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# EFFECTS OF PHORBOL MYRISTATE ACETATE AND CYCLOSPORIN A ON THE PROTEINS OF A LYMPHOKINE PRODUCING CELL LINE, EL4.E1

by

JOSEPH NG

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To my wife, June, and my family, especially Julianna and Joan

## ABSTRACT

The thymoma cell line, EL4.E1, of murine C57 BL (H2 ) origin, secretes a number of lymphokines when stimulated with a tumour premoter, phorbol 12-myristate 13-acetate (PMA). These lymphokines include IL2, IL3, BCGF, EL-TRF and CSF-GM.

The mechanism of action of PMA in these cells is uncertain. Its inductive signal is now known to involve binding to a membrane associated protein kinase C (PK-C). PK-C transduces this signal and is suspected of being activated by the interaction of an endogenous signal with its receptor via the breakdown of phosphoinositide to diacylglycerols. The effects of PMA in a number of tissues and systems include; mitogenic, differentiative and inhibitive action on cellular activity in platelets, lymphocytes, fibroblasts, embryo cells, mast cells and bone marrow cells.

This study showed PMA to specifically alter the protein profile of EL4.E1 cells. This effect was limited to a very low proportion (5%) of the general protein profile of the cells. The majority of the proteins made by EL4.E1 cells were unaffected. The inductive signal (PMA), appearently trigged the activation or inhibition of a part of a pathway which would otherwise respond to an endogenous signal, for instance, a lymphokine. The induction of IL2 was shown to be suppressed selectively by cyclosporin A (CsA). This effect contributes in part to the immuno-suppressive effect of CsA as IL2 plays a central role in the mediation of cellular immunity responses. CsA effects on EL4.E1 cells appear to be as specific as PMA effects.

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## ABBREVIATIONS AND DEFINITIONS

| | |
|---|---|
| Ampholines: | a series of peptides of different isoelectric points; used in forming pH gradients. |
| Antigens: | compounds capable of triggering an immune response usually proteins or carbohydrates. |
| Antibody: | a protein produced as a result of the introduction of an antigen and is capable of specific binding with the antigen that stiimulates its production. |
| ASC: | antibody secreting cell. |
| βME: | beta mercaptoethanol. |
| B cell: | bone marrow derived "bursa-equivalent" lymphocyte in non avian species, precursor of ASC. |
| BCDF: | B cell differentiation factor. |
| BCGF: | B cell growth factor. |
| cDNA: | complementary deoxyribonucleic acid. |
| CsA: | cyclosporin A; a fungi extract which as immunosuppressive properties. |
| CSF-GM: | colony-stimulating factor for granulocytes and macrophages. |
| CTL: | cytotoxic T cell line. |
| 2D PAGE: | two dimensional polyacrylamide gel electrophoresis. |
| $ED_{30}$ IL2 units: | the amount of IL2 activity which causes 30% of maximal response obtained in the same assay with saturating levels of IL2 is defined as 1 unit per ml. |
| EDTA: | ethylenediaminetetraacetic acid. |
| FBS: | foetal bovine serum. |
| HPLC: | high pressure liquid chromatography. |
| hsp: | heat shock protein(s). |
| IEF: | equilibrated isoelectric focussing. |
| IEF-SB: | isoelectric focussing sample buffer. |
| IFNγ: | interferon gamma: immune interferon. |
| IL1, IL2, IL3: | interleukin 1, 2 and 3. |

$^{125}$I-UdR: [$^{125}$I]-5-iodo 2-deoxyribouridine.

Lymphokines: nonimmunoglobulin, soluble secreted glycoproteins of lymphocytes responsible for mediating the multiple effects of cellular immune response.

MHC: major histocompatibility complex; genes encoding cell surface antigenes involved in cell recognition in the immune response.

MW: molecular weight.

mRNA: messenger ribonucleic acid.

NP40: nonidet P40; nonionic detergent.

PAGE: polyacrylamide gel electrophoresis.

PBS: phosphate buffered saline.

pI: isoelectric point.

PK-C: protein kinase C: $Ca^{2+}$, phospholipid dependent protein kinase.

PMA: phorbol 12-myristate 13-acetate; also known as 12-0-tetradecanoylphorbol-13-acetate (TPA).

RHFM: cell growth medium; RPMI 1640 supplemented with, 0.02 mM HEPES (pH 7.3), 20 mM sodium bicarbonate, 0.34 mM sodium pyruvate, $10^{-4}$ $\beta$ME, 10% (v/v) FBS, 100 I.U ml$^{-1}$ Penicillin G potassium and 100 $\mu$g ml$^{-1}$ streptomycin sulphate.

SDS: sodium dodecyl sulphate.

SDS-SB: sample buffer containing SDS for polyacrylamide gel electrophoresis.

T cell: thymus derived lymphocyte.

$T_H$: helper T cell; a T cell subpopulation.

TCA: trichloroacetic acid.

Tumour promoter: an agent capable of inducing tumours when applied following prior treatment with a subthreshold level of carcinogen; itself being noncarcinogenic.

TCF: T cell cytotoxicity factor.

TRF: T cell replacing factor.

Tris-HCl: Tris (hydroxymethyl) amino methane.

# CHAPTER I

# INTRODUCTION

## A. ORGANIZATION AND COMPONENTS OF THE IMMUNE RESPONSE

### 1. *General Introduction*

Immune responses maintain the basic defences against foreign invasions in vertebrates. This protective mechanism is maintained by various leucocytes, including two discrete populations of lymphocytes. One class of lymphocytes, the T lymphocytes (T cells), originate from the bone marrow and mature under thymic influence (hence the designation "T"). T cells mediate cellular immune responses against infectious agents, cancer cells and foreign tissue.

The second class of lymphocytes, B lymphocytes (B cells), mature in an organ called the Bursa of Fabricius in *avians* (hence the designation "B") while in mammals B cells are bone marrow-derived, maturation and differentiation occurring in the secondary lymphoid organs; spleen, lymph nodes and Peyer's patches. B cells carry out humoral immune responses by secreting antibodies specific to foreign antigens, initiating the elimination of these antigens. The humoral response is directed against the extracellular phases of infective agents and resides in the serum of blood.

The maturation of lymphocytes involves two phases, (i) antigen independent and (ii) antigen dependent maturation. This dual system, however, is also dependent upon the role of other accessory cells, macrophages. These function in various capacities involving presentation of antigens to lymphocytes, mediation of physiological changes in an immune response by the secretion of immunostimulatory molecules and the scavenging and removal of infectious agents rendered ineffective by the

lymphocytes.

The recognition of antigens and eventual activation of lymphocytes are regulated by the major histocompatibility complex (MHC). The MHC genes (reviewed by Robertson, 1982; Matsinger, 1982; Hood *et al.*, 1982 and Hood *et al.*, 1983) encode an array of molecules which are expressed on cell surfaces enabling cells of one genetic origin to distinguish between "self" and "non-self". The MHC genetic locus is designated H-2 in the mouse and HLA in humans. The H-2 complex is subdivided into two classes. Class I molecules are expressed on somatic cells which are recognised in conjunction with antigens (for example in viral infections) by T lymphocytes. The specificity of the immune response is thus governed by such a specific designation of targets (by the types of Class I molecules recognised in conjunction with antigens expressed). The resulting response in this case is the generation of cytotoxicity by T lymphocytes. Class II molecules are primarily expressed on B cells and macrophages. These molecules play a role in antigen presentation by macrophages to T lymphocytes as well as in successful T lymphocyte and B lymphocyte interaction in antibody production (reviewed by Howard and Paul, 1983). MHC-dependent regulatory events are referred to as MHC restricted.

As more is known about the immune system of the mouse, the following discussion will pertain to the murine system unless stated otherwise.

## 2. *The T Lymphocyte Population*

Murine T lymphocytes are characterised by a cell surface glycoprotein, Thy1, which is not expressed on either B lymphocytes or macrophages. In addition to mediating cellular immunity responses, T lymphocytes also play a role in the regulation of humoral immunity, and delayed-type hypersensitivity (DTH) responses. These varied responses are not mediated through a multifunctional T cell but

through functional subsets of the T lymphocyte population. These subpopulations are designated T-helpers ($T_H$), which stimulate B cell differentiation and maturation of antigen-specific cytotoxic T cells ($T_C$), and suppressor T cells ($T_S$), which inhibits antibody production (Golub, 1981; Reinherz and Schlossman, 1980). These subpopulations carry cell surface markers termed the Lyt markers in addition to Thy1.

Responses mediated by T lymphocytes may occur either through cell-cell contact or through soluble factors released by T lymphocyte subpopulations. Examples of such soluble factors will be described later in this chapter (section C).

3. *The B Lymphocyte Population*

The murine B lymphocytes are characterised by specific cell surface markers (the Lyb. markers) and surface bound immunoglobulins.

In an immune response, B cells differentiate into antibody-secreting cells (ASC). The final stages of maturation of B lymphocytes are dependent upon soluble factors released by $T_H$ lymphocytes and macrophages (see section C). Resting B lymphocytes express identifiable surface immunoglobulins (mIg), most notably, IgM. Upon activation by antigen and soluble factors from T lymphocytes, resting B lymphocytes differentiate into ASC. An ASC does not undergo further division.

There are 5 major classes of immunoglogulins; IgA, IgD, IgE, IgG and IgM. Each of these classes are further divided into subclasses which will not be discussed here. It suffices to mention that each antibody recognises only one antigen and each ASC secretes antibodies specific to one antigen. Upon maturation into ASC, B lymphocytes often "switch" from IgM synthesis to the synthesis of other Ig classes. The immune system is thus capable of generating an enormous repertoire of antibodies, each with its own antigen specificity. Such diversity is generated by gene

rearrangements. reviewed by Tonegawa, 1983 ).

The signals required to trigger a B cell to differentiate into an ASC include a number of T lymphocyte-derived proteins collectively termed T cell replacing factors (TRF) (Howard and Paul, 1983). TRF initiate the cellular changes required for B lymphocyte differentiation protein synthesis. Another T lymphocyte soluble factor causes proliferation of B cells and is termed B cell growth factor (BCGF). These soluble mediators are discussed in section C of this chapter. B lymphocyte activation may be MHC restricted or non-restricted. MHC restricted B lymphocyte responses involving B-T lymphocyte interaction remains a matter of uncertainty with regard to antigen-surface IgM recognition or soluble factor mediation (reviewed by Howard and Paul, 1983).

## B. INDUCTION AND CELLULAR SECRETION OF PROTEINS

### 1. *Induction and Secretion*

Most mammalian cells are capable of secretion. Some secretory cells are involved in the secretion of soluble factors which have regulatory functions. Not all secretory cells however, secrete in response to a stimulus. Some examples of systems that do are, neural cells which secrete neurohormones and neurotransmitters upon receiving an inducing signal, the endocrine system including the adrenal cortex and medulla, the Islets of Langerhan and the pituitary cells all secrete hormones and peptide hormones in response to the appropriate signal. It is however worth noting that, in these systems, the secreted factors are synthesized and stored within the secretory cells, the products being released in response to an appropriate stimulus.

The inducible release of secreted factors described in this thesis belongs with another category whereby the signal induces the synthesis of proteins and ultimately, (though not inevitably the case), the release of a signal-induced-synthesis protein as

a secreted product. One example of such a system is the B lymphocyte response in immunoregulation (as discussed in section A.3) (reviewed by Howard and Paul, 1983). Another example of signal induced protein synthesis is the heat shock responses (reviewed by Tanguay, 1983).

The "signal hypothesis" proposed by Blobel states that the hydrophobic amino acid sequences often observed at the N terminus (the leader sequence) of a newly synthesised protein facilitates their translocation across membranes. The association and translocation of nascent polypeptides at the microsomal membrane, involves three events. (1) The recognition and binding of a complex, the "signal recognition particle" (SRP) to the leader sequence on the nascent polypeptide.(2) The recognition and binding of a microsome associated "docking protein" to the SRP-nascent peptide complex. (3) The cleavage by a signal peptidase of the leader sequence. Translation is interrupted in the absence of the "docking protein" in microsomes (Meyer *et al.*, 1982; Walter and Blobel, 1982a; Walter and Blobel, 1982b; Walter *et al.*, 1982 and Warren, 1982 - a summary).

2. *Heat Shock Responses*

"What is true for *E. coli* is true for Elephant, only more so."

J. Monod

The heat shock response was first observed in the fruit fly, *Drosophila melanogaster*. Raising the environmental temperature by 5°C from the ambient temperature of many organisms tested, resulted in the induction of a specific set of proteins. This response is termed the heat shock response and applies right across the entire spectrum of organisms from *E. coli* to man. Most investigations into the heat shock response have been done on the *Drosophila* as it represents a convenient and readily available resource. The heat shock response (reviewed in Ashburner

*et al.*, 1979, Schlesinger *et al.*, 1982 and Tanguay, 1983) has been shown to invoke a number of regulatory mechanisms involving transcriptional, control to post translational control. A wide variety of agents including heavy metals, ethyl alcohols, sulfhydryls, amino acid analogs, pyrogens and viruses as well as anoxia can trigger induction of the synthesis of proteins originally identified as heat shock proteins (hsp) (reviewed by Ashburner *et al.*, 1979 and Tanguay, 1983). The heat shock response may thus be more appropriately referred to as a stress response.

A significant feature of the heat shock response is, the coincident occurence of a few major hsp remarkably conserved throughout evolutionary history (Schlesinger *et al.*, 1982). Seven major hsp occur in most systems, four with molecular weights between 22 kilodalton and 27 kilodalton (hence hsp22 to hsp27), hsp68, hsp70 and hsp83 with slight variations in their reported molecular weights. Schlesinger *et al.* 1982 showed that rabbit antibodies raised against hsp70 and hsp89 from chicken embryo fibroblast, cross-reacted with hsp from yeast, dinoflagellates, flies, slime moulds, worms, plants, frog cells, mice and human cells with similar molecular weights. The observed compartmentalization of these various proteins implies roles in the stabilization of cytoskeleton, chromatin protection as well as the autoregulation of the heat shock response (Compton *et al.*, 1978; Craine *et al.*, 1980; DiDomenico *et al.*, 1982 and Schlesinger *et al.*, 1982). The degree and duration of the response is dependent upon the temperature and duration of the temperature shock (Compton *et al.*, 1978). Cells rapidly degrade hsp and heat shock mRNA restoring normal cellular protein synthesis upon removal of the heat shock stimulus. The induction of hsp correlates with the development of thermotolerance in Chinese hamster fibroblasts (Li *et al.*, 1982). Hsp appearance is dependent upon the transcription of new mRNA (Ashburner *et al.*, 1979; Compton *et al.*, 1978; Craine *et al.*, 1981, and DiDomenico *et al.*, 1982), and the preferential translation of hsp mRNA over normal mRNA while the normal mRNA is sequestered rather than

degraded. (DiDomenico *et al.*, 1982; Kruger *et al.*, 1981 and Sorti *et al.*, 1980).

The overall view of the heat shock response is that it is a very specific event involving gene activation. The response, rather than being absolutely specific to heat shock, may actually be a response to stress and other physico-chemical aggressions.

## C. LYMPHOKINES AND CYTOKINES

### 1. *General Introduction*

Lymphokines are defined as non-immunoglobulin secretory glycoproteins of activated lymphocytes. Both lymphokines and cytokines modulate cellular immunity responses by acting on target cells of both lymphoid and non-lymphoid types with stimulatory or inhibitory effects. They exhibit very high specific activity and are actively synthesized and secreted. Their action may be MHC restricted or unrestricted and antigen specific or non-specific (reviewed by Oppenheim, J.J., 1981).

The number of lymphokines and cytokines described and characterized in the past decade are numerous. The following discussion will be confined to such molecules of T cell origin (specifically, $T_H$ cells) from the murine system as it is most relevant to the work described in this thesis.

### 2. *T Lymphocyte Growth and Differentiation Factors*

#### *(a) Interleukin 2 (IL2)*

The lymphokine interleukin 2 (IL2) is physiologically and biochemically well characterized (Farrar *et al.*, 1982). Murine and human IL2 have been purified to homogeneity (Reindeau *et al.*, 1983 and Bŏhleu *et al.*, 1983). IL2 is an MHC unrestricted glycoprotein produced by activated $T_H$ cells and was originally termed T

cell growth factor (TCGF) because it promotes the proliferation of thymocytes (Chen *et al.*, 1976; Farrar *et al.*, 1978; Paetkau *et al.*, 1976).

Murine IL2 is an acidic glycoprotein of native molecular weight 32,000, and 17,000 in its monomeric form. It has multiple isoelectric forms of pI ranging between 3.9-5.0 (Caplan, 1981; Shaw *et al.*, 1978; Gillis *et al.*, 1982; Farrar *et al.*, 1980a; Farrar *et al.*, 1982). The molecular weight of purified murine IL2 as determined by preparative SDS PAGE, is about 21,000 with a specific activity of about 16,000 $ED_{50}$ units $\mu g^{-1}$ protein (Riendeau *et al.*, 1983). The biological properties of murine IL2 include (i) the stimulation of proliferation of thymocytes (Chen *et al.*, 1976; Farrar *et al.*, 1978; Paetkau *et al.*, 1976); (ii) the augmentation of the proliferation and generation of cytotoxic T cells (CTL) by alloantigen-stimulated T cells (Wagner *et al.*, 1978; Farrar *et al.*, 1978 and Bleackley *et al.*, 1982); (iii) the induction of immune interferon (IFN$\gamma$) synthesis by alloantigen-stimulated T cells (Farrar *et al.*, 1981) and (iv) the modulation of ASC responses of B lymphocytes (Farrar *et al.*, 1978 and Watson *et al.*, 1979).

