Effect of Endogenous and Exogenous Interferons on the Differentiation of Human Monocyte Cell Line U937

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INTRODUCTION

In the past few years, evidence has accumulated regarding the possible involvement of IFN3 in cellular regulation, including growth and differentiation [reviewed by Taylor et al. (1)]. Recently, endogenous IFN production (2) has been detected during cell differentiation and the induction of hematopoietic cell differentiation associated with a marked enhancement of IFN-induced 2-5 A synthetase has been reported (3–6). Thus, it has been suggested that interferon plays a general role in growth and differentiation via 2-5 A synthetase induction.

The hematopoietic system represents a unique model for investigation of the biochemical events associated with cellular differentiation. The data concerning the possible role of interferon and 2-5 A synthetase in the process of hematopoietic cell differentiation afford evidence about different actions of the various IFNs on different aspects of cellular differentiation (7–9). Thus, some studies have shown a role of endogenous IFN in inducing growth arrest, a phenomenon constantly coupled with cellular differentiation/maturation (10); other studies, however, provide evidence in favor of a role of IFN-ß as a promoter of growth and differentiation of B lymphocytes: in fact, IFN-ß is capable of inducing synthesis of immunoglobulin by Epstein-Barr virus-transformed human B-cells (11, 12). Some studies suggest that IFN-γ (13–14) or (2′5′) oligoadenylates (15) might help to induce differentiation of the human promonocytic U937 cell line derived from pleural fluid of a patient with diffuse histiocytic lymphoma (16). hydroxy-vit D3, a supposedly physiological inducer, induced complete differentiation of the U937 cells in mature monocytes (17). We have previously shown that this induction of U937 differentiation was associated with a production of interferon in the medium (10 units/ml) and a marked increase in 2-5 A synthetase, which was essentially dependent on an endogenous production of interferon since the enzyme activity was inhibited by anti-IFN antibodies (18). These results were in agreement with a previous study by Yarden et al. (19) showing that the induction of 2-5 A synthetase observed in U937 cells induced to differentiate by phorbol esters mainly results from the autogenous production of IFN-ß.

We decided to reinvestigate these conflicting results by studying the effect of endogenous and exogenous IFN-α, -ß, and -γ on the expressions of 12 differentiation markers and the induction of the major histocompatibility complex during the differentiation of U937 cells.

MATERIALS AND METHODS

Cells and Cell Culture. The human histiocytic lymphoma cell line U937 (16) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Boehringer, Mannheim, Germany). All cells were inoculated at 1 × 10⁶ cells/ml and grew exponentially for 3–4 days. The inducer hydroxy-vit D3 was dissolved in absolute ethanol and added to the cultures at a final concentration of 500 nm. hydroxy-vit D3 reduced the rate of cell proliferation. When the U937 cells were induced with hydroxy-vit D3 on the fourth day of culture, the cells were diluted 2-fold with fresh medium and fresh hydroxy-vit D3 was added in the culture medium to maintain the initial concentration. Preliminary dose-response experiments showed that hydroxy-vit D3 at the doses used is not cytotoxic (cell viability > 95% until the sixth day of culture).

Assay of Properties of Differentiated Cells. To evaluate the differentiation of U937 cells, several tests were performed. Cells with C3 and Fc receptors were detected according to the method of Lotem and Sachs (20) by measuring rosette formation with sheep erythrocytes coated with rabbit anti-sheep erythrocyte antibody and mouse complement or IgG.

Reduction of NBT was assayed using a kit from Sigma (St. Louis, MO) according to the conditions recommended by the manufacturer.

ABSTRACT

The effect of interferons (IFNs) on the differentiation of hematopoietic cells was examined with the human monocytic cell line U937. The differentiation of U937 was induced by hydroxyvitamin D3 and was evaluated through the study of specific markers.

