MECHANISM OF MYELOID DIFFERENTIATION INDUCED BY A DIFFERENTIATION FACTOR ISOLATED FROM RAT LUNG CONDITIONED MEDIUM

DISSERTATION

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A leukemia Differentiation Factor (DF), that induced differentiation of rat leukemia MIA C51 cells, was isolated from endotoxin-stimulated rat lung conditioned media. The DF was purified to apparent homogeneity using ultrogel AcA 44 gel filtration chromatography, DEAE-HPLC and SDS-PAGE. The purified DF activity was associated with a 24 KDa protein band. The amino acid composition of DF was analyzed and showed no similarity to any of the protein factors which have been reported to induce leukemia cell differentiation. Amino terminal sequencing of the purified DF did not detect any specific amino acid signal indicating a blocked N-terminal. Physical properties and receptor binding experiments indicated that this DF is a new myeloid differentiation inducing factor.

In order to investigate the mechanism of action of DF, myeloblastic leukemia MIA C51 cells were incubated with DF and induced to undergo differentiation. Incubation with DF also inhibited the proliferation of these cells. Phorbol diesters, which bind to protein kinase C (PKC), did not affect MIA C51 growth in culture at a concentration (1 μg/ml) that inhibited the proliferation of other cultured cells. On the other hand, dibutyryl cyclic adenosine

monophosphate (Bt_2cAMP) inhibited MIA C51 cells growth in culture. These data suggest that cyclic adenosine 3'; 5' monophosphate (cAMP) is a second messenger in DF-induced signal transduction.

The data from this study showed that DF induced the stimulation of adenylate cyclase activity resulting in an elevation of cellular cAMP levels. At the same time, DF induced a rapid decline in the cellular content of inositol polyphosphates as well as phospholipase C (PLC) activity. Addition of Bt_2cAMP (400 μM) to MIA C51 cells also induced a similar decline in inositol polyphosphates content and PLC activity. The decrease in the intracellular inositol polyphosphate levels was probably the result of an inhibition in PLC activity by DF or Bt_2cAMP. These data suggest that the signal transduction pathway of the DF-induced differentiation is mediated by an increase in cellular cAMP levels which then inactivate PLC and decrease the release of inositol polyphosphates.

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ABBREVIATIONS

AML	Acute myelogenous leukemia
B9	Murine hybridoma cell line
Bt_2cAMP	N^6, O^2-dibutyryl cyclic adenosine 3'; 5' monophosphate
cAMP	Cyclic adenosine 3'; 5' monophosphate
CAPS	3-(cyclohexylamino)-1-propane sulfonic acid
cDNA	Complementary deoxyribonucleic acid
CML	Chronic myelogenous leukemia
CNBr	Cyanogen bromide
CSF	Colony-stimulating factor
DAG	Diacylglycerol
DEAE	Diethylaminoethyl
DF	Differentiation factor
DME	Dulbecco's modification of Eagles medium
DMSO	Dimethyl sulfoxide
FCS	Fetal calf serum
F_{max}	Maximum fluorescence
F_{min}	Minimum fluorescence
HL-60	Human promyelocytic leukemia cell line
HPLC	High performance liquid chromatography
IFN	Interferon
IL	Interleukin
IP	Inositol mono-phosphate
IP_2	Inositol bis-phosphate

IP_3	Inositol tris-phosphate
Kd	Dissociation constant
KDa	Kilodalton
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LT	Lymphotoxin
M1	Murine myelocyte leukemia cell line
M-CSF	Macrophage colony stimulating factor (CSF-1)
MIA C51	Rat myeloblastic (chloroleukemia) cell line
ML	Myeloblastic promyelocytic human leukemia cell line
Mr	Apparent molecular weight
NaN_3	Sodium azide
OAG	L-α-1-oleoyl-2-acetoyl-sn-3-glycerol
PAGE	polyacrylamide gel electrophoresis
P388D1	Macrophage-like murine tumor cell line
PBS	Phosphate-buffered saline
PDBu	Phorbol-12, 13-dibutyrate
PEG	Polyethylene glycol
PIP_2	Phosphatidyl 4, 5 bis-phosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenyl methyl sulfonyl fluoride
PVDF	Polyvinylidene difluoride
R_f	Relative mobility

RLCM	Rat lung conditioned medium
SDS	Sodium dodecylsulphate
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoyl-phorbol-13-acetate
Tris	Tri-(hydroxymethyl) aminomethane
U-937	Human monoblastoid cell line
WEHI-3B	Murine myelomonocytic leukemia cell line

CHAPTER I

INTRODUCTION

Current Understanding of Hematopoiesis: Blood cells are produced in the bone marrow and lymphoid organs by a process known as hematopoiesis, from the greek words "haima" (blood) and "poiein" (to make). The hematopoietic system is broadly organized into three interconnecting compartments. The stem cell compartment renews itself as well as provides differentiated progeny. The transit compartment consists of cells committed to different hematopoietic lineages but which are still able to proliferate and produce mature cells. Finally, the end compartment is the stage of differentiated cells.

In hematopoiesis, a single kind of precursor cell develops into a number of more specialized precursor cells, among them myeloid stem cells and lymphocyte precursor cells, which in turn proliferate and develop to produce the mature cells of the different blood cell lineages. Progenitor or precursor stem cells can each generate clones of up to 10^5 lineage-restricted cells that mature into specialized cells (1, 2). There are different mature specialized cells, such as the red blood cells (erythrocytes) and the different lineages of the white blood cells. Although red blood cells come in only one form, the white blood cells of the immune system include

three different lineages which are granulocytes, monocytes and lymphocytes.

The development of normal myeloid hematopoietic cells is physiologically regulated by specific growth-inducing and differentiation-inducing proteins which are part of a family of regulators called the cytokines. The cytokine family includes the interleukins (ILs), the colony stimulating factors (CSFs), the tumor necrosis factors (TNFs), the interferons (IFNs), the transforming growth factors (TGFs), and the leukemia inhibitory factor (LIF) (3).

Development of Leukemia: The differentiation process of precursor blood cells into mature terminally differentiated cells is involved in the control of blood cell proliferation. A defect in the normal balance between differentiation and proliferation may lead to the development and progression of malignancy. Myeloid leukemia cells, like other cancer cells, have lost control of the coupling between proliferation and differentiation, resulting in the occurrence of an abnormally high proportion of self-replicative cells versus differentiated cells. These replicative leukemia cells are stem cells or cells present at an intermediate stage of maturation.

Possible causes for the development of leukemia may be via unregulated expression of hematopoietic growth factors, defects in growth factor receptors, or deregulation of events that happen downstream from the growth factor-receptor interaction.

***In Vitro* Cell Lines:** Different primary cultured leukemic cells

were established for *in vitro* studies. A human promyelocytic cell line, HL-60, was established from peripheral blood leukocytes of a patient with acute promyelocytic leukemia (4). A human myelogenous leukemia cell line, K562, was established from a patient with chronic myelogenous leukemia (5). Another human myelogenous cell line, KG-1, which is at the stage of myeloblast and promyelocyte stage of differentiation was established from a patient with acute myelogenous leukemia (6). The human myeloid leukemia cell lines ML-1 and ML-3 were established from a patient with acute myelogenous leukemia (7). The human cell line U-937 was established (8), which has monoblastic and immature monocyte characteristics.

Different murine cell lines have also been established. A murine myeloid leukemia cell line, M1, was established from a spontaneous myeloid leukemia in an SL mouse (9), and another leukemia cell line, R453, was established from a C57BL/6 mouse with a Rauscher virus-induced leukemia (10). WEHI-3B myelomonocytic leukemia was induced in a BALB/C mouse injected with mineral oil, and was established as a cell line in liquid culture (11). Also, clone 7M12 originated from a myeloid leukemia obtained after X-irradiation in a SJL/J mouse (12).

Fewer rat leukemia cell lines are available. Rat chloroleukemia, which is a myelogenous leukemia, has been produced by chemical carcinogens and by radiation. Several cell lines have been established by Yunis *et al.* (13), from explants of rat chloroma. One of these cell lines is MIA C51, where cells are predominantly

myeloblasts with some myelocytes.

Hematopoietic Differentiation-Inducing factors: Cultured myeloid leukemia cells as well as myeloid cells from leukemia patients can be induced to differentiate into cells with normal characteristics of macrophages/monocytes or granulocytes. Maturation-arrested cells differentiate when exposed to natural hematopoietic differentiation-inducing factors present in sera or in cell- or organ-conditioned medium, or to a variety of other agents. One of the important biological processes associated with differentiation is the irreversible loss of proliferation potential that occurs during a specific terminal event in differentiation. This process of induction of differentiation of tumor cells constitutes a promising approach to the treatment and therapy of tumor cells generally, and myeloid leukemia cells specifically. Because of its biological significance and therapeutic implications, the differentiation of leukemic cells has been the subject of extensive investigations (14-17). Various myeloid differentiation-inducing factors have been shown to induce the differentiation *in vitro* of several myeloid leukemia cell lines (Table I).

Granulocyte colony-stimulating factor (G-CSF), which stimulates the formation of neutrophilic colonies in soft agar, has proved effective in inducing the differentiation of different human and murine leukemic cell lines. G-CSF has the ability to induce differentiation of a murine myelomonocytic leukemia cell line WEHI-3BD$^+$ (15). G-CSF induced the differentiation of M1 leukemic

TABLE I

Differentiation of Myeloid Leukemia Cells by Different Hematopoietic Regulatory Proteins

Myeloid Leukemic Cell Line	Cell Type	Inducing Factor	Mature Cell Type
M1 (m)	Myelocyte	G-CSF, IL-1α, IL-1β, IL-6, LIF	M/G
7-M12 (m)	Myeloblast	GM-CSF, IL-3	M/G
WEHI-3B (m)	Myelomonocyte	G-CSF, GM-CSF, IL-6	G
HL-60 (h)	Promyelocyte	G-CSF, GM-CSF, TNFα, LT, IFNγ	M/G
U-937 (h)	Monoblastoid Cell	G-CSF, GM-CSF, TNFα, LT, IFNγ	M
P388D1 (m)	Macrophage-like	IL-1α, IL-1β	M
32DC13 (m)	Myeloblast	G-CSF	G
ML-3 (h)	Promyelocyte	TNFα, LT	M/G
ML-1 (h)	Promyelocyte	TNFα, IFNγ, TGFβ	M/G

m, murine; h, human; M, macrophage/monocyte; G, granulocyte

cell line. However, the G-CSF concentration required to induce differentiation in this cell line is higher than for WEHI-3BD+ cells (18, 19). The immature monoblastoid U-937 cells were induced to differentiate by G-CSF into monocyte/macrophage differentiated cells after 3 days of incubation with G-CSF (20). The self renewal of HL-60 cells was reduced by G-CSF (21), and a murine IL-3-dependent 32DC13 cell line differentiates into granulocytes upon exposure to G-CSF (22).

Granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulates the formation of macrophage and neutrophilic colonies in soft agar, also exhibits a capacity to induce the differentiation in WEHI-3BD+ cells (11). The effect of addition of GM-CSF to either HL-60 or U-937 cells was similar to G-CSF actions in both cell lines, because it reduced the self renewal of HL-60 cells (21) and induced the differentiation of U-937 cells (20). In addition, 7M12 cell line was induced to differentiate by both GM-CSF and interleukin 3 (IL-3) (23).

Both recombinant human interleukin-1α and β (IL-1α and β) induced the differentiation of macrophage-like tumor cell line P388D1 toward the monocyte/macrophage phenotype (24). Human IL-1β is also growth inhibitory for M1 cells, and murine IL-1α has been shown to have similar activity (25). Also, IL-1 has been shown to have a growth inhibitory effect on human myeloid cell line K-562 (26).

Interleukin-6 (IL-6) induced a rapid inhibition of proliferation of the murine M1 cells with induction of phagocytic activity and

morphological changes characteristics of mature macrophages (27). IL-6 induced partial differentiation (25% of the cells) of the human U-937 cells, differentiated cells showing monocytic morphology (28). IL-6 also induced the differentiation of WEHI-3BD+ cells (29).

Recombinant human tumor necrosis factor α (TNFα) induced the differentiation of ML-1 human myeloblastic leukemia cells to monocytes (30). Differentiation of HL-60 cells and to a lesser extent ML-3 and U-937 cells into monocyte/macrophage was induced after few days of culture with either TNFα or lymphotoxin (LT) (31).

Leukemia inhibitory factor (LIF) was shown to induce monocytic differentiation of M1 leukemia cells (32). Transforming growth factor β (TGFβ) induced the differentiation of ML-1 human myeloblastic leukemia cells to monocytes (33).

Interferonγ (IFNγ) induced monocytic differentiation in HL-60 and ML-1 cells. However, the monocytic differentiation induced in HL-60 cells was not accompanied by inhibition of cell proliferation (34, 35). U-937 cells can also be induced to differentiate into monocyte/macrophage like-cells by IFNγ (36).

Chemistry of the Differentiation Inducing-Factors

Proteins and genes: Considerable effort have been devoted to study the structural characterization of the different myeloid differentiation-inducing factors. These factors have been described in molecular detail and their genes have been molecularly cloned (Table II).

Structural analysis of granulocyte colony-stimulating factor (G-

TABLE II

Characteristics of the Different Human Cytokines that Induce Differentiation of Myeloid Leukemic Cells

Factor	Molecular Weight (KDa)	No. of Amino Acids		Gene Location (Chromosome No.)
		Total	Mature	
G-CSF	18-22	204	174	17
GM-CSF	14-35	144	127	5
IL-1α	17.5	271	159	2
IL-1β	17.5	269	153	2
IL-3	14-28	152	134 or 140	5
IL-6	23-30	212	184	7
TNFα	17.3	233	157	6
LT	20	205	171	6
LIF	58	202	179	-
IFNγ	40	166	143	12
TGF$_{\beta 1}$	25	391	112	19

CSF) revealed that G-CSF is a glycoprotein that is monomeric in form. The carbohydrate part of G-CSF is linked through O-linkages. Also, the primary sequence of G-CSF contains four cysteine residues which are presumed to form 2 disulfide bridges. Both mouse and human G-CSF cDNA have been isolated (37-39). Mouse and human G-CSFs consist of 208 and 204 amino acid residues respectively, that include a signal peptide of 30 residues. Both human and murine G-CSFs are hydrophobic molecules of similar size with apparent molecular weights of 20 KDa and 25 KDa, respectively. Also, both the murine and human G-CSF genes contain four introns and five exons (40). The gene for human G-CSF is located on chromosome 17 (41).

The human granulocyte-macrophage colony-stimulating factor (GM-CSF) complete gene has been isolated (42). It is 2.5 kb in length, contains 4 exons and three introns (43) and is located on chromosome 5 (44), whereas the murine GM-CSF gene is located on chromosome 11 (45). Both human and mouse GM-CSFs are synthesized as precursors from which 17 residues are cleaved to yield 127 and 124 amino acid residues mature proteins, respectively (46-50). GM-CSF has been purified from mouse lung conditioned medium (51), and from the conditioned medium of a human T-lymphoblast cell line to apparent homogeneity (47). Both human and murine GM-CSFs are glycoproteins with apparent molecular weights of 22 and 23 KDa, respectively. The predicted amino acid sequences for both human and murine GM-CSFs are 54% identical and the nucleotide sequences are 70% conserved (43).

Interleukin-1 (IL-1) consists of two distinct but related

molecules which are IL-1α and IL-1β. Both IL-1α and IL-1β genes are composed of seven exons that are located on chromosome 2 (52, 53). The two forms of IL-1 are synthesized as 31-KDa precursors (HIL-1α is composed of 271 amino acid residues, while HIL-1β is composed of 269 amino acid residues) that are processed to 17.5 KDa mature proteins after proteolysis of more than 100 amino acid residues from the amino terminus to yield 159 and 153 amino acid residues mature HIL-1α and HIL-1β, respectively. On the other hand, murine IL-1α is composed of 270 amino acid residues with a mature protein of 156 amino acid residues, and murine IL-1β is composed of 269 amino acid residues with a mature protein of 152 amino acid residues (3). Both forms of IL-1 share their biological activities and were shown to bind to the same receptor. Human IL-1α is 62% homologous to mouse IL-1α, and human IL-1β is only 26% homologous to human IL-1α (54-56).

Interleukin-3 (IL-3) is a single polypeptide that was purified to homogeneity from WEHI-3BD$^+$ cells conditioned medium (57, 58). IL-3 cDNA clones of mouse (59), gibbon (60) and human (61) have been isolated. The gene for IL-3 has been located to mouse chromosome 11 (45) and human chromosome 5 (44). While the human IL-3 cDNA encodes a polypeptide of 152 amino acid residues, the mouse IL-3 cDNA encodes a polypeptide chain of 166 amino acid residues including a signal peptide of 26 residues and four potential N-glycosylation sites. The homology between mouse and human IL-3 is only 29% at the amino acid sequence level.

Interleukin-6 (IL-6) is a glycoprotein of 23-30 KDa molecular

weight (heterogeneity in size is due to different glycosylation) (62). The human IL-6 gene is located on the short arm of chromosome 7, and the murine IL-6 gene maps to the proximal region of chromosome 5 (63). The human, mouse, and rat IL-6 genes consist of five exons and four introns (64, 65). IL-6 cDNA clones were isolated for human (66), mouse (67), and rat (68). The human 1.3 kb mRNA is translated into a 212 amino acid precursor protein with a signal peptide of 28 amino acid residues. The resulting 184 amino acid protein contains N- and O- possible glycosylation sites. IL-6 is also phosphorylated at several serine residues. Murine IL-6 contains 211 amino acid residues with a signal peptide of 24 residues. Human and murine IL-6 are 65% homologous at the cDNA level and 42% homologous at the protein level, whereas rat IL-6 was 93% and 58% homologous with mature murine and human IL-6 proteins, respectively. Unlike human IL-6, the mouse and rat IL-6 has no potential N-glycosylation sites but several potential O-glycosylation sites are available.

Tumor necrosis factor α (TNFα) was first found in serum of animals primed with Bacillus-Calmette-Guerin (BCG) and then challenged with lipopolysaccharide (LPS) (69). A molecule identical to TNFα and named cachectin (causes cachexia) was isolated from serum of endotoxin-sensitive mice treated with LPS (70). TNFα is a single polypeptide chain of 157 amino acid residues. The genes for human TNFα and murine TNFα are located on chromosomes 6 (71) and 17 (72), respectively. Human TNFα was purified to homogeneity as a 17.0 KDa protein (73). cDNA encoding human TNFα was cloned and was found to be encoding a protein of 233 amino acid residues

(74). The mature protein begins at residue 77, leaving a long leader sequence of 76 amino acid residues. The mature mouse protein is 156 residues long, the rabbit protein lacks two N-terminal residues and is therefore 154 residues long, while the human protein consists of 157 residues (75). The human TNFα has a molecular weight of 17 KDa by SDS-PAGE and 45-60 KDa by gel permeation chromatography (73), suggesting that the native protein is either a dimer or a trimer.

Lymphotoxin (LT) is a homologous protein to TNFα with a 30% amino acid sequence homology (76). TNFα has one disulfide bridge and no methionine residues, whereas LT contains no cysteines and 3 methionines. LT also differs from TNFα by the presence of an asparagine-linked carbohydrate moiety (73). The human TNFα gene, like the LT gene contains 3 introns and 4 exons (3). While the human LT is composed of 205 amino acid residues with a signal peptide of 34 amino acid residues, the mouse LT is composed of 202 amino acid residues with a signal peptide of 33 amino acid residues (3). LT has an apparent molecular weight of 20 KDa in SDS-PAGE, a molecular weight of 60 KDa was estimated by gel permeation chromatography (77).

Leukemia inhibitory factor (LIF) is a single-chain glycoprotein of 58 KDa molecular weight (32). A murine cDNA clone was isolated on the basis of partial amino acid sequence (78), and a human LIF genomic clone was subsequently isolated (79). Human LIF has 78% amino acid sequence identity with murine LIF. While human LIF consists of 202 amino acid residues, the mature protein encodes 179 residues only. At the same time, murine LIF is composed of 178

amino acid residues with a mature protein of 169 amino acid residues. LIF molecule is heavily glycosylated, comprising 65% of the approximate molecular weight as carbohydrate (80). LIF contains 7 possible N-glycosylation sites (Asn-Xaa-Thr/Ser) and three possible O-glycosylation sites (clustered region of OH-amino acids containing adjacent proline residues).

Interferon γ (IFNγ) was originally identified by its antiviral activity (81). It is secreted mainly by activated T lymphocytes following exposure to antigen or mitogens (82). Human IFNγ (Mr = 40 KDa) is a homodimer and consists of 166 amino acid residues with a signal peptide of 23 residues and 2 sites for N-linked glycosylation (83, 84). On the other hand, mouse IFNγ consists of 155 amino acid residues with a signal peptide of 22 amino acid residues. Sequences for mouse (85), bovine (86), and rat (87) IFNγ have also been reported. Human IFNγ is encoded by a single gene and is located on chromosome 12 (88) and murine IFNγ gene is located on chromosome 10 (89). The homology among the species is low (human IFNγ shares 61, 40, and 39% amino acid sequence with the bovine, mouse, and rat respectively) which might be responsible for the lack of cross species reactivity of IFNγ.

