Interferon-induced Differentiation of U937 Cells

COMPARISON WITH OTHER AGENTS THAT PROMOTE DIFFERENTIATION OF HUMAN MYELOID OR MONOCYTELIKE CELL LINES

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ABSTRACT Effects of human fibroblast (β) or leukocyte (α) interferon (IFN) on differentiations of a human histiocytic lymphoma-derived cell line (U937) or promyelocytic leukemia-derived cell line (HL-60) were studied. When cultured with β-IFN (400-1,000 U/ml), U937 cells showed gross morphologic and microscopic changes consisting of clumping, increased cytoplasmic-to-nuclear ratio, enhanced prominence of cytoplasmic granules, and membrane ruffling. After culture with β-IFN, the number of U937 cells reactive with B43.4.1 monoclonal antibody, which is specific for human monocytes, natural killer cells, and neutrophils, increased from <10% of U937 cells to 47%. β-IFN treatment also enhanced antibody-dependent cellular cytotoxicity against chicken erythrocytes by U937 cells. The same morphologic, phenotypic, and functional changes were also observed when U937 were treated with recombinant or natural α-IFN. The effects of α-IFN were totally abolished by anti-α-IFN serum. In contrast, HL-60, which differentiates toward cells of the monocyte lineage in response to phorbol 12-myristate 13-acetate (based on the above criteria), and toward granulocytes in response to dimethyl sulfoxide, did not differentiate when cultured with α- or β-IFN. No consistent relationship between induction of differentiation and changes in phospholipid methylation were observed.

INTRODUCTION

In addition to its antiviral properties, the effects of interferon (IFN)[1] on proliferation of normal or transformed cells and on the function of immunocompetent cells have been extensively studied (1). IFN, like a variety of other agents, is also reported to affect differentiation. For example, IFN may either block (2) or promote (3) dimethyl sulfoxide (DMSO)-stimulated hemoglobin production in Friend erythroleukemia cells, inhibit insulin-dependent differentiation of 3T3 cells into adipocytes (4), or enhance differentiation of mouse macrophages (5).

In human systems, the delay of maturation of monocytes into macrophages by IFN (6) and enhancement of pokeweed mitogen-induced B cell differentiation at low concentrations of IFN and inhibition at high concentration (7) have been reported. Here, we describe differentiation of a human monocyte-like neoplastic cell line after culture with fibroblast or leukocyte IFN and compare its effects with those of other agents known to influence differentiation.

METHODS

Cells. U937 (a monocytelike cell line derived from a patient with histiocytic lymphoma (8), HL-60 (9), and K562 (10) (human myeloid leukemia-derived cell lines) were maintained as suspension cultures in complete medium RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (Biofluids, Inc.), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin (NIH Media Unit), and 10 mM Hepes (Microbiological Associates, Walkersville, MD). Medium was changed on alternate days to maintain a cell density of $\sim 2 \times 10^5$/ml.

Peripheral blood mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (11) from heparinized blood obtained from healthy donors.

Reagents. Phorbol 12-myristate 13-acetate (PMA; Consolidated Midland, Brewster, NY) was stored in acetone at −20°C and diluted with complete medium when used. Final concentration of acetone was <0.1%. DMSO was purchased from Mallinckrodt, Inc. (St. Louis, MO). Fibroblast IFN, (β-IFN), purified to $\sim 3 \times 10^7$ U/mg protein, was obtained from HEM Laboratories (Rockville, MD). Purified leukocyte

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell cytotoxicity; CRBC, chicken erythrocytes; DMSO, dimethyl sulfoxide; IFN, interferon; PMA, phorbol 12-myristate 13-acetate.