The induction of the U937 differentiation was associated with a production of IFN and with a marked increase in (2′5′) oligoadenylate synthetase. Addition of anti-IFN-α/ß antibodies inhibited the enhancement of (2′5′) oligoadenylate synthetase and reduced the inhibitory effect of hydroxyvitamin D3 on cell growth. Nevertheless, neutralization of endogenous IFN excreted during U937 cell maturation did not modify the expression of the differentiation markers examined. Exogenous natural IFN-α, IFN-ß, or recombinant (r) IFN-γ, when added to the culture medium, did not promote a "global" U937 differentiation. Most of the differentiation markers, except for reduction of nitroblue-tetrazolium, were not induced by IFN-α or -ß. However, rIFN-γ was able to induce the appearance of several monocytic membrane markers at an extent comparable or slightly inferior to that elicited by hydroxyvitamin D3. Different effects on the expression of HLA antigens were obtained with comparable or slightly inferior to that elicited by hydroxyvitamin D3. This effect has been associated with the induction of the U937 differentiation was associated with the production of interferon in the medium (10 units/ml) and a marked increase in 2-5 A synthetase, which was essentially dependent on an endogenous production of interferon since the enzyme activity was inhibited by anti-IFN antibodies (18). These results were in agreement with a previous study by Yarden et al. (19) showing that the induction of 2-5 A synthetase observed in U937 cells induced to differentiate by phorbol esters mainly results from the autogenous production of IFN-ß.

Received 3/17/87; revised 9/14/87; accepted 9/30/87.

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1 This research was supported in part by grants from INSERM (U.245, U.91), Centre National de la Recherche Scientifique (U.A.C. 113), Fondation pour la Recherche Médicale, and Associazione Italiana per la Ricerca sul Cancro.

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3 The abbreviations used are: IFN, interferon; 2-5 A synthetase, (2′5′) oligoadenylate synthetase; TPA, 12-O-tetradecanoylphorbol-13-acetate; hydroxy-vit D3, 1α,25 dihydroxyvitamin D3; NBT, nitroblue tetrazolium; rIFN-γ, recombinant γ interferon; FMLP, formyl-methionyl-leucyl-phenylalanine; PHA-LCM, phytohemagglutinin-stimulated leukocyte conditioned medium; poly(I)-poly(C), co-polymer of polyinosinic and polycytidylic acid; HL60, human promyelocytic cell line; TCA, trichloroacetic acid.
The percentage of cells containing intracellular blue-black formazan deposits was determined.

α-Naphthyl acetate esterase activity was examined in cytotoxic freezefracture using a Sigma kit. Binding of monoclonal antibodies to the cell surface antigens was determined by both indirect immunofluorescence and measuring the binding of a second [125I]-labeled antimonunote antibody. The monoclonal antibody MO2 was obtained from Coultertronics France. OKM1 and OKM5 were obtained from Ortho Lab., MAS 072 from Sera-Lab (Cambridge, United Kingdom), TEC MG1, NK1, and M1 from Technogenetics (Milano, Italy), and Leu M4 from Becton-Dickinson (USA). MO2 was a monoclonal antibody, entirely restricted to the monocyte macrophage series. It recognizes a true monocyte antigen because it was resynthesized after protease treatment of monocytes (21). OKM1, TEC M1, and MAS 072 were distinct antigens, specific for monocyte-macrophages (22, 23). OKM5 monoclonal antibody recognizes an antigen present both on mature monocytes and platelets, TEC MG1 monoclonal antibody specifically reacts with an antigen present on the surface of both granulocytic and monocytic cells.

Monoclonal antibodies which specifically recognize HLA classes I and II antigens were obtained from Technogenetics (Milano, Italy). These antibodies were TEC HLA A, B, C; TEC HLA-DR; TEC HLA-D1; TEC anti β2-microglobulin, whose specificity was previously reported (24-26). The secondary antibodies [goat anti-mouse immunoglobulins F(ab')2 fragment] conjugated with fluorescein isothiocyanate were purchased from Cappel Laboratories, Inc., West Chester, PA.

For indirect fluorescence the following procedure was used. The cells were first washed 3 x in Hanks' saline solution and then incubated 60 min at 4°C with 100 μl of an optimal dilution (in Hanks' saline solution containing 1 mg/ml bovine serum albumin of each monoclonal antibody. The cells, washed at 4°C in Hanks' saline solution, were incubated for 60 min at 4°C with fluorescein isothiocyanate-labeled F(ab')2 fragments of immunoabsorbent-purified sheep antibodies against mouse IgGs. After 3 additional washes, the cells were mounted on slides in 50% glycerol in phosphate-buffered saline.