Transforming growth factor β (TGFβ) is a 25 KDa disulfide-linked dimer. Several forms of TGFβ are known which are TGF-β1, TGF-β2, TGF-β1, 2, and TGF-β3. These different forms represent homodimeric and heterodimeric combinations of β1, β2, and β3 polypeptide subunits. The human cDNA clone sequence of TGF-β1 indicates that the monomer is synthesized as the COOH-terminal

112 amino acids of a 391 amino acids precursor. There is a total sequence homology between the respective human, bovine, and porcine mature monomer sequences, while the mouse peptide has a single amino acid substitution (90). TGF-β1 and TGF-β2 consist of two Mr 12.5 KDa polypeptide chains (112 amino acid residues) linked by intermolecular disulfide bridges. There is only 70% sequence homology between TGF-β1 and TGF-β2. The human TGF-β1 gene has been localized to the long arm of chromosome 19 and to chromosome 7 in the mouse (91).

Signal Transduction Mechanisms: Since induction of differentiation constitutes a promising approach to the treatment of cancer and leukemia cells, it is critical to understand the mechanism by which leukemia cells withdraw from the cell division cycle and differentiate when induced by different cytokines. Cellular responses to an external stimulus appears to involve at least three components, a receptor to recognize the hormone, an enzyme to liberate messenger molecules into the cytoplasm and a third component which acts to couple the receptor to its messenger-generating system. Hormones, neurotransmitters, and growth factors bind to specific receptors on the cellular membrane. Occupancy of these receptors induces the production of active messengers which transmit these extracellular signals across the cell membrane.

There are three major signal transduction pathways that are widely utilized by different stimuli. The first pathway involves catalytic receptors (such as macrophage-colony stimulating factor

receptor, epidermal growth factor receptor, insulin receptor, and platelet-derived growth factor receptor) which are tyrosine-specific protein kinases that directly phosphorylate specific target proteins on tyrosine residues. The second pathway triggers changes in cyclic adenosine monophosphate (cAMP) intracellular level, while the third pathway involves inositol phospholipid turnover.

Changes in cellular cAMP levels can be elicited by either affecting cAMP synthesis by adenylate cyclase or hydrolysis by cAMP phosphodiesterase. Many neurotransmitters, hormones and growth factors mediate activation of membrane-associated adenylate cyclase, including granulocyte-colony stimulating factor (G-CSF), epidermal growth factor (EGF), glucagon, and isoproterenol (92-96). On the other hand, inhibition of adenylate cyclase activity has been associated with $\alpha 2$-adrenergic, muscarinic agonists, and opioids receptors (97). In addition, treating cells with a phosphodiesterase inhibitor by using a variety of methylxanthines such as 3-isobutyl-1-methylxanthine can elicit changes in cellular cAMP concentration (96). Changes in cAMP concentration can also be elicited by treating cells with forskolin (adenylate cyclase activator) or with a cell-permeable analog of cAMP such as N6, O2-dibutyryl adenosine 3'; 5'-cyclic monophosphate (Bt_2cAMP).

A number of agents that increase intracellular cAMP by either stimulating adenylate cyclase, inhibiting cAMP phosphodiesterase or being a cell-permeable analog of cAMP, inhibited proliferation of transformed cells as well as induced irreversible morphological differentiation in certain malignant cell lines. Agents that increased

cAMP induced the differentiation of human promyelocytic leukemia HL-60 cells (98), chronic myelocytic K-562 cells and Molt-4 acute T lymphocytic cells (99), inhibited the proliferation of Nb2 lymphoma cells (100), and induced lysozyme activity in a murine leukemia cell line, M1 (101). Cyclic AMP has also been implicated as a differentiation agent in numerous other systems, including the kidney epithelial MDCK cell line (102), breast and colon human cancer cell lines (103), human melanoma cell lines (104), virally transformed fibroblasts (105), and cultured mouse and human neuroblastoma cells (106).

A large group of stimuli exert their biological effects on cells by activating phospholipase C (PLC), which specifically breakdown inositol phospholipids to produce inositol triphosphate (an intracellular messenger that mobilizes Ca^{++}) and 1, 2 diacylglycerol which activates protein kinase C. Mammalian cells contain at least four immunologically distinct isozymes of phospholipase C (PLC) which are PLC-α, -β, -γ, and -δ (107). Increase in inositol phospholipid turnover has been observed in different proliferative cellular responses to a range of neurotransmitters, hormones, and growth factors. Examples of such receptors coupled to inositol phospholipid metabolism are thyroid-stimulating hormone, thyrotrophin-releasing hormone, angiotensin II, thrombin, endothelin, epidermal growth factor, bombesin, platelet-derived growth factor, interleukin-1, and interleukin-2 (108-113).

Activating PLC by an external stimuli is usually followed by activation of protein kinase C (PKC) by the physiological activator of

the enzyme 1, 2 diacylglycerol. The protein kinase C family consists of multiple isozymes with closely related structures. cDNAs encoding 7 different isozymes has been isolated from a rat brain library. These isozymes are referred to as α, β_I, β_{II}, γ, δ, ϵ, and ζ (114).

Activation of PKC can also be induced by phorbol diesters. Phorbol diesters are potent tumor promoters which exert their effects by binding to PKC at the diacylglycerol site and by doing so they mimic the effect of 1, 2 diacylglycerol in the activation of PKC (115-117). Of the active phorbol diesters, the most potent one is 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Phorbol-12,13-dibutyrate (PDBu) is also an active phorbol diester but it is less hydrophobic than TPA.

Phorbol diesters can bring about morphologic, antigenic, biochemical and functional changes in many cultured mammalian cells. Many myeloid leukemia cells were shown to cease proliferation and to differentiate into macrophage-like cells when treated with phorbol diesters. TPA inhibited the proliferation and induced the expression of differentiation markers of many leukemia cell lines, including HL-60 human promyelocytic leukemia cells (118), human K562-4 myeloid leukemia cells (119), the U937 human monoblastic cell line (120), murine macrophage tumor cell M5076 (121), human monocytic THP-1, and the myeloblastic promyelocytic ML-2 human leukemia cells.(123). TPA also induced the expression of differentiation markers of other cell lines including human melanoma cells (123) and human T-lymphoid leukemia cells (124).

While most mammalian cells are responsive to TPA, several TPA-nonresponsive clonal lines were selected for their TPA-resistance. These cells were selected by exposing them to high concentration of TPA for multiple selection rounds, such as variants of the human promyelocytic HL-60 leukemia cells {R-55, R-94, R1B6, PET, DM30, DM90, and HL-60-1E3 (125-130)}. Also, other TPA-unresponsive cells were selected by their inability to respond mitogenically to TPA like variants of the Swiss 3T3 fibroblasts {3T3-TNR-2, and 3T3-TNR-9 (131)}.

Purpose of This Study: It was reported previously that HL-60 cells were induced to differentiate by human lung conditioned medium (132). However, the molecular nature of the differentiation-inducing activity has not been fully characterized. In this report, we observed that rat lung conditioned medium (RLCM) was able to induce the terminal differentiation of cultured rat myeloblastic MIA C51 leukemia cell line which has been established by Yunis *et al.* (13) from explants of rat chloroma. A new leukemia differentiation protein factor (DF) was isolated, and purified from endotoxin-treated rat lung conditioned medium. The biochemical and physical properties of DF were characterized, and its biological functions in stimulating the differentiation of MIA C51 cells were also studied. The mechanism by which MIA C51 leukemic cells differentiate when exposed to DF was also investigated in this study.

MIA C51 cells when transplanted into newborn rats would produce leukemia in those rats. It was reported previously that treatment of

the transplanted newborn rats with rat lung conditioned medium was effective in preventing the development of leukemia in those rats (133). This procedure provides a good *in vivo* model system for investigating the differentiation of leukemia cells. Since MIA C51 cells provide a good *in vivo* and *in vitro* model systems, these cells were used to study the control of proliferation and differentiation of leukemic cells.

In response to DF, cultured MIA C51 cells were shown to undergo terminal differentiation to monocyte/macrophage cells as evidenced by morphological as well as enzyme marker expressions. While control untreated MIA C51 cells showed the morphological characteristics of myeloblast cells, DF-treated MIA C51 cells showed monocyte-like morphology. In addition, treatment of MIA C51 cells with DF enhanced their ability to phagocytize particles and increased their lysozyme and non-specific esterase activities.

CHAPTER II

MATERIALS and METHODS

Assay of Colony-Stimulating Factor Activity: Rat and mouse bone marrow cells were prepared for in vitro soft agar assay as previously described (134). The mouse (C57BL/6J) and rat (Fischer 344) bone marrow cells were isolated by inserting a 21 1/2 gauge needle into the proximal end of the femora and forcing out the cells with 3 ml of ice cold 2X DME media. The cell suspension was thoroughly mixed, and an aliquot was diluted 1:20 in 3% acetic acid and shaken for 1 minute. The number of nucleated cells were counted with a hemocytometer. Bone marrow cells were cultured at a concentration of $1x10^5$ cells/ml (mouse), or $2x10^5$ cells/ml (rat) in the assay medium. A solution of double strength Dulbecco's Modified Eagle's Media was prepared with 20% fetal calf serum, 20% horse serum, 19.8 µg/ml asparagine, 75 µg/ml DEAE-dextran, 100 units/ml pencillin, 100 µg/ml streptomycin and 7.6 mg/ml sodium bicarbonate. The solution was mixed with an equal volume of 0.6% agar and kept at 37°C for 20 min and the cells were then added. One ml of the final cell suspension mixture was pipetted into 35 mm petri dishes containing 0.2 ml of sample at various concentrations. Cells were swirled to ensure an even distribution, then left at room temperature for 30 min to allow the agar to solidify. Dishes were

incubated at 37°C under 7% CO_2 for 6 days. Aggregates of 50 or more cells were counted as a colony under a dissecting microscope.

Cell Culture: The MIA C51 cell line used in this study is a rat chloroleukemia cell line established by Yunis *et al.* (13). Cultured MIA C51 cells proliferate very rapidly with a doubling time of 12 hrs, and cells are predominantly myeloblasts with some myelocytes present. A human cell line U-937 with monoblastic and immature monocyte characteristics (8) was purchased from American Type Culture Collection (ATCC). Both MIA C51 and U-937 cells were maintained in Dulbecco's Modified Eagle's Media supplemented with 10% fetal calf serum (FCS) in a humidified 7% carbon dioxide atmosphere. For growth curve analysis, cultured MIA C51 or U-937 cells were seeded in 35 mm petri dishes ($1x10^5$ cells/ml of MIA C51 cells or $5x10^5$ cells/ml of U-937 cells) containing different concentrations of the sample to be assayed, and incubated in a humidified incubator at 37°C and 7% CO2 for 3 days. Cell counts were performed every 24 hrs with an American Optic Hemocytometer using standard procedures. A murine hybridoma cell line, B9, was selected for its IL-6 dependency (137). The B9 cell line (a generous gift from Dr. Lucien A. Aarden) was maintained in Iscove's Modified Dulbecco's Medium (IMDM), 5% fetal calf serum, 50 μM 2-mercaptoethanol, 1000 U/ml pencillin, 100 μg/ml streptomycin, and supplemented with a source of IL-6.

Dispersed Colony Assay of MIA C51 cells in Soft Agar: MIA C51 cells (500 cells/ml) were added to 0.3% agar-DME media with 10% fetal calf serum, 10% horse serum, 9.9 μg/ml asparagine, 37.5 μg/ml DEAE-dextran, 50 units/ml pencillin, 50 μg/ml streptomycin and 3.8 mg/ml sodium bicarbonate at 37°C. One ml of the final cell suspension mixture was pipetted into 35 mm petri dishes containing different concentrations of the sample in a final volume of 0.2 ml. After the agar solidified, the dishes were incubated for 6 days in a humidified incubator at 37°C and 7% CO_2. The total number of MIA C51 colonies and the number of dispersed MIA-C51 colonies were scored separately. Samples with series dilutions were assayed and only the linear range area was used (135). One unit of DF activity is defined as the activity that induces the formation of one dispersed MIA C51 colony in soft agar under the specified conditions (136).

Preparation of Rat Lung Conditioned Medium: All the steps were done under sterile conditions to prevent any microbial contamination. Rat lung conditioned medium was prepared according to a method described previously by Fojo *et al.* for the preparation of human lung conditioned medium (134). The rats were sacrificed and dissected to remove the lung tissue. The tissue was placed in sterile petri dishes, minced into small pieces, and washed with sterile phosphate buffered saline solution. The tissue was distributed in 2 liter tissue culture roller bottles with 0.1 g of tissue/ml of DME media containing 100 units penicillin, 100 μg

streptomycin, and 5 μg/ml lipopolysaccharide (LPS, Sigma). After incubation at 37°C for 3 days, the tissue debris was removed by centrifugation at 17,600 X g for 10 min, phenylmethyl-sulfonyl fluoride (1 mM) was added as a protease inhibitor, and the conditioned medium thus prepared was frozen for later use.

Chromatography of Rat Lung Conditioned Media on Ultrogel AcA 44 Gel Filtration: One liter of conditioned media was concentrated to 15-20 ml by ultrafiltration with an Amicon concentrator. A column containing Ultrogel AcA 44 (3 x 118 cm, LKB) was pre-equilibrated with 0.01 M Tris-HCl buffer (pH 7.6) containing 0.3 M NaCl, 0.01% NaN_3, and 0.01% PEG. The flow rate was 30 ml/hr, and fractions containing 10 ml/tube were collected. Protein concentration was measured by a UV monitor at A_{280}. Aliquots of samples from fractions 20-60 were desalted by PD-10 column chromatography (Pharmacia), filtered through microporous filters (Millipore) for sterilization, and assayed for CSF and DF activity by the soft agar cloning assay described above.

Ion-Exchange Chromatography of RLCM-DF on DEAE-HPLC: The active fractions (40-45) from the above gel filtration chromatography were pooled, concentrated (4-5 ml, T.P.=30-40 mg), dialyzed and applied to a DEAE-5PW HPLC column (21.5 mm x 150 mm, Waters). The column was pre-equilibrated with 10 mM triethylamine (pH 8.05), and 1mM EDTA. After loading the sample, the column was washed with the equilibration buffer. A linear

gradient of 0 to 125 mM NaCl in the equilibration buffer was applied, and 4 ml fractions were collected. Aliquots from alternate fractions were assayed for protein concentration, desalted, filtered, and assayed for CSF and DF activities.

Purification of DF by SDS-PAGE: Purification of DF to homogeneity was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 14%) according to the procedure of Laemmli (138). To prevent the possible oxidation of methionine residues, 100 mM thioglycolic acid was added to the cathode buffer and allowed to penetrate the gel by pre-running the gel for 30 min at 50 V. Proteins (reduced with β-mercaptoethanol) were then electrophoresed, and the gel was either stained (by Coomassie blue R250 stain or silver stain) or the proteins were elctrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore).

Protein Electroblotting: The gel was soaked in the transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 11, 10% methanol) for 5 min. The PVDF membrane was soaked in 100% methanol for few min followed by a 5 min soak in the transfer buffer (139). Transfer of the proteins to the PVDF membrane was carried out at 200 mA for 45 min in the transfer buffer. The membrane was washed in deionized H_2O for 5 min, stained (with 0.05% Coomassie blue R250 in 40% methanol, and 5% acetic acid), destained (in 50% methanol, and 10% acetic acid), washed in

deionized H_2O, and air dried. The DF band was visualized by Coomassie blue R250 stain, excised with a razor blade, and stored in a microfuge tube at -20°C until used.

CNBr Digestion of the immobilized DF: Each band of DF (immobilized on a PVDF membrane) was treated with 2 mg of cyanogen bromide (CNBr, dissolved in 200 µl of 70% formic acid) for 16 hrs at room temperature. CNBr solution was then transferred to an Eppendorf tube, and allowed to dry in a speed-vac centrifuge. At the same time, an elution solvent (70% isopropanol, and 2 µl/ml of trifluoroacetic acid) was incubated with the PVDF membrane (containing DF) for 2 hrs. The elution solvent was then transferred to the Eppendorf tube containing the CNBr solution, and the membrane was reextracted with another 200 µl of the elution solvent for another 2 hrs. This last elution solvent was then be added the tube containing the two pooled solutions and allowed to dry in a speed-vac centrifuge. The eluted peptides were separated by SDS-PAGE (16.5%) that was pre-run for 30 min at 50 V with a cathode solution containing 100 µM thioglycolic acid. Gels were either stained with silver stain (BIO-RAD), or electophoretically transferred to a PVDF membrane. The PVDF membrane was washed with deionized water, stained, destained, and the band to be sequenced was excised with a razor blade.

Amino Acid Composition Analysis: The amino acid composition of purified DF was determined by hydrolysis at 110°C

for 24 hrs in 100 µl of 6 N HCl (Pierce) (according to the method described by Moore and Stein, 140), in vacuo, in an aluminum block of Pierce Reacti-therm heating module. The ampoule was cooled, opened, and dried overnight in a vacuum dessicator, and redissolved in 100 µl of HPLC grade water. Fifty microliter of the sample was injected into Interaction AA511 column (Pierce), and the amino acids were detected with O-phtaldialdehyde (OPA)/Borate (Pickering).

Amino Acid Sequence Analysis: Excised PVDF membranes containing the DF band was placed in the sequencer cartridge and sequenced on an Applied Biosystems model 475A sequenator (141). The identification of phenylthiohydantoin (PTH)-amino acid derivatives was carried out by reverse-phase HPLC over a C-18 column.

Molecular Weight Determination: The molecular weight of RLCM-DF was estimated by SDS-PAGE (14%) according to the procedure of Laemmli (138). Three DF samples were loaded on the gel, the first sample was heated at 100°C for 5 min with the sample buffer (lane 1), while the other two samples were not heated (lane 2 and 3). After running, the gels for lane 1 and 2 were stained with Coomassie blue stain, while lane 3 was sliced, extracted and the eluates were assayed for DF activity. The molecular weights were estimated from the linear plot of logarithmic molecular weights versus the relative mobilities of protein standards. Protein markers

used were: Phosphorylase B (rabbit muscle) (97.4 KDa, R_f = 0.127); Bovine serum albumin (66.2 KDa, R_f = 0.186); Ovalbumin (hen egg white) (45.0 KDa, R_f = 0.305); Carbonic anhydrase (bovine) (31.0 KDa, R_f = 0.458); Soybean trypsin inhibitor (21.5 KDa, R_f = 0.619); Lysozyme (hen egg white) (14.4 KDa, R_f = 0.754) (BIO-RAD). Standard proteins were located using Coomassie blue stain.

Protease Digestion: A stock solution was prepared for trypsin by dissolving 10 mg of the enzyme in 1 ml of 0.01 M Tris-HCl (pH 8.0) and 0.01 M $CaCl_2$. Aliquots of DF (5000 U) at the DEAE-HPLC stage of purification were prepared in 1 ml samples of the Tris-$CaCl_2$ buffer before adding 50 µg of the enzyme from the stock solution. The solution was incubated at 37°C for 1 hr. At the end of incubation, the sample was chilled on ice immediately and 0.1 ml of fetal calf serum (FCS) was added to the incubation mixture. The samples were dialyzed, sterilized and assayed for DF activity.

B9 Assay for IL-6: A murine hybridoma cell line (B9) was selected for its IL-6 dependency. The B9 assay is based on the fact that IL-6 stimulates proliferation and thymidine incorporation in B9 cell line. B9 cells (1 X 10^5/ml of cultured media) were incubated with different concentrations of either recombinant human IL-6 (National Institute for Biological Standards and Control) or isolated DF for 68 hrs at 37°C in a humidified incubator. The cells were pulsed with 2 µCi of [^{3}H]-thymidine for 3 hrs. Washed cells with PBS were then lysed by the addition of a hypotonic buffer (10 mM

phosphate buffer, pH 7.2), and incorporation of labeled thymidine into nucleic acids was determined after the lysate was treated with 15% TCA for 1 hr. Precipitated material on glass-fiber filters (American Scientific Products) was washed with 15% TCA solution, air-dried, and the filters were then placed in a vial of 5 ml scintillation fluid for determination of radioactivity by liquid scintillation counting (Beckman, LS 5000TD).

Effect of Anti-Rat IL-6 Antibody on DF Activity: Twenty five µl of rabbit serum containing anti-rat IL-6 antibody (a generous gift from Dr. George H. Fey) was added to 3000 units of the DF activity in 1 ml of TNE-buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, and 1 mM PMSF). The solution was incubated for 16 hrs at 4^{o}C (142). To the incubation mixture, 100 µl of Pansorbin (binding capacity of 1.95 mg IgG/ml, Calbiochem) was then added for 2 hrs at room temperature with gentle agitation. The complex was then collected by centrifugation for 5 min at 1000 X g and the supernatant was dialyzed, filtered, and assayed for DF ability to form dispersed MIA C51 colonies in soft agar.

IL-6 Receptor Binding Assay: IL-6 receptor binding assay was performed as described by Nicola *et al.* (143). Briefly, two million KG-1 cells in 120 µl of binding media (RPMI-1640 medium, 2 mg/ml BSA, 5 X 10^4 cpm of ^{125}I-IL-6 [iodinated by the chloramine-T method as previously described (6)], and 20 mM Hepes, pH 7.3) were incubated with various concentrations of recombinant human IL-6 or

DF at 4°C for 8 hrs. After incubation, the reaction mixture was layered onto 200 µl of cold FCS in a 400 µl centrifuge tube and centrifuged at 400 X g for 5 min. The tips were excised and counted in a Packard gamma counter model 5002 (Packard instrumental Co., Meriden, CT). Radioactivity was counted as total binding.