IFN (IFLrA) was produced by recombinant DNA technology as described elsewhere (12). The specific activity of the homogenous IFLrA was $\sim 2 \times 10^8$ U/mg of protein. Naturally occurring leukocyte IFN (α-IFN) and sheep anti-α-IFN antibody were obtained from Interferon Science Inc. (New Brunswick, NJ). The specific activity of α-IFN was 1×10^8 U/mg of protein. Anti-human fibroblast IFN (13) was kindly provided by Dr. J. Y. Djeu of NIH. Anti-chicken erythrocytes (CRBC) rabbit IgG was purchased from Cappel Laboratories, Inc. (Cochranville, PA). Affinity-purified anti-mouse goat IgG was obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). A monoclonal antibody recognizing an antigen shared by human monocytes, NK cells, and neutrophils (B43.4.1) was kindly provided by G. Trinchieri of the Wistar Institute and produced and characterized, as described (14). Monoclonal mouse IgG_{2a} was donated by Dr. R. Asofsky of NIH. OKIa1 monoclonal antibody was purchased from Ortho Pharmaceutical Co. (Raritan, NJ). BRL.1 (anti-monocyte) and anti-Ia monoclonal antibodies were provided by BRL (Gaithersburg, MD).

Culture conditions. Cell line cells were cultured in 24-well tissue culture plates (Costar, 3524, Data Packaging, Cambridge, MA) at an initial concentration of $1-2 \times 10^5$/ml in a total volume of 2 ml with and without inducers. After several days of culture, cells were harvested with a rubber scraper and washed three times with complete medium. Cell counts and viability were determined visually with trypan blue.

Evaluation of cell morphology. 2×10^5 cells in 0.2 ml of complete medium were centrifuged for 7 min at 900 rpm onto microscope slides in a Cytospin centrifuge (Shandon Southern Instrument Inc., Sewickley, PA). Air-dried preparations were fixed for 10 min in methanol and stained for 20 min with Giemsa (Fisher Scientific Company, Fair Lawn, NJ) diluted 1:10 in phosphate-buffered distilled water (pH 7.4).

Rosette assays. Cell surface antigens were examined by an indirect rosette assay (15). 100 μl of cells ($2-3 \times 10^6$/ml) were incubated with an equal volume of monoclonal antibody or monoclonal IgG_{2a} protein (10 μg/ml) at room temperature for 30 min, washed three times, and resuspended in 100 μl of complete medium. 25 μl of a 1% solution of ox erythrocytes conjugated with affinity-purified anti-mouse rabbit IgG using $CrCl_3$ was added, centrifuged for 5 min at 500 rpm, and kept for 1 h at 4°C. The pellet was gently suspended and rosette-forming cells were counted in a hemocytometer. At least 200 cells were counted. Cells that bound three or more ox erythrocytes were defined as positive.

Cytotoxicity assay. Antibody-dependent cell cytotoxicity (ADCC) of cells was assayed by the method of Perlmann (16). In brief, 50 million CRBC were labeled with 150 μCi ^{51}Cr (New England Nuclear, Boston, MA) for 1 h at 37°C, then washed three times with complete medium. 10,000 labeled CRBC in 50 μl of medium, 50 μl anti-CRBC antiserum (4 μg/ml), and 100 μl of medium, with effector cells at concentrations giving three E:T ratios (10:1, 5:1; 1:1) were added in triplicate to individual wells of 96-well round-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT). The plates were incubated for 4 or 18 h. The supernatant from each well was carefully removed using the Titertek automatic harvesting system (Flow Laboratories, Inc., Rockville, MD) and counted in a gamma counter. Supernatants from microwells containing target cells and antibody without effector cells in 0.2 ml of complete medium served as controls for the base-line release of ^{51}Cr. The percentage of cytotoxicity was calculated as follows: % cytotoxicity = (cpm in experimental supernatant) − (cpm in control supernatant)/total cpm in 10^4 target cells × 100. In some experiments, antibody was replaced by complete medium to confirm that the reaction was mediated by antibody. Natural killer cell activity was measured with K562 as target cells as described elsewhere (17).