IL2 production is dependent upon the stimulation of $T_H$ cells by 2 signals (i) by antigen and (ii) by a macrophage derived lymphokine, interleukin 1 (IL1) (Farrar *et al.*, 1980b; Farrar *et al.*, 1980c and Larsson *et al.*, 1980). IL2 plays a central role in a cyclical amplification of cellular immunity by mediating the production of a number of other lymphokines that act on both antigen-activated B and T lymphocyte target cells (Farrar *et al.*, 1982).

### *(b) Interleukin 1 (IL1)*

IL1, also known as lymphocyte activating factor (LAF), was first identified as a T cell proliferation factor. It has a molecular weight of 12,000 to 15,000 (Mizel & Mizel, 1981; Mizel and Rosenstrich, 1979) with an isoelectric point of 4.50

to 4.55 in mice and 6.5-7.5 in humans. The action of IL1 is antigen non-specific and is not MHC restricted. Its cyclic amplification of lymphokine production with IL2 from T cells explains its T cell and B cell proliferation activity.

*(c) T Cell Cytotoxicity Inducing Factors (TCF)*

Two other factors, the T cell cytotoxicity inducing factors (TCF1 and TCF2) have recently been identified in murine T lymphocyte supernatants. These factors are required for the activation of cytotoxic T lymphocytes (Falk *et al.*, 1983). Both TCF1 and TCF2 are distinct from IL2 or IFN$\gamma$ (section C.6 of this chapter) and induce T cell cytotoxicity in synergy with IL2.

3. *B Lymphocyte Growth and Differentiating Factors (BCGF, BCDF, TRF)*

T cell derived lymphokines acting on B cells include a number of identifiable factors called B cell growth factor (BCGF) and T cell replacing factor (TRF). These encompass a number of lymphokines designated B cell differentiation factors (BCDF$\mu$ and BDDF$\gamma$) as well as EL-TRF and B15-TRF (reviewed by Howard and Paul, 1983).

BCGF has a molecular weight of 11,000-15,000 with isoelectric points between pH 6.4 to 8.7 (Farrar *et al.*, 1983; Howard *et al.*, 1982; Thoman and Weigle, 1983). This soluble mediator is mitogenic for activated B cells, has no B cell differentiation properties and is not MHC restricted (Howard *et al.*, 1982). B cell differentiation is attributed to other soluble factors collectively termed TRF. Two separate TRFs designated B15-TRF and EL-TRF (Nakanishi *et al.*, 1983) induce activated B cells to differentiate into ASCs in a non-antigen-specific manner. B cells express cell surface immunoglobulin (IgM). Upon induction by TRF, B cells switch from the synthesis of surface IgM to the secreted form. This involves the expression of a hydrophobic leader sequence and the mechanism of the "signal

hypothesis" described by Blobel. TRF acts on B cells following antigen and BCGF activation of resting $B_r$ cells and induces differentiation to ASC. Clark-Lewis and Schrader (1982) demonstrated that TRF may be separated from IL2. BCDF$\gamma$ (Isakson *et al.*, 1982) and BCDF$\mu$ (Puré *et al.*, 1981) were shown to be responsible for the immunoglobulin isotype expressed in a particular immune response. BCDF$\gamma$, in particular, mediates isotype switching from immunoglobulin $G_1$ negative ($IgG_1$ ) to $IgG_1^{\cdot}$ cells.

4. *Colony-Stimulating Factor (CSF)*

Another target of the T derived cytokines is the haemopoietic progenitor stem cells. CSF generally refers to the three following molecules. (1) CSF-1, also known as CSF-M, stimulates haemopoietic progenitor cells to form macrophage (Stanley, 1979; Stanley and Heard, 1977). (2) CSF-GM stimulates formation of granulocytes and macrophage (Burgess *et al.*, 1977). (3) CSF-G stimulates the outgrowth of granulocytes from progenitor cells (Nicola, *et al.*, 1983). The term CSF originated from the funtional activity of these molecules, that is their ability to cause colony formation from cultures of progenitor cells on a semi-solid medium. Murine CSF-GM can be resolved from IL2 (Clark-Lewis and Schrader, 1982; Hilfiker *et al.*, 1981). A number of other factors also named CSF may have CSF activity as well as other lymphokine or cytokine activity. One such molecule is Interleukin 3 (IL3).

5. *Interleukin 3 (IL3)*

IL3 has a native molecular weight of 23,000. It exhibits a number of biological activities (Ihle *et al.*, 1983b). These include (i) colony-stimulating factor activity, (ii) growth factor activity for a cell line, WEHI-3, (iii) mast cell growth factor activity, (iv) stimulation of outgrowth of 'persistent' (P cells) cells from bone marrow, (v) stimulation of histamine-producing cells, (vi) induction of the

enzyme 20-α hydroxysteroid dehydrogenase and (v) outgrowth of precursor B cells (reviewed by Ihle *et al.*, 1982). IL3 has been purified to homogeneity (Ihle *et al.*, 1983a). Its cDNA has recently been cloned by Fung *et al.* (1984).

6. *Immune Interferon (IFNγ)*

IFNγ, a glycoprotein of molercular weight 25,000, is secreted by activated T lymphocytes. It is an immunoregulatory molecule for T and B lymphocytes and macrophages and augments cytotoxicity against tumour cells (Boraschi *et al.*, 1981; Schultz *et al.*, 1977) as well as enhance antiviral activity (Morahan *et al.*, 1977). The development of cytotoxic T cell activity by IFNγ involves (i) an IL2 dependent regulation of IFNγ production (Farrar *et al.*, 1981) and (ii) an enhancement of proliferation and development of cytotoxic T cells (Farrar *et al.*, 1981).

7. *General Comments*

The lymphokines discussed in this chapter are all important in mediating the immunity response. This involves a cascade of lymphokine action on macrophages, T cells and B cells (as proposed by Farrar *et al.*, 1982) in which $T_H$ cells secreting lymphokines play a central role in the induction of cytotoxicity and ASC responses upon antigen activation.

## D. LYMPHOKINE PRODUCTION BY A MURINE THYMOMA CELL LINE, EL4

1. *Murine Thymoma-EL4*

Murine thymoma (EL4) from C57BL/6($H$-$2^b$) was reported by Farrar *et al.* (1980) and Shimizu *et al.* (1980) to produce IL2 upon induction with PMA. EL4 and spleen derived IL2 are indistinguishable (Caplan, 1982). Some subclones of EL4 cells are a rich source of IL2 as well as a number of other lymphokines described

in section C of this chapter, namely; BCGF (Howard *et al.*, 1982; Farrar *et al.*, 1983), EL-TRF (Nakanishi *et al.*, 1983), IL3 (Ihle *et al.*, 1982; Pearlstein *et al.*, 1983; Fung *et al.*, 1984), CSF-GM (Bleackley *et al.*, 1983, Hilfiker *et al.*, 1981) and TCF2 (Falk *et al.*, 1983).

The experiments in this thesis were carried out on a subclone of the EL4 thymoma. Subcloning by serial dilution, Dr. Havele in this laboratory, isolated a subclone, EL4.E1, which was selected by virtue of its ability to produce IL2 in high titre upon stimulation with PMA. Translation products of mRNA from EL4 in the *X. laevis* oocyte system yields biologically active IL2, CSF-GM and IL3 (Bleackley *et al.*, 1981; Bleackley *et al.*, 1983; Fung *et al.*, 1984). All three factors were detected in the secreted fraction of the *X. laevis* oocytes microinjected with mRNA from EL4 cells. In cell free translation of mRNA from EL4 cells, IL2 activity can be detected in the microsomal fractions (Harnish *et al.*, 1984). This finding agrees with the current understanding of synthesis of secreted proteins as proposed by Blobel. IL2 derived from EL4.E1 cells has been purified to homogeneity (Riendeau *et al.*, 1983).

2. *Phorbol 12-Myristate 13-Acetate (PMA)*

Phorbol esters (tetracyclic diterpenes), found in extracts of croton oil, are potent tumour promoters as tested in a two-stage carcinogenesis protocol (reviewed by Diamond *et al.*, 1980). Application of a subthreshold dose of a carcinogen followed by repetitive application of a non-carcinogenic promoter (PMA or related phorbol esters) results in tumour growth. If the order of initiator (carcinogen) and promoter applications are reversed, tumour formation fails to occur.

PMA, also known as 12-0-tetradecanoylphorbol-13-acetate (TPA), alone or with mitogens also initiates other changes in cells. Newburger *et al.* (1981)

demonstrated that PMA can induce cells of the human promyelocytic leukemia line HL60 to differentiate into "macrophage-like" cells. These cells morphologically and enzymatically exhibit macrophage characteristics but are deficient in functional capacities as mature phagocytic cells. Although PMA has been shown to be mitogenic for some cell types, proliferation-independent differentiation of a clonal cytotoxic T cell line was also demonstrated (Orosz *et al.*, 1983). PMA also induces granulocytes and macrophage colony formation from bone marrow cells of mice (Stuart and Hamilton, 1980). Whether PMA (i) has CSF-GM activity, (ii) induces elaboration and secretion of CSF-GM from a subpopulation of bone marrow cells or (iii) interacts with growth factor receptors hence increasing CSF-GM receptor expression and thus, sensitivity to low levels of CSF-GM, is unclear. Chick embryo myogenic cells are partially driven along their differentiation pathway by PMA. The expression of muscle-specific creatine kinase, desmin and muscle-specific light meromyosin was observed but no fusion nor assembly of myofibrils was evident (Dlugosz *et al.*, 1983). PMA thus appears to co-induce desmin and muscle-specific creatin kinase. This is reversible upon PMA removal. PMA treatment also causes the induction of specific enzymes and proteins, eg. 5'nucleotidase (Newburger *et al.*, 1981), $Ca^{2+}$, phospholipid-dependent protein kinase (Kraft and Anderson, 1983), lymphocyte guanylate cyclase (Coffey and Hadden, 1981) and a 32,000 dalton protein in BALB/c 3T3 mouse fibroblast cells (Hiwasa *et al.*, 1982) as well as in cultured chick embryo muscle cells (Zani and Molinaro, 1983).

The potency of PMA as a tumour promoter has generated a lot of research into its mode of action (reviewed in Diamond *et al.*, 1980 and Nishizuka, 1984). In the last 5 years it has emerged that PMA specifically binds to a membrane protein which co-purifies with a $Ca^{2+}$, phospholipid-dependent protein kinase (PK-C). (Reviewed in Nishizuka, 1984; Ashendel *et al.*, 1983; Castagna *et al.*, 1982; Delclos *et al.*, 1983; Driedger and Blumberg, 1980, Kraft and Anderson, 1983; Kraft *et al.*,

1982; Sando *et al.*, 1982; Shoyab and Todaro, 1980). PK-C is synergistically activated by either PMA or diacylglycerols with $Ca^{2+}$ mobilization resulting in the phosphorylation of seryl and threonyl residues. It is believed that endogenous inducers triggers the hydrolysis of phosphoinositide to diacylglycerols (which has structural similarities to PMA) thus activating PK-C. PK-C (Kraft and Anderson, 1983) is a cytosolic component which associates with membrane fractions in the presence of phorbol esters (Kraft *et al.*, 1982).

It is proposed that the endogenous substrate for PK-C, may actually invoke a cascade mechanism of controlling cellular functions via protein phosphorylation in an independent pathway to that triggered by cyclic adenosine monophosphate (cAMP). Responses evoked by PMA such as enhanced proliferation of lymphocytes and cultured fibroblasts, differentiation of promyelocytes, and secretion by mast cells, leucocytes and platelets at least in part involve $Ca^{2+}$ mobilization (Weinstein, 1983) and PK-C. Nishizuka's group (Kajikawa *et al.*, 1983; Kaibuchi *et al.*, 1983; Yamanishi *et al.*, 1983) demonstrated that a 40,000 dalton protein is the substrate for PK-C. This phosphorylation may well come about via a receptor induced cascade pathway involving $Ca^{2+}$ as well as cyclic guanosine monophosphate (cGMP). The precise interaction of PK-C with cGMP is unclear. Tyrosine kinase activation may also involve PMA and PK-C activity (Gilmore and Martin, 1983).

As mentioned earlier, EL4 cells are induced to secrete lymphokines and cytokines when cultured with PMA. PK-C activity in EL4 thymoma has been demonstrated by Kraft and Anderson (1983) and Kraft *et al.* (1982). The activation of PK-C suggests that the end result of protein phosphorylation may be the activation of a specific subset of genes ("the lymphokine family") which are possibly co-regulated. It was suggested that PMA may cause a $G_1/S$ cell cycle block in EL4 (Farrar *et al.*, 1982). Later studies by Pearlstein *et al.* (1983) on an EL4

subline, EL4-17-1 which responds to PMA stimulation by producing IL2, IL3 and CSF-GM, revealed PMA to exert a non-specific cell cycle block. This finding does not necessarily contradict the former. The studies of Pearlstein *et al.* (1983) evidently show accumulation of PMA treated cells at $G_1$, late S and $G_2$ phases as early as 8 hours after PMA addition while Farrar *et al.* (1982) employed procedures involving the kinetics of nucleic acids and protein synthesis or degradation which may not be entirely reflective of cell cycle status. Pearlstein *et al.* does show that the eventual outcome of a 24 hour exposure to PMA resulted in accumulation of cells at $G_1$. The implication of these findings in relation to PMA activation of PC-K is not known.

### 3. *Cyclosporin A (CsA) Immunosuppression Effects*

Cyclosporin A (CsA) is a relatively new immunosuppressant. Its antilymphocytic and low myelotoxicity features make it clinically the immunosuppressant of choice. CsA also presents a useful tool for the dissection of lymphocyte subpopulations in cellular immune responses.

CsA is an endecapeptide of fungal extract of the *Tolypocadium inflatum* Gams strain. It has a molecular weight of 1202.6 and is a cyclic compound of 11 amino acids one of which was previously unknown. It has been successfully tested in transplantation patients and extensively tested in laboratory animals for its immuno-suppressive properties (Britton and Palacios, 1982; Thomson, 1983 - a review).

The antilymphocytic property of CsA has been extensively investigated following reports by Borel's group on its immuno-suppressive effects in mice and rats (Borel, 1976; Borel *et al.*, 1976; Borel *et al.*, 1977). They demonstrated that its immuno-suppressive properties coupled with its low myelotoxicity makes it preferable

over steroids, and other immune suppressants which are cytotoxic (azathioprine, cyclohexamide and methotrexate). Successful clinical trails have paved the way for the use of CsA in preventing transplantation graft rejection, particularly in kidney and bone marrow transplant patients (reviewed in Britton and Palacios, 1982, European Multicentre Trial Group - Report 1983).

A number of researchers have since reported that CsA acts on specific target sites of lymphocyte populations. Reports that CsA inhibits proliferation of T cells appear to involve the inhibition of lymphokine production at low concentrations of CsA (0.01 to 0.10 $\mu g\ ml^{-1}$) (Bunjes *et al.*, 1981; Bendtzen *et al.*, 1983; Reem *et al.*, 1983; Thomson *et al.*, 1983a; Orosz *et al.*, 1982; Palacios, 1981) as well as the expression of IL2 receptors on responding T cell population at higher concentrations of CsA (1.0 to 5.0$\mu g\ .ml^{-1}$) (Orosz *et al.*, 1982; Larsson, 1980; Palacios, 1981; Palacios and Möller, 1981; Hess, 1982). Inhibition of lymphokine production results in the suppression of delayed hypersensitivity and natural killer cell responses (Thomson *et al.*, 1983b and Gui *et al.*, 1983). CsA shows selective targeting with respect to antigen-specific and non-antigen-specific responses on clonal T cell lines (Orosz *et al.* 1982).

CsA also exhibits antimicrobial (Kirkland and Fierer, 1983; Weinbaum, 1984), antischistosomal (Beudin *et al.*, 1981) and antimalarial (Tommen-Scott, 1981) properties.

## E. OBJECTIVES AND RATIONALE OF WORK PRESENTED IN THIS THESIS

The ability of PMA to induce lymphokine production from EL4 presents a useful system for the study of PMA induction. A number of lymphokines (section C of this chapter) are produced by EL4 upon PMA treatment. It is unclear

whether this induction mechanism involves a "complete reprogramming" of cellular functions or a "specific perturbation" by the induction of subsets of genes in a selective event with minimal perturbation of "house-keeping" cellular functions. This question is addressed in this thesis.

Expression of IL2, CSF-GM and IL3 by EL4 cells after induction with PMA is controlled at the mRNA level (Bleackley *et al.*, 1983; Bleackley *et al.*, 1981; Fung *et al.*, 1984). This study is aimed at investigating the changes on protein biosynthesis in EL4 cells treated with PMA. It would also present an insight into the proportion of cellular events affected by PMA. Accounting for possibilities of post-transcriptional modifications mediated by PMA, an estimated lower proportion of such changes would represent the involvement of control on the mRNA levels as observed with CSF-GM and IL2 mRNAs. Any identifiable PMA inducible product in this study may present clues towards the mechanism of PMA effects. This study may lead to an understanding of the mechanism of PMA induction of lymphokine production (in particular IL2) by EL4 cells.