[^{3}H]PDBu Binding Assay: MIA C51 or U-937 cells ($1x10^6$) were washed twice with PBS, then resuspended in 0.5 ml of binding medium (DME, 20 mM Hepes (pH 7.4), 4 mM glutamine, 1 mg/ml BSA) (144). Different concentrations of [^{3}H]PDBu (specific activity 13.2 Ci/mmol, Dupont-NEN) were added in the absence and presence of 20 µM unlabeled PDBu. The reaction mixture was incubated for 30 min at 37°C in a microfuge tube with gentle shaking. Cells were then washed twice with cold binding medium. The cell pellet was then transferred to a scintillation vial by slicing off the bottom of the tube with a razor blade. Specific binding was determined by subtracting the nonspecific binding from the total cell-bound radioactivity.

Protein Kinase C Assay: Cells ($1x10^7$ of MIA C51 or U-937) were homogenized in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 1 mM PMSF, 30 mM 2-mercaptoethanol). The lysed cells were incubated with 2% Triton X-100 for 30 min at 4°C, followed by centrifugation at 1000 X g for 5 min. Cellular extracts were applied to a 1 ml DE-52 column that had been previously equilibrated with the lysis buffer. The column was then

washed with 10 ml of the lysis buffer. PKC was eluted with the lysis buffer that contains in addition 0.1 M NaCl, and 2 ml fractions were collected. PKC activity was determined by the transfer of $^{32}PO_4$ from [γ-^{32}P] ATP to histone, type V-S (Sigma) (145). Briefly, the reaction mixture contained 50 µl of the DE-52 eluate, 40 µg of histone, 10 mM magnesium acetate, 0.75 mM $CaCl_2$, and 100 µM (γ-^{32}P) ATP in the presence and absence of 25 µg phosphatidyl-serine (Avanti Polar-Lipids, Inc.), and 2 µg of L-α-1-oleoyl-2-acetoyl-glycerol (OAG) (Avanti Polar-Lipids, Inc.) in a final volume of 100 µl. After 10 min incubation at 30°C, the reaction was terminated by the addition of 60 µl of 60 % TCA. An aliquot (80 µl) was spotted onto Whatman P-81 filter paper. The filter paper was then washed in 30% acetic acid for 15 min, 15% acetic acid for 10 min (3 times), and acetone for 5 min. The radioactivity on the dried P-81 filter paper was then determined by a liquid scintillation counter (Beckman, LS 5000TD).

Western Blot Analysis: Protein concentration of the eluted samples from the DE-52 column (0.1 M NaCl) was estimated by the Bradford method (BIO-RAD). Proteins (10 µg) from either MIA C51 or U-937 eluates were resolved by 10% SDS-PAGE at a constant current of 15 mA for 2-3 hrs using the BIO-RAD minigel system. The gel was then either stained with Coomassie blue or electrophoretically transferred to a nitrocellulose membrane in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol for 2 hrs at a constant current of 150 mA with a mini Trans-Blot cell, BIO-RAD. After

transfer, the nitrocellulose membrane was blocked for 1 hr in 3% gelatin added to buffer A (20 mM Tris-HCl (pH 7.6), 0.5 M NaCl, and 0.05% Tween-20). The membrane was then washed (3X) with buffer A and incubated overnight at 4°C with sheep serum containing anti-PKC polyclonal antibody (a generous gift from Dr. Karen L. Leach, The Upjohn Company, Kalamazoo, MC) at a dilution of 1:1000 in buffer A containing 0.1% gelatin. The nitrocellulose membrane was then washed with buffer A followed by a 2 hr incubation with 1:1000 dilution of anti-sheep IgG conjugated to horseradish peroxidase (Sigma) in buffer A and 0.1% gelatin. Immunoreactive bands were visualized by treatment with 4-chloro-1-naphthol.

Determination of Intracellular cAMP Content: MIA C51 cells suspended at 3 X 10^5 cells/ml of culture media in a polypropylene microtube were left to equilibrate for 20 min in a 37°C water bath. Cells were incubated with or without 1500 U of DF for different incubation periods. To terminate the incubation, cells were pelleted in an Eppendorf microfuge at 12000 X g for 20 sec and 0.2 ml of a boiling 1/200 dilution of concentrated glacial acetic acid was added (146). The cells were then boiled for 5 min, centrifuged for 10 min at 12000 X g, and the supernatant was allowed to dry in a speed-vac centrifuge. The dried samples were then resuspended in 200 µl of the assay buffer (0.05 M Na acetate buffer, pH 5.8, with 0.01% thimerosal), and cAMP levels were measured using an Amersham ^{125}I-cAMP RIA kit according to manufacturer's directions.

Adenylate Cyclase Assay: MIA C51 cells were harvested, washed with PBS, resuspended in a lysis buffer (20 mM Tris-HCl, pH 7.0, 0.2 mM EGTA, 0.7 mM EDTA, 0.5 mM PMSF, and 10 mM β-mercaptoethanol), and disrupted by sonication. The homogenate was first centrifuged at 1000 X g for 5 min to remove cell debris then at 12000 X g for 10 min in an eppendorf microfuge at 4°C to form a crude membrane pellet. Adenylate cyclase activity was assayed by measuring the formation of cAMP from [α-^{32}P]ATP by MIA C51 crude membranes. The standard incubation mixture contained 25 mM Tris-HCl, pH 7.8, 0.5 mM EGTA, 1 mM [α-^{32}P]ATP (20 cpm/pmol), 5 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 5 µg/ml pyruvate kinase, 8 µg/ml myokinase, different concentrations of DF, and 3-5 µg of crude membrane protein, used as a source of adenylate cyclase activity, in a final volume of 150 µl (135). The reaction was followed for 30 min at 37°C. To terminate the reaction, 100 µl of 2% SDS, 40 mM ATP and 1.4 mM cAMP was added to the reaction mixture followed by the addition of 250 µl distilled H_2O. The sample was then loaded into 0.75 g of AG 50W-X4 column (BIO-RAD). The column was eluted with deionized H_2O. The first fraction (1 ml of H_2O) contained most of the ATP and ADP. The cAMP fraction was eluted next with 3 ml of H_2O. To the cAMP fraction, 200 µl of 0.3 N $ZnSO_4$ was added followed by 200 µl of 0.3 N $Ba(OH)_2$. After mixing, the tubes were centrifuged and the supernatant was used for quantitation of radioactivity (147). The adenylate cyclase activity is expressed as picomoles of cAMP synthesized/min.

Measurement of [^{3}H]-Inositol Phosphates: MIA C51 cells seeded at 0.7 X 10^6 cells/ml were labeled with 5 μCi/ml of myo-[2-^{3}H] inositol (16.3 Ci/mmol, Amersham) for 24 hrs at 37°C in culture media (148). This will be followed by a three time wash with PBS and incubation of the cells for 1 hr in DMEM supplemented with 35 mg/ml of unlabeled myo-inositol and 10 mM LiCl to inhibit the breakdown of free inositol phosphates to inositol. MIA C51 cells at a density of 2 X 10^6 cells/ml were then incubated either with DF (2000 U/ml), Bt_2cAMP (400 μM), or with no addition for different incubation periods. The reaction was stopped by spinning the cells for 20 sec in an Eppendorf microfuge followed by the addition of 1 ml of 1 : 2 mixture of chloroform : methanol. The cells were homogenized, and the aqueous and organic phases were separated after the addition of 1 N HCl (0.5 ml) and centrifugation of the sample for 10 min at 1000 X g. The aqueous phase was then neutralized with 1 N NaOH before been applied to a 1 ml anion exchange AG1-X4 column (formate form, 200-400 mesh, BIO-RAD). The column was first eluted with 15 ml of H_2O to remove free inositols. Subsequent fractions were eluted with a) 10 ml of 5 mM sodium tetraborate and 60 mM sodium formate, pH 9, which elutes the glycerophosphoinositol, b) 9 ml of 0.2 M sodium formate in 0.1 M formic acid which elutes IP, c) 9 ml of 0.4 M sodium formate in 0.1 M formic acid which elutes IP_2 and d) 9 ml of 1.0 M sodium formate in 0.1 M formic acid which elutes IP_3 (149, 150). An Aliquot from each fraction was then used for quantitation of radioactivity.

Phospholipase C Assay: MIA C51 cells (suspended at 3 X 10^5/ml of culture media in sterile polypropylene microtubes) were allowed to equilibrate for 20 min in a 37°C water bath. Cells were incubated with either DF (1500 U), Bt_2cAMP (400 μM), or with no addition for different incubation periods. The reaction was stopped by pelleting the cells for 20 sec at high speed in an Eppendorf microfuge. Washed cells were sonicated in 400 μl of 20 mM Tris-HCl, pH 7.0, 0.2 mM EGTA, 0.7 mM EDTA, 0.5 mM PMSF and 10 mM β-mercaptoethanol at 4°C. The cell homogenate was centrifuged at 1000 X g at 4°C for 5 min followed by 10 min centrifugation at 12000 X g. The supernatant fraction was used as a source for phospholipase C (PLC) activity. The reaction mixture included 1 mg/ml deoxycholate, 25 mM Tris-HCl, pH 7.0, 3 mM $CaCl_2$, 100 mM NaCl, 10 μM [^{3}H]-PIP_2 (11 cpm/pmol), and 3-5 μg of phospholipase C source in a final volume of 150 μl (151, with minor changes). The reaction was followed for 20 min and stopped with 0.5 ml of chloroform : methanol (1 : 2). The aqueous phase was separated by the addition of 1 N HCl (0.25 ml) followed by centrifugation. An aliquot (400 μl) of the aqueous phase was then used for quantitation of radioactivity.

Intracellular Calcium Measurement: To 1 X 10^6 MIA C51 cells/ml of culture media, 10 μM of Quin-2/AM was added and incubated for 60 min at 37°C. Cells were then washed and resuspended in solution A (150 mM NaCl, 3.7 mM KCl, 3 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 0.4 mM $MgSO_4$, 5.6 mM glucose, 0.1% BSA, 1 mM

$CaCl_2$, and 10 mM Hepes, pH 7.4). Fluorescence of the cells was measured using a spectrofluorimeter in a ratio mode. Standard monochromator settings were 339-nm excitation with 4-nm slits and 492 nm emission with 10 nm slits (152). DF or A23187 ionophore were added to the cells and the fluorescence was detected for 30 min. Maximum fluorescence (F_{max}) was obtained after the addition of 0.1% Triton X-100 to the cell suspension. Meanwhile, minimum fluorescence (F_{min}) was obtained after the addition of 1 mM $MnCl_2$ (quenches fluorescence).

RNA Isolation and Northern Blot: Total cellular RNA was obtained from 3-5 $X10^7$ control or TPA-treated (1 μg/ml) or Bt_2cAMP-treated (400 μM) MIA C51 cells after lysis in 4 M guanidinum isothiocyanate and centrifugation through 5.7 M cesium chloride as described by Chirgwin *et al.* (153). Cellular RNA (20 μg) was separated on 1.0% agarose gels containing 6.7% formaldehyde. Fractionated RNA was transferred to a nylon membrane (Hybond-N, Amersham) in 10 X SSC (1 X SSC = 150 mM NaCl/15 mM Na citrate, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.0) and was fixed to the membrane by exposure to a UV light for 5 min. Membranes were prehybridized at 50°C overnight in 5 X SSC, 2 X Denhardt's solution (2 X Denhardt's = 40 mg Ficoll, 40 mg polyvinylpyrrolidone, and 40 mg BSA in H_2O at a final volume of 100 ml), 50 mM NaH_2PO_4, pH 7.0, 20% formamide, 10% dextran sulfate, 1 mM sodium pyrophosphate, ATP (50 μg/ml), and 500 μg/ml salmon sperm DNA (154). Ten to twenty million cpm of rat *c-fos* probe (Oncogene Science, Inc.) was

added to 5 ml of prehybridization solution and the membrane was hybridized at 50°C for 12-16 hrs. The membrane was washed sequentially in 2 X SSC/0.1% SDS at 25°C, 37°C, and 50°C and exposed to X-ray film (Kodak XAR-5) using an intensifying screen at -80°C for 17-72 hrs.

CHAPTER III

RESULTS

Purification and Characterization of DF: The purification of a leukemia differentiation factor (DF) from LPS-stimulated rat lung conditioned medium is summarized in Table III. Approximately one liter of rat lung conditioned media (RLCM) was concentrated by ultrafiltration. The total protein concentration of the concentrated RLCM was 900 mg with a total activity of 18 million DF units. Both the differentiation activity (as assayed by its ability to induce dispersed colony formation of MIA C51 cells in soft agar) and the CSF activity were detected at fractions 40-45 on gel filtration chromatography with an apparent molecular weight of 20-30 KDa (Fig.1A, Fig.1B). This overlap between DF and CSF activity was due to the similarity in their molecular weight. The concentrated DF-active fractions had a total protein concentration of 41 mg with a total activity of 12 million DF units. At this stage, the yield was 67% with a 15-fold purification.

The DF-active fractions pooled from AcA 44 Ultrogel filtration chromatography were concentrated, dialyzed, and applied to an ion exchange DEAE-HPLC column. As shown in Fig.2A, the DF activity was eluted at 40-60 mM NaCl. Two CSF active regions were detected which were eluted at 70-100 mM NaCl (Fig.2B). Although some CSF

TABLE III

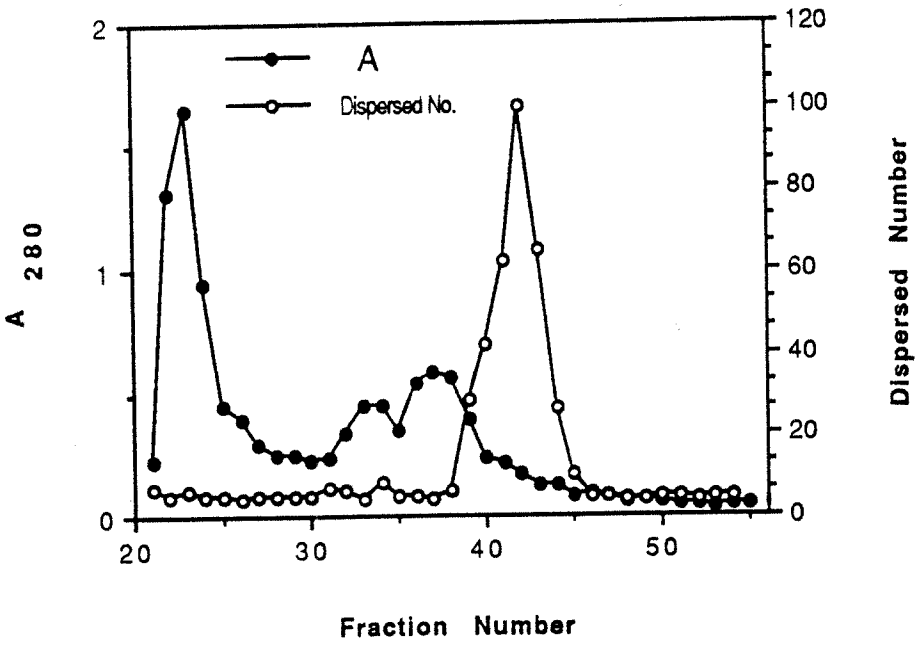
Purification of a Differentiation Factor from Rat Lung Conditioned Medium

Step	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Conditioned media	9.0×10^2	1.8×10^7	2.0×10^4	1	100
AcA 44 column	4.1×10	1.2×10^7	2.9×10^5	15	67
DEAE-HPLC	3.4	3.0×10^6	8.8×10^5	44	17

One DF unit is defined as one dispersed MIA C51 colony in the soft agar cloning assay.

Fig.1 Gel Filtration Chromatography of Rat Lung Conditioned Media: One liter of rat lung conditioned media was concentrated (15-20 ml) and applied on an ultrogel AcA 44 gel filtration chromotography column (3 x 118 cm). The column was eluted with 0.01 M Tris-HCl, pH 7.6, which contains 0.3 M NaCl. Fractions 20-60 were assayed for dispersed colony assay of MIA C51 cells in soft agar (A), and for colony-stimulating factors activity (B).

A.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Dispersed No.
A 280
Dispersed Number
Fraction Number
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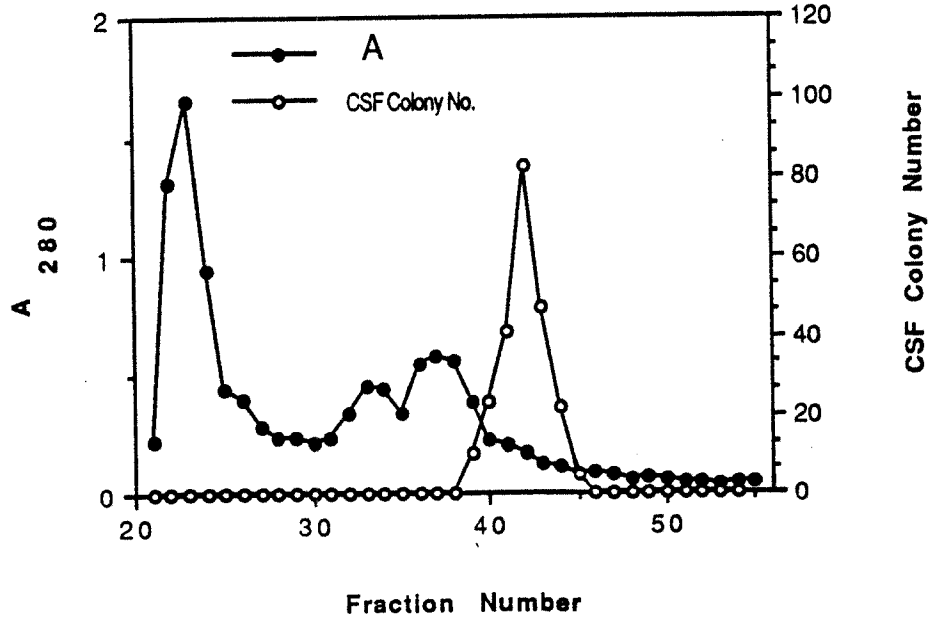
2
20
30
40
50
0
20
40
60
80
100
120

B.


A
CSF Colony No.
A 280
CSF Colony Number
Fraction Number
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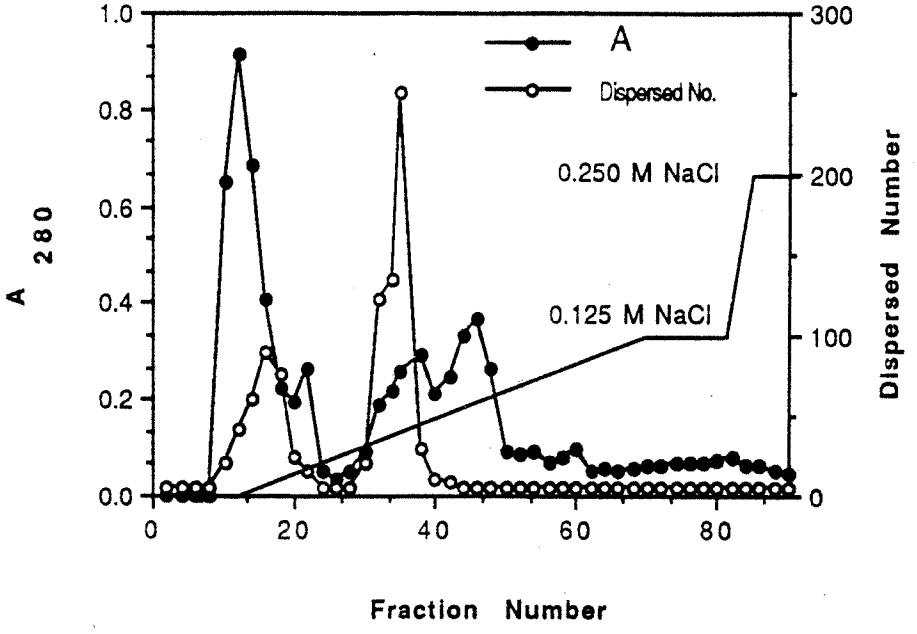
1
2
20
30
40
50
0
20
40
60
80
100
120

Fig.2 DEAE-HPLC Ion-Exchange Chromatography: DF-Active fractions from AcA 44 gel filtration chromatography (Fig.1A, 40-45) were pooled, concentrated (4-5 ml), dialyzed, and applied to a DEAE-5PW high pressure liquid chromatography (HPLC) column (Waters). A linear gradient of 0 to 125 mM NaCl in 10 mM triethylamine (pH 8.05) and 1 mM EDTA was used to elute the column. The fractions were assayed for dispersed colony formation of MIA C51 cells in soft agar at 10 µl (A), and for colony-stimulating factors activity at 200 µl (B).

A.


