Mechanisms of Action of Mycophenolic Acid

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Abstract

Mycophenolic acid (MPA) is an immunosuppressive drug that inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH). IMPDH is the rate-limiting enzyme in the *de novo* pathway of guanosine nucleotide synthesis. There are two pathways for the synthesis of guanosine nucleotides in all cells, the *de novo* pathway and the salvage pathway. Proliferative responses of lymphocytes to mitogens were found to be dependent solely on the activity of the *de novo* pathway to provide sufficient levels of guanosine nucleotides, because the salvage pathway is unable to supply the increased levels of guanosine nucleotides that the cells require. Therefore inhibition of IMPDH by MPA inhibits the *de novo* pathway and depletes guanosine nucleotides from lymphocytes resulting in an inhibition of proliferative responses.

Guanosine nucleotides are essential for many different cellular processes and the effects of MPA on cellular functions can not be explained simply by an inhibition of proliferation in T cells. Therefore we have considered the effect of MPA on two different cellular functions that may be involved in the immunosuppressive effect of MPA. We have investigated the glycosylation of cell surface proteins and also the activation and DNA binding of transcription factors in a T and an endothelial cell line in the presence of MPA (CEM-C7 and ECV304 cells respectively).

Using flow cytometry we have been able to detect the effect of MPA treatment on cell surface expression of fucose and mannose in CEM-C7 and ECV304 cells. MPA treatment of resting and mitogen stimulated CEM-C7 T cells had no effect on fucose and mannose expression. However in resting and TNFα stimulated ECV304 cells MPA inhibited the expression of both fucose and mannose, but had a more significant effect on fucose expression.

The effect of MPA on AP-1 and NF-κB activity was also investigated using a reporter gene approach in CEM-C7 and ECV304 cells. MPA was found to decrease TNFα stimulated NF-κB activity in T cells, which was subsequently found to be due to decreased cellular proliferation. However, MPA caused a significant potentiation of PMA stimulated AP-1 activity in T cells. In contrast, there was no effect of MPA on either AP-1 or NF-κB activity in endothelial cells.

Gel shift assays on whole cell extracts were utilised to investigate the effect of MPA on transcription factor DNA-binding using AP-1, CREB, and NF-κB specific probes. The DNA-binding ability of all the transcription factors was inhibited by MPA in resting or Con A stimulated T cells but was unaffected in either resting or TNFα stimulated endothelial cells. Gel shifts of PMA stimulated CEM-C7 cultures treated with MPA indicate the presence of an additional complex compared to cultures treated with MPA alone suggesting this complex may contribute to the increased AP-1 activity detected in the reporter gene assays.

Our results therefore suggest that MPA treatment has important consequences not only involving the disruption of cell-cell interactions but also effects on cell signalling pathways. In addition, the results implicate AP-1 as an essential transcription factor induced in T cells in response to MPA, which may mediate the effects of MPA on cellular differentiation.

Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university or other institute of learning.

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The Author

The author graduated in Medical Science (BMed Sc Honours) from The University of Birmingham in 1995. Until October 1998 he has worked in the Immunology Research Group at the University of Manchester, School of Biological Sciences where he has conducted work on the immunosuppressive drug mycophenolic acid and its mechanisms of action. In February 1999 he is beginning a postdoctoral position at The University of Miami School of Medicine where he will be applying the techniques learned during his PhD into investigating the role of mucin molecules in tumour development and progression.

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I will miss you all but if you fancy a cheap holiday in the sun you know where I am.

This thesis is dedicated to my mum and dad.

I hope your investment has paid off!!

Thanks for everything I couldn't have done it without you.

List of Abbreviations

AP-1	Activator protein 1	IFNγ	Interferon gamma
APC	Antigen presenting cell	Ig	Immunoglobulin
ADCC	Antibody dependent cell mediated cytotoxicity	IMPDH	Inosine monophosphate dehydrogenase
ATGAM	Antithymocyte gamma globulin	IP_3	Inositol 1,4,5-trisphosphate
CD	Cluster of differentiation	JNK	Jun N-terminus kinase
Con A	*Concanavalin A*	LAT	Linker for activation of T cells
CREB	cAMP response element binding protein.	LFA-1	Leucocyte function associated antigen 1
CsA	Cyclosporin A	LTA	*Lotus tetragonolobus*
CTLA-4	Cytotoxic T lymphocyte antigen 4	LPS	Lipopolysaccharide
		mAb	monoclonal antibody
DAG	Diacylglycerol	MAPK	Mitogen activated protein kinase
EBV	Epstein Barr virus		
ERK	Extracellular signal related kinase	MHC	Major Histocompatibility Complex
FKBP	FK506 Binding protein		
GIT	Gastrointestinal tract	MMF	Mycophenolate mofetil
GNA	*Galanthus nivalis*	MPAG	Mycophenolic acid glucuronide
HEV	High endothelial venule		
HLA	Human Leucocyte Antigen	NF-AT	Nuclear factor of activated T cells
HuPBMC	Human peripheral blood mononuclear cell	NF-κB	Nuclear factor kappa B
		PHA	Phytoheamaglutinin
HUVEC	Human umbilical vein endothelial cells	PMA	Phorbol 12-Myristate 13-acetate
ICAM-1	Intercellular adhesion molecule 1	PI-3K	Phosphatidylinositol 3-kinase

PIP_2	Phosphatidyl inositol 4,5-bisphosphate
PLC	Phospholiase C
PLCγ1	Phospholipase C γ 1
PTK	Protein tyrosine kinase
PTPase	Protein tyrosine phosphatase
SAPK	Stress activated protein kinase
SRE	Serum response element
SRF	Serum response factor
TNFα	Tumour necrosis factor
TRE	TPA response element
UEA1	*Ulex europaeus I*
VCAM-1	Vascular cell adhesion molecule 1
VFA	*Vicia faba*
VLA-4	Very late antigen 4

Introduction

1 Allograft Rejection

The success of an allotransplant into an individual is dependent on proteins of the Major Histocompatibility Complex (MHC). These molecules, present on the surface of all nucleated cells, are important as they act to present peptides to circulating immune cells that will then respond if the peptide is seen as foreign (Krensky *et al*, 1990). There are three types of MHC of which classes I and II are the most important in transplantation. Class I molecules are found on almost all nucleated cells therefore they are important in determining the specificity of attack against allogeneic tissues and virus-infected cells by cytotoxic T lymphocytes. Under normal circumstances the groove is occupied by polypeptides of normal cellular constituents derived from intracellular degradative pathways. In a transplanted tissue, depending on the genetic disparity, a different array of the same cellular constituents will be presented on the surface due to slight differences in shape and charge of the MHC groove.

Class II MHC molecules are expressed principally on cells of the immune system such as antigen presenting cells, macrophages, B cells and T helper cells. An exception to this rule is activated endothelial cells that are also able to express MHC II (Westphal *et al*, 1997). It is thought that the antigenic stimuli that activate rejection reactions against many grafts are class II antigens within the tissue itself or present on passenger leucocytes within the graft.

Minor histocompatibility antigens play a more prominent role in transplants between siblings with identical MHC (Colvin, 1990). These antigens are peptides of donor proteins, which can be processed and presented by self-MHC and recognized as foreign by recipient T cells.

1.1 Direct and Indirect Allograft Recognition

T cells are extremely sensitive to fine structural differences within the MHC and therefore play a central role in the specific immune response to an allograft. There are two distinct pathways of allograft recognition, the direct pathway and the indirect pathway (Sayegh, Watschinger and Carpenter, 1994; Sykes, 1996; Warrens, Lombardi

and Lechler 1994). The direct pathway involves T cell recognition of intact allo-MHC molecules on the surface of donor cells, while indirect recognition requires presentation of donor antigens shed from the graft and taken up by recipient APCs and presented to T cells. During the process of indirect allorecognition these antigens may include peptides derived from processed donor MHC. T cells primed by the direct pathway probably play a more dominant role in acute rejection while T cells primed by the indirect pathway are thought to be particularly important in chronic rejection (Sayegh, Watschinger and Carpenter, 1994).

Graft rejection, classified into four types depending on the speed of rejection (Kirkpatrick, 1987), is a complex process relying on a variety of effector mechanisms directed against antigenic disparities between the donor and recipient (Häyry *et al*, 1984). Both cell-mediated and antibody mediated mechanisms are involved in allograft rejection (Hall, 1991). The cellular component involves highly specific T cell-mediated processes and also natural killer cell (NK) and macrophage. The effects of antibody production include complement activation and antibody dependent cell-mediated cytotoxicity (ADCC).

1.2 Hyperacute Rejection

Hyperacute rejection occurs within a few minutes to a few days after transplantation. Such rejection episodes occur in patients who have previously been sensitised to donor antigens and thus possess preformed antibodies. Immediate graft rejection occurs so quickly that the graft blood supply is immediately compromised due to complement and antibody deposition on the arterial wall.

Accelerated Cellular Rejection is a form of hyperacute rejection that occurs within a few days of transplantation due to the presence of presensitised T cells.

1.3 Acute Rejection

These rejection episodes generally begin around one week post-transplantation but may not develop for several weeks if the patient is on intensive immunosuppressive

therapy. Acute rejection includes both a cellular and humoral response against the graft.

1.4 Chronic Rejection

Although the incidence of acute rejection has decreased over the years, the process of chronic rejection still limits long-term graft survival (Häyry *et al*, 1993, Häyry, 1996). Chronic rejection is a slow process taking months or years to develop leading to a gradual loss of function of the graft. The most common histologic feature is always a persistent, low-grade inflammation in the vascular periphery and subendothelium and concentric generalised intimal thickening (Häyry *et al*, 1997). Low-grade cell-mediated rejection, as a result of cytokine secretion by lymphocytes and macrophages in the vascular endothelium, and also antibodies or antigen-antibody complexes deposited in the grafted tissue are involved in the process. IgG and complement have been found lining the walls of allograft blood vessels (Whitley, Kupiec-Weglinski and Tilney, 1990). Continual damage to the graft endothelium causes the cells to secrete growth factors in an attempt to repair the damage. Consequently over time smooth muscle and endothelial cell proliferation and migration is induced leading to intimal hyperplasia (Häyry, 1996). Current immunosuppressive regimens generally have little effect on the progression of this process.

2 T Cell Activation – Costimulation

2.1 T Cell Receptor and Accessory Molecules

The critical receptor for T cell activation is the T cell receptor (TCR), which is composed of α/β or γ/δ chains that are non-covalently associated with the CD3 complex at the cell membrane. The CD3 complex consists of γ, δ, ε and the associated η and ζ chains, which are splice variants that are functionally distinct from the CD3 chains (Frank *et al*, 1991). The CD3 complex consists of CD3ε-CD3γ, CD3ε-CD3δ heterodimers and a homo- or heterodimer of the ζ chain with either, η or FcεRIγ chains although most TCRs contain ζ homodimers (Frank *et al*, 1991, Zenner *et al*, 1995). The cytoplasmic chains of the CD3 complex are responsible for coupling the TCR to the intracellular signalling machinery, as the TCR has no intrinsic signalling capacity.

T cell activation is controlled by the T cell receptor in combination with additional signals provided by accessory molecules on the antigen presenting cell (APC) (Janeway and Bottomley, 1994). T cell receptor stimulation in the absence of adequate costimulation fails to generate T cell responses. For example, naïve T cells require at least two signals in order to proliferate and differentiate into effector cells (Van Gool *et al*, 1996). The first signal is sent via the TCR and its co-receptors CD4 and CD8 that serve this essential co-receptor function in T cells, by binding to membrane-proximal regions of MHC II and I respectively and transducing extracellular signals to intracellular signalling pathways within the T cell. The TCR recognises antigenic peptides bound to MHC molecules on the surface of APC's while co-receptors on the surface of the T cell are important for enhancing the sensitivity of the cell to activation and therefore increasing the signals transduced through the TCR (Weiss and Litman, 1994). The second important signal is delivered by ligands on the APC interacting with specific receptors on the T cell surface to provide costimulatory signals to complement TCR engagement.

2.2 CD28

CD28 is one of the best-characterised costimulatory receptors on T cells. CD28 and its homologue CTLA-4 provide one of the most dominant costimulatory pathways for T cell activation (Chambers and Allison, 1997; Lenshow, Walunas and Bluestone, 1996; Linsley and Ledbetter, 1993). CD28 is expressed on the majority of T cells (all $CD4^+$ cells and 50% $CD8^+$ cells) while CTLA-4 expression is restricted to activated T cells (June *et al*, 1994; Lindsten *et al*, 1993). Both molecules interact with their ligands CD80 (B7-1) and CD86 (B7-2) present on the surface of APCs although CTLA-4 has a 10-fold higher affinity than CD28 (June *et al* 1994). CTLA-4 can modulate T cell activation resulting in downregulation and "switching off" of the immune response (Fallarino, Fields and Gajewski, 1998). CTLA-4 is upregulated during T cell activation in order to down-regulate the immune response (Saito, 1998) and its expression can be induced by anti-CD28 mAb (Lindsten *et al*, 1993). CD28 delivers a positive costimulatory signal to the T cell by enhancing cytokine production and causing T cell proliferation and activation. It also augments T cell survival by enhancing the expression of anti-apoptotic molecules such as Bcl-x_L (Boise, Noel and Thompson, 1995; Linsley and Ledbetter, 1993). CD80 and CD86 are differentially expressed, with expression being controlled by cell-cell interactions, cytokines and also the state of activation of the cell (Van Gool *et al*, 1996; Sansom and Hall, 1993).

2.3 CD40

The receptor for CD40L is another important costimulatory molecule called CD40. CD40 is expressed on professional APCs as well as T cells, B cells, macrophages, eosinophils, endothelial cells and fibroblasts and influences many aspects of the immune response (Stout and Suttles, 1996; Foy *et al*, 1996; Van Gool *et al*, 1996; Van Kooten and Banchereau, 1996, 1997). CD40 interacts with CD40L present on activated T cells - primarily $CD4^+$ but also some $CD8^+$ - mast cells, eosinophils and activated B cells. Triggering of CD40 on B cells is essential for growth, isotype switching and immunoglobulin (Ig) synthesis (Banchereau *et al*, 1994). The importance of CD40 in B cell activation is highlighted in patients with hyper IgM

syndrome who have a mutation in CD40L expression on their T cells. B cells in these patients produce normal IgM levels in response to T cell-dependent antigens but they are unable to switch to other classes of immunoglobulin (Ig) (Callard *et al*, 1993). It has been proposed that the interaction of naïve T cells expressing CD40L, with APCs expressing CD40, leads to upregulation of CD80/86 on the APC, which can then deliver the important costimulation required by the T cell to become fully active (Grewal and Flavell, 1996; Yang and Wilson, 1996).

CD40-CD40L interactions between T cells and endothelial cells have been shown to be able to induce endothelial cell activation leading to upregulation of adhesion molecule expression suggesting another way in which T cells may augment inflammatory responses (Yellin *et al*, 1995; Larsen and Pearson, 1997).

2.4 Adhesion Molecules

Adhesion molecules help strengthen cell-cell interactions and also facilitate migration of leucocytes into inflammatory or alloantigenic sites (Springer, 1990; Heeman *et al*, 1994a). They have also been found to play a role in signal transduction and help to costimulate T cell activation (Richardson and Parsons, 1994; Schaller and Parsons, 1994). During the process of transplanting an organ, the stress caused by the surgical procedure, ischaemia or reperfusion may lead to the upregulated expression of adhesion molecules on endothelial cells making the transplanted organ more prone to immunologic attack (Azuma *et al*, 1994a). Adhesion molecule upregulation on both endothelial cells and infiltrating cells correlates with rejection episodes (Heeman *et al*, 1994b; Azuma and Tilney, 1994).

2.4.1 CD2

CD2 (LFA-2) is expressed on the surface of T cells, and binds to its ligand LFA-3 (CD58) on haemopoietic and non-haemopoietic cells, an interaction which is thought to enhance T cell antigen recognition (Selvaraj *et al*, 1987; Davis and van der Merwe,

1996). Following T cell activation there is an increase in adhesion between CD2 and LFA-3 (Springer, 1990). It is thought that the main effect of CD2 is the enhancement of T cell antigen recognition is by bringing together the plasma membranes of the T cell and APC to a distance optimal for TCR-peptide-MHC interaction. CD2 and the TCR appear to share signalling components, it has been reported that fine-tuning of CD2 co-receptor function appears to be regulated by transmembrane regions of TCR-associated ζ-chains (Von Bonin, Ehrlich and Fleischer, 1998).

2.4.2 Integrins

The integrin family of adhesion molecules contributes to several different stages of the immune response (Albelda and Buck, 1990; Springer, 1990). The interaction between LFA-1 and its ligands ICAM-1, -2, -3 is one of the most dominant adhesion systems underlying T cell:APC interactions. LFA-1 (also known as CD11a/CD18) expression is restricted to leukocytes while ICAM-1 is expressed on a wide variety of cell types including dendritic cells and B cells. Not only is this ligand/receptor pair important for strengthening interactions between the T cell and APC but it also provides an important costimulatory signal to T cells (van Seventer *et al*, 1990; Van Kooyk and Figdor, 1997; Damle *et al*, 1992; Kanner *et al*, 1993). The interaction of LFA-1 with its other ligand ICAM-2 and VLA-4 with its ligand VCAM-1 are also able to generate costimulatory signals to increase T cell responsiveness (Damle, Klussman and Aruffo, 1992; Damle *et al*, 1993).

The consequence of these co-ordinated receptor-ligand interactions is to modulate transcription factor activation and the expression of various genes that regulate proliferation, differentiation and alter the function of various lymphoid and non-lymphoid cells.

3 T Cell Signal Transduction

3.1 T Cell Receptor Signalling

The T cell receptor (TCR) has no intrinsic protein tyrosine kinase activity yet it is able to activate cytoplasmic protein tyrosine kinase (PTKs) (Weiss and Littman, 1994). Signal transduction by the TCR occurs via the CD3 γ, δ, ε chains and the ζ chains, which contain immunoreceptor tyrosine-based activation sequence motifs (ITAMs) within their cytoplasmic tails. These motifs consist of the amino acid sequence $D/EX_7D/EX_2YX_2LIX_7YX_2L/I$ where X is any amino acid (Zenner *et al*, 1995). One ITAM sequence is present in each of the CD3 chains while there are 3 copies in the ζ chains, two copies in the η chains and one copy in the γ chains. The major function of ITAM sequences is to interact with intracellular signalling molecules. Tyrosine residues within the ITAM sequence are phosphorylated following TCR stimulation and can then recruit cellular protein tyrosine kinases which contain SH2 domains (See figure 1) (Chan and Shaw, 1995).