The proportion of stained cells was scored in incident light with a Leitz standard universal fluorescent microscope equipped with a set of filters for narrow fluorescence. For the quantitative assay of monoclonal antibodies binding to the cells, the cells were first incubated as for indirect immunofluorescence. After 3 washes with cold Hanks' saline solution, cells were incubated for 60 min at 4°C with 0.2 ml of [125I]-labeled F(ab')2 sheep anti-mouse immunoglobulins appropriately diluted (50 ng/ml). At the end of the incubation, the cells were layered on a cushion of dibutyl phthalate oil (density, 1.025) and then centrifuged at 10,000 x g for 2 min. The supernatant and most of the oil were removed by aspiration, and the radioactivity in the cell pellet was measured in a gamma counter. In order to measure the "nonspecific" binding of [125I]-labeled F(ab')2 sheep anti-mouse immunoglobulins to U937 cells, monoclonal antibodies were replaced by mouse myeloma protein TEPC 183 (IgM-K). Lysosome was assayed turbidimetrically (27) in cells lysed with 0.5% Triton X-100 (Sigma) using egg-white lysozyme (Sigma) as standard. Results are expressed in units per microgram of egg lysozyme.

Generation of superoxide was measured by the ability of this anion to reduce ferricytochrome C (28). The stimulus for generation was provided by TPA. One ml of Hanks' balanced salt solution (pH 7.40) containing 5 x 107 cells, 0.12 mM ferricytochrome C (Sigma) and, where appropriate, 100 ng freshly diluted TPA with or without 60 μg superoxide dismutase (Sigma), was incubated for 60 min at 37°C. The reaction was stopped by submerging the tubes in an ice bath. Cells were pelleted by centrifugation at 4°C and 500 x g for 5 min and the absorbance for the supernatant was then determined at 550 nm in a Gilford 240 spectrophotometer. The quantity of superoxide generated was expressed as the A/60 min x 5 x 107 cells which is inhibited by 60 μg superoxide dismutase.

Assay for FMLP Receptors. Two million U937 cells were incubated in 15 nm [3H]FMLP (New England Nuclear, Boston, MA) in a total volume of 0.2 ml in the presence or absence of 10 μM unlabeled FMLP (Sigma). After 3 h at 4°C, the cell suspensions were rapidly filtered onto glass fiber discs (Whatman GF/C; Whatman, Inc., Clifton, NJ) and washed with 30 ml of 4°C phosphate-buffered saline. Radioactivity on the discs was measured by liquid scintillation spectroscopy. Specific binding was defined as the total amount of labeled FMLP bound minus the amount bound in the presence of an excess of unlabeled cold FMLP. Specific binding measured as cpm was converted to sites per cell.

Assay of 2-5 A Synthetase Activity. The procedure used has already been described (29). Briefly, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM magnesium acetate, 10% (v/v) glycerol, and 0.5% (v/v) of NP40. The lysate was centrifuged at 10,000 x g for 10 min. The supernatant was added to a suspension of poly(I)-poly(C) agarose in the same buffer. After 10 min of enzyme binding to the poly(I) -poly(C) ligand, the supernatant was discarded by centrifugation. The poly(I) -poly(C) agarose beads were washed twice with the same buffer.

To the pellet of poly(I)-poly(C) agarose, 10 μl of an incubation mixture were added to make final concentrations, in 25 μl, of 3 mM [α-32P]ATP (0.15 μCi/incubation), 20 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.1 mg/ml creatine kinase and 6 mM phosphocreatine. The mixture was incubated at 37°C for 2 h. The reaction products were analyzed by thin-layer chromatography on polyethyleneimine-cellulose; 3 μl of each mixture was spotted and the chromatogram developed with 2 M Tris-HCl, pH 8.6.

Spots corresponding to ATP and (2'5') oligoadenylenates located by autoradiography were cut and their radioactivity was determined by Cerenkov counting. One unit of 2-5 A synthetase was defined as the amount incorporating 1 nmol AMP residues into 2'5' oligoadenylenates/min at 37°C.

Protein concentrations were determined according to the method of Spector (30).

[3H]Thymidine Incorporation. DNA synthesis was measured as [3H]-thymidine incorporation into acid-precipitable materials. Two μCi of [methyl-3H]thymidine (40 Ci/mmol; New England Nuclear) were added to U937 cells grown in liquid suspension for 2 h at 37°C. The cells, washed 3 times in cold phosphate-buffered saline, were lysed by incubation for 10 min at 37°C in 0.1% sodium dodecyl sulfate (Bio-Rad Laboratories). One hundred μl 0.1% sodium dodecyl sulfate for 1 x 106 cells were added and to this an identical volume of cold 10% TCA was added. After 10 min at 4°C, the TCA precipitates were collected on 2.4 cm Whatman GF-C filters and washed 3 times with 5 ml cold 10% TCA. The filters were then dried, treated for 10 min at 37°C with 0.5 ml NCS solubilizer (Amersham/Searle), and counted in a PPO-POPOP-toluene scintillation mixture.