Phospholipid methylation assay. Phospholipid methylation was measured by the incorporation of [^{3}H]methionine into chloroform-methanol extractable lipids of intact cells (18). Briefly, cells were incubated at 37°C in 5% CO_2 at 5×10^6/ml in a final volume of 0.4 ml of RPMI 1640 with 2% fetal bovine serum and 400 pmol [methyl-^{3}H]methionine (sp act 80 Ci/mmol, New England Nuclear). The reaction was stopped by removing the medium and adding 0.5 ml of 10% cold trichloroacetic acid containing 10 mM methionine. The 27,000-*g* pellet was washed twice, then extracted with chloroform-methanol 2:1, and washed with methanol/0.1 M KCl (1:1). An aliquot of the chloroform phase was removed and counted in a β-counter. The remaining chloroform phase was dried over sodium sulfate, evaporated to dryness, and separated by thin-layer chromatography on silica gel G with a mobile phase of chloroform/methanol/acetic acid/water (50:25:7:3).

RESULTS

Morphology and growth patterns. When U937 cells were cultured in the presence of IFN (400–1,000 U/ml), DMSO (1% vol/vol), or PMA (6–30 nM), gross morphologic changes consisting of clumping of cells and adherence to the plastic surfaces were observed. These were similar to those changes observed after exposure of U937 cells to supernatants of mixed lymphocyte cultures (19). These effects were most prominent in the presence of PMA, where they were seen within 1 d of culture; 90% of U937 cells formed clumps after 6–7 d of culture with PMA (30 nM). The effects of IFN or DMSO were more subtle and variable during the initial days of culture, but after 6–7 d of culture, 30–50% of β-IFN (1,000 U/ml)-treated cells and 40–50% of DMSO-treated cells showed clumping.

Treatment with each of these agents resulted in slower growth of U937 cells (Fig. 1). In the presence of inducers, cell proliferation was inhibited 76% by PMA and 42% by IFN after 7 d of culture. Cultured U937 cells always retained >90% viability.

When cells were examined microscopically, differences were also apparent within 1 d in PMA-treated cultures. IFN-treated cells exhibited identical changes but were maximal on day 7. As shown in Fig. 2, differentiated cells were larger and were characterized by increased cytoplasmic/nuclear ratio, paler cytoplasm, more prominent granules, a greater degree of vacuolization in the cytoplasm, and ruffled plasma membranes. The number of cells in mitosis decreased (not shown) and occasional multinucleate giant cells were encountered (Fig. 2, *C* and *D*), which were rarely observed in untreated cultures.

Appearance of new cell antigens on differentiated

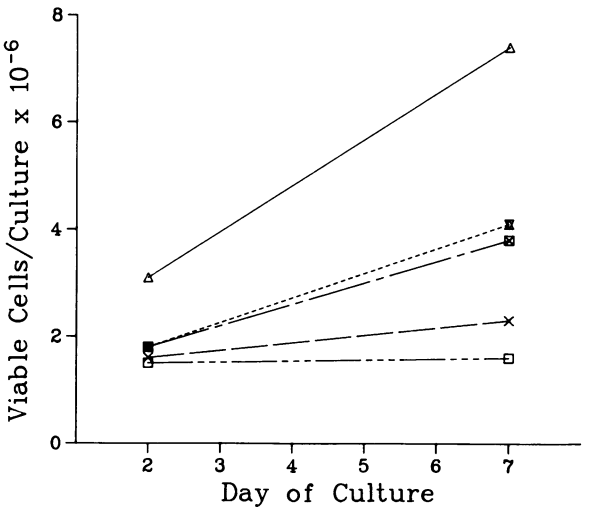

FIGURE 1 Growth of U937 cells in culture with or without inducers. U937 were cultured at an initial concentration of 2×10^5/ml in either PMA (30 nM; □), DMSO 1% vol/vol (×), β-IFN (400 U/ml; ××), IFNLrA$_2$ (400 U/ml; ⊠), medium (△). Data are presented as the mean of four different experiments.

cells. Using indirect rosette techniques, we could not detect the presence of monocyte-associate antigens on U937 cells, as measured by reactivity with monoclonal antibodies against B43.4.1, BRL.1, or Ia-like antigens. A small population of U937 cells (<10%) reacted with B43.4.1 (Table I). When treated with either β-IFN or recombinant α-IFN (400 U), a portion of cells reacted with B43.4.1, similar to that seen after DMSO or PMA treatment (Table I). Ia antigens or other monocyte antigens remained undetectable in treated cells.