Four families of protein tyrosine kinases are involved in TCR signalling these are src family PTKs, Csk, tec and syk family PTK's (Qian and Weiss, 1997; Zenner *et al*, 1995; Mustelin and Burn, 1993). Members of the Src family involved in TCR signalling include lck, fyn and yes, their catalytic activity correlates with autophosphorylation at a conserved tyrosine residue while phosphorylation of residues in their C-terminus suppresses their activity. Lck is associated with CD4 and CD8 and is therefore brought close to the ITAM motifs following TCR/CD4 engagement of MHC. Fyn interacts with the TCR complex via CD3, but is thought to be less important than lck in TCR signalling. Mice with the fyn gene disrupted have no gross T cell alterations except that thymocytes are refractile to stimulation through the TCR while peripheral T cells mature normally and reacquire significant signalling capabilities (Appleby *et al*, 1992). The syk family members syk and ZAP-70 are expressed solely in T cells and NK cells and become recruited to the CD3 and ζ chain complex following TCR stimulation where they are rapidly tyrosine phosphorylated.

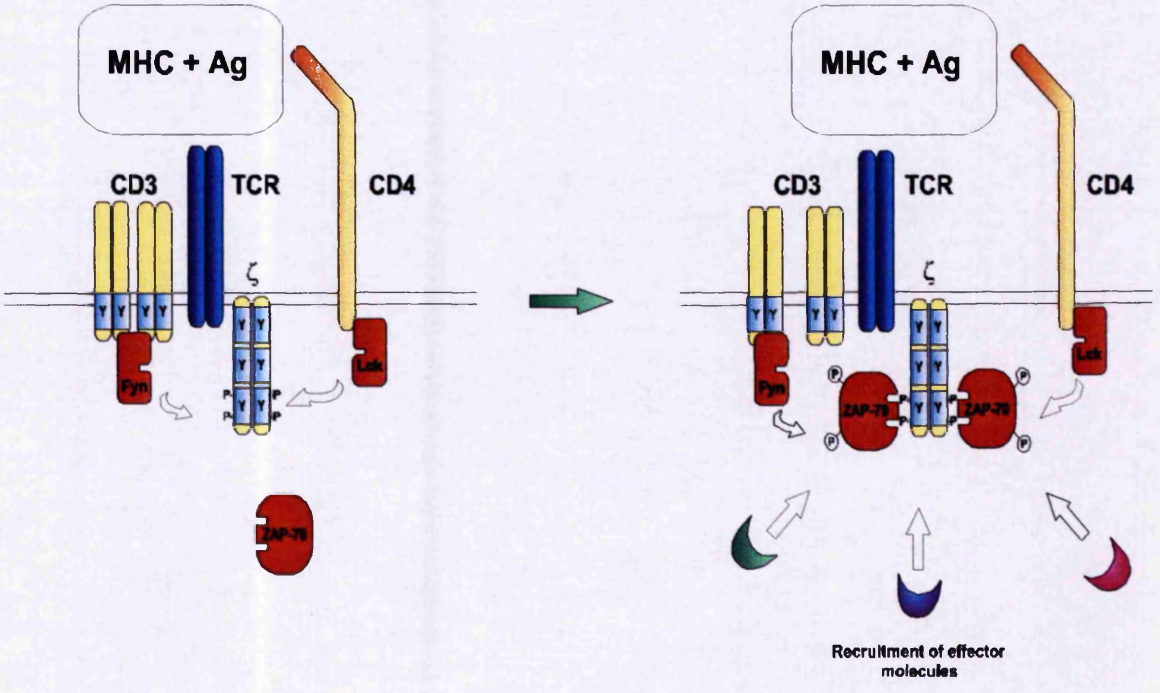


Figure 1. Interactions between the TCR and tyrosine kinases. Following interaction between the TCR and antigen in the context of MHC, along with the binding of CD4 to the same MHC molecule, co-receptor associated lck or TCR associated fyn phosphorylate ITAM motifs. Phosphorylation of both tyrosine residues within the ITAM sequence mediates ZAP-70 binding to the phosphorylated receptor. This permits phosphorylation of ZAP-70 by lck or fyn to provide docking sites for other SH2-containing effector molecules. (Figure adapted from Chan and Shaw 1995 and Mustelin and Burn 1993)

Syk can associate with the TCR independent of activation and tyrosine phosphorylation unlike ZAP-70, which requires tyrosine phosphorylation of ITAM motifs (Zenner *et al* 1995). The recruitment is dependent on the SH2 domains of ZAP-70 associating with the tyrosine phosphorylated ITAM motifs of the TCR-associated ζ-chains (Chan *et al,* 1992). Csk is a negative regulator of TCR signalling that phosphorylates the carboxy-terminal regulatory tyrosine residues of lck and fyn downregulating their catalytic activity (Mustelin and Burn, 1993). CD45, a protein tyrosine phosphatase, is responsible for continuously counteracting the action of Csk by dephosphorylating these c-termini. CD45 is required for optimal activation of T cells in response to PMA stimulation, which suggests a role for Src-related kinases in PKC signalling pathways (Czyzyk *et al,* 1998). Cells lacking CD45 expression have an increased threshold for TCR signalling.

The initial step in TCR signalling is tyrosine phosphorylation of ITAM sequences, which is mediated preferentially by lck (Qian and Weiss, 1997). Phosphorylated ITAMs are then able to recruit ZAP-70 to the plasma membrane via its SH2 domain. ZAP-70 is subsequently phosphorylated by lck or fyn which regulates its catalytic activity.

CD4 and CD8, which are essential co-receptors for T cell activation, bind to MHC II and I respectively allowing simultaneous binding of TCR and CD4/8 to the same MHC molecule. Both CD4 and CD8 bind lck via their cytoplasmic domains, an interaction essential for effective signal transduction, which increase the kinase activity of Lck upon CD4/8 ligation (Micelli, Von Hogen and Parnes, 1991). Lck therefore plays 2 roles in T cell activation the first involving its kinase activity which acts early in the signalling pathway leading to ITAM phosphorylation on CD3 and ζ chains. Secondly interaction of the SH2 domains of lck with phosphotyrosine residues on CD3 anchors CD4/lck to the TCR complex increasing the efficiency of TCR signal transduction. Binding of CD4 to the same MHC molecule augments T cell receptor signalling by up to 100-fold (Weiss and Littman, 1994).

Following TCR stimulation, many cytoplasmic and membrane proteins become tyrosine phosphorylated and multiple protein complexes form between TCR-induced phosphoproteins and SH2 domain containing effector tyrosine kinases (Cantrell, 1996) which initiate downstream signalling pathways (see figure 2). Adaptor proteins, which have no intrinsic enzymatic activity, are involved in these interactions and are

therefore crucial for integration of signal transduction pathways following antigen receptor ligation (Peterson *et al*, 1998).

3.1.1 Activation of Phospholipase C

TCR activation induces PLC activity (Cantrell, 1996; Fraser, Straus and Weiss, 1993) leading to hydrolysis of PIP_2 to IP_3 and DAG. IP_3 elevates intracellular calcium and DAG activates some isoforms of protein kinase C. This therefore produces activation of PKC isoforms and serine/threonine phosphorylation of their substrates and an increase in intracellular calcium. It is thought that TCR induction of PLC activity is due to tyrosine phosphorylation of PLCγ1 (Cantrell, 1996). PLCγ associates with another TCR-induced phosphoprotein important for localizing PLCγ to the plasma membrane where its substrates are located. Elevation of intracellular calcium influences calmodulin dependent events such as activation of calcinuerin, a calcium/calmodulin dependent phosphatase responsible for the activation of the transcription factor NF-ATc. This pathway is the target for the immunosuppressive drugs cyclosporin A and FK506, which will be discussed later. PKC activation leads to the direct serine phosphorylation and activation of Raf (Carroll and May, 1994). Activated Raf is then able to activate downstream signalling cascades and in particular MAP kinase cascades (Howe *et al*, 1992). It was also shown that PMA stimulation of T cells, which activates PKC directly, is also able to activate Ras as well which then activates many different substrates (see later section) (Izquierdo *et al*, 1992).

3.1.2 Activation of Phosphatidylinositol 3-Kinase (PI-3K)

TCR activation is also able to activate the Phosphatidylinositol 3-Kinase (PI-3K) pathway (Cantrell, 1996, Ward; June and Olive, 1996; Ward *et al*, 1992) although engagement of CD28 is required for optimal activation of this pathway (August and Dupont, 1994). PI-3K is a heterodimer consisting of a regulatory 85kDa subunit which contains SH2 domains for interactions with phosphotyrosine residues, and a catalytic 110 kDa subunit (Kapeller and Cantley, 1994).

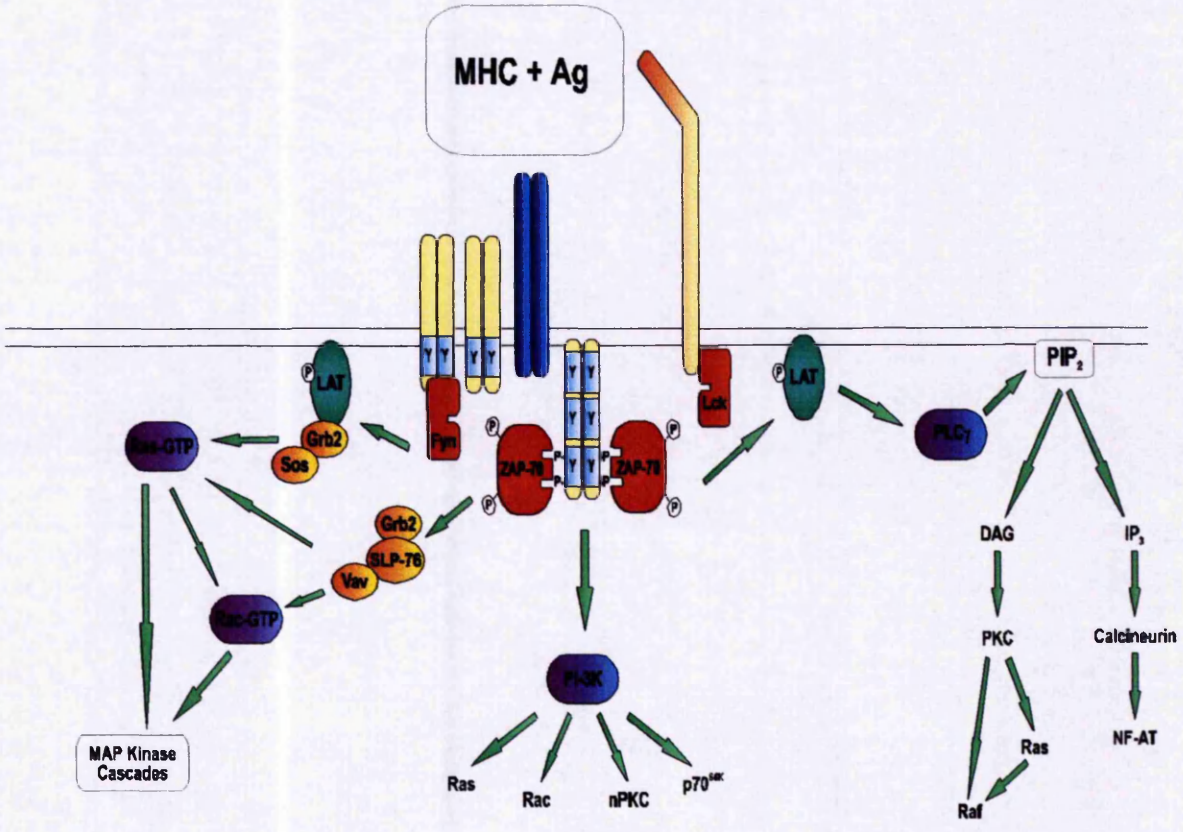


Figure 2. Activation of intracellular signalling pathways following TCR activation. Following TCR engagement protein tyrosine kinases such as ZAP-70, lck and fyn become activated leading to the phosphorylation of LAT, which couples the TCR to phospholipase C γ1 (PLCγ1) and Ras regulated signalling pathways. Activation of PI-3K leads to the activation of the effector molecules Ras, Rac, PKC isofroms and $p70^{S6K}$. Figure adapted from Ward, June and Olive 1996, Mustelin and Burn 1993 and Cantrell 1996.

Two other less well characterised kinases also exist, a PI-3K activated by G-protein βγ subunits and a mammalian PtdIns-specific PI-3K. PI-3K generates 3 products PIP, PIP_2 and PIP_3, they have all been found to accumulate in response to ligation of different receptors including the TCR, CD28, CTLA-4 and CD4. PI-3K couples to CD28 and CTLA-4 via interaction with its SH2 domain and a phosphotyrosine motif in the cytoplasmic tail of the 2 molecules. The p85 subunit of PtdIns 3-kinase is coupled to the TCR and CD4 via association with tyrosine phosphorylated TCR ζ and ε chains involving lck and fyn (Ward, June and Olive, 1996). Binding of fyn SH3 domains to proline rich sequences on PI-3K are thought to regulate its activity by mechanisms independent of tyrosine phosphorylation of PI-3K.

There are a number of downstream targets for PI-3K but there are three particularly important for T cell activation. Protein kinase C (PKC) isozymes α, β, δ, ε, η, θ and ζ, which have been implicated in the control of many signalling pathways including those mediated by p21ras and MAP kinases. The specific PKC isoform involved is poorly understood as different isoforms can activate different transcription factors (Genot, Parker and Cantrell, 1995). A second target is those pathways involved in AP-1 regulation, PI-3K may be involved in regulating the signalling cascades that control ERK or JNK activation and therefore regulation of c-fos and c-jun. PI-3K can directly modulate Ras pathways and also Rac has been implicated as a downstream effector for PI-3K. The third possible target is p70S6 kinase ($p70^{s6k}$) a mitogen activated serine/threonine kinase, which is activated by PI-3K via threonine phosphorylation, it is an important protein involved in protein synthesis and cell cycle progression.

3.1.3 Ras Activation

TCR stimulation also induces Ras activation (Cantrell, 1996; Henning and Cantrell, 1998, Downward *et al*, 1990) due to interactions with guanine nucleotide exchange factors such as sos and vav and also GTPase activating proteins e.g. $p120^{GAP}$ and neurofibromin. Guanine nucleotide exchange factors and GTPase-activating proteins are important for controlling Ras activation by determining the balance between the rate of hydrolysis of bound GTP and rate of exchange of bound GDP for cytosolic

GTP (Cantrell, 1996). Stimulation of the TCR leads to a rapid activation of $p21^{ras}$ (Downward *et al*, 1990).

Grb2 is an adaptor protein that plays a key role in Ras activation. Sos is constitutively associated with GRB2, an adaptor protein that contains SH2 and SH3 domains (Buday *et al*, 1994). The SH3 domain of GRB2 is important for binding to proline rich regions of Sos while the SH2 domain can bind to phosphotyrosine residues on receptors or adaptor proteins (Peterson *et al*, 1998). TCR activation leads to phosphorylation of a 36-kDa protein that forms a complex with GRB2/Sos (Sieh *et al*, 1994; Buday *et al*, 1994), therefore linking the TCR to the Ras pathway, which correlates with Ras activation. This protein called LAT (linker for activation of T cells) (Peterson *et al*, 1998) is an integral membrane protein, which is a substrate for syk and ZAP-70, that contains tyrosine residues in its cytoplasmic tail which are docking sites for the SH2 domain of GRB2. The binding of Sos via GRB2 to membrane associated proteins does not affect Sos activity but is thought to act by simply relocating Sos from the cytoplasm to the membrane where Ras is present (Buday *et al*, 1994). LAT is thought to act as a bridging protein (Peterson *et al*, 1998) because it can also associate with PLCγ1 and complex with GRB2 following TCR activation as well as the 85kDa subunit of PI-3K (Zhang *et al*, 1998).

ZAP-70 has also been shown to interact with Vav through the phosphorylation of the adaptor molecule SLP-76 allowing it to bind Vav (Da Silva *et al*, 1997). Vav can also activate members of the rac/rho family of GTPases and shows selectivity for Rac over Ras (Henning and Cantrell, 1998). Vav is tyrosine phosphorylated following TCR activation, which increases its guanine nucleotide exchange activity for Ras (Henning and Cantrell, 1998). Tyrosine phosphorylated Vav associates with tyrosine phosphorylated SLP-76 via SH2 domain interactions, this complex then associates with GRB2 in a complex also found to contain pp36/38 as well (Tuosto, Michel and Acuto, 1996). Vav is also activated by ligation of CD28 with B7-1 or B7-2 in the absence of TCR ligation. Vav is therefore activated by the initial T cell and APC interaction. This interaction regulates the cellular location of Vav to promote its interaction with its substrates. Vav also interacts with PI-3K, which is thought to either modify Vav activity or control its cellular localisation (Henning and Cantrell, 1998). Vav knockout animals have T cells (and B cells) that respond poorly to stimulation by the antigen receptor but T cells were still stimulated by phorbol ester

and ionomycin suggesting that Vav is critical for receptor triggered T cell proliferation (Zhang *et al*, 1995).

Another pathway for Ras activation occurs via a PKC-dependent pathway (Ohtsuka, Kaziro and Satoh, 1996; Izquierdo *et al*, 1992) involving TCR activation of PLCγ1 by PTKs and subsequent PKC activation. This second route for Ras activation may involve regulation of GAPs, as guanine nucleotide exchange rates are unchanged, causing the phosphorylation of proteins that regulate $p120^{GAP}$ or through $p21^{rap1}$, another GTP-binding protein (Downward, Graves and Cantrell, 1992).

Ras exerts its biological function via at least 3 different effector pathways; one involving a serine/threonine kinase Raf-1, one involving PI-3K and one involving a guanine nucleotide stimulator for Ras (Henning and Cantrell, 1998). Activated Ras interacts with Raf-1 a serine/threonine kinase that regulates MAP kinase cascades (Cantrell, 1996). Membrane localisation of Raf by Ras is essential for the activation of Raf (Stokoe *et al*, 1994). Raf-1 interacts with activated $p21^{Ras}$ and couples it to the MAP kinases. Raf-1 is phosphorylated and can then activate MAP kinase kinases e.g. MEK1, which can then stimulate MAP kinases ERK1 and ERK2 (Robinson and Cobb, 1997) which are able to activate transcription factors. An important transcription factor is Elk-1 that interacts with the serum response factor (SRF) to regulate fos gene expression. Fos proteins are important components of the AP-1 complex and therefore important activators of cytokine gene expression. AP-1 activity is also regulated by Jun gene expression, the activation of a different MAP kinase pathway leads to Jun kinase activation (JNK1 and 2). Activation of JNKs augments the transcriptional activity of c-jun which positively autoregulates its own transcription (Angel *et al*, 1988). TCR stimulation leads to the activation of JNK1 and 2 which is important as it was found to be a point of convergence for both CD28 and TCR signalling pathways, full activation of JNK was only achieved by simultaneous stimulation of the TCR and CD28 (Su *et al*, 1994).

