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THE UNIVERSITY OF ALBERTA

EFFECTS OF CYCLOSPORINS ON LYMPHOKINE GENE EXPRESSION

by

KAREN MEEROVITCH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EFFECTS OF CYCLOSPORINS ON LYMPHOKINE GENE EXPRESSION submitted by Karen Meerovitch in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry.

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Date ..June 20, 1986.....

To Ben, and my parents.

ABSTRACT

The tumor promoter phorbol diester 12-myristate 13-acetate induces the synthesis and secretion of a number of lymphokines from T-lymphocytes, while the immunosuppressive agent Cyclosporin A blocks the expression of lymphokines by these cells. These effects were studied in the mouse T-lymphoma cell line EL4, the human T-leukemia line Jurkat, and in normal human peripheral blood T lymphocytes. Induction of expression of genes encoding lymphokines such as interleukin-2 in EL4, Jurkat and peripheral blood lymphocytes and granulocyte-macrophage colony stimulating factor in EL4 cells under various conditions was examined by cytoplasmic dot (cytodot) hybridization technique using ^{32}P-labeled interleukin 2 and granulocyte macrophage colony-stimulating factor synthetic oligonucleotide and cDNA probes. Cyclosporin A inhibited the accumulation of interleukin 2 and granulocyte macrophage colony-stimulating factor mRNAs in induced cells. Various cyclosporin A analogues were found to differentially inhibit lymphokine mRNA, thus illustrating that cyclosporin A reactivity is related to a specific structural portion of its molecule, *i.e.*, the hydroxyl group in the first amino acid. Another immunosuppressive drug, dexamethasone, was found to inhibit interleukin 2 mRNA accumulation in human peripheral blood lymphocytes but not in human and mouse tumor cell lines.

The cytodot technique was used as a means to determine the cyclosporin A concentration in the serum of a cyclosporin A-treated renal allograft recipient; the concentration was calculated from the degree of inhibition of interleukin 2 mRNA in EL4 cells in the presence of cyclosporin A-containing serum. The values obtained with this technique confirmed those obtained by the radioimmunoassay performed at the hospital.

Interleukin 2 mRNA accumulation in stimulated EL4 cells was found to peak at 12 to 16 hours and decline thereafter. Removal of the induction signal, phorbol myristate acetate, by washing and reculturing the cells in fresh medium at 12 hours,

resulted in a rapid decline in the levels of interleukin 2 mRNA as compared to control levels in the constant presence of phorbol myristate acetate. Addition of the protein synthesis inhibitor cycloheximide to cells at different times after induction with phorbol myristate acetate was initiated, resulted in a rapid accumulation ('superinduction') of interleukin 2 mRNA. In contrast, addition of cyclosporin A after induction, resulted in a rapid decay of interleukin 2 mRNA [illegible]aling a half-life of 1.5 to 2 hours. These 2 events suggested that regulation of [illegible]kin 2 mRNA [illegible] in EL4 cells may be under the control of a repressor that selectively destabil[illegible]kin 2 mRNA. Simultaneous addition of cycloheximide and cyclosporin A resulted in superinduction of interleukin 2 mRNA. Conventional pulse-labeling of EL4 cells with ^{3}H-uridine demonstrated that (i) cyclosporin A does not perturb the turnover of bulk poly(A)$^+$ cellular mRNAs and (ii) the majority of bulk poly(A)$^+$ cellular mRNAs were long lived.

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ABBREVIATIONS AND DEFINITIONS

Antibody:	an immunoglobulin molecule capable of combining specifically with a known substrate (antigen).
Antigen:	a molecule capable of stimulating an immune response.
B cell:	one of two major classes of lymphocytes. B cells derive from the Bursa of *Fabricius* in birds or the bone marrow of mammals and respond to antigen by differentiating into antibody producing cells.
BCDF:	B cell differentiation factor.
BCGF:	B cell growth factor.
BSF-1:	B cell stimulating factor-one.
cDNA:	complementary deoxyribonucleic acid.
Con A:	concanavalin A - a plant substance that binds to sugar residues on cell surfaces and stimulates T cells to proliferate — a lectin.
cpm:	counts per minute.
CsA:	cyclosporin A — a cyclic undecapeptide isolated from fungal extracts which has immunosuppressive properties.
CSF:	colony-stimulating factor.
DMSO:	dimethyl sulfoxide.
EDTA:	ethylenediaminetetraacetic acid.
ED_{30}:	the amount of IL2 activity which causes 30% of maximal response in the same assay with saturating levels of IL2 is defined as 1 unit per ml.
FCS:	fetal calf serum.
GM-CSF:	granulocyte macrophage colony-stimulating factor.
IFN-α, IFN-β, IFN-γ:	interferon alpha, beta and gamma.
IL1, IL2, IL3:	interleukin one, two and three.
IL2 R:	interleukin 2 receptor.
LAK:	lymphocyte activated killer cell.

lymphokines:	soluble secreted glycoproteins of lymphocytes having diverse effects on other cells.
mAP:	messenger affinity paper.
MeBmt:	(4R)-4-[(E)-2-butenyl]-4, N-dimethyl-L-threonine, amino acid 1 of cyclosporin A.
MHC:	major histocompatibility complex.
MIF:	migration inhibition factor.
MLR:	mixed lymphocyte reaction — the proliferative response of allogeneic lymphocytes when cultured together.
mRNA:	messenger ribonucleic acid.
NK:	natural killer cell.
NP-40:	nonidet P40 — nonionic detergent.
PBL:	peripheral blood lymphocytes.
PBS:	phosphate buffered saline pH 7.2.
PHA:	phytohemagglutinin — a plant lectin that agglutinates animal cells and stimulates lymphocytes, mostly T cells, to proliferate.
pI:	isoelectric point.
PMA:	phorbol diester 12-myristate 13-acetate.
RNA:	ribonucleic acid.
rRNA:	ribosomal ribonucleic acid.
SDS:	sodium dodecyl sulphate.
SDS-PAGE:	sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
SSC:	sodium chloride, sodium citrate.
SSPE:	sodium chloride, sodium phosphate, ethylenediamine tetraacetic acid.
TCA:	trichloroacetic acid.
T cell:	a class of lymphocytes derived from the thymus capable of responding to thymus dependent antigens and MHC gene

products.

T_{CTL}: cytotoxic T lymphocyte.

T_{DTH}: delayed type hypersensitivity T.

TE: tris ethylenediaminetetraacetic acid.

T_H: helper T lymphocyte.

TRF: T cell replacing factor.

T_S: suppressor T lymphocyte.

CHAPTER I

INTRODUCTION

A. OVERVIEW OF THE IMMUNE RESPONSE

1. *The Cellular Basis of Immunity*

The immune response in higher animals has evolved as a defence mechanism against foreign antigens. Various types of cells participate in response to a foreign antigen to elicit an immune response. The major cells involved are the lymphocytes and macrophages. Lymphocytes are divided into two major categories according to the site at which they mature *i.e.* B-lymphocytes (B referring to the Bursa of Fabricius of birds or to the bone marrow in mammals) and T-lymphocytes (T referring to the thymus).

(a) B Lymphocytes

Upon activation with antigen, the B lymphocytes mature and synthesize and secrete free antibody, specific to foreign antigen, into the blood and other body fluids (humoral immunity). The final stages of maturation of B lymphocytes depend on soluble factors released by helper T lymphocytes and macrophages (see Section A.2). The secreted antibody acts, for example, by coating bacteria to enhance their phagocytosis or by binding to, and neutralizing, bacterial toxins.

(b) T Lymphocytes

T-cells include an array of cell subsets. Some cell subsets mediate important regulatory functions that help, T-helper cells (T_H), or suppress, T-suppressor cells (T_S). Immune responses by the production of soluble factors including lymphokines (see Section A.2 below). Other cell subsets are directly involved in effector functions, such as the cytotoxic destruction of antigen-bearing cells, cytotoxic T-cells (T_{CTL}). In

addition, T-cells are involved in other cellular immunity reactions, including delayed sensitivity and contact sensitivity (T_{DTH}).

(c) Macrophages and Other Cells

Besides B- and T-lymphocytes, other cell types participate in immunity by carrying out crucial effector functions such as phagocytosis, vasodilation and processing of antigen during immune induction. Thus, polymorphonuclear neutrophils, eosinophils, basophils and mast cells participate in the inflammatory response mediated by antibodies. The macrophages and macrophage-related cells such as the dendritic cells of the spleen, the epithelial Langerhans cells and the specialized epithelial cells, such as those found in the thymus, play a central role as auxillary cells in the initiation of the immune response and in the generation of T-cell memory. These cells serve to present antigen, in the context of 'self', to the helper T-cells. 'Self' is defined by an array of glycoprotein molecules which are encoded by the genes of the major histocompatibility complex (MHC) (reviewed in the murine system by Hood *et al.*, 1983) and are expressed on cell surfaces, thus enabling cells of one genetic origin to distinguish between 'self' and 'non-self'. Helper T-cells recognize class-II molecules expressed on B-cells and macrophages and cytotoxic T-cells recognize class-I molecules expressed on all somatic cells.

These immune cells occur in organized tissues and organs, including the spleen, the lymph nodes, the Peyer's patches of the intestine and the tonsils. In addition, a very substantial proportion of the lymphocytes and macrophages is present as a recirculating pool of cells in the blood and lymph.

2. *Helper T-Cells*

(a) Inductive Mechanisms

The induction of a T-cell immune response to a foreign antigen requires the activation of T lymphocytes with receptors for the specific antigen. The human T-cell antigen receptor has been shown to be a 90-kilodalton (kD) polymorphic heterodimer of α and β chains, each of 40- to 50-kD, associated with at least three 20- to 28-kD non-polymorphic polypeptide chains identified by the T3 monoclonal antibody (Haskins *et al.*, 1983; Yanagi *et al.*, 1984; Meuer *et al.*, 1983a; Van der Elsen *et al.*, 1984). Similarly, the mouse T-cell antigen receptor has been characterized and cloned (Allison *et al.*, 1982; Hedrick *et al.*, 1984; Van der Elsen *et al.*, 1985). T-cell activation is initiated after antigens, mitogens, such as phytohemagglutinin (PHA) or concanavalin A (Con A), or antibodies, interact with this complex T-cell antigen receptor (Palacios, 1982; Meuer *et al.*, 1983b). When the receptor interacts with antigen in the context of products of a major histocompatibility locus, and with the macrophage-derived product, termed lymphocyte-activating factor (Gery *et al.*, 1972), and now referred to as interleukin-1 (IL1) (Aarden *et al.*, 1979), the T-cells express the gene encoding the lymphokine interleukin-2 (IL2), previously termed T-cell growth factor (Morgan *et al.*, 1976; Smith, 1980a). The observation that the macrophage product, IL1, is a required signal for the mitogen-induced IL2 product is well documented (Shaw *et al.*, 1980; Larsson *et al.*, 1980b; Smith *et al.*, 1980b; Möller (ed.), 1982). Activation through the T-cell receptor complex is associated with an increase in intracellular calcium (Weiss *et al.*, 1986) and the activation of protein kinase C, with translocation of this enzyme from the cytoplasm to the plasma membrane (Nishizuka, 1984). These induction signals can be by-passed by the use of the calcium ionophore A23187, which increases intracellular calcium, and by phorbol myristatic acetate, which activates protein kinase C (Rosenstreich *et al.*, 1979; Farrar *et al.*, 1982; Kraft and Anderson, 1983; Truneh *et al.*,

1985).

To exert its biological effect, IL2 must interact with specific high affinity membrane receptors (Robb *et al.*, 1981b; Greene and Leonard, 1986). Resting T cells do not express IL2 receptors (IL2R), but after activation with an antigen or mitogen, the gene for the receptor is transcribed and the protein expressed on the T-cell surface (Leonard *et al.*, 1985). Once this process is initiated, activated T cells are clonally expanded as long as IL2 continues to be present. The failure to either produce the growth factor or its receptor results in a failure of the T-cell immune response. The interaction of Ag with its receptor is also involved in the production of other lymphokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), γ-interferon, and B-cell growth factor-I (BCGF-1 or BSF). These factors will be discussed in more detail in the next section.

(b) Lymphokines Produced by Helper T-Cells

(i) Interleukin-2

Although all subclasses of T-cells have been shown to release IL2 under the appropriate conditions, helper T-cells appear to be the major source of IL2 (Pfizenmaier *et al.*, 1984). The lymphokine IL2 has been well characterized both physiologically and biochemically (Farrar *et al.*, 1982). The fractions with IL2 activity purified from human, ape, rat and murine cells were found to be secreted as single polypeptide chains with molecular weights of 19,000-22,000 as determined by gel permeation chromatography and 15,000-17,000 by SDS-PAGE (Caplan *et al.*, 1981; Schreier *et al.*, 1980; Henderson *et al.*, 1983; Robb and Smith, 1981a). Although murine IL2 behaved as a molecule of 30,000-40,000 molecular weight by gel filtration, this behavior was attributed to non-covalent dimerization of a 15,000-17,000 molecular weight subunit (Paetkau *et al.*, 1984). Both human and mouse IL2 can be isolated in various forms differing by

amounts of glycosylation, leading to heterogeneity for each species. Human and mouse IL2 proteins themselves are different, the mouse IL2 having an intrinsic pI near 5.0, and the human IL2, near 8.2. Both mouse and human IL2 are hydrophobic and stable (Shaw *et al.*, 1978; Gillis *et al.*, 1982; Paetkau *et al.*, 1984). IL2 is detected by a variety of biological assays, such as stimulation of Con A-induced proliferation of thymocytes (DiSabato *et al.*, 1975; Paetkau *et al.*, 1976) and stimulation of *in vitro* proliferation of cytotoxic T lymphocyte lines (Morgan *et al.*, 1976; Gillis *et al.*, 1978). The specific activities of mouse and human IL2, expressed in terms of ED_{30} (see Abbreviations), are 10,000-15,000 and 5,000-8,000 units per μg protein, respectively (Riendeau *et al.*, 1983). The concentration of IL2 giving 30% maximal responses in cell culture are in the order of 3-10 x 10^{-12} M (Paetkau *et al.*, 1984). Although IL2 is well recognized as a growth factor for activated T cells, it has other biological properties. This factor has been reported to induce thymocyte proliferation (Paetkau *et al.*, 1976; Farrar *et al.*, 1978), to augment the proliferation and generation of cytotoxic cells by alloantigen-stimulated T-cell populations (Wagner and Röllinghoff, 1978; Farrar *et al.*, 1978) and to induce the synthesis of immune interferon (IFN γ) by alloantigen-stimulated T cells (Farrar *et al.*, 1981). IL2 has been shown to amplify antibody-forming B-cell responses by inducing T-cells to release B-cell differentiation factors (BCDF) (Inaba *et al.*, 1983) and B-cell growth factors (BCGF) (Howard *et al.*, 1983). IL2 may also have a direct effect on B-cells beyond that exerted indirectly through induction of B-cell specific lymphokines (Waldmann *et al.*, 1984; Korsmeyer *et al.*, 1983). In addition, IL2 may play a direct role in stimulating non T-cell immune responses, *e.g.* to increase the natural killer cell (NK) and lymphocyte-activated killer cell (LAK) activities (Henney *et al.*, 1981).

Recently, the genes encoding human and mouse IL2 have been molecularly cloned (Taniguichi *et al.*, 1983; Kashima *et al.*, 1985), and the cDNAs expressed in eukaryotic cells. The entire gene has now been sequenced (Fujita *et al.*, 1983; Holbrook

et al., 1984) and localized to human chromosome 4 (Siegel *et al.*, 1984). The human cDNA encodes biologically active IL2 which would consist of 153 amino acids including a 20 amino acid signal sequence. The longest mouse cDNA insert encodes a polypeptide of 169 amino acids (Kashima *et al.*, 1985). Of particular interest is the presence of highly conserved sequences in the 5'-flanking regions of the human and mouse IL2 gene (Fuse *et al.*, 1984). Whereas the coding region shows nucleotide homology of 72% between the two genes, the 5' upstream region spanning about 500 base pairs (bp) shows 85% homology (Fuse *et al.*, 1984). It is likely that such sequences are involved in controlled expression of the IL2 genes in activated T-lymphocytes (Chapter 1C, Section 2).

(ii) Colony-Stimulating Factors

T-cell-derived glycoproteins are also required to stimulate the proliferation of the hematopoietic cell lineages and modulate some aspects of the functional activity of these cells. Since these regulatory glycoproteins were first identified by their ability to stimulate precursor cells to form colonies of progeny cells, they are referred to as the colony-stimulating factors (CSFs). The best characterized of these CSFs are those controlling granulocyte-macrophage (GM) populations, (GM-CSFs) (Metcalfe, 1985). In addition, CSFs are not limited to the regulation of GM differentiation but they are also able to cause proliferation of a wide range of other cell types, including erythroid, megakaryocytic, eosinophil, mast, stem and multipotential cells. This molecule is referred to as Multi-CSF. Interleukin 3 (IL3) is included in the Multi-CSF complex, but is of T-lymphocyte origin and does not activate erythroid and stem cell populations (Metcalf, 1986).

Murine GM-CSF was purified to homogeneity as a glycoprotein of molecular weight 23,000 D (Burgess *et al.*, 1977). Based on amino-terminal amino acid sequence data oligonucleotide probes were used to isolate cDNAs for GM-CSF (Gough *et al.*,

1984; Gough *et al.*, 1985). Sequencing of these cDNA clones indicate that the mature GM-CSF polypeptide contains 124 amino acids. Mouse genomic GM-CSF exists in single-copy form, and the gene is localized on chromosome 11 (Gough *et al.*, 1984). Clones of cDNA for human GM-CSF have been isolated (Wong *et al.*, 1985; Lee *et al.*, 1985). Sequencing of these cDNA clones indicated that the precursor GM-CSF polypeptide contains 127 amino acids. In the protein coding region there is 70% nucleotide homology with the equivalent region of murine GM-CSF, but there is no species cross-reactivity between murine and human GM-CSF (Metcalf, 1986).

Similarly, Multi-CSF (IL3) has been purified to homogeneity (Ihle *et al.*, 1982). Estimates of molecular weight ranged from 23,000 to 30,000 D, possibly due to a varying carbohydrate content. Clones of cDNA for murine IL3 have been isolated (Fung *et al.*, 1984; Yokota *et al.*, 1984). The IL3 polypeptide appears to contain 140 amino acids. The chromosomal gene for murine IL3 has been characterized and shown to be present in a single copy form (Miyatake *et al.*, 1985a) and on the same chromosome as GM-CSF. Many T-lymphocytes have the ability to synthesize both GM-CSF and Multi-CSF; exposure of such clones to antigens or inducers, such as Con A, can cause an increase of transcription of both genes, suggesting some type of common induction process (Kelso *et al.*, 1986).

(iii) Immune Interferon

Interferons are a heterogeneous family of proteins: IFNs are induced by viral infection or bacterial stimulation and produced by leukocytes and fibroblasts (IFN-α and IFN-β, respectively). Immune interferon, also known as interferon-gamma (IFN-γ), is induced by specific antigens or mitogens and produced by stimulated lymphocytes. Helper T-lymphocytes predominantly produce IFN-γ (Trinchieri and Perussia, 1985), however upon appropriate stimulation NK cells can become potent IFN-γ producers (Trinchieri *et al.*, 1984). IFN-γ is a glycoprotein with 2 active species of molecular

weights 20,000 and 25,000 identified by SDS-PAGE (Yip *et al.*, 1982). The 146 amino acid sequence of mature murine IFN-γ was derived from the nucleotide sequence of the cloned cDNA (Gray and Goeddel, 1983). The human genomic DNA sequence of the IFN-γ gene is situated on chromosome 12 (Trent *et al.*, 1982) and there is evidence for only one gene. Thus, the 2 components of different molecular weight are due to post-transcriptional processing. The availability of homogeneous recombinant IFN-γ has enabled much progress to be made in the study of the biology of IFN-γ (Gray *et al.*, 1982).

In addition to its antiviral activity, IFN-γ has other biological effects including inhibition of cell proliferation, and various immunoregulatory actions (Friedman and Vogel, 1983). IFN-γ has profound effects on macrophages, stimulating their phagocytic and tumoricidal activities as well as inducing the increased expression of certain cell surface proteins, including the class-I and class-II MHC antigens (Steeg *et al.*, 1982). It has been found that murine IFN-γ treatment results in increases in the levels of mRNA for Class-I and Class-II MHC genes (Nakamura *et al.*, 1984; Paulnock-King *et al.*, 1985). In addition, IFN-γ acts as a B cell maturating factor and stimulates cytotoxic T cell responses (Farrar *et al.*, 1981).

(iv) B Cell Growth and Differentiation Factors

As mentioned previously, several antigen-nonspecific, genetically unrestricted factors derived from T cells have been shown to play a role in the regulation of B cell responses (Howard and Paul, 1982). One such factor, designated B-cell growth factor (BCGF-1) appears to be required for the proliferation of a subset of B cells after interaction with antigen or antiimmunoglobulin antibodies. BCGF-1 on its own activates resting B cells, and is therefore called B-cell stimulatory factor (BSF-1). BCGF has a molecular weight of 11,000-15,000 D with isoelectric points between pH 6.4 to 8.7 (Howard and Paul, 1982). Recently, cDNA clones have been isolated encoding a murine

T-cell product that has mast cell and T-cell growth activities, distinct from IL3 and IL2, as well as B-cell stimulating activities. These include induction of class II antigen on resting B cells, enhancement of IgE and IgG1 production and costimulation of anti-IgM-activated B cells. These are all properties of BSF-I (Lee *et al.*, 1986; Noma *et al.*, 1986). The nucleotide sequence from the cDNA predicts that the precursor polypeptide consists of 120 amino acids with a molecular weight of 14,000 (Lee *et al.*, 1986). Lee *et al.* (1986) and Noma *et al.* (1986) have designated this factor encoded by their cDNA clones as "interleukin-4".

A second series of factors, called B cell differentiation (BCDF) or T cell-replacing factors (TRF), is involved in the terminal maturation of proliferating B cells into immunoglobulin secreting cells. Upon induction with TRF, B cells undergo isotype switching (Möller [ed.], 1984).

(v) Summary

Mitogen- or antigen-initiated lymphocyte activation processes involve a concert of not only cellular membrane interactions and antigen-presentation events, but also a series of amplification signals mediated by lymphokines. Distinct lymphokines either bind to common target cells and induce identical functional cellular changes, or they bind to, and activate different cells involved in a cascade of interactions that eventually result in activation and promotion of the cytotoxic T-cell and antibody-forming B-cell responses (Farrar *et al.*, 1982).

Because IL2 production is a pivotal event in the cascade of antigen non-specific events, the study of this growth factor becomes most important in the understanding of the transduction of growth-promoting signals.

B. EFFECTS OF CYCLOSPORIN A (CsA) ON THE IMMUNE SYSTEM

1. *Historical Review*

Cyclosporin A (CsA), a relatively new immunosuppressive drug, was discovered by Borel in 1970 (Borel, 1982). This compound was extracted from two fungal strains, *Cylindrocapon lucidum* Booth and *Tolypocladium inflatum* Gams. It was found to have little antifungal activity, but had a low degree of toxicity. This prompted further investigation and a pharmacological screening program. Initial studies by Borel demonstrated that CsA had profound immunosuppressive activity (Borel, 1976a; Borel *et al.*, 1976b). CsA treatment caused an inhibition of antibody formation to T-dependent antigens, encouraged a prolongation of skin graft survival, and the suppression of delayed-type hypersensitivity skin reactions (Borel, 1976a; Borel *et al.*, 1976b; Borel *et al.*, 1977). Additional studies revealed that CsA failed to inhibit the antibody response to lipopolysaccharide antigens, suggesting that the action of CsA was limited to T lymphocyte-dependent responses (Borel *et al.*, 1976b). The role of CsA as a potent suppressor of organ allograft rejection has been demonstrated in many instances in a wide variety of species (Calne, 1979; White and Calne, 1982). This improved transplantation success rate for kidney, heart, liver and bone marrow (Tutschka *et al.*, 1979) was due to the unique property of CsA in sparing B cells, myelo- and hematopoietic elements.

2. *Effects on T-Lymphocyte Responses*

Initial studies revealed several important facts regarding the effects of CsA on lymphocyte responses. First, CsA inhibited lymphocyte proliferation in response to mitogen and alloantigen stimulation (White *et al.*, 1979). Second, in the primary mixed-lymphocyte response (MLR), the induction of cytolytic T-lymphocytes (CTL) was inhibited by CsA (Hess and Tutschka, 1980; Wang *et al.*, 1981). In contrast, the generation

of suppressor cells was not affected by CsA (Kupiec-Weglinski *et al.*, 1984; Hess *et al.*, 1981). Third, CsA had to be present at culture initiation or shortly thereafter in order to observe an inhibition of mitogen-induced T-cell activation (Hess and Tutschka, 1980). It was reported that the proliferative responses to mitogens *in vitro* were inhibited by CsA by blocking receptor-binding sites for antigen, OKT3 (monoclonal antibody to the T-cell receptor), Con A, PHA, or class-II MHC antigen (Palacios and Möller, 1981; White *et al.*, 1979; Britton and Palacios, 1982; Chapter 1B, Section 3). However, recent studies with cloned helper and cytolytic T-cell lines clarified the effects of CsA on single cell populations of T lymphocytes (Shevach, 1985). CsA has been shown to inhibit the proliferation of cloned-helper T-cells (Herold *et al.*, 1986; Orosz *et al.*, 1982) and cloned cytolytic T cells in one report (Orosz *et al.*, 1982) but not in another (Herold *et al.*, 1986). This discrepancy was probably due to the differences in systems. It has now been accepted that the effect of CsA is mediated, for the most part, by its ability to block IL2 production (Chapter 1B, Section 4). This has been demonstrated in a variety of systems (Bunges *et al.*, 1981; Kaufmann *et al.*, 1984; Britton and Palacios, 1982; Wagner, 1983). This may explain the observed inhibition of the generation of CTL by indirect suppression of helper T-cell function in mixed lymphocyte populations.

Stimulation of T-lymphocyte growth by IL2 or PMA, on the other hand, is not sensitive to CsA (Bunges *et al.*, 1981; Orosz *et al.*, 1982; Shevach, 1985). No direct effect of CsA on IL2 receptor expression was demonstrated using a monoclonal antibody to the IL2 receptor (Miyawaki *et al.*, 1983).

3. *Cellular Site of CsA Action*

The exact cellular site at which CsA disrupts lymphokine production remains uncertain. While it was initially suggested that plasma membrane receptors for CsA on mouse and human lymphocytes were present (Ryffel *et al.*, 1980; Ryffel *et al.*, 1982), LeGrue *et al.* (1983) showed that CsA does not bind to specific drug sites but, because

it is highly hydrophobic, it partitions readily into lipid bilayers. Alternatively, it was suggested that CsA interferes with the binding of the OKT3 and mitogen receptors (Palacios and Moller, 1981). However, it was found by Colombani *et al.* (1985a), that there was no influence of OKT3 monoclonal antibody pretreatment on dansylated-CsA binding. Furthermore, the dansylated-CsA was found to accumulate in both the membranes and cytoplasm, but not in the nuclei of cloned T-cells (Colombani *et al.*, 1985a). An uncharacterized 15 kD cytoplasmic protein that binds to CsA, called cyclophilin, was isolated from a murine thymic lymphoma (BW5147), and was also found in a variety of murine tissues, as well as in the calf thymus (Merker and Handschumacher, 1984; Handschumacher *et al.*, 1984). It is difficult to postulate a relationship between cyclophilin and the mechanism of action of CsA, since cells found to be resistant to growth inhibition by CsA contained the similar protein. However, it is possible that the sensitivity to CsA may relate to the rate of production or turnover of the binding component.

