAE by cytochemical staining on HL-60 cells cultured for 5 days in the presence of the indicated concentrations of 1,25(OH)2D3 and, in the absence of 1,25(OH)2D3, of: no other inducer (O); 2 U/ml rIFN-γ (γ); 10 U/ml rTNF (Δ); 50 U/ml rTNF (A); 100 U/ml rTNF (B); and 500 U/ml rIFN-γ (○).

The expression of transferrin receptor, recognized by antibody SE9, on HL-60 cells was decreased by rTNF and by 1,25(OH)2D3 or their combination, but not by rIFN-γ (Table 1). Analysis by two-color immunofluorescence (Fig. 3) showed that, after induction with both rTNF and 1,25(OH)2D3, the OKM1 and B52.1 differentiation antigens were expressed on the same subpopulation of HL-60 cells, different from that expressing the transferrin receptor. Simultaneous analysis of cell cycle (propidium iodide, DNA staining) and indirect immunofluorescence indicated that most of the cells expressing OKM1 and B52.1 antigens are in the G1 phase of the cell cycle, whereas the transferrin receptor is expressed predominantly on cycling cells in S- or G2 phase (not shown).

Expression of OKM1 and B52.1 differentiation antigens was also induced on ML3 cells by rTNF and 1,25(OH)2D3, alone and in combination; the proportion of cells induced to express the antigens was higher than in the HL-60 cell line (Table 2). HLA-DR antigen expression (antibody B33.1) on ML3 cells was induced by rIFN-γ, rTNF, and, in a higher proportion of cells, by the two factors together but not by 1,25(OH)2D3; induction of HLA-DR antigen expression by rIFN-γ or rTNF was partially prevented by 1,25(OH)2D3. Expression of the transferrin receptor on ML3 cells was partially inhibited by high concentrations of 1,25(OH)2D3, but an effect of rTNF was observed only in combination with 10−8 M 1,25(OH)2D3.

With U937 cells, expression of the OKM1 antigen induced by 1,25(OH)2D3 was potentiated when rIFN-γ or, more so, both rIFN-γ and rTNF were present but not when only rTNF was present (Table 3). In the absence of 1,25(OH)2D3, a combination of both rIFN-γ and rTNF was necessary to induce OKM1 antigen on a significant proportion of the cells. The B52.1 antigen was induced only by high concentrations of 1,25(OH)2D3 in combination with rIFN-γ or with a combination of rIFN-γ and rTNF. No significant changes in the expression of transferrin receptors on U937 cells was observed, except for a modest reduction when rIFN was added, alone or in combination with the other inducers.

Expression of FcR and Induction of Ab-CMC Activity. rIFN-γ induced high-affinity FcR for monomeric IgG on HL-60 cells and this effect was slightly potentiated by rTNF (Table 4). 1,25(OH)2D3 slightly decreased the expression of the receptor, but did not prevent its induction by rIFN-γ. On ML3 cells the high-affinity FcR was only modestly induced by 100 U/ml rIFN-γ, but more efficiently induced by rTNF or a combination of rIFN-γ and TNF (Table 4 and Fig. 4). 1,25(OH)2D3 also induced expression of the receptor on ML3 cells and potentiated its induction by rTNF. Like HL-60 cells, U937 cells were induced to express the high-affinity FcR by rIFN-γ and the induction was potentiated by rTNF. 1,25(OH)2D3 induced a modest increase of FcR on U937 cells; the highest expression of FcR was observed on cells induced with both 1,25(OH)2D3 and rIFN-γ. As shown in Fig. 4 for ML3 cells, the expression of the high-affinity FcR on the three cell lines, unlike that of the OKM1 and B52.1 antigens, was not restricted to a subpopulation of cells, but is expressed, to varying extents, on all cells in the cultures.

Ab-CMC activity in HL-60 cells was induced, in agreement with our previous results (37), by rIFN-γ and, synergistically, by a combination of rIFN-γ and rTNF (Fig. 5A). 1,25(OH)2D3 induced some cytotoxic activity and its effect was potentiated by both rIFN-γ and rTNF (Fig. 5B). Analysis by isobologram analysis (not shown) of the induction of Ab-CMC activity of HL-60 cells by combinations of different concentrations of rTNF and 1,25(OH)2D3 (Fig. 2B) demonstrates that the two compounds act synergistically in inducing Ab-CMC activity, whereas no synergistic effect is observed with rIFN-γ. Most Ab-CMC activity is induced in ML3 cells by rTNF, but not by rIFN-γ (Fig. 5C). 1,25(OH)2D3 does not induce Ab-CMC activity in ML3 cells but potentiates the induction by rTNF (Fig. 5D). In U937 cells rIFN-γ, rTNF, and 1,25(OH)2D3 are modest inducers of Ab-CMC (Fig. 5, E and F); strong cytotoxic activity is, however, mediated by U937 cells cultured in the presence of both 1,25(OH)2D3 and rIFN-γ.
of the two compounds (Fig. 6B). Dose response analysis of the effects of the combination of rTNF and 1,25(OH)₂D₃ on ANAE activity showed a clear synergistic effect (Fig. 2D). rIFN-γ, which has only a modest ability to induce ANAE activity in HL-60 cells, potentiated the effect of rTNF, but not that of 1,25(OH)₂D₃ (Figs. 2D and 6B).