These changes were observed within 1 d with each of the inducers (data not shown) and continued throughout the culture period. In addition, both types of IFN tested enhanced reactivity with B43.4.1, with the relative proportion of rosette-forming cells increasing each day to a maximum of 47% (Table I). Similar experiments were performed in parallel using HL-60, since differentiation of this line towards macrophages by PMA or towards myeloid cells by a variety of other agents has been described. β-IFN, in doses up to 400 U/ml, induced no expression of B43.4.1 in this cell line, whereas PMA induced B43.4.1 antigen expression (14% at day 7).

Functional analysis of U937 cells. U937 cultured with the various inducers were examined at days 1, 2, 4, and 7 for their cytotoxic activities against antibody-coated CRBC, a function associated with monocytes (Fig. 3). PMA differentiated U937 cells into cytotoxic effector cells after 1 d of culture, and cells expressed maximum cytotoxic activity as early as day 4. U937 cells stimulated with DMSO expressed B43.4.1 antigen and morphological changes as described above, but demonstrated low cytotoxicity against CRBC. Cells cultured with 400 U β-IFN expressed cytotoxicity at day 1, which increased over the course of 7 d.

To substantiate that the effect of IFN was in fact not due to other contaminant proteins, we compared β-IFN and homogenous IFLrA with respect to their ability to induce ADCC activity by U937 cells (Fig. 4). Both had almost the same capacity to differentiate U937 cells into ADCC effector cells. In one experiment, limited by the quantity of antiserum available to us, the effect of β-IFN was abolished in a dose-dependent manner by its pretreatment with anti-human β-IFN (data not shown). In other experiments, we used α-IFN (natural), because of the availability of specific antiserum in large quantities. α-IFN had almost the same capacity to promote the expression of CRBC-ADCC activity by U937 at 400–1,000 U/ml as other interferon preparations tested. Representative data of three different experiments of U937 cultured for 6 d with IFN are shown in Table II. Anti-α-IFN or sheep serum had slight growth inhibitory activities when tested against U937, but they had no activity in promoting differentiation of U937 as examined by CRBC-ADCC. Expression of CRBC-ADCC induced by β-IFN was only partially inhibited by anti-α-IFN to an extent that was also observed with control sheep serum. In contrast, expression of CRBC-ADCC activity and growth-inhibitory activity induced by α-IFN were completely abolished by treatment with specific antiserum (1:100) with less effect at decreasing concentrations.

Activated monocytes as well as peripheral blood mononuclear cells are known to be cytotoxic to erythrocytes (20). Therefore, the specificity of the ADCC activity by U937 cells cultured with or without inducers was examined. In a 4-h ^{51}Cr-release assay, cells cultured with β-IFN or PMA did not express cytotoxic activity without antibody (Fig. 5).

Spontaneous cytotoxic activity against K562 was examined. We observed no activity by U937 cells and HL-60 cells after cultivation for 4 or 7 d with inducers in a 4-h assay. In an 18-h ^{51}Cr-release assay, U937 cells cultured with PMA did express a small amount of cytotoxic activity against K562 cells (8.6% ^{51}Cr release at an E/T ratio of 100:1), but cells cultured with other inducers did not express cytotoxic activity (data not shown).