A
Dispersed No.
0.250 M NaCl
0.125 M NaCl
A 280
Dispersed Number
Fraction Number
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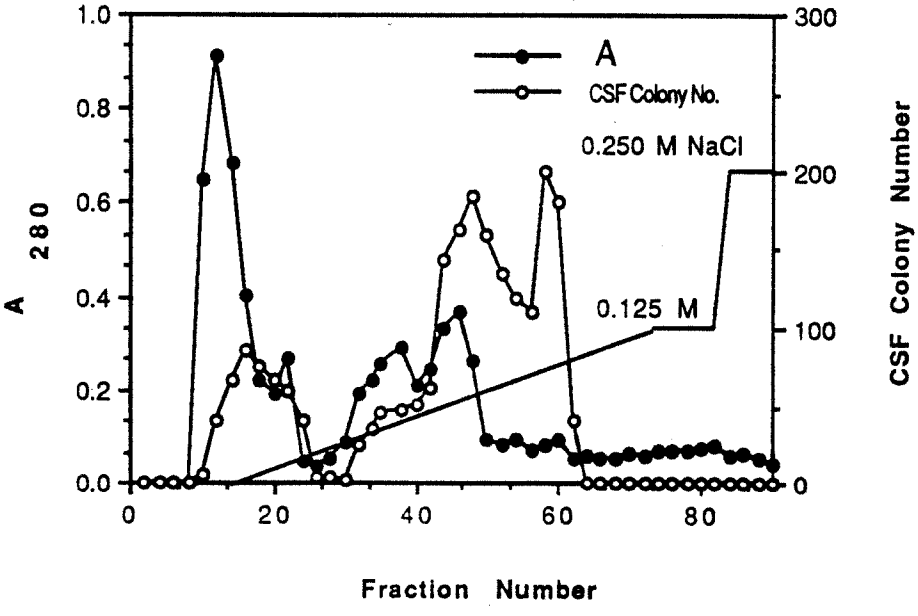
0.8
0.6
0.4
0.2
0.0
300
200
100
0
0
20
40
60
80

B.


A
CSF Colony No.
0.250 M NaCl
0.125 M
A 280
CSF Colony Number
Fraction Number
1.0
0.8
0.6
0.4
0.2
0.0
300
200
100
0
0
20
40
60
80

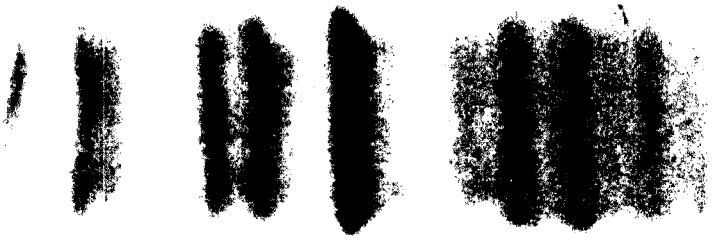
activity is still present in the DF-active fractions, chromatography on ion exchange DEAE-HPLC column was effective in separating most of the CSF activity from the DF activity which indicated that the DF activity was due to a distinct factor that was not associated with the CSF activity. Concentrated DF-active fractions had a total protein concentration of 3.4 mg with a total DF acitvity of 3 million DF units and 3 x 10^4 CSF units.

Further purification of DF was performed by polyacrylamide gel electrophoresis (SDS-PAGE, 14%). Assay of the extracted proteins for the DF activity have indicated that the DF activity was associated with a 24 KDa protein band (Fig.3). After electrophoresis, the proteins were electrophoretically transferred to a PVDF membrane. The 24 KDa band was excised and used either for amino acid composition analysis, or for amino terminal sequencing.

The amino acid composition analysis of DF is shown in Table IV. Based on the amino acid composition analysis the minimum molecular weight of native DF was 18.7 KDa (assuming the total number of amino acid residues is 152), which is smaller than that estimated by SDS-PAGE (24 KDa). Moreover, amino acid composition analysis showed the presence of one methionine residue only.

Amino terminal sequencing of the excised DF band did not give any specific amino acid signal. When the excised DF band was subjected to CNBr treatment, two apparent cleavage peptides were detected by silver stain (a large peptide of 14 KDa Mr and a smaller peptide). (Fig.4 D). After electrophoresis, the cleaved peptides were transferred to a PVDF membrane. Coomassie blue stain detected only

Fig.3 Molecular Weight Determination of DF: For the determination of DF molecular weight, DF was loaded onto a 14% SDS-PAGE. After electrophoresis, the gel was sliced into strips 2.2 mm in width, minced, extracted in PBS containing 1 mg BSA/ml, dialyzed, and assayed for dispersed colony formation of MIA C51 cells in soft agar. The standard protein markers used were: Phosphorylase B (97.4 KDa, R_f = 0.127); BSA (66.2 KDa, R_f = 0.186); Ovalbumin (45.0 KDa, R_f = 0.305); Carbonic anhydrase (31.0 KDa, R_f = 0.458); Soybean trypsin inhibitor (21.5 KDa, R_f = 0.619); and Lysozyme (14.4 KDa, R_f = 0.754). The molecular weights were estimated from the linear plot of logarithmic molecular weights versus the relative mobilities of standard proteins. Also, SDS-PAGE (14%) profile of concentrated DF-active fractions from DEAE-HPLC is shown.



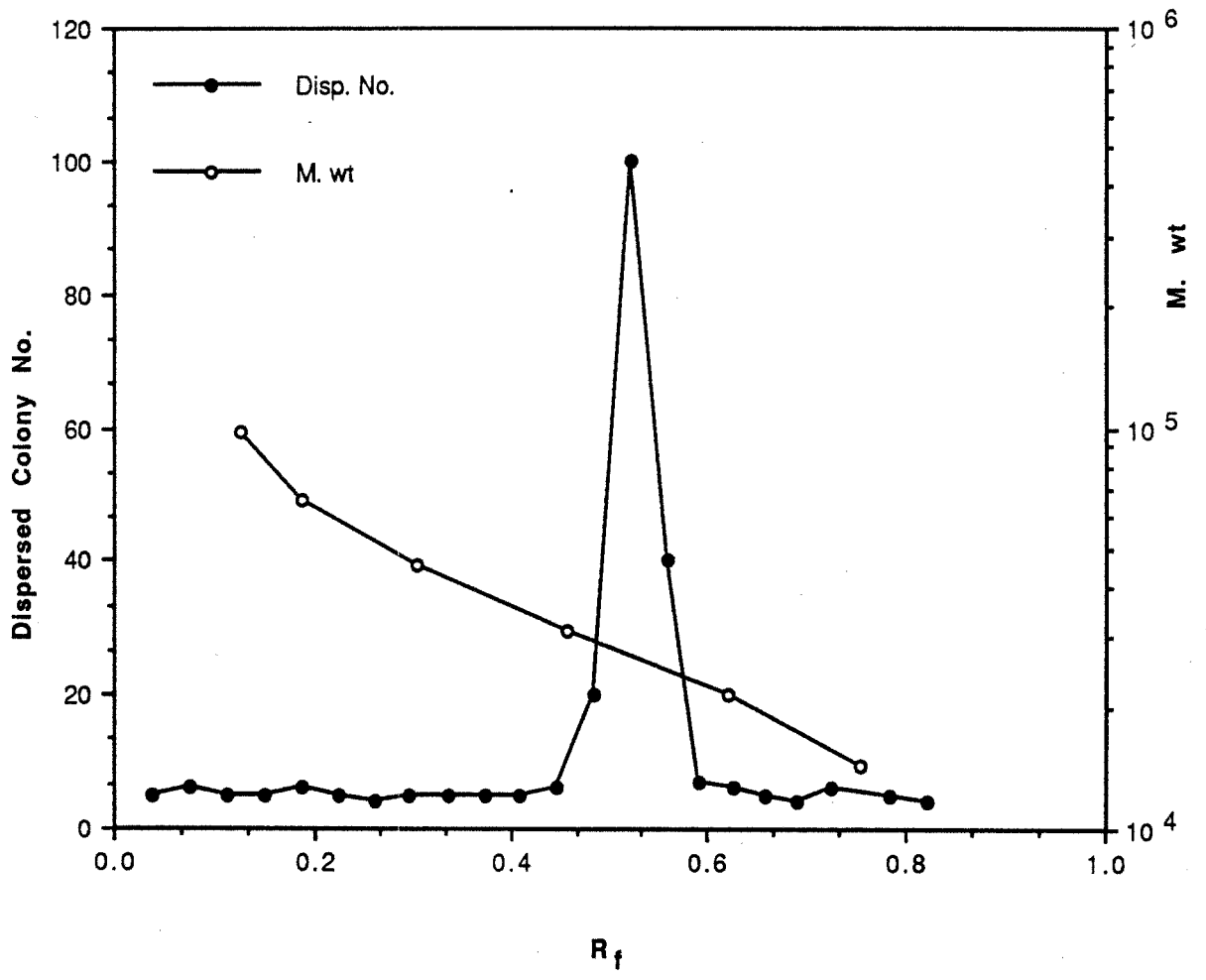

Disp. No.
M. wt
Dispersed Colony No.
M. wt
120
100
80
60
40
20
0
10^6
10^5
10^4
0.0
0.2
0.4
0.6
0.8
1.0
R_f

TABLE IV

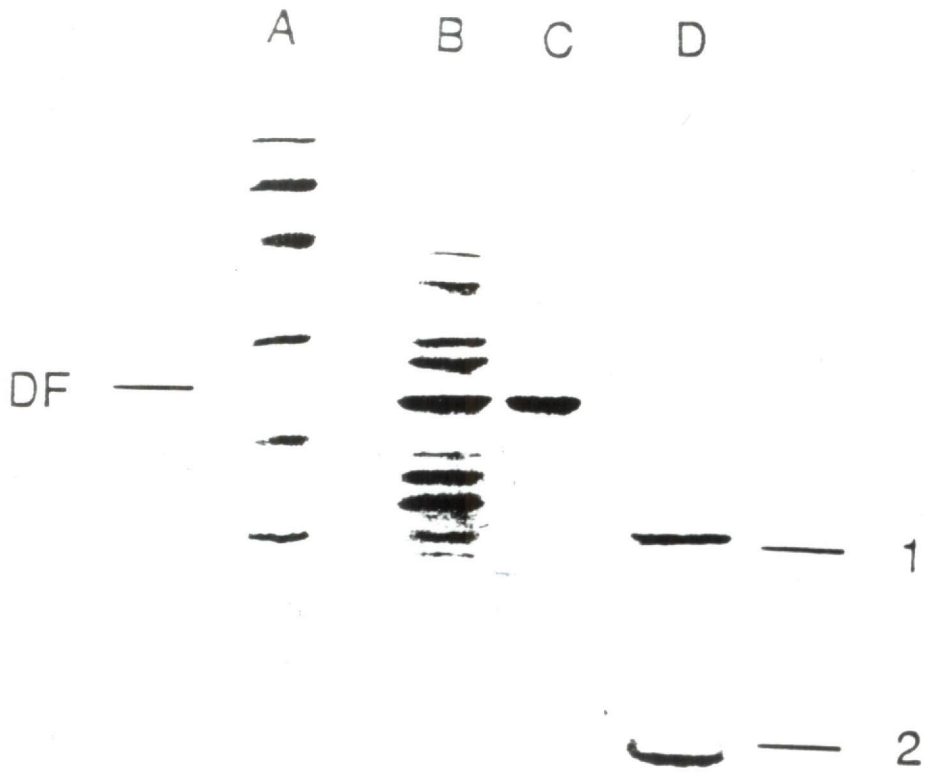
Amino Acid Composition of DF

Amino Acid Residue	DF Normalized Mole Ratio
Asx	11.2
Thr	7.9
Ser	10.8
Glx	17.5
Pro	ND
Gly	22.0
Ala	14.8
Cys	ND
Val	13.0
Met	1.0
Ile	8.0
Leu	16.9
Tyr	0.0
Phe	8.2
His	6.1
Lys	8.3
Trp	ND
Arg	7.2

ND, not determined

Purified DF was hydrolyzed at 110°C for 24 hrs in 100 µl of 6 N HCl in vacuo, dried in vacuo and redissolved in 100 µl of HPLC grade water. Sample was then injected into Interaction AA511 column and the amino acids were detected with OPA.

Fig.4 Effect of CNBr Treatment on DF: SDS-PAGE (16.5%) profile of: A) the standard protein markers used {Phosphorylase B (97.4 KDa); BSA (66.2 KDa); Ovalbumin (45.0 KDa); Carbonic anhydrase (31.0 KDa); Soybean trypsin inhibitor (21.5 KDa); and Lysozyme (14.4 KDa)}, B) concentrated DF-active fractions from DEAE-HPLC column chromatography, C) eluted DF band from PVDF membrane, and D) cleaved peptide fragments of DF by CNBr.


A
B
C
D
DF
1
2

the 14 KDa peptide. Sequencing of the 14 KDa peptide failed to detect any specific amino acid signal indicating that this peptide is the N-terminal fragment of the protein. Further effort is needed to sequence the other peptide.

DF was sensitive to proteolytic digestion by trypsin. More than 90% of DF activity was lost after trypsin digestion indicating the proteinacious nature of DF (Table V).

Effect of DF on MIA C51 cells: In response to DF, cultured MIA C51 cells undergo terminal differentiation to monocyte/macrophage cells as evidenced by morphological as well as enzyme marker expressions. While control untreated MIA C51 cells showed the morphological characteristics of myeloblast cells, DF-treated MIA C51 cells showed monocyte-like morphology. In addition, treatment of MIA C51 cells with DF enhanced their ability to phagocytize particles and increased their lysozyme and non-specific esterase activities (155).

MIA C51 cells form compact colonies in soft agar. When an inducer (such as DF) is added to the culture medium, these cells form dispersed colonies in soft agar. The formation of dispersed colonies in soft agar has been seen in many systems including bone marrow granulocyte-macrophage progenitor cells. Similar dispersed colony formation is also seen when WEHI-3BD+ cells are treated with G-CSF (156).

Addition of DF to MIA C51 cells in culture inhibited the proliferation of MIA C51 cells in a concentration dependent manner

TABLE V

Trypsin Digestion of DF

Treatment	Dispersed Colony No.	Compact Colony No.
1. DF + FCS	112	5
2. DF + FCS + Trypsin	117	4
3. DF + Trypsin + FCS	20	132

The data are the average of duplicate samples.

DF (5000 U/ml of 0.01 M Tris-HCl, pH 8.0, and 0.01 M $CaCl_2$) was incubated with (3) or without (1, 2) 50 µg of trypsin at 37°C for one hour. At the end of the incubation, samples were chilled on ice immediately followed by the addition of 0.1 ml of FCS. After the addition of FCS to tube number 2, 50 µg of trypsin was added. Samples were then dialyzed, sterilized, and assayed for DF activity.

(Fig.5). A concentration of 10000 U/ml inhibited the proliferation of MIA C51 cells completely and resulted in cell death.

The relationship between DF and IL-6: DF stimulated the proliferation of an IL-6-dependent murine hybridoma B9 cell line as measured by [^{3}H]-thymidine incorporation assay (Fig.6). Maximum incorporation of thymidine into B9 cells was obtained at a DF concentration of 400 units/ml. The B9 assay is specific for IL-6 and is not influenced by a variety of other cytokines except for murine IL-4 which shows some activity in this assay (157).

At the same time, recombinant human IL-6 induced the formation of dispersed MIA C51 colonies in soft agar (similar to DF action). As shown in Fig.7, IL-6 at a concentration higher than 0.5 U/ml but lower than or equal to 10 U/ml induced dispersed colony formation of MIA C51 cells in soft agar in a dose-dependent manner. IL-6 also inhibited the proliferation of MIA C51 cells in cell culture in a concentration dependent manner (Fig.8). Maximal inhibition of cell growth was obtained at an IL-6 concentration of 100 U/ml.

This similarity in action between IL-6 and DF suggested a relationship between the two proteins. Incubation of DF with a polyclonal rabbit anti-rat IL-6 serum followed by immuno-precipitation did not affect DF activity. The DF activity in the supernatant of the sample that was incubated with the antibody against rat IL-6 and immunoprecipitated was similar to the control activity, similar to the activity of the sample that was incubated with the antibody but not immunoprecipitated, and similar to the

Fig.5 Effect of DF on MIA C51 cell proliferation: Rat chloroleukemia MIA C51 cells (1 X 10^5 cells/ml of culture media) were incubated with different concentrations of DF for 3 days. Every 24 hrs cell number was counted using an American Hemocytometer by a standard procedure.

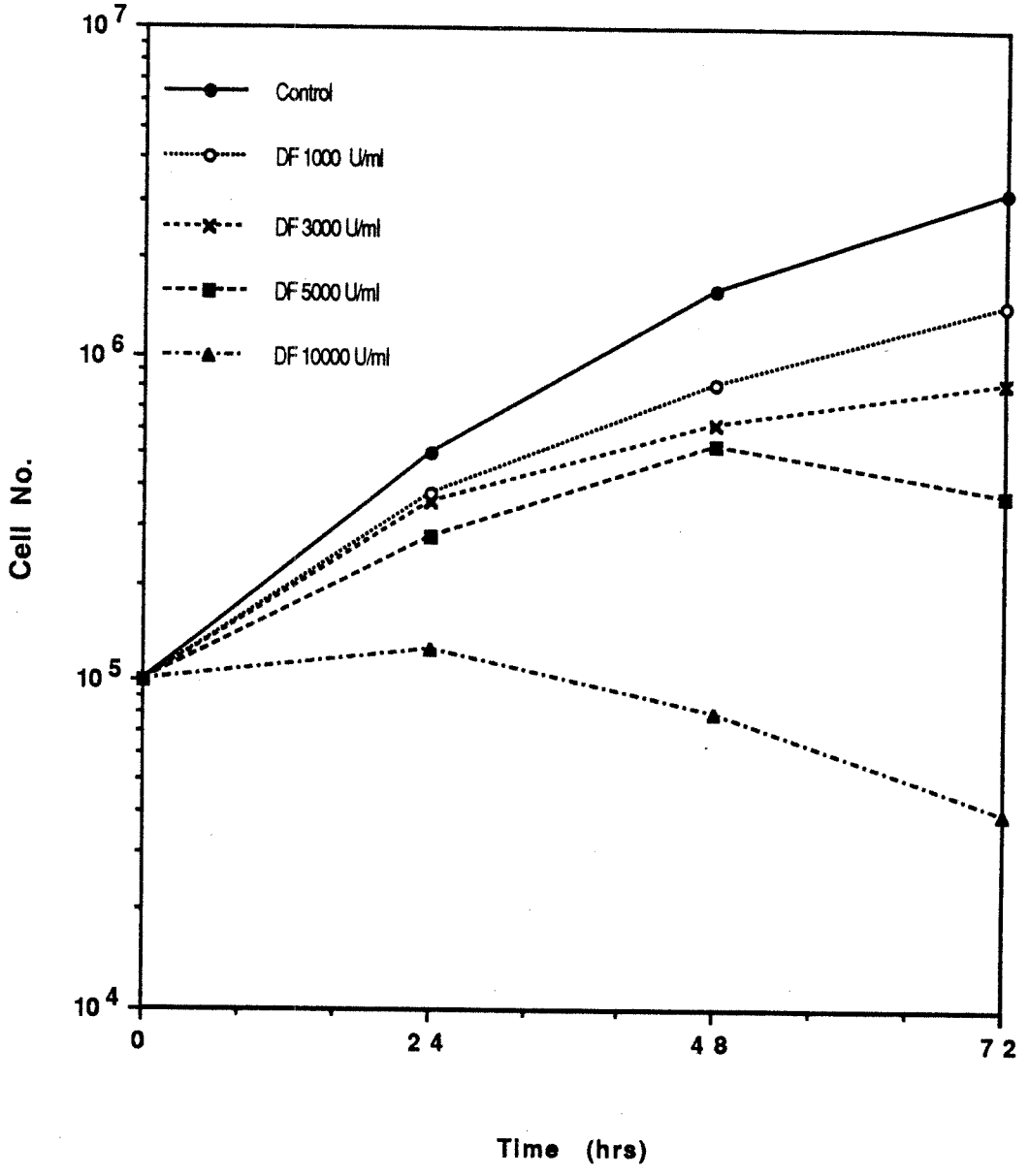

10 7
10 6
10 5
10 4
Cell No.
Control
DF 1000 U/ml
DF 3000 U/ml
DF 5000 U/ml
DF 10000 U/ml
0
2 4
4 8
7 2
Time (hrs)

Fig.6 Effect of DF on the Proliferation of B-9 Cells: Cultured B-9 cells (1 X 10^5 cells/ml of culture media) were incubated with or without different concentrations of DF for 68 hrs. Cells were then pulsed with 2 μCi of ^{3}H-thymidine for 3 hrs and the radioactivity in the nuclei was counted.

