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Lymphokine Inducing "Terminal Differentiation" of the Human Monoblast Leukemia Line U937: A Role for γ Interferon

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The human monoblast leukemia line, U937, is growth-inhibited and induced to develop markers of mature monocytes by lymphokine preparations. Lymphokine is cytostatic and induces expression of Fc receptors in U937 and in myelomonocytic leukemic lines RC-2A and KG-1, but does not have these effects on T- and B-lymphocytic lines. In addition to previously described properties, including complement receptors, phagocytosis, and antibody-dependent cellular cytotoxicity (ADCC), Mac-1 and Mac-3 surface antigens defined by monoclonal antibodies are induced on U937 cells by lymphokine and phorbol ester. The Mac-1 surface component appears to have a regulatory role in differentiation of the monocyte lineage line, since antibodies to this antigen block the induction of Mac-3 antigen. The lymphokine activity was concentrated by salt precipitation and characterized by ion-exchange and size chromatography. Fractions of about 40,000 daltons were responsible for growth inhibition and induction of Fc receptors and Mac-1 antigen in U937 cells. However, ADCC was not induced in U937 by individual fractions of lymphokine, suggesting that this cytotoxic capacity may be regulated by a lymphokine of a different size, which is only effective after initial maturation steps. Since γ-interferon is present on the 40K size range of lymphokine, the possibility that interferon is a differentiation modulator for the monoblast cells was investigated. Highly purified γ-interferon (10^7 U/mg protein) at 10–300 U/ml inhibited growth and induced Fc receptors in U937 similar to the effect of lymphokine. The Fc-receptor-inducing activity of lymphokine was inhibited by a neutralizing monoclonal antibody to γ-interferon, suggesting that this differentiation factor in lymphokine is γ-interferon.

THE RECENT INTEREST in biologic response modifiers as tools in cancer therapy has focused on physiologic regulators of the leukemic process. Lymphokine (LK) factors induce differentiation markers and/or block cell growth in certain human myeloid and monoblast leukemic cell lines.[1-15]

The U937 cell line derived from pleural fluid of a patient with diffuse histiocytic lymphoma has the properties of monoblasts or immature monocytes: weakly positive for esterase, peroxidase, lysozyme, endogenous pyrogen, phagocytosis, and receptors for immunoglobulin Fc and for complement.[6-8] The cells have receptors for human IgE[9] and are hosts for the growth of yellow fever virus, which shows enhanced infection via IgG antibody binding.[10] Upon stimulation with LK or tumor promoter phorbol myristic acetate (PMA), the cells acquire new receptors for Fc, complement, and chemotactic factor, responses to chemotactants, antibody-dependent and nonspecific cytotoxicity to vertebrate cells, and microbicidal activity to an intracellular parasite.[1,5,11-13]

In this article we describe the induction of macrophage-restricted surface antigens in U937 cells in parallel with other markers and characterize LK activity that induces "terminal differentiation" in certain human myeloid cell lines. Most of the activity in LK for inducing Fc receptors in U937 cells could be identified with γ-interferon, as highly purified γ-interferon induces this marker, and a monoclonal antibody, which specifically neutralizes the biologic effects of γ-interferon, neutralizes the induction of Fc receptors by lymphokine.

MATERIALS AND METHODS

Cell Lines

Human monocytic/myeloid leukemia lines U937, KG-1, and RC-2A;[5] leukemic T-cell lines JURKAT, CCRF-CEM, and Peer;[14] B-cell lines DAUDI, BM-NH,[15] and RPMI 8866;[16] and erythroleukemia-like line K562[17] have been described. Murine macrophage line RAW264.10 was used as a positive control for Mac surface antigens.[18] Lines were grown in RPMI 1640 medium containing 10% fetal bovine serum.