Regarding HL-60, cells cultured with PMA (30 nM) expressed low ADCC activity against CRBC (4.5%) in a 4-h ^{51}Cr-release assay, but cells cultured with DMSO (1% vol/vol) or β-IFN (400 U/ml) did not express ADCC activity even after 7 d culture. HL-60 cultured for 7 d did not express spontaneous cytotoxic activity

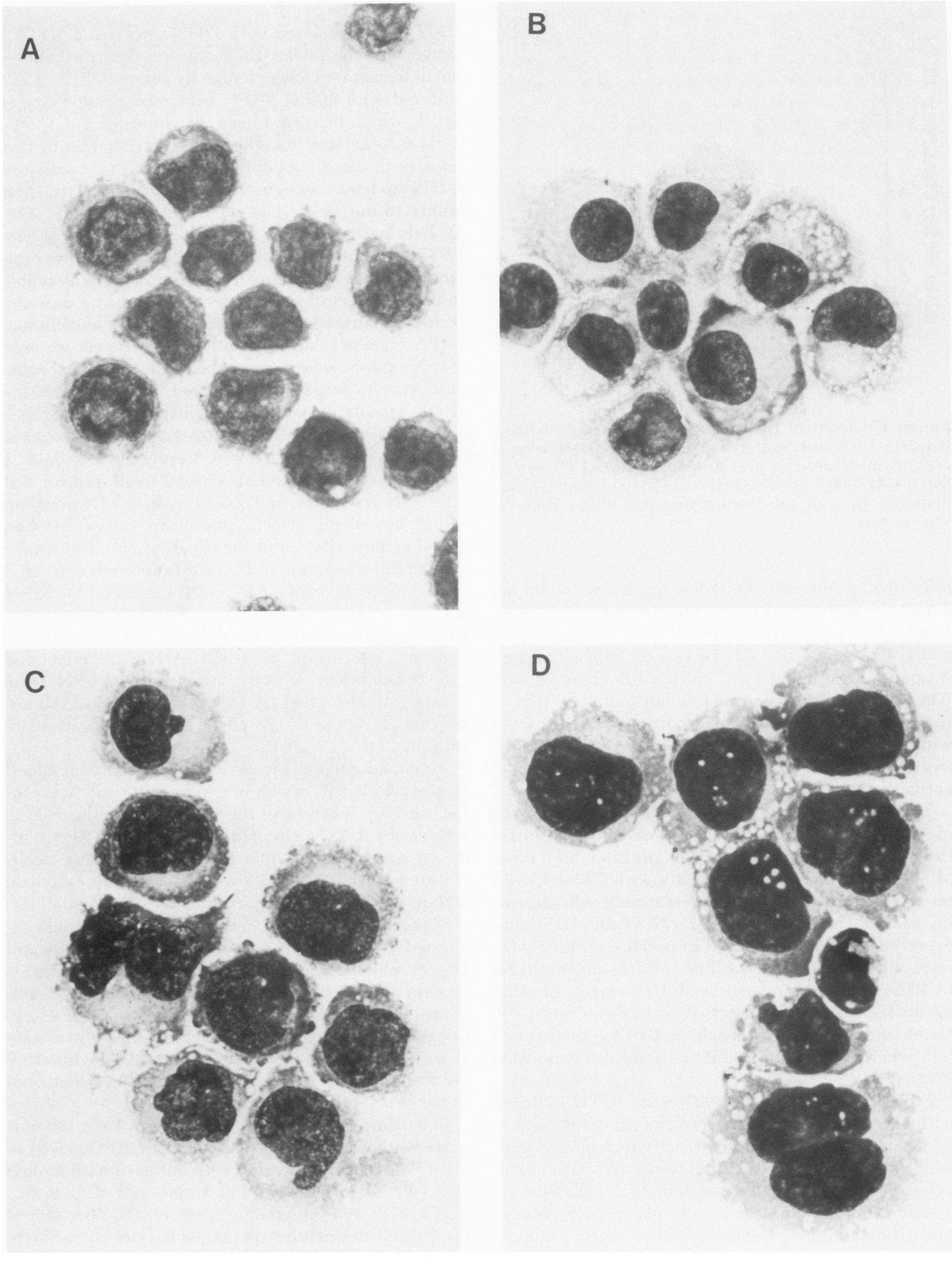
A
B
C
D

TABLE I

Appearance of B43.4.1 Reactive Cells after Cultivation of U937 Cells with Various Stimulators (Percentage of Positive Cells per Culture)