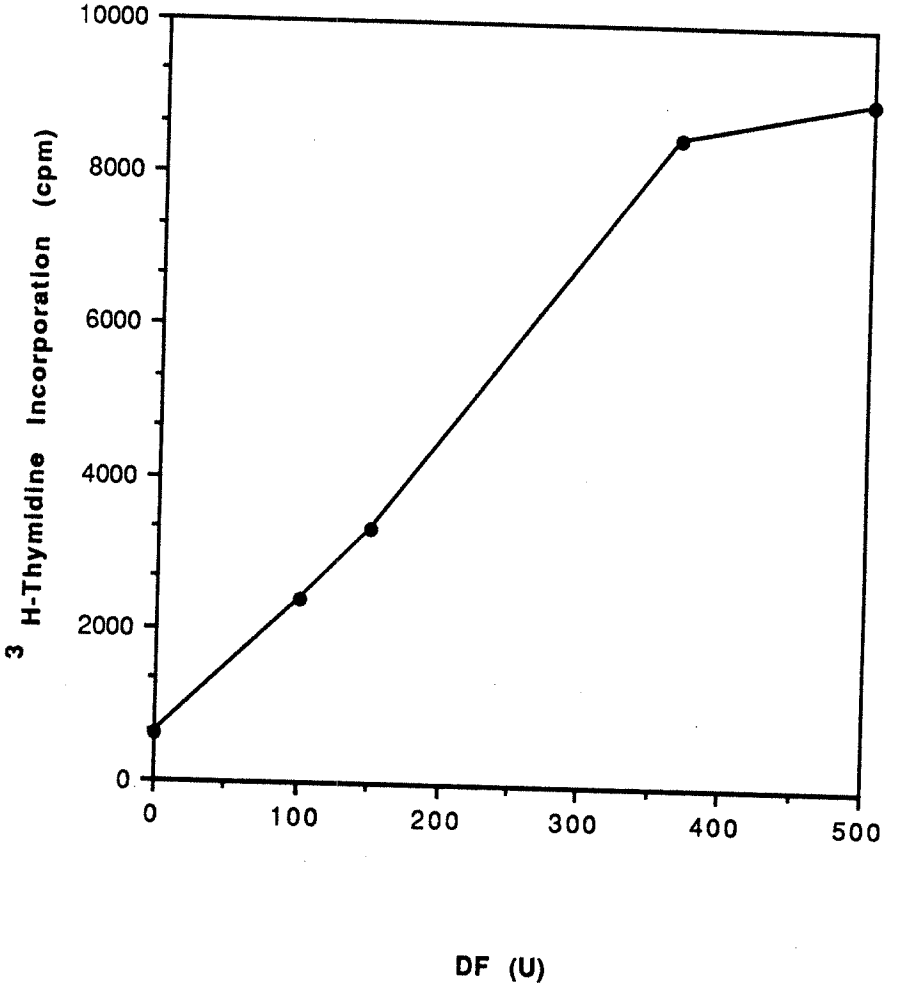

10000
8000
6000
4000
2000
0
0
100
200
300
400
500
^{3}H-Thymidine Incorporation (cpm)
DF (U)

Fig.7 Effect of rIL-6 on Dispersed Colony Formation of MIA C51 cells in soft agar: MIA C51 cells (500 cells/ml) were cultured in 0.3% agar-DME culture media. The culture plates were incubated for 6 days with or without different concentrations of rhIL-6 (0-10 U) at 37°C and 7% CO_2 in a humidified incubator. The total number of colonies and the number of dispersed colonies were scored separately.

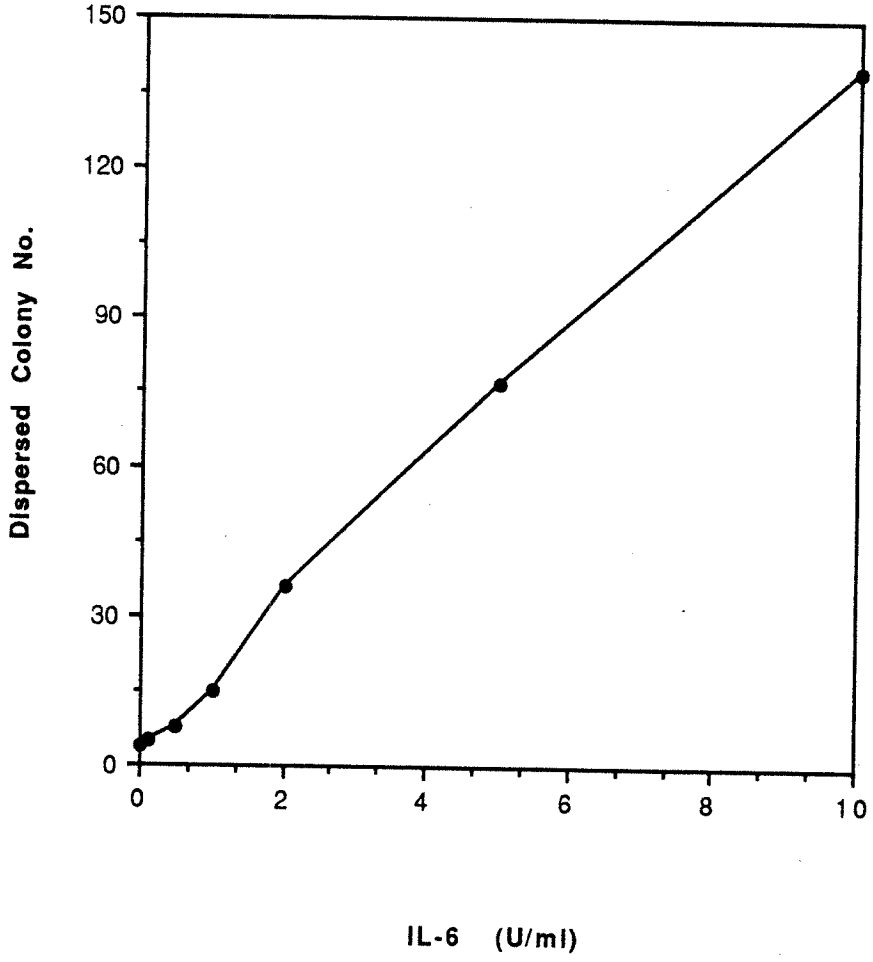
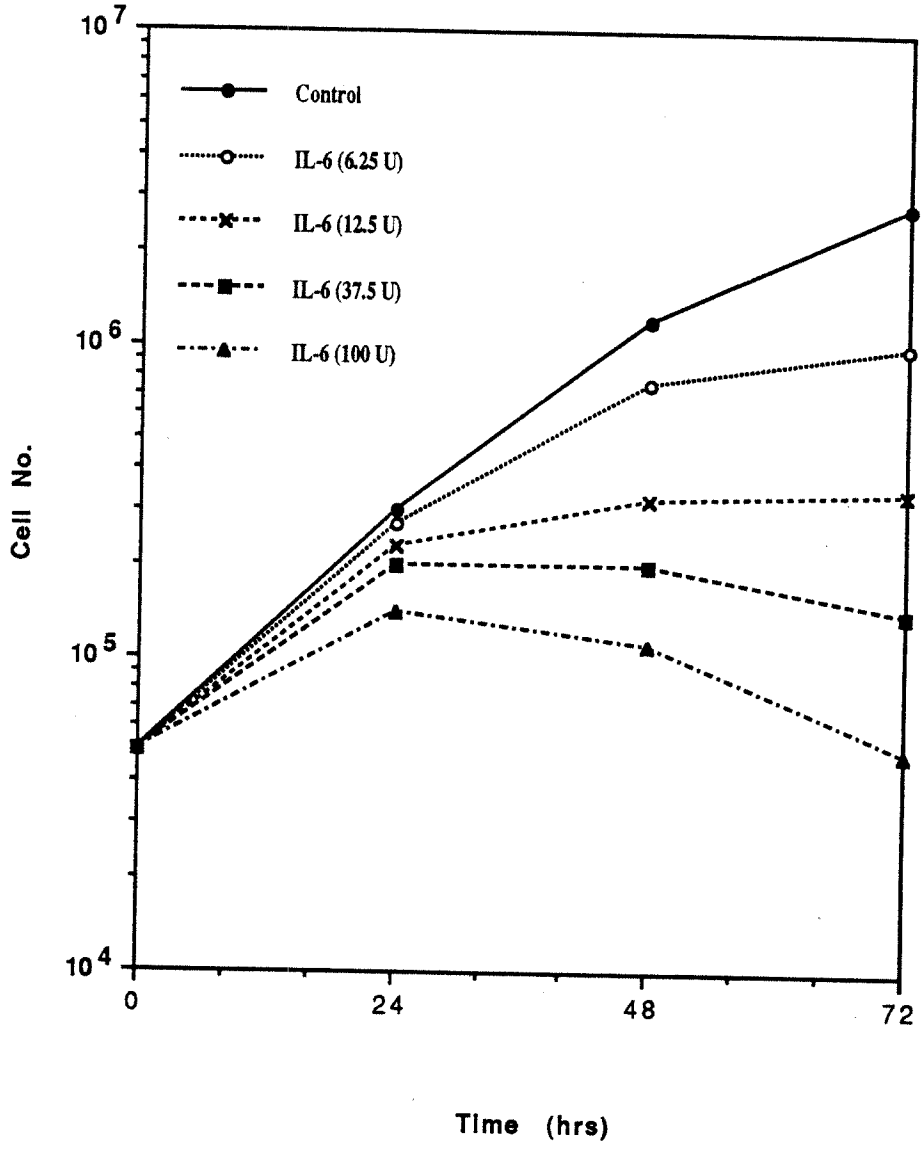

150
120
90
60
30
0
Dispersed Colony No.
0
2
4
6
8
10
IL-6 (U/ml)

Fig.8 Effect of rIL-6 on MIA C51 cell proliferation: Rat chloroleukemia MIA C51 cells (1 X 10^5 cells/ml of culture media) were incubated with different concentrations of IL-6 (0-100 U) for 3 days. Every 24 hrs cell number was counted using an American Hemocytometer using a standard procedure.


10^7
10^6
10^5
10^4
Cell No.
Control
IL-6 (6.25 U)
IL-6 (12.5 U)
IL-6 (37.5 U)
IL-6 (100 U)
0
24
48
72
Time (hrs)

activity of the sample that was incubated only with Pansorbin without the antibody (Table VI). However, a positive control (rat IL-6, not available) is needed to confirm the validity of the assay.

In order to investigate whether IL-6 and DF were related and bind to the same receptor, a binding competition experiment was performed. While DF failed to compete with ^{125}I-IL6 for binding to KG-1 cells (Fig.9 B), unlabeled IL-6 did compete with ^{125}I-IL-6 for binding to KG-1 cells (Fig.9 A). A dose-dependent inhibition of the binding of ^{125}I-IL-6 by unlabeled IL-6 was found. Maximal inhibition of IL-6-binding was reached at an IL-6 concentration of 10 ng.

Mechanism of Action of DF: As mentioned earlier two major signal transduction pathways are widely utilized by different stimuli in addition to the signalling pathway that involves catalytic receptors, which are tyrosine-specific protein kinases. One pathway triggers changes in cAMP intracellular levels, while the other involves inositol phospholipid turnover which also activates protein kinase C (PKC). In an effort to study the actual mechanism of action of DF, we investigated the effect of two types of chemicals on MIA C51 cell growth in soft agar and in culture media. The two chemicals that were used were Bt_2cAMP (a cAMP analog) and phorbol diesters (which mimic the effect of diacylglycerol in activating PKC).

The effect of Bt_2cAMP on the cell growth of MIA C51 cells was investigated. As shown in Fig.10, MIA C51 cell growth was slightly inhibited at a Bt_2cAMP concentration of 50 μM. At higher concentrations of Bt_2cAMP, the inhibition of cell growth was more

TABLE VI

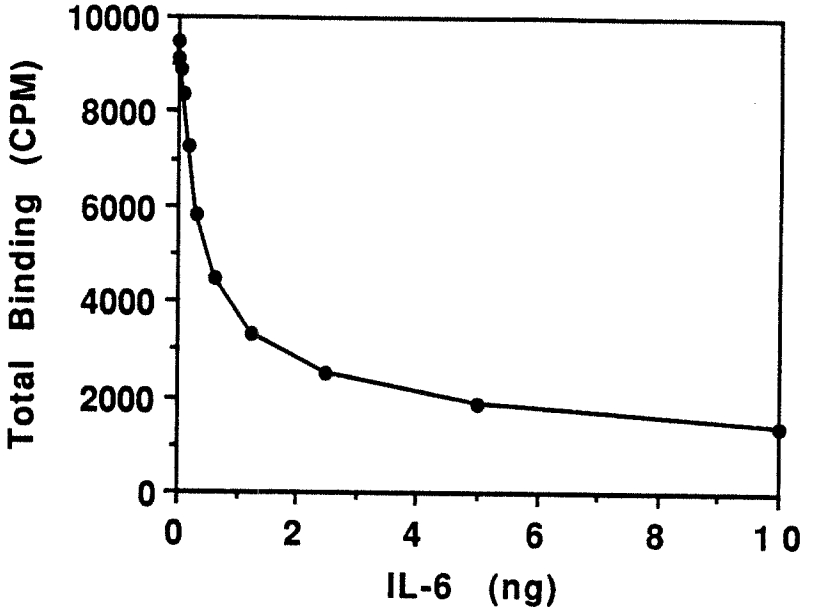
Effect of Anti-Rat IL-6 Antibody on DF Activity

Sample	DF Activity (Dispersed Colony No.)
DF (3000 U/ml)	180
DF + Antibody	182
DF + Pansorbin	169
DF + Pansorbin + Antibody	173

Twenty five µl of rabbit serum containing anti-rat IL-6 antibody was added to 3000 U DF activity in 1 ml of TNE-buffer (20 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA and 1 mM PMSF). The solution was incubated for 16 hrs at 4°C followed by immuno-precipitation. The supernatant was assayed for the differentiation activity by assaying for dispersed colony formation of MIA C51 cells in soft agar. The results are the average of duplicate experiments.

Fig.9 Inhibition of ^{125}I-IL-6 Binding to KG-1 Cells: Two million KG-1 cells /120 µl of binding media was incubated for 8 hrs at 4°C with increasing amounts of cold rhIL-6 (A) or DF (B). After incubation, cell bound radioactivity was counted. The results are the average of duplicate experiments.

A.


Total Binding (CPM)
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
IL-6 (ng)

B.

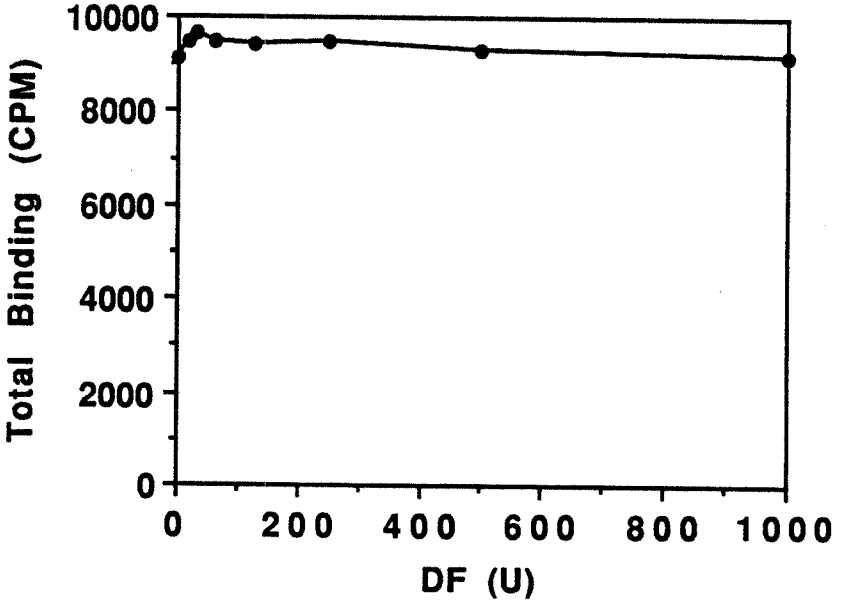
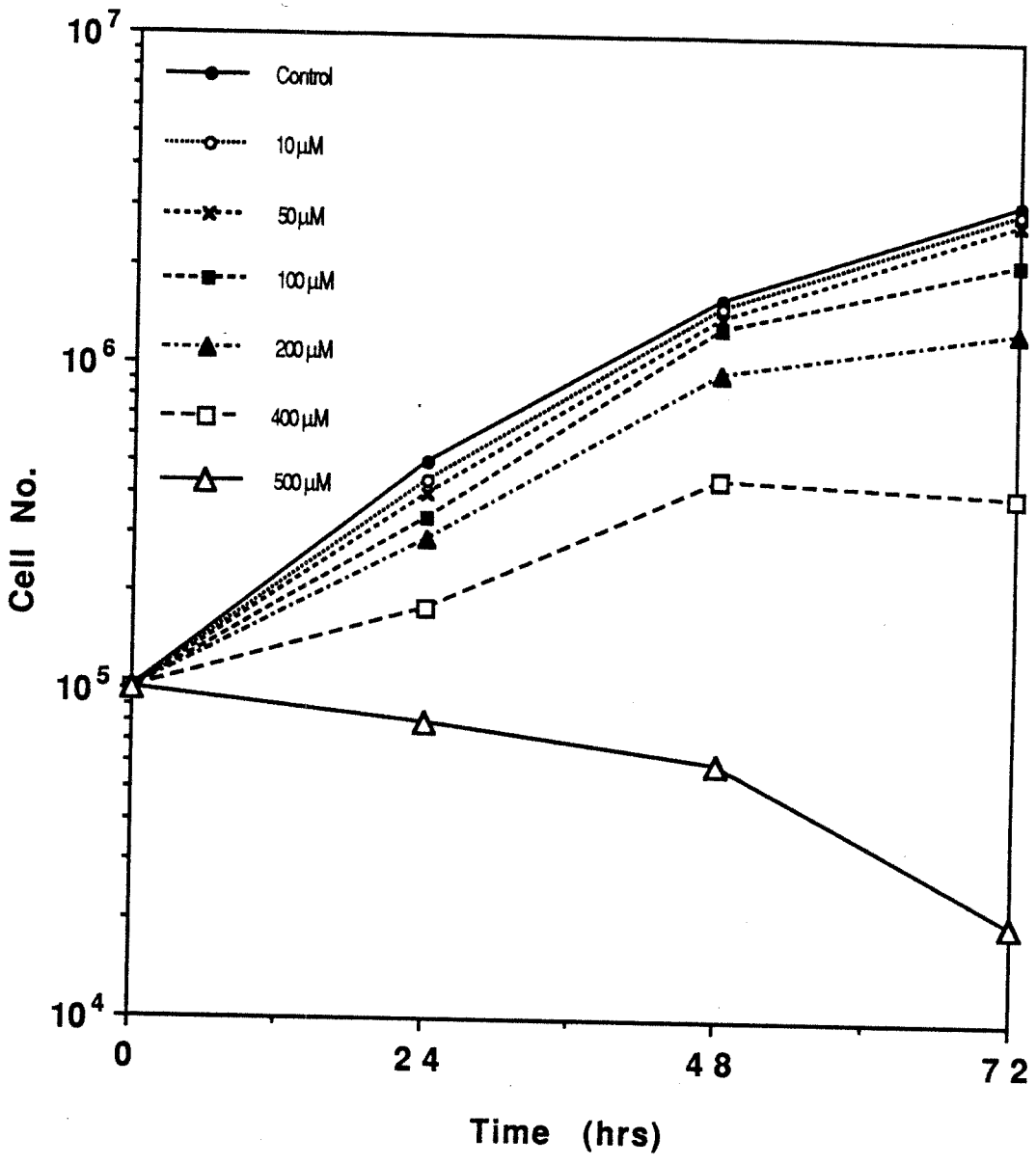

Total Binding (CPM)
10000
8000
6000
4000
2000
0
0
200
400
600
800
1000
DF (U)

Fig.10 Effect of Bt_2cAMP on MIA C51 cell proliferation: MIA C51 cells (1 X 10^5 cells/ml of culture media) were incubated with different concentrations of Bt_2cAMP for 3 days. Every 24 hrs cell number was counted using an American Hemocytometer using a standard procedure.


10^7
10^6
10^5
10^4
Cell No.
Control
10 µM
50 µM
100 µM
200 µM
400 µM
500 µM
0
24
48
72
Time (hrs)

evident. Addition of 500 μM Bt_2cAMP caused complete inhibition of cell growth and cell death. Since Bt_2cAMP is metabolized by cellular esterases into cAMP and butyric acid, more effort is needed to determine the effect of butyric acid on MIA C51 growth in culture, and soft agar.

As shown in Fig.11, Bt_2cAMP at a concentration higher than 50 μM also induced dispersed colony formation of MIA C51 cells in soft agar in a dose-dependent manner. Bt_2cAMP at a concentration higher than 300 μM induced inhibition of MIA C51 proliferation in soft agar. So, both Bt_2cAMP and DF inhibited the proliferation of MIA C51 cells in cell culture and induced the formation of dispersed colony in soft agar in a dose-dependent manner.

Treatment of MIA C51 cells with phorbol diesters produced a different response from that obtained by Bt_2cAMP. As shown in Fig.12 (A and B), the addition of a concentration less than or equal to 1 μg/ml of TPA or 10 μg/ml of PDBu had no effect on the proliferation rate of MIA C51 cells. However, the proliferation rate of MIA C51 cells was 70% inhibited by the addition of 3 μg/ml or higher concentration of TPA (Fig.12B). The difference in potency between PDBu and TPA was mainly due to the fact that PDBu is less hydrophobic and therefore less active than TPA. On the other hand, both TPA and PDBu (0.5 μg/ml) were effective inhibitors of U-937 cell proliferation (Fig.13 A and B).

The resistance or tolerance of MIA C51 cells for such a high concentration of phorbol diesters was not expected. A possible reason for this resistance might be that MIA C51 cells have lower

Fig.11 Effect of Bt_2cAMP on Dispersed Colony Formation of MIA C51 cells in soft agar: MIA C51 cells (500 cells/ml) were cultured in 0.3% agar-DME culture media. The culture plates were incubated for 6 days with or without different concentrations of Bt_2cAMP (0-300 μM) at 37°C and 7% CO_2 in a humidified incubator. The total number of colonies and the number of dispersed colonies were scored separately.