IFNs and Anti-IFN Antibodies. IFN-α was purchased from Institut Pasteur Production, Rueil Malmaison, Paris, France, IFN-β was donated by UPISA Laboratoire, France, and recombinant IFN-γ was a gift from Biogen. Their respective specific activities were 106, 107, and 108 units/mg protein. Anti-IFN-α was kindly provided by Dr. K. Berg, Denmark, and anti-IFN-β by Dr. A. Billiau, Belgium. The recombinant IFN-7, incubated with test supernatants, and subsequently washed, and counted in a gamma scintillation counter, is proportional to the amount incorporating 1 μmol AMP residues into 2'5' oligoadenylenates/min at 37°C.

Preliminary dose-response experiments showed that IFNs at the doses used (10-500 units/ml) were noncytotoxic for U937 cells; at higher concentrations (1000 or more units/ml) all the types of IFN (α, β, or γ) were significantly cytotoxic.

Interferon-γ Assay. IFN-γ was measured by a radioimmunoassay (IMRX; Centocor, Inc., Malvern, PA) based upon the use of polystyrene beads coated with a mouse monoclonal antibody specific for human IFN-γ, incubated with test supernatants, and subsequently washed, and of a second monoclonal antibody to human IFN-γ labeled with 125I that reacts with IFN-γ bound to the beads. The radioactivity bound to the beads, quantified in a gamma scintillation counter, is proportional to the concentration of IFN-γ in the specimen, within the working range of the assay. Results were expressed in units calculated using a preparation of recombinant IFN-γ as standard reference.

RESULTS

Effect of Anti-α and -β Interferon Antibodies on the Differentiation of U937 Cells Induced by hydroxy-vit D3. Cellular differentiation is characterized by 2 related phenomena: (a) progres-
sive and coordinate acquisition of a panel of specific markers; 
(b) progressive loss of cellular growth capacities. Since inter-
feron is produced during differentiation of U937 induced by 
hydroxy-vit D3 (18), the question raised was on the role that interferon may play in inducing the process of cellular differ-
entiation. IFN-γ was not detectable by a very sensitive radio-
immunoassay in the culture medium of control as well as of 
hydroxy-vit D3 treated U937 cells. Thus, we tested whether the addition of both hydroxy-vit D3 and IFN antibodies could 
modify the induction of differentiation of U937 cells. In order 
to evaluate the differentiation of U937 cells, several differentia-
tion markers were investigated: α-naphthyl acetate esterase 
activity, NBT reduction, lysozyme activity, FMLP receptors, 
and M02, OKM5, M1 and MG1 membrane antigens recogn-
ized by monoclonal antibodies. These markers are expressed 
little or not at all in undifferentiated promonocytic U937 cells 
but are highly expressed in mature monocytes. We have previ-
ously shown that hydroxy-vit D3 induced both complete differ-
entiation of U937 cells into mature monocyte and a very marked 
increase in 2-5 A synthetase (18). Thus, 2-5 A synthetase 
(interferon marker) can be considered as a biochemical marker 
of cell status and differentiation in the monocyte line. This 
enhancement of 2-5 A synthetase activity was dependent on 
endogenous interferon production because addition of anti-
IFN-β antibodies reduced the enzyme level to that of nonind-
cuced cells (Table 1). The doses of antibodies used could 
neutralize 20 times the interferon level detected in the medium 
culture (10 units/ml) of U937 induced to differentiate by hy-
droxy-vit D3.

Our results clearly showed that the addition of anti-IFN 
antibodies neutralized all the endogenous IFN produced by 
hydroxy-vit D3 induced U937 cells. However, the anti-IFN-β 
antibodies added contemporaneously with hydroxy-vit D3 were 
unable to inhibit the appearance of the monocytic, membrane, 
or cytoplasmic markers induced by hydroxy-vit D3 (Table 1). 
Similarly, the contemporaneous addition of hydroxy-vit D3 and 
a neutralizing anti IFN-γ antibody did not prevent the appear-
ance of monocyte markers induced by hydroxy-vit D3 (data not 
shown). Thus, endogenous interferons are not necessary for the 
induction of most of the differentiation markers examined.