3.2 CD28 Signalling

CD28 is also able to induce signals within T cells, but via pathways that are distinct from those delivered by the TCR complex (Fraser, Straus and Weiss, 1993; Rudd,

1996; Ward *et al*, 1993). It is also able to cause proliferation and cytokine production of resting human T cells, when stimulated by a specific mAb, without requirement for TCR co-engagement (Ward *et al*, 1992). Stimulation of CD28 lowers the activation threshold of T cells and increases response longevity. One of the main functions of CD28 is to augment the immune response by upregulating IL-2 production because the IL-2 promoter contains a CD28 response element required for the CD28-mediated activation of IL-2 transcription (Shapiro *et al*, 1997). CD28 lacks intrinsic tyrosine kinase activity and therefore requires phosphorylation. Lck and fyn were found to be associated with the CD28 signalling complex (Hutchcroft and Bierer, 1994) and are therefore responsible for this phosphorylation. There is an activation-dependent stimulation of protein tyrosine kinase activity in CD28 immune complexes; CD28 is phosphorylated at a conserved phosphotyrosine residue within its cytoplasmic tail that serves as a binding site for SH2 containing proteins (August and Dupont, 1994). This occurs following CD28 cross-linking with anti-CD28 mAb or B7 ligation (Truitt *et al* 1994). CD28 interacts with three intracellular proteins – PI-3kinase, ITK, and GRB2/SOS an interaction which is dependent on the phosphorylation of tyrosine residues on CD28 by lck and fyn (Raab *et al*, 1995). PI-3K binds to tyrosine phosphorylated CD28 and is therefore anchored to the membrane by this interaction, this increases its kinase activity, allowing it to act on its substrates to produce D3-phosphoinositides (August and Dupont, 1994; Truitt, Hicks and Imboden, 1994). The homologue of CD28, CTLA-4 despite being classed as an inhibitory receptor, has also been shown to bind PI3-kinase in a manner similar to CD28 therefore providing evidence that CTLA-4 is coupled to intracellular signalling pathways (Schneider *et al*, 1995). CD28 is also capable of binding to the SH2 domain of Grb-2, which is bound to Sos. CD28 activates an array of downstream signalling molecules initiated predominantly by activation of Ras, Rac/cdc42 that activate MAPK, SAPK/JNK and p38 kinases (Kaga *et al*, 1998; Rudd, 1996). CD28 is also able to activate sphingomyelinase pathways generating phosphatidylcholine and ceramide (Edmead *et al*, 1996; Boucher *et al*, 1995) providing additional signalling pathways. Ceramide has many downstream targets, which also include activation of Ras/Raf, JNK and PKCζ. Stimulation of T cells with CD3 and CD28 or CD3 with ceramide enhances the activity of p21-activated kinase and MEK kinase 1. The Rac/cdc42 pathway was therefore postulated to transduce and facilitate cross-talk between CD28 costimulatory

signals and the TCR signal (Kaga *et al*, 1998). CD28 ligation is necessary for AP-1 activity and the CD28 response element within the IL-2 promoter contains an AP-1-NF-AT binding site. Therefore following T cell activation TCR signalling via calcineurin-calmodulin pathways would provide NF-AT while CD28 ligation would contribute AP-1 which could then complex with NF-AT and bind the promoter.

Following TCR activation a number of important nuclear complexes are induced which are important for lymphokine gene expression (Fraser, Straus and Weiss, 1993) including NF-κB, AP-1, NF-AT. The signalling cascades initiated by TCR transmit the signals to the nucleus culminating in transcription factor activation, for example the Ras/Raf pathway induces c-fos transcription (Karin, 1995), c-fos is an important member of the AP-1 complex involved in the induction of many immediate early genes.

4 Prevention of Rejection

4.1 Tissue Matching

Graft rejection can be prevented or at least delayed significantly by matching of MHC antigen. The degree of matching of class I antigens in kidney transplants has been shown to lead to different graft survival times (Festenstein, McCloskey and Yacoub, 1990). Rejection episodes in patients following heart transplantation has also been shown to correlate with HLA-DR matching (Smith *et al*, 1995). There was found to be a significant association between improved graft survival and HLA-DR mismatching over one, five, and ten years after transplant.

One technique to help prevent rejection is to type peripheral blood lymphocytes from proposed recipients using specific anti-sera against MHC molecules (Kirkpatrick, 1987). It relies on the fact that antisera against known MHC molecules will react with antigens present on the lymphocyte surface and sensitise them so they are destroyed when complement is added to the culture. This can thus determine which MHC molecules are present on the cell surface and the most compatible donor can then be selected.

4.2 *Immunosuppression*

T cells play a fundamental role in the process of graft rejection. Upon activation by antigen they proliferate and secrete various cytokines and proinflammatory mediators that influence the subsequent activation of B cells, macrophages, NK cells and cytotoxic T lymphocytes. Therefore inhibition of alloreactive T cells by immunosuppressive drugs is essential for inhibiting the immune response and preventing allograft rejection. Most of the currently used immunosuppressive therapies have their limitations, for example cyclophosphamide can produce haemorrhagic cystitis while cyclosporin can cause nephrotoxicity, fibrosis and hypertension (Pankewycz *et al*, 1996). Other adverse effects associated with immunosuppression include an increased susceptibility to infection by fungi and

viruses. There is therefore a need for new immunosuppressive drugs with reversible, lymphocyte selective, antiproliferative effects that are free of serious side effects. Some of the drugs currently available are described below. They are usually given in combination with steroids, which are non-specific anti-inflammatory agents, and/or azathioprine a non-specific inhibitor of DNA synthesis, which allows drug dosages to be reduced in order to decrease potential side effects.

4.2.1 Cyclosporin and FK506

Cyclosporin A (CsA) and FK506 are two drugs that both inhibit early events in Th cell activation leading to inhibition of IL-2 production (Schreiber and Crabtree, 1992). They act within several hours of T cell activation inhibiting transcription of early cytokine genes, for example IL-2, -3, -4, TNF and IFNγ. Effects on T cells are dependent on the inhibition of IL-2 secretion as addition of exogenous IL-2 rescues the cells from the antiproliferative effect of CsA and FK506. CsA and FK506 bind to the intracellular proteins cyclophilin and FK binding protein (FKBP) respectively (Wiederrecht, 1993). These two proteins belong to a family called immunophilins, which possess prolyl-cis-trans isomerase activity (Galat, 1993) although this activity is unrelated to the immunosuppressive actions of the two drugs. Binding of the drug inhibits the isomerase activity but the actual effect of the drug is to form an inhibitory complex with the immunophilin that can now interact with other proteins (Flanagan *et al*, 1991; Schreiber and Crabtree, 1992). The major target for the drug-immunophilin complex is the calcium/calmodulin regulated serine/threonine phosphatase calcineurin (Clipstone and Crabtree, 1993). Immunophilin-drug complexes formed by both CsA and FK506 bind with high affinity to calcineurin, which is ubiquitously expressed, and inhibit its activity (Fruman *et al*, 1992). Calcineurin is necessary for the assembly of a transcription factor called nuclear factor of activated T cells (NF-AT) which is involved in the expression of many cytokine genes. NF-AT is composed of two subunits, a T cell specific pre-existing cytosolic component (NF-AT1) (Rao, 1994) and an inducible ubiquitously expressed nuclear component (NF-ATa) (Flanagan *et al*, 1991) that contains members of the AP-1 family. NF-AT is phosphorylated in resting cells but upon activation, the increase in intracellular calcium concentration after TCR

stimulation leads to activation of calcineurin phosphatase activity. Calcineurin is able to bind directly to NF-AT1, causing it to be rapidly dephosphorylated (Jain *et al*, 1993; Navia, 1996), which allows it to translocate to the nucleus where it binds to the DNA response element along with the nuclear subunit NF-ATa. A CD28 mediated signalling pathway is also present that can activate NF-AT mediated IL-2 transcription but by a CsA resistant pathway (Ghosh *et al*, 1996). It is by inhibition of calcineurin-regulated dephosphorylation of NF-AT1 that CsA and FK506 inhibit T cell activation (Wiederrecht *et al*, 1993; Clipstone and Crabtree, 1993; Thomson, Bonham and Zeevi, 1995). CsA and FK506 have also been shown to partially inhibit NF-κB responses (Mattila *et al*, 1996; Mattila, 1990) because calcineurin can stimulate NF-κB transcription by enhancing the inactivation of IκB which produces an increase in nuclear NF-κB DNA binding (Frantz *et al*, 1994).

4.2.2 Rapamycin

Rapamycin (Rapa) is a macrolide antibiotic that works by blocking T cell responses to IL-2 and other cytokines (Abraham and Wiederrecht, 1996). It binds to the immunophilins, but is only able to bind to FKBP. However, rapamycin-FKBP complexes are unable to bind and inhibit calcineurin phosphatase activity. Instead the rapamycin-FKBP complex interacts with its target called the mammalian target of rapamycin (mTOR) and interferes with the ability of cells to progress from G1 to S phase of the cell cycle (Abraham and Wiederrecht, 1996; Flanagan and Crabtree, 1993). The antiproliferative effects of rapamycin are not restricted to lymphocytes because it also inhibits hepatocyte, endothelial cell, fibroblast and smooth muscle cell proliferation (Gregory *et al*, 1993). Rapamycin inhibits the activation of the 70-kDa S6 kinase family ($p70^{s6k}$), a family of proteins induced by growth factors in T cells (Calvo *et al*, 1992). $P70^{s6k}$ regulates the multiple phosphorylation of 40s ribosomal protein S6, which in turn regulates translation of a class of mRNAs containing oligopyrimidine tracts at their translational start site that encode many of the components of the protein synthetic apparatus (Pullen and Thomas, 1997). The ability of rapamycin to interfere with cell cycle progression is thought to be due to effects on the cell cycle inhibitor Kip1, which inhibits G1 cyclin-cdk2 complexes (Abraham and

Wiederrecht, 1996). Rapamycin is thought to inhibit the downregulation of Kip1 protein following IL-2 stimulation maintaining G1 cyclin-cdk2 complexes saturated with Kip1 (Nourse *et al*, 1994).

4.2.3 Leflunomide

Leflunomide inhibits dihydroorotate dehydrogenase (DHODH) an enzyme in the *de novo* pathway of UMP synthesis. Its antiproliferative effects are due to inhibition of pyrimidine synthesis as they are reversed by exogenous uridine (Brazelton and Morris, 1996; Cao *et al*, 1995; Cherwinski *et al*, 1995b, c; Cao *et al*, 1995). Lymphocytes are more sensitive to the effects of leflunomide due to them having limited amounts of DHODH compared to normal cells and also resting lymphocytes have smaller pyrimidine pools which are required to increase dramatically (8-fold) following T lymphocyte stimulation (Fairbank *et al*, 1995). Leflunomide is therefore a potent inhibitor of T lymphocyte proliferation (Chong *et al*, 1993, 1996; Cherwinski *et al*, 1995a, b). Leflunomide also inhibits proliferation and antibody secretion in B cells and decreases the tyrosine phosphorylation of JAK3 and STAT6, induced by IL-4 receptor stimulation (Brazelton and Morris 1996; Siemasko *et al*, 1998). Leflunomide has also been shown to inhibit the activity of fyn and lck in both Jurkat and CTLL-4 cells (Xu *et al*, 1995).

4.2.4 Monoclonal Antibodies

Antibody therapy is used for the induction of immunosuppression allowing the withholding of immunosuppressants until stable allograft function is established (Perico and Remuzzi, 1997). For example OKT3 (an anti-CD3 antibody) or antithymocyte globulin (a polyclonal anti-lymphocyte antibody) can be given in the immediate post-transplant period. They both cause the depletion of T cells from the circulation. Because of the crucial role for IL-2 in the initial T cell activation, anti-IL-2 receptor antibodies have been used successfully in an attempt to reduce the incidence of rejection (Waldman and O'Shea, 1998). Monoclonal antibodies against

adhesion molecules have also been exploited as potential anti-rejection therapies (Perico and Remuzzi, 1997; Berlin *et al*, 1992; Heeman *et al*, 1994b; Paul *et al*, 1993). Studies using antibodies to different costimulatory molecules have shown promising results in prolonging graft survival time (Pearson *et al*, 1997; Hancock *et al*, 1996; Woodward *et al*, 1996; Sayegh *et al* 1995; Larsen *et al* 1996).

5 Mycophenolic Acid

5.1 Purine nucleoside biochemistry

The importance of intact purine nucleotide synthesis pathways in lymphocytes, which are required for normal immune responses, is highlighted by the genetic deficiencies of purine nucleoside phosphorylase and adenosine deaminase. In these conditions patients display severe T- or T- and B- lymphocyte depletion and impaired responses to mitogens (Gudas *et al*, 1978; Giblett *et al*, 1972). Mitogen stimulated T cells require increased *de novo* synthetic pathways to provide additional ribonucleotide precursors necessary for RNA and DNA synthesis, repletion of energy stores, second messenger precursors and purine and pyrimidine sugars for membrane lipid synthesis and glycosylation. In mitogen stimulated T lymphocytes, pyrimidine and purine ribonucleotide pools expand disproportionately. Purine concentrations increase two-fold but the greatest increase is seen in pyrimidine nucleotides which increase up to 8-fold. This may be necessary for the expansion in membrane biosynthesis (Fairbank *et al*, 1995).

In normal mammalian cells, guanine and adenine nucleotides are manufactured from small precursor molecules through a *de novo* pathway or by recycling purine bases through a salvage pathway (See figure 3) (Sievers *et al*, 1997; Allison *et al*, 1993a; Allison and Eugui, 1993; Stryer, 1988). Phosphoribosyl-1-pyrophosphate (PRPP) is synthesised from ATP and ribose 5-phosphate. The committed step in *de novo* purine synthesis is then the formation of 5-phosphoribosylamine from PRPP and glutamine. This then undergoes a series of reactions culminating in the formation of inosine monophosphate (IMP), the purine base of inosine monophosphate is called hypoxanthine. IMP is the precursor for both AMP and GMP. For the synthesis of guanine nucleotides it must first be converted to xanthosine monophosphate (XMP) before being converted to GMP. The salvage pathway for purine synthesis is a much simpler route and involves the transfer of the ribose-phosphate moiety of PRPP directly onto free guanine bases, a reaction that requires the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRTase).

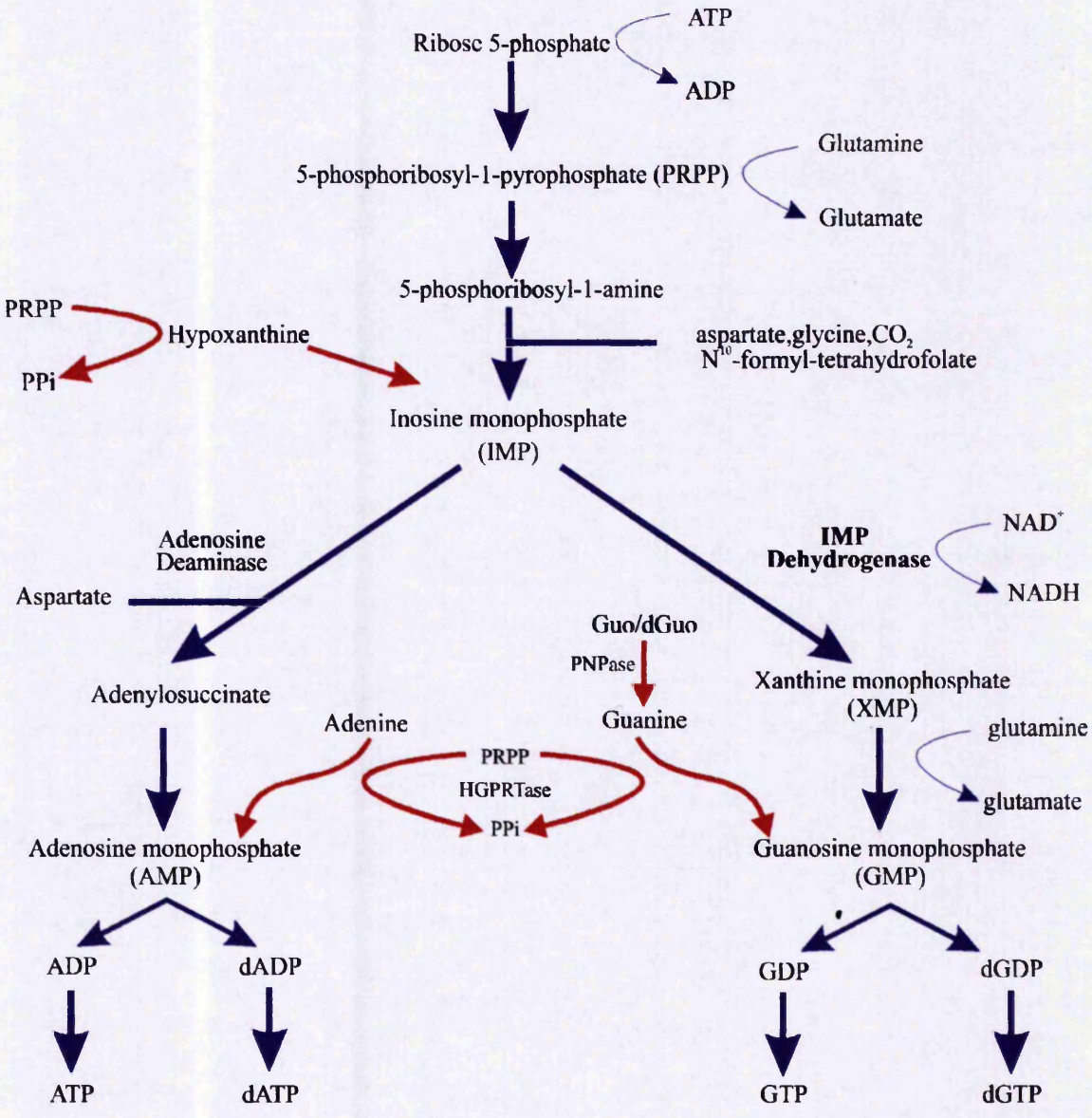


Figure 3. Pathways for the synthesis of purine nucleotides. The diagram shows the central position for IMPDH in GMP synthesis. MPA inhibits the activity of IMPDH and therefore the supply of guanosine nucleotides via the *de novo* pathway. The salvage pathway is highlighted by the red arrows and the *de novo* pathway by the blue arrows. Figure adapted from Sievers *et al* 1997.