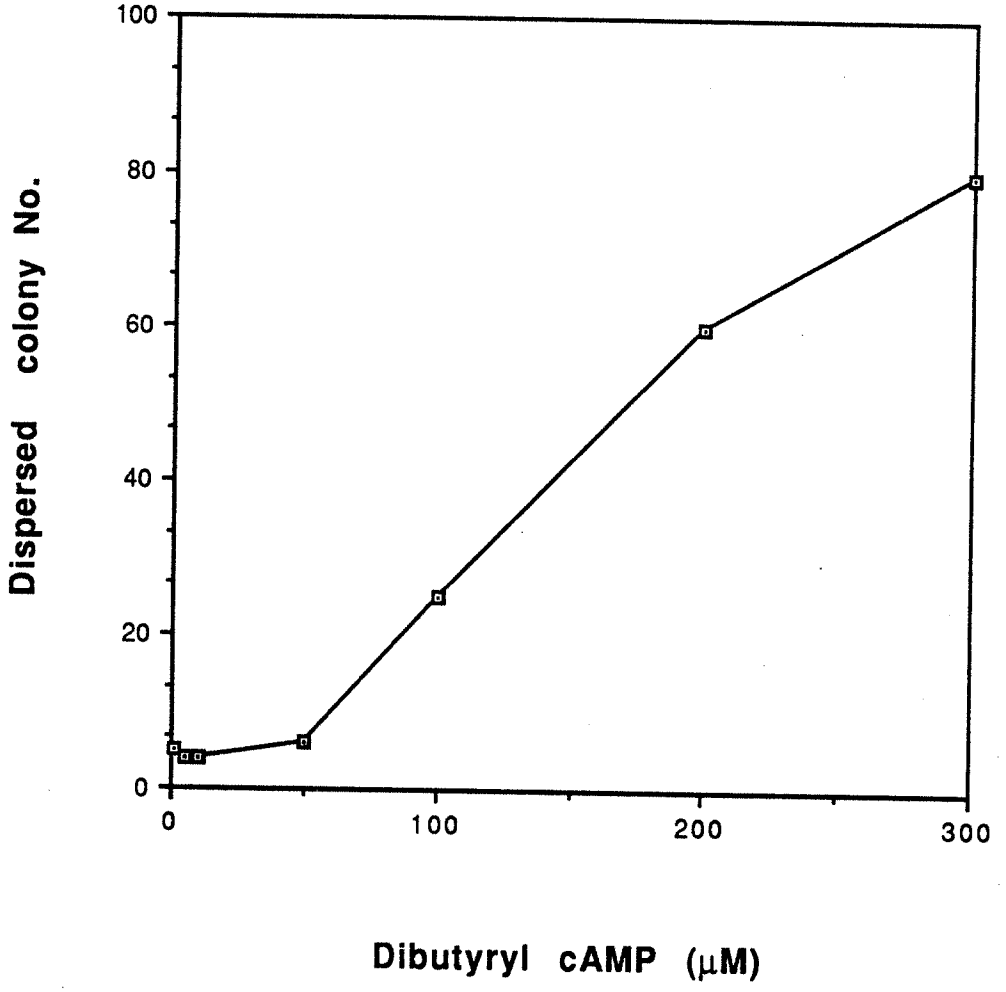
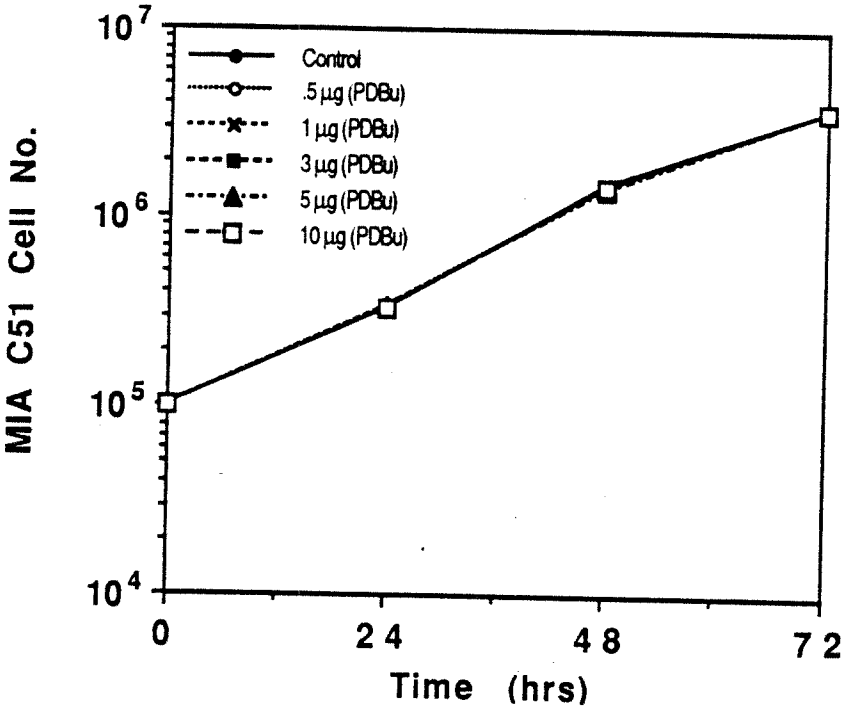

Dispersed colony No.
100
80
60
40
20
0
0
100
200
300
Dibutyryl cAMP (μM)

Fig.12 Effect of PDBu and TPA on MIA C51 cell proliferation: Rat chloroleukemia MIA C51 cells (1 X 10^5 cells/ml) were incubated for three days with different concentrations of either PDBu (A) or TPA (B). Cell number was counted every 24 hrs.

A.


MIA C51 Cell No.
10^7
10^6
10^5
10^4
Control
.5 µg (PDBu)
1 µg (PDBu)
3 µg (PDBu)
5 µg (PDBu)
10 µg (PDBu)
0
24
48
72
Time (hrs)

B.

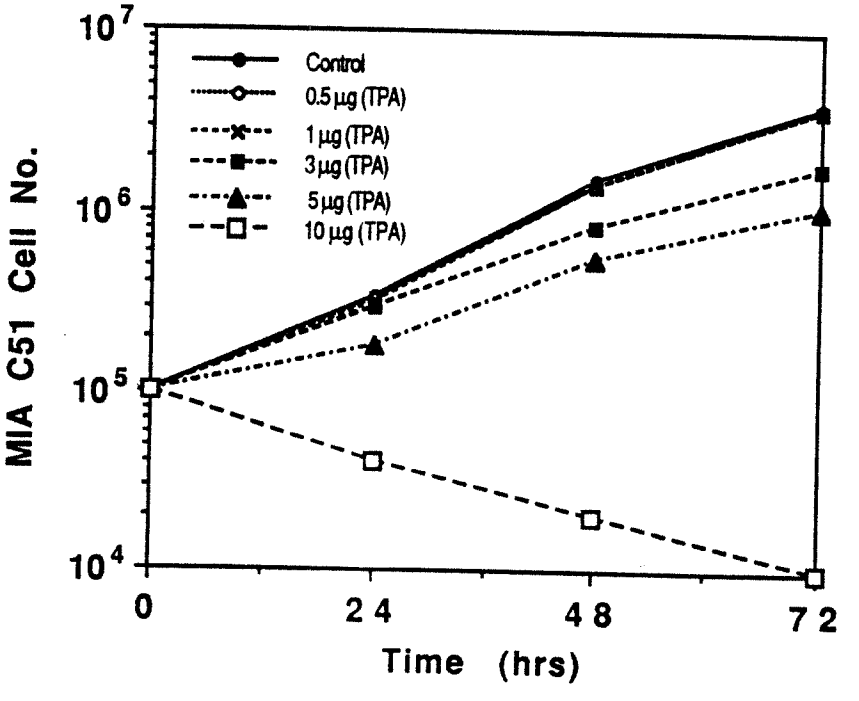
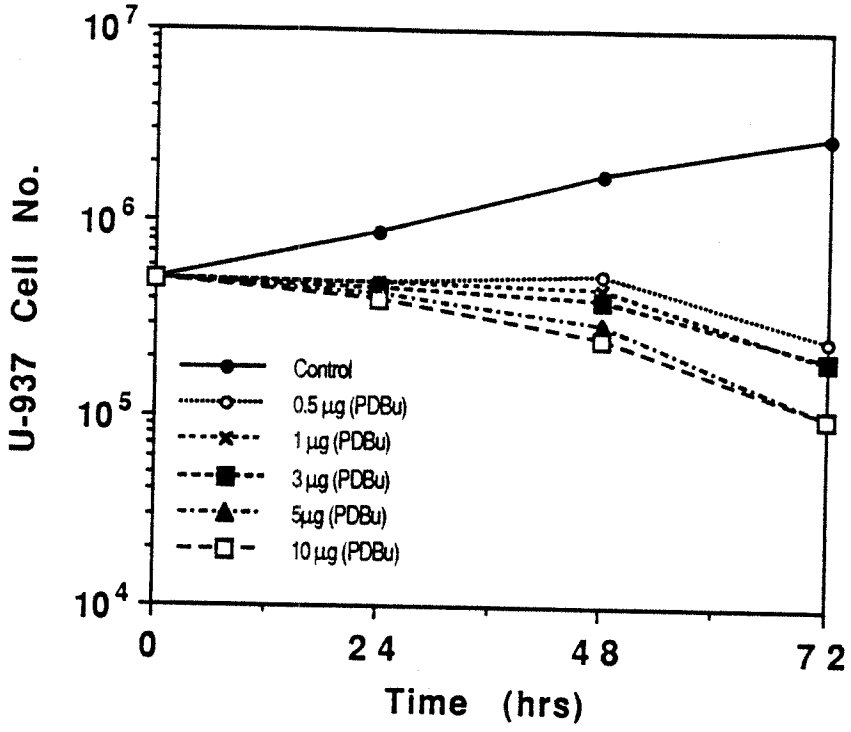
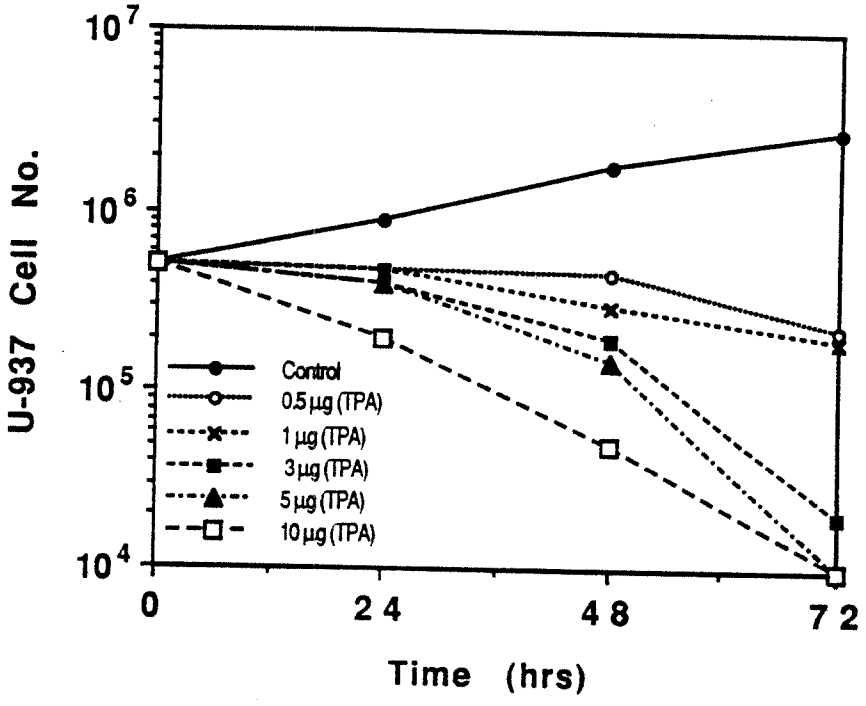

MIA C51 Cell No.
10^7
10^6
10^5
10^4
Control
0.5 µg (TPA)
1 µg (TPA)
3 µg (TPA)
5 µg (TPA)
10 µg (TPA)
0
24
48
72
Time (hrs)

Fig.13 Effect of PDBu and TPA on U-937 cell proliferation: Human monoblastoid U-937 cells (5 X 10^5 cells/ml) were cultured for three days with different concentrations of either PDBu (A) or TPA (B). Cell number was counted every 24 hrs.

A.


10^7
10^6
10^5
10^4
U-937 Cell No.
Control
0.5 µg (PDBu)
1 µg (PDBu)
3 µg (PDBu)
5µg (PDBu)
10 µg (PDBu)
0
24
48
72
Time (hrs)

B.


10^7
10^6
10^5
10^4
U-937 Cell No.
Control
0.5 µg (TPA)
1 µg (TPA)
3 µg (TPA)
5 µg (TPA)
10 µg (TPA)
0
24
48
72
Time (hrs)

number of phorbol diester receptors compared to other cell lines which are sensitive to phorbol diesters such as U-937 cells. Using a [^{3}H]PDBu binding assay, we have determined that the phorbol diester-sensitive U-937 cells have about 14 fold higher number of phorbol diester receptors as compared to MIA C51 cells (Table VII).

PKC is the known high affinity receptor for phorbol diesters. PKC activity for both MIA C51 cells and U-937 cells was assayed. Figure 14 clearly shows a major difference in PKC activity between MIA C51 cells and U-937 cells. The PKC activity in U-937 cells is about 11 fold higher than the PKC activity of MIA C51 cells. This difference in PKC activity was comparable to the difference in the number of phorbol diester receptors (Table VII).

Immunoblot analysis, with sheep serum containing anti-PKC antibody of the 0.1 M NaCl eluate from a DE-52 column for both MIA C51 cells and U-937 cells is shown in Fig.15. The immunoblot analysis showed a clear 76-78 KDa PKC band in U-937 cells (lane D) but not in MIA C51 cells (lane C). The identity of a 66 KDa protein band in lane C is unknown, howewer the similarity in molecular weight of the 66 KDa band with the molecular weight of PKCζ (67.7 KDa) raises the possibility that the 66 KDa band is PKCζ. Since, anti-PKCζ antibody is commercially available, the presence of PKCζ in MIA C51 cells could be determined.

Treatment of MIA C51 cells with DF produced more than a 2.5 fold increase in the cellular level of cAMP within few min of DF addition, and then declined over the the next few min. The highest level of cAMP was reached after 10 min of DF addition (0.8 pmol/ 10^6 cells,

Fig.14 PKC Activity of MIA C51 and U-937 Cells: The cellular extract from ten million cells of either MIA C51 or U-937 cell lines was loaded onto a 1 ml DE-52 column. PKC activity was eluted at 0.1 M NaCl. PKC activity was determined by the transfer of $^{32}PO_4$ from [γ-^{32}P] ATP to Histone, V-S (Sigma). One unit of PKC is defined as the amount of activity that causes the incorporation of 1 nmole of phosphate into histone per 10 minutes. PKC activity is expressed as units per ten million cells.

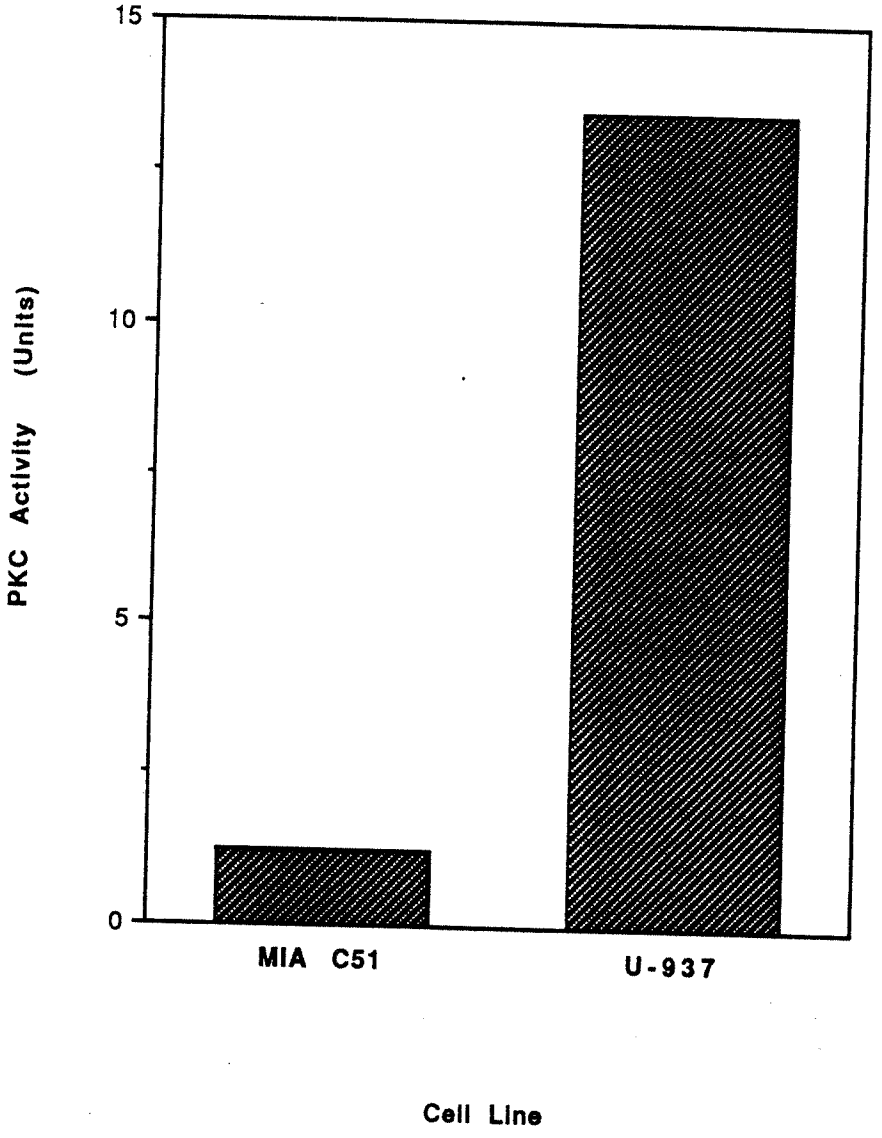

15
10
5
0
PKC Activity (Units)
MIA C51
U-937
Cell Line

TABLE VII

Measurement of Phorbol Diester Receptors in Intact MIA C51 and U-937 Cells

[^{3}H]PDBu (nM)	Specifically Bound [^{3}H]PDBu (pmol/10^6 Cells) MIA C51	U-937
4.8	0.010	0.140
9.1	0.020	0.310
27.3	0.053	0.810
54.6	0.117	1.638

1 X 10^6 MIA C51 or U-937 cells were incubated with different concentrations of [^{3}H]-PDBu with and without 20 μM unlabeled PDBu for 30 min at 37°C. The cell pellet was then washed twice with cold (4°C) binding media, and the radioactivity in the cell pellet was quantitated.

Fig.15 SDS-PAGE and Western Blot Analysis of MIA C51 and U-937 Proteins: Cellular proteins were resolved by 10% SDS-PAGE. Ten µg of the eluted proteins (0.1 M NaCl) from the DE52 column were loaded per lane. The protein profile as visualized by Coomassie blue is shown in lane A for MIA C51 cells and lane B for U-937 cells. The molecular weight markers are bovine albumin (66.0 KDa), ovalbumin (45.0 KDa) and lysozyme (from chicken egg white, 14.3 KDa). Also shown is the immunoblot analysis of the eluted MIA C51 proteins (lane C) and U-937 proteins (lane D) using sheep serum containing anti-PKC antibody (1:1000 dilution).

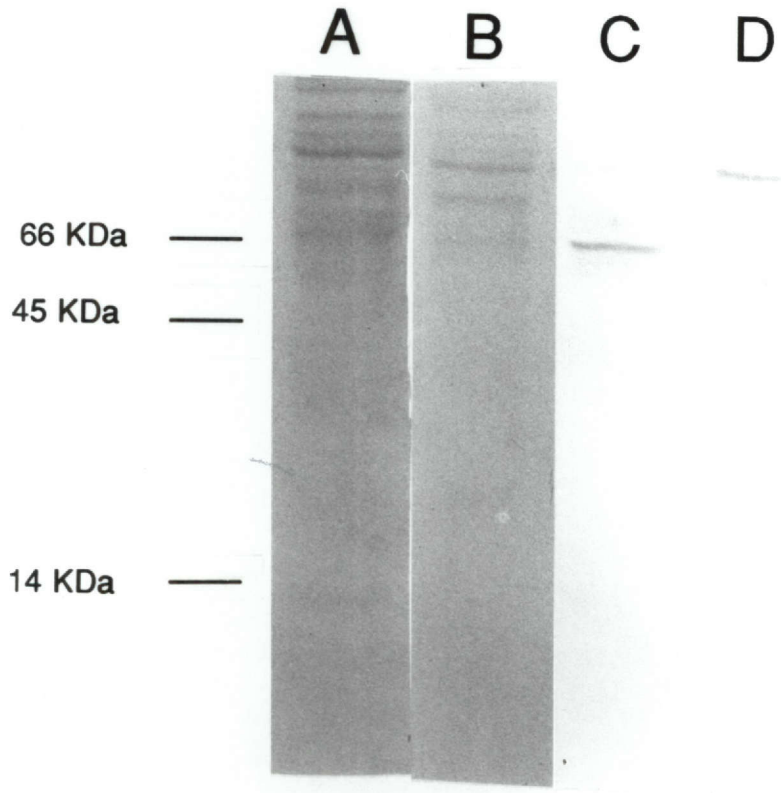

A
B
C
D
66 KDa
45 KDa
14 KDa

Fig.16).