Days	Medium	DMSO	PMA	β-IFN	IFLrA
		1% vol/vol	*30 nM*	*400 U/ml*	*400 U/ml*
2	8	36	55	34	29
7	10	40	71	47	44

when cultured with β-IFN, DMSO, or PMA, even in an 18-h ^{51}Cr assay.

Effect of inducers of differentiation on phospholipid methylation. Recent studies have reported a decrease in phospholipid methylation in certain mouse (21) or human cells (22) stimulated to differentiate. Some IFN preparations also inhibit phospholipid methylation by peripheral blood mononuclear cells (23). We examined the effect of IFLrA on incorporation of the labeled methyl group of [^{3}H]methionine into phospholipids of U937 or HL-60 cells, taking each as an example of an IFN-responsive or nonresponsive line, respectively. As shown in a representative experiment (Table III), IFLrA caused in each a comparable decrease in phospholipid methylation, compared with control cells, in the two lines, despite the inability to induce differentiation in HL-60 cells. Similar results were seen when cells were stimulated with DMSO. PMA treatment, which is able to induce differentiation of both lines towards monocytes, caused a minimal increase in phospholipid methylation in each cell type in the experiment described. In five other experiments, not described here, PMA treatment in doses capable of inducing U937 differentiation, resulted in no significant change in phospholipid methylation by U937 cells.

DISCUSSION

We report that IFN, both leukocyte and fibroblast, differentiated U937 cells into cells with certain characteristics of monocyte-macrophages. Differentiation events were documented in morphologic, phenotypic, and a functional (ADCC) change. Induction of differentiation by IFN-containing preparations could not readily be ascribed to contamination by other substances, since highly purified recombinant IFN (IFLrA) was also used. In addition, the effect of α- or β-IFN was reversed by its pretreatment with specific anti-

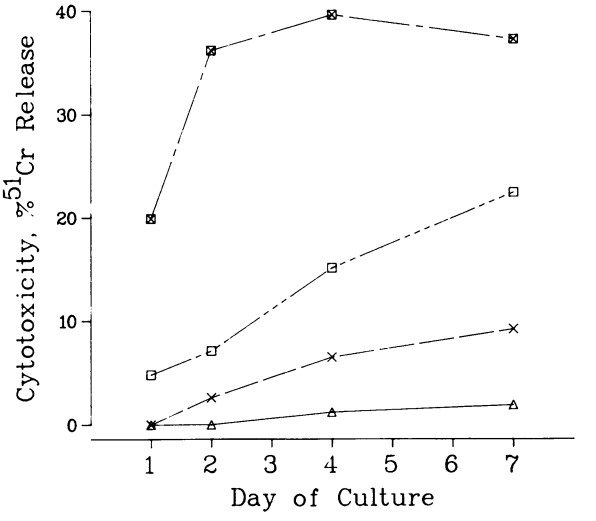

FIGURE 3 ADCC activity of U937 cultured with or without inducers. PMA 30 nM (⊠), DMSO 1% vol/vol (×), β-IFN (400 U/ml; □), medium (△). Conditions of culture are the same in Fig. 1. E:T ratio was 10:1. Representative data from one of three separate experiments are shown depicting the mean of cytotoxicity of three replicate samples, the standard error of which was always <5%.

IFN heteroantiserum. U937 cells previously were found differentiated by supernatants of mixed-lymphocyte culture (19), known to contain immune (γ) IFN. It is likely that all three classes of IFN have this property, and we are presently evaluating the direct effect of γ-IFN in our system.