Inhibition of Cell Growth during Differentiation. Induction of 
cellular differentiation is usually associated with a loss of the 
proliferative potential. Thus, hydroxy-vit D3 induced a reduc-
tion of cell growth of U937 cells which became particularly 
evident after the second day of culture (Fig. 1A).

Hydroxy-vit D3 reduced by 55% the rate of cell growth of 
U937 cells and the addition contemporaneously of anti-IFN-α/ 
β antibodies to the culture medium to hydroxy-vit D3 reduced 
the inhibitory effect of hydroxy-vit D3 on cell growth. The 

### Table 1 Effect of anti-β interferon antiserum on differentiation of U937 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>α-Naphthyl acetate esterase (% positive cells)</th>
<th>NBT reduction (% positive cells)</th>
<th>Lysozyme (μg/mg of protein)</th>
<th>M02 binding (% positive cells)</th>
<th>MAS 072 binding (% positive cells)</th>
<th>2-5 A synthetase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1</td>
<td>4</td>
<td>1.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.98 ± 0.07</td>
</tr>
<tr>
<td>Anti-β antiserum</td>
<td>0.2</td>
<td>3</td>
<td>1.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td>1α,25 (OH)₂-vitamin D₃</td>
<td>85</td>
<td>95</td>
<td>2.3</td>
<td>59</td>
<td>47</td>
<td>4.5 ± 0.15</td>
</tr>
<tr>
<td>1α,25 (OH)₂-vitamin D₃ and anti-β antiserum</td>
<td>82</td>
<td>90</td>
<td>2.3</td>
<td>55</td>
<td>49</td>
<td>1.02 ± 0.13</td>
</tr>
</tbody>
</table>

**Fig. 1.** A, effect of anti-α and anti-β IFN antibodies on the induction of growth inhibition during differentiation. U937 cells were exposed to hydroxy-vit D3 (×), hydroxy-vit D3 + anti-α and anti-β IFN antibodies (△), medium control (○), and assayed for growth inhibition; average value from 5 separate experiments. The difference in growth rate between anti-IFN treated cells and control cells was statistically not significant (P > 0.1), while the difference between hydroxy-vit D3 or hydroxy-vit D3 + anti-IFN antibody treated and control cells is statistically significant (P < 0.01). B, effect of exogenous IFN-β and rIFN-γ on cell proliferation. U937 cells were grown in the absence (○) control) or in the presence of rIFN-γ (△), 250 units/ml; □, 1000 units/ml, and IFN-β (△, 10 units/ml; △, 100 units/ml). Results are the mean of 5 separate experiments.
contribute to the inhibition of cell growth. This hypothesis is in accordance with the well-established fact that interferons have a marked antiproliferative effect. Thus, addition of as little as 10 units/ml IFN-β induced an inhibition of U937 growth similar to that elicited by hydroxy-vit D3, while rIFN-γ exhibited an inhibitory activity only at high doses, as shown in Fig. 1B.

The effects of IFNs on [3H]thymidine incorporation into U937 cells were also tested (Fig. 2). In control cells a peak of [3H]thymidine was observed 1 day after subculture in fresh medium. This early [3H]thymidine incorporation peak was inhibited by IFN-β or rIFN-γ as well as by hydroxy-vit D3; furthermore, no cooperative effect in inhibiting DNA synthesis in U937 cells was observed in the presence of both hydroxy-vit D3 and IFNs.

Effect of Exogenous α, β, or γ Interferons on Induction of U937 Differentiation. The effect of exogenous interferons on the induction of U937 differentiation was investigated by incubating U937 cells, respectively, in the presence of IFN-α, -β, or -γ and following the evolution of a series of differentiation markers (Table 2).

We observed that most monocytic markers were not expressed under the action of exogenous natural IFNs with the exception of NBT reduction and Fc receptors. Moreover, the effects of interferons on these 2 markers were different depending on the nature of the IFN used. The expression of “NBT reduction” was higher in U937 cells treated by IFN-α or -β than by rIFN-γ. The 2-5 A synthetase was strongly enhanced by IFN-α or -β, but very little by IFN-γ. The expression of Fc receptors was increased by rIFN-γ only. In addition, rIFN-γ was able to induce the appearance of several monocytic markers (mostly membrane markers) in U937 cells at a level comparable to that elicited by hydroxy-vit D3 (Table 2).