Cyclophilin has been purified to homogeneity from bovine thymocytes. It binds CsA with a dissociation constant of 2×10^{-7} M (240 ng ml^{-1}), is sulfhydryl dependent, unstable at 56°C and at pH 4 or 9.5, and sensitive to trypsin digestion. The first 46 out of 117 residues have been identified and were found to have no sequence homology to any other proteins in the National Biomedical Research Foundation data base (Handschumacher *et al.*, 1984). Recently, it has been observed that purified cyclophilin has immunologic cross-reactivity with calmodulin, as determined in a calmodulin radioimmunoassay involving a high-affinity polyclonal antiserum (Colombani *et al.*, 1985b). It has also been shown that dansylated-CsA binds to calmodulin, and *vice versa*. In addition, calmodulin inhibitors R24571 and W-7 competitively inhibit the binding of CsA to cloned T lymphocytes (Colombani *et al.*, 1985b). It is important to note that cyclophilin may be a structural analog to calmodulin or may co-purify with it, which could account for the cross-reactivity in the calmodulin radioimmunoassay.

Calmodulin is a ubiquitous calcium binding protein that plays an important role in regulating calcium-dependent cell-functions (Means and Dedman, 1980). *In vitro* models of T-cell activation have demonstrated that proliferation in response to mitogens (PHA or Con A), the OKT3 monoclonal antibody, or the calcium ionophore A23187, results in an increase in intracellular free calcium ($[Ca^{2+}]_i$) (Chapter 1A, Section 2a). In contrast, phorbol esters have no effect on $[Ca^{2+}]_i$ in a cloned human T cell line (Oettgen *et al.*, 1985) or in non-transformed T lymphocytes (Tsien *et al.*, 1982). CsA has been shown to inhibit the proliferative response of human lymphocytes induced by Con A and A23187 (Kay *et al.*, 1983a,b), but to have no effect on the responses of human T lymphocytes to stimulation by PMA (Sugawara and Ishizaka, 1983; Shevach, 1985). The conclusion that CsA affects T cell activation by affecting $[Ca^{2+}]_i$ or phorbol ester-mediated stimulation of protein kinase C activity has been disproved. CsA was observed not to affect the increase in intracellular calcium secondary to mitogen stimulation of T lymphocytes (Metcalfe, 1984; Wiskocil *et al.*, 1985), nor to block the activation signalling regulated by PMA-induced activation of protein kinase C (Fidelus and Laughter, 1986; Manger *et al.*, 1986). The observations that calmodulin inhibitors, trifluoroperazine (TFP) or W-7 inhibited both human and murine T-lymphocyte proliferation in response to alloantigen or mitogen in a dose-dependent manner (Cheung *et al.*, 1984; LeGrue and Munn, 1986), and that W-7 inhibited IL2 mRNA production in TPA plus A23187 stimulated human tonsillar lymphocytes (Yamamoto *et al.*, 1985), are fortuitous with respect to CsA being a calmodulin-specific antagonist. The fact that CsA and calmodulin inhibitors both block the induction of IL2 mRNA is not sufficient evidence to assume that CsA's action is mediated via calmodulin. Finally, only one calmodulin-dependent enzyme activity (*in vitro* brain phosphodiesterase) was shown to be inhibited by CsA (Colombani *et al.*, 1985b) from a number of fundamental cellular activities regulated by calmodulin.

4. *Effects on Lymphokine Gene Expression*

Recent investigations using cDNA probes for lymphokines have indicated that, in both tumor and normal cells, the inhibition of lymphokines by CsA occurs at a pretranslational level. CsA was shown to inhibit IL2 mRNA production in PMA plus mitogen-stimulated Jurkat cells (a human leukemic T cell line) (Elliott *et al.*, 1984; Krönke *et al.*, 1984; Wiskocil *et al.*, 1985). Similarly, CsA was shown to inhibit IL2 mRNA synthesis in PMA stimulated EL4 cells (a murine lymphoma T cell line) (Granelli-Piperno *et al.*, 1984). In addition to IL2, CsA has also been shown to inhibit IFN-γ production in thymocytes and T lymphocytes (Reem *et al.*, 1983) and production of IFN-γ mRNA in Jurkat cells (Wiskocil *et al.*, 1985). In normal T cells, CsA was also shown to inhibit the synthesis of IL2 and IFN-γ mRNAs (Granelli-Piperno *et al.*, 1986). In cloned helper and cytolytic T cell lines, CsA was shown to inhibit the synthesis of IL2, IL3, and γIFN mRNAs (Herold *et al.*, 1986).

It is also likely that CsA affects the generation of other soluble immunologically active factors *e.g.*, macrophage inhibitory factor (MIF) (Thompson *et al.*, 1983) and IL1 (Bunges *et al.*, 1981). However, CsA does not affect the synthesis of lymphokines in all instances, since cell lines constitutively producing IL2 (the primate MLA 144) or IL3 (WEHI-3) are not affected by the presence of CsA (Bouwer and Hinreds, 1983). Furthermore, three lines of evidence suggest that CsA does not affect the expression of the IL2 receptor, (i) responsiveness to IL2 of cloned helper and cytolytic T cells was not affected by CsA (Herold *et al.*, 1986); (ii) anti-Tac binding to Jurkat is not inhibited by CsA (Krönke *et al.*, 1984) and (iii) CsA does not inhibit IL2 receptor mRNA in stimulated PBL (Shaw *et al.*, submitted). In addition, CsA does not inhibit the expression of another inducible gene, HT-3, in induced Jurkat cells (Krönke *et al.*, 1984) suggesting that CsA exerts a selective inhibition on induced genes in T lymphocytes.

The biochemical mechanism by which CsA mediates its action is still unclear. However, the observation that the effect of CsA may in fact reside at the level of IL2 gene transcription opens up the possibility of further studies on lymphokine gene expression.

C. REGULATION OF EUKARYOTIC GENE EXPRESSION

1. *General Principles*

In general, eukaryotic gene regulation can occur at each step that is required for mRNA formation (transcription) and protein synthesis (translation). The variety of gene control sites include (i) RNA transcription, (ii) RNA processing and transport and (iii) mRNA stability and accessibility to ribosomes (Darnell, 1982). The following discussion will concentrate on the levels of control of mRNA concentration, and not the biochemical mechanisms underlying the controls.

(i) mRNA Transcription. mRNA transcription is initiated when RNA polymerase II recognizes a conserved 8-10 nucleotide region ('TATA' box) located 25-30 nucleotides upstream from the RNA start site. This regulatory region is important for establishing the variation of transcription rates. Recently, factors have been described that control the decision of RNA polymerase II to initiate transcription from the promoter region. Such 'trans-acting' proteins have been identified for a number of systems including SV40 transcription (Dynan and Tjian, 1983) immunoglobulin transcription (Singh *et al.*, 1986) and adenovirus transcription (Kovesdi *et al.*, 1986). To measure transcriptional activity *in vitro* assays must be used in which nuclei are isolated and previously initiated growing RNA chains are elongated in the presence of radioactive nucleoside triphosphates. It is then possible, with cDNA clones as probes, to measure differential transcription rates of specific genes. Eukaryotic gene expression is frequently controlled at the transcriptional level.

(ii) RNA Processing. The sequence of events that convert a primary RNA transcript to a mature mRNA are collectively known as 'RNA processing'. Soon after initiation of an RNA chain, a m'Gpp residue (cap) is added to the first nucleotide. At the 3' end of the transcript, a poly(A) segment is added post-transcriptionally to heterogeneous nuclear RNA (hnRNA). At least part of the signal for poly(A) addition is provided by the AAUAAA sequence found 10-25 nucleotides upstream from the poly(A) addition site. The poly(A) segment is complexed with specific proteins and this ribonucleoprotein complex is required for efficient transport of the message from the nucleus to the cytoplasm. The final processing steps, which also occur in the nucleus, require removal of non-coding intervening sequences by a mechanism termed 'splicing'.

The function of the 5'-cap is to protect the mRNA from degradation as well as to act as a recognition signal for ribosomes and thus promote efficient translation. The poly(A) segment is not required for translation, but is required to protect the mRNA from cytoplasmic degradation. The control of mRNA processing can be achieved either through differential selection of poly(A) addition sites (*e.g.*, heavy chains of the immunoglobulins [Rogers *et al.*, 1980]) or variations in the splicing patterns (*e.g.*, adenovirus nuclear RNA [Shaw and Ziff, 1980]).

(iii) RNA Stability. After the mRNA has reached the cytoplasm, the fates of mRNA molecules differ. Evidence exists for a wide range of half lives for different specific mRNAs in the same cells. For example, adenovirus-2 mRNAs in rat cells turn over more rapidly (35 and 100 min) than the bulk of poly(A)-containing mRNAs (4 to 5 hr) (Wilson *et al.*, 1978). In addition, different half lives for the same mRNAs in the same cell under different circumstances have been observed. During erythroblast differentiation in mammals, the developing cell makes many different mRNAs including globin mRNA. Globin mRNA constitutes about 10% of the population and has a half-life of 17 hr. During the final cell divisions the cell becomes a highly specialized

reticulocyte in which 90% of the cellular messages are globin mRNA, with the same half-life of 17 hr. This can be accounted for only by the specific destruction of all other long-lived mRNA components (Bastos *et al.*, 1977).

There are examples of hormone-dependent changes in half lives of specific mRNAs. When mammary gland organ cultures are stimulated with prolactin, there is an accumulation of casein mRNA. This accumulation is associated with a 17-25 fold increase in the mRNA half-life, but only a 2-3 fold increase in its transcriptional rate (Guyette *et al.*, 1979). A second reported instance of mRNA stabilization occurs with oestrogen treated roosters. The half-life of vitellogenin and VLDL mRNAs during the first week of oestrogen treatment is >24 hr. However, upon oestrogen withdrawal, the vitellogenin and VLDL mRNAs are destabilized to a half-life of 3 hr (Wiskocil *et al.*, 1980). Thus the effective cytoplasmic concentration of a specific mRNA can be determined by several factors including the rate of gene transcription, the efficiency of processing of the primary transcript and the stability of the mature mRNA.

2. *Regulation of Lymphokine Gene Expression*

When helper T-cells are activated by lectin and antigen, various lymphokines are induced at high levels (Chapter 1B, Section 2). Therefore, many lymphokine genes may contain specific sequence(s) required for this inducible expression. A comparison study using the 5'-flanking region sequences of IL2, IL3, IFN-γ and GM-CSF revealed no significant consensus sequences shared by all lymphokine genes but did indicate the presence of several homologies (Miyatake *et al.*, 1985b). A GC-rich region, preceding the TATA box, was found in mouse IL3, mouse and human IL2 and mouse GM-CSF but not in the human IFN-γ gene. However, there are sequence homologies between mouse IL3 and mouse GM-CSF, and between mouse GM-CSF and human IFN-γ (Miyatake *et al.*, 1985b). In another study, a consensus sequence, 5'-GPuGPuTTPyCA Py-3', was found in the 5'-flanking regions of murine GM-CSF, Multi-CSF, and IL2. This

sequence is also present in the human IFN-γ gene, albeit in the reverse orientation (Stanley *et al.*, 1985). This consensus sequence may play a role in the coordinated induction of these genes.

Coordinate induction has been demonstrated for IL2 and IFN-γ in PHA and PMA stimulated Jurkat cells (Wiskocil *et al.*, 1985). Although it has been demonstrated that IL2 can enhance IFN-γ production (Farrar *et al.*, 1981; Torres *et al.*, 1982; Kasahara *et al.*, 1983) and IL2 R expression (Depper *et al.*, 1985; Malek and Ashwell, 1985) in cultures of normal T cells, it has also been shown that this probably occurs only in suboptimally stimulated T-cells (Krönke *et al.*, 1985). In fact, nuclear transcription assays in human T-cells stimulated with PHA and PMA revealed that IFN-γ and IL2 R gene transcription occurred before that of IL2 (Krönke *et al.*, 1985).

Most mouse and human lymphokine genes such as IL2 (Fujita *et al.*, 1983), IFN-γ (Gray and Goeddel, 1983) and GM-CSF (Stanley *et al.*, 1985; Miyatake *et al.*, 1985b) are composed of 4 exons and 3 introns, except IL3 (Miyatake *et al.*, 1985a) which consists of 4 exons and 5 introns. Gene activation in eukaryotic cells requires that the configuration of the DNA be "exposed" to allow for RNA polymerase and/or regulatory molecules to bind and initiate the synthesis of new RNA chains. Such regions would be preferentially digested with DNase. In accord with this view, a particular DNase hypersensitive site at the 5'-side of the IFN-γ gene has been found in the chromatin of activated T-cells, but not in inactivated T-cells (Hardy *et al.*, 1985). This result suggests that signals required for IFN-γ gene activation (transcription) exert their effect at least in part at the 5'-flanking regions of the gene.

Activation of the IL2 gene has been demonstrated by means of oocyte-translation systems (Elliott *et al.*, 1984; Granelli-Piperno *et al.*, 1984), Northern blotting (Elliott *et al.*, 1984; Wiskocil *et al.*, 1985) and nuclear transcription assays (Krönke *et al.*, 1985). A kinetic analysis has shown that the appearance of IL2 and IFN-γ mRNAs in

induced Jurkat cells was transient, such that IL2 and IFN-γ mRNAs reached maximum levels at 4 to 6 hours after induction and were barely detectable by 12 hr (Clarke *et al.*, 1984; Krönke *et al.*, 1984; Wiskocil *et al.*, 1985). Similar, though slower, kinetics of IL2 mRNA in PHA-activated human tonsillar lymphocytes have been reported (Efrat *et al.*, 1982; Efrat *et al.*, 1984). The progressive decline in the IL2 mRNA could be the result of shut-off of active IL2 mRNA synthesis and/or the inactivation or breakdown of the mRNA. Efrat and Kaempfer (1984) have shown that treatment of induced T-cells with cycloheximide, an inhibitor of protein synthesis, resulted in a retardation of the down regulation of IL2 mRNA with the concomitant increase ('superinduction') in mRNA accumulation. This suggests that the expression of the IL2 gene might be under the control of a repressor protein. This negative control element could act as a repressor of transcription or as a factor accelerating IL2 mRNA degradation. It has been reported that the transcription rate of the IL2-gene in superinduced cells does not exceed that observed in normally induced cells (Krönke *et al.*, 1985; Efrat and Kaempfer, 1985). In a series of "run-on" experiments, Krönke *et al.* (1984) noted that nuclear transcription of IL2 continues for at least 15 hr following stimulation of Jurkat at a time when mRNA levels are decreasing. This result, and the fact that the superinduction phenomenon appears to be in part a result of mRNA stabilization suggests that non-transcriptional regulatory mechanisms are involved in the rapid fall of IL2, and presumably IFN-γ, transcripts following activation. Such non-transcriptional regulatory mechanisms could include degradation of the transcribed RNA (Chapter 1C, Section 1). Further understanding of the mechanisms that result in the turning on and off of the IL2 genes will be important for understanding lymphokine gene regulation.

D. THESIS OBJECTIVE

The objective of this study was to investigate molecular mechanisms important in the process of lymphokine gene regulation and therefore in overall immune responsiveness. The experimental system used was the induction of lymphokines by PMA in murine and human T-lymphocyte tumor cells. These cells are induced to synthesize and secrete lymphokines by the same signals as normal T-lymphocytes. Since the immunosuppressive agent CsA is known to suppress lymphokine production at the transcriptional level (Elliott *et al.*, 1984), but not to affect the general synthesis of cellular genes (Paetkau *et al.*, 1985), it was used as a modulator of lymphokine production.

To achieve the overall objective, it was first necessary to assess the ability of Cs to inhibit lymphokine mRNA production (*i.e.*, IL2 and GM-CSF) in a variety of cell types. For this, a rapid method had to be devised or adopted to isolate and quantitate lymphokine mRNAs. The study of IL2 gene regulation, using CsA as a tool to specifically perturb the gene expression of lymphokines, constituted the main objective of this research.

CHAPTER II

MATERIAL AND METHODS

A. CELL CULTURE

1. *Cell Culture Medium*

The cell culture medium consists of RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) with the addition of 20 mM. sodium bicarbonate, 0.34 mM sodium pyruvate, 0.02 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid) (Sigma, St. Louis, Mo.) adjusted to pH 7.3, and supplemented with 100 I.U. ml^{-1} penicillin G potassium (10×10^4 I.U. l^{-1}, Ayerst Hospital Pharmacy, Montreal, Quebec) and 100 μg ml^{-1} streptomycin sulfate (Gibco). This medium is referred to as RH.

RH with 10^{-4} M β-mercaptoethanol (Baker) is referred to as RHM. RHM with 10% (v/v) heat inactivated fetal calf serum is referred to as RHFM.

Cells grown in RHFM were incubated under 5% CO_2 in air at 37°C and under 100% humidity.

All cell culture work was performed with either COSTAR tissue culture plates (Belco Glass Inc., Vineland, N.J.) or Nunc and Corning tissue culture plates, flasks and centrifuge tubes (Gibco Laboratories, Grand Island, N.Y.)

2. *Cell Lines*

EL4.E1 is a subclone of the murine T-cell lymphoma described by Farrar *et al.* (1980) which produces high levels of IL2 when stimulated with phorbol diesters. The subclone JEH.3B of the human T-cell leukemia line (Jurkat) was described by Gillis and Watson (1980) which also generates IL2 upon stimulation with phorbol diester plus mitogen.

EL4 and Jurkat cell variants were grown in RHFM to a maximum density of 2 x 10^6 cells ml^{-1}. Cell density was determined by scoring on a hemacytometer after staining dead cells with 0.7% eosin. Fresh cultures were seeded at a density of 2 x 10^4 cells ml^{-1}. High producers of IL2 from the EL4.E1 or Jurkat. 3B cell lines were frozen at a cell density of 1 x 10^6 cells ml^{-1} in RHFM containing 10% dimethylsulfoxide (DMSO) (Fisher Scientific, N.J.) and 20% FCS (Gibco fetal calf serum) in a 2 ml pro-vial (Cooke Laboratories, Alexandria, Virginia) and stored liquid nitrogen.

Frozen stocks were periodically thawed at 37°C, diluted in 5 mls RHFM, centrifuged at 250 x g for 5 minutes and resuspended at a density of 5 x 10^4 cells ml^{-1}.

3. *Isolation of Peripheral Blood Lymphocytes*

Peripheral blood mononuclear cells were isolated either from the concentrated buffy coats (the white blood cell layer when fresh heparinized blood is sedimented at 400 x g) of normal human donors or from fresh blood collected in 17 x 10 mm heparinized vacutainer tubes (Becton, Dickinson & Co., Mississauga, Ontario) from a healthy human donors, by means of sedimentation on Ficoll-Paque (Pharmacia Inc., N.J.). The blood was carefully overlaid on Ficoll and centrifuged at 1000 x g for 20 min. The interface was collected and the cells pelleted at 250 x g for 10 min. The cells were washed three times in RHFM and resuspended to a final concentration of 10^6 cells ml^{-1} in RHFM.

4. *Reagents*

Samples of cyclosporin A (CsA), cyclosporin C (CsC) and cyclosporin D (CsD), kindly donated by Dr. F. Pasutto (Department of Pharmacy, University of Alberta) were dissolved in DMSO at a concentration of 1 mg ml^{-1} and diluted to the desired concentrations in the culture medium. Cyclosporin samples were stored frozen at -20°C.

Four different analogues of cyclosporin A synthesized in the laboratory of Dr. J. Vederas (Department of Chemistry, University of Alberta), were received from him as a gift. These analogues were dissolved in DMSO and then diluted to a working stock of 100 mg ml^{-1} in RH. Serial threefold dilutions were made from the stock and stored at -20°C.

Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol (1 mg ml^{-1}) and diluted with media to a 5 μg ml^{-1} stock. Concanavalin A (Con A) (Sigma) was made up in RH at a concentration of 2 mg ml^{-1} and filter-sterilized. Cycloheximide (Sigma Chemical Co.) was made up in phosphate-buffered saline (PBS) at a concentration of 2 mg ml^{-1}. All these reagents were stored at -20°C.

Dexamethasone (K-line Pharmaceuticals, Downview, Ontario) and melphalan (Sigma) were both diluted in media to a concentration of 500 μg ml^{-1} and stored at 4°C.

5. *Stimulation of Cells to Produce Lymphokines*

In order to induce IL2 synthesis, EL4.E1 cells at a concentration of 10^4 ml^{-1} in a Costar 6-well plate (5 ml total) were stimulated with 15 ng ml^{-1} PMA. Supernatants were harvested by centrifugation (250 x g for 5 min) after 24 hours when optimal levels of IL2 activity were obtained. For maximal IL2 mRNA production, cells were harvested 12 to 16 hours after stimulation (Bleackley *et al.*, 1981). To induce the optimal synthesis and secretion of lymphokines from the Jurkat cell line, it was necessary to use both PMA (15 ng ml^{-1}) and the mitogen Con A (30 μg ml^{-1}). Human peripheral blood lymphocytes (PBL) were stimulated in the same way as Jurkat cells. In the case of Jurkat cells maximal IL2 mRNA synthesis occurred 4 to 6 hours after stimulation, whereas with PBL, IL2 mRNA was detectable after 12 hours (observed

results).

In order to obtain a complete inhibition of lymphokine mRNA production, 100 ng ml^{-1} of CsA were routinely used for all the cell lines studied. This had no effect on cell viability throughout the incubation period (observed results, Elliott *et al.*, 1984; Krönke *et al.*, 1984).

6. *Biological Assay for IL2 Activity*

The standard assay cell MTL2.8.2 is an IL2-dependent subclone of a murine cytotoxic T cell line (CTL) described by Bleackley *et al.* (1982). This assay measured the ability of metabolically active MTL2.8.2 cells to reduce the tetrazolium dye 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazon product, as described by Mosmann (1983).

Triplicate, independent serial dilutions (usually threefold) of cell-free supernatants to be assayed were prepared in 96-well, flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) in a 50 μl volume of RHFM. MTL2.8.2 cells, (5×10^4 in 100 μl RHFM) were added to each well, and the cultures incubated for 48 hours. Four to six hours before measurement, 10 μl of a 5 mg ml^{-1} aqueous solution of the MTT dye was added. At the time of harvest, 30 μl of 10% N-lauroylsarcosine (Sarkosyl) (Sigma) was added and the trays were additionally incubated for 30 min at 37°C in order to solubilize the proteins. To solubilize the dye and bleach the phenol red color of the cultures, 100 μl of 0.04N HCl in 2-propanol were added, and the samples were mixed vigorously with a multipipettor. The microtiter plates were then directly analyzed in a Dynatech MR600 ELISA plate reader by using 570 and 630 nm as test and reference wavelengths, respectively. The activity of IL2 was expressed as a percentage of an internal positive control.

B. ISOLATION AND CHARACTERIZATION OF RNA

1. *Dot Blot Analysis of Cellular Cytoplasmic RNA*

Relative levels of cellular cytoplasmic mRNA were determined by the cytoplasmic dot hybridization method described by White and Bancroft (1982). Cells (2-5 x 10^6) were pelleted by centrifugation (250 x g, 5 min) resuspended in 1.0 ml of PBS and repelleted by centrifugation in a sterile 1.5 ml tube (15,000 x g, 15 sec) in an Eppendorf Model 5412 Centrifuge (Brinkman). After resuspension in 45 μl of ice-cold 10 mM Tris (pH 7.0), 1 mM disodium ethylene tetraacetic acid (EDTA) (referred to as TE), cells were lysed by addition of two 5 μl aliquots of 5% Nonidet P-40 (NP-40) (Sigma Chemical Co.) with 5 min of mixing on ice between the additions. Following the pelleting of nuclei, (15,000 x g, 2 min), 50 μl of the supernatant were transferred to a sterile 1.5 ml tube containing 30 μl of 20X SSC (0.15M NaCl, 0.015M trisodium citrate) (SSC) plus 20 μl of 37% (w/w) formaldehyde (J. Baker, Chemical Co., Phillipsburg, N.J.). The mixture was then incubated at 60°C for 15 min and stored at -70°C.

For analysis, 5-20 μl aliquots of each sample (approximately 2.0 x 10^6 cell equivalents) were serially diluted (usually two-fold) with 15X SSC in a 96 well microtitre plate to yield a final volume of 150 μl. All 150 μl of each dilution were applied with suction to a 4 mm diameter spot on a nitrocellulose sheet (BA45, 0.45 μM) supported on No. 470 paper employing a 96-hole Minifold apparatus (Schleicher and Schuell). The nitrocellulose sheet was then baked (80°C, 2 hours) *in vacuo* to fix cytoplasmic macromolecules and stored in a dry place until ready to prehybridize.

2. *Northern Analysis of Cellular mRNA*

Isolation of cellular cytoplasmic mRNA for Northern analysis was similar to that described above, with the following modifications. After cells were washed in PBS and centrifuged as described, the pellets were resuspended in 90 μl of ice-cold TE, and lysed by the addition of two 10 μl aliquots of 5% NP-40 with 5 min incubation on ice between the additions. Following pelleting of nuclei as described, 100 μl of the supernatant were transferred to a sterile 1.5 ml tube containing 300 μl TE, 20 μl 10% (w/v) sodium dodecyl sulfate (SDS) (Biorad Laboratories), and 10 μl of 2 mg ml^{-1} proteinase K (Bethesda Research Laboratories). The mixture was digested at 37°C for 15 min, the RNA precipitated at -20°C overnight by the addition of sodium acetate (pH 5.2) to a final concentration of 0.3M and 2.2 volumes of 95% ethanol. Total RNA was recovered from the ethanol by centrifugation at 15,000 x g for 15 min at 4°C. The pellet was dried and dissolved in 20-30 μl sterile 25 mM EDTA, 0.1% SDS, and the concentration of RNA determined by fluorimetry (Morgan *et al.*, 1979).

Gels for Northern analysis of mRNA consisted of 0.8% agarose (BioRad) in buffer containing 20 mM 3-[N-morpholino] propane sulfonic acid, pH 7.0 (MOPS) (Sigma), 5 mM sodium acetate, 1 mM EDTA, 1% formaldehyde and 1.5 μg ml^{-1} ethidium bromide. RNA samples (6-20 μg) were denatured by incubating at 55°C for 15 minutes in 20 μl of the same buffer containing 6.5% formaldehyde and 50% deionized formamide (Fluka, Germany), before being cooled to 4°C and loaded onto the gel. Gels were electrophoresed for about 10 hr at 30 volts, soaked in 20X SSC for 0.5 hr, and transferred to BA85 nitrocellulose filters (Schleicher and Schuell) which had been soaked for 1 hr in 15X SSC. Nitrocellulose filters were then baked in an 80° vacuum oven for 2 hr, and stored in a dry place until ready to prehybridize.

3. *Isolation of poly(A)⁺ RNA by Messenger Affinity Paper*

Poly(A)⁺ RNA was separated from total cytoplasmic RNA according to the procedure of Wreschner and Herzberg (1984). A diazonium-activated paper is chemically coupled with poly(U), giving it a very high affinity for poly(A) sequences. The poly(A)-containing messenger RNA affinity paper (mAP) was obtained from Amersham (Oakville, Ontario) and the protocol recommended by the manufacturers was followed. Briefly, mAP was cut into 1 cm^2 pieces with sterile scissors and wetted in 2X SSPE (0.15M NaCl, 0.01M monosodium phosphate, 1 mM EDTA). Aliquots of 10 μl of an RNA solution were placed on the mAP pieces that were placed on several layers of Whatman 3 mm filter paper. This was followed by washings of the mAP pieces separately in 5 mls of 0.5M NaCl (three times, 10 min each) in a Costar 6-well dish, and finally in 5 mls of 70% (v/v) ethanol for 2 min. The air-dried mAP pieces were then transferred into 1 ml of distilled water in a sterile 15 ml Falcon tube for 5 min at 70°C; this released the poly(A)⁺ RNA. The mAP was removed and discarded. Poly(A)⁺ RNA was precipitated with 1/10 vol of 3M sodium acetate and 3 vol of ethanol (-20°C).