PRPP is therefore essential for guanosine nucleotide synthesis by either salvage or *de novo* pathways. The activities of two key enzymes in the de novo pathway, PRPP synthetase and ribonucleotide reductase, are regulated by nucleotide levels (Allison *et al*, 1993a; Allison and Eugui, 1993). PRPP synthetase is inhibited by adenosine nucleotides and activated by guanosine nucleotides while the activity of ribonucletide reductase is decreased by dATP and stimulated by dGTP. Therefore adequate supplies of guanosine nucleotides are required for the activity of these enzymes.

Resting lymphocytes meet their metabolic requirements by salvage pathways of purine nucleotide synthesis. But upon stimulation with mitogen (e.g. PHA) there is an increase in salvage pathway activity but more importantly there is also activation of de novo purine biosynthesis (Barankiewicz and Cohen, 1987). This higher activity of de novo synthesis in proliferating lymphocytes compared to resting cells might be due to failure of the salvage pathway to provide sufficient nucleotides (Marijnen *et al*, 1989). Deoxyguanosine is effectively activated to dGTP by salvage pathways but the dGTP pool generated has limited or no access to DNA replication (Nguyen and Sadée, 1986). Cellular dGTP pools have virtually no access to DNA replication, so guanine must be recruited from ribonucleotide precursor pools e.g. GDP, GMP so that there is a small functional dGTP-DNA precursor pool. Using guanine tracers in S-49 lymphoma cells, [^{14}C] guanine was shown to be rapidly incorporated (<5min) into DNA indicating that there is only a small functional guanine-DNA precursor pool (Duan and Sadée, 1987). This is in contrast to adenine nucleotide pools where there was a 30-minute delay before [^{3}H] adenine was incorporated into DNA. Other studies have shown that it is the level of GTP that is essential for DNA synthesis. It has been shown that the depletion of GTP rather than dGTP produces the toxic effects of mycophenolic acid, a potent inhibitor on inosine monophosphate dehydrogrenase, in S-49 cells (Cohen, Maybaum and Sadée, 1981) and also in CEM cells (Nguyen, Cohen and Sadée, 1983).

The importance of the *de novo* pathway in human lymphocytes is highlighted by the fact that patients suffering from Lesch-nyhan syndrome characterised by the absence of HGPRT, an essential enzyme in salvage pathways of purine synthesis, still have normal T lymphocyte proliferation and function (Allison *et al*, 1975). Patients have abnormal B cell function, suggesting this pathway may be important in B cells, and

suffer from mental retardation and have a tendency for self mutilation suggesting that the brain has very little *de novo* purine biosynthesis

5.2 Inosine monophosphate Dehydrogenase (IMPDH)

IMPDH is the rate-limiting enzyme in *de novo* synthesis of guanine nucleotides. It catalyses the NAD^+-dependent oxidation of inosine monophosphate to xanthosine monophosphate at the branch point in the purine metabolic pathway. IMPDH is therefore essential for providing the necessary precursors for DNA and RNA synthesis. IMPDH activity is mediated by two isoenzymes denoted IMPDH type I and type II both of which contain 514 amino acids and they have a high degree of sequence homology with 84% identity (Natsumeda and Carr, 1993). IMPDH activity is tightly linked with cellular proliferation and is also associated with malignant transformation (Jackson and Weber, 1975; Huberman, Glesne and Collart, 1995). A decrease in the level of IMPDH mRNA was observed in lymphocytes relative to leukaemic cell lines, which showed an increase in expression of type II IMPDH mRNA (Collart *et al*, 1992). IMPDH activity plays a role in regulating the maturation process and declining levels of IMPDH protein and activity are consistent with differentiation to a mature state (Kiguchi *et al*, 1990). Inhibitors of IMPDH, such as MPA, have been shown to induce terminal differentiation in different tumour cell lines (Kiguchi *et al*, 1990), and also inhibit DNA synthesis by arresting cells in G1 (Laliberté *et al*, 1998). This effect is dependent on decreasing the level of guanosine nucleotides, as it is reversible by the addition of exogenous guanosine. Inhibitors of IMPDH are potent inducers of maturation in HL-60 cells a promyelocytic cell line (Knight *et al*, 1987; Sokoloski Blair and Sartorelli, 1986; Collart and Huberman, 1990). Guanosine nucleotide depletion has been shown to induce terminal differentiation in HL-60 cells (Sokoloski *et al*, 1993). Calcium binding proteins MRP8 and MRP14 form a non-covalently linked complex that is induced during terminal differentiation of cultured human monomyelocytic leukaemic cells. This complex inhibits casein kinase II and is thought to mediate the growth inhibition necessary for terminal differentiation. In HL-60 cells cultured with MPA, MRP8 and MRP14 expression was induced, which was due to an increase in transcription

initiation (Warner-Bartnicki *et al*, 1993). The same effect was also seen with 1,25-$(OH)_2$ Vit D_3 which also induces differentiation but by completely different mechanisms.

Decreasing the levels of cellular guanosine nucleotides produces an increase in the steady state level of IMPDH mRNA suggesting that IMPDH expression is itself regulated by the level of purine end products (Glesne, Collart and Huberman, 1991). It appears that IMPDH gene expression is regulated post-transcriptionally in response to fluctuations in intracellular guanine nucleotides. Treatment of HL-60 cells with MPA decreases the levels of guanosine ribonucleotides, which causes an increase in IMPDH mRNA levels. The changes in IMPDH gene expression were shown to be due to alterations of IMPDH mRNA in the nucleus suggesting a post-transcriptional regulation of expression.

Different levels of the 2 isoforms of IMPDH are found in different tissues (Senda and Natsumeda, 1994), high levels of type I IMPDH are found in kidney, pancreas, colon, peripheral blood lymphocytes and foetal heart, brain and kidney. The type II transcript was found to be higher than type I but far less variable. Previous reports suggested that type II mRNA was specifically increased in mitogen stimulated lymphocytes while the expression of type I mRNA remained constitutive (Nagai, Natsumeda and Weber, 1992). However, more recent reports show that there is an increase in mRNA levels of both IMPDH type I and type II (Dayton *et al*, 1994). In human leukaemic cell lines there were increased amounts of both type I and type II enzymes. But the increase in IMPDH activity observed did appear to be due to specific upregulation of type II IMPDH which was predominantly expressed in leukaemic cells (Konno *et al*, 1991; Nagai *et al*, 1991). In contrast, in normal lymphocytes/leucocytes type I mRNA is the dominant species (Natsumeda and Carr, 1993).

Despite the high degree of sequence homology it appears that the two isoforms are differentially regulated. Regulation of IMPDH type I is determined by the use of three alternative promoters (Gu, Spychala and Mitchell, 1997) which gives rise to three different sized transcripts. Given the previous reports suggesting the importance of IMPDH type II it may well be that appropriate regulation of IMPDH type I gene expression is important in T cell mitogenic responses. Differential regulation at the

transcriptional level from the different promoters may give rise to highly tissue- or cell-specific regulation.

IMPDH type II, located on chromosome 3 (Glesne *et al*, 1992), on the other hand is transcribed from a single promoter and its transcriptional upregulation may involve events such as secondary protein-protein interactions and/or post-translational modifications of pre-bound transcription factors (Zimmerman *et al*, 1997). Analysis of the IMPDH type II promoter (Zimmerman, Spychala and Mitchell 1995) has suggested also that a major transcriptional component may be responsible for upregulation of type II IMPDH, several important regulatory sites were found in the 5'-flanking region which may be important for IMPDH type II expression.

MPA is a potent, non-competitive reversible inhibitor of IMPDH that has been shown to be a more potent inhibitor of IMPDH type II, which has a 4.8-fold lower K_i than the type I isoform (Carr *et al*, 1993). The selective inhibition of the inducible type II isoform may thus avoid any potential toxicity caused by inhibition of the constitutive type I isoform. MPA binds non-competitively to IMPDH with respect to its substrates and in the inhibited state the reduced NADH cofactor has been released, but XMP has not been produced resulting in MPA and an IMP intermediate being simultaneously bound in the active site of the enzyme (Sintchak *et al*, 1996). Upon inhibition of IMPDH by MPA, the GMP pool is the only pool that is largely depleted. Inhibition of IMPDH by MPA causes an immediate and dramatic inhibition of DNA synthesis before guanosine nucleotide pools other than GMP are depleted (Nguyen and Sadée, 1986). In Molt F4 human malignant lymphoblasts 0.5mM MPA was found to maximally inhibit IMPDH activity inhibiting cell growth and depleting guanine nucleotides (Stet *et al*, 1994)

5.3 MPA Pharmacology

MPA is administered as mycophenolate mofetil (MMF) the morpholinoethylester prodrug of MPA, which has an increased bioavailability compared with MPA due to enhanced absorption (Lee *et al*, 1990; Sievers *et al*, 1997; Shaw *et al*, 1995). After administration MMF is rapidly de-esterified to MPA by the liver (Bullingham, Nicholls and Hale, 1996) and then undergoes metabolism by glucuronidation in the

liver although extrahepatic glucuronidation also occurs in the renal tubular cells and gut wall as well (see figure 4). MPA glucuronide (MPAG) is the sole metabolite of MPA and has no activity (Bullingham, Nicholls and Hales, 1996). It is eliminated in the urine and also excreted into bile (Sweeney, Hoffman and Esterman, 1972) where it undergoes extensive hepatic recirculation and is reconverted to MPA by intestinal microflora by β-glucuronidase activity. Plasma concentrations are therefore found to peak twice following administration of a single dose of MPA (Bullingham, Nicholls and Hales, 1996). This may also be the reason for one of the main side effects of MPA, which are gastrointestinal tract (GIT) disturbances probably due to elevated levels of MPA found in the GIT system (Platz *et al*, 1991a).

In the circulation MPA is extensively bound to human serum albumin (HSA) (Nowak and Shaw, 1995), an interaction that has a substantial effect on its interaction with its targets. As concentrations of HSA increase the degree of MPA binding increases therefore decreasing the free MPA fraction and therefore the degree of IMPDH inhibition. This has implications for people with altered liver and kidney function who will experience altered levels of HSA and will therefore need to have the dose of MPA carefully monitored. MPA interactions with HSA are reversible, it has been shown to be displaced from HSA by high concentrations of MPAG and also salicylate. These compounds will therefore increase the free fraction of the drug.

5.4 Effects on Cellular Proliferation and Functions

MPA is a potent inhibitor of nucleic acid synthesis (Franklin and Cook, 1969) and suppresses proliferative responses of human peripheral blood B and T lymphocytes in response to mitogens and allogeneic lymphocytes (Eugui *et al*, 1991). GTP depletion was found to inhibit DNA synthesis due to an inhibition of primer RNA synthesis (Catapano *et al*, 1995), a process thought to be highly sensitive to intracellular GTP concentration. Short RNAs of 8-10 nucleotides (primer RNAs) are synthesized at the replication fork and are then elongated by DNA polymerase to form RNA-primed DNA fragments. These RNA primers have either GTP or ATP almost exclusively at their 5' termini. GTP is also preferred over ATP due to its ability to form three hydrogen bonds therefore enhancing the stability of the primer-DNA interaction.

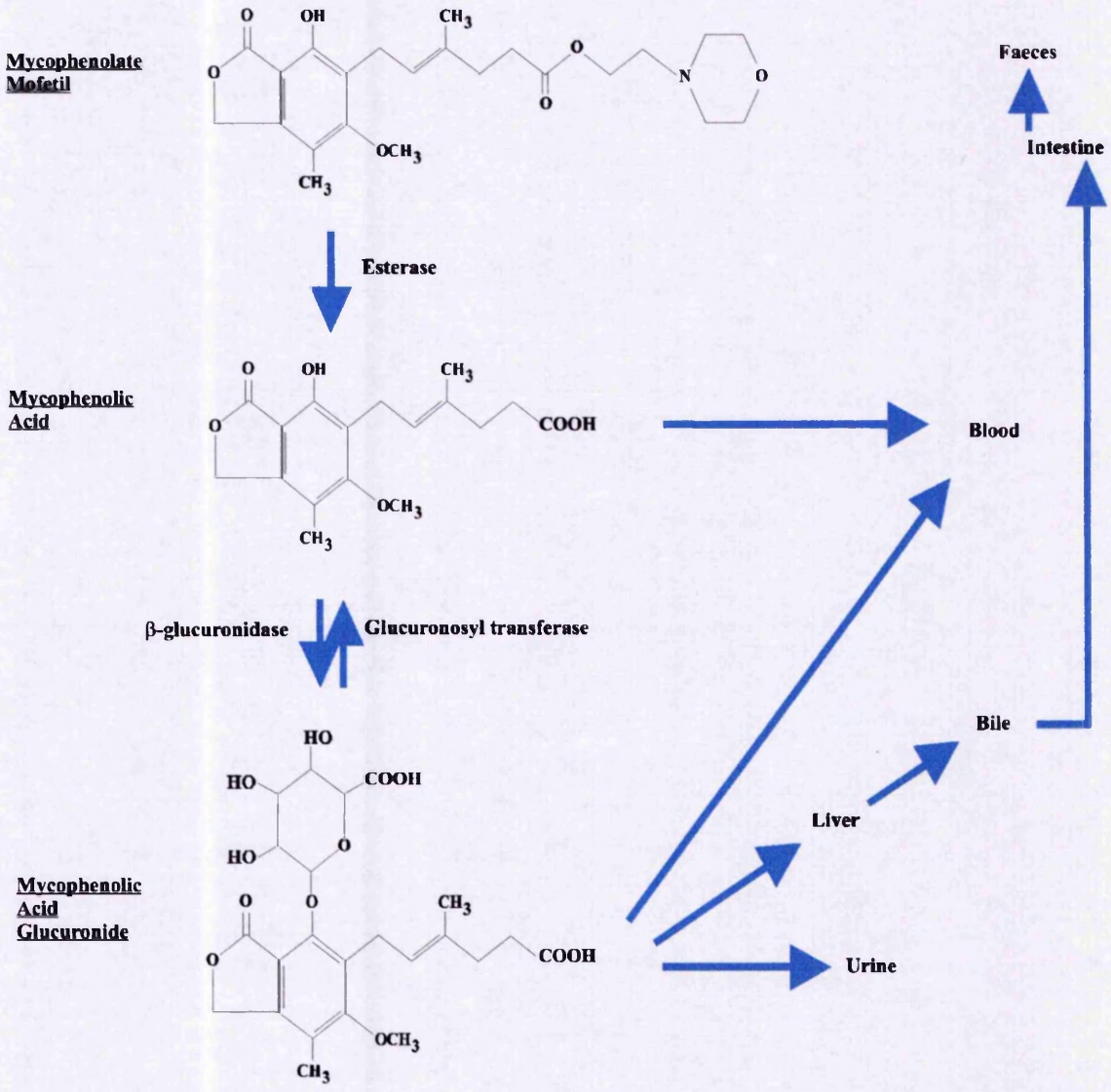


Figure 4. The metabolism and excretion of mycophenolic acid. The diagram shows the structure of mycophenolate mofetil and its derivatives including the sites of interconversion and methods of excretion. MMF is rapidly de-esterified in the liver to form MPA, which then undergoes glucuronidation to form MPA glucuronide. MPAG is then excreted in the urine or into the bile, where it undergoes hepatic recirculation. (Figure adapted from Allison and Eugui 1994).

Synthesis of RNA-primed DNA fragments is therefore sensitive to levels of guanosine nucleotides. This is thought to be the principal effect on lymphocytes exerted by MPA.

MPA is a potent inhibitor of lymphocyte proliferation inhibiting the proliferative response of lymphocytes to mitogens (Eugui *et al*, 1991) an effect that is reversible by the addition of exogenous guanosine or deoxyguanosine. MPA markedly depletes pools of GTP and dGTP after only 6 hours of culture and it is still inhibitory to mixed lymphocyte cultures when added later, even after 72 hours, suggesting it affects late events following antigenic stimulation.

Cell cycle arrest induced by MPA depleting guanosine nucleotides produces a decrease in the expression of cyclins D3, D2 and CDK6 and maintenance of high levels of $p27^{Kip1}$ an inhibitor of cell cycle progression (Laliberté *et al*, 1998).

Fibroblasts and endothelial cells are less sensitive to MPA than lymphocytes, but at higher concentrations MPA has also been shown to inhibit their proliferation (Eugui *et al*, 1991). MPA inhibits growth factor induced proliferation of vascular smooth muscle cells and endothelial cells (Mohacsi *et al*, 1997) highlighting their dependence on the *de novo* pathway for purine synthesis. This finding is important as smooth muscle cell and endothelial cell hyperplasia and growth factor production are important mediators of chronic rejection (Azuma *et al*, 1994).

These *in vitro* experiments have also been performed *in vivo* where MPA has been shown to significantly reduce intimal thickening following balloon catheter arterial injury in rats (Gregory *et al*, 1995) which is used as a model of the response of vessels to immune injury caused by rejection. MPA has also been shown to be able to suppress the generation of cytotoxic T cells in mice immunized with allogeneic cells (Eugui, Mirkovich and Allison, 1991a, b).

Decreases in GTP concentration has been shown to be critical in differentiation induced by MPA in leukaemic cells and restoration of GTP pools blocked this differentiation (Sokoloski, Blair and Sartorelli, 1986).

Macrophages are only moderately sensitive to MPA as they are non-proliferative and therefore have no requirement for DNA synthesis. MPA does appear to induce other metabolic effects such as the induction of differentiation of human macrophage precursor cell lines and inhibition of monocyte adhesion to the endothelium (Laurent *et al*, 1996). The effect of MPA (0.1-1μM) on monocytes is an inhibition of

proliferation followed by an induction of differentiation into macrophages (Waters, Webster and Allison, 1993). This may be important because monocytes produce inflammatory cytokines such as TNFα and IL-1. Differentiation of monocytes into macrophages is accompanied by a restriction of their capacity to produce IL-1 but the acquisition of the ability to produce IL-1ra (Roux-lombard, Modoux and Dayer, 1989). Drug-induced acceleration of the differentiation of monocyte-macrophage lineage cells could therefore modify the balance of pro-inflammatory cytokines versus cytokine antagonist production and thus exert long-acting anti-inflammatory effects.
Experiments with rat mast cells found MPA to decrease the release of 5-HT from granules in cells sensitised with IgE and triggered with antigen (Mulkins, Ng and Lewis, 1992). This effect was also reversible with exogenous guanosine. But IgE receptor mediated PGD_2 production was unaffected by MPA suggesting that adequate levels of guanosine nucleotides are only required for granule exocytosis in mast cells. Antigen-stimulated secretion by rat basophilic leukaemia cells is inhibited by depleting cells of GTP with MPA (Smith Wilson, Deanin and Oliver, 1991) an effect the authors propose to be due to inactivation of G proteins required for this secretion. There have also been several other reports of interference of G-protein function by depletion of cellular guanine nucleotide levels (Rizzo *et al*, 1990).