**DISCUSSION**

Myeloid cell lines as well as fresh normal and leukemic myeloid cells are induced to differentiate along the monocytic lineage by IFN-γ (12, 16), TNF (36, 37), and 1,25(OH)₂D₃ (7, 9–11). On myeloid cell lines, IFN-γ alone is a relatively poor inducer of differentiation (13, 16), effectively increasing expression of only high-affinity FcR and class II HLA-DR antigens. However, IFN-γ synergizes with TNF or 1,25(OH)₂D₃ in inducing complete antigenic, morphological, and functional differentiation of myeloid cell lines (16, 20, 21, 36, 37). On fresh leukemic or normal bone marrow myeloid cells IFN-γ is an effective inducer of differentiation (12), possibly through induction of TNF (48) or 1,25(OH)₂D₃ (23) production by monocyte/macrophages contaminating the fresh cell suspension. Our data confirm that 1,25(OH)₂D₃ at concentrations of 10⁻⁹ to 10⁻⁷ M induces monocytic differentiation of the three human myeloid cell lines HL-60, ML3, and U937, and that rIFN-γ potentiates the effect of 1,25(OH)₂D₃. Moreover, we show that rTNF is much more active than rIFN-γ in potentiating the effect of 1,25(OH)₂D₃ and that the combination of the two inducers is synergistic in inducing expression of at least...
INDUCTION OF DIFFERENTIATION BY TNF AND 1,25(OH)2D3

No 1,25(OH)2D3

10^{-8}M 1,25(OH)2D3

rIFNγ

100 U/ml

rTNF

100 U/ml

rTNF

100 U/ml

rIFNγ

100 U/ml

Log fluorescence intensity

Fig. 4. Expression of high-affinity FcR for monomeric IgG on ML3 cells cultured for 5 days in the presence of the indicated combinations of 1,25(OH)2D3, rTNF, and rIFN-γ. Indirect immunofluorescence with murine monoclonal IgG2a B147.2 was used for detection of FcR. Histograms are as described in the legend of Fig. 2.

some of the differentiation markers analyzed. This synergistic effect is reminiscent of that described by Amento et al. (49), using PHA-CM and 1,25(OH)2D3 on U937 cells; no detectable levels of IFN activity were present in those supernatants, although a lymphokine that synergized with 1,25(OH)2D3 was present. PHA-CM does contain IFN as well as LT, a cytokine with differentiation-inducing activity identical to that of TNF (36, 37, 50).

At concentrations easily attained upon in vivo leukocyte stimulation, rTNF potentiates with variable potency the effect of 1,25(OH)2D3 in all three myeloid cell lines tested. Consistent with previous reports (20, 21), 1,25(OH)2D3 inhibits myeloid cell proliferation, and its antiproliferative effect is potentiated by IFN-γ and, as described here, by TNF. Several observations indicate that the decreased proliferative capability of the cells is a consequence of the differentiation induced rather than a direct toxic effect of either 1,25(OH)2D3 or TNF. For instance, induction of monocyte surface antigens and enzymatic and functional activities is observed on ML3 cells at concentrations of 1,25(OH)2D3 that do not significantly affect proliferation (10^{-8} M). The expression of the transferrin receptor recognized by antibody 5E9 and present on proliferating cells of most lineages is reduced concomitantly with a decrease in the proliferation of the cells. The two-color immunofluorescence experiments and the analysis of cell cycle and surface antigen expression demonstrate that two subpopulations of HL-60 cells are present in the cultures induced with the two compounds: one population stops proliferating, accumulates in G1, loses expression of the transferrin receptors, and expresses both the OKM1 and the B52.1 differentiation antigens; the other population continues to proliferate and to express transferrin receptors but does not acquire the markers of differentiated cells. The ratio between the two subpopulations depends on the concentration of the inducers; with the experimental conditions used in this study, differentiation of all cells and complete arrest of growth were not observed. We previously reported identical findings for differentiation of HL-60 cells induced by PHA-CM (39).

Induced expression by 1,25(OH)2D3 of all markers of differentiation tested, i.e., myelomonocytic surface antigens, ANAE activity, ability to reduce NBT, and to mediate Ab-CMC, is potentiated by rTNF. Unlike the 1,25(OH)2D3 mediated enhancement of la antigen expression reported for the murine WEHI-3 cell line (22), this compound prevented the induction of HLA-DR antigens by rIFN-γ and rTNF on ML3 cells. With this cell line, both 1,25(OH)2D3 and rTNF has a more pronounced differentiation-inducing effect, as indicated by the large proportion of cells induced to express differentiation markers and by the stronger induction of some of these markers (e.g., FcR for monomeric Ig) and of functions possibly dependent on them (Ab-CMC). By contrast, the expression of the high-affinity FcR for monomeric Ig and of functions possibly dependent on them (Ab-CMC). By contrast, the expression of the high-affinity FcR for monomeric Ig and of functions possibly dependent on them (Ab-CMC).
patients are of the CD4(+) helper T-cell subset, an efficient producer of LT (53) and TNF (54). A pathological role for the synergy of TNF or LT and 1,25(OH)2D3 in inducing hypercalcaemia could also be envisaged in situations of reactive activation of lymphocytes and macrophages such as in tuberculosis or sarcoidosis (55). Although the possible therapeutic application of TNF and 1,25(OH)2D3 is suggested by their synergistic induction of differentiation and growth arrest in leukemic cells, the fact that both compounds induce hypercalcaemia, and may act synergistically in doing so, should caution against their use until more studies are conducted. The model described here offers the opportunity to study in detail the mechanism(s) by which IFN-γ and TNF cooperate with other compounds to induce differentiation in myeloid cells.

ACKNOWLEDGMENTS

We thank J. Faust and P. Kmetz for assistance at the cytofluorometer, M. Hoffman for editing, and M. Kaplan for typing the manuscript.

REFERENCES


Fig. 6. Effect of 5 days culture in the presence of 1,25(OH)2D3, rTNF, and rIFN-γ, as indicated, on the ability of HL-60 cells to reduce NBT and to express ANAE activity.


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