4. *Probes*

Oligonucleotide probes were synthesized by P. Barr (Chiron, Emeryville CA.) on an Applied Biosystems nucleotide synthesizer. They were based on published sequences for human (Taniguchi *et al.*, 1983) and mouse (Fuse *et al.*, 1984) IL2 and murine GM-CSF (Gough *et al.*, 1983). Cloned cDNA probes for mouse IL2, GM-CSF and human IL2 were isolated from cDNA libraries (in λgt 10) from PMA-stimulated EL4.E1 cells or PMA and Con A-stimulated Jurkat cells respectively by John Elliott in Dr. V. Paetkau's laboratory (University of Alberta). The screening probes for murine and human IL2 were the human IL2-34, which cross-reacts with murine IL2 by virtue of 25 out of 28 identities at the 3' end of the probe, as well as murine and human

specific oligonucleotide probes described in detail in Shaw *et al.* (Shaw *et al.*, 1986, submitted). Murine GM-CSF cDNA clones were identified using the mixture of 3 oligonucleotides mouse GM-30.1, -.2, and -.3 The characteristics of the oligonucleotide and cDNA probes are shown in Table 1 (taken from Shaw *et al.*, 1986).

5. *Labeling of Probes and Hybridization*

Synthetic oligonucleotide probes (usually 100 ng) were 5'-end labeled with [γ-^{32}P]-ATP (New England Nuclear, 2900 Ci nmol^{-1}) using 300-400 units ml^{-1} T4 polynucleotide kinase (Pharmacia, Dorval, Québec) in buffer containing 0.1M Tris.Cl (pH 7.6), 0.2M $MgCl_2$, 10 mM dithiothreitol, 0.2 mM spermidine (37°C, 30 min) to a final specific activity of 3-6 x 10^8 cpm μg^{-1}. The cDNA probes were nick translated in the presence of [α-^{32}P]-dCTP (New England Nuclear, 3000 Ci mmol^{-1}) using a kit from Bethesda Research Laboratories (BRL, Gaithesburg, Maryland), to a final specific activity of 0.8-4 x 10^8 cpm μg^{-1}.

Filters were prehybridized for 6-18 hr at 42°C in a solution containing either 20% (v/v) (for oligonucleotide probes) or 50% (v/v) (for full length cDNA probes) formamide, 50 mM sodium phosphate, pH 6.8, 2 mM sodium pyrophosphate, 100 μM ATP (Sigma), 5X Denhardts solution (0.02% [w/v] each of ficoll, polyvinylpyrollidone and BSA), 5X SSC, 0.1% (w/v) SDS, 2.5 mM EDTA and 100 μg ml^{-1} heat denatured salmon sperm DNA (Sigma) and 100 μg ml^{-1} yeast tRNA (Boehringer Mannheim, Dorval, Quebec) as described by Maniatis *et al.*, 1982. Hybridization to ^{32}P-labeled DNA probes was carried out in the same solution, for 18 hr at 42°C, at a final concentration of 0.5 - 2 x 10^6 cpm ml^{-1}. Filters which had been hybridized with synthetic oligonucleotide probes were washed for 1.5 hr at 42°C in 2X SSC/0.1% (w/v) SDS. Filters hybridized with cDNA probe were washed for 0.5 hr at 42°C in 2X SSC/0.1% (w/v) SDS, followed by two washes for 0.5 hr each at 55°C in 2X SSC/0.1% (w/v) SDS and 0.2 x SSC/0.1% SDS, respectively. Filters were wrapped in "Saran wrap" and

TABLE 1

DESCRIPTION OF PROBES

Probe	Length	Region Encoded	Sequence
Synthetic Probes:			
Human-IL2-34	34	C terminus of human and murine IL2	(5')AGTTAGTGTTGAGAT-GATGCTTTGACAAAAGGTA
Murine-GM-43.13	43	Amino acids 263-272, primary translation product, murine GM-CSF	(5')GCTCGAATATCTTCAG-GCGGGTCTGCACACATGTTA-GCTTCTT
Murine-GM-43.14	43	Amino acids 90-104, primary translation product, murine GM-CSF	(5')TTCAAGGCGCCCTTGA-GTTTGGTGAAATTGCCCCGT-AGACCCT
Murine-GM-43.15	43	Amino acids 105-118, primary translation product, murine GM-CSF	(5')CGGAGTTGGGGGGCAG-TATGTCTGGTAGTAGCTGGC-TGTCATG
Cloned cDNA Probes:			
Human-pIL2.1	820	Human IL2 cDNA clone	Entire coding region plus 100 nucleotides of 5' UT and 260 nucleotides of 3' UT
Murine-pIL2.1	960	Murine IL2 cDNA clone	Entire coding region plus 130 nucleotide of 5' UT and 320 nucleotides of 3' UT
Murine-pGM.1	900	Murine GM-CSF cDNA clone	Entire coding and 3' UT regions, plus 50 nucleotides of 5' UT.

The table summarizes the properties of both synthetic oligonucleotide probes and cloned cDNA sequence probes. The number at the end of the designations of the synthetic probes indicates the length (if more than one probe of that length was made, they are identified as .1, .2 etc.).

exposed to film (Kodak X-Omat AR) with 2 intensifying screens for 1 to 3 days at -70°C.

Hybridized probe was removed by either boiling the filters for 10 min in distilled water or washing for 1-2 hr at 65°C in 50 mM Tris.Cl (pH 8.0), 2 mM EDTA, 0.5% (w/v) sodium pyrophosphate, 0.02% (w/v) each of BSA, Ficoll and polyvinyl pyrrolidone.

6. *Quantitation of Results*

The amount of RNA hybridized to a particular DNA dot was detected by auto-radiography as described above. A quantitative determination of the amount of RNA bound to a dot was made by cutting out the dot and measuring its radioactivity in a scintillation counter in 5 ml Scintimix (1 gal. scintillation grade toluene plus 1 15 g GAL-PAK [New England Nuclear]). When low levels of radioactivity were bound that could not be accurately determined by scintillation counting, then the relative amounts of bound radioactivity were determined by scanning the autoradiograph with a Dynatech MR600 ELISA plate reader at a wavelength of 570 nm as a test wavelength.

C. BIOSYNTHETIC LABELING OF RNA MADE BY EL4.E1 CELLS

1. *Pulse Labeling*

0.5 - 1.0 x 10^6 EL4.E1 cells ml^{-1} were suspended in a bulk culture in RHFM with 2 μCi ml^{-1} [^{3}H]-uridine (NEN, 26.5 Ci $mmol^{-1}$) plus deoxyinosine (P-L Biochemicals Ltd., Milwaukee, WI.) to a final concentration of 0.4 mM, with or without 15 ng ml^{-1} PMA. Cells were incubated for 16 hr (unless otherwise stated) at 37°C, under 100% humidity and 5% CO_2 in air. After the incubation period, the cells were washed three times in warm RHFM and resuspended at the same concentration in 5 ml RHFM with or without varying concentrations of "cold" uridine (Sigma), (a) with or

without 15 ng ml^{-1} PMA, and (b) with PMA plus 100 ng ml^{-1} CsA. Over a 24 hr "chase" period, duplicate samples were pooled and harvested by centrifugation at 250 x g for 5 mins. The cell pellets were washed in PBS and ^{3}[H]-RNA were recovered from the cells as described in Materials and Methods Section B.2. Where indicated, poly(A)$^+$ mRNA was isolated as described in Materials and Methods, Section B3. A portion of the volume containing the released poly(A)$^+$ RNA was directly used for fluorimetry readings and further mixed with 10 ml of Aquasol (NEN) and counted. In this fashion the specific activity (cpm μg^{-1}) of poly(A)$^+$ RNA was calculated.

2. *Measurement of Incorporation of Radioactivity*

Radioactivity incorporation was determined at various times throughout the pulse and "chase" by three consecutive 15-minute precipitations at 4°C of nucleic acids on 3 mM Whatman filter discs in 5% (v/v) TCA and 5 mM sodium pyrophosphate, followed by rinsing in cold 95% ethanol. The filter discs were dried under a heat lamp and the radioactivity was counted in Scintimix.

CHAPTER III

EFFECTS OF SOME IMMUNOSUPPRESSIVE DRUGS ON IL2 AND GM-CSF mRNA PRODUCTION BY TUMOR AND NORMAL CELLS

A. INTRODUCTION

Cyclosporin A (CsA) is a potent immunosuppressant that has been shown to act selectively on the T-cell arm of the immune response to inhibit the production of lymphokines (Chapter I, Section B). It has been shown that CsA acts to inhibit lymphokines at the transcriptional level (Elliott *et al.*, 1984; Krönke *et al.*, 1984; Wiskocil *et al.*, 1985; Granelli-Piperno *et al.*, 1986). Glucocorticosteroids, and steroid hormones in general, are also widely used clinically as immunosuppressants and anti-inflammatory agents. Their immunosuppressive effects may in part be due to their ability to inhibit metabolic activities in both unstimulated and mitogen-stimulated human lymphocytes (Smith *et al.*, 1977). A synthetic glucocorticosteroid, dexamethasone, has been shown to have a dose-dependent inhibitory effect on interleukin 2 (IL2) production in stimulated lymphocytes (Gillis *et al.*, 1979; Larsson, 1980a). Glucocorticosteroids are also important modulators of eukaryotic gene expression.

Failure to detect lymphokine production in stimulated lymphocytes might be due to the rapid reutilization or degradation of these proteins. Alternatively, this observed inhibition could occur at the level of transcription, translation or post-translational processing of the lymphokines (Chapter I, Section C). In an attempt to distinguish between these possibilities, experiments reported in this chapter were designed to quantitate levels of lymphokine mRNA. The availability of synthetic and cloned cDNA probes for mouse and human IL2 and mouse GM-CSF provided a means with which to analyze the effects of immunosuppressants on lymphokine mRNA production. For the purpose of replicate determinations of dose-response curves, the cytoplasmic dot hybridization

technique (White and Bancroft, 1982) was employed. This permitted the analysis of mRNA levels in numerous samples and required minimal manipulation prior to analysis, and should reduce losses of RNA or its degradation by nucleases. Quantitation of relative cytoplasmic mRNA levels with this technique would make it possible to study the abilities of immunosuppressants such as CsA and dexamethasone to inhibit lymphokine synthesis at the mRNA level.

B. RESULTS

1. *Chemistry of Cyclosporin A (CsA)*

The structure of CsA (Figure 1a) was determined by chemical degradation as well as by X-ray crystallographic analysis of an iodo derivative (Reügger *et al.*, 1976; Petcher, 1976). Ten of the eleven ring members are derivatives of known aliphatic amino acids: they are α-amino butyric acid (Abu) in position 2, sarcosine (Sar) in position 3, N-methylleucine (MeLeu) in positions 4, 6, 9 and 10, valine (Val) in position 5, alanine (Ala) in position 7, D-alanine (D-Ala) in position 8, and N-methylvaline (MeVal) in position 11. The amino acid in position 1 is novel. It is (4R)-4-[(E)-2-butenyl]-4, N-dimethyl-L-threonine and abbreviated as MeBmt. This amino acid has the polar features of an N-methyl-L-threonine which is substituted at the carbon chain by butenyl and methyl groups. CsA has a molecular weight of 1202.6D, is neutral, and due to the fact that 7 of 11 amino acids are N-methylated, is quite hydrophobic.

2. *CsA Inhibits the Accumulation of IL2 mRNA in Induced EL4.E1 cells*

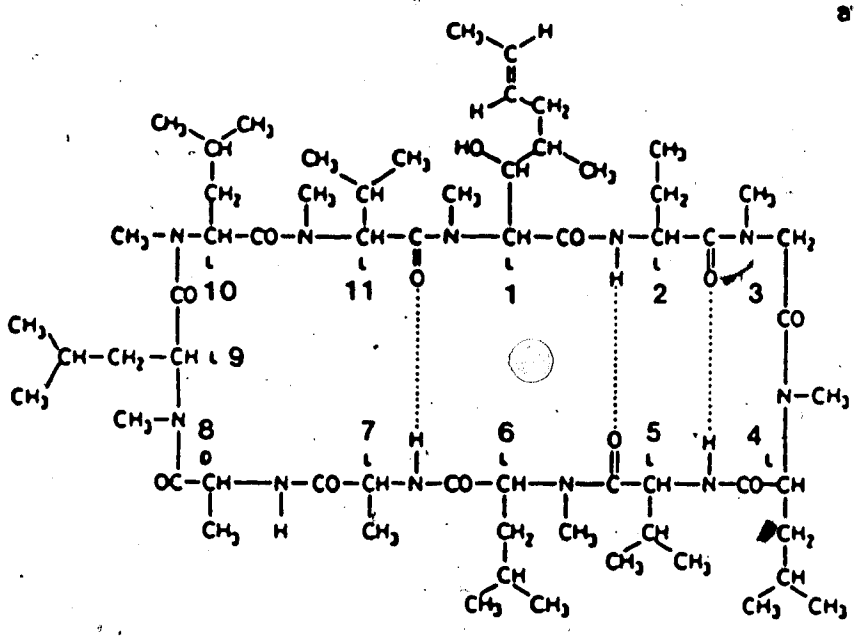
A synthetic oligonucleotide, specific for IL2 mRNA, was employed as a probe to detect murine IL2 mRNA. The IL2 mRNA levels were determined in cloned uninduced EL4.E1 cells or in EL4.E1 cells induced with PMA (15 ng ml^{-1}) for 12 hr in the presence or absence of varying concentrations of CsA. Cytoplasmic RNA was isolated from

FIGURE 1

(A) CHEMICAL STRUCTURE OF CYCLOSPORIN A

(B) AMINO ACID SUBSTITUTIONS OF A FEW NATURALLY OCCURRING CsA ANALOGUES

a



b

Name	Cyclosporin A contains	Position	Derivative contains
Cs B	Abu	2	Ala
Cs C	Abu	2	Thr
Cs D	Abu	2	Val
Cs G	Abu	2	NorVal

each cell population and analyzed by the cytoplasmic dot hybridization technique with end-labeled probe for IL2 mRNA. As shown in Figure 2, IL2 mRNA was detectable in PMA-positive samples, whereas low levels of IL2 mRNA was detected in uninduced cells (-PMA). CsA at a concentration of 30 ng ml^{-1} maximally inhibited IL2 mRNA production. As concentrations of CsA were decreased, increasing amounts of IL2 mRNA were detected. Conventional Northern blot analysis was done to confirm the specificity of the probes for IL2 mRNA transcripts. It should be noted that the concentration of CsA observed to completely inhibit IL2 and GM-CSF mRNA (Figure 3) corresponds to the concentrations used to suppress immune functions in renal transplant patients and is therefore pharmacologically relevant.

3. *CsA Analogues Differentially Inhibit IL2 mRNA Accumulation in EL4.E1 Cells*

Several naturally occurring analogues of CsA have been reported (Traber *et al.*, 1977), and they usually differ in their chemical structure only at one amino acid (Figure 1b). Two of these analogues, CsC and CsD, were available to test for their potencies relative to CsA.

As shown in Figure 2, CsC at a concentration of 100 ng ml^{-1} and CsD at a concentration of 300 ng ml^{-1} maximally inhibited the accumulation of IL2 mRNA in induced EL4.E1 cells. The protocol for analysis was the same as in the previous section. These data suggest that CsA is approximately 3 times more potent than CsC, and 10 times more potent than CsD in reducing IL2 mRNA levels. The relative immunosuppressive potencies of these 3 compounds correspond to *in vivo* immunosuppression data (Borel, personal communication).

4. *Cytodots can be Quantitated by Scanning the Autoradiogram*

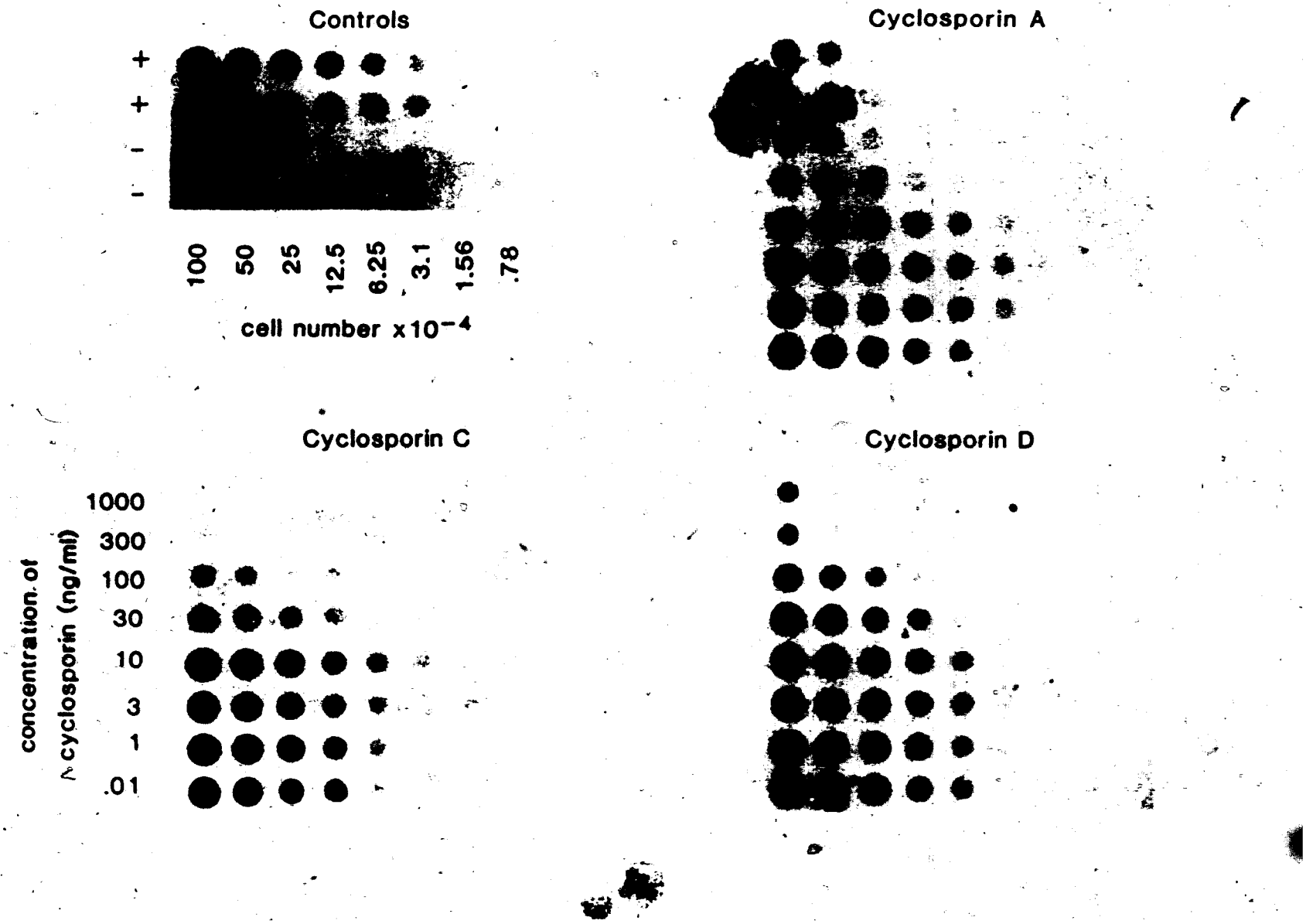
In order to quantitate the dose-response effect of CsA on IL2 mRNA, the autoradiogram (Figure 2) was scanned in an ELISA plate reader at a wavelength of

FIGURE 2

DOSE-DEPENDENT INHIBITION OF IL2 mRNA

ACCUMULATION IN EL4.E1

EL4.E1 cells (5×10^6 cells ml^{-1}) were cultured in the presence of PMA (15 ng ml^{-1}) and various concentrations of CsA, CsC or CsD for 12 hr. Total cytoplasmic RNA was isolated, as described in Materials and Methods (Chapter 2B, Section 1), transferred to nitrocellulose and hybridized to end-labeled synthetic IL2 34-mer. Samples are representative of 10×10^5 cell equivalents serially diluted 2-fold. The controls are cells cultured with (+) and without (-) PMA. The dilutions in the control panel apply to all panels. Drugs were added only to stimulated cells.


Controls
+
+
-
-
100
50
25
12.5
6.25
3.1
1.56
.78
cell number x10⁻⁴
Cyclosporin A
Cyclosporin C
concentration of cyclosporin (ng/ml)
1000
300
100
30
10
3
1
.01
Cyclosporin D

570 nm. A dilution of cell number was picked (*e.g.*, 25 x 10^4 cell equivalents) in the range where absorbance units were linear with respect to cell equivalent number, and thus proportional to RNA concentration. The absorbance units of that particular dilution were used to generate a percentage of the amount of mRNA remaining in the presence of CsA over a positive control (minus CsA). As shown in Figure 3, a dose-response curve depicts a 50% inhibition of IL2 mRNA at 14 ng ml^{-1} of CsA, and at 63 ng ml^{-1} of CsD. This corresponds to the approximate results obtained from the cytodot as shown in Figure 2.

5. *Selectivity of CsA*

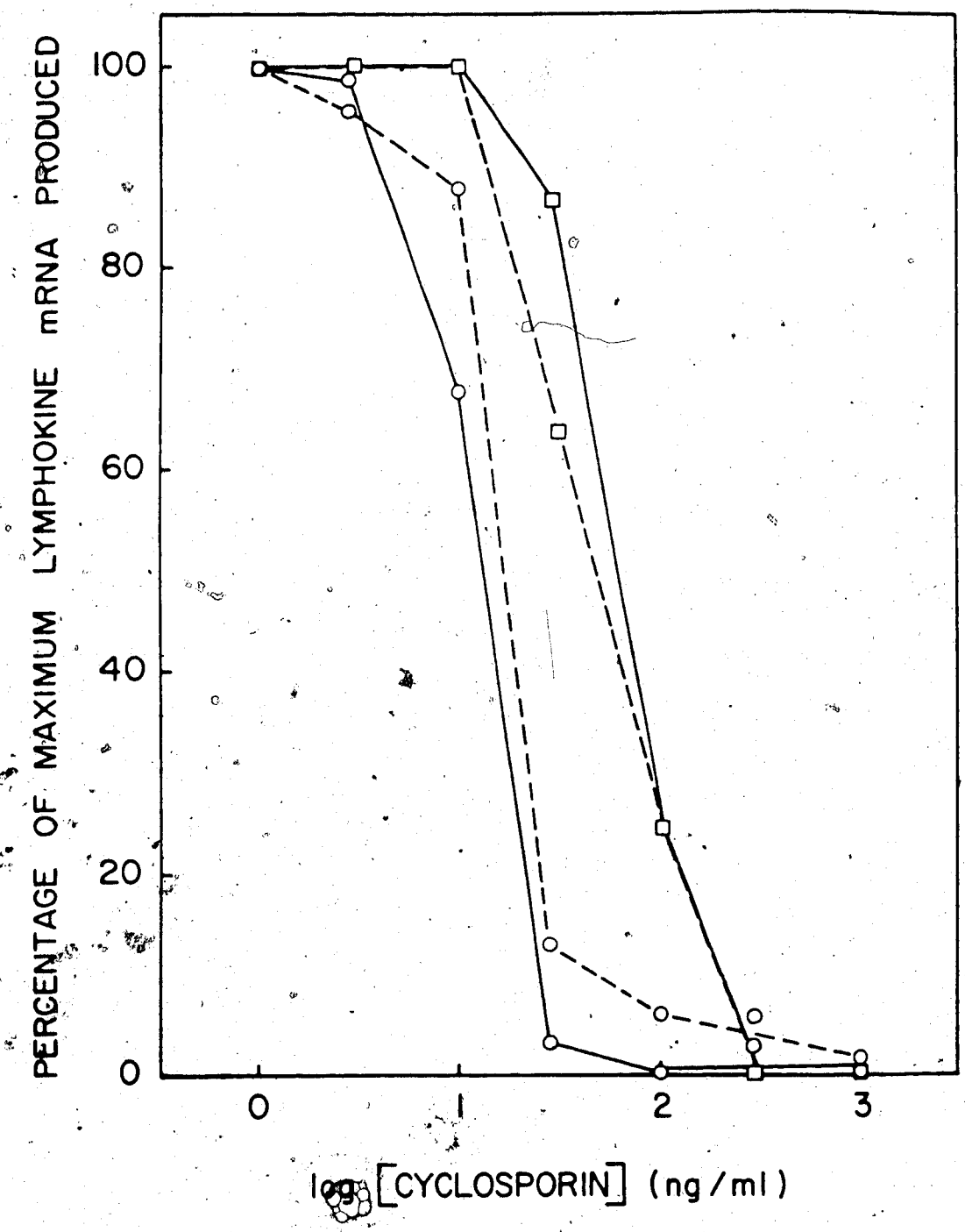
CsA did not diminish cell viability or the overall synthesis of DNA, RNA or proteins in either unstimulated or stimulated EL4.E1 cells (J. Ng, unpublished data). Thus CsA did not appear to exert a general inhibitory action on cell metabolism. To further examine the possible selectivity of CsA inhibition, cytodot samples from induced EL4.E1 cells treated with various concentrations of CsA, CsC or CsD (as described for Figure 2) were hybridized to the synthetic GM-CSF probe. The cytodots were analyzed quantitatively and the results of CsA- and CsD-induced inhibition of GM-CSF are shown in Figure 3. The results show that GM-CSF was induced and CsA and CsD blocked this induction at the mRNA level, at concentrations almost identical to IL2. This suggests that the effects of CsA and CsD on these 2 lymphokines are related to common intracellular mechanisms.

It was shown by Shaw *et al.* (1986) and Wisckocil (1985) that another lymphokine, γ-interferon (γ-IFN) is also induced by PMA and Con A in human PBL and in Jurkat cells, and turned off by CsA at the mRNA level. CsA does not, however, inhibit the production of IL2-receptor mRNA in PMA- and Con A-induced human PBL and Jurkat cells (Shaw *et al.*, submitted); it therefore appears that its inhibiting effect does not extent to all the induced mRNA's. Moreover, CsA has no effect on the

FIGURE 3

QUANTITATIVE ANALYSIS OF THE EFFECTS OF CsA AND CsD ON THE INDUCTION OF mRNA FOR IL2 AND GM-CSF IN EL4.E1 CELLS

Cells were induced with PMA to which various concentrations of CsA (—o—) or CsD (—□—) were added, as described in the legend to Figure 2. The IL2 (solid lines) or GM-CSF (dashed lines) RNA levels were quantitated by scanning the autoradiograms in an ELISA plate-reader. The absorbance units at 570 nm of 2.5 x 10^5 cell equivalents are expressed as a percentage of a fully induced positive control (100%).


PERCENTAGE OF MAXIMUM LYMPHOKINE mRNA PRODUCED
100
80
60
40
20
0
0
1
2
3
log [CYCLOSPORIN] (ng/ml)

synthesis of mRNAs for the T-cell receptor β-chain or actin, which are consistently expressed in Jurkat and EL4.E1 cells (Shaw *et al.*, submitted). Thus, CsA-induced inhibition of lymphokine gene expression appears to be a selective feature.

6. *CsA, CsC and CsD Inhibit IL2 Production in Murine EL4.E1 Cells and Human PBL*

Cell-free supernatants from EL4.E1 cells stimulated with PMA, or human PBL stimulated with PMA plus Con A, were assayed for IL2 activity as described in Materials and Methods (Chapter 2A, Section 6). Typically, the EL4.E1 cell line produced 500 to 1000 units of IL2, whereas fresh human PBL produced only 20-50 units. As shown in Figures 4 and 5, CsA induces a dose-dependent inhibition of IL2 activity in stimulated EL4.E1 cells and PBL. Analogues of CsA, CsC and CsD similarly produce a dose-dependent inhibition of IL2 in both cell types at concentrations resembling those observed for IL2 mRNA in EL4.E1 cells. Moreover, the observed potencies of CsC and CsD, as compared to that of CsA, are reproduced in the biological assay. These results serve to illustrate the fact that the inhibitory effect of CsA on the induction of lymphokine mRNA, as determined by cytodot analysis (Figure 2), reflects an inhibition of biologically active IL2. In addition, Figure 5 demonstrates that this effect is not specific to the EL4.E1 cell line, but is also observed in primary cultures of human PBL.