Binding of ^{125}I-Labeled Monoclonal Antibodies (MoAb) to Cells

Rat M1/70, M3/38, and M3/84 MoAb to mouse antigens Mac-1, Mac-2, and Mac-3, respectively, were purified and labeled with ^{125}I as described previously.[18] ^{125}I-labeled MoAb was stored in a solution containing 1% bovine serum albumin carrier protein and 4 mg/ml human γ-globulin (Miles Laboratories, Elkhart, IN) to block binding by Fc receptors. Cells (10^6 in 0.1 ml phosphate-buffered saline—PBS) were incubated 60 min at 4°C with 10 μl ^{125}I-MoAb (approximately 70 ng/150,000 cpm). Cells were then washed 4 times and were assayed for remaining radioactivity. This time period and amount of antibody were shown to give plateau

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Table 1. Expression of Mac-1, Mac-2 and Mac-3 Antigens on Human Hematopoietic Cell Lines

Line	Type	Mac-1	Mac-2	Mac-3
U937	Monoblast leukemia	—†	—	—
U937-LK*		5,230 ± 1,660 (2,020–10,440)	—	4,610 ± 1,520 (1,470–7,980)
U937-PMA*		8,180 ± 1,210 (3,560–10,700)	—	5,900 ± 1,590 (2,890–8,350)
RC-2A	Myelomonocytic leukemia	—	—	—
KG-1	Myeloblast leukemia	—	—	—
K562	Erythroleukemia	—	—	—
JURKAT	T leukemia	—	—	—
CEM	T leukemia	—	—	—
Peer	T leukemia	—	—	—
DAUDI	B lymphoma	—	—	—
BM	B lymphoblast	—	—	—
RPMI 8866	B lymphoblast	—	—	—
RAW 264	Murine macrophage	15,110 ± 6,009 (3,330–23,000)	7,870 ± 2,348 (3,310–11,210)	4,570 ± 1,088 (3,100–8,500)

Counts per minute (cpm) bound to 10^6 using 70 ng ^{125}I-labeled antibody (about 150,000 cpm), as described in Materials and Methods. Mean of at least three determinations ± SE and range in parentheses. Binding of each ^{125}I-MoAb was blocked over 90% by 1 μg/ml unlabeled homologous MoAb but not by heterologous MoAb.

*Cells incubated 6 days with 20% LK or 3 ng/ml PMA.

†Mean not significantly different from 0, $p > 0.05$.

levels of binding to murine macrophage cell lines and to induced U937 cells. Incubations without cells showed 100–400 cpm, and these backgrounds were subtracted.

Rosette Assays for Fc Receptor and Mac-1

Cell receptors for immunoglobulin Fc were assayed by rosetting with IgG-coated erythrocytes.[5] Mac-1 antigen was detected by incubating 10^6 cells in 0.1 ml phosphate-buffered saline with 1 μg/ml MoAb for 20 min at room temperature, washing, incubating 20 min at room temperature with 1:100 dilution rabbit anti-rat IgG serum (Cappel Laboratories, Cochranville, PA), washing, and rosetting with protein-A-coated erythrocytes, as described previously.[19]

LK Preparation and Fractionation

LK was prepared from peripheral blood mononuclear cells by culturing 2 days with 1% PHA-M (DIFCO Laboratories, Detroit, MI) in RPMI 1640 medium containing 0.25% bovine serum albumin.[20] Leukemic monoblast-inducing factor was fractionated from LK by ammonium sulfate precipitation, purified by passing through a DE-52 ion-exchange column at 0.07 *M* NaCl, and size fractionated on an AcA 44 Ultrogel column (LKB Products, Rockland, MD) as described previously for purification of interleukin-2 (IL-2).[20] The 2-day LK contained no myeloid colony-stimulating activity (CSA).[5]

Assay for Antibody-Dependent Cellular Cytotoxicity (ADCC)[5]

Effector cells were incubated at 25:1 with ^{125}IUdR-prelabeled mouse myeloid leukemia M1 in 20-hr incubations in the presence of 10^{-3} dilution of rabbit anti-mouse spleen serum. Background radiolabel release of 5%–11% by targets alone ± antiserum, or by targets plus U937 effector cells without antiserum, was subtracted.