5.5 Effects on Cytokine Production.

MPA was found to have no effect on IL-1 production by human peripheral blood monocytes induced by LPS (Eugui *et al*, 1991). It was also shown that following stimulation of human peripheral blood mononuclear cells with LPS or PMA + ionomycin in the presence of MPA (10^{-6}M) there was no effect on IL-4, -5, -10, IFNγ, TNFβ, TNFα or GM-CSF production. The exception was at 48 hours when there was a significant decrease in IL-3 production (Nagy, Andersson and Andersson, 1993). By contrast following stimulation with super antigen (*S.aureus* enterotoxin A) a more physiological stimulus, which occurs via the T cell receptor, at 48 hours MPA inhibited significantly all cytokine production.
Similar effects were seen in rats receiving maintenance MMF following renal allografting. The production of all lymphocyte and macrophage derived cytokines was

inhibited throughout the 32-week follow-up period (Nadeau, Azuma and Tilney, 1996). In a separate experiment, MPA was shown to have no effect on cytokine production by Con A stimulated mouse spleen cells. The levels of IL-2 and IL-10 mRNA were the same as controls (Lemster *et al*, 1992).

In mice injected with allogeneic cells, MMF caused a significant decrease in IFNγ production. However in mice injected with LPS, MMF produced considerably less inhibition of IFNγ production (Lui *et al*, 1998). The effect on IFNγ production in response to allogeneic cells was probably due to effects on T cell proliferation leading to a decrease in the net IFNγ production while IFNγ production in response to LPS is a T cell independent polyclonal response. This explains why MMF only had a small effect on LPS-induced IFNγ production as it inhibits lymphocyte proliferation and therefore clonal responses, but spares polyclonal responses.

5.6 Effects on Antibody Production

MPA can inhibit antibody formation by polyclonally activated human B cells (Eugui *et al* 1991; Eugui, Mirkovich and Allison, 1991b; Smith *et al*, 1998). MPA was shown to be highly effective at blocking human tetanus-specific IgG memory responses of human splenocytes (Burlingham *et al*, 1991). Exposure to equine derived antithymocyte immunoglobulin frequently results in production of anti-ATGAM antibody production thus lowering the efficacy of the treatment. But concomitant administration of renal transplant patients with MMF and ATGAM leads to a decreased incidence and reduced titre of anti-ATGAM IgG antibody formation compared with azathioprine controls (Kimball *et al*, 1995). In a separate study, renal transplant patients were given influenza vaccine and then their antibody responses were measured. Patients receiving MMF instead of azathioprine were found to have suppressed humoral responses to influenza. MMF has also been used to successfully treat a patient with IgA nephropathy (Nowack, Birck and van der Woude, 1997). Experiments in mice have shown MPA to inhibit immunoglobulin formation in response to sheep erythrocytes (Eugui, Mirkovich and Allison, 1991a). In models of hyperacute rejection using rats presensitised with skin grafts prior to heart allografting,

rats receiving MPA had similar levels of antibody as controls (Knechtle *et al*, 1992), suggesting MPA has no effect on the antibodies themselves only on their production.

5.7 MPA and Protein Glycosylation

Glycoproteins are proteins containing oligosaccharide chains covalently attached to specific amino acid residues within the polypeptide chain. The carbohydrate chains vary between different glycoproteins, ranging from disaccharides to complex oligosaccharide structures (Hughes, 1983). There are two types of carbohydrate linkage to proteins; N-linked glycoproteins have carbohydrate chains attached to asparagine residues, while O-linked glycoproteins have the carbohydrate chain linked to serine, threonine, hydroxylysine or hydoxyproline residues. An individual glycoprotein may contain more than one carbohydrate unit attached at different positions either by N- or O- linkages or both. Glycosylation is species- and cell-specific, but protein structure is the major determinant of the extent to which a given protein is glycosylated.

Glycoprotein synthesis involves the transfer of sugar residues from carrier molecules to an acceptor molecule, which is the growing oligosaccharide chain. The carrier molecules for the monosaccharides are nucleotides (see below):

Sugar	Activated Form
Galactose (Gal)	UDP-gal
Mannose (Man)	GDP-man
N-acetylglucosamine (glcNAc)	UDP-glcNAc
N-acetylgalactosamine (galNAc)	UDP-galNAc
Fucose (Fuc)	GDP-fuc
N-acetylneuraminic acid (Sialic acid)	CMP-neuNAc
Xylose (Xyl)	UDP-xyl
Glucuronic Acid	UDP-glcUA

N-glycan synthesis involves a common lipid intermediate called dolichol. Oligosaccharide sugars are attached to dolichol before transfer to the polypeptide

chain (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985). The initiation of precursor oligosaccharide synthesis always involves the transfer of UDP-glcNAc to dolichol phosphate followed by the transfer of a second glcNAc from UDP-glcNAc. A β-mannosyl residue then attaches to the donor, this time being GDP-man. The next steps are the transfer of a further eight mannose and three glucose residues to form a branched structure. This oligosaccharide moiety is then transferred en bloc to asparagine in the polypeptide chain. The sequence Asn-X-serine/threonine (Ser/Thr) is always found internally in the glycosylated polypeptide chain.

The oligosaccharide then undergoes extensive modification known as processing after transfer to the polypeptide. Soon after attachment to the polypeptide, the glucose residues are removed followed by six mannose residues to leave a core consisting of man_3-$glcNAc_2$. In the course of processing the stable man_3-$glcNAc_2$ components of mature glycoproteins are formed. N-glycans generally fall into two categories, high mannose and complex, they both have an inner core consisting of man_3-$glcNAc_2$ but high mannose glycans contain additional mannose residues (usually 2-6) while complex oligosaccharides carry other sugars including glcNAc, gal, fuc, sialic acid.

O-glycan synthesis involves the direct transfer of sugars from nucleotide intermediates to the polypeptide, the sugars being linked to either serine or threonine residues. O-linked glycans vary in size from monomers to oligomers of up to 20 constituents (Lis and Sharon, 1993). Larger O-glycans are common in mucins, which are common in epithelial secretions and as membrane constituents (Hughes, 1983). Typical constituents of O-glycans are GlcNAc accompanied by Gal, GlcNAc, L-fuc, various sialic acid or sulphate. The most common O-glycosidic link is that between glcNAc and ser/thr without any other sugars attached to it, such glycoproteins are virtually always found in nucleoplasmic and cytoplasmic compartment (Haltiwanger *et al*, 1992). T lymphocyte activation has been shown to induce rapid changes in the levels of O-linked GlcNAc present on nuclear and cytosolic proteins suggesting it plays an important role in T cell activation (Kearse and Hart, 1991).

Glycoproteins are important constituents of lymphocyte cell surfaces where they act as receptors and ligands involved in immune regulation. T cell activation has been shown to lead to changes in the expression of cell surface carbohydrates (Sutton, Stoddart and Hutchinson, 1992).

MPA has been reported to inhibit the transfer of mannose residues to glycoproteins (Allison *et al*, 1993b) using fluorescent lectins specific for mannose. This is thought to be due to the dependence of both mannose and fucose on GTP for their transfer to glycoproteins. Adhesion molecules are extensively glycosylated and MPA was shown to decrease the incorporation of ^{3}H-mannose into VLA-4 (Allison *et al*, 1993b). It was shown that lymphocyte-endothelial cell attachment between PHA activated T cells or IL-1 activated endothelial cells treated was decreased by treatment of either cell type with MPA. This is probably due to decreased expression of the particular ligand on the activated cells or possibly by interfering with glycosylation alters the affinity for the molecules for their ligands (Allison *et al*, 1993b). In sarcoma 180 cells, MPA treatment led to a decrease in the rate of incorporation of [2-^{3}H] mannose and fucose into glycoproteins (Sokoloski and Sartorelli, 1985). Intracellular GTP levels were decreased by 80% with no change in the levels of ATP, but there was an increase in UTP levels. In monocytes treated with MPA, they retain normal cytokine and cytotoxicity responses but they showed a decrease in the mannosylation of high molecular weight glycoproteins and a decrease in adhesion specifically to laminin with no significant effects on collagen or fibronectin adhesion (Laurent *et al*, 1996). These results are in contrast to the effects seen with expression of cell adhesion molecules on MPA treated TNFα-induced human umbilical vein endothelial cells (HUVEC) (Hauser *et al*, 1997). Pretreatment of HUVECs with MPA before TNFα activation lead to an increase in binding of U937 cells as well as an increase in VCAM-1 and E-selectin surface expression. The effect was though to be due to increased mRNA stability for VCAM-1 and E-selectin. Similar results have also been obtained with MPA causing an upregulation of ICAM-1 expression on IFNγ-stimulated, as well as resting, HUVECs despite the fact that GTP levels were decreased by about 70% (Bertalanffy *et al*, 1998). In a separate experiment looking at the *in vivo* effects of MMF on lymphocyte binding to allografts opposite results were found (Heeman *et al*, 1996). Grafts in MMF treated animals survived longer than controls and had better preserved graft structure. Cellular infiltration was also decreased by MMF, particularly macrophages and neutrophils, but with similar numbers of T cells. There was decreased binding of lymphocytes to the graft but ICAM-1 and VCAM-1 expression was almost identical between MMF and controls while LFA-1 was

decreased. This was interpreted as an effect of MMF on lymphocytes only and not endothelial cells.

5.8 Inhibition of Proliferative Arteriopathy

Vascular injury which occurs during the process of chronic rejection is associated with a proliferative arteriopathy caused by proliferation of smooth muscle cells and fibroblasts, leading to arterial intimal thickening. Both humoral and cellular mechanisms are thought to be involved in the pathogenesis of the process. Various inflammatory cells contribute to the intimal hyperplasia as they infiltrate the vascular wall causing the production of inflammatory cytokines, growth factors and upregulation of adhesion molecules (Azuma *et al*, 1995).

The effects of MPA on this process of progressive arteriopathy have been investigated in both rejecting rat kidney allografts (Azuma *et al*, 1995) and also in arteries after mechanical injury by balloon catheter (Gregory *et al*, 1995). After injury by balloon catheter, dead smooth muscle cells, endothelial cells and platelets release an array of growth factors to initiate smooth muscle cell activation and proliferation. This leads to significant arterial intimal thickening with failure of the endothelium to regrow. However treatment with MPA plus rapamycin suppressed the progressive intimal thickening and endothelial regrowth was seen. The development of intimal thickening is thought to depend on bFGF that may play a key role in the early response to vascular smooth muscle cell injury.

In chronically rejecting kidneys there is usually a gradual functional decline associated with vascular obliteration, glomerulosclerosis, tubular atrophy and fibrosis. There is a macrophage infiltration into glomeruli and around vessels plus growth factor and cytokine expression as well as increased adhesion molecule expression. MPA treatment of rat renal allografts (Azuma *et al*, 1995) dramatically decreased the macrophage and lymphocyte infiltrate allowing the graft to remain almost normal in appearance. There was also a much smaller deposition of IgG and C3, which are thought to contribute to intimal proliferation and arterial obliteration, in MPA treated recipients. It was also shown that MPA was effective regardless of when treatment was initiated whether it was started at the time of engraftment or after eight weeks.

In a rat arterial stenosis model of vascular injury, which shows similar changes to that occuring during the rejection process, MMF was shown to reduce significantly the total vessel wall thickness as well as the neointimal thickness (Fraser-Smith. Rosete and Schatzman, 1995). MPA also significantly decreased the adventitial inflammation seen in a rat aortic transplant model of chronic rejection (Räisänen-Sokolowski, Myllärniemi and Häyry, 1994).

This effect of MPA in preventing arterial smooth muscle cell proliferation, apart from rapamycin, makes it unique amongst immunosuppressive drugs.

5.9 Effects of MPA on EBV-Transformed Lymphocytes

One of the potential consequences of immunosuppression is an increased risk of lymphoma development, particularly polyclonal B cell lymphomas with Epstein Barr Virus (EBV) being implicated in the majority of cases (Starzl *et al*, 1984). In one study (Savoie *et al*, 1994) EBV^- patients pretransplant experienced primary EBV infection within 3 months after transplantation and developed a higher EBV infected cell burden in peripheral blood than in patients who were EBV^+ pretransplant. It was also found that patients who were EBV^- pretransplant had a higher risk of developing post-transplant lymphoproliferative disease compared to patients who were EBV^+ pretransplant. Usually EBV-transformed lymphocyte proliferation is kept under control by cytotoxic T cells, which cause regression of this EBV-driven outgrowth. In immunocompromised and immunosuppressed patients, T cell mediated surveillance is inhibited so that transformed B cells are allowed to proliferate uncontrolled (Young *et al*, 1989). It has been shown that CsA abolishes the regression of EBV outgrowth and also causes an increase in the frequency of spontaneous transformation of cultured lymphocytes to EBV virus genome positive lymphoblastoid cells (Rickinson *et al*, 1984; Crawford *et al*, 1981). By contrast MPA has been shown to inhibit proliferation of newly infected or established EBV-transformed cell lines (Alfieri, Allison and Kieff, 1994) and therefore has the benefit of reducing the risk of development of EBV-related lymphoproliferative disease.

MPA has also been shown to be useful in the treatment of other viral infections. For example it has been shown to be effective for the treatment of herpes simplex virus

(HSV) infection when given in combination with the current anti-viral drugs acyclovir, gancyclovir and PCV (Neyts, Andrei and de Clercq, 1998; Pancheva, Roeva and Remichkova, 1997). These drugs are phosphorylated by viral and cellular kinases to triphosphorylated metabolites, which inhibit viral DNA polymerases. It was therefore proposed that by decreasing endogenous levels of dGTP with MPA there would be less competition for the drugs and therefore better inhibition of viral DNA polymerases. This has important implications because immunosuppression increases the risk of viral infection, but MPA may be able to potentiate the effects of the antiviral drugs, which are administered to treat the disease.

MMF has also been shown to be effective in avoiding *Pneumocystis carinii* infection when compared with FK506, rapamycin and steroids (Oz and Hughes, 1996). *Pneumocystis carinii* infection is a life-threatening disease for immunocompromised patients and it was found that rats injected daily with FK506, dexamethasone or rapamycin had increased susceptibility to *P.carinii* pneumonitis while MPA treated rats stayed free from infection. This is another potentially beneficial effect of MPA for the treatment of transplant patients.

5.10 MPA and Nitric Oxide Production

MPA has been shown to decrease cytokine-induced nitric oxide (NO) biosynthesis in mouse and rat vascular endothelial cells (Senda *et al*, 1995). NO is synthesized from L-arginine by nitric oxide synthase. There are two types of NOS in mammalian cells, constitutive NOS whose activity is $Ca2^{+}$/calmodulin and NADPH-dependent, and inducible NOS which is induced by cytokines and is NADPH/BH_4-dependent as well as Ca2+/calmodulin dependent. This inducible isoform releases larger amounts of NO for longer periods after stimulation than the constitutive isoform. Several cofactors are required for NOS activity such as BH_4, which is synthesized from GTP by the activity of GTP cyclohydrolase I (Hatakeyama, Harada and Kagamiyama, 1992). This cofactor is rate limiting for cytokine-inducible NOS activity. It was found therefore that MPA, via IMPDH inhibition and GTP depletion reduces BH_4 levels and thus decreases the NO production. Recovery of NO production could be achieved by the addition of exogenous guanosine. Production of NO by endothelial cells and

macrophages at sites of inflammation could have damaging effects on the tissues so this additional effect of MPA on NO production could act further to increase the chance of graft survival.

5.11 MPA and Psoriasis

MPA has been used investigationally for the treatment of psoriasis since 1971 (Epinette *et al*, 1987) because lymphocytes are the predominant inflammatory cell type found in psoriatic lesions (Haufs *et al* 1998). Such lesions also have an increased rate of cellular proliferation and MPA may therefore also affect keratinocyte proliferation. The epidermis also has a high β-glucuronidase activity and is therefore capable of reactivating MPA glucuronide to MPA (Gomez, Michaelover and Frost, 1977). Long-term studies have shown MPA to be an effective psoriasis suppressant however not all patients responded to treatment (Epinette *et al*, 1987). Oral MMF has been used to successfully treat a patient with severe psoriasis such that the condition cleared up, with no need for further treatment, after three months of treatment (Haufs *et al*, 1998). MMF has also been shown to be effective in the treatment of other skin disorders such as bullous pemphigoid (Böhm *et al*, 1997).

5.12 MPA and Drug Interactions

Experiments *in vitro* have shown that FK506 in combination with MPA was able to inhibit cell proliferation to the level seen with 10-fold higher concentrations of FK506 when used alone (Thomson *et al*, 1993). The effect of MMF in combination with other immunosuppressants was also determined in vivo in renal transplant patients (Zucker *et al*, 1997a, b). There was little effect of MPA on FK506 pharmacokinetics, but there was a significant increase in trough MPA levels and MPA AUC values of patients receiving tacrolimus (FK506) and MMF when compared with patients receiving the same dose of MMF with CsA. Patients receiving tacrolimus and MMF also averaged significantly lower levels of MPAG than patients receiving CsA and

MMF. The reasons for these effects of FK506 on the pharmacokinetics of MPA remain to be determined.

The effect of food and antacids on MMF pharmacokinetics was also investigated in rheumatoid arthritis patients (Bullingham *et al*, 1996). Feeding caused a decrease in MPA C_{max} and a decrease in t_{max} but with no change in overall AUC. The secondary plasma MPA peak that occurs at 6hrs post-dose when fasting was delayed with food to around 8hrs. MPAG C_{max} was found to increase with feeding with no change in t_{max}. Antacids containing aluminium and magnesium were found to decrease both C_{max} and AUC for both MPA and MPAG. It was concluded though that the differences seen were small and unlikely to have clinically significant effects

5.13 MPA and Side Effects

Most adverse effects seen with MMF are mild and rarely require discontinuation of treatment (Silverman Kitchin *et al*, 1997; Sievers *et al*, 1997). The most common side effects of MMF are gastrointestinal disturbances, symptoms are dose dependent and patients usually respond to dose division with smaller doses given more frequently. Symptoms associated with these disturbances include nausea, diarrhoea and abdominal cramps. MPA can also produce genitourinary symptoms such as urgency, frequency, dysuria and burning although the symptoms decrease after the first year of treatment. Reversible dose-related haematologic effects including anaemia, leukopenia, neutropenia and thrombocytopenia have also been reported. There is also an increased risk of opportunistic infection although not exceeding acceptable limits and characteristic for immunosuppressed patients (Sollinger *et al*, 1992b). There has been one report of MMF causing pulmonary toxicity, which can simulate opportunistic pneumonia (Gross *et al*, 1997) and may lead to severe pulmonary fibrosis. Occasional neurologic side effects including weakness, tiredness, headache and tinnitus may also occur but the symptoms seem to decrease after several years of therapy.