7. *Differential Inhibition of Lymphokine mRNA by Synthetic CsA Analogues*

Quantitation of relative mRNA levels with the cytodot technique is both sensitive and biologically relevant. However, NP-40 lysis of cells results in some variability in mRNA levels in the same concentration of cells from day to day. This is usually circumvented by duplicate or triplicate sampling. The results indicate that both titration with different concentrations of cyclosporin and with different numbers of cells are

FIGURE 4

DOSE-DEPENDENT INHIBITION OF IL2 PRODUCTION

IN EL4.E1 CELLS BY CsA, CsC AND CsD

Cell-free supernatants of EL4.E1 cells induced with PMA (15 ng ml^{-1}) and treated with various concentrations of CsA (—o—), CsC (—Δ—) or CsD (—□—) for 15 hr were assayed for IL2 activity as described in Materials and Methods, Chapter 2A, Section 6. The activity of IL2 was expressed as a percentage of a positive control. The positive control was the amount of IL2 produced from a PMA-stimulated culture of EL4.E1 cells in the absence of any drugs.

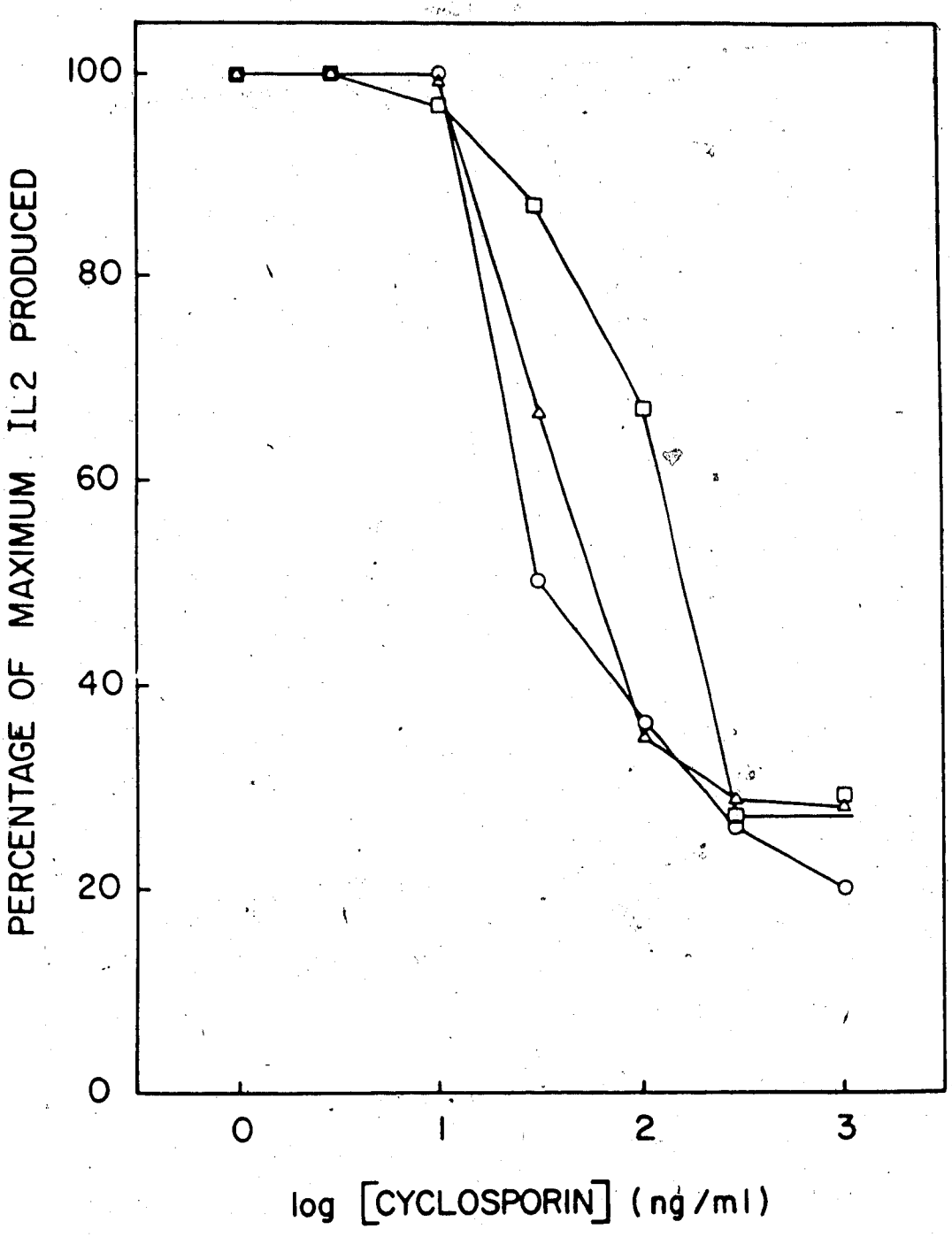
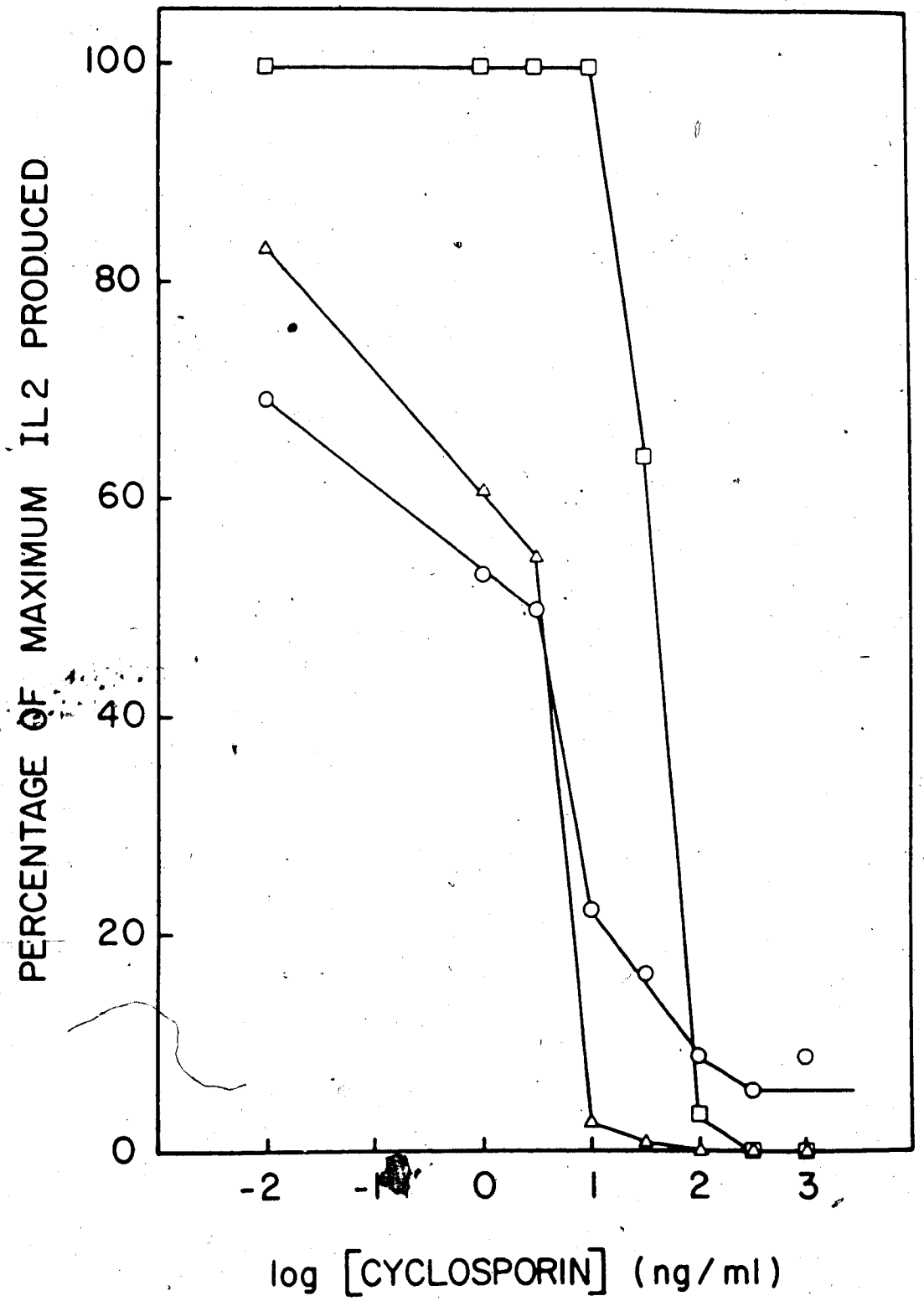

PERCENTAGE OF MAXIMUM IL2 PRODUCED
100
80
60
40
20
0
0
1
2
3
log [CYCLOSPORIN] (ng/ml)

FIGURE 5

DOSE-DEPENDENT INHIBITION OF IL2 PRODUCTION

IN HUMAN PBL BY CsA, CsC AND CsD

Human PBL were isolated from fresh buffy coat as described in Materials and Methods, Chapter 2A, Section 3, and stimulated for 24 hr in the presence of PMA (15 ng ml^{-1}) and Con A (30 μg ml^{-1}) and treated with various concentrations of CsA (—o—), CsC (—Δ—) or CsD (—□—). Cell-free supernates were assayed for IL2 activity as described in Materials and Methods, Chapter 2A, Section 6. Human IL2 is active on murine cells (Gillis *et al.*, 1982)


PERCENTAGE OF MAXIMUM IL2 PRODUCED
100
80
60
40
20
0
-2
-1
0
1
2
3
log [CYCLOSPORIN] (ng/ml)

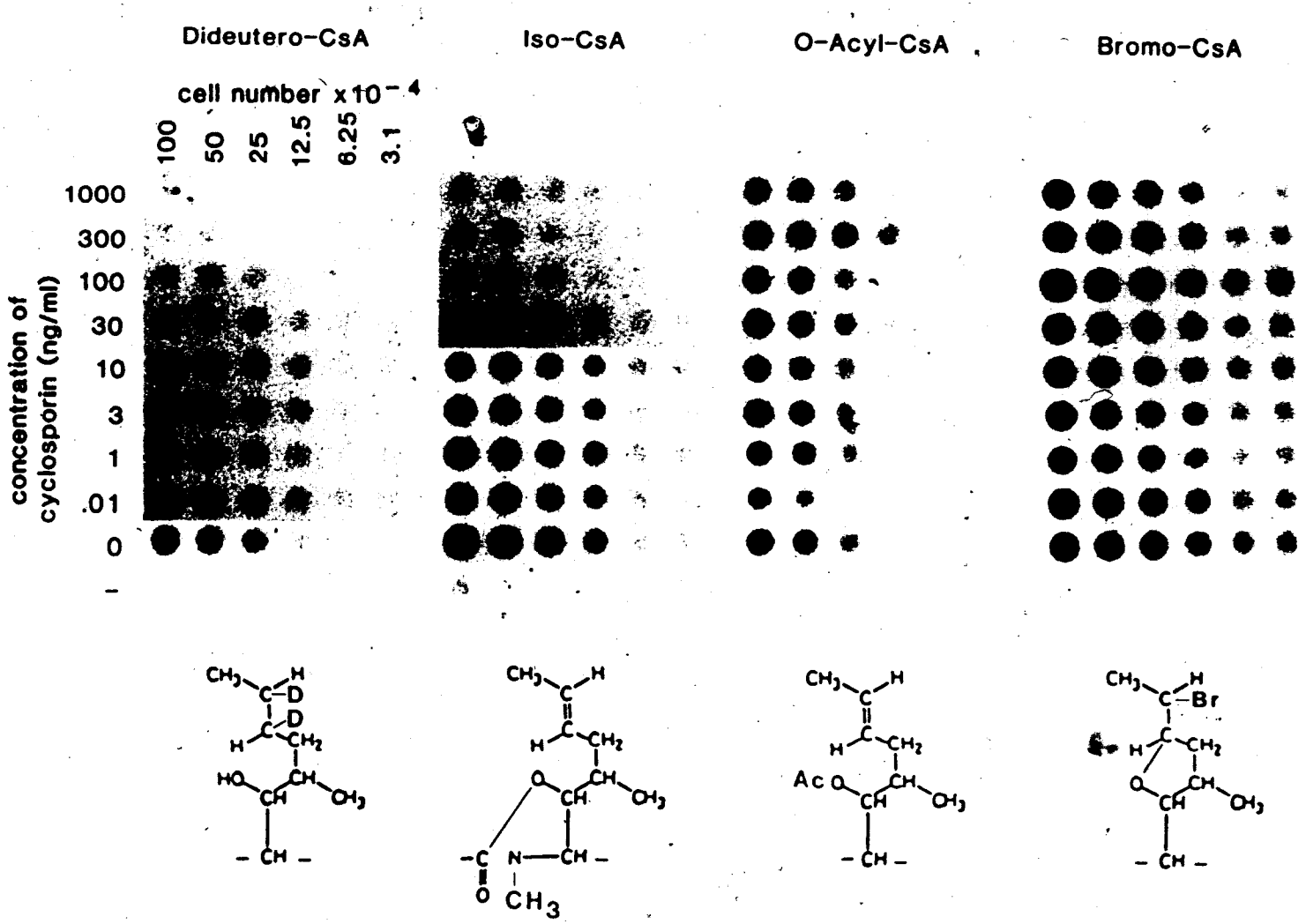
consistent (Figures 2, 4 and 5). As well, it has been shown that there is a direct correlation between IL2 mRNA levels from cytodots and IL2 activity in stimulated cell supernatants (Figures 2 and 4). Therefore, the cytodot technique provides a useful assay for examination of specific aspects of the CsA structure required for activity in suppressing lymphokine mRNA. As a consequence of the rigid conformation of the cyclosporin skeleton, the unusual amino acid, MeBmt, in position 1, is directed quasi-perpendicular to the plane of the peptide ring (Wenger, 1985). This could result in it being involved in interactions with a receptor. Modifications in this amino acid were synthesized by Dr. J. Vederas (Chemistry Department, University of Alberta) in order to determine which feature(s) of MeBmt were essential for its activity. Figure 6 shows the structure of 4 such CsA analogues. These analogues were added in varying concentrations to PMA-stimulated EL4.E1 cells. The mRNA from cytoplasmic extracts of cells were hybridized to a synthetic IL2 34-mer probe. As shown in Figure 6, PMA induced IL2 mRNA synthesis, however the variability observed between samples reflects the actual amounts of IL2 mRNA produced as confirmed by the amount of IL2 activity assayed from the supernatant from PMA-stimulated cells (data not shown). Saturation of the double bond in Dideutero-CsA resulted in a dose-dependent inhibition of IL2 mRNA, with complete inhibition around 100 ng ml^{-1}. Similarly, Iso-CsA retained its immunosuppressive activity, maximally inhibiting IL2 mRNA at 100 ng ml^{-1}. These 2 analogues were seen to have activity comparable to that of native CsA. In contrast, O-Acyl-CsA and Bromo-CsA were much less potent than CsA in inhibiting IL2 mRNA. The variability in IL2 mRNA levels observed vertically in O-Acyl-CsA reflects the limitation of the cytodot technique addressed above. Bromo-CsA seemed to be completely inactive while O-Acyl-CsA could partially inhibit mRNA accumulation at 1000 ng ml^{-1}. Identical results were observed when the levels of GM-CSF mRNA were measured (data not shown). These results suggest that the unusual MeBmt chain is intimately involved in the biological activity of the cyclosporin molecule.

FIGURE 6

EFFECT OF CsA ANALOGUES ON THE ACCUMULATION

OF IL2 mRNA IN EL4.E1 CELLS

RNA samples from uninduced (-) and induced (o) EL4.E1 cells, or induced in the presence of various concentrations of CsA analogues for 12 hr, were prepared and analyzed by the cytodot techniue exactly as described in the legend to Figure 2.


Dideutero-CsA
Iso-CsA
O-Acyl-CsA
Bromo-CsA
cell number x10⁻⁴
100
50
25
12.5
6.25
3.1
concentration of cyclosporin (ng/ml)
1000
300
100
30
10
3
1
.01
0
–

8. *Effect of CsA Analogues on IL2 Production in Jurkat and Human PBL*

In order to exclude the possibility that the effects of the different CsA analogues were not specific to the murine EL4.E1 tumor cell line, they were tested on the human Jurkat Leukemia cell line and fresh human PBLs. These two cell types were induced with PMA and Con A in the presence of 0.01 to 1000 ng ml^{-1} of a CsA analogue. Cells were cultured for 24 hr and the supernants were collected and assayed for IL2 activity. Concentrations of CsA analogues up to 1000 ng ml^{-1} had no effect on the viability of the cultured cells nor on the bioassay cells (data not shown). Figures 7 and 8 show the effect of the different analogues on IL2 production in stimulated Jurkat cells or human PBLs respectively. In both cell types, the Dideutero-CsA and Iso-CsA showed a dose-dependent inhibition of IL2 activity, while O-Acyl-CsA and Bromo-CsA had little or no effect on IL2 activity. These results are comparable to those observed at the mRNA level for IL2 and GM-CSF. This suggests once again that the inhibitory effect of CsA is exerted on biologically active mRNA. These results demonstrate that modifications of CsA in position 1, result in a differential inhibition of IL2 production in other cell types, and are not specific to a murine tumor cell line.

9. *Effect of Bromo-CsA on the Dose-Dependent Inhibition of CsA on IL2 Production in EL4.E1 Cells*

In order to assess further the functional importance of the MeBmt side chain of CsA, EL4.E1 cells were stimulated with PMA and incubated with various concentrations of CsA in the presence of 1 μg ml^{-1} or 0.1 μg ml^{-1} Bromo-CsA. At 24 hr, cell-free supernatants were collected and assayed for IL2 activity. The percentage of IL2 remaining in the presence of CsA, as compared to a fully induced control sample, is shown in Figure 9 as a function of CsA concentration. The curve resembles the characteristic dose dependent inhibition of IL2 production by CsA (Figures 4 and 5). CsA at high concentrations (30-1000 ng ml^{-1}) in the presence of Bromo-CsA, completely inhibited IL2

FIGURE 7

EFFECT OF CsA ANALOGUES ON IL2 PRODUCTION

IN JURKAT CELLS

5×10^4 Jurkat cells were stimulated with PMA (15 ng ml^{-1}) and Con A (30 μg ml^{-1}) in the presence of various concentrations of Dideutero-CsA (—o—), O-Acyl-CsA (—•—), Iso-CsA (—Δ—) or Bromo-CsA (—□—). After 24 hr incubation, the cell-free supernatants were collected and assayed for IL2 activity as described in Materials and Methods, Chapter 2A, Section 6. The activity of IL2 was expressed as a percentage of a positive control (100%). The symbol (—■—) is a consequence of (—•—) and (—□—) coinciding at the same point.

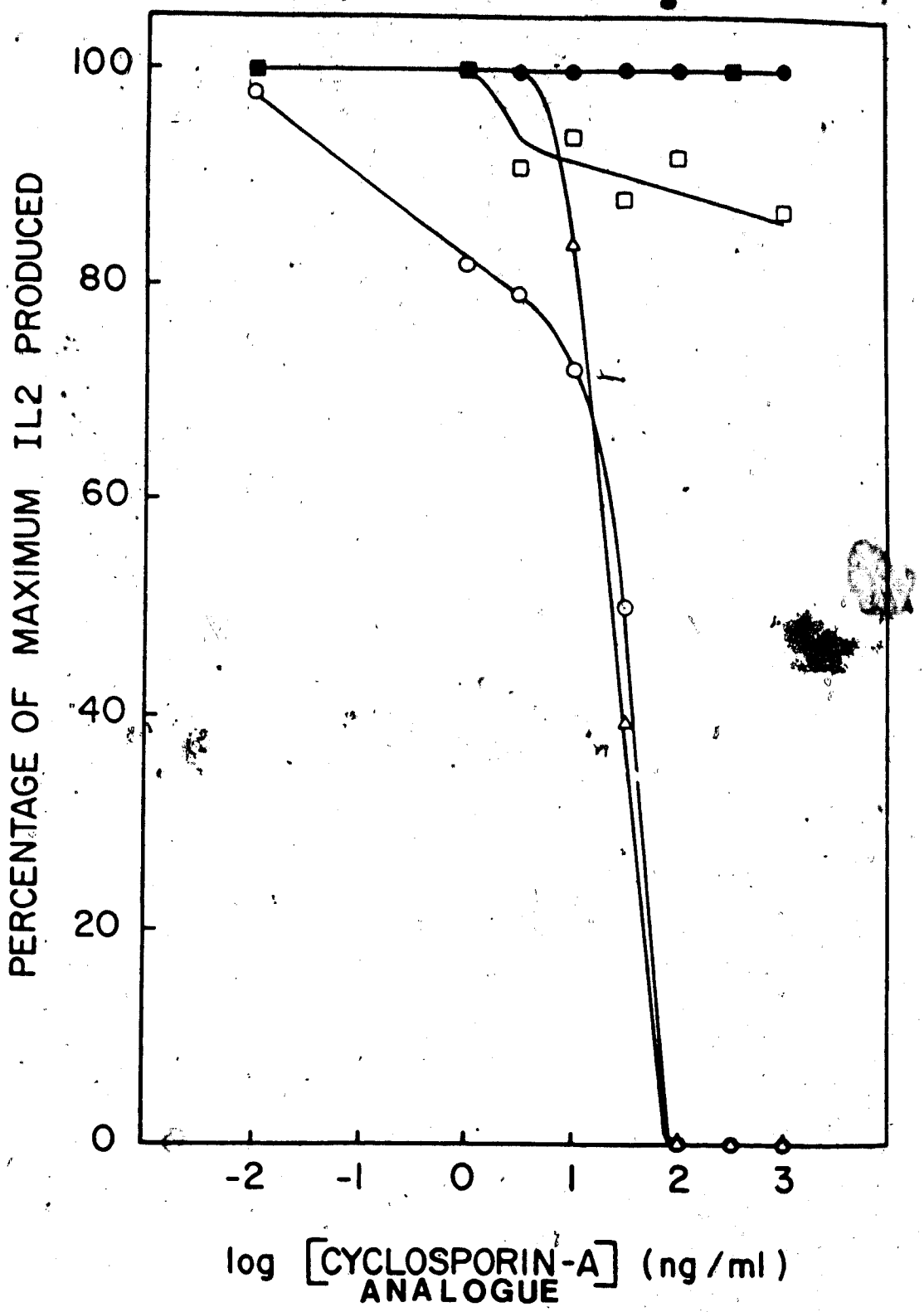

PERCENTAGE OF MAXIMUM IL2 PRODUCED
100
80
60
40
20
0
-2
-1
0
1
2
3
log [CYCLOSPORIN-A] (ng/ml)
ANALOGUE

FIGURE 8

EFFECT OF CsA ANALOGUES ON IL2 PRODUCTION IN HUMAN PBL

5×10^6 human PBL were stimulated with PMA (15 $ng \cdot ml^{-1}$) and Con A (30 $\mu g\ ml^{-1}$) in the presence of various concentrations of Dideutero-CsA (—o—), O-Acyl-CsA (—●—), Iso-CsA (—Δ—) or Bromo-CsA (—□—). After 24 hr incubation, the cell-free supernatants were collected and assayed for IL2 activity as described in Materials and Methods, Chapter 2A, Section 6. The activity of IL2 was expressed as a percentage of a positive control (100%).