Interferon, Assays, and Monoclonal Antibody

Human γ-interferon at a specific activity of 10^7 U/mg protein was purified from staphylococcal enterotoxin-induced lymphokine, as described previously[21] (B. Y. Rubin, S. L. Anderson, W. A. Martimucci, and S. K. Millet, submitted for publication). A murine IgG monoclonal antibody[21] that neutralizes the antiviral and anticellular effects of γ-interferon, but not α- or β-interferon, was used as hybridoma culture media. Interferon titers were assayed in vesicular stomatitis virus-infected WISH cells by inhibition of the cytopathic effect as compared to a laboratory γ-interferon standard.[22]

RESULTS

Induction of Antigens Identified by Monoclonal Antibodies (MoAb) to Mac-1 and Mac-3

Table 1 shows that a number of human T, B, myeloid, and null cell lines do not express Mac antigens identified by rat MoAb to these murine macro-

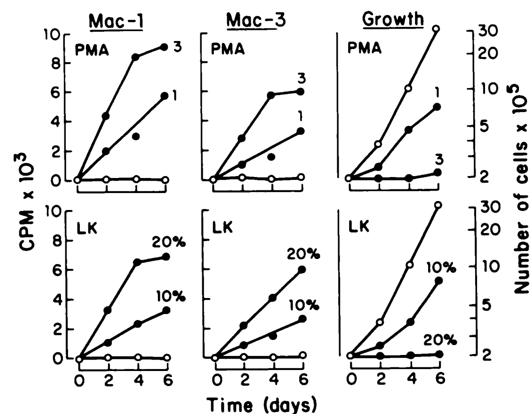

Fig. 1. Induction of Mac-1 and Mac-3 antigens and growth inhibition of U937 cells. Cells were incubated at 2 × 10^5/ml with 1 or 3 ng/ml PMA (upper) or 10% or 20% (v/v) LK (lower), and on various days, the cell number was determined and Mac-1 and Mac-3 expression measured as in Table 1. Cultures were diluted 1:2 with medium on day 3; cell growth is expressed per 2 × 10^5 cells in initial culture. Open circles indicate control values.

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Table 2. Inhibition of Induction of Mac-3 Antigen on U937 Cells by Anti-Mac-1

Inducer	Addition	Addition After Induction	Mac-1 (cpm)	Percent Control*	Mac-3 (cpm)	Percent Control*
0	0		120 ± 40		0	
PMA	0		6,090 ± 740	100	4,430 ± 710	100
PMA	M1/70		†		980 ± 60	22
PMA	M3/84		5,660 ± 300	93	†	
PMA		M1/70	†		4,650 ± 540	105
PMA		M3/84	6,010 ± 220	99	†	
LK	0		5,110 ± 140	100	3,810 ± 560	100
LK	M1/70		†		320 ± 40	8
LK	M3/84		5,430 ± 370	106	†	
LK		M1/70	†		3,630 ± 150	95
LK		M3/84	4,990 ± 480	98	†	

U937 cells were incubated 6 days with 3 ng/ml PMA or 20% LK, plus 1 μg/ml MoAb at time 0 or for 1 hr after the 6-day induction, as indicated, washed, and assayed for Mac-1 and Mac-3 antigens as in Table 1. Normal rat IgG at 1 and 10 μg/ml did not affect induction of the antigens (not shown). Control LK (PMA added at the end of lymphocyte culture) was inactive.

*Percent of induced antigen level.

†Detection of antigen by ^{125}I-MoAb at day 6 was blocked by the presence of unlabeled MoAb of the same specificity added at day 0 or day 6 of culture.

phage-restricted antigens. However, incubation of U937 monoblast leukemic cells with lymphokine or PMA for 6 days induced the expression of Mac-1 and Mac-3 antigens. The induction of these antigens was slow, requiring a number of days to develop full expression, and induction was correlated with growth inhibition of the cell line (Fig. 1). Mac-2 antigen was never seen on human cells in these experiments.