In a separate experiment we followed the kinetics of induction of monocytic membrane markers induced by rIFN-γ, hydroxy-vit D3, and both of these compounds added together. This experiment showed that (a) most of membrane monocytic markers were fully expressed from the fourth to sixth day of culture on U937 cells grown under all the above-mentioned conditions and (b) the contemporaneous addition of rIFN-γ and hydroxy-vit D3 resulted in an additive stimulatory effect in the induction of all membrane monocytic markers studies (Fig. 3).

Table 2. Effect of α, β, and γ interferons on differentiation of U937 cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control IFN-α</th>
<th>IFN-β</th>
<th>rIFN-γ</th>
<th>α,25 (OH)2-Vitamin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT reduction (% positive cells)</td>
<td>3</td>
<td>90</td>
<td>90</td>
<td>45</td>
</tr>
<tr>
<td>a-Naphthyl acetate esterase (% positive cells)</td>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>8</td>
</tr>
<tr>
<td>Superoxide generation (O2-generated-A550/2.5 x 10^4 cells/h)</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Lysozyme receptors (% positive cells)</td>
<td>0.9</td>
<td>1.4</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Fc receptors (% positive cells)</td>
<td>7–8</td>
<td>7–8</td>
<td>7–8</td>
<td>61–64</td>
</tr>
<tr>
<td>MO2 binding (% positive cells)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>43</td>
</tr>
<tr>
<td>OKM1 binding (% positive cells)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>3</td>
</tr>
<tr>
<td>TEC M1 binding (% positive cells)</td>
<td>2</td>
<td>1.5</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>OKM5 binding (% positive cells)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>58</td>
</tr>
<tr>
<td>TEC MG1 (% positive cells)</td>
<td>21</td>
<td>20</td>
<td>23</td>
<td>79</td>
</tr>
<tr>
<td>FMLP receptors [sites/cell x 10^3]</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>2-5 A synthetase activity (units/mg)</td>
<td>0.90</td>
<td>5.5</td>
<td>5.5</td>
<td>1.2</td>
</tr>
<tr>
<td>vitamin D3</td>
<td>385.5</td>
<td>454.0</td>
<td>587.9</td>
<td>792.5</td>
</tr>
<tr>
<td>Vitamin D2</td>
<td>540.5</td>
<td>540.0</td>
<td>587.9</td>
<td>792.5</td>
</tr>
</tbody>
</table>

**Fig. 2.** Effect of hydroxy-vit D3, exogenous IFN-β, and rIFN-γ on [3H]-thymidine incorporation into U937 cells. U937 cells were grown in the absence or presence of hydroxy-vit D3 or different concentrations of exogenous IFN-β and rIFN-γ, respectively. The effects of individual and combined treatments on [3H]-thymidine incorporation into U937 cells were also tested (Fig. 2). In control cells a peak of [3H]-thymidine was observed 1 day after subculture in fresh medium. This early [3H]-thymidine incorporation peak was inhibited by IFN-β or rIFN-γ as well as by hydroxy-vit D3; furthermore, no cooperative effect in inhibiting DNA synthesis in U937 cells was observed in the presence of both hydroxy-vit D3 and IFNs.

**Fig. 3.** Kinetics of induction of membrane monocytic markers on U937 cells grown in the presence of rIFN-γ and/or hydroxy-vit D3. U937 cells grown in the presence of either rIFN-γ (100 units/ml, left), hydroxy-vit D3 (250 ng/ml, middle), or both rIFN-γ and hydroxy-vit D3 (right) were analyzed at different days of culture for the presence of membrane monocytic markers. The expression of these markers was analyzed by means of specific monoclonal antibodies and the binding of these antibodies to the cells was measured using a 125I-labeled F(ab')2 donkey antimouse immunoglobulin as a secondary antibody.
Effect of Exogenous α, β, or γ Interferons on Induction of Major Histocompatibility Complex during Differentiation of U937 Cells. Control uninduced U937 cells express class I HLA antigens (100% positive cells), while only a minority of these cells express class II HLA antigens [25 ± 5 (SD) and 10 ± 4% of the cells were HLA-DR and HLA-DCI positive, respectively]. However, in previous studies, U937 cells before differentiation have been reported to exhibit only or low expression of class I HLA antigens [19]; there is much variation between U937 clones and this may explain this discrepancy. Induction of the differentiation of U937 cells by hydroxy-vit D₃ or by TPA is accompanied, with similar kinetics for both inducers, by a marked enhancement of the expression of HLA-DCI antigens, and by no modification of HLA-ABC antigens expression, as shown in Fig. 4. Hydroxy-vit D₃ induced a slight decrease of the expression of HLA-DR antigens; however, no change was observed during TPA induction. The modifications in the expression of HLA antigens mostly occurred during the passage from the second to sixth day of culture, which corresponds to the time when monocytic markers appear on U937 cells. In contrast, rIFN-γ induced in U937 cells a marked increase in the level of HLA-DCI antigen expression and any significant change in HLA-DR and HLA-ABC antigen expression (Table 3). Moreover, the expression of HLA-DCI antigens, as compared to the effect of hydroxy-vit D₃ alone, was highly enhanced by the contemporaneous addition of hydroxy-vit D₃ and rIFN-γ. This treatment modified the kinetics of HLA-DCI antigens induction since maximal HLA-DCI expression was observed at days 2–4 of culture, while in cells grown with hydroxy-vit D₃ only peak expression of HLA-DCI antigens occurred at day 6 of culture (Fig. 4). This stimulatory effect of rIFN-γ on HLA-DCI expression seems to be specific for U937 cells since control experiments performed on other leukemic cell lines, K562, HEL, and HL-60 showed no HLA-DCI antigens induction by rIFN-γ (data not shown). Additional experiments showed that rIFN-γ clearly enhances the expression of HLA-DCI antigen in normal human monocytes (data not shown). The addition of IFN-α or -β elicited a moderate enhancement of HLA-ABC antigen expression and no significant modification in the level of expression of both HLA-DR and -DCI antigens (Table 3).