This system also presented an opportunity to determine the effects of CsA on the production of IL2 in EL4 cells.

# CHAPTER II

# MATERIAL AND METHODS

## A. CELL CULTURE

### 1. *Cell Culture Media and Conditions*

The cell culture medium consists of RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) supplemented with 20 mM sodium bicarbonate, 0.34 mM sodium pyruvate, 0.02 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid), pH 7.3 (Sigma, St. Louis, Mo.), 100 I.U $ml^{-1}$ Penicillin G potassium (10X $10^6$ $IU.l^{-1}$, Ayerst Hospital Pharmacy, Montreal, Quebec) and 100 $\mu g$ $ml^{-1}$ streptomycin sulphate (Gibco). This is referred to as RH.

RH containing $10^{-4}$ M $\beta$-mercaptoethanol (Baker) is referred to as RHM.

RHM containing 10% (v/v) heat inactivated foetal bovine serum (FBS from Gibco) is referred to as RHFM.

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

Unless otherwise stated, all cell culture work was performed with either COSTAR tissue culture plates from Bellco Glass Inc., Vineland, N.J. or Nunc and Corning tissue culture plates, flasks and centrifuge tubes from Gibco Laboratories, Grand Island, N.Y.

### 2. *Growth and Subcloning of EL4.E1 Cells*

EL4.E1 cells 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 7.0% eosin. Fresh cultures were seeded at a density of 2.5 x $10^4$ cells $ml^{-1}$. EL4.E1 cells were subcloned by limiting dilutions at the concentrations of 5.00 cells $ml^{-1}$, 2.50 cells $ml^{-1}$ and 1.25 cell $ml^{-1}$ in 96 well microtitre plates (200 µl per well). The plates were screened using an inverted microscope for wells with colonies.

Cloning efficiencies were calculated from the equation based on the Poisson distribution term $P_0 = exp(-EN)$, $P_0$ being the proportion of wells with no clones, efficiency (E) was calculated from the average number of cells per well (N).

3. *Growth of MTL.2.8.2 Cells*

The MTL.2.8.2 cell line is an IL2 dependent subclone of a murine cytotoxic T cell line (CTL) described by Bleackley *et al.* (1982). MTL.2.8.2 cells were grown in IL2 supplemented RHFM. These adherent cells were detached from the culture flasks by replacing the growth medium with 20 mM EDTA in $Ca^{2+}$ and $Mg^{2+}$ free phosphate buffered saline (PBS) for 5 min. The cells were pelleted by centrifugation 250 g for 5 mins and resuspended in RHFM. New cultures were seeded at 4 x $10^4$ cells $ml^{-1}$ and grown for 3 days in IL2 supplemented RHFM before being used in IL2 assays.

4. *Generation of IL2*

EL4.E1 cells were harvested by centrifugation at 250 g for 15 mins and washed with RHFM. These were resuspended at $10^6$ cells $ml^{-1}$ in fresh RHFM containing PMA (from Sigma) for 24 hours. Cell free supernatants containing IL2 were harvested by centrifugation at 250 g for 5 mins and if not used immediately were stored at -20°C.

## 5. *Biological Assay for IL2 Activity*

The IL2 assay as described by Bleackley *et al.* (1981) is based on the proliferation of MTL 2.8.2 cells in response to the growth factor (IL2). Serial dilutions of cell-free supernatants to be assayed were pipetted into flat bottomed 96 well Nunc plates in 100 $\mu$l per well aliquots. $10^4$ MTL.2.8.2 per 100 $\mu$l were added per well and incubated for 16 hours. 50 $\mu$l of "labelling mix" containing 1.5 $\mu$Ci ml$^{-1}$. $^{125}$Ideoxyribouridine ($^{125}$I-UdR) purchased from NEN, Dorval, Quebec, 2 x $10^{-2}$ $\mu$g ml$^{-1}$ fluorodeoxyribouridine, 0.4 mM 2'-deoxyinosine in RHFM, was added to each well. To facilitate uptake of $^{125}$I-UdR by the cells, a further 4 hour [illegible]ion period was necessary. The cells were then detached from the bottom of the plates with [illegible] mM EDTA in $Ca^{2+}$,$Mg^{2+}$-free-PBS as described in section A.2 of this chapter and harvested onto glass fibre filters with repeated isotonic saline washes using Titretek multiple sample harvester. These samples were counted on an LKB Rackgamma $\gamma$-counter. All samples were assayed in triplicate and the data expressed as a plot of the percent maximum response (obtained in the same assay with saturating levels of IL2) versus the dilutions of each sample.

# B. L-[$^{35}$S]-METHIONINE BIOSYNTHETIC LABELLING OF PROTEINS MADE BY EL4.E1 CELLS

## 1. *L-[$^{35}$S]-Methionine Biosynthetic Labelling of EL4.E1 Cells*

Proteins made by EL4.E1 cells were biosynthetically labelled using a pulse chase protocol. In each experiment, the cells were incubated at $10^6$ cells ml$^{-1}$ for 12 hours in either (a) RHFM only, (b) 20 ng ml$^{-1}$ PMA in RHFM, (c) 50 ng ml$^{-1}$ CsA in RHFM or (d) 20 ng ml$^{-1}$ PMA and 50 ng ml$^{-1}$ CsA in RHFM. Prior to the pulse labelling, the cells were washed three times in methionine-free RPMI 1640 (Gibco Select Amine Kit) containing $10^{-4}$ M $\beta$ME, 1% (v/v) FBS, 20 ng ml$^{-1}$

phenyl methyl sulphonyl fluoride (a) with or without PMA, (b) including CsA, with or without PMA. This is referred to as the "cold labelling-medium". Pulse labelling was done in 200 $\mu$l cultures in microtest-tubes containing $10^7$ cells $ml^{-1}$. 800 $\mu$Ci $ml^{-1}$ L-[$^{35}$S]-methionine (900-1100 Ci $mmol^{-1}$., NEN) in the "cold labelling-medium". Labelling conditions were: 35' min pulse, at 37°C, 100% humidity, 5% $CO_2$ in air with periodic resuspension of the cells by gently tapping the tubes every 10 mins. The pulse was terminated with addition of an equal volume of chase-medium which was made up of the "cold labelling-medium" containing 30 $\mu$g $ml^{-1}$ cold L-methionine representing a 250-fold molar excess of cold methionine over L-[$^{35}$S]-methionine. Over a 4 hour chase period, the cells were resuspended at 25 min intervals, at the end of which, the cells and supernatants were separated by centrifugation at 250 g for 5 mins. Cell-free supernatants were stored frozen at -20°C. Cell pellets were washed twice in PBS and resuspended with 5% (v/v) Nonidet P40 (NP40 from Sigma) in PBS and vortexed. Cellular debris was removed by centrifugation at 1600 g for 15 mins and the soluble fractions stored at -20°C. All media were prewarmed to 37°C before use. CsA (a gift from Dr. F. Pasutto, Department of Pharmacy, University of Alberta, Sandoz lot #OL 27-400N) was made up in DMSO at 1 mg $ml^{-1}$.

Radioactivity incorporation was determined by precipitation of proteins on 3 MM Watman filter discs in 5% (v/v) trichloroacetic acid (TCA); 10 min wash at 4°C, 10 min wash in boiling TCA and 10 min wash at 4°C. The filter discs were dried and counted in Scintimix from NEN.

## 2. *Treatment of Samples for Electrophoresis*

Cellular and secreted proteins labelled with L-[$^{35}$S]-methionine were precipitated by addition of five volumes reagent grade acetone at -20°C and left at -20°C for 15 mins. Precipitates were pelleted at 16,000 g for 15 mins and washed twice with,

-20°C acetone.

Protein pellets were resuspended in isoelectric focussing sample buffer (IEF-SB) consisting of 8 M urea (ultra pure from Canadian Scientific Products), 2.0% (v/v) NP 40, 2.0% (w/v), Ampholines (LKB), and 5% (v/v) $\beta$ME. Unless otherwise stated, the samples were loaded on to IEF gels immediately.

## C. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

### 1. *Equilibrated Isoelectric Focussing (IEF)*

Equilibrated IEF gels were of dimensions 2 mm x 10 cm tubes. The gels were prepared as described by O'Farrell (1975) containing 8 M urea, 4% (v/v) NP40, 4% (w/v) acrylamide (BIORAD), and 2% (w/v) ampholine (LKB). The 2% (w/v) ampholine in the gels consisted of either (a) 2% (w/v) pH 3.5 to 10.0, or (b) 0.7% (w/v) pH 3.5 to 10.0, 1.3% (w/v) pH 2.5 to 4.0 mixture resulting in gels with pH profiles ranging between pH 4.0 to 7.0 and pH 3.0 to 5.0 per 10 cm gel length, respectively.

Samples were loaded in 50 $\mu$l aliquots with 500,000 dpm per gel and overlayed with IEF-SB (see section B.2 of this chapter) diluted with an equal volume of purified water. In each experiment, 'blank gels' loaded with IEF-SB only were sliced into 1 cm strips after focussing. The ampholines were eluted from these fractions in 0.5 ml of 0.1 M KCl for 40 mins and the pH was determined.

### 2. *SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)*

Discontinuous slab SDS PAGE was performed according to the method described by Laemmli (1970) with the following modifications.

The stacking gel was of 4% (w/v) acrylamide and the separating gel was a gradient gel with a 7.5% (w/v) to 18.0% (w/v) acrylamide gradient as well as a

0.2% (w/v) to 3.75% (w/v) glycerol gradient : a final mixture of 0.375 M Tris-HCl pH 8.8 and 0.1% (w/v) SDS. Electrolyte for both electrodes was 0.05 M Tris-HCl pH 6.8, 0.375 M glycine and 0.1% (w/v) SDS. Final concentration of sample buffer (SDS-SB), apart from containing 5% (w/v) SDS and 15% (v/v) glycerol, was as described by Laemmli (1970)

The tube gels were loaded with the more acid end of each gel to the right hand edge of each slab gel after standing at room temperature for 15-20 mins in an SDS equilibrating buffer consisting of 10% (w/v) glycerol, 5% (v/v) $\beta$ME, 2.3% (w/v) SDS and 62.5 mM Tris-HCl pH 6.8. Equilibrated IEF gels with samples may be stored frozen at -20°C in this buffer. $^{14}C$-molecular weight markers (BRL) were loaded in the sample buffer (SDS-SB) described above.

Unless stated otherwise, gels were fixed in 50% (v/v) methanol, 10% (v/v) glacial acetic acid and permeated with NEN Enlightning for fluorography on Kodak-Eastman XAR-5 X-ray film for 12-24 hours.

3. *Silver Staining of Two Dimensional (2D) PAGE Gels*

The silver staining technique used was a modification of that described by Oakley *et al.* (1980).

The gels to be silver stained were fixed in 50% (v/v) methanol. Purified water was used for washing the gels and making up the solutions. The crucial step was the liberal washing of the gels with purified water at the stages indicated below, with constant agitation.

The gel was

(i) fixed in 50% (v/v) methanol for an hour,

(ii) was rehydrate and washed with 3 x 200 ml changes of water to remove methanol,

(iii) immersed in 10% (w/v) unbuffered glutaraldehyde (Fisher) for 30 mins to enhance silver staining,

(iv) washed extensively with water for at least 4 hours (or overnight) with a minimum of eight 300 ml changes,

(v) immersed in <u>ammoniacal silver reagent</u> (see below) in the dark for 15 mins,

(vi) washed extensively in water with five 200 ml changes for 20 mins,

(vii) developed with 0.005% (w/v) citric acid and 0.019% (w/v) formaldehyde to the required intensity.

The development of, the silver stain could be stopped by replacing the developer with 50% (v/v) methanol and 10% (v/v) glacial acetic acid. Gels could be stored indefinately in a sealed bag containing 50% (v/v) methanol.

<u>Ammoniacal Silver Reagent</u>: This was made up just before use by slowly adding 4 mls of 20% (w/v) silver nitrate to 1.4 mls of ammonium hydroxide (88) and 21 mls of 0.1 M NaOH with constant aggitation. It was then made up to 100 mls with purified water.

Gels could be destained with the following solution (freshly made up) if over-developed. Six mls of 0.2 M potassium ferricynide was added to 12 mls of 2 M sodium thiosulphate and made up to 200 mls with water. The destaining could be achieved by gently aggitating the gels in the solution either to completion or to the required intensity. The reaction could be stopped by washing extensively with

water or 50% (v/v) methanol. The gels could be restained from step (v) after washing off all the destaining solution, or after rehydration and washing, if 50% (v/v) methanol was used.

# CHAPTER III

# BIOSYNTHETIC LABELLING OF PROTEINS MADE BY EL4.E1 CELLS

## A. INTRODUCTION

The object of the investigations in this chapter was to determine the feasibility of studying the effects of PMA and CsA on the protein biosynthesis by EL4.E1 cells. PMA induces IL2 production in EL4.E1 cells. A host of lymphokines that are co-induced by PMA (chapter I, section C.1.) in EL4.E1 cells include CSF-GM, BCGF, TRF, TCF2, and IL2. As covered in Chapter I (section C), CsA appears to inhibit IL2 production and data supporting such an inhibition (Elliott *et al.*, 1984, manuscript submitted) suggest that a study on the effect of these agents (CsA and PMA) on biosynthesis of proteins may present clues to the specificity of their mode of action in this cell line.

The kinetic studies described in this chapter were designed to determine the optimal conditions required to stimulate EL4.E1 cells with PMA. Exprimental conditions designed to optimise biosynthetic labelling of these cells with L-[$^{35}$S]-methionine. A series of pilot experiments were designed to test the feasibility of labelling these cells in a pulse-chase experiment. A pulse-chase protocol was decided upon because a pulse labelling at a point when the cells are actively synthesising proteins on treatment with PMA and CsA, and a following chase, would allow these labelled proteins to be secreted. Thus it would be possible to study the final effect of the agents on these proteins at steady state by two dimensional gel analysis.

Experiments by Dr. B Caplan (Caplan 1982 -Ph.D Thesis) revealed that production of IL2 by EL4.E1 cells increased over the first twenty-four hours culture, reached a plateau at a high level after 24 hours and remained at t: level for as long as 72 hours. The maximal rate of IL2 production within the twenty-four hours was an indication of the cells actively responding to PMA stimulation, thus the experiments in this study were confined to this period.

EL4.E1 cells (chapter II, section A.3) were grown in 10% (v/v) FBS in cell growth medium (RHFM). In the presence of 10% (v/v) FBS in RHFM, cell-free supernatants contain 6 mg $ml^{-1}$ of proteins from the serum. A high concentration of serum proteins may interfere with the visualization of secreted proteins (made by EL4.E1 cells) on polyacrylamide gels. A study on the serum level requirement over the 24 hour period was thus necessary.

## B. RESULTS

### 1. *Subcloning of EL4.E1 Cells*

The clonal cell line EL4.E1 had been in culture for two years. It typically produced high titres of IL2 (Dr. C. Havele, unpublished data) when stimulated with PMA. To determine the stability of the cell line with respect to IL2 production, a subcloning experiment was carried out.

From 26 clones obtained in the 0.25 cell per well culture (Table 1), 12 were picked at random and maintained in culture. These were designated E1.1 to E1.12. Over a period of 3 months, the cultures were tested for levels of IL2 production several times. In response to PMA stimulation of these subclones, IL2 levels were comparable within experimental variation to the parental EL4.E1 cell line, as averaged over 4 experiments (Table 2).

TABLE 1

CLONING EFFICIENCY OF EL4.E1 CELLS

| Average Cells/Well | No. Wells | Number Positive | Proportion Negative | Efficiency |
|---|---|---|---|---|
| (N) | | | ($P_0$) | (E) |
| 1 | 96 | 70 | 0.27 | 1.3 |
| 0.5 | 96 | 41 | 0.57 | 1.1 |
| 0.25 | 96 | 26 | 0.73 | 1.3 |

Subcloning of EL4.E1 cells by serial dilution was performed as described in section A.2 of Mate ials and Methods. The cloning efficiency (E) was calculated from the proportion o negative wells ($P_0$) and the average number of cells expected per well (N) using i Poisson Distribution term, $P_0 = \exp(-EN)$

## TABLE 2

### IL2 PRODUCTION BY SUBCLONES OF EL4.E1 CELLS

| Clones | Average IL2 $ED_{30}$ $ml^{-1}$ ± SE (n = 4) |
|---|---|
| EL4.E1 | 2400 ± 566 |
| E1.1 | 2506 ± 583 |
| E1.2 | 2450 ± 212 |
| E1.3 | 2015 ± 545 |
| E1.4 | 1950 ± 636 |
| E1.5 | 2500 ± 707 |
| E1.6 | 2850 ± 200 |
| E1.7 | 2051 ± 707 |
| E1.8 | 2202 ± 586 |
| E1.9 | 2549 ± 614 |
| E1.10 | 2099 ± 246 |
| E1.11 | 2150 ± 369 |
| E1.12 | 1900 ± 453 |

Subclones of EL4.E1 cells (designated E1.1 to E1.12) were stimulated with PMA and the cell-free supernatants were harvested after 24 hours in culture as described in section A.4 of Materials and Methods. IL2 assays (chapter II, section A.5) were performed on the supernatants and the IL2 levels were compared with those of the parental EL4.E1 cell line.