There are two possible mechanisms by which DF could elevate cellular cAMP concentration, DF could either stimulate cAMP synthesis by stimulating adenylate cyclase, or it could inhibit cAMP hydrolysis by inhibiting cAMP phosphodiesterase. The effect of DF on the adenylate cyclase activity of the isolated MIA C51 crude membrane preparation was examined. As shown in Fig.17, DF stimulated adenylate cyclase activity in a dose-dependent manner. DF at a concentration of 1000, 1500, and 4000 units stimulated adenylate cyclase activity 1.5-, 1.8-, and 2.6-fold, respectively.

In order to investigate the possible involvement of other signal transduction pathways in DF action, we studied the effect of DF addition to MIA C51 cells on inositol levels. Figure 18 shows that DF caused a rapid decrease in the levels of inositol mono-, bis-, and tris-phosphates (IP, IP_2, and IP_3, respectively). Within 6 min of DF addition, the levels of IP, IP_2, and IP_3 were 86%, 66%, and 56% of untreated control cells, respectively.

As mentioned above, DF also induced an elevation in intracellular cAMP levels. The relationship between induced-cAMP elevation and the reduction of inositol levels was studied by investigating the effect of Bt_2cAMP on inositol levels. As shown in Fig.19, Bt_2cAMP at a concentration of 400 μM also reduced inositol levels. Within 6 min of Bt_2cAMP addition, the levels of IP, IP_2, and IP_3 were 63%, 60%, and 42% of untreated control cells, respectively. These results suggest that the reduction in inositol levels is mediated through a mechanism that is related to cAMP elevation.

Fig.16 Effect of DF on Intracellular cAMP Level: Rat chloroleukemia MIA C51 cells (3×10^5) were incubated at 37°C in a microfuge tube with or without DF (2000 U) for different incubation periods. To terminate the reaction, 200 µl of boiling 1:200 dilution of concentrated glacial acetic acid was added to pelleted cells. The cells were then boiled for 5 min and centrifuged. The supernatant was allowed to dry, and the residue was resuspended in the assay buffer. cAMP level was measured by an Amersham ^{125}I-cAMP kit. The results are the average of duplicate samples, and the standard deviations for all the points is less than 0.01 pmol of cAMP.

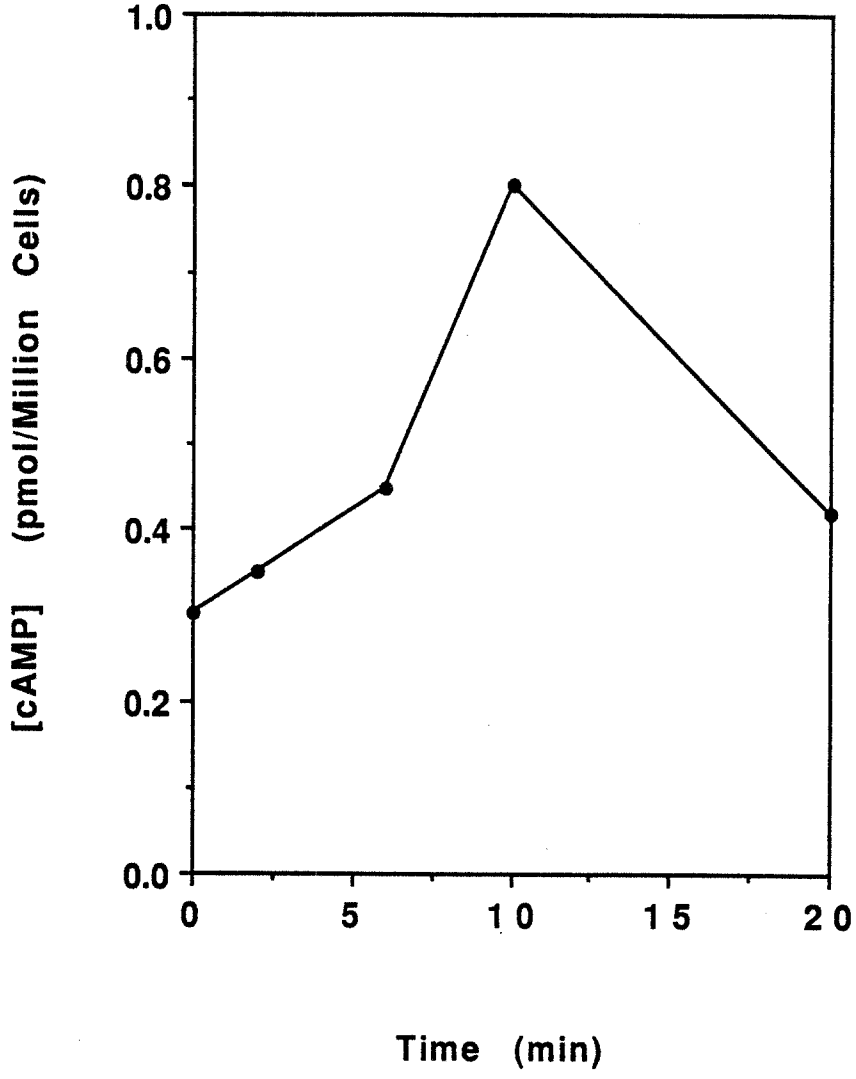

1.0
0.8
0.6
0.4
0.2
0.0
[cAMP] (pmol/Million Cells)
0
5
10
15
20
Time (min)

Fig.17 Effect of DF on the Adenylate Cyclase Activity: 3-5 μg of MIA C51 membrane proteins were incubated with and without different concentrations of DF for 30 min at 37°C in a reaction mixture. The reaction was stopped by 2% SDS, 40 mM ATP, and 1.4 mM cAMP. The cAMP fraction was then separated from ATP and ADP on AG 50W-X4 column eluted with H_2O. The results are the average of duplicate samples.

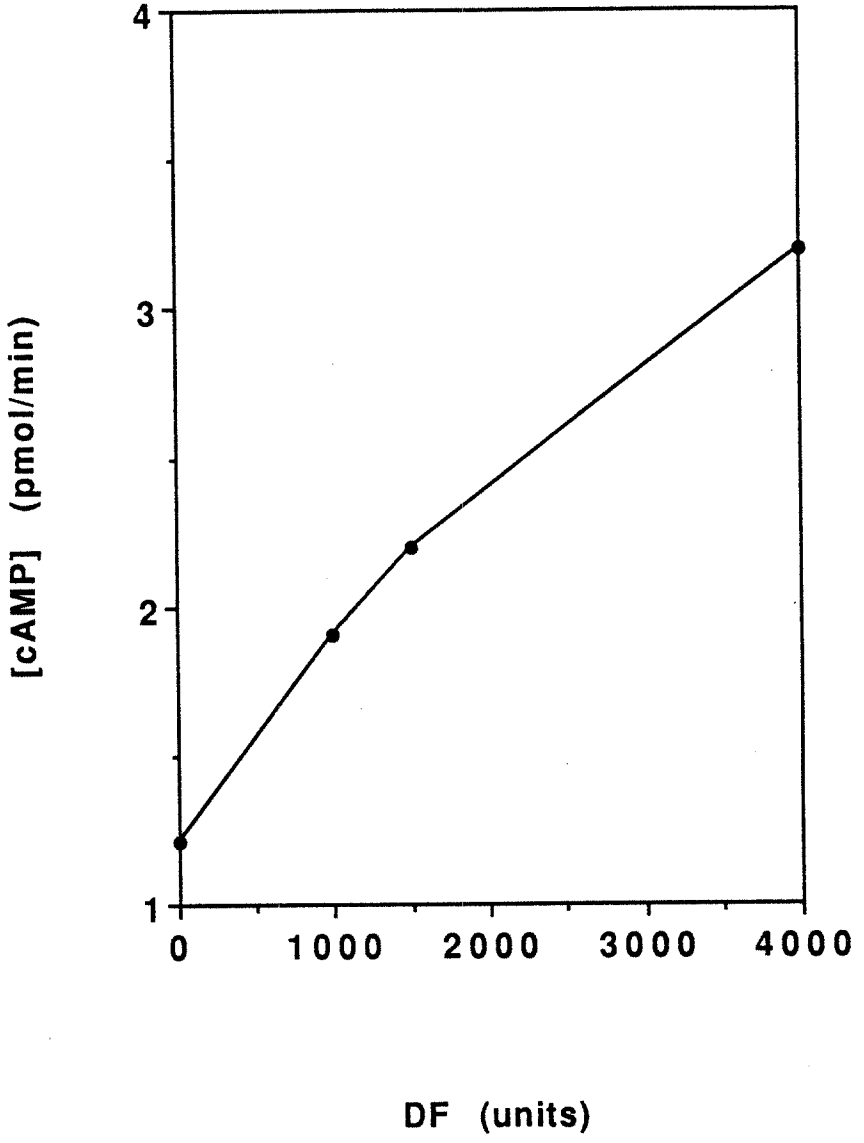

4
3
2
1
0
1000
2000
3000
4000
[cAMP] (pmol/min)
DF (units)

Fig.18 Effect of DF on MIA C51 Inositol Levels: Labeled MIA C51 cells (2 X 10^6) with Myo-[2-^{3}H]-inositol were incubated with or without DF (2000 U) at 37°C for different incubation periods. The reaction was terminated after the addition of 1 ml of 1:2 mixture of chloroform : methanol to pelleted cells. This was followed by the addition of 1 N HCl (0.5 ml) and centrifugation in order to separate the aqueous phase from the organic phase. Neutralized aqueous phase was then applied to a 1 ml AG1-X4 column (BIO-RAD), eluted with different concentrations of sodium fomate, which was used to separate the different inositol phosphates.

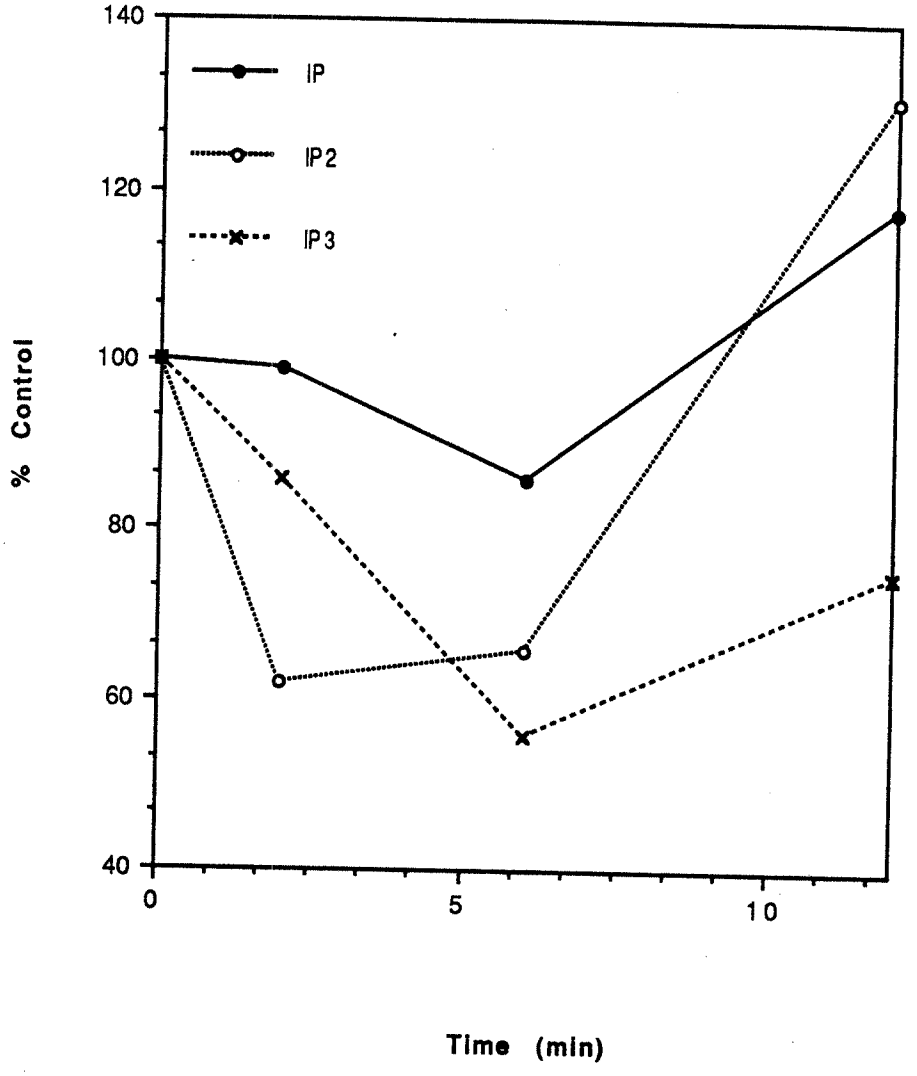
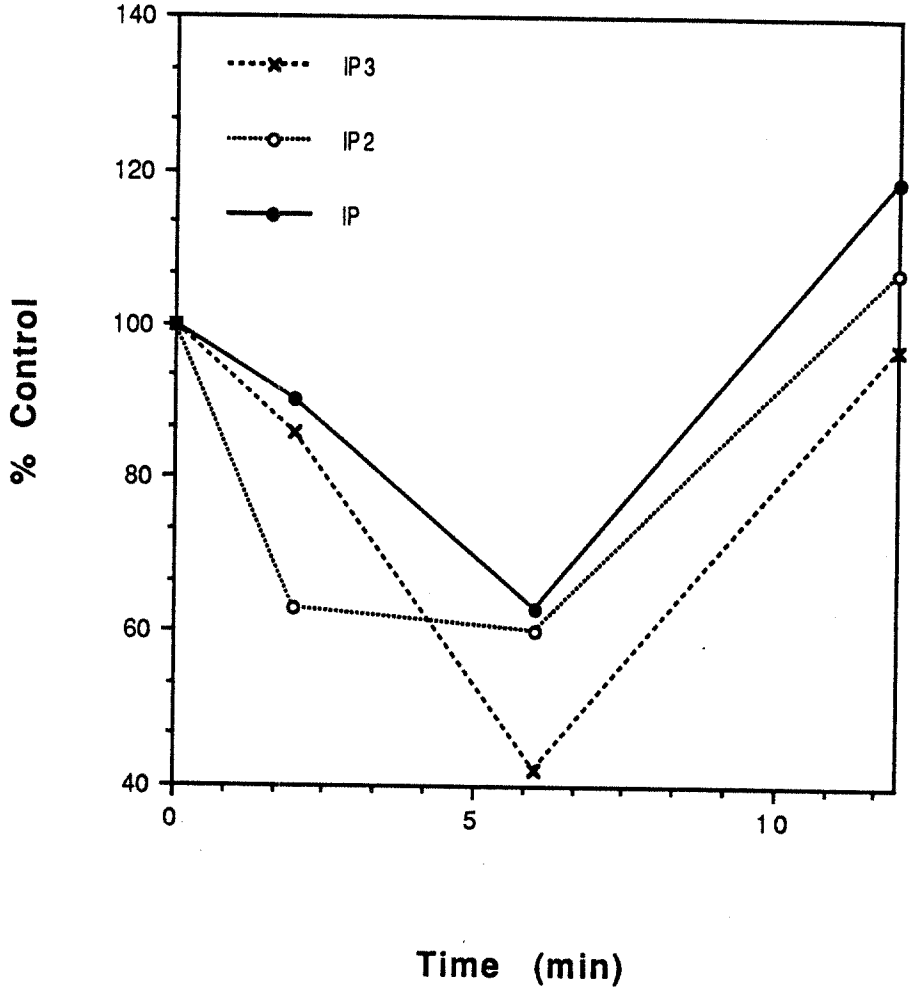

140
120
100
80
60
40
0
5
10
IP
IP2
IP3
% Control
Time (min)

Fig.19 Effect of Dibutyryl cAMP on MIA C51 Inositol Levels: Labeled MIA C51 cells (2 X 10^6) with Myo-[2-^{3}H]-inositol were incubated with or without Bt_2cAMP (400 μM) at 37°C for different incubation periods. The reaction was terminated after the addition of 1 ml of 1:2 mixture of chloroform : methanol to pelleted cells. This was followed by the addition of 1 N HCl (0.5 ml) and centrifugation in order to separate the aqueous phase from the organic phase. Neutralized aqueous phase was then applied to a 1 ml AG1-X4 column (BIO-RAD) which was used to separate the different inositol phosphates.


140
120
100
80
60
40
0
5
10
IP3
IP2
IP
% Control
Time (min)

There are several possible mechanisms by which cAMP could decrease inositol levels. One of these mechanisms involves a direct reduction in phospholipase C activity. In order to determine if this is a possible mechanism, MIA C51 cells were treated with and without DF, after which phospholipase C activity was assayed using phosphatidyl inositol 4,5 bisphosphate (PIP_2) as a substrate. As shown in Fig.20, DF addition to MIA C51 cells induced a rapid decrease in phospholipase C activity within few min. Phospholipase C activity was minimal at 10 min (only 19% of the control activity). Treating MIA C51 cells with Bt_2cAMP (400 μM) for different time periods induced a similar reduction in phospholipase C activity (only 22% of the control activity) but this response was within 2 min of Bt_2cAMP addition.

Treatment of MIA C51 cells with different concentrations of DF for 30 min did not produce any apparent change in intracellular Ca++ levels (Table VIII). The ionophore A23187 has been shown to enhance the passage of divalent cations across biological membranes. As expected, addition of the Ca++ ionophore A23187 (1 μM) to MIA C51 cells produced a large increase in the intracellular Ca++ level.

Enhanced transcription of the *c-fos* proto-oncogene has been associated with the TPA-mediated signal transduction. As shown in Fig.21, treatment of MIA C51 leukemia cells with TPA (1 μg/ml) for 0.5, 1, and 2 hours did not induce the transcription of *c-fos* proto-oncogene. At the same time, incubation of MIA C51 cells with Bt_2cAMP (400 μM) for 0.5 or 1 hr induced the transcription of *c-fos* proto-oncogene.

Fig.20 Effect of DF or Bt_2cAMP Treatment on PLC Activity: MIA C51 cells (3 X 10^5 cells/ml of culture media) were incubated either with no addition, DF (1500 U), or Bt_2cAMP (400 μM) for different incubation periods. At the end of the incubation, the reaction was stopped by pelleting the cells at high speed for 20 sec followed by washing, homogenizing, and spinning the homogenate for 10 min at 12000 X g. 3-5 μg of the supernatant proteins were incubated with [^{3}H]-PIP_2, in a reaction mixture, for 20 min at 37°C. To terminate the reaction, 0.5 ml of 1:2 mixture of chloroform: methanol was added. The aqueous phase was separated from the organic phase by the addition of 0.25 ml of 1 N HCl and centrifugation. An aliquot of the aqueous phase was then used for quantitation of the radioactivity.