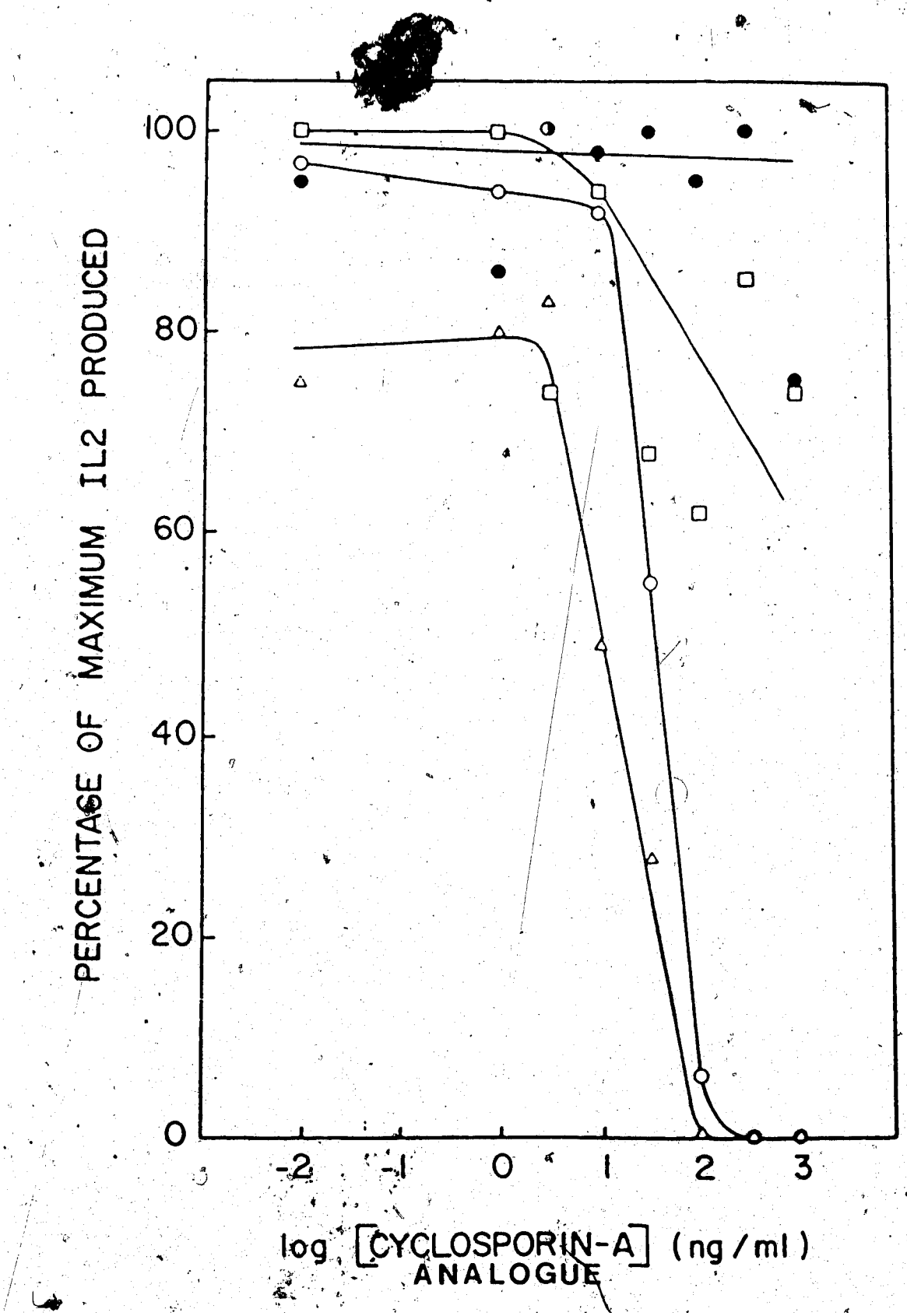
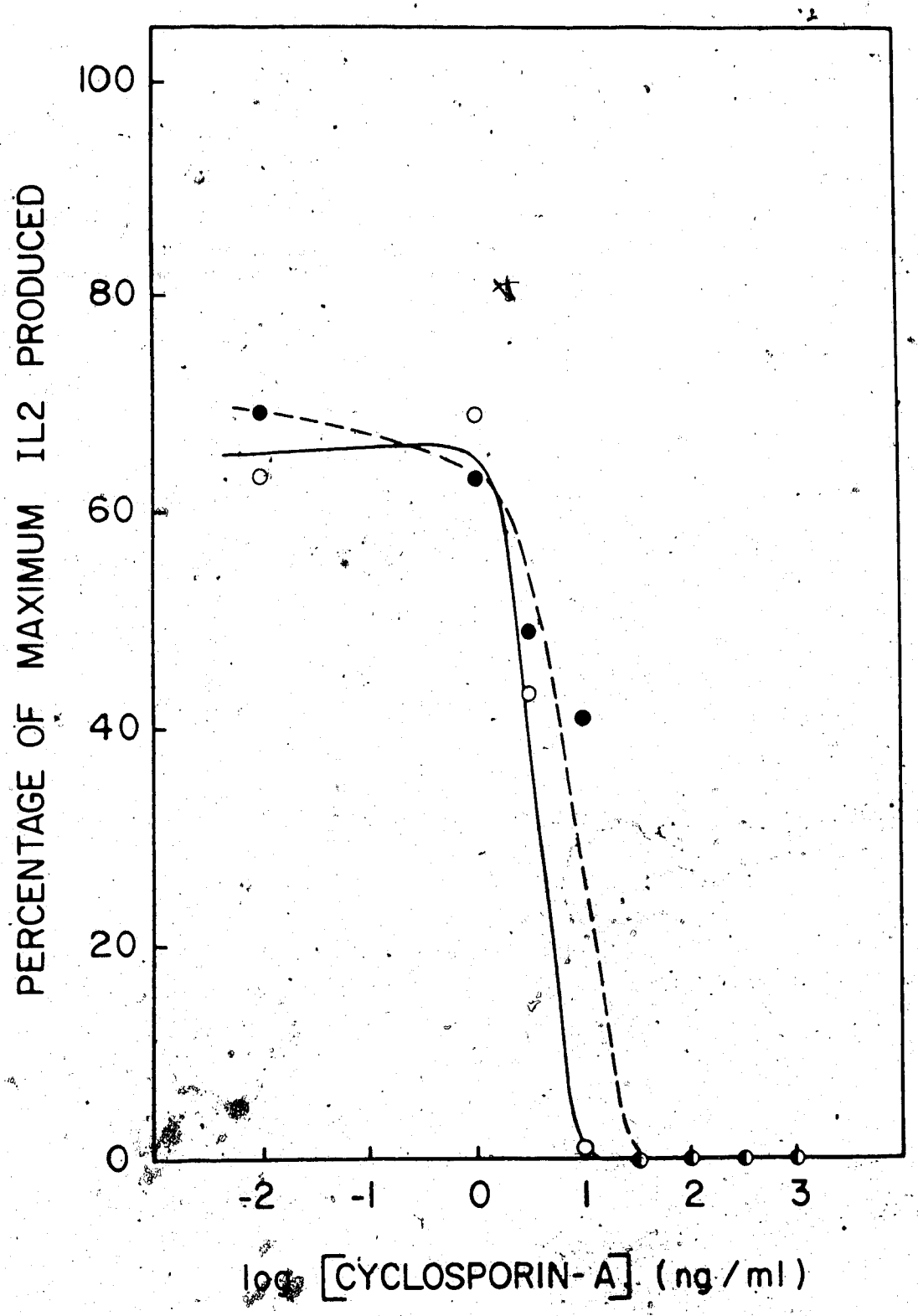

PERCENTAGE OF MAXIMUM IL2 PRODUCED
100
80
60
40
20
0
-2
-1
0
1
2
3
log [CYCLOSPORIN-A] (ng/ml)
ANALOGUE

FIGURE 9

EFFECT OF CsA ON IL2 PRODUCTION IN EL4.E1 CELLS IN THE PRESENCE OF BROMO-CsA

Cell-free supernatants of PMA-stimulated EL4.E1 cells treated with various concentrations of CsA and either 1000 ng ml^{-1} Bromo-CsA (—o—) or 100 ng ml^{-1} Bromo-CsA (—•—) for 24 hr were assayed for IL2 activity as described in Materials and Methods, Chapter 2A, Section 6. The activity of IL2 was expressed as a percentage of a positive control.


PERCENTAGE OF MAXIMUM IL2 PRODUCED
100
80
60
40
20
0
-2
-1
0
1
2
3
log [CYCLOSPORIN-A] (ng/ml)

production in EL4.E1 cells. As the concentration was lowered, increasing amounts of IL2 production were observed. It was shown by cytodot analysis (Figure 2), that very low concentrations of CsA (*e.g.*, 0.01 ng ml^{-1}) had no inhibitory effect on the accumulation of IL2 mRNA. However, it is apparent from Figure 9 that only 70% of maximal IL2 production was attained in the presence of 0.01 ng ml^{-1} of CsA. This was not due to an inhibitory effect of CsA, but rather to fluctuations occasionally observed in the assay that detects IL2 protein levels.

As the presence of 1 μg ml^{-1} or 0.1 μg ml^{-1} of Bromo-CsA did not appear to compete with CsA in its ability to inhibit IL2 production, it appears that modification of the MeBmt side chain by either the cyclization of the ring or the addition of the Bromine group renders the molecule inactive. Perhaps, this side chain is important for recognition of a CsA receptor.

It is clear that selective modification of the CsA structure renders it less active in its ability to inhibit IL2 mRNA accumulation and activity. A summary of results is given in Table 2. In order to compare the immunosuppressive activities of the analogues to the activity of CsA, the former are expressed as their concentration required to cause a 50% inhibition of IL2 mRNA synthesis.

10. *Effects of Other Immunosuppressive Drugs and Anti-Cancer Agents on the Induction of IL2 mRNA*

In addition to CsA, other drugs such as glucocorticosteroids and anti-cancer agents are used clinically to immunosuppress patients. In most cases, such treatments cause severe damage to lymphoid tissues.

Since IL2 plays a major role in the proliferation and activation of T lymphocytes (Chapter 1A, Section 2), and its production can be quantitatively measured at the mRNA level (Chapter 3B, Section 4), the effects of 2 immunosuppressants

TABLE 2

EFFECT OF CYCLOSPORIN ANALOGUES ON IL2 mRNA ACCUMULATION AND IL2 ACTIVITY

Cyclosporin	Concentration (ng/ml) required for 50% inhibition of IL2 production					
	EL4		JURKAT		h-PBL	
	mRNA	Activity	mRNA	Activity	mRNA	Activity
CsA	10	30	nd	20	3	3
CsC	30	56	nd	50	10	4
CsD	100	158	nd	125	100	40
Iso-CsA	10	2	nd	30	10	10
Dideutero-CsA	20	10	nd	25	10	30
Dihydro-CsA	20	nd	nd	nd	nd	nd
O-Acyl-CsA	1000	30	nd	≥1000	1000	≥1000
Bromo-CsA	≥1000	300	nd	≥1000	≥1000	1000

The table summarizes the effects of different cyclosporin analogues on both IL2 mRNA and activity in three cell types. The concentrations required for 50% inhibition of IL2 mRNA or activity are taken from dose-response curves derived from quantitative analysis of cytodots or IL2 assays, respectively. Activity refers to the amount of IL2 assayed in the supernatants of activated cells as described in Materials and Methods (Chapter 2A, Section 5).

nd; not determined.

dexamethasone and 6-mercaptopurine, and 2 anti-cancer agents, cyclophosphamide and melphalan, on the induction of IL2 mRNA in tumor and normal cells were examined.

EL4.E1 cells were stimulated with PMA for 13 hr in the presence of various concentrations of melphalan (up to 5 μg ml^{-1}), cyclophosphamide (up to 200 μg ml^{-1}), dexamethasone or 6-mercaptopurine, (both up to 5 μg ml^{-1}). The mRNA levels were analyzed by cytodots using IL2 34-mer as probe. No cytotoxic effects were observed at the highest concentrations of the drugs studied (data not shown), nor were there any inhibiting effects on the IL2 mRNA accumulation in EL4.E1 cells in the presence of the highest concentrations of the drugs, as seen in Table 3.

Moreover, when dexamethasone or melphalan were tested on the Jurkat cell line, no inhibition of IL2 mRNA synthesis was observed. To examine the possibility that these drugs may not be active on tumor cell lines, the same dilutions of drugs were added to PMA- and Con A-stimulated normal human PBL. After 20 hr incubation, cytoplasmic RNA was isolated for cytodot analysis, and hybridized to synthetic IL2-34-mer. Interestingly, only dexamethasone appeared to have a slight inhibitory effect on IL2 mRNA production in human PBL. This selective effect of dexamethasone on cell type is demonstrated in Figure 10. In EL4.E1 and Jurkat cells, IL2 mRNA was present at control levels in the presence of all concentrations of dexamethasone tested. However, in PBL, 5000 ng ml^{-1} of dexamethasone caused a slight inhibition of the accumulation of IL2 mRNA. The supernatants from stimulated cultures treated with dexamethasone were assayed for IL2 activity and the following results were obtained: At 5000 ng ml^{-1} of dexamethasone there were 22 IL2 units ml^{-1}, at 1000 ng ml^{-1}, 25 units ml^{-1}; at 500 ng ml^{-1}, 26 units ml^{-1}; at 200 ng ml^{-1}, 28 units ml^{-1}; at 40 ng ml^{-1}, 34 units ml^{-1}; at 8 ng ml^{-1}, 44 ng ml^{-1} which reflected control levels [illegible]. Thus the IL2 activity in the supernatants suggest that there is a dose-d[illegible]on of IL2 production by dexamethasone.

TABLE 3

EFFECT OF DIFFERENT IMMUNOSUPPRESSIVE DRUGS ON THE INDUCTION OF IL2 mRNA IN MOUSE AND HUMAN T CELL LINES AND NORMAL PBLs

Drug	EL4	JURKAT	h-PBL
6-Mercaptopurine			
5000 ng ml^{-1}	+	nd	+
Cyclophosphamide			
200 μg ml^{-1}	+	nd	+
Dexamethasone			
200 ng ml^{-1}	+	+	+
1000 ng ml^{-1}	+	+	—
Melphalan			
200 ng ml^{-1}	+	+	+
1000 ng ml^{-1}	+	+	+

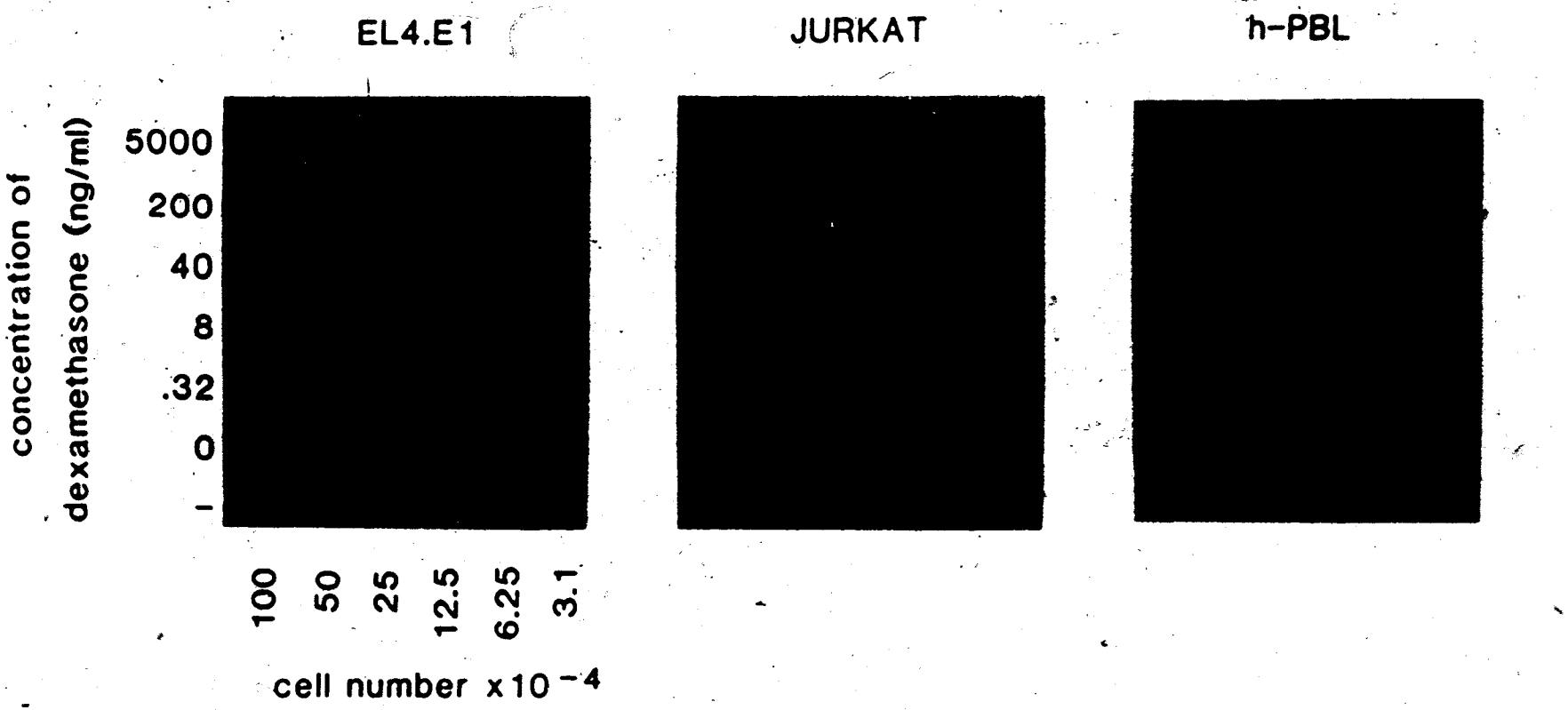
The symbols (-) and (+) refer to the absence or presence of IL2 mRNA respectively, in PMA stimulated EL4.E1 cells or PMA plus Con A stimulated Jurkat and PBLs, that were treated with various drugs. The concentration of drugs tested varied from 0.32 ng ml^{-1} to a maximum described in the text. The concentrations shown in the table were selected to illustrate whether or not the drugs had an effect on IL2 mRNA production.

nd; not determined.

FIGURE 10

INHIBITORY EFFECT OF DEXAMETHASONE ON IL2 mRNA ACCUMULATION IN HUMAN PBL BUT NOT ON TUMOR CELL LINES

5 x 10^6 EL4.E1 cells, Jurkat cells or human PBL were stimulated with 15 ng ml^{-1} PMA (and 30 μg ml^{-1} Con A for Jurkat and human PBL) and treated with various concentrations of dexamethasone. EL4.E1 and Jurkat cells at 13 hr and PBL at 20 hr were harvested and cytoplasmic RNA extracted as described in Materials and Methods, Chapter 2B, Section 1. Cytodot filters were hybridized to ^{32}P-labeled synthetic IL2-34 mer.


EL4.E1
JURKAT
h-PBL
concentration of dexamethasone (ng/ml)
5000
200
40
8
32
0
-
100
50
25
12.5
6.25
3.1
cell number x10 -4

11. *Human Serum Inhibits IL2 mRNA Accumulation in Stimulated EL4.E1 Cells*

CsA is a potent suppressor of rejection of kidney, liver, heart, heart-lung, and bone marrow transplants (White & Calne, 1982). Its widespread use in transplantation surgery is hindered by its toxicity (especially nephrotoxicity) and by a lack of knowledge of the optimal dosage to be administered to the patient. Thus, a strong correlation was found between CsA levels in the blood serum and clinical post-transplantation complications (Keown *et al.*, 1981; Irschik *et al.*, 1984; Rogerson *et al.*, 1985). The avoidance of nephrotoxic complications, while maintaining acceptable immunosuppressive CsA serum levels, requires a fast, reliable and reproducible CsA-monitoring method as an important element in post-renal transplantation therapy.

A commercially available radioimmunoassay (RIA) kit from Sandoz Products is currently most widely used for this purpose. Unfortunately the CsA concentration, as measured with the RIA kit are found to be substantially higher than when measured with high performance liquid chromatographic (HPLC) techniques (Carruthers *et al.*, 1983). This difference may be due to the antibody, present in the RIA kit, which may cross-react with several CsA metabolites that are closely related to the parent compound. Little is known of the contributions of these cyclosporin metabolites to the activity spectrum of the drug (Shran & Abisch, 1984).

Since, as summarized above, the monitoring of CsA concentrations in the blood or serum of transplant patients is not totally reliable at present, the development of a bioassay method that would reliably determine such concentrations quantitatively, would appear to be both urgent and important.

As shown above, CsA inhibits, in a dose-dependent fashion, the induction of IL2 at the mRNA level in EL4.E1 cells, and this correlates with the degree of immunosuppression. It was proposed that, the CsA concentration in patient serum could

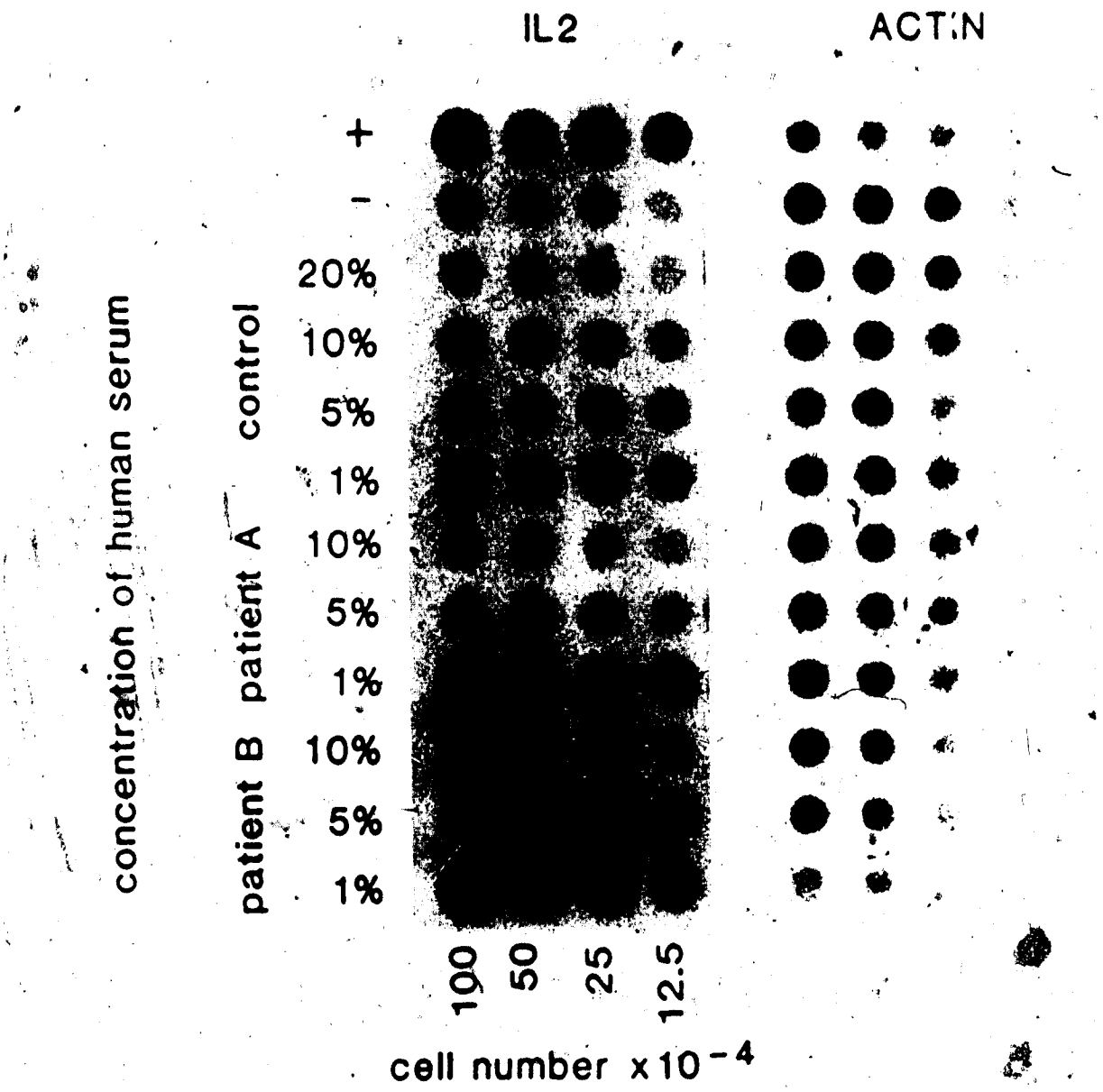
be rapidly assessed by means of the cytodot analysis through the ability of CsA-containing serum to inhibit IL2 mRNA accumulation in stimulated EL4.E1 cells; the degree of inhibition could be read off a standard curve.

In order to test this hypothesis it was first necessary to determine the effect of human serum on IL2 mRNA production in stimulated EL4.E1 cells. Human serum samples from renal allograft recipients were obtained through Dr. Kovithavongs (Transplantation Unit, University of Alberta Hospital). Serum samples were taken prior to the administration of CsA ('day 0' sample) and then periodically post-CsA treatment. Serum was obtained from fresh heparinized blood that was clotted at 4°C. All serum samples were heat-inactivated at 56°C for 30 min. The 'day 0' serum samples from 2 patients and from a healthy donor, were added at various concentrations to PMA-stimulated EL4.E1 cells and incubated for 16 hr. Cytoplasmic RNA samples were transferred to nitrocellulose and hybridized to nick-translated IL2-cDNA or actin-DNA probe as a control. The effects of human serum on IL2 mRNA production are shown in Figure 11. PMA induced significant levels of IL2 mRNA in these cells and this induction was inhibited by high concentrations of human sera. However, the concentration required for inhibition varied from individual to individual. Possibly, the serum promoted a generalized inhibition or degradation of RNA although there was no measurable effect on the viability of cells during the treatment period (data not shown). Therefore, after removing the IL2 probe, the filters with RNA were hybridized with a labeled actin gene probe, as shown in Figure 11. The levels of actin mRNA accumulation are relatively consistent with the exception of the PMA positive control. This could be a result of the variability of mRNA levels addressed in Section 7 above, or else PMA might have a direct affect on reducing actin mRNA accumulation. The latter is unlikely since all other samples in the presence of serum were also stimulated with PMA. Since there was no significant decrease in the actin mRNA that could account for the magnitude of IL2 mRNA inhibition, no evidence was obtained for

FIGURE 11

CONCENTRATION-DEPENDENT INHIBITION BY HUMAN SERUM ON THE INDUCTION OF IL2 mRNA IN EL4.E1 CELLS

2×10^4 EL4.E1 cells were stimulated with (+) or without (-) PMA (15 ng ml^{-1}) or with PMA and 20, 10, 5 or 1% heat inactivated human serum. After 16 hr incubation, cells were harvested and RNA extracted for cytodot analysis. The filter was first hybridized to nick-translated murine IL2-cDNA probe. The IL2 probe was removed by washing at 65°C as described in Materials and Methods, Chapter 2B, Section 5, and then the filters hybridized to nick-translated actin-F cDNA probe.


IL2
ACTIN
concentration of human serum
patient B patient A control
+
-
20%
10%
5%
1%
10%
5%
1%
10%
5%
1%
100
50
25
12.5
cell number x10 -4

non-specific serum-induced RNA degradations. These results show that human serum inhibits IL2 mRNA accumulation in a concentration-dependent fashion, and that 1% (or 5% as is the case of patient B) is the maximum concentration of serum that could be added to EL4.E1 cells that did not inhibit IL2 mRNA production.

12. *Determination of CsA Concentration in the Serum of a Renal Allograft Recipient*

The concentration of CsA in the serum of patient B (see previous section) was determined. Twelve serum samples taken on different days during a 24 day post-transplantation follow-up period were available for testing. The serum was heat-inactivated and added at 1 or 5% concentration to PMA-stimulated EL4.E1 cells. For a standard curve stimulated EL4.E1 cells were incubated in the presence of various dilutions of CsA in the presence of 1% "day 0" serum.

At 12 hr the cytoplasmic RNA was isolated and transferred to nitrocellulose filters, or cytodots. The filters were hybridized to IL2-cDNA probe.

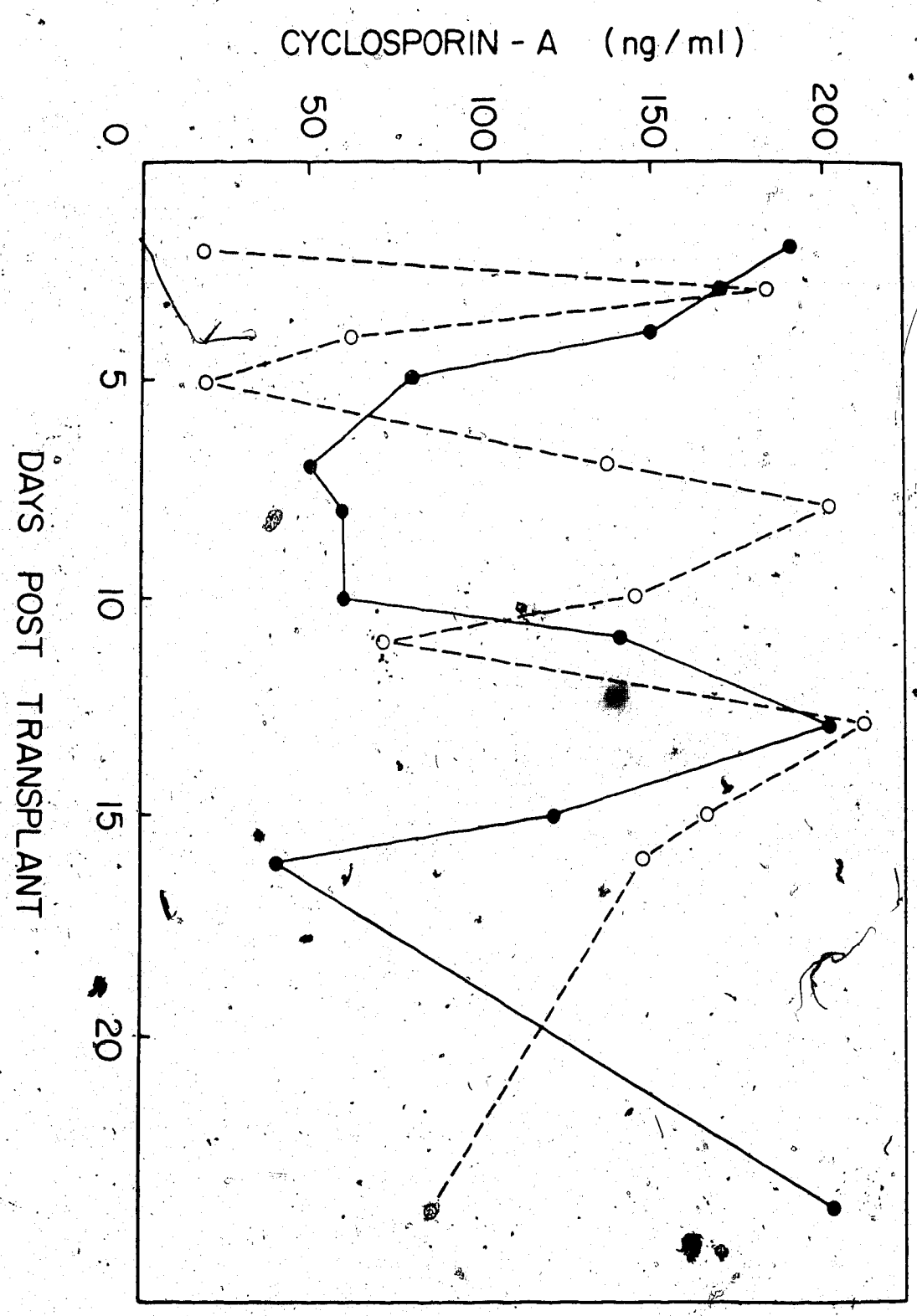
Quantitation of IL2 mRNA was achieved by counting the radioactive dots in a scintillation counter in 5 mls of Scintimix. A standard curve representing the cpm of IL2 mRNA versus the concentration of CsA was drawn (not shown). Determination of the CsA-concentration in the serum samples was achieved by the use of this standard curve. Figure 12 depicts a graph of CsA concentration in the serum of patient B during a 24 day period.