2. *Kinetics of IL2 Production by EL4.E1 Cells*

Stimulation of EL4.E1 cells with PMA led to a rapid increase in IL2 levels after a 4 hour lag. The maximal rate of IL2 increase occured between 8 to 24 hours after PMA addition (Figure 1). Experiments involving production of IL2 in different FBS concentrations in RHFM revealed that, the levels of serum did not affect IL2 production dramatically (Figure 2), 2200 $ED_{50}$ units $ml^{-1}$ being produced in 10% (v/v) FBS and 1900 $ED_{50}$ units $ml^{-1}$ being produced in 1% (v/v) FBS. Removal of serum from the medium resulted in a decrease to 1100 $ED_{50}$ units $ml^{-1}$ in serum free medium.

To determine the requirement for PMA over a 24 hour period, a kinetic study (Table 3) was performed in which cells were cultured with or without PMA for various intervals. Removal of PMA after 12 hours resulted in approximately 50% reduction of IL2 levels compared to a secondary culture, containing PMA for a further 12 hour incubation period (Table 3 supernatants C1 and C2 respectively).

Cultures F and G also showed that when PMA was omitted from these secondary cultures after 16 hours in PMA, IL2 levels declined. Control cultures D1, D2, E1, and E2 showed that replating the cells for various intervals with or without PMA resulted in a decrease in IL2 levels whenever PMA was not included in the medium.

The most significant observation is that whenever, the cells were cultured in PMA-free medium for a period of time, a four hour lag in IL2 production occured upon replating those cells in PMA. These four hours could represent the half-life of the mRNA for IL2.

The results suggested that PMA is required in culture throughout the 24 hour incubation period to maintain a high titre of IL2 in the supernatant.

FIGURE 1

PRODUCTION OF IL2 BY PMA STIMULATED EL4.E1

CELLS IN 10% FBS (FOETAL BOVINE SERUM)

Cell free supernatants from a 24 hour PMA stimulation of EL4.E1 cells were harvested (chapter II,section A.4) at various time intervals. IL2 assays as described in section A.5 of Materials and Methods were carried out at a 1:100 dilution of the cell free supernatants. IL2 levels were expressed as the percentage maximal proliferation response to a saturating level of a crude IL2 preparation in the same assay. The figure shows the data from two separate experiments (△-△, ●-●) and a control with unstimulated cells (■-■).

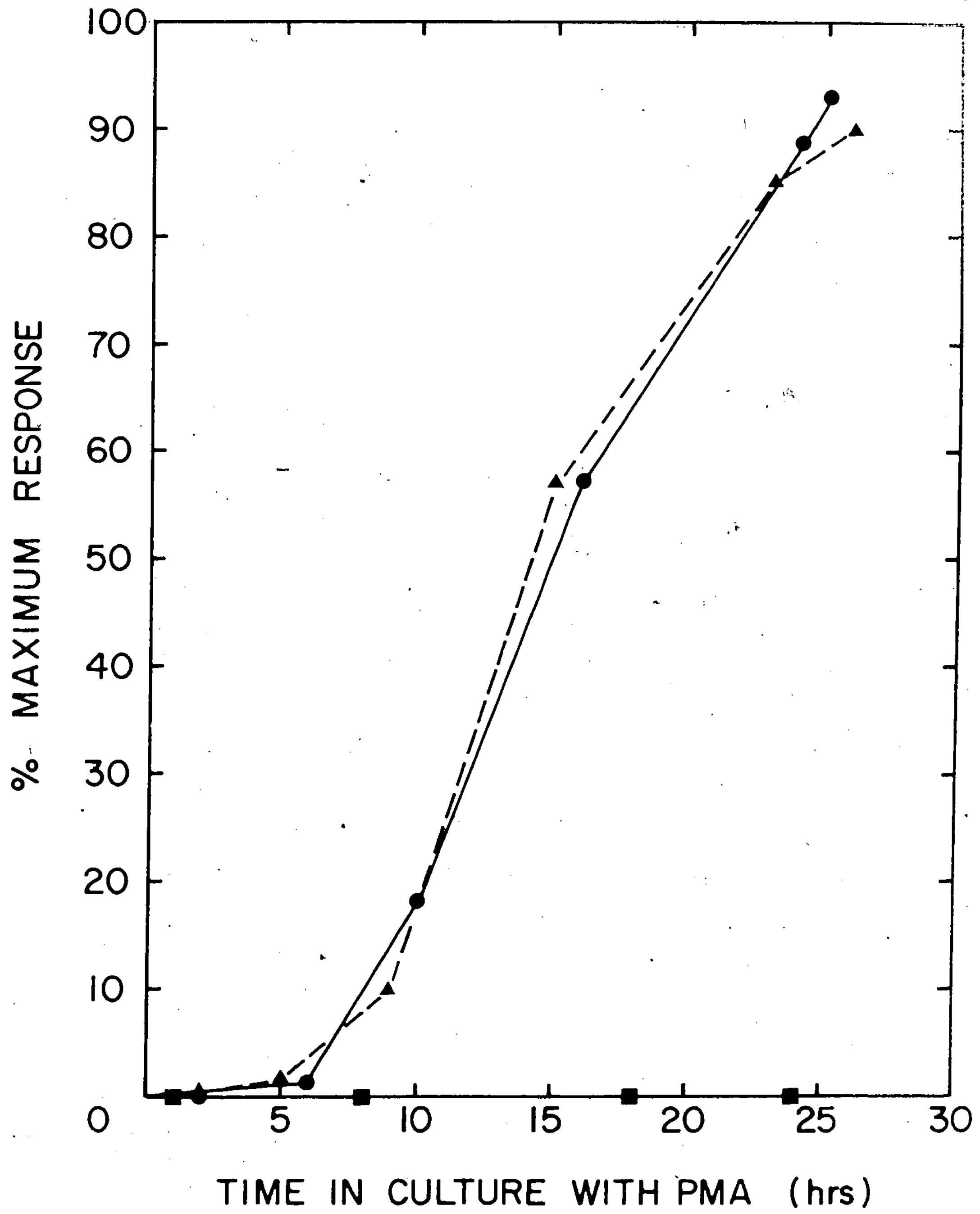
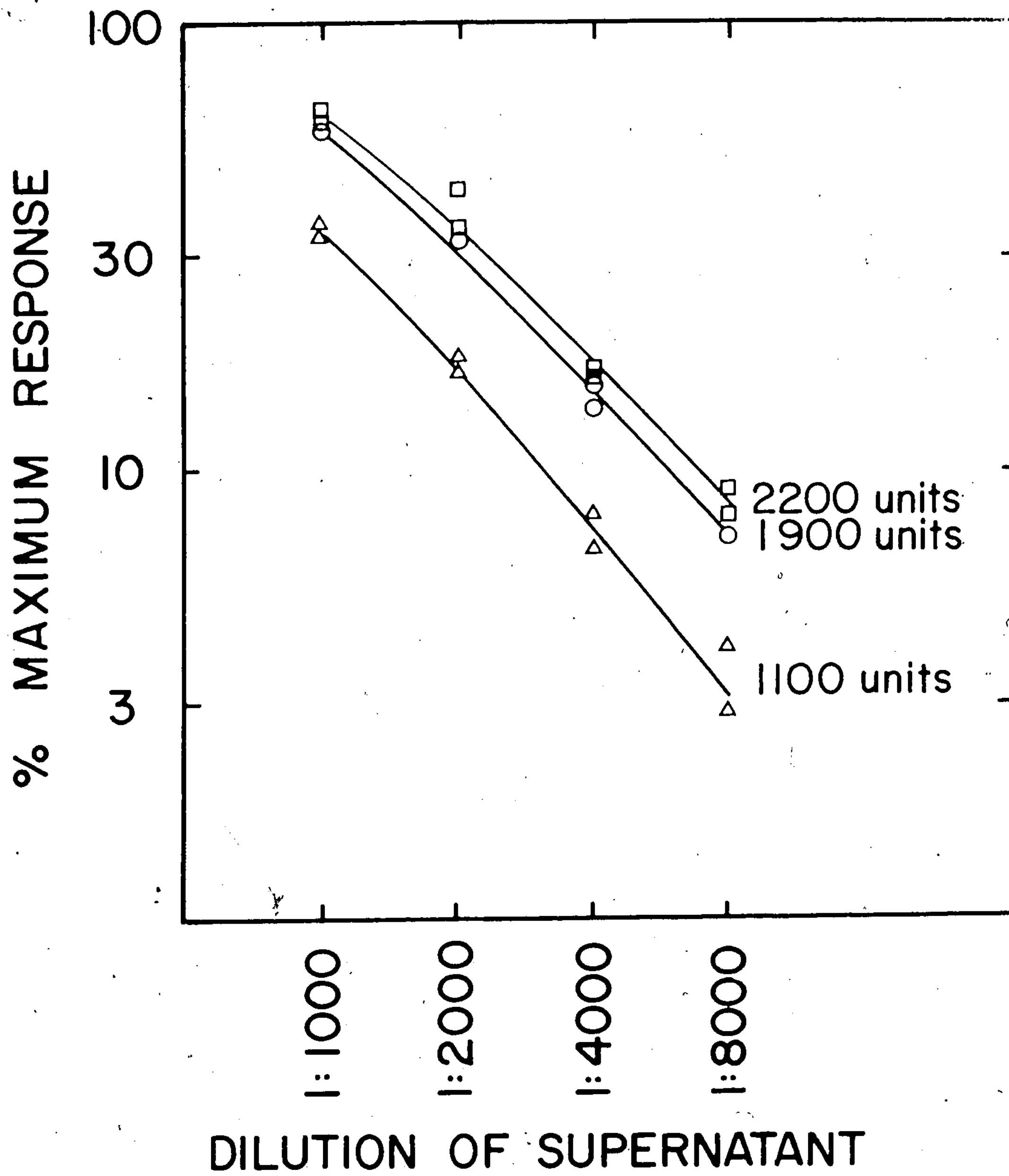

% MAXIMUM RESPONSE
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
TIME IN CULTURE WITH PMA (hrs)

FIGURE 2

IL2 PRODUCTION BY PMA STIMULATED EL4.El CELLS

IN VARYING CONCENTRATIONS OF SERUM IN THE MEDIUM.

IL2 production by EL4.El cells in varying levels of FBS, 10% ( ) 1% (o) and serum free (Δ) medium. PMA stimulation over a 24 hour period generated IL2 as described in section A.4 of Materials and Methods. The cell free supernatants from 24 hour cultures were assayed for IL2 activity by serial dilutions in RHFM.


100
30
10
3
% MAXIMUM RESPONSE
2200 units
1900 units
1100 units
1:1000
1:2000
1:4000
1:8000
DILUTION OF SUPERNATANT

## TABLE 3

## EFFECTS OF REMOVAL OF PMA AT VARIOUS INTERVALS OVER A 24 HR PERIOD ON IL2 PRODUCTION

| | | Cultures | IL2 $ED_{50}$ units $ml^{-1}$ |
|---|---|---|---|
| A. | | $10^6$ EL4.E1 cells $ml^{-1}$ in 20 ng $ml^{-1}$ PMA x 24 hrs | 2010 |
| B. | | $10^6$ EL4.E1 cells $ml^{-1}$ in 20 ng $ml^{-1}$ PMA x 12 hrs | 1062 |
| C. | | $10^6$ cells $ml^{-1}$ from B in | |
| | 1. | Fresh medium + 20 ng $ml^{-1}$ PMA x 12 hrs | 850 |
| | 2. | Fresh medium only x 12 hrs | 450 |
| | 3. | Fresh medium + 20 ng $ml^{-1}$ PMA x 4 hrs | 375 |
| | 4. | Fresh medium only x 4 hrs | 312 |
| D. | | $10^6$ cells $ml^{-1}$ from C3 in | |
| | 1. | Fresh medium + 20 ng $ml^{-1}$ PMA x 8 hrs | 500 |
| | 2. | Fresh medium only x 8 hrs | 225 |
| E. | | $10^6$ cells $ml^{-1}$ from C4 in | |
| | 1. | Fresh medium + 20 ng $ml^{-1}$ PMA x 8 hrs | 470 |
| | 2. | Fresh medium only x 8 hrs | 75 |
| F. | | $10^6$ EL4.E1 cells $ml^{-1}$ in 20 ng $ml^{-1}$ PMA x 16 hrs | 1450 |
| G. | | $10^6$ cells from F in | |
| | 1. | Fresh medium + 20 ng $ml^{-1}$ PMA x 8 hrs | 463 |
| | 2. | Fresh medium only x 8 hrs | 250 |
| H. | | $10^6$ EL4.E1 $ml^{-1}$ in medium only x 24 hrs | 0 |

3. *Effects of Cyclosporin A (CsA) on IL2 Production*

CsA had been shown to inhibit IL2 production in lymphocytes (Bunges *et al.*, 1981; Britton *et al.*, 1982; Elliott *et al.*, 1984, manuscript submitted). Observations of Dr. J. Elliott (personal communication, U. of A.) demonstrated that 50 ng $ml^{-1}$ CsA produced maximal (99%) inhibition of IL2 production by EL4.E1 cells. Cells pretreated with PMA were also inhibited from producing IL2 when replated in medium containing PMA in the presence of CsA.

4. *Survival of EL4.E1 Cells after a High Cell Density Pulse ($10^7$ cells $ml^{-1}$) in Low Methionine Concentration*

For reason of economy, the biosynthetic labelling of proteins was carried out in a small volume at high cell density ($10^7$ cells $ml^{-1}$). An arbitrary pulse time of 45 mins was decided upon. It was necessary to determine the status of these cells after such a treatment.

EL4.E1 cells at a density of $10^7$ cells $ml^{-1}$ were cultured in 0.1 nM L-methionine made up in RPMI 1640, 1% (v/v) FBS, $10^{-4}$ $\beta$ME, 20 ng $ml^{-1}$ PMSF, 5% $CO_2$ in air, 37°C, 100% humidity for 45 mins. The high cell density pulse condition was terminated by addition of an equal volume of chase medium as described in Materials and Methods (section B.1). The number of dead cells was determined with eosin staining and scored with a hemacytometer. The results showed that 90% of the cells remained viable up to 6 hours after the high cell density pulse indicating that the pulse-chase experimental conditions were acceptable.

5. *Production of IL2 after Incubation of Cells at High Density*

To determine if subjecting EL4.E1 cells to what may be unfavourable conditions affected their biological function, IL2 production in response to PMA stimulation was assesed. After subjecting the cells to the pulse-labelling conditions described in chapter III, section B.4, the IL2 levels in the supernatants were assayed at various times after termination of pulse-labelling conditions. Figure 3 illustrates that IL2 production increased with incubation time after termination of a 45 min pulse, indicating that the cells were not adversely affected by the conditions of the pulse-chase experiment.

6. *Incorporation of L-[$^{35}$S]-Methionine into EL4.E1 Cells Over Various Pulse Labelling Intervals*

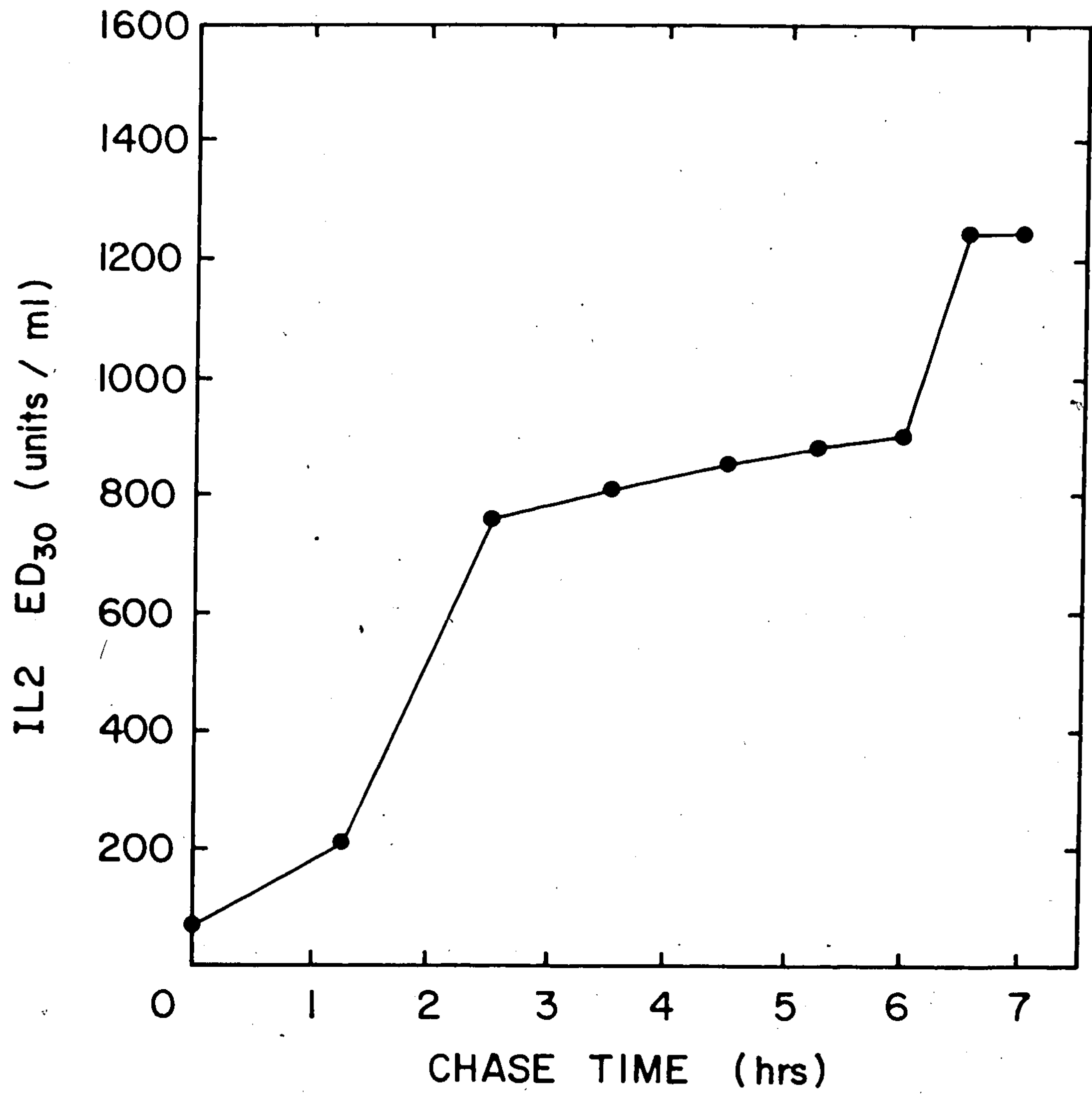
On establishing that the conditions proposed for pulse labelling were not adverse to the cells, the time interval for optimal uptake of the label was be determined.