Inhibition of Antigen Induction in U937 Cells by Antibody to Mac-1

Because Mac-1 and Mac-3 are induced on U937 cells, we tested whether these antigenic structures had any role in the process of differentiation. U937 cells were incubated with inducing agents in the presence of antibody to Mac-1 or Mac-3. Table 2 shows that M1/70 anti-Mac-1 blocked induction by PMA or LK of Mac-3 antigen on U937 cells by 78%–92%. Anti-Mac-1 did not block the detection of Mac-3 on previously induced U937 cells. In contrast, M3/84 anti-Mac-3 did not affect the induction of Mac-1 antigen. Another rat MoAb, M5/113, of the same isotype as M1/70,[18] and normal rat immunoglobulin did not interfere with induction of Mac-1 or Mac-3 antigen (not shown). Thus, the Mac-1 surface antigen appears to have a regulatory role in human monocyte differentiation.

Specificity of Monoblast Differentiation Factor

LK at 20% v/v completely inhibited the growth of U937 and partially blocked the growth of myelomonocytic leukemia line RC-2A and myeloid leukemia line KG-1 (Table 3). In contrast, proliferation of T- and B-cell leukemic lines, Epstein-Barr virus positive (EBV$^+$) normal lymphoblast cells, and erythroleukemia K562 was not affected by LK. In parallel with

Table 3. Specificity of Monoblast Differentiation Factor

Cell Line	Growth Inhibition (%)			Fc Receptor (% Positive)			
	LK (%): 10	20	40	0	10	20	40
Myeloid							
U937	66*	98*	100*	8	21*	38*	42*
RC-2A	6	23*	42*	2	11*	22*	27*
KG-1	12	40*	63*	1	4	15*	18*
T cell							
CEM	0	0	4	2	0	2	2
Peer	2	0	2	ND†	ND	ND	ND
JURKAT	1	3	3	14	16	17	15
B cell							
DAUDI	0	0	0	0	0	1	0
BM	0	0	2	4	2	0	3
Erythroleukemia							
K562	2	3	3	ND	ND	ND	ND

Cells were incubated 6 days with LK and assayed for Fc receptors and cell number, as in Fig. 1.

*Significantly different from control, $p < 0.05$.

†ND, not done.

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growth inhibition of the myeloid-related leukemic lines, expression of Fc receptors was induced by LK in the myeloid lines but not in the other hematopoietic lines.

Molecular Size of LK That Induces Terminal Differentiation in U937

We and others previously found that unfractionated lymphokine could induce cell surface receptors on U937 as well as antibody-dependent cytotoxic capacity.[1,5,12] To determine whether these macrophage characteristics are induced by a single factor, we fractionated the LK by charge and size chromatography. Essentially all the growth-inhibitory activity for U937 and material inducing Fc receptors and Mac-1 antigen were found in the fraction not binding to DEAE cellulose at 0.07 *M* NaCl, pH 7.8. The DEAE fraction was further fractionated on an Ultrogel AcA 44 column. Figure 2 shows that size fractions of about 40,000 daltons could induce Fc receptors and Mac-1 antigen, coincident with growth inhibition of U937. In two additional experiments of size fractionation, these three activities eluted together. In contrast to induction of these markers, no ADCC to tumor targets could be detected in cultures of U937 that had been incubated with individual fractions from the size column. Table 4 shows that maximum expression of Fc receptors and Mac-1 antigen and growth inhibition of U937 occurs with 20%–40% v/v of both LK and its 40K fraction. In contrast, ADCC is induced only by the unfractionated LK.