Superimposed is the CsA-concentration determined by means of RIA by the hospital staff. The graphs generated by both techniques are similar in two ways; (i) there are fluctuations in the CsA concentration during the course of investigation and (ii) the maximal concentration detected was 200 ng ml^{-1}. The limitation of the bioassay is that concentrations of CsA less than 20 ng ml^{-1} cannot be detected since these concentrations produce IL2 mRNA levels equivalent to control values. Similarly

FIGURE 12

DETERMINATION OF THE CsA CONCENTRATION IN THE SERUM OF A RENAL ALLOGRAFT RECIPIENT

2.5 x 10^6 EL4.E1 cells were stimulated in the presence of 1% human serum (from patient B, Figure 11). After 13 hr incubation, cells were harvested and RNA extracted for cytodot analysis. Similarly, 2.5 x 10^6 EL4.E1 cells were stimulated in the presence of 1% day '0' serum (see text) and various concentrations of CsA. RNA was extracted at 13 hr and analyzed as above. The filters were hybridized to nick-translated IL2 cDNA probe. IL2 mRNA levels were quantitated by counting the radioactive dots in Scintiverse. Concentrations of CsA in the serum samples (—o—) were determined from a standard curve of CsA concentration versus IL2 mRNA levels expressed as cpm. The concentration of CsA determined by the hospital staff are also shown (—•—).


CYCLOSPORIN - A (ng / ml)
0
50
100
150
200
DAYS POST TRANSPLANT
5
10
15
20

concentrations of CsA greater than 300 ng ml^{-1} cannot be quantitated since at these concentrations, IL2 mRNA is at background levels.

C. DISCUSSION

1. *Structure-Activity Relationships of CsA*

The effects of CsA and other immunosuppressive drugs on lymphokine gene expression was studied, using synthetic oligonucleotide or cDNA probes specific for mouse and human IL2, or mouse GM-CSF, in a cytoplasmic RNA dot-blot technique. The work reported in this chapter demonstrates that induction of EL4.E1 murine tumor T-cells with PMA leads to increased transcriptional activity of the IL2 and GM-CSF genes, whereas in uninduced EL4.E1 cells, IL2 and GM-CSF-specific transcripts were not identified. Similar, although lower, IL2 mRNA accumulation in PMA- and Con A activated Jurkat leukemic T-cells and human PBLs, but not in uninduced cells, was observed. Thus, the IL2 and GM-CSF mRNA accumulation observed in induced cells reflects increased transcriptional activities of the IL2 and GM-CSF genes that result in biologically active mRNAs.

It was previously reported that CsA abrogates IL2 production by activated T lymphocytes (Bunjes *et al.*, 1981; Hess *et al.*, 1982; Orosz *et al.*, 1984). The results of this study demonstrate that IL2 and GM-CSF mRNA accumulation in induced EL4.E1, Jurkat or human PBLs was diminished by CsA in a dose-dependent manner. However, it was not possible to determine from this study if CsA acted by blocking lymphokine transcription at initiation, processing, stability or translation stage. The induction of IL2 (Elliott *et al.*, 1984) and IFN-γ (Wiskocil *et al.*, 1985) mRNAs by PMA, and suppression by CsA, was previously demonstrated in Jurkat cells, and of IL2 mRNA in EL4 cells (Granelli-Piperno *et al.*, 1984). Furthermore, it has been shown that CsA acted by blocking IL2 mRNA transcription in isolated nuclei from stimulated Jurkat cells

(Krönke *et al.*, 1984) as well as from stimulated EL4.E1 cells (Shaw *et al.*, 1986). However, these reports do not exclude the possibility of additional effects of CsA on IL2 mRNA processing, stability or translation. Additional studies of the ability of CsA to modulate lymphokine gene expression will be reported in the next chapter.

One of the objectives of the work described in this chapter was to determine the chemical characteristic of CsA responsible for its immunosuppressive activity *in vitro*. During the course of this work it was reported elsewhere (Wenger, 1985) that the biological activity of CsA for the most part is associated with the unusual side chain in position 1 as well as amino acids in position 2, 3 and 11. The compounds were characterized using pharmacologic models previously used for testing cyclosporins (Borel *et al.*, 1976a,b; Borel *et al.*, 1977). CsA analogues were obtained that had modifications in the MeBmt group. They were tested for their ability to inhibit IL2 or GM-CSF mRNA accumulation *in vitro*, relative to that of CsA. This approach is very different from that of Wenger (1985). The concentration required for a 50% inhibition of IL2 mRNA in stimulated EL4.E1 or human PBLs is summarized in Table 2. The CsA analogues with modifications in position 1 or 2 were not found to have more enhanced immunosuppressive properties as compared to the parent molecule. Modification of the hydroxy group in the unusual side chain in position 1 as in O-Acyl-CsA and Bromo-CsA, rendered these molecules approximately 100 times less potent as compared to CsA. The saturation of the double bond in Bromo-CsA could also have contributed to the observation that Bromo-CsA was inactive. However, as shown in Dideutero or Dihydro-CsA, saturation of the double bond alone only rendered these molecules somewhat less active than CsA. Thus, Bromo-CsA might be rendered completely inactive by a combined effect of modifying the hydroxy group and saturating the double bond. Acidic treatment of CsA in the absence of water effects an N,O-acyl migration of the methylvalyl moiety and furnishes Iso-CsA. This reaction is reversible (L. Trimbell, personal communication) and thus explains the observation that Iso-CsA is as active as CsA

despite the modification of the hydroxyl group. Therefore, these results suggest that irreversible modification of the hydroxyl group in the MeBmt side chain of CsA, results in a loss of immunosuppressive activity. To further test this conclusion, it would be of interest to synthesize an analogue that contains only the parent amino-acid threonine of MeBmt. With the cytodot technique it would be possible to determine if this hydrophobic amino-acid was necessary for immunosuppressive activity. However, as important as MeBmt seems to be, the biological activity is associated with a larger portion of the structure as shown by modification of amino acid in position 2. Naturally occurring isomers CsC and CsD are respectively 3 and 10 times less potent than CsA, in inhibiting IL-2 mRNA in stimulated EL4.E1 cells. However, the differences in potencies for CsA and CsC are much less significant as compared to CsD when repeatedly tested on other cell types. The conclusion is that CsC is as potent as CsA but that substitution of α-amino butyric acid in position 2 of CsA for valine as in CsD, renders the molecule much less active. Amino acids in position 3 and 11 are important because they provide steric bulk (Wenger, 1985).

The results presented here suggest that a free hydroxy group is essential for CsA-mediated immunosuppression. The implication is that hydrogen bonding is necessary for CsA action, perhaps by binding to a membrane receptor. The nature of a CsA receptor is, however, unclear as detailed in Chapter 1B, Section 3.

Interestingly, the results presented here on the relative abilities of CsA analogues to inhibit the induction of two lymphokine mRNAs, correspond to the specificity of cyclophilin for binding CsA. Analogues that were unable to inhibit lymphokine mRNA also had affinity for cyclophilin (Handschumacher *et al.*, 1984). These results suggest that the inhibition of lymphokine mRNA in stimulated T cells by CsA, may be a result of CsA binding to an intracellular component, and that upon modification of CsA's structure results in a loss of biological activity.

2. *Dexamethasone-Mediated Inhibition of IL2 mRNA*

The work in this chapter (Section 10) demonstrated that, of four anti-inflammatory or anti-cancer drugs, only dexamethasone induced a significant inhibition of IL2 mRNA in stimulated human PBLs. The lack of an effect of 6-mercaptopurine, cyclophosphamide or melphalan on inhibition of IL2 mRNA may be a result of their inability to act *in vitro*. In fact, it appears that the alkylating action of cyclophosphamide requires the metabolizing of the drug *in vivo* by the mixed function oxidase enzymes of liver microsomes, which produce several active metabolites. *in vivo* metabolizing might also be required for melphalan. 6-Mercaptopurine, the active compound of azothioprine, is an anti-inflammatory agent that is most often used in combination with CsA to maintain immunosuppression and prevent inflammation following organ transplantation. The results observed that 6-mercaptopurine does not inhibit IL2 mRNA production in normal PBL-stimulated with PMA and Con A, indicate an alternative mechanism for its mode of action *in vivo*.

Dexamethasone was observed to cause a partial inhibition of IL2 mRNA production in induced human PBLs, but not in induced murine or human T cell lines. Generally, dexamethasone acts by stimulating the synthesis of specific mRNA, such as mouse mammary tumor virus RNA (Ringold *et al.*, 1983), metallothionen (Hager and Palmiter, 1981) and α_1-acid glycoprotein (Vannice *et al.*, 1984) mRNAs. Dexamethasone has also been shown to inhibit the synthesis of mRNA for propiomelanocortin (Roberts *et al.*, 1982), α_1-fetoprotein (Bélanger *et al.*, 1981) IL2 and IFN-γ (Arya *et al.*, 1984) and IL2 receptor (Larsen and Grabstein, 1985). It is these latter effects that result in a glucocorticoid suppression of the immune response. Arya and co-workers observed that 1 μM dexamethasone (392 ng ml^{-1}) inhibited IL2 and IFN-γ in PMA plus TPA stimulated human PBL and only minimally inhibited IL2 mRNA in Jurkat cells. The results presented here are in direct agreement with Arya *et al.* (1984).

The mechanism for steroid hormone action is initiated to act by binding to soluble cytoplasmic receptors; this is followed by transportation of steroid-receptor complexes to the nucleus. The nuclear complexes bind with high affinity to specific DNA sites adjacent to regulated genes, to reduce or enhance their transcription. This mechanism does not account for the lack of effect observed in stimulated Jurkat cells, since they too contain dexamethasone receptors (Arya *et al.*, 1984). However, the fact that Jurkat cells are of neoplastic origin, might mean that they have undergone gene rearrangements which have abolished regulatory sites for dexamethasone-receptor complexes. Alternatively, the receptor may be able to bind dexamethasone but be altered in such a way that the complex can no longer bind to regulatory regions on the DNA. It might be that dexamethasone affects the synthesis or function of IL-1 produced by macrophages, as it has been reported that glucocorticoids render human T lymphocytes unresponsive to IL1, thus preventing IL2 production (Palacios and Sugarawa, 1982). In contrast, corticosteroids reportedly suppress the production of IL1 by murine macrophages (Snyder and Unanue, 1982). If the effect of dexamethasone is mediated via IL-1, this could explain the result observed with Jurkat cells, since it is a clonal T-helper cell line that does not require exogenous IL1. This result further supports the usefulness of the cytodot assay, in that it is possible to measure and biologically assess the gene expression of a specific cell in a heterogeneous population of cells.

3. *Clinical Application of the Cytodot Assay*

The availability of a rapid and quantitative assay to measure IL2 mRNA levels for clinical use is important. Monitoring of CsA-concentration in allograft recipient sera by the ability of the serum to inhibit IL2 mRNA production in cloned T-helper cell lines would be such an assay. The preliminary results reported in this chapter predict that such a tool could prove useful, but the following considerations have to be taken into account. (i) Normal human serum suppresses IL2 mRNA accumulation. This has

previously been shown with human (Cooperbrand *et al.*, 1968; Matsushima *et al.*, 1984) and mouse (Hardt *et al.*, 1981; Hsu *et al.*, 1981; Viet *et al.*, 1973) sera. However, it has been shown that the inhibition by murine serum of the proliferation of IL2-dependent cells is not specific for IL2 since this effect was observed with other cells not dependent on IL2 and also murine serum did not inactivate IL2 *in vitro* (Hooton *et al.*, 1985). If normal human serum does not specifically inhibit IL2 mRNA, then this assay for measuring CsA concentrations in serum would be feasible. A titration curve would be necessary to determine which concentration to use. (ii) It appears from Figure 11 that different individual's sera inhibit IL2 mRNA production to the same extent at different concentrations. Thus, to be clinically applicable to a variety of individuals, preliminary tests must be performed in each case to determine which concentration of serum to use in the assay. (iii) The limit of accurate measurement of CsA in the serum determined with the bioassay is in the order of 20 ng ml^{-1} to 300 ng ml^{-1} (Chapter 3B, Section 12). This limitation has also been reported for the RIA at 65 ng ml^{-1} (Rogerson *et al.*, 1985) and for HPLC at 32 ng ml^{-1} (Carruthers *et al.*, 1983). In this respect, the bioassay is more sensitive. In general, the technique is interesting but not readily acceptable for clinical use. The results could be available in no less than 3 days after sampling, therefore daily monitoring with this technique would not be advantageous. Interestingly though, patient B studied in this work, rejected a renal graft. The bioassay results confirm the RIA values that the concentration of CsA was generally less than 200 ng ml^{-1}. It has been reported that CsA concentrations less than 200 ng ml^{-1} are associated with a higher frequency of rejection (Irschik *et al.*, 1984). The results presented here emphasize the correlation between cyclosporin levels and clinical complications.

CHAPTER IV

FORMATION AND DECAY KINETICS OF IL2 mRNA IN EL4.E1 CELLS

A. INTRODUCTION

The objective of the work reported in this chapter was to investigate the possibility of post-transcriptional control of IL2 expression (Chapter 1C, Section 2) by examining the stability of IL2 mRNA in relation to the stability of bulk cellular mRNAs. The EL4.E1 cell line used for this study is clonal, thus the accumulation of IL2 mRNA and its subsequent decline result from intracellular events and not from regulatory signals originating in another cell type. Early experiments on the stability of mRNA made use of protocols in which actinomycin D was added to block the synthesis of new messengers, so that decay of pre-existing mRNA could be followed. However, according to Singer and Penman (1972), actinomycin D had secondary effects on translation initiation. Thus it is possible that mRNA remains stable in the presence of actinomycin D, because of the loss of regulatory molecules. In fact, there are many reports of increased enzymatic activities after the addition of actinomycin D to cells actively synthesizing specific enzymes (Tomkins *et al.*, 1972).

Since actinomycin D blocks all nuclear transcription, theoretically it would inhibit the synthesis of molecules that control the accumulation and decay of IL2 mRNA. In attempting to determine the half-life of IL2 mRNA, normal clearance mechanisms must be retained. Therefore, because the immunosuppressive drug CsA has been shown to inhibit the synthesis of IL2 mRNA (Elliott *et al.*, 1984; Krönke *et al.*, 1984; Granelli-Piperno *et al.*, 1986) and its effect is selective, *i.e.*, it does not inhibit the transcription rate of non-lymphokine genes (Chapter 3B, Section 5), CsA is a valuable tool to study further IL2 gene expression at the molecular level. Since CsA blocks transcription of the IL2 gene without perturbing other cellular functions, it was used to monitor the decay

of IL2 mRNA in EL4.E1 cells. The half-life of bulk cellular mRNAs was determined by conventional pulse-labelling of cells with ^{3}H-uridine.

B. RESULTS

1. *Kinetics of IL2-mRNA Accumulation in Stimulated EL4.E1 Cells*

The kinetics of appearance of mRNA for IL2 in EL4.E1 cells stimulated with PMA, as followed by cytoplasmic RNA dot blot hybridization, are illustrated in Figure 13. For quantitative analysis, the autoradiogram was scanned on an ELISA plate reader and optical densities of the spots were used to generate a graph. Levels of RNA become detectable by the 4th hr following stimulation, and then the values fall into a sigmoidal curve. Not shown in this figure is the decline in the accumulation of IL2 mRNA following attainment of [redacted] levels at approximately 16 to 24 hr. The kinetics of induction of IL2 mRNA in PMA stimulated EL4.E1 cells agree with the kinetics of accumulation of IL2 in the culture medium (J. Ng, Effects of PMA and CsA on a T cell line, M.Sc. Thesis, Edmonton, 1984). The mRNA levels precede extracellular IL2 activity by about 4 hr.

2. *PMA Requirement for IL2 mRNA Synthesis*

To determine whether PMA was required continuously or only initially to induce the synthesis of IL2 mRNA, a kinetic study was performed in which EL4.E1 cells were cultured with and without PMA after a 12 hour preincubation period with PMA. Removal of PMA at 12 hr resulted in a dramatic loss of IL2 mRNA accumulation as compared to a culture in which PMA was left for a further 6 hr incubation period (Figure 14). Approximately 50% of IL2 mRNA decayed in 2 hr following a 1 hr lag period. These results demonstrate that, in order to maintain high levels of IL2 mRNA, PMA must be present in the culture system throughout the induction period. In addition, the observation that the removal of the induction signal results in a rapid decline

FIGURE 13

KINETICS OF CYTOPLASMIC ACCUMULATION OF IL2 mRNA

DURING INDUCTION OF EL4.E1 CELLS

5×10^6 EL4.E1 cells were induced with 15 ng ml^{-1} PMA. Total cytoplasmic RNA was extracted at various time intervals after induction, as described in Section B.1 of Materials and Methods. Cytoplasmic dot blots were hybridized to nick-translated IL2 cDNA probe and the autoradiograms quantitated by scanning on an ELISA plate-reader at 570 nm. The optical density (O.D.) units correspond to 2.5×10^4 cell equivalents with a negative control (-PMA) subtracted.

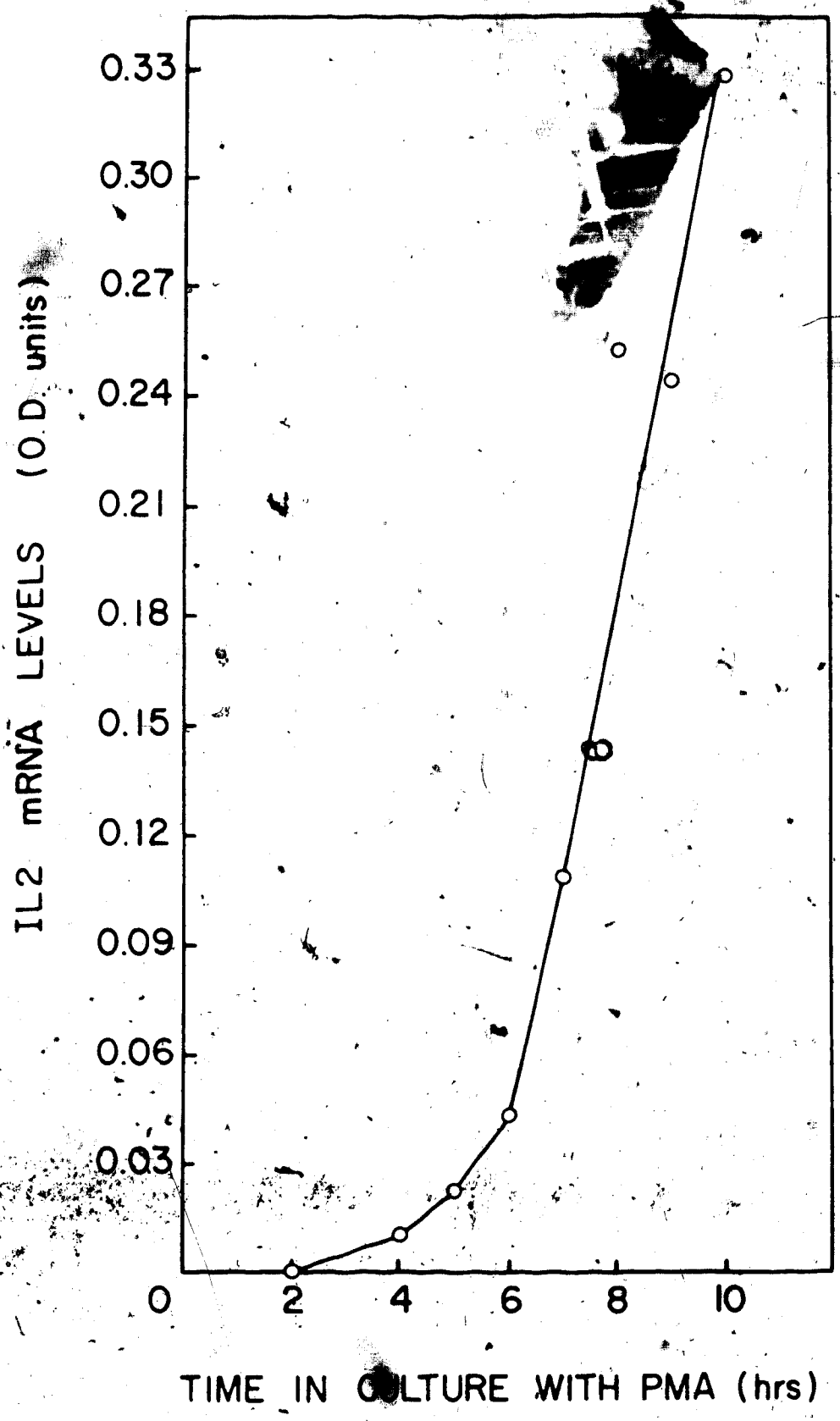
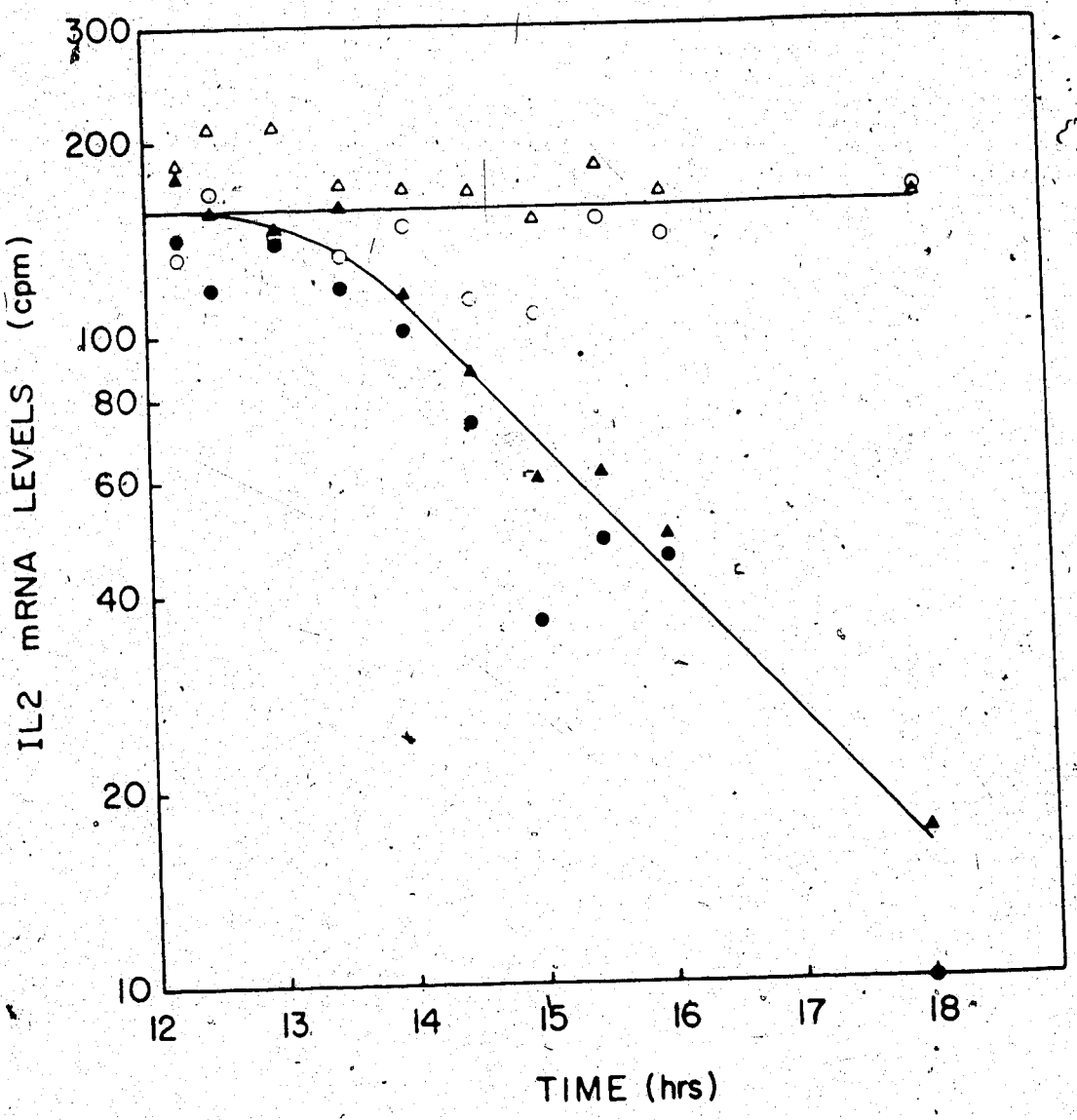

0.33
0.30
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0
2
4
6
8
10
IL2 mRNA LEVELS (O.D. units)
TIME IN CULTURE WITH PMA (hrs)

FIGURE 14

EFFECT OF REMOVING PMA AFTER A 12 HR STIMULATION

IN EL4.E1 CELLS ON THE ACCUMULATION OF IL2 mRNA

EL4.E1 cells were induced in the presence of PMA (15 ng ml^{-1}) for 12 hr. At that time, the cells were washed three times in warm medium and recultured at the same density (10^6 cells ml^{-1}) with (open symbols) or without (filled symbols) fresh PMA. Cells were further incubated for 6 hr. Total cytoplasmic RNA was extracted at various time intervals after the wash as described in Materials and Methods, Chapter 2B, Section 1. RNA was transferred to nitrocellulose filters and hybridized to nick-translated IL2 cDNA probe. Quantitation of IL2 mRNA levels were performed by counting the radioactive dots corresponding to 4 x 10^5 cell equivalents in 5 ml Scintiverse, and subtracting the counts of a negative control sample. The figure shows the data from two separate experiments (circles and triangles).


300
200
100
80
60
40
20
10
IL2 mRNA LEVELS (cpm)
12
13
14
15
16
17
18
TIME (hrs)

in the accumulation of IL2 mRNA, suggests that this mRNA has a short half-life. These results support the concept that the production of IL2 in T cells is regulated at the level of transcription (Efrat and Kaempfer, 1982; Clark *et al.*, 1984).

3. *Superinduction of IL2 mRNA*

Prior studies have indicated that cycloheximide, if added with the mitogen, does not alter lymphokine mRNA levels, but blocks the down regulation of IL2 mRNA occurring at later time points (Efrat and Kaempfer, 1984; Efrat and Kaempfer, 1985). To determine if this phenomenon also occurs in a murine tumor T-cell line, EL4.E1 cells were stimulated with PMA for 10 or 24 hr during which period they were exposed to cycloheximide for various lengths of time, or not exposed to cycloheximide. Figure 15 depicts the kinetics of accumulation of IL2 mRNA under various conditions of induction. The presence of cycloheximide from the time of stimulation (0 hr) resulted in no observable accumulation of IL2 mRNA at 10 hr (Figure 15B) and much less than the control (PMA) at 24 hr (Figure 15A). Similarly, CsA added at time 0 resulted in no observable accumulation of IL2 mRNA at 24 hr (Figure 15A). However, the presence of cycloheximide in the 6 to 10 hr, 8 to 10 hr or 10 to 24 hr time interval led to significant superinduction of IL2 mRNA levels relative to the controls incubated only with PMA (Figure 15B). Identical results were obtained for GM-CSF mRNA (data not shown), suggesting that common intracellular mechanisms regulate lymphokine mRNAs in EL4.E1 cells. It should be pointed out that cycloheximide had no effect on the expression of the IL2 receptor in PMA plus Con A-stimulated Jurkat cells or human PBLs, nor on the constitutive expression of the T-cell receptor β chain in EL4.E1 cells (Paetkau *et al.*, 1985). This indicates that cycloheximide-mediated superinduction does not reflect merely a generalized stabilization of cellular mRNA.