In the experiment of Figure 4, samples were taken at 10 mins intervals after initiation of pulse-labelling conditions determined previously and the incorporation of radioactivity into proteins in the total reaction was determined. Figure 4 illustrates that $^{35}$S incorporation into high molecular weight material increased between 0 to 45 mins of incubation in both unstimulated and PMA-stimulated cultures. Incorporation of label (30%) in both cultures reached a plateau after about 45 mins. At this point there may be a number of cellular events involving the degradation and recycling of proteins and amino acids. A pulse-time of 35 mins was therefore decided upon.

## FIGURE 3

### KINETICS OF IL2 PRODUCTION BY 5 x $10^6$ EL4.E1 CELLS $ML^{-1}$ AFTER 45 MINUTES AT $10^7$ CELLS $ML^{-1}$

Aliquots of cell-free supernatants were harvested at various time intervals and assayed for IL2 activity as described in Materials and Methods (sections A.4 and A.5). IL2 levels are expressed as $ED_{30}$ units $ml^{-1}$.



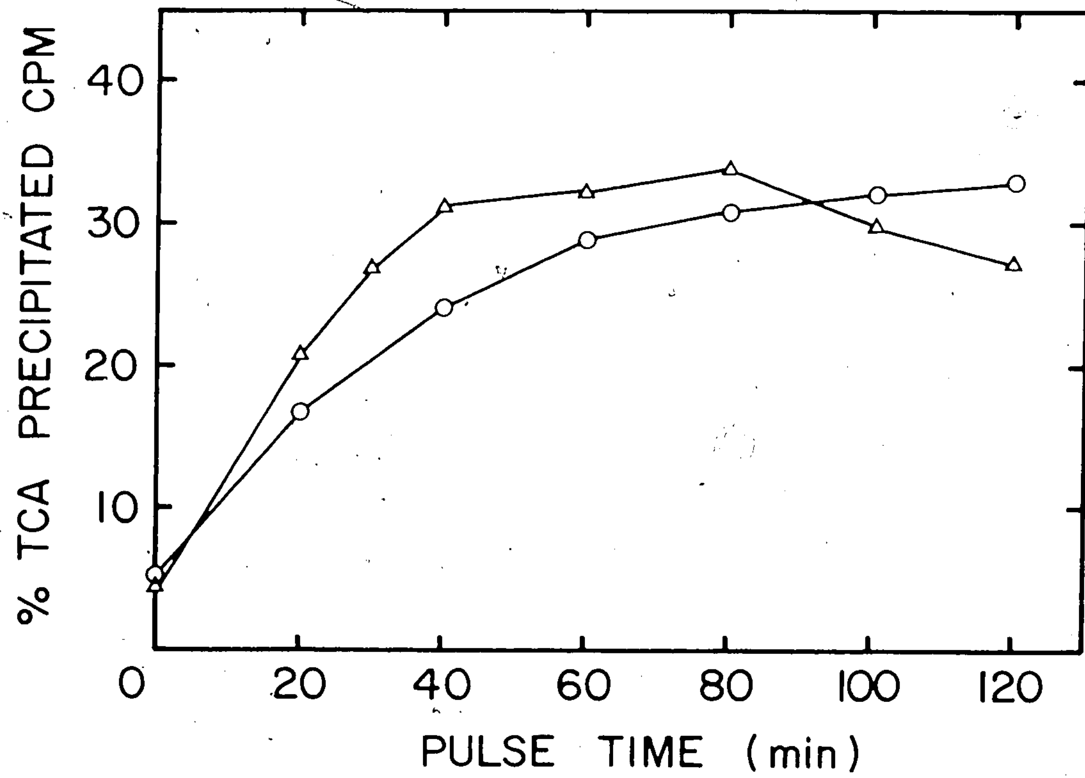
1600
1400
1200
1000
800
600
400
200
IL2 ED30 (units / ml)
0
1
2
3
4
5
6
7
CHASE TIME (hrs)

## FIGURE 4

## L-[$^{35}$S]-METHIONINE INCORPORATION INTO EL4.E1 CELLS OVER VARIOUS PULSE INTERVALS

EL4.E1 cells at $10^7$ $ml^{-1}$ were incubated with 200 $\mu$Ci $ml^{-1}$ L-[$^{35}$S]-methionine and cultured under conditions described in Materials and Methods (B.1). At various time points, samples were taken and label incorporation into macromolecules was determined as described in Materials and Methods (B.1) after each sample was made 5% (w/v) SDS and vortexed.

Results from two cultures, PMA-stimulated (-o-) and unstimulated (-Δ-), are shown in the figure. The data points represent an average of two experiments for each culture. The vertical axis represents the percent TCA precipitable cpm of the total cpm in each reaction.


% TCA PRECIPITATED CPM
40
30
20
10
0
20
40
60
80
100
120
PULSE TIME (min)

### 7. *Label Incorporation at Various L-[$^{35}$S]-Methionine Concentrations*

The specific activity of L-[$^{35}$S]-methionine labelled protein was increased to minimize the amount of time required for fluorography. In a 35 min pulse labelling experiment under the conditions previously described (Material and Methods, section B.1), the concentration of label was increased and the incorporation of label was monitored to determine if high concentrations of label adversely affected its uptake.

In Figure 5 it may be observed that both PMA-stimulated cultures and unstimulated cultures showed the same proportion of label incorporation (as demonstrated by TCA precipitable cpm) as the label concentration was increased. With a label concentration of 800 $\mu$Ci ml$^{-1}$, labelled proteins of very high specific activity (20-30 $\mu$Ci ug$^{-1}$ protein) were obtained.

### 8. *Distribution of Label in a Pulse-Chase Labelling of Proteins Made by EL4.E1 cells*

Table 4 shows the distribution of TCA precipitable label in two compartments: cellular material and cell-free supernatant. Ninety percent of the total incorporated label was found in the cellular compartment and 6% in the cell-supernatant regardless of whether the cells were stimulated or not.

## C. DISCUSSION

The results presented here demonstrate that the cell line was stable after two years of culture under the conditions specified in chapter II, section A.2 with respect to IL2 production in response to PMA stimulation. This was shown by the various subclones of EL4.E1 cells exhibiting comparable levels of IL2 in the supernatants. The cells respond to PMA induction by producing IL2 over a twenty-four hour period after a four hour lag period. Maximal rate of IL2 production

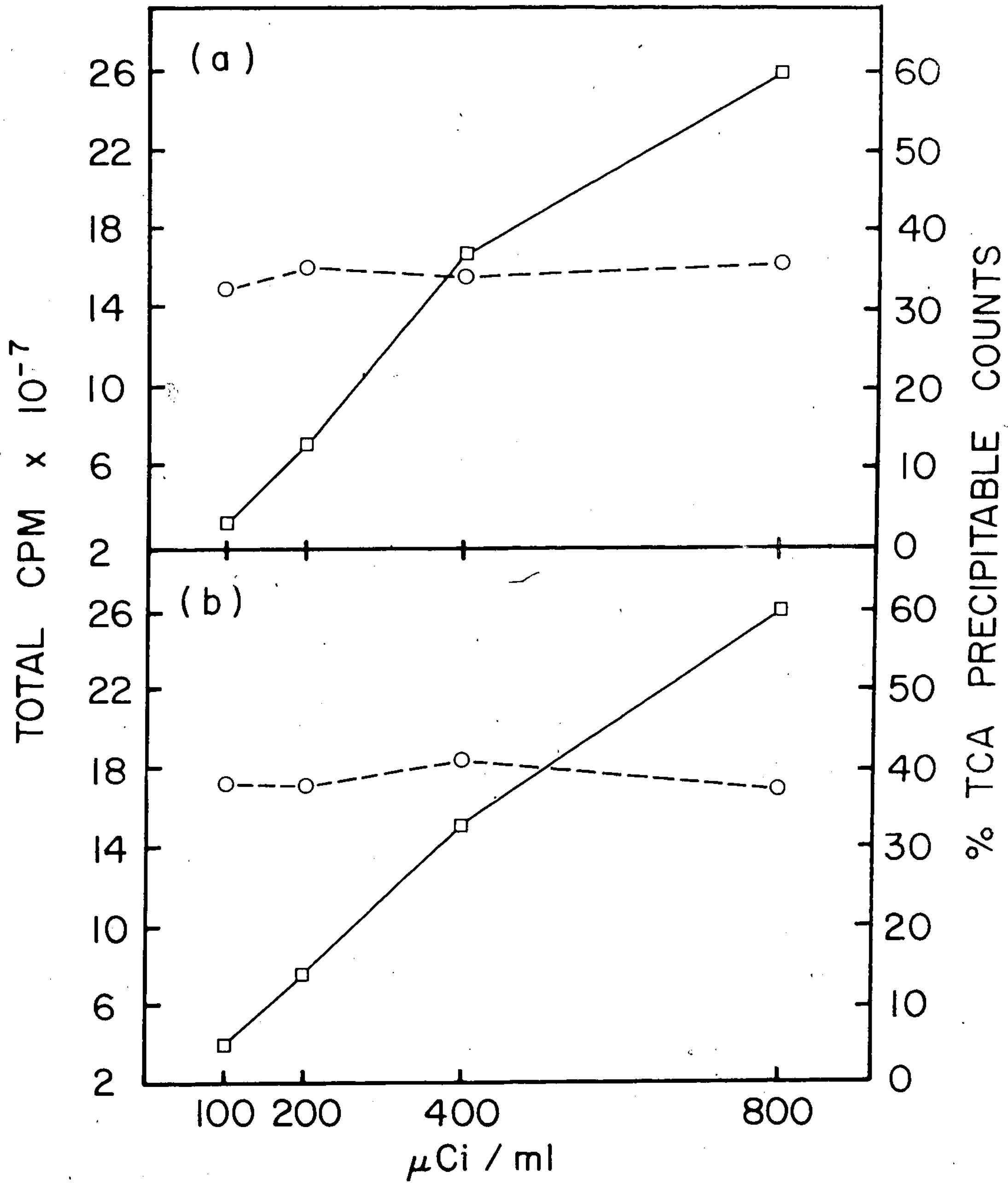
## FIGURE 5

## L-[$^{35}$S]-METHIONINE INCORPORATION AT VARIOUS CONCENTRATIONS OF LABEL BY EL4.E1 CELLS IN A 35 MIN PULSE

EL4.E1 cells at a density of $10^7$ cells $ml^{-1}$ were incubated under the condition described in Materials and Methods (section B.1) with increasing concentration of L-[$^{35}$S]-methionine from 100 $\mu$Ci $ml^{-1}$ to 800 $\mu$Ci $ml^{-1}$.

After a 35 mins pulse the samples were made 5% (w/v) SDS and vortexed. TCA precipitation of macromolecules was carried out as described in Materials and Methods, section B.1 and the incorporation of label was determined. The experiment was done with both PMA-stimulated cultures (Figure 5a), and with unstimulated cultures (Figure 5b). Each point in the figure represents an average of two separate experiments.

The total cpm $ml^{-1}$ in the reaction ( -- ), is represented on the left vertical axis and the percent TCA precipitable cpm of the total cpm in the reaction (o--o) on the right vertical axis.


(a)
(b)
26
22
18
14
10
6
2
60
50
40
30
20
10
0
TOTAL CPM x 10^-7
% TCA PRECIPITABLE COUNTS
100 200 400 800
μCi / ml

occurred after about 12 hours incubation with 20 ng $ml^{-1}$ PMA. Since the levels of IL2 production doubled between 0 and 1%, and then remained constant regardless of the serum concentration, 1% (v/v) FBS was used in subsequent experiments. This enabled the loading of secreted proteins made by EL4.E1 cells on a gel without interference from serum proteins. PMA appeared to be required continuously over a 24 hour period to maintain a high level of IL2 in the supernatant. In further experiments, PMA was always included in the growth medium of PMA-treated cells.

The experiments designed to determine the optimal conditions for biosynthetic labelling with L-[$^{35}$S]-methionine for analysis by PAGE resulted in the following parameters being decided upon: 800 $\mu$Ci $ml^{-1}$ L-[$^{35}$S] methionine, $10^7$ cells $ml^{-1}$, and 1% (v/v) FBS, a pulse-labelling period of 35 mins and a chase of 4 hours.

TABLE 4

DISTRIBUTION OF L-[$^{35}$S] METHIONINE INCORPORATED BY EL4.E1 CELLS

| Chase time (hrs) | % TCA Precipitable cpm | | $ED_{50}$ units $ml^{-1}$ IL2 | |
|---|---|---|---|---|
| | Cells ± SE | Supernatant ± SE | +PMA | -PMA |
| 0 | 26.2 ± 0.4 | 1.0 ± 0.5 | 80 | 0 |
| 1 | 26.3 ± 0.2 | 1.4 ± 0.2 | 150 | 0 |
| 2 | 26.8 ± 0.1 | 1.8 ± 0.4 | 400 | 0 |
| 3 | 27.4 ± 0.4 | 2.0 ± 0.5 | 675 | 0 |
| 4 | 28.0 ± 0.5 | 2.0 ± 0.3 | 800 | 0 |

Pulse-chase labelling of EL4.E1 cells 200 $\mu$Ci $ml^{-1}$ of L-[$^{35}$S]-methionine was done as elaborated in section B.1 of Materials and Methods. Hourly samples were taken during the chase period and the TCA precipitable cpm in each compartment were determined in as described chapter II, section B.1. Supernatants were also tested for IL2 activity as described (chapter II, section A.5). The percent TCA precipitable cpm was calculated from the proportion of TCA precipitable cpm from the total cpm in each reaction. The average total incorporation of label was 30.1 ± 0.4% (n = 4).

# CHAPTER IV

# TWO DIMENSIONAL GEL ANALYSIS OF BIOSYNTHETICALLY LABELLED PROTEINS MADE BY EL4.E1 CELLS

## A. INTRODUCTION

Two dimensional polyacrylamide gel electrophoresis (2D PAGE) involves isoelectric focussing (IEF) of protein samples in one dimension and SDS PAGE in the second dimension (O'Farrell, 1975). The advantage of 2D PAGE analysis over either of the above techniques alone is the higher resolution well as the ability to predict the molecular weight and the isoelectric point (pI) of a protein. This method was used analysis of the proteins from EL4.E1 cells biosynthetically labelled under the following conditions; (a) unstimulated cells, (b) CsA-treated cells, (c) PMA-treated cells and (d) PMA and CsA-treated cells. It was hoped that this particular analysis would reveal the identities of the lymphokines, IL2, IL3, CSF-GM, BCGF, EL-TRF, and TCF2 discussed in Chapter I, section C.1. As some of the pI's of these lymphokines were known, it was necessary to establish a pH range in IEF gels in order to encompass these molecules. Using this method, the chemical perturbation of the cells by PMA and CsA with respect to protein biosynthesis in general, and IL2 in particular, could be observed. This would reveal if the effects of PMA and CsA involve a major reorganization of cellular functions or a more specific event involving few changes within the cell.

Such a study could reveal some general trends on the induction of specialised functions by differentiated cells. T-lymphocytes secrete lymphokines upon receiving an inducing signal. PMA in this context could be analogous to normal immunological signals.

## B. RESULTS

### 1. *Isoelectric Focussing and 2-Dimensional Gel Electrophoresis of HPLC-purified IL2*

Figure 6 demonstrates the pH profile of both IEF gel systems used. Between different sets of experiments, the pH profile were reproducible with these equilibrated gels and hence the pI's of proteins equilibrated on these IEF gels could be predicted with relative confidence.

HPLC purified IL2 (a gift from Dr. Riendeau, Department of Biochemistry, University of Alberta) was subjected to 2D PAGE and the gel was silver stained. In Figure 7, HPLC purified IL2 from EL4 supernatant has a molecular weight of 21.5-21.8K with charge heterogeneity ranging between pI 3.90 and pI 4.50. Three species are evident, with molecular weights and pI's (from right to left) a) 21.8K, pI 3.95, (b) 21.8K, pI 4.30, (c) 21.5K, pI 4.48. Charge heterogeneity confered by different degrees of glycosylation may explain the shift in molecular weight and pI.