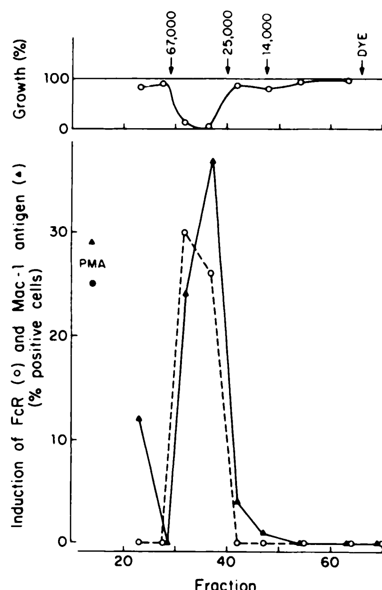

Fig. 2. Size of LK factor that induces differentiation and growth inhibition. LK was concentrated by ammonium sulfate precipitation, and the material passing through a DE-52 column at 0.07 *M* NaCl (0.01 *M* Tris, pH 7.8) was applied to an Ultrogel AcA 44 column. Fractions were incubated at 20% (v/v) with U937 cells for 6 days to induce Fc receptors and Mac-1 antigens, measured by rosette formation. Control cell numbers increased 13.8-fold. Material causing differentiation and growth inhibition eluted in a broad peak at about 40,000 daltons. No significant ADCC was detected in cells incubated with any fraction (U937 stimulated with unseparated LK had 31% ± 3% cytotoxicity).

Table 4. 40,000-Dalton Factor Induces Macrophage Markers and Growth Inhibition But Not ADCC in U937

Inducer	Concentration (%)	FcR (%)	Mac-1 (%)	Growth Inhibition (%)	ADCC (%)
0		5	2	0	0
Unfractionated LK	10	16*	26*	47*	17*
	20	35*	46*	95*	35*
	40	37*	41*	98*	32*
40,000 Fraction	10	8	17*	26*	0
	20	28*	40*	89*	0
	40	34*	42*	99*	0

Cells were cultured 6 days with LK or 40,000-dalton fraction and assayed for macrophage properties and growth inhibition, as in Fig. 2.
*Statistically significant, $p < 0.05$.

Since γ-interferon is found in the size range of the LK differentiation factor, we assayed the LK for interferon levels and tested the effect of purified γ-interferon on U937 cells. As shown in Table 5, highly purified γ-interferon induced Fc receptors in the monoblast cells as well as lymphokine. In three experiments, the minimum amount of γ-interferon inducing a significant level of Fc receptors was 1–10 U/ml. The LK contained about 200 U/ml of inter-

Table 5. Identification of γ-Interferon as the Major Activity in Lymphokine-Inducing Fc Receptors

Inducer	Antibody (NU)	Fc Receptor (%)	Growth Inhibition (%)
0	0	2	0
γIFN			
1 U/ml	0	11	24
10	0	39	18
100	0	76	44
300	0	86	68
10	200	3	*
100	200	7	*
LK			
1%	0	27	12
10%	0	30	28
20%	0	53	60
40%	0	57	72
40%	100	23	*
40%	1,000	9	*

Cells were incubated with purified γ-interferon (IFN), lymphokine (LK), or monoclonal anti-γ-interferon at the indicated neutralizing units (NU)/ml shown, and assayed for Fc receptors, as in Fig. 1. Interferon titer of the lymphokine was 200 U/ml.
*Not measured because the antibody preparation alone inhibited growth by about 20%.

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feron, which was demonstrated to be γ-interferon by virtue of its neutralization by a monoclonal antibody specific for γ-interferon. The γ-interferon content of the LK was sufficient to account for the latter's differentiation effect on U937 cells. In support of this, a neutralizing monoclonal antibody almost completely inhibited the Fc-receptor-inducing activity of LK, as well as that of purified γ-interferon (Table 5).

DISCUSSION

These results show that new differentiation antigens, Mac-1 and Mac-3, appear on the LK-treated U937 cells in parallel with other markers of mononuclear phagocytes. Changes in U937 surface antigens have been described previously. Waldrep et al.[23] reported a new antigen on LK-activated U937 cells, detected by a heterologous antiserum, which reacted with peripheral blood monocytes only after they had undergone maturation in culture. Griffin et al.,[24] using MoAb, found that U937 cells were induced by LK to express MY-3 and MY-4 antigens, which are present mainly on normal monocytes. Mac-1 antigen is found on human monocytes, granulocytes, and a proportion of "null" lymphocytes, including some NK cells.[25] In the mouse, expression of Mac-3 antigen is restricted to macrophages.[26] We showed that all murine macrophage cell lines tested were positive for Mac-1, Mac-2, and Mac-3 antigens.[5] The murine myeloblast line, M1, could be induced by a variety of agents to express Mac-1 and Mac-3, but not Mac-2 antigens,[5] in a similar manner to the results reported here.