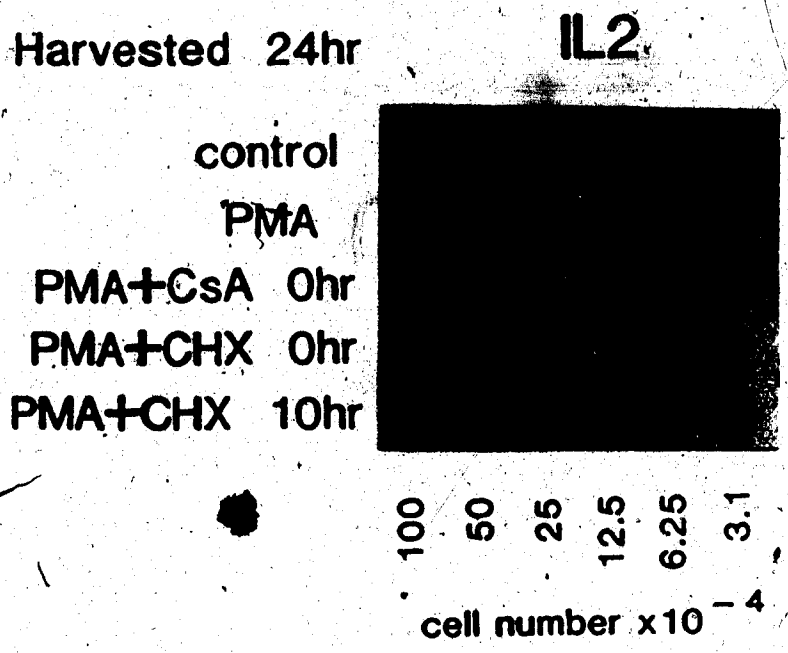
Addition of cycloheximide upon stimulation blocked the induction of IL2 and GM-CSF mRNAs completely. Therefore, protein synthesis is required for the induction

FIGURE 15

SUPERINDUCTION OF IL2 mRNA

Cycloheximide (20 $\mu g\ ml^{-1}$) was added to 5 x 10^6 EL4.E1 cells at the time of stimulation with PMA (15 $ng\ ml^{-1}$) or 6, 8 or 10 hr after PMA induction as indicated. At 10 or 24 hr cells were harvested and cytoplasmic RNA extracted as described in Materials and Methods, Chapter 2B, Section 1. RNA was transferred to nitrocellulose and hybridized to nick-translated IL2 cDNA probes. The autoradiogram is shown in A. Quantitation of the autoradiographic spots were achieved by scanning on an ELISA plate-reader at 570 nm. The optical density (O.D.) units, shown in B, correspond to the dilution which represents 25 x 10^4 cell equivalents.

A



B

time (hr)			
PMA	cycloheximide	harvest	IL2 mRNA levels[a]
-	-	10	0
0	-	10	0.13 ± .01
0	0	10	0
0	6	10	0.90 ± .01
0	8	10	1.00 ± .01
0	-	24	0.26 ± .01
0	10	24	>1.44

[a] O.D. units per 2.5 x 10^4 cell equivalents.

of lymphokine mRNA synthesis. When only cycloheximide was added to EL4.E1 cells, there was no observable accumulation of IL2 mRNA (data not shown). This suggests the requirement for *de novo* protein synthesis for IL2 mRNA induction. However, once transcription was initiated, the steady state levels of IL2 mRNA were increased by the addition of cycloheximide. The extent of superinduction was approximately 8 to 10-fold (Figure 15). This effect of cycloheximide may be due in part to stabilization of the mRNA, coupled perhaps to an increase in transcription.

4. *Kinetics of IL2 mRNA Decay and Superinduction in the Presence of Cyclosporin A and Cycloheximide*

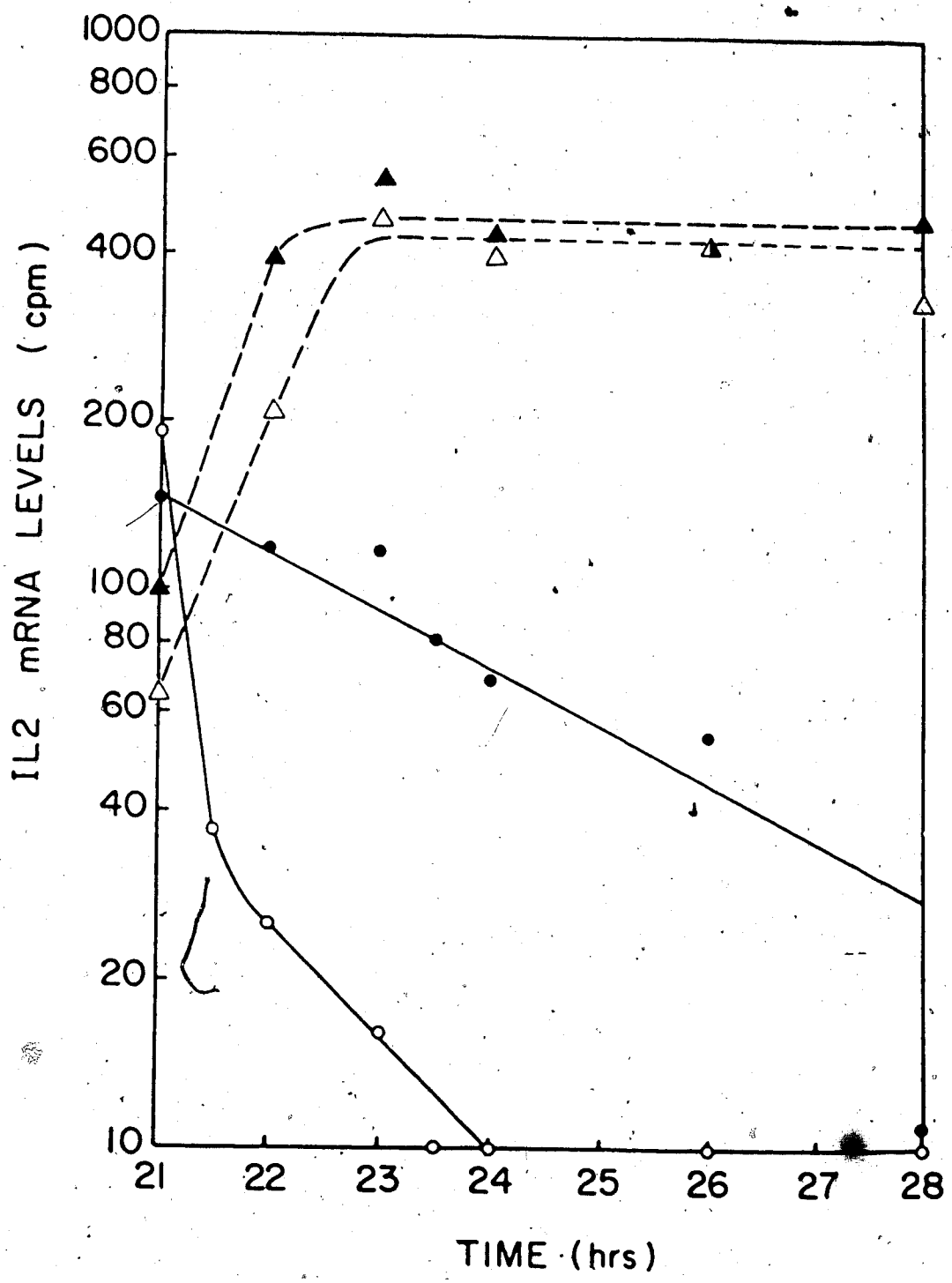
CsA has been previously shown to reduce IL2 mRNA levels by inhibiting its transcription rate in isolated nuclei (Krönke *et al.*, 1984; Shaw *et al.*, submitted). It is this effect of CsA that was taken advantage of for the following experiments. CsA was used as a tool to inhibit the on-going transcription of the IL2 gene, thus enabling the determination of the half-life of the pre-existing IL2 mRNA in the cytoplasm without effecting normal mRNA clearance mechanisms.

EL4.E1 cells were stimulated with PMA for 20 hr after which the induced cells were transferred to individual wells that did or did not contain CsA. Cytoplasmic RNA was extracted from the cells before and at various times points after CsA treatment. Cytoplasmic RNA dot blot hybridization to nick-translated IL2-cDNA probe was performed. The kinetic curves of RNA degradation, as determined by scintillation counting of the radioactive dots, are shown in Figure 16. The IL2 mRNA levels in the absence of CsA represent the normal IL2 mRNA decay rate. The presence of CsA at 20 hr after PMA induction resulted in a dramatic loss of IL2 mRNA accumulation which, after a 1 h lag, became undetectable after 3.5 to 4 hr. It remains unclear from Figure 16 whether the mRNA decay curve in the presence of CsA is truly exponential. Figure 17 represents the percent survival of IL2 mRNA in the presence of CsA as

FIGURE 16

KINETICS OF CsA-MEDIATED IL2 mRNA DECAY AND CYCLOHEXIMIDE-MEDIATED IL2 mRNA SUPERINDUCTION IN STIMULATED EL4.E1 CELLS

EL4.E1 cells (10^6 cells ml^{-1}) were stimulated with PMA (15 ng ml^{-1}) for 20 hr. At this time either nothing (—●—), 100 ng ml^{-1} CsA (—o—), 20 μg ml^{-1} cycloheximide (—▲—) or 100 ng ml^{-1} CsA plus 20 μg ml^{-1} by cycloheximide (—Δ—) were added to one-quarter of the cells and further incubated for 8 hr. At several time intervals after the drug additions, RNA was extracted as described in Materials and Methods, Chapter 2B, Section 1. The RNA was transferred to nitrocellulose filters and hybridized to nick-translated IL2 cDNA probe. Quantitation of IL2 mRNA levels were achieved by counting the radiolabeled spots corresponding to 5 x 10^4 cell equivalents in Scintiverse, and subtracting a negative control (-PMA).


1000
800
600
400
200
100
80
60
40
20
10
IL2 mRNA LEVELS (cpm)
21
22
23
24
25
26
27
28
TIME (hrs)

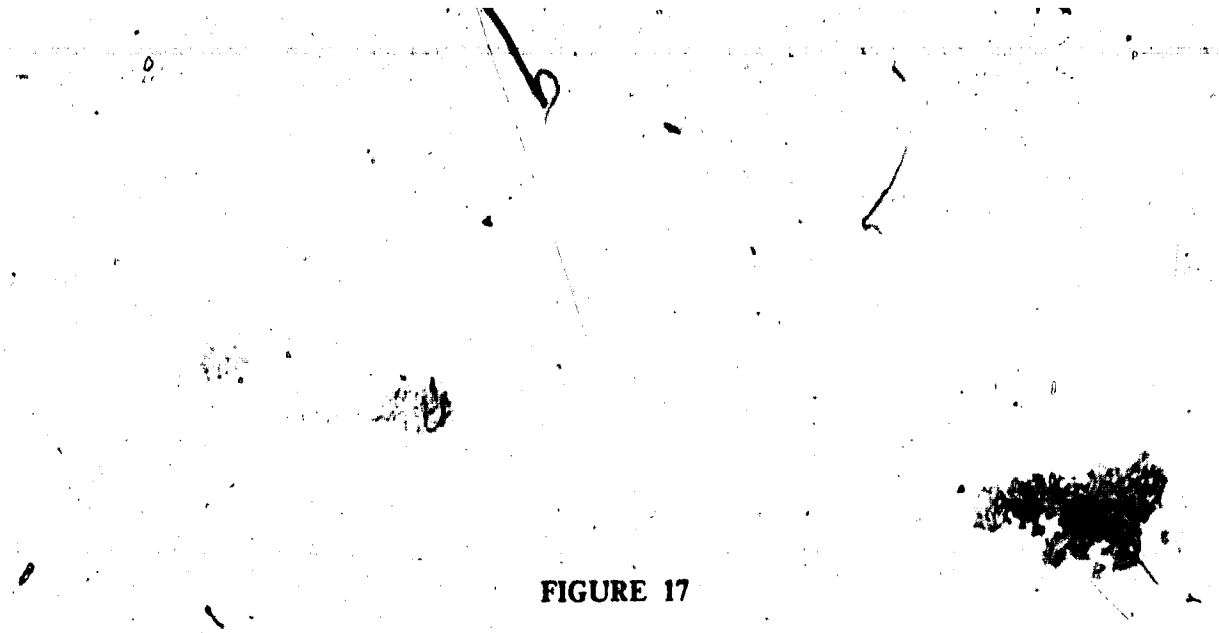
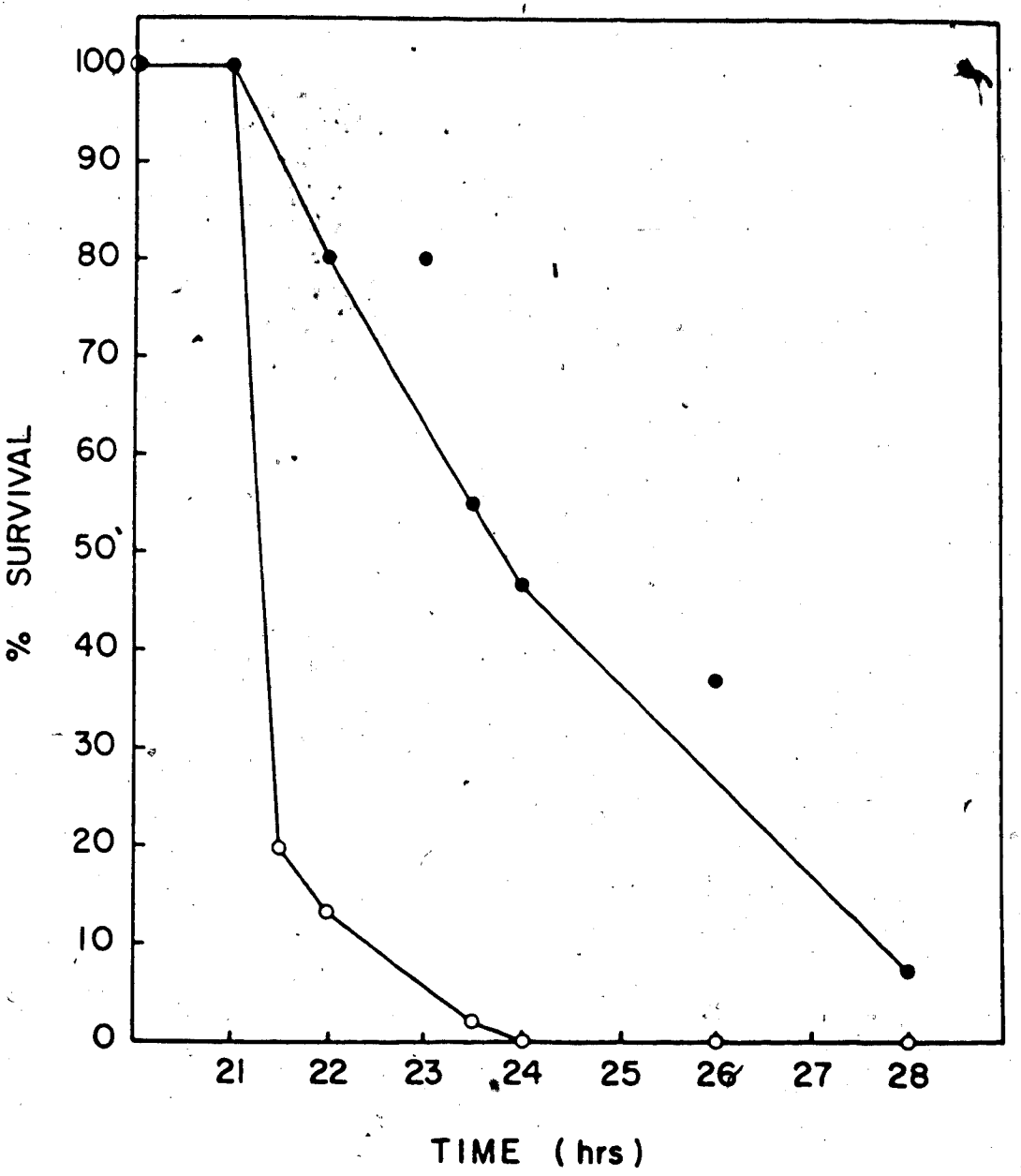


FIGURE 17

SURVIVAL CURVES OF IL2 mRNA IN THE ABSENCE AND PRESENCE OF CsA

IL2 mRNA from EL4.E1 cells was quantitated as described in the legend to Figure 16. Zero time of CsA treatment (100 ng ml^{-1}) was used as a reference for 100% survival. Curves for half-lives of IL2 mRNA in the presence (—o—) and absence (—•—) of CsA are shown.


100
90
80
70
60
50
40
30
20
10
0
% SURVIVAL
21
22
23
24
25
26
27
28
TIME (hrs)

compared to the control.

As shown in Figure 15, addition of cycloheximide at various times after induction resulted in an increase in the accumulation of IL2 mRNA. To evaluate the kinetics of IL2 mRNA superinduction, EL4.E1 cells were stimulated with PMA for 20 hr and then cycloheximide was added. Cytoplasmic RNA was extracted at 1 hr intervals, and analysed by dot blot hybridization to nick-translated IL2 cDNA probe. Quantitation of the data was achieved by scintillation counting of the radioactive dots. When cycloheximide was added at 20 hr there was a dramatic rise in IL2 mRNA levels (Figure 16). After 3 hr in the presence of cycloheximide, there was an approximate 5-fold increase in the accumulation of IL2 mRNA and this was observed to persist for a further 5 hr.

The combined effect of cycloheximide and CsA was also tested in this experiment. EL4.E1 cells were stimulated with PMA for 20 hr at which time both cycloheximide and CsA were added. Cytoplasmic RNA was extracted at 1 hr intervals and analysed as described above. The combined effect of cycloheximide and CsA led to an increase in IL2 mRNA accumulation. However, this rise was not identical to that observed with cycloheximide alone. The additional presence of CsA resulted in a slightly lower increase in IL2 mRNA accumulation (Figure 16).

Figure 16 shows that (i) addition of CsA after 20 hr of PMA stimulation resulted in a rapid decay of IL2 mRNA accumulation as compared to the normal decay rate of the message during shut-off, (ii) the addition of cycloheximide after 20 hr of PMA stimulation resulted in a rapid accumulation of IL2 mRNA and the subsequent stabilization of the message at this superinduced level and (iii) that the addition of cycloheximide plus CsA after 20 hr of PMA stimulation resulted in a superinduction of IL2 mRNA production although somewhat less than with cycloheximide alone.

5. *Messenger Decay Kinetics*

The reduction in IL2 mRNA accumulation upon CsA treatment observed above, reflects a post-transcriptional down-regulation of the message. To rule out the possibility that CsA exerts any additional effects on the processes that control mRNA accumulation, the kinetics of decay of bulk cellular mRNAs were determined in the absence and presence of CsA.

(a) Kinetics of Labeling with Uridine

Exponentially growing cells can incorporate ^{3}H-uridine into RNA. Messenger RNA decay can be monitored directly by measuring the decreasing counts in cytoplasmic poly(A)-containing RNA. EL4.E1 cells, at a density of 10^6 cells ml^{-1}, were cultured in the presence of 1 μCi ml^{-1} (4×10^{-3}M) ^{3}H-uridine. At various time intervals, aliquots of the total cell culture were spotted onto Whatman 3 mm filter paper and nucleic acids were precipitated in 5% TCA. Quantitation of label incorporated was achieved by counting the filters in Scintiverse. As shown in Figure 18, incorporation of ^{3}H-uridine, continued for 24 hr. However, the TCA precipitated cpm were not observed to increase significantly during the 24 hr pulse. This could be a result of degradation of the nucleoside. In an attempt to address this possibility an excess of cold uridine or deoxyinosine was added. Deoxyinosine is a substrate for enzymes that degrade uridine (V. Paetkau, personal communication). The addition of 0.5 μM cold uridine had no appreciable effect on the incorporation of ^{3}H-uridine into TCA-precipitable nucleic acids, but the addition of 0.4 mM deoxyinosine resulted in a dramatic increase in the amount of ^{3}H-uridine incorporated into nucleic acids. The incorporation was observed to increase rapidly during the first 5 hr and to increase at a slower rate during the next 19 hr.

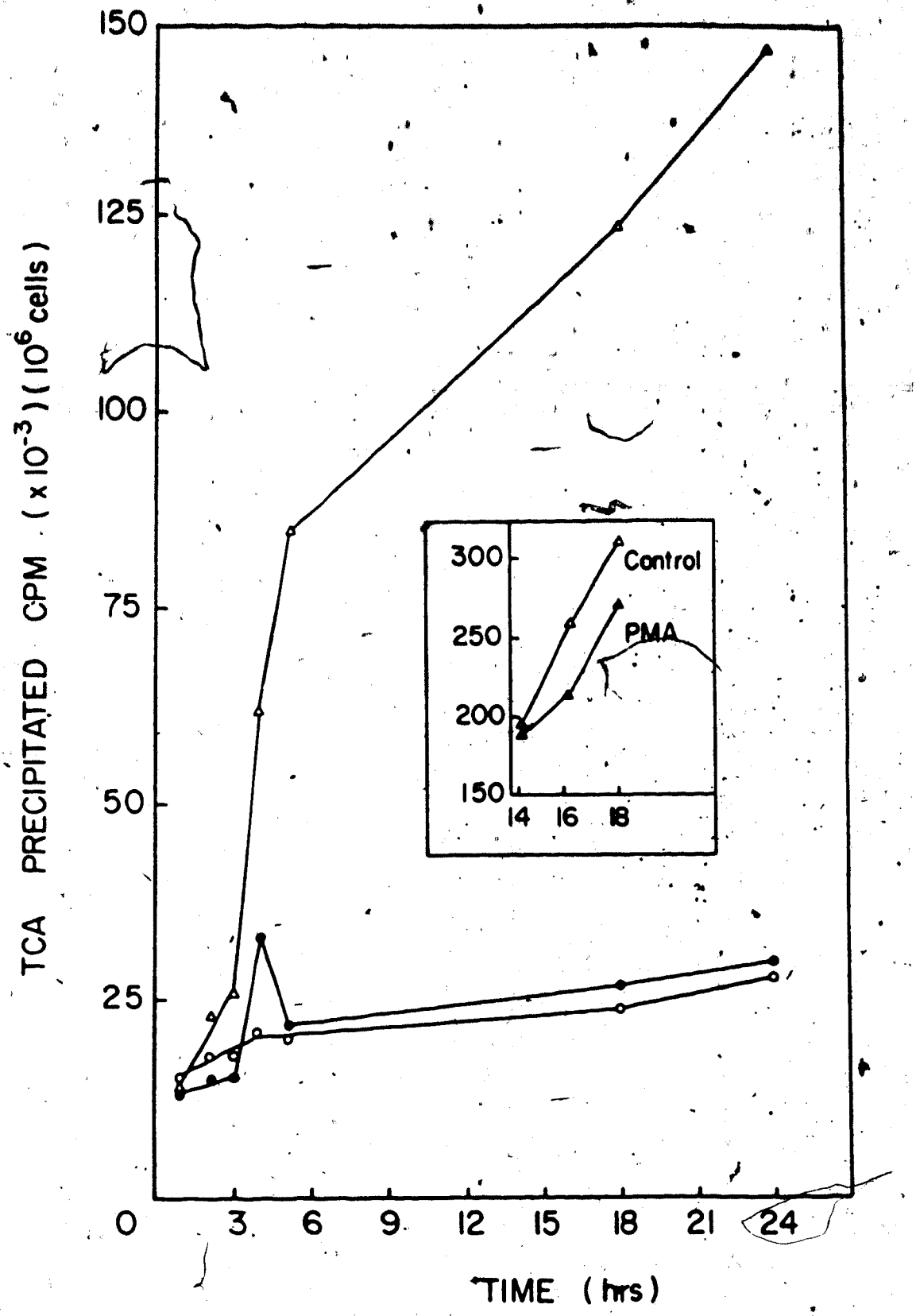
To rule out the possibility that ^{3}H-uridine was incorporated into DNA, the percentage of ^{3}H-RNA in the TCA-precipitated material was determined. RNA was

FIGURE 18

KINETICS OF ^{3}H-URIDINE INCORPORATION INTO

TCA-PRECIPITATED MATERIAL FROM EL4.E1 CELLS

EL4.E1 cells (10^6 cells ml^{-1}) were cultured in the continuous presence of 1 μCi ml^{-1} ^{3}H-uridine (—o—) with the addition of 0.5 μM uridine (—●—) or 0.4 mm deoxyinosine (—Δ—). At the indicated times, 0.05 ml aliquots of the total cell culture were applied to Watman filter paper and precipitated in 5% TCA as described in Materials and Methods, Chapter 2C, Section 2. The inset shows TCA precipitated counts into unstimulated (—Δ—) or PMA stimulated (15 ng ml^{-1}) (—▲—) EL4.E1 cells in the continuous presence of 2 μCi ml^{-1} ^{3}H-uridine from a separate experiment.


150
125
100
75
50
25
0
3
6
9
12
15
18
21
24
TIME (hrs)
TCA PRECIPITATED CPM . (x 10⁻³) (10⁶ cells)
300
250
200
150
14
16
18
Control
PMA

extracted from cell cultures labeled with ^{3}H-uridine, in the absence and presence of deoxyinosine, as described in Materials and Methods, Section B.2. A portion of the RNA was precipitated directly onto filter discs in TCA. Another portion from the same sample was subjected to mild alkaline hydrolysis at 37°C overnight, before precipitation with TCA. The samples in the absence and presence of deoxyinosine consisted of 73 and 74% ^{3}H-RNA to TCA-precipitated counts after 18 hr and 63 and 65% respectively after 24 hr. The portions of RNA that were subjected to alkaline hydrolysis retained 0.76 ± 0.57% (n=4) of the counts compared to the TCA-precipitated counts. These results suggest that 99% of the counts in extracted total RNA are sensitive to alkaline hydrolysis, and that very little of the label was incorporated into non-RNA material. The inset in Figure 18 shows that both PMA-stimulated and unstimulated cultures incorporate the label (as determined by TCA precipitable cpm) to the same extent. A labeling time of 14-16 hr was therefore decided upon to achieve biosynthetic labeling of long-lived RNA components in the cell.