An artifact of staining can be seen in the region of the 68K molecular weight marker.

### 2. *Pulse-Chase Labelling and 2D PAGE of Proteins made by EL4.E1 cells*

(a) <u>IL2 Production by EL4.E1 cells in a Pulse-Chase Labelling Experiment</u>

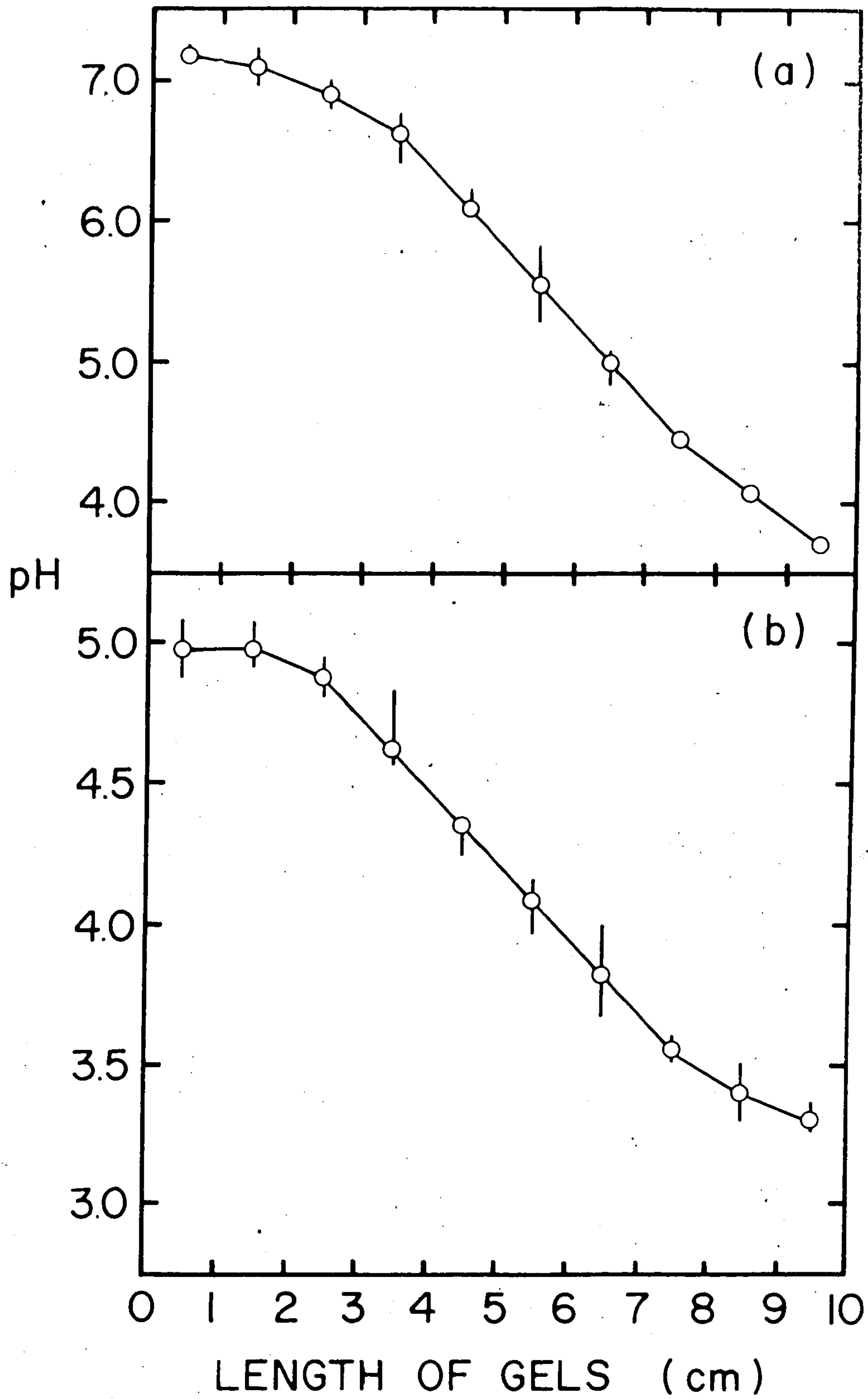
The results of Table 5 demonstrates that CsA inhibited the production of IL2 by PMA-stimulated EL4.E1 cells. At 50 ng $ml^{-1}$, CsA inhibited IL2 production by 98% after 12 hours.

After a pulse labelling of the cells, the supernatants from a 4 hours chase showed that elaboration of IL2 by EL4.E1 cells stimulated with PMA was 99%

FIGURE 6

pH PROFILE OF IEF GELS

IEF was performed as described in section C.1 of Materials and Methods. Two gel systems were used resulting in gels with pH profiles ranging between (a) pH 4.0-7.0 and (b) pH 3.0-5.0. The focussed gels were sliced into 1 cm strips as described in chapter II, section, C.1 and the pH of the eluted ampholines determined. The vertical line at each data point on the graphs represents the range of pH observed in six experiments and each data point represents the mean in six experiments.


(a)
(b)
pH
7.0
6.0
5.0
4.0
5.0
4.5
4.0
3.5
3.0
0 1 2 3 4 5 6 7 8 9 10
LENGTH OF GELS (cm)

## FIGURE 7

2D PAGE OF HPLC PURIFIED IL2 FROM EL4.E1 CELLS

2D PAGE of HPLC purified IL2 was performed as described in chapter II, sections C.1 and C.2. The sample in 41% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, was made 1% (w/v) SDS, dried down under vacuum and resuspended in IEF-SB (chapter II, section B.2).

Silver staining of the gel (chapter II, section C.3) is a modification of the procedure described by Oakley *et al.* (1980).

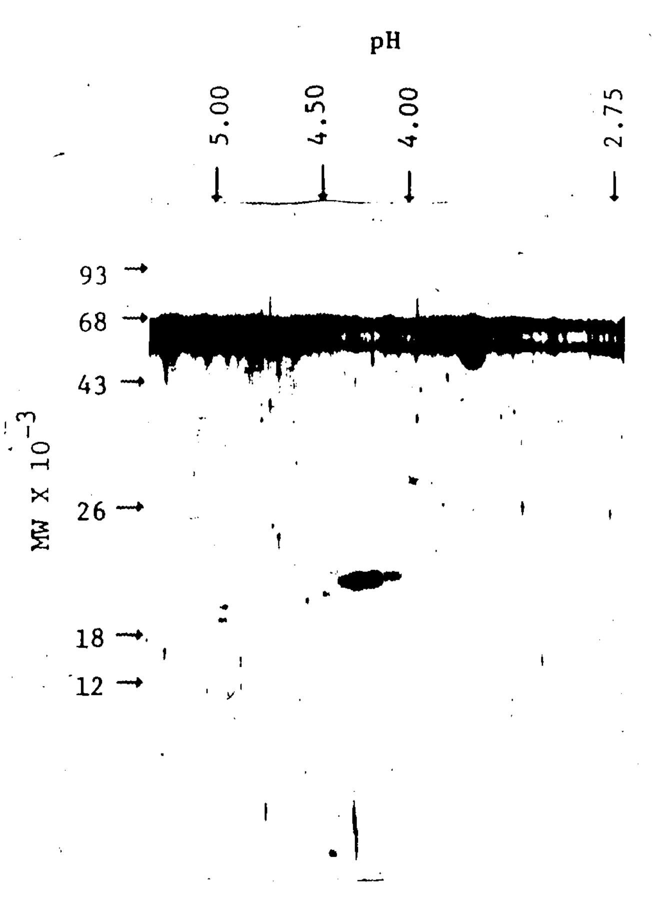

pH
5.00
4.50
4.00
2.75
93
68
43
26
18
12
MW X 10-3

TABLE 5

IL2 ASSAY ON SUPERNATANTS FROM EL4.E1 CELLS

IN A PULSE CHASE EXPERIMENT

| Supernatant Samples | IL2 $ED_{50}$ units $ml^{-1}$ |
|---|---|
| 12 hours after initiation of cultures | |
| RHFM control | 0 |
| Untreated cells | 0 |
| CsA-treated cells | 0 |
| PMA-treated cells | 740 |
| PMA and CsA-treated cells | 14 |
| 4 hours after pulse labelling of cultures | |
| Untreated cells | 0 |
| CsA-treated cells | 0 |
| PMA-treated cels | 750 |
| PMA and CsA-treated cells | 5 |

Supernatants were harvested 12 hours after initiation of cultures and stored at -20°C. The cells were washed and pulsed with L-[$^{35}$S]-methionine as described in Materials and Methods (section B.1). After a 4 hour chase, supernatant samples were harvested as described in Materials and Methods (section A.4).

inhibited by CsA.

(b) <u>Proteins made by EL4.E1 cells under the influence of PMA and/or CsA</u>

The effects of PMA and CsA on the expression of proteins by EL4.E1 cells were studied by 2D PAGE. PMA and CsA present concurrently or separately results in changes in the two dimensional profile of proteins made by EL4.E1 cells. Cells were incubated under the following conditions and both secreted and intracellular proteins analysed:

(1) RHFM only (Figures 8A, 9E and 10I)

(2) 50 ng $ml^{-1}$ of CsA (Figures 8B, 9F and 10J)

(3) 20 ng $ml^{-1}$ of PMA (Figures 8C, 9G and 10K)

(4) 20 ng $ml^{-1}$ of PMA and 50 ng $ml^{-1}$ of CsA (Figures 8D, 9H and 10L)

The mRNAs from EL4.E1 cultured under conditions 1, 3 and 4 were also isolated and the cell-free translated products were analysed by 2D PAGE (Figures 11M, 11N and 11O). These profiles were reproducible in three separate experiments. The proteins of interest are identified on each plate, for example, S17, C2 or t3. The secreted proteins (S), cellular proteins (C) and proteins translated from mRNA *in vitro* (t) in various experiments were compared to determine their relative differences under each condition described above.

As an indication of the reliability of the molecular weights and pI on these gels, the molecular weight and pI of cellular actin (a major protein of mammalian cells) was determined. On these gels, actin occured in two forms with a molecular weight of 44,500 and pIs of 5.4 ± 0.02 and 5.7 ± 0.02. This agreed with published data on the molecular characteristics of cellular actin (Garrels and Gibson, 1976). The pI 5.4 species was the most intensely labelled form.

## FIGURE 8

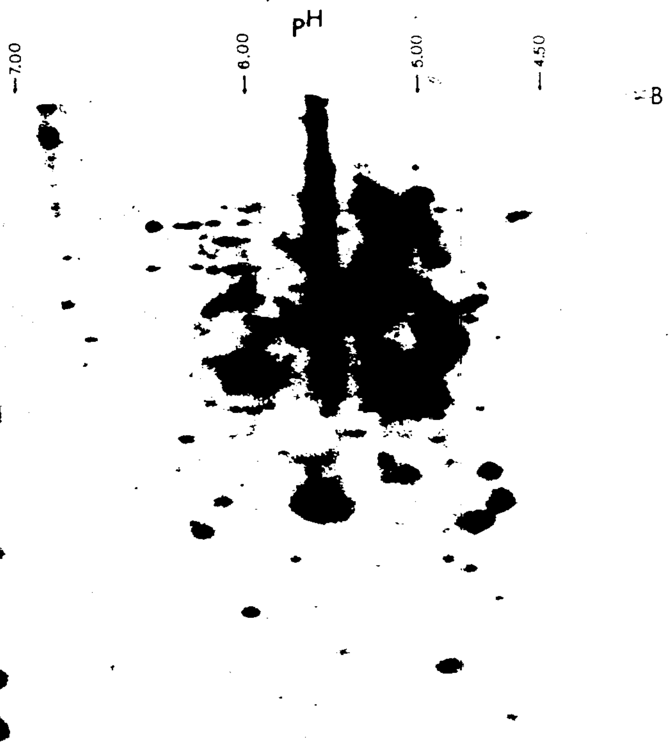
## 2D PAGE OF SECRETED PROTEINS MADE BY EL4.E1 CELLS

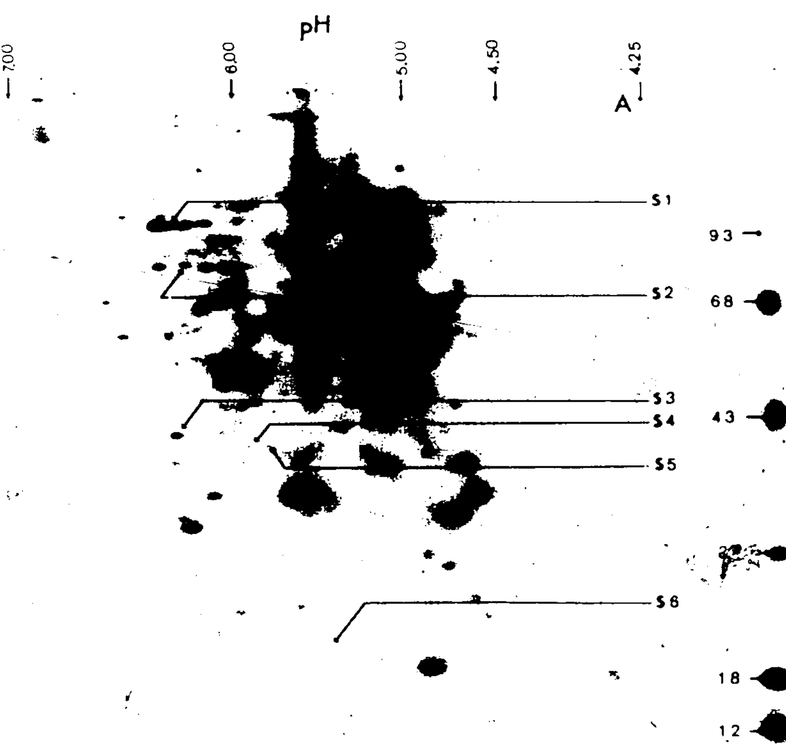
## (MIDDLE pH RANGE)

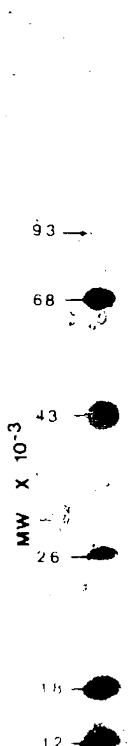
L-[$^{35}$S]-methionine labelled proteins from cell-free supernatants were prepared as described in Materials and Methods (section B.1 and B.2). IEF gels contained 2% (w/v) ampholine pH 3.5-10.0 (chapter II, section C.1) resulting in gels with pH ranging between 4.00 and 7.00 (Figure 8A). 2D PAGE was performed as described in Materials and Methods (section C.1 and C.2).

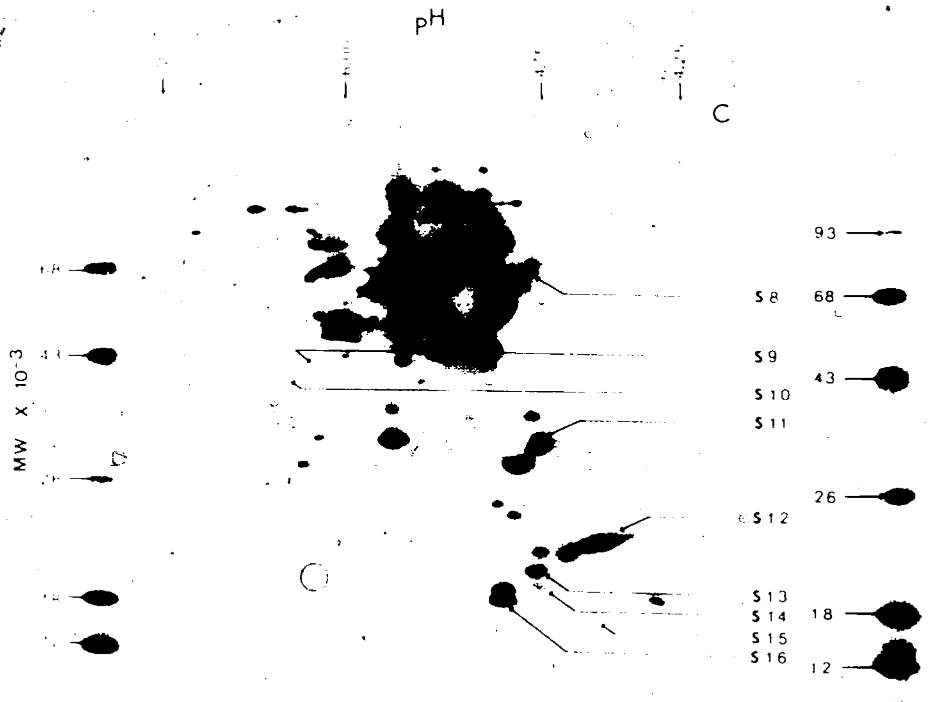
The fluorograms of these gels represent the 2D PAGE profiles of secreted proteins from the cell-free supernatants of

(A) Untreated EL4.E1 cells.
(B) CsA-treated EL4.E1 cells.
(C) PMA-treated EL4.E1 cells.
(D) PMA and CsA-treated EL4.E1 cells.