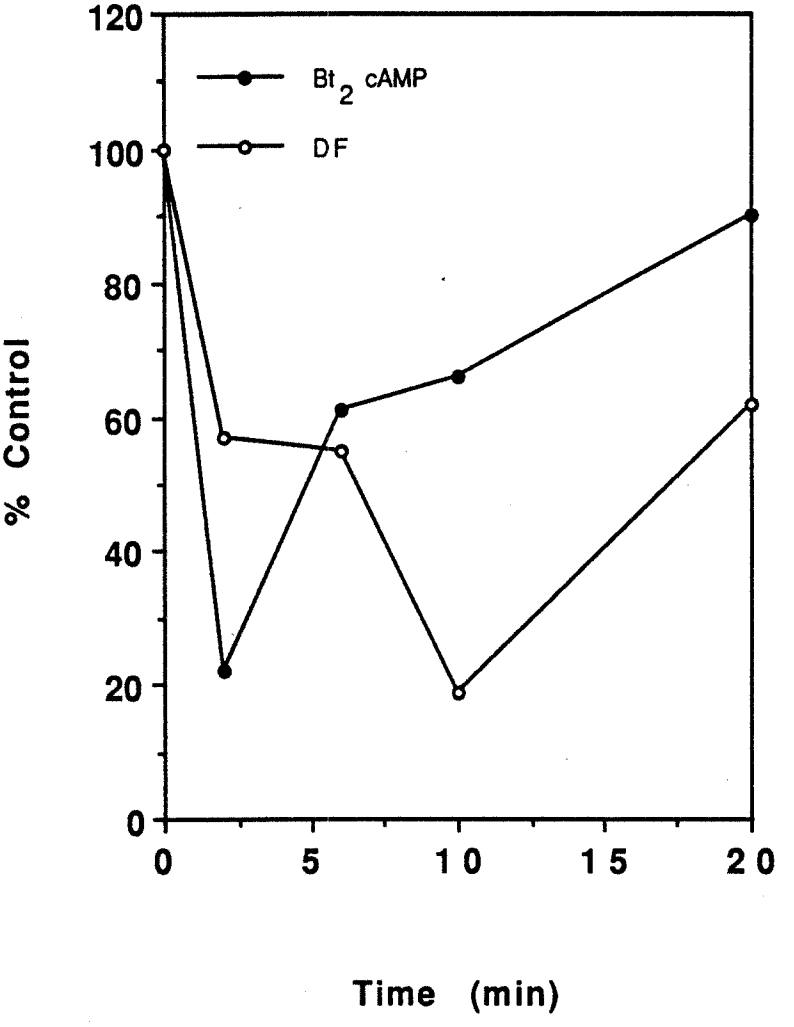

120
100
80
60
40
20
0
% Control
Bt2 cAMP
DF
0
5
10
15
20
Time (min)

TABLE VIII

Intracellular Ca^{++} Levels in Control and Treated MIA C51 Cells

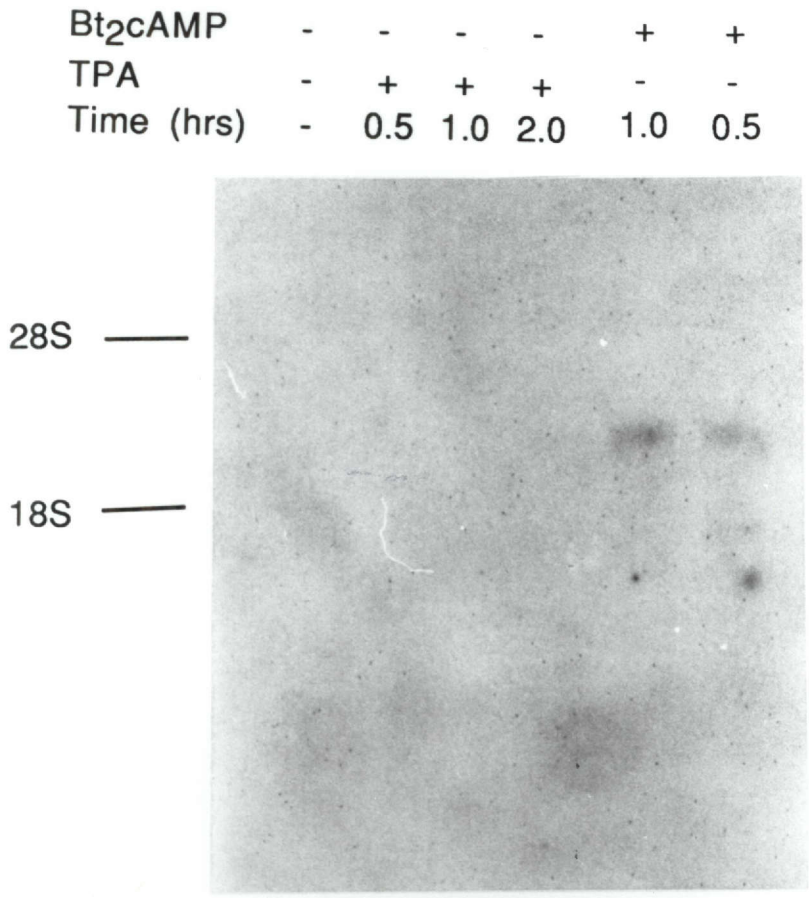
Treatment	$[Ca^{++}]$ (nM)
No addition	68.4
DF (200 U)	66.5
DF (400 U)	68.4
DF (800 U)	67.1
DF (1000 U)	70.3
A23187 (1 μM)	487.7

$1x10^6$ MIA C51 cells were incubated with 10 μM quin2/AM in DME for 60 min. Cells were then washed and resuspended in a simplified solution that contained 1.0 mM Ca^{++}, and the fluorescence was measured. Cells were treated either with different concentration of DF, A23187 ionophore, or with no addition. After cell treatment, fluorescence was measured for 30 minutes. Calcium concentration was calculated according to the following formula:

$[Ca^{++}] = Kd\ (F - F_{min})/(F_{max} - F)$

Kd = 115 nM, F_{max} was obtained after lysing the cells with 0.1% Triton X-100, F_{min} was obtained after the addition of 0.5 mM $MnCl_2$.

Fig.21 Northern Blot Analysis of *c-fos* Oncogene mRNA in MIA C51 Cells: Northern blot analysis of MIA C51 cellular mRNA, in different tissue culture conditions, hybridized to rat *c-fos* probe. Lane 1 is control untreated MIA C51 cells; lane 2, 3, and 4 are TPA-treated (1 μg/ml) MIA C51 cells for 0.5, 1, and 2 hrs respectively; lane 6, and 7 are Bt_2cAMP-treated (400 μM) MIA C51 cells for 1 and 0.5 hrs respectively. Each lane was loaded with 20 μg of total RNA.


Bt2cAMP - - - - + +
TPA - + + + - -
Time (hrs) - 0.5 1.0 2.0 1.0 0.5
28S
18S

CHAPTER IV

DISCUSSION

A differentiation factor (DF) for leukemia cells has been isolated and purified to apparent homogeneity from rat lung conditioned media. DF was characterized as a 24 KDa protein factor. Although the DF activity was associated with a 24 KDa DF band, it is possible that the protein responsible for the DF activity was not the apparent 24 KDa protein band but another protein that is present in undetectable level. In order to determine if this is the case, further study is needed to obtain a partial amino acid sequence of the 24 KDa protein band. A peptide with the same amino acid sequence will then be synthesized and an antibody will be raised against that peptide. If indeed the 24 KDa protein band is DF, the antibody will then be able to immunoprecipitate both the 24 KDa protein band and the DF activity associated with it.

Since the attempts to sequence DF at the amino-terminal were not successful, it is most likely that the amino-terminal residue of DF is blocked. The nature of this blockage is not known. In order to get a partial amino acid sequence, the 24 KDa protein was cleaved with cyanogen bromide (CNBr) which produced two apparent peptides as predicted from the amino acid composition analysis which detects the presence of one methionine residue only. Attempts to

sequence the larger 14 KDa peptide were not successful, which suggests that the amino terminal of this peptide is the blocked amino terminal of DF. Further effort on the isolation and sequencing of the small fragment is needed in order to obtain partial amino acid sequences for molecular cloning.

While both DF and the colony-stimulating activities (CSFs) were similar in their molecular weights, they differed in their binding to DEAE-HPLC column. The DF activity eluted from a DEAE-HPLC column at 40-60 mM NaCl, whereas most of the CSF activity eluted at 70-100 mM NaCl. Although some CSF activity was present in the DF-active fractions, it only constituted 1% of the DF activity (DF-active fractions contained $3x10^6$ DF units and $3x10^4$ CSF units). Because most of the experiments described in this study were conducted using the partially purified DF after DEAE-HPLC at a concentration of 1000-3000 DF units, it is unlikely that 10-30 units of CSF activity present in the DF-active fractions will produce the observed effects. The presence of this low CSF activity in the DF-active fractions could be due to the intrinsic activity of DF to stimulate the formation of hematopoietic colonies. Several interleukins, such as IL-1, IL-3, and IL-6 (158), have also shown this colony-stimulating activity.

DF and interleukin-6 (IL-6) are similar in their actions on rat MIA C51 cells and murine hybridoma B9 cells. In addition to DF actions on MIA C51 cells, DF stimulated the proliferation of IL-6-dependent B9 cells. The B9 assay is specific for IL-6, and is not influenced by a variety of other cytokines except for murine IL-4 which shows some

activity in this assay (157). The stimulation of proliferation of B9 cells by the DF-active fractions could be due to the functional similarity of DF and IL-6. At the same time, recombinant human IL-6 induced the dispersed colony formation of MIA C51 cells in soft agar and inhibited the proliferation of MIA C51 cells in culture media. Also, IL-6 is a glycoprotein of 23-30 KDa molecular weight which is similar to the molecular weight of DF (24 KDa). The similarities of DF and IL-6 in fuctions and molecular weight raises the question whether the two proteins are identical.

Since the IL-6 binding assay was performed on human KG-1 cells, it is important to know whether rat IL-6 would bind to the human IL-6 receptor. There is no available data concerning the binding of rat IL-6 to the human cells. However, it was reported that ^{125}I-labelled recombinant human IL-6 was able to bind to sinusoidal membranes from rat liver (159), and human IL-6 was able to bind to mouse receptors (160). Both human and murine IL-6 can also stimulate the proliferation of a murine hybridoma B9 cell line. Since rat IL-6 is 93% homologous to murine IL-6, it is expected that rat IL-6 would also stimulate the proliferation of B9 cells. These observations thus suggest that the human, mouse, and rat IL-6s all can bind to the same receptor on KG-1 cells. This study showed that DF failed to compete and inhibit human ^{125}I-IL-6 binding to KG-1 cells which suggests that DF is different from rat IL-6. The amino acid composition of DF is also different from the amino acid composition of rat IL-6 (68). While, the amino acid composition analysis of DF predicted the presence of one methionine, thirteen

valine, eight threonine, and six histidine residues, rat IL-6 cDNA showed the presence of four methionine, nine valine, seventeen threonine, and two histidine residues. These results appear to indicate that DF and rat IL-6 are different protein factors that, although probably functionally related, are not identical.

Interleukin-1α and β (IL-1α and IL-1β) which have similar estimated molecular weights (12-18 KDa) are different from the estimated molecular weight of DF (24 KDa). While DF was eluted at 40-60 mM NaCl from DEAE-HPLC column, human IL-1β failed to bind to DEAE-Sepharose (161) and murine IL-1 (from P3881 murine macrophage cell line) was eluted at a broad range of 75-200 mM NaCl with the peak activity occurring at 160 mM NaCl (162). There is no apparent similarity in the amino acid composition of DF with either human IL-1α, or human IL-1β (163). These facts support the idea that DF and IL-1 (α and β) are different protein factors.

Tumor necrosis factor α (TNFα) and lymphotoxin (LT, TNFβ) occur naturally in dimeric or trimeric forms. Human TNFα activity in gel permeation chromatography was associated with an apparent molecular weight of 45 $\pm$ 6 KDa while that of human LT was eluted with an apparent molecular weight of 60 KDa. Similarly, human interferon γ (IFNγ) has a molecular weight of 40 KDa (164). On the other hand, DF was eluted from Ultrogel AcA 44 gel filtration chromatography with an apparent molecular weight of 20-30 KDa. The amino acid composition of DF is not similar to human TNFα (73), human LT (73), or rat IFNγ (87). These facts support the idea that DF, TNFα, LT, and IFNγ are different proteins.

The elution profile of leukemia inhibitory factor (LIF) from the DEAE column is different from that of DF (40-60 mM NaCl). Two peaks of LIF activity were separated on DEAE-Sepharose column. Most of LIF activity (which was called LIF-A) failed to bind to a DEAE ion-exchange column, and the other (LIF-B) was eluted between 150 mM to 250 mM NaCl (165). The molecular weight of LIF was estimated to be 58 KDa which is much larger than the estimated molecular weight of DF (24 KDa), and the amino acid composition of rat LIF is different from the amino acid composition of DF (166). These facts indicate that DF and rat LIF are different protein factors.

Transforming growth factor β (TGF-β) is a dimer composed of two subunits linked by intermolecular disulfide bridges. While reduced and nonreduced DF (with β-mercaptoethanol) migrated as a 24 KDa protein in SDS-PAGE, reduced TGF-β migrated as 12.5 KDa protein and the non-reduced TGF-β migrated as a 25 KDa protein. DF was shown to bind to DEAE-HPLC ion exchange chromatography, whereas TGF-β failed to bind to DEAE column but it was purified on a CM-Trisacryl M cation exchange column (167). The amino acid composition of human TGF-β1 and DF is different (168). Therefore, it is reasonable to conclude that DF is distinct from the TGF-β family.

These observations suggest that the DF isolated and purified from rat lung conditioned media is different from the known differentiation-inducing factors which include CSFs, IL-1α, IL-1β, IL-6, TNFα, LT, IFNγ, LIF, and TGF-β. The characterization of DF as a new differentiation-inducing factor for myeloid leukemia cells

provides an important tool for the *in vitro* and *in vivo* investigation of leukemia cell differentiation.

The physiological role of DF remains to be established. However, DF could be responsible for preventing the formation of leukemia cells by keeping in check the development of out of phase differentiating cells. DF could also interact with other hematopoietic growth factors as part of the cytokine network in regulating hematopoiesis.

In this study, a rat myeloblastic leukemia MIA C51 cell line was characterized as a phorbol diester resistant cell line as evidenced by: (1) the inability of phorbol diesters to inhibit MIA C51 cells proliferation; (2) reduction of phorbol diester receptor number; (3) low PKC activity; (4) failure to induce *c-fos* expression.

While TPA failed to induce *c-fos* transcription in MIA C51 cells, addition of Bt_2cAMP to these cells did induce *c-fos* transcription. Induction of *c-fos* proto-oncogene expression by Bt_2cAMP has also been demonstrated in other cell lines. For example, induction of rat pheochromocytoma PC12 cell line differentiation to neurites by Bt_2cAMP was associated with a rapid expression of the *c-fos* gene (169). Also, exposure of THP-1 monocytic leukemia to either Bt_2cAMP or cholera toxin resulted in an increase in *c-fos* mRNA level (170).

Previous studies by different investigators had shown that the cellular content of PKC activity from various cell lines and tissues is different. If one unit of PKC activity is defined as the amount of activity that causes the incorporation of 1 nmol of phosphate into

histone per 10 min per 10^7 cells, then the total PKC activity in: 1) B lymphoid 70Z/3 cell line is 6.5 units (171); 2) IL-3-dependent FDC-P1 cells is 30 units (145); 3) rat pinealocytes is 100 units (172); 4) neutrophils is 1500 units (173); 5) platelets is 2500 units (173); 6) and U-937 cell line is 13.2 units (this study). At the same time, the total cellular PKC activity in MIA C51 cells is only 1.2 units. The reason for this low PKC activity in MIA C51 cells is not known but it seems to be due to the low level of PKC protein in this cell line.

Anti-PKC antibody detected a 76-78 KDa PKC protein in U-937 cells but not MIA C51 cells, whereas a 66 KDa protein band was detected in MIA C51 cells. The identity of the 66 KDa protein is unknown. However, it is possible that the 66 KDa protein is PKCζ which has a similar molecular weight as reported previously (174). The PKCζ activity was shown to be dependent on phospholipids and it was assayed in the presence of 8 μg phosphatidyl serine and 0.8 μg diolein (174). The PKC activity reaction mixture used in this study contained 25 μg phosphatidyl serine and 2 μg OAG which should be able to detect the PKCζ activity. However, very low PKC activity was detected in MIA C51 cells suggesting that the 66 KDa protein band is probably not PKCζ. Further studies with anti-PKCζ antibody (GIBCO BRL) and western blot analysis should confirm this assumption.

The molecular basis for the TPA-resistance of the mutant cells of HL-60 cell line or the Swiss 3T3 fibroblast is still unclear. But, most mutants had no change in the levels of PKC enzyme. This study has showed that the resistance of MIA C51 cells to phorbol diesters

was associated with low number of phorbol diester receptors. Similarly, the resistance of some cell lines at certain conditions to TPA was associated with a decrease in the number of phorbol diester receptors. HL-60 TPA-resistant R1B6 cells were found to have a reduced concentration of phorbol ester receptors in the cytosol compared to the parental HL-60 cell, when grown in the presence of phorbol diesters (127). A reversible resistant variant of Friend erythroleukemia cells to TPA was also found to contain only 20% of the parental (TPA-sensitive) receptor number (175). At the same time, when ML-1 cells were cultured in serum-free medium they displayed a decreased responsiveness to TPA-induced differentiation accompanied with a reduction in total TPA receptor number compared with cells cultured in the presence of fetal bovine serum (176). Also, when U-937 cells were grown in lipoprotein deficient serum they were found to have a reduced responsiveness to TPA accompanied with a reduction in particulate PKC activity (177).

This study has indicated that MIA C51 cells are resistant to phorbol diesters, and contain low levels of PKC activity. Further experiments are necessary to determine if the low level of PKC in MIA C51 cells is responsible for their resistance to phorbol diesters. For example, these cells can be transfected with a PKCγ cDNA plasmid. If the low level of PKC is responsible for the insensitivity of MIA C51 cells to phorbol diesters, the introduction of PKCγ, which is a phorbol diester responsive isozyme, by transfection should restore the phorbol diester responsiveness. The effect of phorbol diesters on the inhibition of proliferation of these cells and

the expression of *c-fos* oncogene can be observed as indicators for their phorbol diester responsiveness.

The mechanism of action of DF on MIA C51 cells was studied. This study has showed that treatment of MIA C51 cells with the DF-active fractions had stimulated the activity of adenylate cyclase which in turn increased the intracellular cAMP levels. Increased cAMP levels seem to inhibit phospholipase C activity which reduces the cellular inositol phosphate levels.

Cyclic AMP is involved as a second messenger in the inhibition of proliferation of transformed cells as well as induction of irreversible morphological differentiation in different malignant cell lines. Agents that increased intracellular cAMP levels were shown to induce the differentiation of different myeloid leukemia cell lines as well as numerous other cancer cells. Similar to previous studies, induction of differentiation of MIA C51 cells by DF was associated with an increase in cAMP levels. In addition, Bt_2cAMP was shown to be effective in inhibiting MIA C51 cells growth in culture and in inducing the dispersed colony formation of MIA C51 cells in soft agar.

The DF-receptor complex does not directly stimulate the activity of adenylate cyclase. Rather, the activated receptor of DF stimulates a guanyl-nucleotide binding protein (G protein) which carries the signal to the adenylate cyclase molecule. Two kinds of G proteins are known to be coupled to the adenylate cyclase molecule. They are the stimulatory G protein (G_S) which stimulates the adenylate cyclase activity, and the inhibitory G protein (G_i) which inhibits the

adenylate cyclase activity. ADP ribosylation of G_s by cholera toxin activates the G_s component resulting in the stimulation of the adenylate cyclase. ADP ribosylation of G_i by pertussis toxin inactivates the G_i component resulting also in the stimulation of the adenylate cyclase molecule. The mechanism of activation of adenylate cyclase by DF is not known. Further experiments are needed to determine if the activation of adenylate cyclase molecule by DF is mediated by the activation of G_s or the inhibition of G_i.

The increase in inositol phospholipid turnover has been observed in different proliferative cellular responses to neurotransmitters, hormones, and growth factors. In many cell types, the signal that induces the turnover of inositol phospholipids and promote cellular proliferation was antagonized by the signal that produces cAMP. Elevations in cAMP concentration have been shown to inhibit receptor-mediated hydrolysis of inositol phospholipids and inositol phosphates generation in platelets (178, 179), lymphocytes (180), neutrophils (181), natural killer cells (182), C6Bu1 glioma cells (183), insulin secreting islets (184), kidney cells (185), smooth muscle cells (186), glomerulose cells (97), and NCB-20 neurotumor cells (187). Such an effect by cAMP on inositol phospholipids breakdown and cellular proliferation is demonstrated in the mechanism of action of DF which decreased the cellular inositols concentration and inhibited the proliferation of MIA C51 cells.

Similar to the effect of DF on MIA C51 inositol levels, previous studies have demonstrated that different stimuli can also cause a decrease in phosphatidyl inositol turnover and inositol levels.

However, the involvement of cAMP in their mechanism of action is not known. Addition of retinoic acid to HL-60 cells induced an early reduction in the production of inositol phosphates (188). Also, 24 hrs following induction of granulocytic differentiation of HL-60 cells by DMSO the cellular content of inositol phosphates decreased significantly (148). In addition, a significant decrease in inositol phospholipid turnover and IP_3 cellular levels were detected within 2 hrs of DMSO addition to friend murine erythroleukemia cells (189). From these reports, it can be generalized that induction of differentiation of leukemia cells is accompanied by the decrease of inositol phosphates. Since the increase of inositol phosphates is associated with cell proliferation, the decrease of inositol phosphates could be the mechanism involved in the inhibition of proliferation as observed in this study.

Stimulation of adenylate cyclase activity by DF caused an increase in the synthesis of cAMP which in turn increased the intracellular cAMP level and activated a cAMP-dependent protein kinase. It is known that cAMP-dependent protein kinase catalyzes the transfer of terminal phosphate from ATP to specific serine or threonine residues of selected proteins in the target cell. Covalent phosphorylation of these proteins in turn regulates their activity (190). The mechanism of the DF-induced inhibition of PLC activity remains to be established. Activation of cAMP-dependent protein kinase by DF may have regulated the activity of PLC by phosphorylation. Inhibition of inositol phospholipid hydrolysis due to increased cAMP concentration was shown to be correlated with the

specific phosphorylation of PLC-γ by cAMP-dependent protein kinase. Treatment of C6Bu1 glioma cells with cAMP-elevating agents caused inhibition of PLC activity and increased serine phosphorylation in PLC-γ (183). It is possible that the activation of cAMP-dependent protein kinase by DF have caused the phosphorylation of PLCγ.

Understanding the mechanism of induction of differentiation of myeloid leukemia cells into terminal differentiated cells is critical for leukemia treatment and therapy. This study has shown for the first time that a differentiation factor-induced rise in cellular cAMP levels, due to activation of adenylate cyclase, inhibited phospholipase C activity which in turn reduced cellular inositol phosphates level.

In summary, a leukemia Differentiation Factor (DF), which induces the differentiation of a rat myeloblastic MIA C51 leukemia cell line, was purified to apparent homogeneity from rat lung conditioned medium. Rat myeloblastic MIA C51 leukemia cell line was characterized as a phorbol diester-resistant cell line. The signal transduction pathway of the DF-induced differentiation is mediated by stimulation of adenylate cyclase activity, which increased the intracellular cAMP level, and inhibition of PLC activity.

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