Unlike CsA and FK506, nephrotoxicity and hepatotoxicity are infrequent (Platz *et al*, 1991a).

5.14 Mycophenolic Acid Effects in Vivo

Several trials have been performed to determine the *in vivo* effects of MPA in both humans and animals and shown that MMF is effective *in vivo* at reducing the incidence of transplant rejection in both humans and animals (Sievers *et al*, 1997). Many studies have been performed to evaluate the use of MMF in renal, liver and cardiac transplant patients (Pirsch and Sollinger, 1996). Immunosuppressive effects of MPA were investigated using renal allografts in dogs (Platz *et al*, 1991b). Triple therapy of 20mg/kg/day MPA with CsA and prednisone produced a significant increase in survival time compared with controls receiving CsA and prednisone alone. This combination also allowed a reduction in the dosage of CsA and prednisone administered. Side effects of MPA seen were minimal but an increase in dosage was associated with gastro-intestinal tract symptoms such as gastritis and diarrhoea. A study to test the safety and tolerance in patients receiving primary cadaver kidneys was carried out (Sollinger *et al*, 1992a) which showed mycophenolate mofetil to produce no major adverse effects or toxicity. In separate studies of renal cadaveric kidney transplantation (Sollinger, 1995; Behrend, Lueck and Pichlmayr, 1997; Pichlmayr *et al*, 1995), MMF produced a significant decrease in acute rejection episodes compared with azathioprine when given in combination with CsA and steroids. The time to first rejection episode was also significantly longer in MMF treated patients. Successful long-term rescue was achieved in 69% of human patients showing signs of allograft rejection resistant to other forms of therapy (Sollinger *et al*, 1993). The result of long-term MPA treatment (2 years) was also investigated (Deierhoi *et al*, 1993) and MPA was found to be generally well tolerated in renal transplant recipients at doses of up to 3,500 mg/day for periods of up to two years. Few side effects were observed and also few infections, with eight infections in five patients out of a total of forty-nine in the original trial. The use of steroids as part of triple therapy regimens produces multiple side effects. It was found that it was possible to gradually taper the dose of prednisolone over a period of 2-4 months while maintaining CsA and MMF, without leading to increases in acute rejection episodes and with continued stable renal function (Grinyo *et al*, 1997). MMF is also capable of reversing on going rejection in refractory kidney transplant rejection due to the failure of conventional anti-rejection therapy (Sollinger *et al*, 1992b; Sollinger *et al*, 1993).

Patients receiving simultaneous pancreas-kidney transplants have extremely high incidences of acute rejection (Stegall *et al*, 1997). The use of MMF in combination with either FK506 or CsA decreased rejection significantly compared with historical treatments of CsA and azathioprine, but the follow-up period has so far been short, therefore longer periods of observation are required. MMF in combination with FK506 has also been shown to be effective in the treatment of acute-steroid-resistant rejection following kidney transplant (Carl *et al*, 1998). Patients developed stable renal function although there was an increase in opportunistic infections. Similar beneficial effects have been shown for acute steroid-resistant liver rejection (Platz *et al*, 1997).

MMF is beneficial in the treatment of a rat model of chronic rejection (Azuma *et al* 1995). MMF treated recipients had normally functioning kidneys with decreased macrophage and lymphocyte infiltration and decreased levels of serum IgM and IgG.

MMF prolonged heart allograft survival in both rat (Morris *et al*, 1990) and primate heterotopic heart allograft models (Morris *et al*, 1991). In human heart transplant recipients, MMF has been shown to be safe and well tolerated, when used in combination with CsA and corticosteroids, to produce significant immunosuppression (Ensley *et al*, 1993). When substituted for azathioprine it resulted in decreased incidence of rejection and allowed decreases in the doses of corticosteroids used.

In models of cardiac xenograft rejection MMF has also been shown to result in a reduced vascular rejection and prolonged xenograft survival (O'Hair, McManus and Komorowski, 1994). Islet xenotransplantation has also been effectively prolonged with MMF immunosuppression (Beger and Menger, 1997).

MMF given in combination with FK506 has been shown to be effective in prevention of acute liver rejection (Platz, Mueller and Neuhaus, 1997; Eckhoff *et al*, 1998). MMF was also shown to be effective in the treatment of therapy-resistant liver rejection with twenty-one out of twenty-three patients responding to treatment (Klintmalm *et al*, 1993). The ability of MMF to prevent composite tissue graft rejection was also investigated; composite tissue allografts are highly antigenic and often require two to three times greater immunosuppressant doses than for organ transplants. It was demonstrated that a combination of low, sub-therapeutic doses of MMF and CsA prevented rejection in 89% of rat limb allografts followed for 231-254 days (Benhaim *et al*, 1996).

6 Aims and objectives

Many of the immunosuppressive actions of MPA are not accounted for by its antiproliferative properties and there are many aspects of the mode of action of MPA that remain to be resolved. The effects of guanosine nucleotide depletion appear to have many more implications than inhibiting lymphocyte proliferation and the effects on other aspects of cellular activation require a much greater understanding. The general hypothesis addressed in this thesis was that the effect of MPA is not merely due to its antiproliferative actions, but that it may have more subtle immunosuppressive effects as well. By using various techniques I have explored different avenues of the mechanism of action of MPA at both the cellular and molecular level. One aim was to investigate more thoroughly the effect of MPA on cell surface protein glycosylation in both lymphocytes and endothelial cells with the objective of determining whether the effect of MPA is specific to a particular cell type. The second aim was to study the effect of GTP depletion on intracellular signalling pathways to determine whether MPA selectively affects the activation and function of different transcription factors.

These experiments will therefore help define more specifically the mechanism(s) of action of MPA and allow better immunosuppressive regimens to be designed.

Methods

1 Materials

All materials and solutions used in the following experiments are described in the accompanying text.

2 Cell Culture

Cell culture was conducted in Class II safety cabinets with unidirectional laminar down-flow (MDH Intermed).

2.1 Cell Lines

CEM-C7a cells were kindly provided by Dr Ged Brady, School of Biological Sciences, University of Manchester. Rosalind Gunby (and Dr Ged Brady) also kindly provided the stably transfected CEM-C7a cell lines NRE-27 and TRE-8. ECV304 (human umbilical vein endothelial cells) and Jurkat E6.1 cell lines were obtained from The European Collection of Animal Cell Culture (ECACC).

2.2 Culture Medium

All cell culture reagents were obtained from Sigma (Poole Dorset, UK) unless otherwise stated. Cell culture plastics were obtained from Costar, again unless otherwise stated.

Jurkat E6.1 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 2mM glutamine, 1X non-essential amino acids and 100units penicillin/0.1mg/ml streptomycin.

CEM-C7 cells were cultured in suspension in Optimem (Gibco brl) supplemented with 5% fetal calf serum, 100units penicillin, 0.1mg/ml streptomycin and 2mM glutamine.

ECV304 cells were cultured as adherent monolayers in Medium 199 supplemented with

10% foetal calf serum, 100units penicillin/0.1mg/ml streptomycin solution and glutamine 2mM.

3 Routine Maintenance of Cell Lines

All cell cultures were maintained at 37°C in 5% CO_2 and passaged every 3-4 days. Adherent monolayers were washed with phosphate buffered saline (PBS) and trypsinised into single cell suspension by treatment with trypsin-EDTA (Gibco) before passage.

Cell counts were performed by trypan blue exclusion, briefly 20µl of cell suspension was diluted in 180µl trypan blue (0.04% in PBS)(Sigma) and viable cells counted using a Neubauer haemocytometer (0.1mm)

4 Dissection of Rat Lymph Node Cells

Some experiments in the earlier work used rat lymph node cells (LNCs) dissected from female PVGRT1^{u}7B rats aged 8-10 weeks. Briefly, rats were killed by inhalation of ether and the mesenteric, cervical and axilliary lymph nodes were dissected out and place in universal tubes, kept on ice, containing Hanks Balanced Salt Solution (HBSS, supplemented with 1% fetal calf serum). Under sterile conditions, the lymph nodes were forced through a wire mesh into a petri dish containing HBSS (1% fetal calf serum). The suspension was transferred to a universal tube, the volume made up to 25mls with 1% HBSS, and centrifuged at 1500rpm for 5mins (MSE Mistral 2000). The supernatant was removed and the cell pellet resuspended in 20ml 1% HBSS and centrifuged again as before. This was repeated once more and the cell pellet finally resuspended in 10ml RPMI (supplemented as for Jurkat cells plus 1mM sodium pyruvate, 10mM hepes solution) and counted by trypan blue exclusion (as above).

5 Separation of Human Peripheral Blood Mononuclear Cells

Initial work also used human peripheral blood mononuclear cells (HuPBMC) purified from the blood of normal healthy volunteers in the laboratory. Blood was removed into vacutainers containing EDTA (Beckton Dickinson) then layered on top of an equal volume of Histopaque (Sigma) in Falcon tubes. Tubes were centrifuged at 3000 rpm for 20mins (MSE Mistral 3000) and the buffy coat, containing the mononuclear cells, was removed into a new tube with a Pasteur pipette. RPMI medium (supplemented as for rat LNCs) was added up to 25mls and the tubes centrifuged at 1500rpm for 10mins (MSE Mistral 2000) to remove excess histopaque. Cell pellets were then washed twice by resuspending in 10ml RPMI and centrifuging at 1500rpm for 5mins before resuspending in a final volume of 10mls for counting by trypan blue exclusion as before.

6 Measuring Cellular Proliferation by ^{3}H-thymidine Incorporation

Cells at $1x10^{6}$ cells/ml were cultured in 96-well flat bottom plates in the presence of various treatments for 72hr in a humidified incubator (37°C, 5% CO_2). Cells were pulsed for the final 24hrs by adding 1μCi of ^{3}H-thymidine (Amersham) into each well. The Cells were then harvested (Dynatech Multimash 2000) onto filter paper and dried in scintillation vials (Sarstedt) in an oven (37°C) for 1-2hrs. 2ml of scintillation fluid (Optiphase Hi-safe, Fisons chemicals) was added to each vial and thymidine incorporation measured using a scintillation counter (Beckman LS1801) as described by the manufacturers.

7 Analysis of Cell Surface Glycoproteins by Flow Cytometry

Cells at $1x10^{6}$cells/ml were cultured for 24, 48 or 72hr at 37°C, 5% CO_2 in a humidified incubator in 24 well plates (suspension cells) or 12-well plates (adherent cells). Before staining the cells, cultures were counted by trypan blue exclusion using a haemocytometer and $5x10^{5}$ cells removed into FACS tube (Alpha Laboratories). Tubes

were centrifuged at high speed for 1minute (Sorvall cell wash centrifuge). The cell pellet was washed twice by resuspending in 500µl of DAB-2-azide (500mls DAB containing 2% FCS, 20mM sodium azide) with centrifugation as before between washes. After the final wash, 20µl of FITC conjugated lectin (see appendix B) at the optimal dilution was added to the pellet which was then vortexed, before covering and incubating on ice for 30 minutes. The cells were harvested by centrifugation as before and washed in 500µl DAB-2-azide 3 times as before. After the final wash the cells were resuspended in 200µl PBS and 200µl 2% formaldehyde (2% formaldehyde diluted from 40% stock solution with PBS) to fix them before analysing (Beckton Dickinson Facscan).

8 Determination of Transcription Factor Binding by Electrophoretic Mobility Shift Assay (EMSA)

8.1 Preparation of Whole Cell Extracts

2ml of cells ($1x10^6$ cells/ml) were cultured for 24, 48, 72 hours at 37°C, 5% CO_2 in a humidified incubator in tissue culture flasks (Costar). Cells were harvested into 25ml universal tubes and centrifuged at 1500rpm for 5 min (MSE Mistral 2000). The pellet was washed twice by resuspending in PBS and centrifuged as before. The cell pellet was resuspended in 50µl of extraction buffer (20mM hepes pH7.8, 450mM NaCl, 0.2mM EDTA, 25% glycerol, 0.5mM DTT, 0.5mM PMSF) and transferred to 0.5ml Eppendorf tubes. The samples were then frozen (dry ice/liquid nitrogen) and thawed (37°C water bath) three times before centrifuging for 10 minutes in a microcentrifuge (MSE Microcentaur, 13000rpm). Supernatants were aliquoted and stored at -70°C.

8.2 Bradford Assay for Estimation of Protein Concentration

The protein concentrations of the whole cell extracts were determined by Bradford assay against standards of known protein concentrations (2μg, 4μg, 8μg, 10μg, 15μg, 20μg) prepared from Bovine serum albumin (BSA 1mg/ml)(Sigma). All volumes were made up to 800μl using dilution buffer (25mM tris-HCl pH7.9, 5mM $MgCl_2$, 0.1mM EDTA, 100mM KCl, 10% glycerol, 1mM DTT 0.2 mM PMSF) and then 200μl dye reagent (Biorad) was added. The samples were vortexed briefly and left to stand for 5mins before transferring to disposable cuvettes (Sarstedt) and measuring the absorbance at 595 O.D (Pharmacia Ultrospec III). A standard curve was calculated from the BSA standards and the concentration of protein in the unknown samples determined from this.

8.3 Preparation of Radioactive Probes

Consensus oligonucleotides (Promega, see appendix B) were labelled with [^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Promega). Labelling reactions contained:

Consensus oligonucleotide (1.75pmol/ml)	2μl
T4 Polynucleotide kinase	1μl
T4 Polynucleotide Kinase Buffer	1μl
[^{32}P] ATP (3000Ci/mmol at 10mCi/ml)	1μl
dH_2O	5μl

Reactions were incubated at room temp. for one hour before purifying the radiolabelled oligonucleotide from unincorporated [^{32}P]ATP by sephadex G50 column chromatography. Briefly, a 1ml syringe was plugged with non-absorbent cotton wool, and filled with sephadex G-50 (suspended in TE Buffer). The syringe was inserted through a hole in the lid of a 15ml Falcon tube then centrifuged at 1500rpm for 5min

(MSE Mistral 2000) to compact the sephadex G-50. More G-50 was added to the column in a similar manner until a volume of 0.8ml was obtained. The oligonucleotide labelling reaction was added to the top of the column and centrifuged for 5mins at 3000rpm (MSE Mistral 3000). Radiolabelled oligonucleotide was collected in a 1.5ml Eppendorf tube placed underneath the column in the 15ml Falcon centrifuge tube. The volume collected in the Eppendorf was made up to 100µl with TE buffer (10mM Tris-HCl pH8.0, 1mM EDTA pH8.0) and stored at -20°C.

8.4 Binding Reactions

Binding reactions were prepared as follows: 2µl 5x binding buffer (20% glycerol, 5mM $MgCl_2$, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 250mM Tris-HCl pH 7.5), 1µl radiolabelled probe, 1µl Poly[(dI-dC•dI-dC)] (2µg/µl) (Sigma), 7µg whole cell extract and distilled water up to a total volume of 10µl. Reactions were incubated at room temp. for 1hr before adding 2µl of loading buffer (250mM Tris-HCl pH7.5, 0.2% bromophenol blue, 40 % glycerol) to each tube before loading onto the gel.
The amount of extract used was 7µg, which was always kept constant.

Four control reactions were always included to assess the specificity of the probe. The Cold probe in competition assays was added in excess.

	Negative control	Positive control	Specific competitor	Non-specific competitor
5x binding buffer	2µl	2µl	2µl	2µl
HeLa Cell extract	-	1µl	1µl	1µl
Hot probe	1µl	1µl	1µl	1µl
Cold Probe	-	-	1µl	-
Cold "other" probe	-	-	-	1µl
Poly(dI-dC)	1µl	1µl	1µl	1µl
Distilled water	up to 10µl	up to 10µl	up to 10µl	up to 10µl

8.5 Gel Preparation

A 9% polyacrylamide gel was prepared containing 15ml Ultrapure Protogel 37.5:1 (National Diagnostics), 5ml 10xTBE (890mM Tris base, 890mM Boric Acid, 32mM EDTA), 30ml dH_2O, 250µl 10% ammonium persulphate (sigma), 90µl TEMED (Biorad). The gel was poured into the casting apparatus (Genetic Research Instrumentation) and left to polymerise for 1 hour. The gel was transferred to the electrophoresis tank (Genetic Research Instrumentation) and pre-run for 30mins at 100v in 1x TBE buffer before the binding reactions were loaded. Samples were resolved at room temp. at 150v until the dye front reached two-thirds of the way down the gel. The gel was fixed in 10% acetic acid and 10% methanol for 20mins before drying onto 3mm Whatman filter paper at 80°C for 1-2hrs under vacuum. The gel was exposed to autoradiographic film (Kodak Biomax MR-1) in a developing cassette (Kodak X-Omatic) overnight at –70°C. Films were developed using an automated developer (Kodak M35-M X-OMAT Processor).

9 Molecular Cloning and DNA Manipulation

9.1 Bacterial Transformation

9.1.1 Preparation of Competent Cells

E.coli DH5α (Promega) cells were streaked onto L-agar plates (5g Tryptone, 2.5g NaCl, 2.5g yeast extract, 0.5g glucose, 5g agar in 500ml dH_20) and grown overnight at 37°C. Single colonies were transferred into 5ml LB broth (as for L-agar but without 5g agar) and shaken vigorously (250-275rpm) in an orbital shaker overnight at 37°C. The 5ml culture was added to 100ml of pre-warmed LB broth and shaken until culture turbidity was equal to 0.6 (O.D. at 550nm). The cultures were chilled on ice for 5mins before centrifugation for 5mins at 6000rpm at 4°C (Beckman centrifuge JA110 rotor). The bacterial pellet was gently resuspended in 50ml ice cold TfB1 (30mM potassium

acetate, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% v/v glycerol, pH adjusted to 5.8 with 0.2M acetic acid and then filter sterilised) and chilled on ice for 5mins. The cells were centrifuged for 5mins at 6000 rpm 4°C and resuspended in 5ml ice-cold TfBII (10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% v/v glycerol, pH adjusted to 6.5 with KOH and filter sterilised) and placed on ice for 30mins. 100µl aliquots of cells were snap frozen in pre-chilled (dry ice) 2.5ml nunclon screw cap cryotubes, and stored at -70°C.