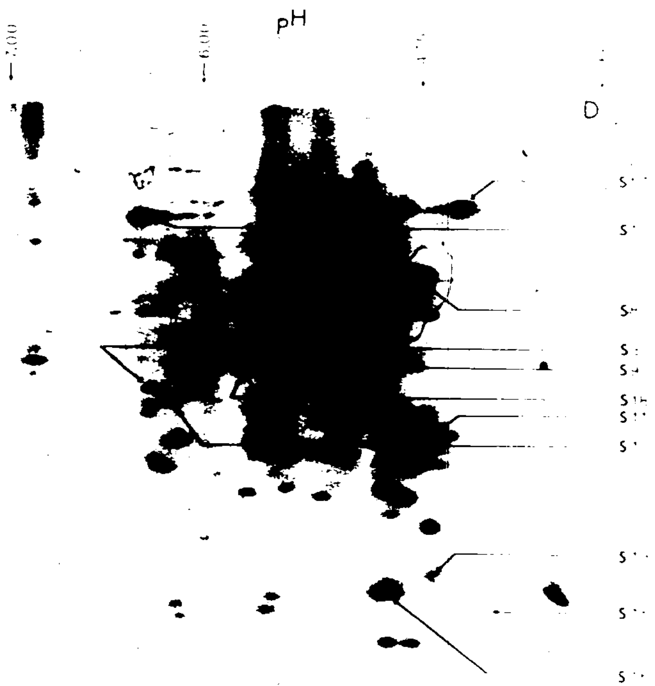

pH
7.00
6.00
5.00
4.50
B
93
68
43
18
12


pH
7.00
6.00
5.00
4.50
A
4.25
S1
S2
S3
S4
S5
S6


93
68
43
26
12
MW X 10-3



MW X 10-3
pH
C
S8
S9
S10
S11
S12
S13
S14
S15
S16
93
68
43
26
18
12

7.00
6.00
pH
D

FIGURE 9

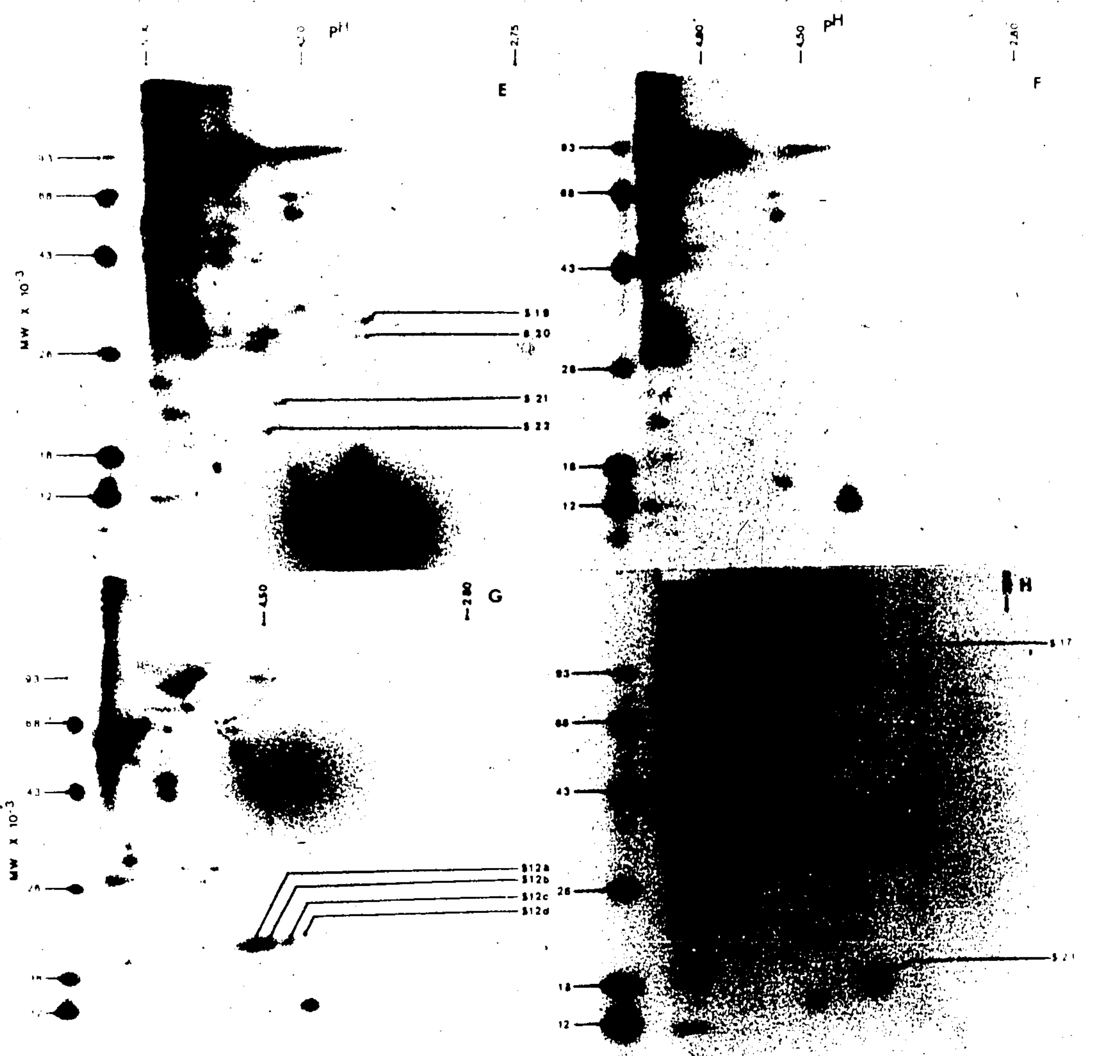
2D PAGE OF SECRETED PROTEINS MADE BY EL4.E1 CELLS

(LOW pH RANGE)

L-[$^{35}$S]-methionine labelling of secreted proteins in the cell- free supernatants of EL4.E1 cells was performed (chapter II, section B.1) and prepared for 2D PAGE (chapter II, section B.2). The IEF gels contained a 0.7% (w/v) ampholine, pH 3.5 to 10.0 and 1.3% (w/v) ampholine, pH 2.5 to 4.0 mixture resulting in gels with pH ranging between pH 2.75 and 5.00 (Figure 9E). 2D PAGE was performed as described in sections C.1 and C.2 of Materials and Methods.

The fluorograms of these gels represent the 2D PAGE profiles of secreted proteins from the cell-free supernatants of

(E) Untreated EL4.E1 cells.
(F) CsA-treated EL4.E1 cells.
(G) PMA-treated EL4.E1 cells.
(H) PMA + CsA-treated EL4.E1 cells.



E
F
G
H
pH
2.75
4.80
4.50
2.80
MW x 10-3
93
68
43
26
18
12
S 19
S 20
S 21
S 22
S12a
S12b
S12c
S12d
S 17

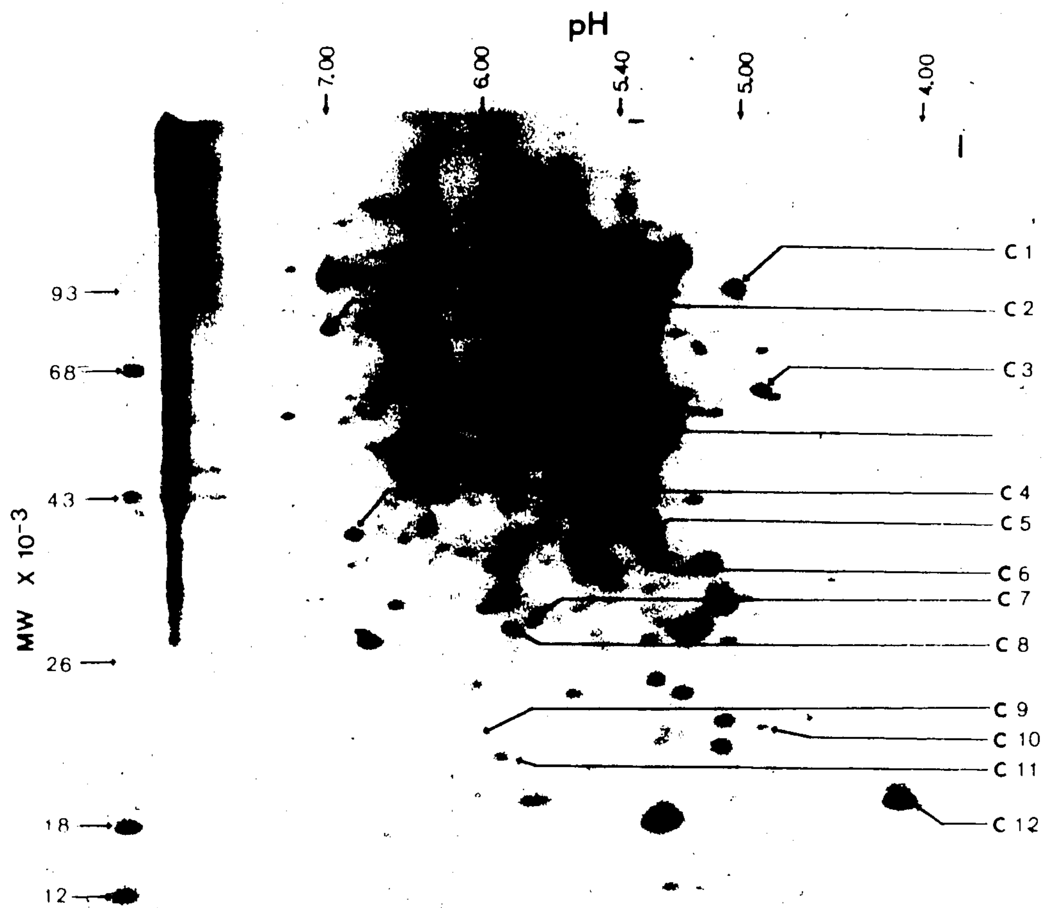
## FIGURE 10

### 2D PAGE OF THE CELLULAR PROTEINS MADE BY EL4.E1 CELLS

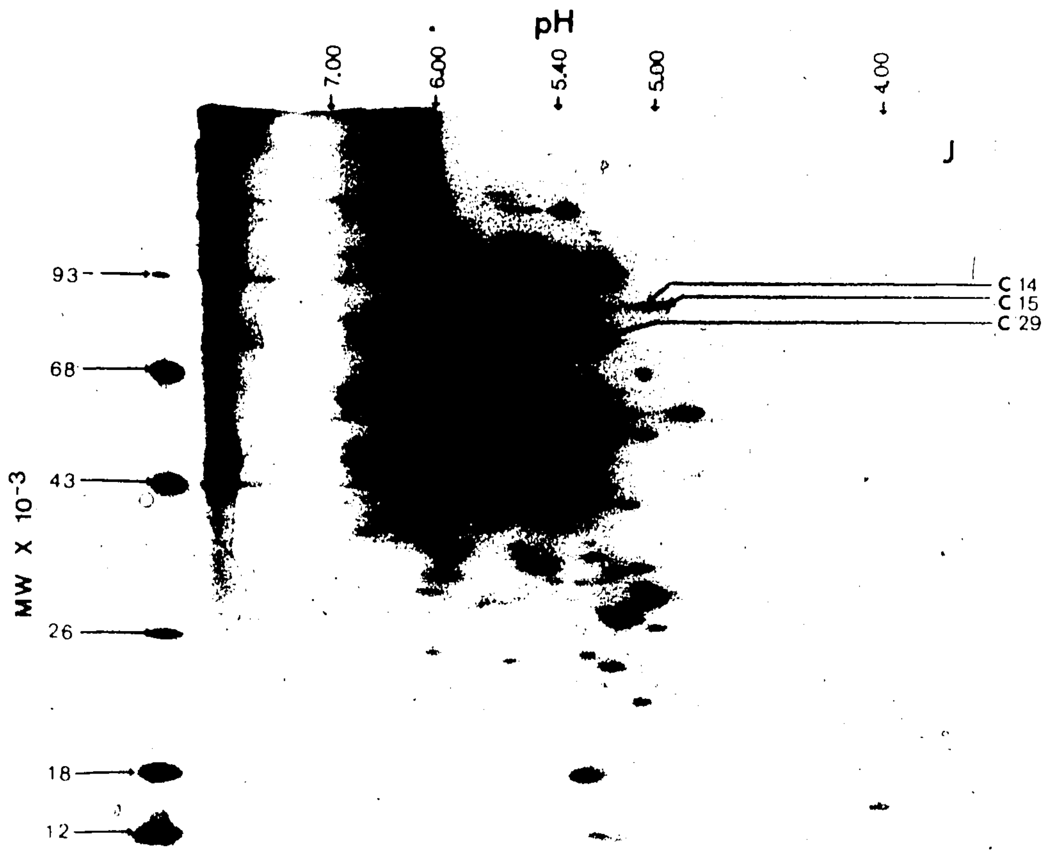
L-[$^{35}$S]-methionine labelled cells (section B.1 of Materials and Methods) were prepared for 2D PAGE as described in Materials and Methods (sections B.2, C.1 and C.2). IEF gels contained 2% (w/v) ampholine pH 3.5 to 10.0 resulting in gels with pH ranging between 4.00 and 7.00 (see Fig. 8A).

The fluorograms of these gels represent the 2D PAGE profiles of cellular proteins synthesized by

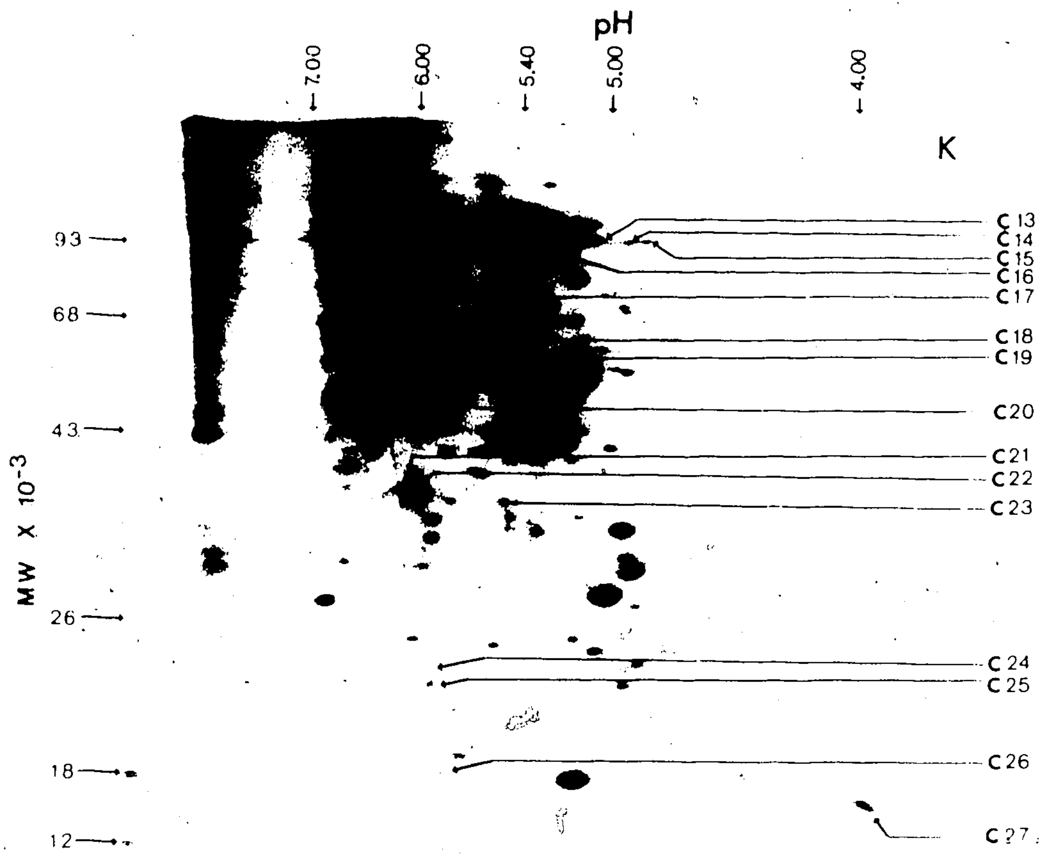
(I) Untreated EL4.E1 cells.
(J) CsA-treated cells.
(K) PMA-treated cells.
(L) PMA + CsA-treated cells.