Interestingly, the addition of PHA-leukocyte conditioned medium, which is known to contain one or more compounds capable of inducing the differentiation of U937 cells [14], induced a moderate increase in the expression of HLA-ABC antigens and a marked enhancement of HLA-DR antigen expression associated with only a very moderate stimulation of HLA-DCI antigen expression (Fig. 5). Furthermore, the addition of anti IFN-γ antiserum to PHA-LCM at a concentration capable of neutralizing all IFN-γ contained in PHA-LCM prevented the enhancing effect of PHA-LCM on HLA-DCI antigen expression but did not modify the stimulatory action on HLA-DR antigen expression (data not shown). Taking all these data into account, it appears clearly that in U937 cells, class II HLA antigens, HLA-DR and HLA-DCI, are submitted to differential regulation: HLA-DCI antigen modulation in U937 cells have shown that it is different from tumor necrosis factor, interleukin-1 and -2.
IFNs and Monocytic Differentiation

IFNs exert pleiotropic effects on myelomonocytic cells (31). It was shown that IFN-γ is capable of enhancing the expression of class II HLA antigens (32), of inducing or enhancing the expression of the high-affinity Fc receptor for monomeric IgG (33), of inducing myeloid cells from established lines, from normal bone marrow, or from patients with chronic or acute myelogenous leukemia, and of expressing a series of surface markers, enzymes, and functional activities characteristic of cells differentiating along the monocytic pathway (34). However, evidence concerning this last point is conflicting. Thus, early reports afforded some evidence that IFN-α and -β (13) or IFN-γ (14, 35, 36) was able to induce the differentiation of U937 cells to mature monocytes. In the aforementioned studies the monocyte-inducing properties of IFNs were evaluated by investigation of only 1 to 3 monocye-specific markers. In the present study, this question was reevaluated, investigating the effect of endogenous and exogenous IFNs on the expression of 12 differentiation markers in U937 cells. Previous studies have shown that the production of endogenous interferon (IFN-β) as well as the activity of (2′S)-oligoadenylate synthetase markedly increases when murine (Friend erythroblast leukemia) or human leukemic (U937) cell lines are induced to terminal maturation (2, 7–10, 18, 19). This endogenous IFN is mostly represented by IFN-β since its effect is neutralized by anti-IFN-β antisera, while IFN-γ is undetectable. Attempts have been made to suggest that this endogenous IFN could play a role in some cellular and biochemical processes leading to cellular differentiation. We showed that immunological neutralization of endogenous IFN produced by U937 cells induced with hydroxy-vit D₃ did not inhibit the appearance of maturation markers of U937. These results strongly suggest that endogenous IFN does not play a major role in the induction of cellular differentiation of U937 cells. Our results clearly showed that, contrary to hydroxy-vit D₃, exogenous IFNs were unable to induce a marked enhancement of all these markers. Thus, IFN-α and -β were capable of inducing only an important enhancement of NBT reduction and 2-5 A synthetase activity. These results afford clear evidence that IFN-α and -β were unable to induce the differentiation of U937 cells to mature monocytes. A different conclusion has to be drawn for rIFN-γ since this compound, although not being a "global" inducing agent of the differentiation of U937 cells, is capable of inducing the appearance of several membrane monocytic markers on these cells, in a similar manner with respect to hydroxy-vit D₃.