9.1.2 Plasmid Transformation of Competent Cells

1µl of plasmid DNA solution was added to 80µl DH5α competent cells, thawed on ice, in pre-chilled Eppendorfs and incubated on ice for at least 30mins. The bacteria were heat-shocked by placing in a water bath at 42°C for 90secs before further chilling on ice for 2mins. 4 volumes of SOC recovery medium was added to each tube (2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, autoclaved then add 20mM $MgCl_2$ + $MgSO_4$, 20mM glucose). Tubes were then incubated for 1hr with aeration (225-250rpm on an orbital shaker) at 37°C. 100µl of transformed cells was spread onto L-agar plates containing ampicillin (100µg/ml)(Sigma) and incubated at 37°C overnight.

9.2 Plasmid Preparation

9.2.1 Mini-Prep of Plasmid DNA (Boil-Lysis Method)

This method typically produces small amounts of plasmid DNA (1-5µg) from a 3-5ml culture for use when screening bacterial colonies for those with a particular construct following insertion of a DNA fragment into a plasmid.

One colony from the transformation experiment was picked into 2ml LB broth containing ampicillin (100µg/ml) (Sigma) for 4-5 hours at 37°C with aeration (250rpm on an orbital shaker). 1ml of the culture was removed into an Eppendorf tube and

centrifuged at 13000rpm in a microcentrifuge (MSE Microcentaur) for 15 seconds. The pellet was resuspended in 250µl STET buffer (8% v/v sucrose, 0.5% v/v triton-x-100, 50mM EDTA, 10mM Tris-HCl pH8.0) plus 15µl lysozyme (Sigma)(freshly made in TE buffer at 10mg/ml) by vortexing. Samples were placed in a boiling water bath for 40 seconds then immediately centrifuged for 10mins in a microcentrifuge at 13000rpm. The supernatant was transferred into a fresh Eppendorf containing 30µl 3M sodium acetate and 420µl isopropanol, vortexed, then placed on ice for 15mins. Samples were centrifuged for 15mins at 13000rpm in a microcentrifuge, the supernatants were then aspirated and the DNA pellet left to air-dry for 5mins. The pellet was resuspended in 80µl TE buffer plus RNAase (10mg/ml).

For experiments to check if the correct DNA insert had been ligated into the plasmid, 6-16 µl of the suspension was digested with 1µl of the appropriate restriction enzymes plus 2µl of buffer at 37°C for 1-2hrs. The samples were then run out on a 0.8% agarose gel to determine if the plasmid contained the correct insert.

9.2.2 Maxi-Prep for Plasmid Preparation

To obtain larger quantities of high purity plasmid DNA for use in transfections, caesium chloride gradient centrifugation was used. 500ml L-broth (100µg/ml ampicillin) cultures of *E.coli* DH5α were grown overnight in 2.5L flasks with shaking (270rpm on an orbital shaker) at 37°C. The following day, cultures were harvested by centrifuging at 2000xg (Beckman JA10 rotor in Beckman high-speed centrifuge J2-MC) for 10 mins at 4°C. The pellet was gently resuspended in 10ml resuspension buffer (25mM Tris-HCl (pH8.0), 50mM glucose, 10mM EDTA) containing 10mg/ml fresh lysozyme, and incubated at room temp. for 10minutes before adding 20ml lysis buffer (0.2M NaOH, 1% w/v SDS prepared fresh each time from stocks of 5M NaOH and 10% (w/v) SDS). The mixture was inverted several times and incubated on ice for 10 minutes, then 10mls 3M potassium acetate solution (3M, pH5.5) was added and incubated for a further 15mins on ice before centrifuging at 25,000g for 30mins at 4°C in Beckman JA-17 rotor (Beckman J2-MC). The supernatant was poured through gauze into fresh tubes and 0.6 volumes of isopropanol were added. This solution was centrifuged at

20,000xg, 4°C for 30mins in JA-17 rotor and the supernatant discarded. The pellet was washed with 70% ethanol and centrifuged at 20,000g, 4°C for 10mins in JA-17 rotor. The pellet was air-dried then redissolved in 10mls TE buffer (pH 8.0). Exactly 10g of CsCl (Sigma) were added to the TE buffer and mixed gently until completely dissolved then 0.3ml of ethidium bromide solution (10mg/ml Sigma) was added to the solution. Any precipitating lipid was removed by brief centrifugation at 2000xg for 5 minutes at room temp. in JA-17 rotor. The DNA/CsCl/EtBr solution was transferred to Beckman optiseal ultracentrifuge tubes with a syringe and avoiding the formation of air bubbles. The tubes were balanced in pairs to within 0.1g of each other, topping up with CsCl solution (10mg/ml in TE Buffer) if necessary. Tubes were sealed and centrifuged at 450,000xg, 15°C for 16-20hrs in a Beckman ultracentrifuge L-80. The tubes were then removed and clamped to a stand before removing the plasmid DNA (lower band), avoiding the genomic DNA, into a 1.5ml Eppendorf tube using a needle and syringe. The plasmid DNA solution was mixed with an equal volume of water saturated isopropanol (water saturated with CsCl) and vortexed before centrifuging for 5mins at 13000rpm (MSE microcentaur bench top centrifuge). The upper organic layer was discarded and an equal volume of water saturated isopropanol added then centrifuged again. This was repeated until the upper organic layer became colourless. The CsCl was removed by diluting with 2 volumes dH_2O and 6 volumes room temp. 100% ethanol, the samples were placed at -70°C to precipitate the DNA for 1hr. The solution was centrifuged at 15000g for 30mins at 4°C (Beckman JA-17 rotor) and the pellet washed with 70% ice-cold ethanol and vortexed before centrifuging again and repeating the ethanol wash. After the second wash the pellet was air-dryed and resuspended in 1ml dH_2O. The DNA concentration was then determined by spectrophotometry (OD 260) (Pharmacia Ultrospec III).

9.2.3 Isolation/Purification of DNA

9.2.3.1 DNA Extraction from Whole Blood

Blood was removed from normal healthy volunteers into EDTA containing vacutainer tubes (as before) and the DNA extracted using the Promega Wizard Genomic DNA

Purification Kit following the manufacturers instructions. Briefly, 300µl of blood was placed into a 1.5ml sterile Eppendorf containing 900µl of Cell Lysis Solution. The tube was inverted 5-6 times and incubated at room temp. for 10mins before centrifuging at 13000rpm for 20secs (MSE Microcentaur). The supernatant was removed and the pellet resuspended by briefly vortexing the tube. 300µl of Nuclei Lysis Solution and 1.5ml of Rnase solution was added to the resuspended pellet and mixed by pipetting up and down. The solution was incubated for 15mins at 37°C and left to cool before proceeding to the next step. 100µl of Protein Precipitation Solution was added to the tube and vortexed vigorously for 10-20secs and then centrifuged at 13000rpm (microcentrifuge) for 3mins at room temp. The supernatant was transferred to a clean 1.5ml Eppendorf containing 300µl of room temp. isopropanol and gently mixed by inversion until white threads of DNA appeared. The tube was then recentrifuged at 13000rpm for 1min, the supernatant removed, and 300µl of room temp. 70% ethanol added. After centrifuging for a further minute at 13000rpm, the supernatant was again removed and the DNA pellet left to air dry for 10-15mins. 100µl of DNA Rehydration Solution was added to the tube and the DNA rehydrated by incubating at 65°C for one hour, the DNA was then stored at 4°C until required.

9.2.3.2 Phenol/Chloroform Extraction – Ethanol precipitation

DNA was extracted from solutions by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Tubes were then vortexed briefly and spun at high speed for 15secs in a microcentrifuge (MSE Microcentaur) and the top aqueous layer containing the DNA removed into a clean tube. To increase DNA extraction, 100ml of TE buffer (pH 8.0) was added to the remaining organic phase then vortexed and centrifuged as before. One-tenth of the volume of 3M sodium acetate was then added to the solution containing the DNA and mixed by vortexing, before adding 2-2.5 volumes of ice-cold 100% ethanol and vortexing again. The tube was then placed in dry ice/freezer (-70°C) for at least 30mins, and spun in a microcentrifuge at 13000rpm for 15mins. The supernatant was removed and 1ml of room temp. 70% ethanol added. The tube was inverted several times and centrifuged as before then the

supernatant removed and the pellet left to dry. Once dry, the pellet was dissolved in an appropriate volume of dH_2O.

10 Cloning of the IL-2 Promoter

10.1 Polymerase Chain Reaction

10.1.1 Location and Sequence of Primers

Two pairs of primers (Genosys) were designed to amplify the promoter region of the human IL-2 promoter from –361 bp to –48 bp relative to the peptide start codon. The ends of the two primers were designed so that they contained restriction sites that would allow cloning into a suitable vector when the two are cut with the appropriate enzymes.

Primer 1

XhoI

5' – CCGCTCGAGACTTTTGTATCCCCACCCCC – 3'

Primer 2

HindIII

5' – GGGTTCGAAAGGGAACTCTTGAACAAGAGATGC – 3'

10.1.2 PCR Reaction Components

The PCR reaction consisted of DNA template (500ng), 100pM of each primer, and dH_2O up to a final volume of 50µl. 50µl of master mix was then added on top of this, which contained 2.5U Bio-X-act DNA polymerase (Bioline), 1xOPTI buffer (Bioline), 200µM dNTPs, 1.5mM $MgCl_2$ and dH_2O to a final volume of 50µl.

10.1.3 Cycle Conditions

The PCR was conducted in a Techne Thermal Cycler PHC-3 using the following cycle conditions:

Denaturation	94°C	5mins	1cycle
Denaturation	94°C	1min	35 cycles
Annealing	65°C	30secs	
Extension	72°C	30secs	
Final Extension	72°C	10 mins	1cycle

10.2 Restriction Enzyme Digestion

Restriction enzyme digestions were performed in 50µl reaction volumes using the appropriate buffer supplied by the manufacturer (New England Biolabs). Reactions generally consisted of no more than 100ng/µl DNA and 0.1 volumes of enzyme. Digests were incubated between 2-3hours and 48 hours before heat inactivation at 70°C for 10-15minutes. Plasmid digests prior to cloning had 20units of calf intestinal phosphatase (New England Biolabs) added for the final 30 minutes of digestion to prevent the complimentary ends from re-ligating during the ligation reaction.

10.3 Ligation

DNA fragments were joined using T4 DNA ligase (Gibco). A range of ratios of vector to insert (1:1, 1:3, 1:6) were combined with 1x T4 ligation buffer (supplied by manufacturer), 1u T4 DNA ligase and made up to a final volume of 10µl with dH_2O. Reactions were incubated for 12-16 hours at 15°C.

10.4 Cloning of PCR Products

PCR products were gel purified and cloned into Poly-Gal vector kindly provided by Maurice Needham (Zeneca Pharmaceuticals). Both vector and PCR product were cut with the respective enzymes to provide them with complimentary ends. This allows both to be ligated together placing the Il-2 promoter upstream of the Lac Z reporter gene (see figure 5).

11 DNA Sequencing

11.1 Manual DNA sequencing

Manual sequencing was performed by the "Sanger" or "chain-termination" method using T7 Sequenase v2.0 (USB) sequencing kit and the radioactive nucleotide α-[^{35}S]dATP (Amersham).

11.1.1 Template Preparation

0.5-3μg of plasmid DNA, prepared by CsCl maxi-prep, was denatured in a 0.5 ml Eppendorf containing:

0.5-3μg DNA	μl (up to 7μl)
dH_2O	μl (to adjust vol. to 13μl)
Plasmid Denaturing Reagent	5μl
Primer (0.5-2pmol)	1μl
Total volume	13μl

The reagents were mixed thoroughly and then incubated at 90-100°C for 5mins and chilled on ice before adding 2μl of Plasmid Reaction Buffer. The template/primer/buffer mixture was incubated at 37°C for 10mins then chilled on ice.

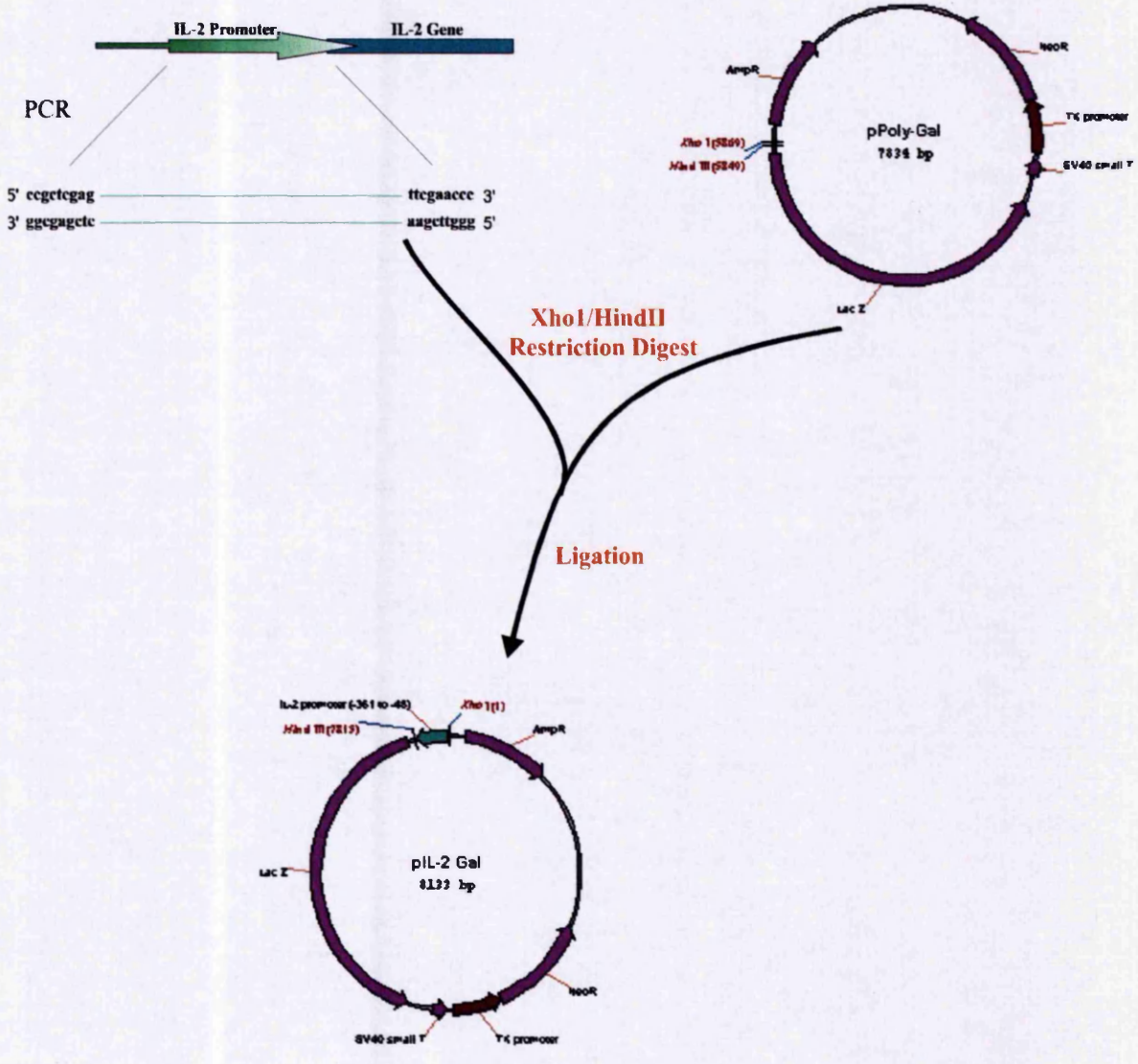


Figure 5 – Cloning of the human IL-2 promoter. Schematic representation of the method for cloning the human IL-2 promoter region. Following PCR amplification of the IL-2 promoter from extracted human DNA (-361 to –48bp), both PCR product and vector were cut with XhoI (24hr) and HindIII (48hrs). Plasmid and PCR fragments were then ligated before checking for insertion by growth of transformed bacteria on agar plates containing ampicillin.

11.1.2 Labelling Reaction

The labelling reactions were set up by adding the following reagents to the ice-cold annealed DNA mixture (15µl):

DTT (0.1M)	1µl
Labelling mix (diluted 1:5 in dH_20)	2µl
[^{35}S]dATP	0.5µl
T7 sequenase plasmid sequencing formulation	2µl

The reaction components were mixed thoroughly by pipetting and incubated at 37°C for 5mins. 1µl of manganese buffer was added to each tube after 5mins to emphasise bands close to the primer

11.1.3 Termination Reactions

4.5µl of labelling reaction was added to prewarmed tubes containing one of four dideoxynucleotide termination mixes; either ddATP, ddCTP, ddTTP, ddGTP. The reaction components were mixed by pipetting and incubated at 37°C for 5mins. Addition of 4µl of stop solution stopped the reaction, then the samples were heated to 75°C for 2mins immediately before loading onto the sequencing gel (3µl per lane).

11.1.4 Gel Preparation

Sequencing gels were prepared 24hrs prior to loading with 26.7ml Protogel (as before), 50g urea and 5ml 20X glycerol tolerant buffer (560mM Tris base, 1.74M taurine, 10mM $Na_2EDTA.2H_2O$), the total volume was adjusted to 100ml with dH_2O. When ready to pour, 1ml of 10% ammonium persulphate and 25µl of TEMED was added. Gels were cast into cleaned sequencing glass plates (Flowgen 33cm x 40cm), an inverted shark-tooth comb inserted and the gel allowed to polymerise.

11.1.5 Gel Electrophoresis

Prior to loading, the sequencing gel was pre-run at 60w for 30mins in 1X glycerol tolerant buffer. 3µl of sample was loaded per well and the gel run at 60w in 1X glycerol tolerant buffer until the dye front reached the bottom of the gel. The glass plates were separated and the gel soaked for 15mins in 5% acetic acid, 15% methanol. The gel was transferred to Whatman 3M filter paper and dried under vacuum at 80°C. The dried gel was then exposed to Kodak autoradiographic film (Kodak Biomax MR-1) for 3 days at room temp. in a developing cassette. Films were developed using an automated developer (Kodak M35-M X-OMAT Processor).

11.2 Automated DNA Sequencing

Samples, prepared by CsCl maxi-prep, were sequenced using the ABI Big Dye DNA sequencing kit (Perkin Elmer Applied Biosystems) that uses fluorescently labelled dideoxynucleoside triphosphates. Reactions were prepared containing:

Sequencing Primer	3.2µl (at 2pM/µl)
Plasmid DNA	2.5µl (at 100ng/µl)
Big Dye sequencing mix	8.0µl
dH_2O	6.3µl

Reactions were mixed well and centrifuged briefly before placing a layer of oil on top. Samples were amplified in a thermal cycler (Perkin Elmer DNA Thermal Cycler 480) for 30 cycles consisting of 96°C for 1min 30sec and 60°C for 5mins.