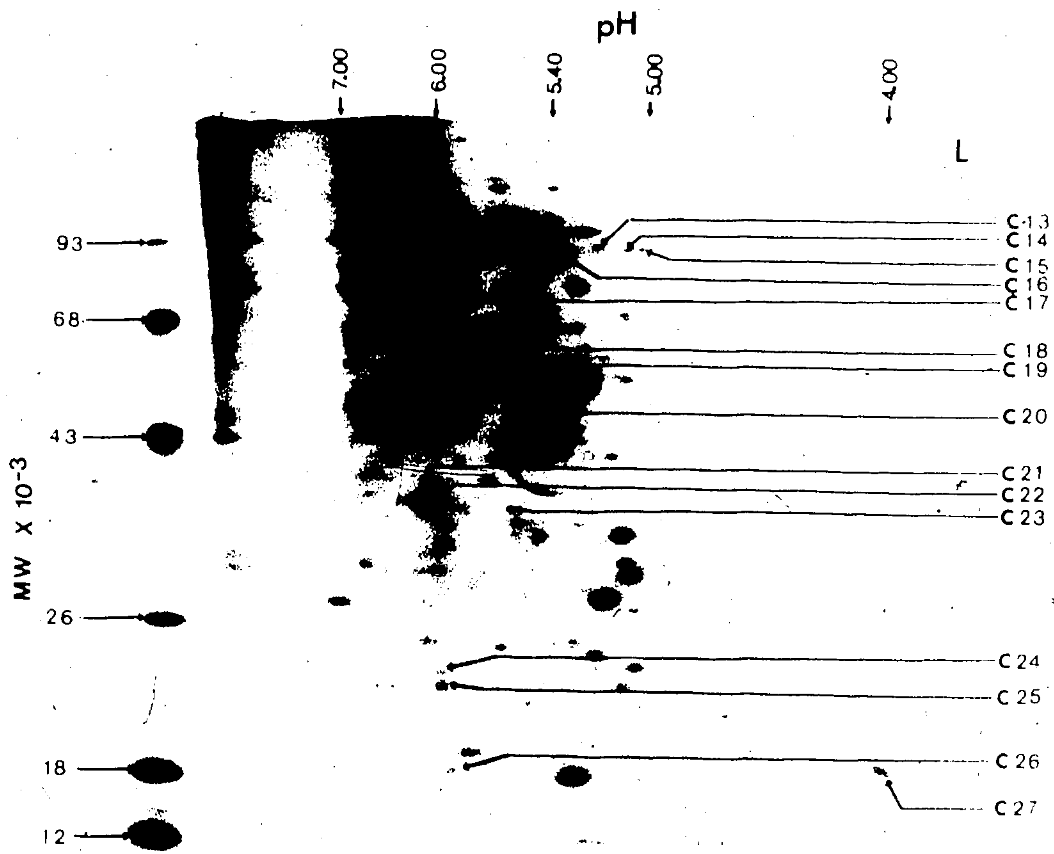


pH
7.00
6.00
5.40
5.00
4.00
MW X 10-3
93
68
43
26
18
12
C 1
C 2
C 3
C 4
C 5
C 6
C 7
C 8
C 9
C 10
C 11
C 12



pH
7.00
6.00
5.40
5.00
4.00
J
93
68
43
26
18
12
MW X 10-3
C 14
C 15
C 29


pH
7.00
6.00
5.40
5.00
4.00
K
MW X 10-3
93
68
43
26
18
12
C 13
C 14
C 15
C 16
C 17
C 18
C 19
C 20
C 21
C 22
C 23
C 24
C 25
C 26


pH
7.00
6.00
5.40
5.00
4.00
L
MW X 10-3
93
68
43
26
18
12
C 13
C 14
C 15
C 16
C 17
C 18
C 19
C 20
C 21
C 22
C 23
C 24
C 25
C 26
C 27

Cells treated with CsA only (Figures 8B and 9F), showed no changes in the secreted protein profile from the untreated controls (Figures 8A and 9E). In the presence of PMA, or PMA and CsA however, very specific changes were observed. When comparing untreated cells with PMA-treated cells, the effects of PMA was limited to a very small number of proteins seen in the profiles of secreted proteins (Figures 8A and 8C) and cellular proteins (Figures 10I and 10K). Approximately 5% of the total number of spots were "turned off" by PMA and 5% were "turned on". IL2 was identified on Figures 8C and 9G as S12 by virtue of its charge and molecular weight. Four species S12a, S12b, S12c, and S12d could be identified on Figure 8C with the following molecular weights and pIs a) 21.0K pI 4.50, b) 21.4K pI 4.75, c) 21.5K pI 4.40 and d) 21.8K pI 4.28. By estimation from the specific activity of IL2 (16,000 units $\mu g^{-1}$ of HPLC purified IL2) (Riendeau *et al.*, 1983), average IL2 activity in the supernatant (800 units $ml^{-1}$), and equivalent volume loaded per gel (100 $\mu l$), the amount of IL2 in the gel is about 5 ng. This represents 0.6% of the total protein concentration in an equivilent volume of serum-free supernatant (8.0 $\mu g$ $ml^{-1}$. This correlated well with the proportion of IL2 (0.8%) in the supernatant of PMA-treated cells calculated from the 2D PAGE data in Figure 8C.

Cultures containing PMA as well as CsA showed reduced levels of IL2 (Figures 8D and 9H). It can be seen however, that other PMA-inducible proteins, S14 (Figures 8C and 8D) and C17 (Figures 10K and 10L) were also affected by CsA. The majority of PMA-inducible proteins were not affected by CsA. The appearance of S17, S18 and S23 (Figure 8D and 9H) when both agents were present, the reappearance of S1, S3 and C2 (Figures 8D, 10L) in cultures when CsA was added to PMA containing cultures, and the occurrence of only one distinguishable 73K protein (C29, Figure 10J) in CsA containing medium suggested that, the mechanism of action of both agents could be related. C14 and C15

(Figures 10J, 10K and 10L) appeared to be induced by both agents together or PMA alone but not by CsA alone. These observations revealed that, the majority of both intracellular and secreted proteins were unaffected by CsA or PMA. A specific effect is exerted by both agents rather than a gross non-specific perturbation of cellular biosynthesis of proteins. CsA affected only about 2.5% of the entire number of proteins detected in contrast to the 5% by PMA.

### (c) PMA and CsA Effects on mRNA Levels

The effects of CsA and PMA observed on Figures 8, 9 and 10 could have been due to their effects on the post-translational modifications of proteins.

To determine if PMA and/or CsA exerted controls at the mRNA level, wheat germ-translated products from mRNA of variously treated EL4.E1 cells (generous gifts from Dr. D. Harnish and Dr. J. Elliott, Department of Biochemistry, University of Alberta) were subjected to analysis by 2D PAGE (Figures 11M, 11N and 11O). Correlation of these proteins with the proteins from EL4.E1 cells was not possible because of the probable occurrence of incomplete translation products as well as differences in post-translational modification. The synthesis of t1, t2, t3, t4 and t5 was affected by treatment of the cells with PMA and/or CsA. Both agents affected the levels of some mRNAs in the cell.

## C. DISCUSSION

Table 6 assigns the proteins affected by PMA and CsA in these experiments into categories based upon their presence (/) or absence (x) under each experimental condition as seen on the gels.

## FIGURE 11

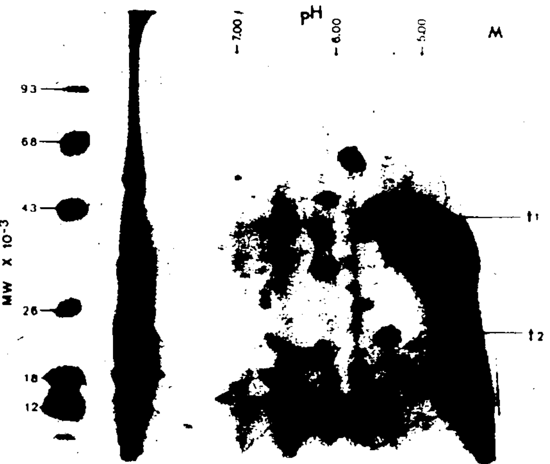
## 2D PAGE OF CELL-FREE TRANSLATION PRODUCT OF mRNA FROM EL4.E1 CELLS

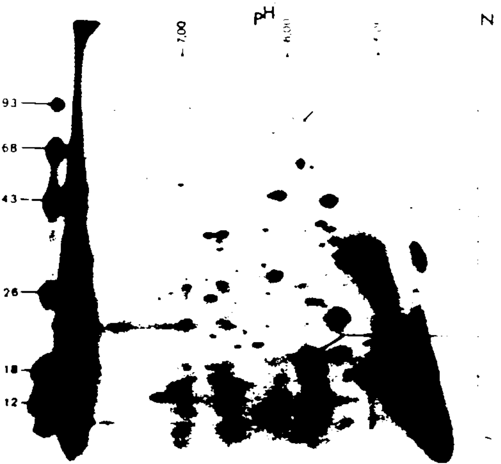
Cell-free translation products of mRNA from EL4.E1 cells (generous gifts of Dr. D. Harnish and Dr. J. Elliott, University of Alberta) were prepared by the wheat germ translation method (Riendeau *et al.*,1983). The proteins from the wheat germ translations were precipitated as described in section B.2 of Materials and Methods and prepared for 2D PAGE.

2D PAGE was performed (chapter II, section C.1 and C.2) with IEF gels containing 2% (w/v) ampholine pH 3.5 to 10.0 resulting in gels with pH ranging between 4.00 and 7.00.

Fluorograms of gels represent 2D PAGE profiles of the translation products of mRNA isolated from

(M) Untreated EL4.E1 cells.
(N) PMA-treated EL4.E1 cells.
(O) PMA and CsA-treated EL4.E1 cells.


W
pH
7.00
6.00
5.00
MW X 10-3
93
68
43
26
18
12


Z
pH
7.00
6.00
93
68
43
26
18
12

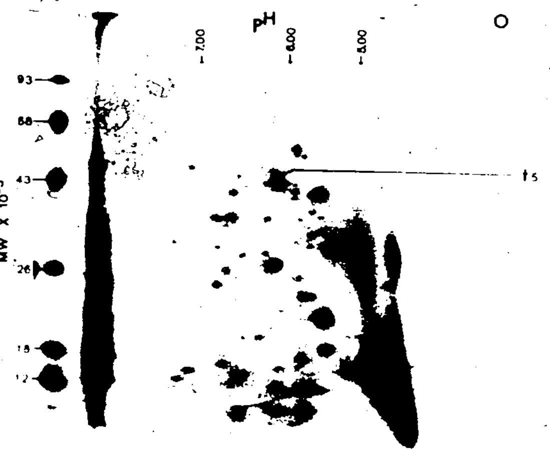

O
pH
7.00
6.00
5.00
MW X 10-3
93
68
43
26
18
12

TABLE 6

CATEGORIES OF PROTEINS AFFECTED BY PMA AND CsA TREATMENT

6(a)

| Categories | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| Untreated cells | x | x | x | x | / | / | x |
| CsA-treated cells | / | x | x | / | / | / | x |
| PMA-treated cells | x | / | x | / | x | x | / |
| PMA + CsA-treated cells | x | x | / | / | / | x | / |

6(b)

| Categories | H | I | J |
|---|---|---|---|
| Untreated cells | x | x | / |
| PMA-treated cells | x | / | x |
| PMA + CsA-treated cells | / | x | x |

The categories of proteins affected by the agent PMA and CsA were charted according to the relative enhanced (/) or diminished(x) intensity of that particular protein in each fluorogram. Table 6(a) charts the categories of proteins from intact EL4.E1 cells and Table 6(b), the categories of proteins from wheat germ-translated mRNA of variously treated EL4.E1 cells.

Taking into account all possible combinations, protein t5 in category H may correlate to only one category (C) in Table 6a; t5 may thus be one of S17, S18 or S23. Similarly, category I corresponds to category B with t3 and t4 being possibly any two of S12, S14 and C17 while category J with t1 and t2 corresponds to a whole host of possibilities in category F.

A direct comparison of identity could not be made between the wheat germ-translated proteins and the proteins synthesized by the intact cells. This is due the occurrrence of differences in post-translational modifications of proteins and incomplete translation products in the wheat germ translation reaction.

Table 7 lists some examples of proteins sensitive to PMA and CsA treatment in various categories defined in Table 6. S12 as mentioned earlier is IL2. It is present when PMA is added but not when CsA is concurrently present in the culture (category B). S12 could have the same identity as t4 in category I from the wheat germ translation of mRNA from EL4.E1 cells.

TABLE 7

EXAMPLES OF PROTEINS IN EACH CATEGORY OUTLINED IN TABLE 6

| Category | Protein | M.W.x10$^{-3}$ | pI |
|---|---|---|---|
| A | C29 | 73.0 | 5.25 |
| B | S12 | (a) 21.0 | 4.55 |
| | | (b) 21.4 | 4.47 |
| | | (c) 21.5 | 4.40 |
| | | (d) 21.8 | 4.28 |
| C | S17 | 100.0 | 4.50 |
| | S23 | 18.0 | 4.25 |
| D | C16 | 84.0 | 5.30 |
| E | S3 | 39.0 | 6.75 |
| | C2 | 70.0 | 7.00 |
| G | C17 | 64.0 | 7.00 |
| H | t5 | 48.0 | 6.20 |
| I | t3 | 83.5 | 5.00 |
| | t4 | 21.0 | 5.00 |
| J | t1 | 37.5 | 5.45 |
| | t2 | 23.7 | 5.00 |

Category F represents proteins affected by PMA. These were "turned off" whenever PMA was present in the culture and was unaffected by the presence or absence of CsA. These were S2, S3, S4, S5, S6, S19, S20, S21, S22, C1, C3, C4, C5, C6, C7, C8, C9, C10, C11 and C12.

Category G represents "PMA-inducible" proteins unaffected by CsA. These include S7, S8, S9, S10, S11, S13, S15, S16, C13, C18, C20, C21, C22, C23, C24, C25, C26 and C27.

CHAPTER V

# CONCLUSIONS

PMA causes noticeable morphological changes and induces the production of lymphokines in EL4.E1 cells. The results presented in this thesis indicate that PMA and CsA effects in EL4.E1 cells involved a selective event rather than a total "reprogramming" of cellular functions.

Table 8 summarizes the total number of proteins found in each of the categories mentioned in Chapter IV(C) (Table 6) and the percentages of the total number of proteins observed in these systems.

The effects of CsA on PMA-treated cells accounted for about 2% of the 5% of changes observed to be caused by PMA treatment alone. Both agents induced minimal changes in the protein biosynthesis pattern of EL4.E1 cells. The observed retention of the general 2D PAGE profiles of proteins from CsA or PMA-treated cells suggested that both these agents affected specific pathways. PMA is known to work through a PK-C (Chapter I, section C.2). The pathway activated by PMA via PK-C is believed to be one aspect of an activation pathway involving synergy with $Ca^{2+}$ activation as well. Nishizuka and colleagues showed that the response in platelets to endogenous activation by thrombin could not be observed with PMA or the $Ca^{2+}$ ionophore (A23187) alone. PMA and A23187 together, however mimicked the response of platelets to thrombin activation (Kaibachi *et al.*, 1983; Yamanishi *et al.*, 1983). Such a synergy has also been demonstrated in rat neutrophils (Kajikawa *et al.*, 1983). Nishizuka proposed that the endogenous extracellular signal evoked both pathways upon activation of its receptor. It is not known what effects A23187 would have upon EL4.E1 cells induced by PMA. The activation of PK-C is probably involved in PMA induction of lymphokine production by EL4.E1 cells. The

TABLE 8

THE NUMBER OF PROTEINS FOUND IN EACH CATEGORY DEFINED IN TABLE 6a AND THE PERCENT OF THE TOTAL NUMBER OF PROTEINS THESE REPRESENT

| Categories | A | B | C | D | E | F | G | Total number of proteins ±SE (n=3) |
|---|---|---|---|---|---|---|---|---|
| Number of secreted proteins | - | 2 | 3 | - | 2 | 8 | 8 | 130 ± 7 |
| Number of cellular proteins | 1 | 1 | 0 | 3 | 1 | 11 | 11 | 370 ± 10 |
| % of total proteins | 0.20 | 0.60 | 0.60 | 0.60 | 0.60 | 3.80 | 3.80 | |

The number of proteins were estimated by visually scoring the number of spots on each fluorogram.

transduction of signal via PK-C activation appeared to be extremely specific in this instance.

IL2 was observed to be specifically induced by PMA stimulation of EL4.E1 cells. CsA inhibited this induction as demonstrated by both IL2 assays and 2D PAGE. The immunosuppressive action of CsA is at least in part due to its inhibition of IL2 production (Bunjes *et al.*, 1981; Britton and Palacios, 1982). Cell-free translation of mRNA from EL4.E1 cells (Elliott *et al.*, 1984, manuscript submitted) revealed the absence of translatable IL2 mRNA from CsA-treated, PMA-induced cells. PMA induction in the absence of CsA yields translatable mRNA for biologically active IL2. This study does not reveal if PMA and CsA control of IL2 expression occurs at the transcription or post transcription level. On average, 3% of the total number of proteins observed by 2D PAGE of cell-free translation product of mRNA from EL4.E1 cells were affected by both PMA and CsA. Both agents thus showed a selective effect on the levels of some mRNA species.

The specificity of PMA effects observed in this study could be mediated through the PK-C. This membrane associated protein is activated by an endogenous signal, which probably occurs upon the signal interacting with its specific receptor. According to the model proposed by Nishizuka ([illegible]) this endogenous signal not only activates PK-C but also causes an increase in cytosolic $Ca^{2+}$, which acts synergistically with the kinase to trigger a physiological response. The changes observed in the protein profiles of PMA-treated cells by 2D PAGE analysis could represent a partial response similar to that described by Kaibuchi *et al.* (1983) and Yamanishi *et al.* (1983) for serotonin release from platelets. CsA effects on PMA-treated cells probably acts through a pathway independent of PK-C or more profound changes would have been observed by 2D PAGE of proteins from EL4.E1 cells.

It was recently reported that PMA specifically induced a set of eight proteins which were also induced by ultra violet irradiation or mitomycin C treatment of cultured human fibroblast cells (Schropp *et al.*, 1984). These were detected by 2D PAGE analysis of cellular proteins pulse-labelled with L-[$^{35}$S]-methionine. It was proposed that these proteins could possibly be involved in the repair of DNA damage caused by ultra violet irradiation or PMA. These proteins did not correspond to any of the PMA-inducible proteins observed in EL4.B1 cells.

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