No effect of IFN was seen on myeloid cell line (HL-60) differentiation, despite the line's demonstrated ability to differentiate towards monocytes, judged by the same criteria employed to detect U937 differentiation. Recently we have observed induction of 2'-5' oligo(A) synthetase (24), an enzyme known to be induced by IFN treatment, in extracts of IFN-treated HL-60 or U937 cells (A. Schmidt. Manuscript submitted for publication). This indicates that IFN can bind to and act on HL-60 cells, although, for as yet undetermined reasons, differentiation is not induced.

Our findings raise the possibility that some of the in vivo effects of IFN administration, including its antineoplastic properties, might be attributable to its influences on differentiation. For example, a report of the disappearance of symptoms after β-IFN treatment of a patient with Hodgkin's disease (25), which is assumed to be of histocytic-monocytic origin, might be attributed to these effects.

FIGURE 2 Morphology of U937 cells after 6 d in culture with (*A*) medium alone; (*B*) 6 nM PMA, day 1; (*C*) α-IFN (1,000 U/ml); (*D*) β-IFN (1,000 U/ml), each × 1070.

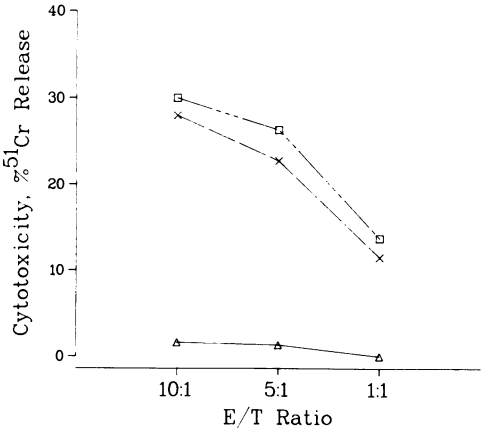

FIGURE 4 Comparison of β-IFN and IFLrA with respect to induction of U937 ADCC activity. β-IFN (400 U/ml; □), IFLrA (400 U/ml; ×), medium (△). Data shown represent one experiment of two performed with similar results.

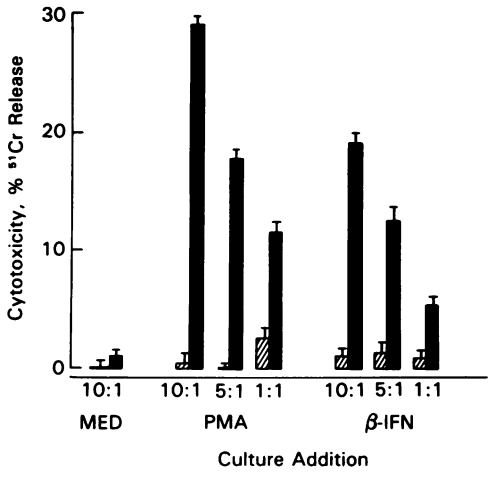

FIGURE 5 ADCC by U937 cells induced to differentiate by β-IFN or PMA. U937 cells were treated for 7 d with β-IFN (400 U/ml) or PMA (30 nM) as described in Methods. Cytotoxicity was tested at three E/T ratios (10:1, 5:1, 1:1) for PMA or β-IFN treated target effector cultures and only one E/T ratio (10:1) for control cultures. ^{51}Cr-labeled CRBC targets were tested in the presence (■) or absence (▨) of anti-CRBC antibody. MED, medium.

Effects of PMA on differentiation of other cells have been observed and like interferon, have been found to vary according to the cell type used. For example, conversion of 3T3 cells to adipocytes, with and without insulin, has been reported to be inhibited by PMA (26). Spontaneous differentiation of Friend erythroleukemia cells was also inhibited by PMA (27). Differentiation of myeloid leukemia cells was induced by PMA in human (28) and mouse (29) cells. These findings seem to be correlated with recent findings that showed that PMA induces myeloid progenitor cells to form colonies of the monocyte/macrophage type, and, conversely, that it inhibited the formation of colonies by early erythroid progenitor cells (30).