(b) Kinetics of Chase After 16 Hr of Labeling with ^{3}H-Uridine

Table 4 shows the effect of washing out the ^{3}H-uridine after 16 hr labeling period. EL4.E1 cells at a density of 10^6 cells ml^{-1}, were incubated with ^{3}H-uridine (2 μCi ml^{-1}) One sample contained PMA, the other did not. After 16 hr of incorporation, both stimulated and unstimulated cultures were centrifuged and resuspended in fresh medium 3 times. After the wash, the stimulated culture was divided into two. Fresh PMA was added to one culture and fresh PMA plus CsA to the other. The unstimulated culture was free of PMA. At various times during the chase an aliquot of each sample was precipitated onto filter discs with TCA. As shown in Table 4, no significant decrease of label in precipitated cpm was observed in any of the samples during the first 24 hr of chase. The amounts of TCA-precipitated material were very similar regardless of whether the cells were stimulated or not, or even if CsA was present. This

TABLE 4

EFFECT OF WASHING OUT ^{3}H-URIDINE

Chase time (hr)	TCA-precipitated cpm		
	+PMA	PMA+CsA	-PMA
0.5	7430	6853	nd
2	8515	6214	5289
3	6021	8445	nd
4	7048	7115	7354
6	6274	7059	6431
24	4928	5487	6096

EL4.E1 cells were incubated with ^{3}H-uridine (2 μCi ml^{-1}) for 16 hr, in the absence or presence of PMA (15 ng ml^{-1}). At 16 hr the cells were washed 3 times in RHFM. The stimulated culture was divided into two; to one fresh PMA was added, to the other fresh PMA plus CsA (100 ng ml^{-1}) were added. The unstimulated culture remained in the absence of PMA. The cultures were incubated at 37°C for a further 24 hr. At various times indicated, 50 μl aliquots were spotted onto Watman 3 mm filter discs and precipitated with 5% TCA.

nd; not determined.

result suggested that there may be re-utilization of the labeled precursors into newly synthesized RNA. In an attempt to effect the chase more efficiently, various concentrations of cold uridine were added after the wash. Table 5 shows the results from an experiment in which unstimulated EL4.E1 cells at a density of 10^6 cells ml^{-1}, were labeled for 16 h in the presence of 2 μCi ml^{-1} of ^{3}H-uridine. At this time, the cells were washed 3 times and resuspended to the original density in the presence of various concentrations of cold uridine. During the chase period, aliquots from each culture were precipitated onto filter discs with TCA. Concentrations up to 10 mM of cold uridine did not seem to affect the turnover of ^{3}H-RNA during a chase followed for 23 hr. These results suggested that competing out the label with cold uridine does not affect the degradation of total cpm incorporated into RNA, obviating the need for such a protocol. The results obtained eliminate the possibility that there was a breakdown of RNA and re-utilization of the labeled nucleotides. This is in agreement with earlier findings in exponentially growing HeLa cells (Holtzman *et al.*, 1966). These results imply that a significant fraction of the total RNA population has a turnover rate that is slower than 23 hr.

(c) Turnover of ^{3}H-poly(A)$^+$ RNA in EL4.E1 Cells

The results presented in sections (a) and (b) show that a direct determination of mRNA half-life is possible. EL4.E1^o cells, at a density of 10^6 cells ml^{-1}, were labeled for 16 hr with 2 μCi ml^{-1} ^{3}H-uridine, in the absence or presence of PMA. At 16 hr, the cells were washed 3 times and resuspended to the same density. The stimulated cells were divided into 2 separate wells. To one half, only fresh PMA was added, to the other, fresh PMA plus CsA were added. The unstimulated cells were resuspended in the absence of fresh PMA. At various intervals after the wash, 5 x 10^6 cells from each of the three samples were harvested and total cytoplasmic RNA was extracted as detailed in Materials and Methods, Section B.2. Each sample of RNA was ethanol-precipitated

TABLE 5

EFFECT OF ADDING COLD URIDINE TO THE CHASE

Cold uridine concentration	TCA-precipitated cpm		
	1 hr	4 hr	23 hr
0	9219	5822	7178
1 nM	6489	6552	7310
10	7141	5011	7651
100	6237	3648	7756
1 μM	7446	5964	7510
10	6461	5666	6304
100	6408	5588	7050
1 mM	8131	5438	6843
10	7659	6650	5733
blank	734	436	664

EL4.E1 cells were incubated with ^{3}H-uridine (2 μCi ml^{-1}) for 16 hr, in the absence of PMA. At 16 hr the cells were washed three times in RHFM. The cells were resuspended to that original density and replated into fresh wells containing various concentration of cold uridine. The cultures were incubated at 37C for a further 24 hr. At various times indicated, 50 μl aliquots were spotted onto Watman 3 mm filter discs and precipitated with 5% TCA. The blank refers to cells that were not pulsed with ^{3}H-uridine but were treated in the same fashion.

and resuspended in a small volume (25 μl) 25 mM EDTA/0.1% SDS. A portion of the total RNA (3 μl) was diluted to 50 μl. One fifth was used to quantitate the concentration by means of fluorimetry. Three-fifths were counted in 10 ml aquasol to determine the total cpm in ^{3}H-RNA.

Of the original 25 μl of total RNA, 10 μl was applied to mAP as described in Materials and Methods, Section B.3. The poly(A)$^+$ RNA was released into 1 ml of sterile water. 600 μl were used to quantitate the concentration of poly(A)$^+$ RNA by fluorimetry and then subsequently dissolved in 10 mls aquasol to determine the cpm remaining in the poly(A)$^-$ fraction. The percentage of poly(A)$^+$ mRNA in the total RNA population, as calculated either from the concentration in μg or from the cpm, was 4.81 $\pm$ 1.68% (n=25) and 4.98 $\pm$ 1.76% (n=25) respectively. The specific activities (cpm μg^{-1}) for the ^{3}H-total and ^{3}H-poly(A)$^+$ RNAs were calculated and are presented in Figure 19. As indicated, the additional presence of CsA during the chase in the stimulated cultures had no effect on the overall decay curves of the ^{3}H-total or poly(A)$^+$ RNAs. Therefore, in the presence of PMA, the changes in specific activity of the poly(A)$^+$ RNA with time could be fitted with two straight lines. The steeper sloped line had a half-life of 1.5 hr, the other had a half-life in the order of tens of hours. The specific activity of the unstimulated cultures (-PMA) appeared to turnover more rapidly than the stimulated cultures.

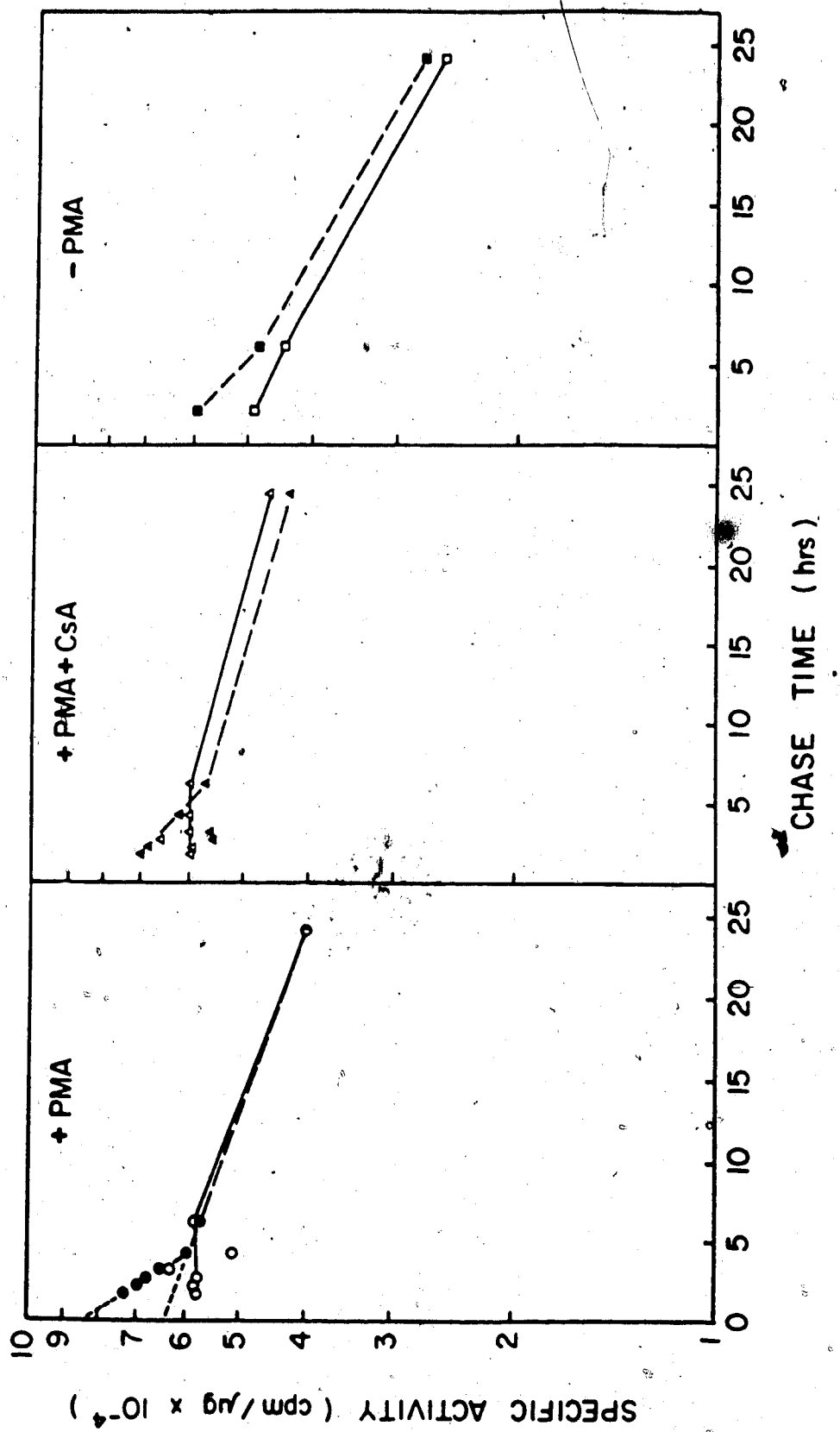
Extrapolation to zero time of the two lines in Figure 19 indicated the initial amounts of the two components. The intercepts at zero time give an initial ratio of radioactivity in the fast-decaying component to the slow-decaying component, *i.e.*, 25% of the labeled message is in the rapidly decaying species and 75% in the slowly decaying species.

The remainder of the ^{3}H-poly(A)$^+$ RNA released from mAP was denatured and transferred to nitrocellulose as for dot blots. The filter was hybridized with nick-

FIGURE 19

TURNOVER OF TOTAL AND POLY(A)$^+$ RNA

EL4.E1 cells (10^6) at a density of 5 x 10^5 cells ml^{-1} were stimulated with PMA (15 ng ml^{-1}) and labeled with ^{3}H-uridine (2 μCi ml^{-1}) in the presence of deoxyinosine (0.4 mM). Unstimulated EL4.E1 cells (3 x 10^7) at the same density, were also labeled with ^{3}H-uridine (2 μCi ml^{-1}) in the presence of deoxyinosine (0.4 mM). After 16 hr of incorporation, the cells were centrifuged and resuspended in fresh medium. Cell density was kept at 5 x 10^5 cells ml^{-1}. After the wash (zero time) the stimulated culture was divided into 2. Fresh PMA was added to one culture (circles), and to the other fresh PMA plus CsA (100 ng ml^{-1}) (triangles). The unstimulated cells remained in medium free of PMA (squares). At intervals after the labeling, portions of each sample were removed for extraction of cytoplasm and isolation and analysis of mRNA (see Materials and Methods, Chapter 2B, Sections 2 and 3 and Chapter 2C, Section 1). The specific activities of total (open symbols) and poly(A)$^+$ RNA (closed symbols) for each portion are plotted on a logarithmic scale as a function of time.


- PMA
+ PMA + CsA
+ PMA
SPECIFIC ACTIVITY (cpm / µg x 10⁻⁴)
CHASE TIME (hrs)
0
5
10
15
20
25
1
2
3
4
5
6
7
9
10

translated IL2-cDNA probe. Quantitation of the autoradiogram was achieved by scanning with a densitometer. Figure 20 depicts the decay of IL2 mRNA levels in the presence of CsA added at 16 hr. After a 1.5 hr lag, the IL2 mRNA decays with a half-life of 1-1.5 hr, as expected. The control IL2 mRNA levels in the continued presence of PMA, remained constant (data not shown). These results illustrate that while IL2 mRNA is rapidly decaying in the presence of CsA, the overall rate of bulk cellular poly(A)$^+$ RNA decay in the presence of CsA is very slow. Therefore, CsA does not seem to have any additional effects on the rate of bulk cellular RNA turnover.

C. DISCUSSION

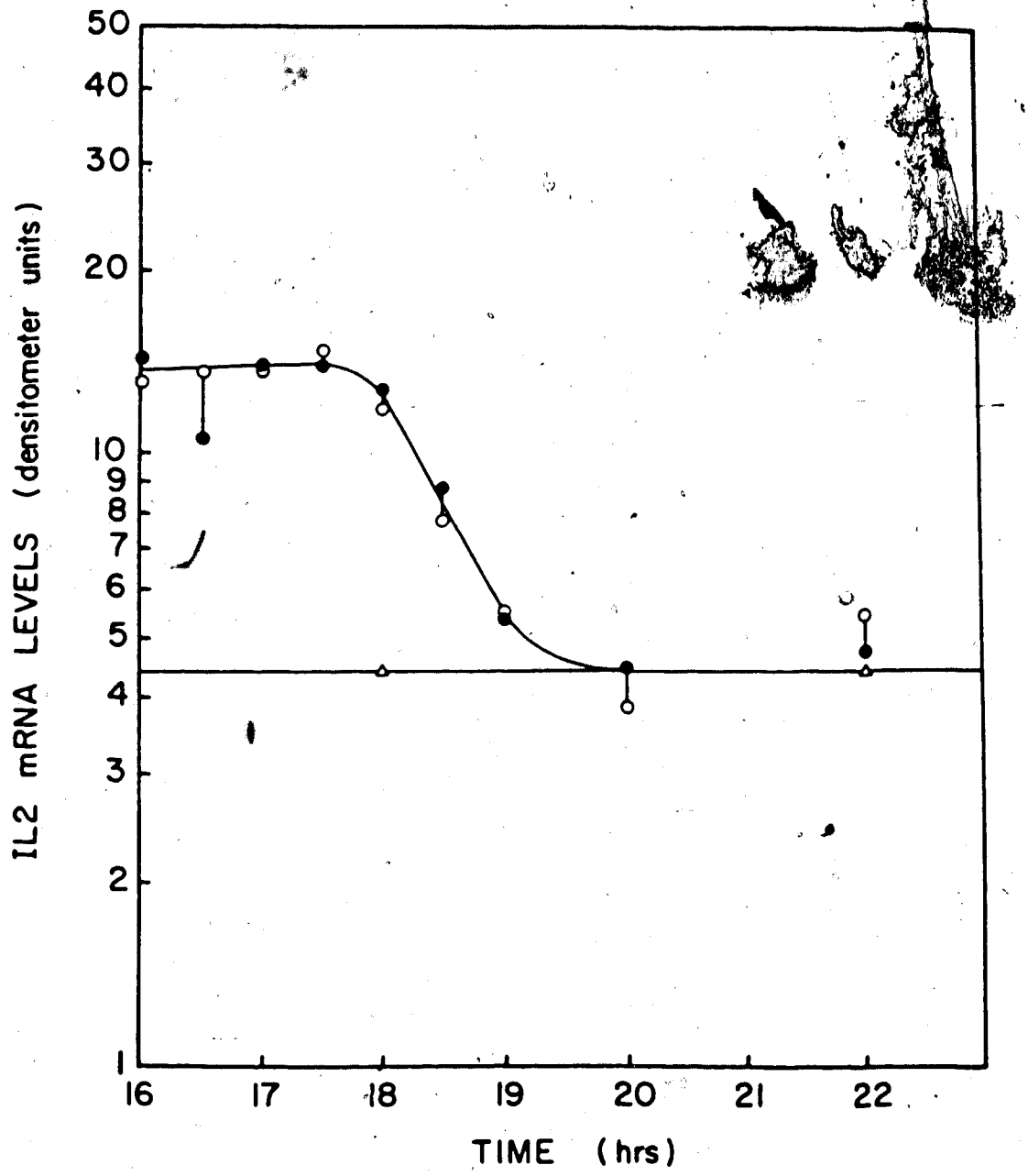
The results presented in Sections 1 and 2 demonstrated that (i) IL2 mRNA is induced by PMA in EL4.E1 and (ii) removal of PMA after induction results in a rapid decline of IL2 mRNA following a 1 hr lag. This suggests that there is a mechanism for rapidly turning off IL2 mRNA upon removal of the stimulus. This is consistent with the recent report by Watts and McConnell (1986). In studying antigen presentation to T cells by supported planar membranes, they found that IL2 production is rapidly turned off upon removal of T cells from the supported planar membrane. These results could be explained by a direct affect on gene transcription rates and/or a post-transcriptional mechanism accelerating IL2 mRNA processing or degradation. The sequence of events associated with induction of IL2 gene expression after PMA treatment has not yet been defined. PMA is known to activate the plasma membrane-associated protein kinase C (Nishizuka, 1984), but a direct role of this in increasing transcription has not yet been demonstrated (Rabin *et al.*, 1986).

In an attempt to study further the regulation of IL2 mRNA accumulation and decay, cycloheximide was added at various intervals to induced EL4.E1 cells to inhibit protein synthesis. Two important observations are illustrated in Figure 15. First, addition

FIGURE 20

DECAY OF IL2 mRNA DURING A CHASE IN CsA

Total RNA (16.2 ± 2.9 μg [n=24]) from all three samples described in the legend to Figure 20 were immobilized on nitrocellulose in duplicate and hybridized to nick-translated IL2 cDNA probe. mRNAs were quantitated by densitometry scanning of the autoradiographs. IL2 mRNA levels from PMA plus CsA (—o— and —●—) and control (—Δ—) samples are shown as a function of time.


IL2 mRNA LEVELS (densitometer units)
50
40
30
20
10
9
8
7
6
5
4
3
2
1
16
17
18
19
20
21
22
TIME (hrs)

of cycloheximide at the time of stimulation blocked the induction of IL2 mRNA. This clearly indicates that activation of the IL2 gene in EL4.E1 cells is a secondary response *i.e.*, prior protein synthesis is necessary. In contrast, PBL do not require prior protein synthesis for IL2 mRNA induction (Krönke *et al.*, 1985; Paetkau *et al.*, 1986). The discrepancy in the results between tumor cell lines and PBL may be explained by the fact that PBL, consisting of mixed cell populations, could presumably have received inductive signals from other cell types prior to stimulation in culture.

The second observation supported by Figure 15 is that, following the first hours of induction, addition of cycloheximide resulted in a superinduction of IL2 mRNA. The superinduction of IL2 mRNA is consistent with the concept that a labile protein regulates the level of the IL2 mRNA (Efrat and Kaempfer, 1984). The superinduction phenomenon has been demonstrated with a variety of mRNAs such as β-IFN in poly (I:C) activated human fibroblasts (Sehgal and Gupta, 1980; Raj and Pitha, 1981), c-*myc* in Con A activated mouse spleen cells (Kelly *et al.*, 1983), IL1 in PMA induced P388D cells (Mizel and Mizel, 1981), tyrosine aminotransferase in cortisol induced rat livers (Hofer and Sekeris, 1978), histones in HeLa cells (Stimac *et al.*, 1983), JE and KC mRNAs in PDGF stimulated 3T3 cells (Cochran *et al.*, 1983) and c-*fos* in TPA stimulated monocytic cell lines (Mitchell *et al.*, 1985). The results from these studies suggested that the cycloheximide-mediated mRNA superinduction is mediated by a post-transcriptional mechanism. However, in other systems cycloheximide-mediated superinduction has been shown to involve stimulation of the transcription rate *e.g.*, β-actin gene in mouse-embryo-derived AKR-2B cells (Elder *et al.*, 1984), β-IFN gene in Chinese hamster ovary cells (Ringold *et al.*, 1984; Maroteaux *et al.*, 1983), MHC-class II invariant chain gene in the pre-B cell line K (Rahmsdorf *et al.*, 1986), human-Ig-genes transfected into mouse L cells (Isahara, 1984) and human-Ig-genes in a pre-B cell line (Wall *et al.*, 1986). This effect may be a consequence of inhibiting the production of a labile protein whose action regulates gene transcription in these systems.

Superinduction of the IL2 gene in EL4.E1 cells is due to post-transcriptional mechanisms, since the transcription rate was not found to increase in the presence of cycloheximide (Paetkau *et al.*, 1986; Krönke *et al.*, 1985). Presumably, superinduction of IL2 mRNA is a result of stabilization of the message. This is not the case for other messages in EL4.E1 cells, *i.e.*, IL2R, T cell receptor β-chain, or actin (Paetkau *et al.*, 1986). We would therefore predict that IL2 mRNA has relatively short half-life.

The purpose of the experiment in Figure 16 was to investigate the half-life of IL2 mRNA. CsA was added to induced EL4.E1 cells to inhibit on-going transcription of the IL2 gene (see Chapter 1C, Section 2), and the decay of pre-existing IL2 mRNAs was monitored. The results illustrate a 1 hr lag before IL2 mRNA rapidly decays with a half-life of approximately 1.5 hr. Also shown in Figure 16 are the kinetics of IL2 mRNA superinduction in the presence of cycloheximide. Within 3 hr after treatment with cycloheximide there is at least a 5-fold increase in IL2 mRNA accumulation. These results predict that a labile or rapidly turning over protein is involved in the post-transcriptional regulation of IL2 mRNA. This protein is presumably involved in IL2 mRNA degradation since blocking protein synthesis with cycloheximide causes a dramatic increase (superinduction) in IL2 mRNA levels. CsA might act directly by binding to regulatory regions on DNA or indirectly by inducing trans-acting factors required for transcription. Alternatively, this speculative CsA-induced factor might affect RNA degradation. Simultaneous addition of cycloheximide and CsA resulted in superinduction of IL2 mRNA, suggesting that the CsA effect required protein synthesis.

In order to determine the turnover rate of bulk cellular poly(A)$^+$ RNA EL4.E1 cells were induced in the presence of ^{3}H-uridine for 16 hr. The protocol was adopted in order to label all cellular RNAs in the cell. At 16 hr, the cells were washed and recultured with fresh PMA or fresh PMA plus CsA. At various time points after the wash, an aliquot of each culture was TCA (5%) precipitated. In addition, total and

poly(A)$^+$ RNA were extracted. The observation that the TCA-precipitable counts did not turnover within 24 hr of chase, suggested that the bulk cellular RNA population was long lived (Table 5). Determination of the specific activity of total ^{3}H-RNA confirmed this observation. The fact that the specific activity of the control (-PMA) culture appeared to decay, is probably due to new RNA synthesis during continued cell growth. In contrast PMA-stimulated EL4.E1 cells are arrested in G_1 phase and therefore do not divide. The specific activities of the ^{3}H poly(A)$^+$ RNAs in the PMA samples suggest that there are 2 components with different half-lives in the cell. These results are consistent with 2 reports in the literature. Singer and Penman (1973) found 2 components with half-lives of 7 and 24 hr in HeLa cells and Spradling *et al.* (1975) found 2 classes of cellular messages with half-lives of 1.2 and 21 hr in cultured insect cells. These results suggest the presence of short lived and long lived components of mRNA in cells. It has been reported that rRNA in mammalian cells is very stable (Singer and Penman, 1973). However, the observation that the long-lived component of the ^{3}H poly(A)$^+$ RNA decays with the same slope as the total ^{3}H-RNA (Figure 19) suggests that the poly(A)$^+$ RNA is possibly contaminated with rRNA. Therefore, the half-life of the long-lived component is perhaps over-estimated. It can be concluded from this experiment that there appear to be short-lived mRNAs in EL4.E1 cells and that the additional presence of CsA does not alter the apparent half-lives of bulk cellular poly(A)$^+$ RNAs.

With the same RNA, it was shown that the additional presence of CsA did result in a rapid decay of IL2 mRNA following a 1.5 hr lag, compared to the control (Figure 20). If this result could be extended to other cell lines, and to other lymphokine genes, it suggests that lymphokine mRNAs are short-lived and regulated by alterations in their stabilities. The presence of rapidly decaying mRNAs in different cells have been observed, and summarized in Table 6. The interesting feature is the relative stabilization of a truncated c-*myc* mRNA. Such differences in mRNA stabilities reflect

TABLE 6

EXAMPLES OF mRNA HALF-LIVES

mRNA	System	Half-Life	Reference
βIFN	Human fibroblast	30-60 min	Raj and Pitha, 1981
Total Cellular poly(A)⁺ RNA		10 hr	
c-*myc*	HeLa cells	15 min	Dani *et al.*, 1984
GPDHase		8 hr	
Truncated c-*myc*	Murine plasma cell tumor	60-140 min	Piechaczyk *et al.*, 1985;
Normal size c-*myc*		15-35 min	Eick *et al.*, 1985
Histone	Mouse myeloma cells		Sittman *et al.*, 1983
	Non-dividing	15 min	
	Dividing	1 hr	
Ad2 E1A	Rat embryo cells	35 min	Wilson *et al.*, 1978;
Ad2 E1B		100 min	Wilson and Darnell, 1981
Bulk mRNA		4.5 hr	

the intrinsic properties of the RNA molecule and not their cellular contexts. While histone mRNAs are rapidly destabilized in the presence of DNA synthesis inhibitors, Morris *et al.* (1986) found that by replacing the 5'-flanking region of human H3 histone mRNA for the leader from a hsp70 mRNA, the fusion mRNA remained stable following inhibition of DNA synthesis. This strongly suggest that the 5' non-coding region of mRNAs might be an important regulatory target for their degradation. The observation of sequence homologies and consensus sequences in the 5' regulatory region of lymphokine genes (Chapter 1C, Section 2) suggests a mechanism for post-transcriptional regulation of lymphokine gene expression similar to that suggested for histone and c-*myc* mRNAs. The mechanism would imply that IL2 mRNA would be rapidly induced upon antigenic stimulation and that upon removal of the stimulation, IL2 mRNA rapidly disappears. Therefore the induction signal provides a responsive system, both for enhancement and diminuition of IL2 production. IL2 mRNA would thus be maintained at low steady state levels, a property essential for an immunoregulatory signal.

CHAPTER V

CONCLUSIONS

The objective of the experiments described in this thesis was to study the effect of cyclosporins on lymphokine gene expression. In the first part of the work, the amount of IL2 mRNA synthesized was used to measure the immunosuppressive properties of various cyclosporins. In the second part, CsA was used as a tool to perturb IL2 gene expression in an attempt to study IL2 gene regulation.

Using the cytoplasmic dot hybridization technique to quantitatively measure lymphokine mRNA levels, several novel findings were demonstrated. (i) The ability of CsA, CsC and CsD to inhibit IL2 mRNA synthesis correlated with immunosuppression. (ii) The hydroxyl group in the first amino acid of CsA was essential for its ability to inhibit IL2 and GM-CSF mRNAs. (iii) Normal human serum inhibited the transcription of IL2 mRNA in stimulated EL4 cells. (iv) The CsA concentration in transplant patients' sera can be monitored through the ability of CsA-containing serum to inhibit IL2 mRNA accumulation in stimulated EL4 cells. (v) The accumulation of IL2 mRNA is dependent on the continued presence of the induction signal. (vi) The short half-life of IL2 mRNA was calculated, following the addition of CsA, to be 1.5 hr after a 1.5 hr lag period in stimulated EL4 cells. In addition, this latter ability of CsA in effecting IL2 mRNA degradation was specific; CsA had no effect on the turnover rate of bulk cellular poly(A)$^+$ mRNA in EL4 cells.

The general conclusion that can be drawn from the data presented in the first part of this work is that an *in vitro* assay that quantitates lymphokine mRNA levels, can be used to screen a variety of immunosuppressive drugs. Moreover, the technique provides a practical application for screening previously uncharacterized synthetic or natural agents that may have immunosuppressive properties. The clinical application has

already been addressed in this work. Although the conclusions drawn have been described, future studies of this nature could be useful in determining the immune status of patients about to undergo transplant surgery. This technique could be applied as a preliminary monitoring tool that would predict the individual susceptibility to CsA, and thus provide a rational means with which to administer drug dosages.

The general conclusion that can be drawn from the data in the second part of this work is that IL2 gene expression is highly regulated in T cells. This regulation has been shown to be mediated by as yet uncharacterized factors, or proteins, that act at both transcriptional and post-transcriptional levels. To investigate the post-transcriptional control of IL2 expression, the stability of its mRNA was examined. This was achieved by the ability of CsA to inhibit IL2 gene transcription. The results of the study provided a value for the half-life of IL2 mRNA of 1.5 hr. The logical implication is that lymphokine mRNAs are short-lived, however future studies in determining half-lives of other lymphokine and non-lymphokine mRNAs are necessary to be able to reach this conclusion. The requirement for continuous stimulation for expression of the IL2 gene and the short half-life of IL2 mRNA are characteristics of gene products that are important regulatory molecules.

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