The function of most serologically recognized macrophage surface structures is unknown. Mac-1 antigen on murine macrophages and human neutrophils is, or is closely associated with, the complement receptor CR3.[27] In the present experiments, anti-Mac-1 antibody blocked the induction of Mac-3 antigen on U937 cells (Table 2), as was seen during induction of murine M1 myeloblast cells.[18] This indicates that as Mac-1 antigen becomes expressed on macrophage lineage cells during maturation, it may have a regulatory role in the process of differentiation.

An LK that induces differentiation markers and inhibits growth in U937 cells coincides with fractions that did not bind to DEAE cellulose and had an apparent size of 40,000 daltons (Fig. 2). This agrees with the study of Olsson et al.,[4] in which several types of LK contained material not binding to DEAE-Sephadex and of about 40,000 daltons that induced differentiation and cytostasis of the promyelocytic leukemia line HL-60. In some preparations, these authors also found differentiation factors eluting from the ion-exchange column at 0.2 *M* NaCl and of 25,000 molecular weight. Myeloid CSF was separable from differentiation factor in the 4-day LK of Olsson et al.[4] and was not present in our 2-day LK.

Both the unseparated LK[5] and its 40,000-dalton fraction induced Mac-1 antigen, Fc receptor (Fig. 2), complement receptor, and nonspecific phagocytosis (not shown) in U937 cells. However, the capacity of U937 for ADCC to tumor targets was stimulated by the initial LK but not by the fractionated material (Table 4). This suggests that U937 cells may need several factors, one for inducing Fc receptors and perhaps other properties of mature monocytes, and another factor, acting later, that specifically induces ADCC.

In the murine system, we showed that an LK that induces antibody-dependent killing in peritoneal macrophages and macrophage cell lines was different from an activity that induces nonspecific killing of sarcoma targets.[28] We have recently found that the ADCC-stimulating activity and the nonspecific activating factor could be distinguished by size chromatography, being of 60,000 and 50,000 apparent molecular weight, respectively.[29] U937 cells may be a suitable model for the assay of several LK factors that have different biologic effects in the regulation of maturation and expression of certain monocyte markers.

We have identified γ-interferon as the major Fc-receptor-inducing protein in LK (Table 5). Our results are similar to reports that γ-interferon stimulates the expression of Fc receptors in defective murine macrophages.[30] Interferons are well known for their cell growth inhibitory effects.[31] Human α- and β-interferons were recently reported to enhance differentiation of the HL-60 promyelocytic cell line by several inducing agents, although alone, the interferons did not induce differentiation or inhibit cell growth.[32] Inducers of differentiation in human myeloid leukemia include proteins other than interferon, since conditioned medium of the human bladder carcinoma line 5637 induces U937[5] and supernates of the hepatoma line SK-Hep induce HL-60,[33] but these materials have no interferon activity (B. Y. Rubin and J. Gabrilove, unpublished results). Further experiments are necessary to determine if interferon can induce the whole range of differentiation properties associated with U937 or if it is restricted to a few specific markers.

The evidence for defined endogenous proteins, as well as for other agents, with capacity to induce in vitro the differentiation of myeloid leukemic cell lines and suppress their in vivo leukemia-inducing potential is well established in murine experiments.[34-36] These leu-

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kemic cells can be induced by corticosteroids and microbial products to make their own differentiation factor.[37] The in vivo induction of a similar differentiation factor in patients receiving endotoxin therapy has been described.[38] Although the reported inhibition by interferon of normal myleopoiesis in in vitro essays[31,39] suggests caution, the use of biologic response modifiers, such as interferons, other differentiation proteins, and vitamin analogues,[40] in combination with chemotherapy, offers the possibility of a new approach to treatment of myeloid leukemias that are currently refractory to conventional therapy.

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