The phenotypic changes induced by IFN, as well as PMA or DMSO in U937 cells, might be considered to represent activation, as has been observed for in-

TABLE II
Effect of Specific Antibody on α-IFN-induced Differentiation of U937 Cells (Day 6)

	CRBC-ADCC*	Cell numbers recovered
		%
Medium	−0.5±0.9	100
β-IFN	27.6±3.2	19
+Ab (1:100)	20.0±1.4	19
+Ab (1:1,000)	27.2±1.1	21
+Ab (1:10,000)	21.1±2.7	17
+Sheep‡ (1:100)	16.6±1.6	18
α-IFN	30.1±2.5	22
+Ab (1:100)	0.5±0.8	57
+Ab (1:1,000)	4.7±0.8	40
+Ab (1:10,000)	9.2±0.9	27
+Sheep (1:100)	14.5±1.1	15
Ab (1:100)	0.6±0.6	53
Sheep (1:100)	0.5±0.7	53

α- or β-IFN were treated with either anti α-IFN or normal sheep serum for 30 min at room temperature. These treated or untreated preparations of IFN were added to the culture based on the initial IFN concentration of 1,000 U/ml.

* E:T ratio 10:1, mean±SE of triplicate assays.

‡ Normal sheep serum.

TABLE III
Effect of IFN, PMA, and DMSO on Phospholipid Methylation in U937 and HL-60

Cell	Stimulus	Methyl group incorporation into phospholipids
		pmol/10⁶ cells/h
U937	Medium	3.77±0.39*
U937	DMSO (*1%*)	3.31±0.18
U937	PMA (*6 nM*)	4.11±0.09
U937	IFLrA (*1,000 U/ml*)	3.15±0.02
HL-60	Medium	2.52±0.07
HL-60	DMSO (*1%*)	2.37±0.08
HL-60	PMA (*6 nM*)	2.62±0.01
HL-60	IFLrA (*1,000 U/ml*)	2.38±0.01

* ±SD of duplicate.

duction of chemotactic peptide receptors on U937 treated with lymphokine (31). Possibly, the phenomena of activation and differentiation are related in U937 cells. In the examples cited here, U937 cells acquired new antigenic characteristics as well as new functional activities characteristic of mature monocytes.

The mechanisms of differentiation and enhancing effects of IFN or other inducers remain unclear. Several investigators have reported a decrease of phospholipid methyltransferase activity in mouse (21) (M1 cells) and in human (22) myeloid leukemia cells (HL-60) early in differentiation. This enzyme is thought to be activated by various biological stimuli (32). We could not observe changes in the functional activity of methyltransferase by any of three inducers that correlate with the ability of cell-line cells to undergo differentiation (or not) in response to IFN, as well as the other agents, in either U937 or HL-60. The discrepancy between our data using PMA and those of others may be ascribed to the different (i.e., lower) doses we used, since in our hands doses above 100 nM were often toxic, and to possible differences in the concentration of methionine in the media used, since we have shown the dependence of effects on lipid methylation on methionine concentration (33). However, at concentrations of inducers (notably IFN, but also TPA) where we could observe similar morphological and functional changes, indicative of differentiation, methyltransferase activity remained almost the same. These findings make less likely the hypothesis that early decreases in phospholipid methylation are associated with commitment to differentiation.

The system described here, where biologically active agents with a potentially wide variety of disparate biochemical effects induced differentiation along different pathways, represents a unique opportunity to investigate the control mechanisms governing these events. We are presently investigating methylation of other cell constituents aside from phospholipids, other aspects of phospholipid metabolism, and pathways involved in regulation of RNA synthesis that could potentially be altered by IFN and other agents which induce differentiation of monocytelike cells.

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