The immunological neutralization of endogenous IFN by anti-IFN-α/β antisera partially reduced the inhibition of cellular proliferation observed in U937 cells induced with hydroxy-vit D₃. On the other hand, exogenous IFN-β or rIFN-γ blocked [³H]thymidine incorporation, occurring after subculture of U937 cells in fresh medium, in accordance with previous observations (37, 38). These results suggest that endogenous IFN may represent one of the factors which contribute to the loss of cellular proliferation observed when hematopoietic cells are induced to terminal differentiation. This conclusion is conceptually in line with recent studies (2, 10, 39, 40), suggesting that endogenous IFN works in a feed-back loop which limits growth and ensures a negative control of cell growth.

Furthermore, we showed that exogenous IFNs may modulate the expression of HLA antigens of U937 cells: the effect of IFN-α and -β is more pronounced on class I HLA antigens, while the action of rIFN-γ is restricted to class II HLA-DC antigens. These data are apparently at variance with other results showing that rIFN-γ enhance HLA-DR antigen expression in U937 cells (41). However, this discrepancy could be tentatively related to the heterogeneity of U937 for the expression of HLA-DR antigens (42). Furthermore, our data on the modulation of HLA antigen expression by rIFN-γ in U937 cells are of some potential interest since several studies on other cell types, such as fibroblasts (32), vascular endothelium (43), or keratinocytes (44) showed that rIFN-γ markedly enhances HLA-DR and only moderately enhances HLA-DC antigens.

Moreover, there is clear evidence that rIFN-γ modulates the expression of both HLA-DR and -DC antigens in normal human monocytes cultured in vitro (45–47). Thus, modulation of HLA-DC antigens by rIFN-γ may represent a particular feature of monocyctic cells; this phenomenon seems to have a physiological role since the membrane expression of HLA-DC plays a key role in antigen presentation by monocytes (48). Furthermore, the observation that PHA-leukocyte conditioned medium is capable of selectively enhancing HLA-DR antigens expression leads us to the obvious conclusion that one or more substances different from IFN-γ contained in PHA-LCM is/are capable of modulating HLA-DR antigens in U937 cells. This conclusion is directly supported by the observation that the addition of a neutralizing anti-IFN-γ antibody prevents the stimulatory effect of PHA-LCM on HLA-DC antigen expression but does not modify the enhancement of HLA-DR antigen expression. This observation is consistent with recent studies showing that the expression of Ia antigens on mouse lymphoid B-cells (49) and human diploid fibroblasts or large bowel adenocarcinoma cells (50) was increased by a lymphokine other than IFN-γ.

In spite of the fact that IFN-γ is not a "global" inducing agent because there are several monocyte characteristics which are not inducible by IFN-γ treatment, the effect of this substance on the induction of surface antigens, enzymes, and functional activities characteristic of cells differentiating along the monocytic pathway may have important biological significance. In line with this statement, it is of some interest to underline that several macrophage functions such as tumor cell cytolyis, antibody-dependent cell-mediated cytotoxicity, and phagocytic activity are known to increase after rIFN-γ treatment (31). It is tempting to suggest that the induction of some monocytic membrane components by IFN-γ plays a role in the above-mentioned activities. The inducing effect of IFN-γ on Fc monomeric receptor for IgG is in agreement with previous studies (33, 51). IFN-γ may also induce IgE receptor expression on U937 cells (52). Furthermore, recent observations have shown that tumor necrosis factor exhibits a synergistic effect with IFN-γ in inducing the monocytic differentiation of either U937 or HL-60 cells (53).

Finally, the observation that hydroxy-vit D₃ and rIFN-γ exhibit a cooperative effect in inducing the synthesis of monocytic membrane markers of U937 cells is in accordance with a recent study performed on the HL 60 cell line (54) suggesting that these 2 compounds may act through different mechanisms in stimulating the synthesis of monocytic membrane proteins during monocytic differentiation.

Acknowledgments

The authors wish to thank Drs. K. Berg and A. Billiau for anti-α and anti-β IFN antibodies, Biogen for rIFN-γ, and C. Denolf and V. Martinez for secretarial assistance.

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