The products were then purified by ethanol precipitation; the whole reaction mix excluding the layer of oil was removed into a tube containing 2µl 3M sodium acetate (pH 4.6) and 50µl 95% ethanol. The mixture was vortexed briefly and left at room temp. to precipitate for 15mins then spun in a microcentrifuge (MSE Microcentaur) at high speed for 20mins. The supernatant was then removed and 250µl of 70% ethanol

added, the tube was again vortexed briefly and centrifuged at high speed for 5min. The supernatant was removed and the pellet left to dry before sequencing.

12 Recombinant Plasmids

A number of plasmids were used to study the actions of mycophenolic acid on transcription factor activation.

12.1 Reporter Gene Constructs

The following plasmids, pCMV-gal, p4xTRE-Gal, pEX-Gal and pAC-373, were kindly provided by Maurice Needham (Zeneca Pharmaceuticals). The plasmid pTRE-Gal has a tetrad repeat of the TPA response element (TRE, GTGACTAA) and the pEX-Gal construct has three NF-kB response elements (GGAAAGTCCCC), for detailed maps see appendix A.

13 Transient Transfection

During transient transfections, exogenous DNA is not integrated into the cellular DNA but is maintained for short periods of time external to the chromosomes.

13.1 Calcium Phosphate Transfection

Calcium phosphate transfection relies on the formation of calcium phosphate-DNA co-precipitates, which absorb to the cell surface and are subsequently endocytosed.

Cell preparation. Adherent cells for transfection were seeded 24hrs prior to transfection at a density such that they reach 80% confluence in 3-4 days. The medium was changed the morning of the transfection at least an hour before transfecting. All volumes are for transfection in 10cm dishes and can be scaled up accordingly.

Transfection

***$CaPO_4$ Solution A*:** 500µl HBS solution (50mM HEPES, 274mM NaCl, pH 7.1 with NaOH then filter sterilise) and 10µl 70mM sodium phosphate (made by mixing equal volumes of 70mM Na_2HPO_4 and NaH_2PO_4 fresh each time) added to the HBS solution just before mixing.

***Solution B*:** 15µg plasmid DNA made up to 440µl with dH_2O and 60µl 2M $CaCl_2$ (added just before mixing)

Solution B was added slowly dropwise, to solution A while at the same time continuously bubbling air through solution A to aid mixing. The solution was vortexed and allowed to precipitate for 20mins at room temp. then applied dropwise to the cells. The precipitate was incubated with the cells for 5-6hrs at 37°C then the cells glycerol shocked by adding 2mls of 25% glycerol shock medium (culture medium + 25% v/v glycerol, filter sterilized) for 1minute. The dish was flooded with 10ml PBS and removed, the PBS wash was repeated twice more to remove $Ca^{2+}PO_4$ precipitate before applying 10ml fresh medium as normal and incubating overnight. Cells were then seeded into 96 well plates (see over).

13.2 Transient Transfection with Lipofectamine

Suspension cells were counted and the concentration adjusted to $1.25x10^7$ cells/ml by centrifugation (1500 rpm, 5mins) and washed in Optimem (Gibco brl), to remove any antibiotics, before resuspending in the appropriate volume of Optimem. All volumes are for transfection in a 6 well plate, DNA was added to the cells by preparing the two solutions A and B.

***Solution A*:** 2µg DNA, 6µl Plus reagent and Optimem (up to a total volume of 100µl), incubate at room temp. for 15 minutes.

***Solution B*:** 16µl Lipofectamine (Gibco), 84µl Optimem.

Solution B was added to each well first then after 15 mins solution A was added and mixed gently then left for a further 15mins at room temp. Next 800µl of cells at $1.25x10^7$ cells/ml were added and incubated at 37°C for 5hrs. After 5hrs add 5mls of 0.625% complete RPMI and leave overnight at 37°C.

Adherent cells were plated out 24hr prior to transfection, the cells were counted and the concentration adjusted to $2x10^5$ cells/ml with culture medium. Cells were then plated at $2x10^6$ cells per flask in a T75 flask (Costar) and incubated overnight at 37°C. The following day the medium was removed from the cells and replaced with 5ml Optimem. The DNA was then added to the cells by combining the two solutions A and B.

> ***Solution A***: 15µg DNA, 25µl Plus Reagent and Optimem up to 625µl and incubate at room temp. for 15mins.
>
> ***Solution B***: 85µl Lipofectamine, 540µl Optimem

Solution B was added to solution A, mixed gently then incubated again for a further 15mins at room temp. and then added dropwise onto the cells. The DNA was incubated for 5hrs in a humidified incubator at 37°C before removing the solution and washing the cells twice with PBS to remove excess lipid. After the final wash the PBS was replace with culture medium.

Seeding into 96-Well Plates. The following day transfected cells were counted by trypan blue exclusion and resuspended in 0.5% phenol red free complete medium adjusting the cell concentration. Adherent cells were first washed with PBS and trypsinised to a single cell suspension with trypsin-EDTA (Gibco), the trypsin was then neutralised with 0.5% phenol red free medium before counting. Cells were centrifuged (1500rpm, 5mins) and the pellet resuspended in phenol red free medium at $2x10^6$ cell/ml (suspension cells) or $1.0x10^6$ cells/ml (adherent cells). 50µl of cells was added to each well of a 96 well plate giving a final concentration of $1x10^5$ cells/well.

For experiments using stably transfected cell lines, cells were simply counted and adjusted to $2x10^6$ cells/ml before adding 50µl of cell suspension per well.

13.3 Treatments.

Various treatments were added to the cells in either 10µl or 25µl volumes and they were diluted accordingly to account for this. MPA (stock 10^{-1}M in DMSO) was diluted to the appropriate working concentration in 0.5% phenol-red free culture medium (Sigma). 12-phorbol-myristic acetic acid (PMA) (stock 10mg/ml in DMSO), human recombinant tumour necrosis factor α (TNFα) (stock 500,000U/ml in PBS) (both Calbiochem), guanosine (stock 0.1M) (Sigma) were also diluted to the appropriate working concentrations in 0.5% phenol red free medium.
Transfected cells were incubated for the appropriate length of time, in the presence of the various treatments, before assaying for reporter gene activity.

13.4 Reporter Gene Expression Assays - β-Galactosidase

The prokaryotic lac-z gene within the polyGal plasmid encodes β-galactosidase, which efficiently metabolises galactose and its analogues, such as chlorophenolred-β-D-galactopyranoside (CPRG) (Boehringer Mannheim). Enzymatic metabolism of CPRG results in a colourimetric shift from yellow to red, which can be detected at a wavelength of 570nm. The introduction of response elements upstream of this gene provides a sensitive reporter system for monitoring lac z gene expression mediated by different transcription factors.
Transfected cells plated at $1x10^5$ cells/well in 96-well plates were assayed for β-galactosidase activity. 100µl of CPRG cocktail was added to each well using a multi-channel pipette (7µl 10x Z buffer (600mM Na_2HPO4, 400mM NaH_2PO4, 100mM KCl, 10mM $MgSO_4.7H_2O$, 500mM β-mercaptoethanol, adjust to pH 7.0 with 1M NaOH), 2µl 10% SDS, 7µl 50mM CPRG, 82µl dH_2O). The plate was incubated at 37°C and

the optical density (570nm) measured using a plate reader (Dynatech MR 7000) using Dynex Revelation 3.2 software.

13.5 Determining Protein Concentration in Reporter Gene Assays

To determine the effect of MPA on protein levels during the treatment period, samples were cultured in exactly the same way as they had been for the reporter gene assay but assayed for protein concentration (Micro BCA Protein Assay Kit, Pierce). Briefly, because of interference by phenol red with the assay, the samples were first removed from each well and spun at 13000rpm for 5mins in a microcentrifuge (Microcentaur) before removing the supernatant. The pellet was resuspended in 150µl of sterile PBS and returned to a 96-well plate. A range of standards of known concentration were prepared using BSA diluted in PBS and placed on the plate. The BCA working reagent was prepared using the solutions supplied by the manufacturer and 150µl added to each well. The plate was then shaken on a plate shaker for 30sec before incubating for 2 hrs at 37°C. After 2 hrs, the plate was removed from the incubator and left to cool to room temp. before reading on a plate reader at 570nm (Dynatech MR 7000) using Dynex Revelation 3.2 software. A standard curve was plotted using the BSA standards and then the protein concentrations in the unknown samples determined from this.

Results

1 Preliminary Observations

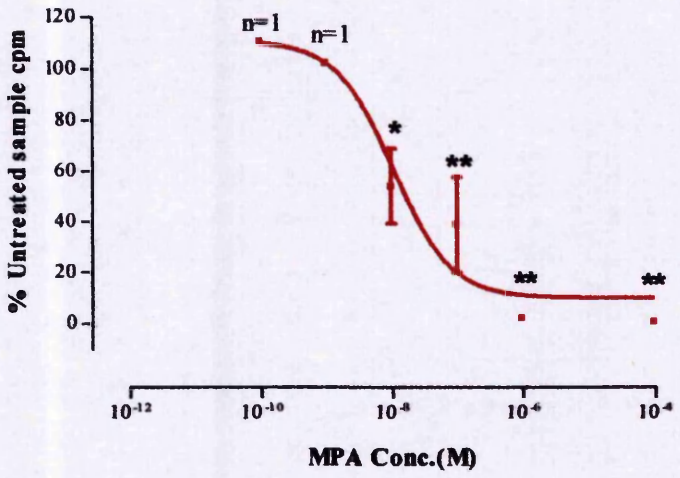
Initial work was performed using rat lymph node cells (LNCs) because of their frequent use as experimental models in many different settings, including previous experiments with MPA both *in vivo* and *in vitro*. As can be seen in figure 6A, MPA was shown to be a potent inhibitor of rat LNC proliferation stimulated by a mixed lymphocyte reaction. Proliferation was significantly inhibited at concentrations of MPA as low as $1x10^{-8}$M. Rat LNCs cells were then used in an to attempt to reproduce previous published worked which looked at the effect of MPA on cell surface protein glycosylation (Allison *et al*, 1993b) using fluorescently labelled lectins and detected by flow cytometry. Different lectins specific for either mannose or fucose residues attached to glycoproteins were used to attempt to identify alterations in their incorporation into cell surface glycoproteins. However, after several months of study involving a systematic approach to cell activation, the choice of lectin and its concentration as well as other parameters, we were unable to detect any effect caused by MPA on the binding of any of the lectins to Con A stimulated rat LNCs (figure 7).

It was then decided subsequently, to more accurately reproduce previous experiments, to use human peripheral blood mononuclear cells (HuPBMC). The cells were isolated from normal healthy human volunteers, to determine if the lack of an effect by MPA previously was due to species differences. MPA was found to be a potent inhibitor of Con A stimulated HuPBMC proliferation (figure 6B). Figure 8 shows the effect of MPA on the binding of fluorescently labelled lectins to Con A stimulated HuPBMCs. As before, following an extensive study considering various different variables we were in contrast to previous reports, unable to detect any differences in the binding of fucose and mannose specific lectins to Con A stimulated HuPBMCs following MPA treatment.

The experiments with rat LNCs and HuPBMCs described above are not performed with totally pure populations but are mixed populations of cells. Therefore the use of specific cell lines would allow us not only to look at an isolated cell population but also to compare between the effects of MPA on different cell types. For this reason two different cell lines were chosen, CEM-C7 T cells, a T lymphoblastoid cell line (Foley *et al* 1965) and ECV304 cells, a human umbilical vein endothelial cell line

(Takahashi *et al* 1990; Takahashi and Sawasaki 1992). These cell lines were used for the majority of the remainder of work shown below to compare the effects of MPA on cells of lymphoid and non-lymphoid origin.

A.



B.

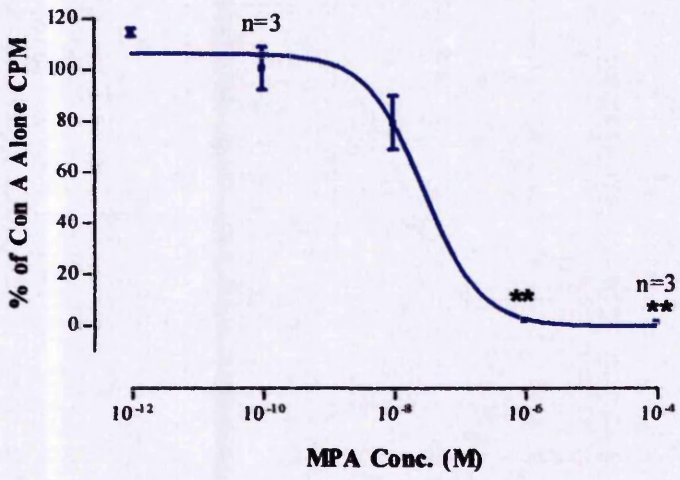


Figure 6. The effect of MPA on Con A stimulated HuPBMC proliferation and rat LNC proliferation in a mixed lymphocyte reaction. The figures show the effect of MPA on rat lymph node cells stimulated via a mixed lymphocyte reaction (figure A) and Con A stimulated human peripheral blood mononuclear cells (figure B). The results shown are the means of four individual experiments except where indicated and are plotted as the % of the cpm from stimulated cells alone. Mean stimulated control values were 30211.6 cpm (4467.3 unstim.) and 57783.1cpm (1831.66 unstim.) for rat LNC and HuPBMCs respectively. * and ** denotes $P<0.05$ and <0.01 respectively one-way ANOVA followed by Dunnetts multiple comparisons test.

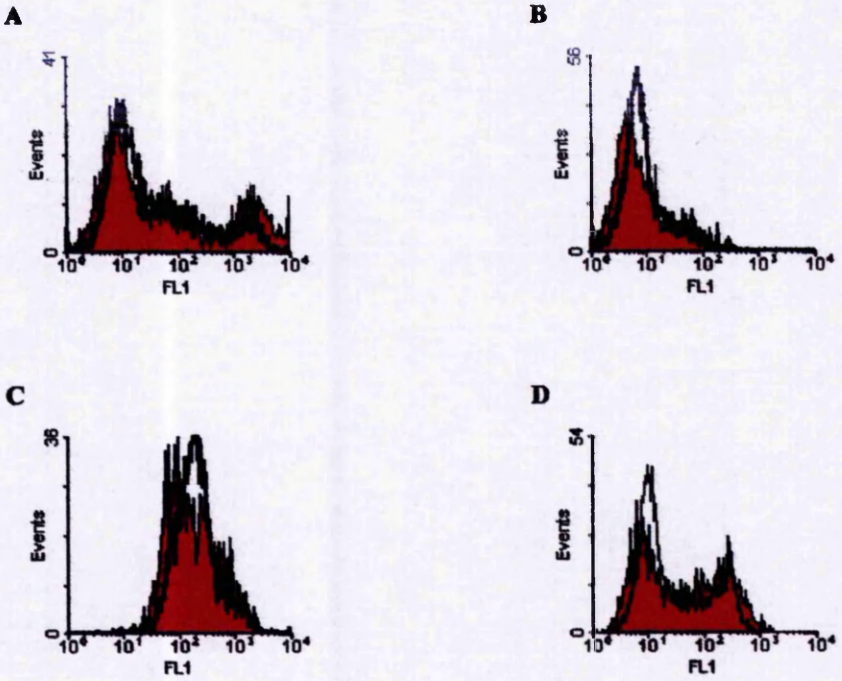


Figure 7. The Effect of MPA on lectin binding to Con A stimulated rat lymph node cells. Rat LNCs were cultured for 72hr in the presence of Con A and MPA ($1x10^{-6}$M) before removing for FACs analysis with different lectins. The figures show the effect of MPA (black line) on lectin binding compared with Con A stimulated cells alone (filled red trace). Figures A and B are the fucose-specific lectins UEA1 and LTA respectively while figures C and D are the mannose-specific lectins VFA and GNA respectively. The figures shown are representative of three individual experiments (only 1 experiment with GNA).

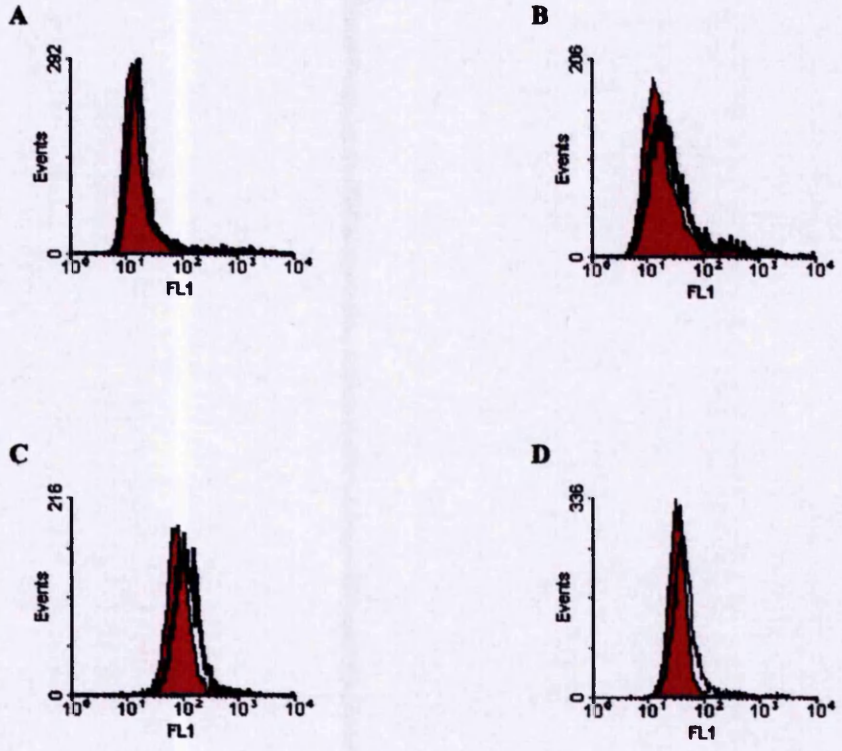


Figure 8. The Effect of MPA on lectin binding to Con A stimulated human peripheral blood mononuclear cells. HuPBMCs were cultured for 72hr in the presence of Con A and MPA ($1x10^{-6}$M) before removing for FACs analysis with different lectins. The figures show the effect of MPA (black line) on lectin binding compared with Con A stimulated cells alone (filled red trace). Figures A and B are the fucose-specific lectins UEA1 and LTA respectively while figures C and D are the mannose-specific lectins VFA and GNA respectively. The figures shown are representative of three individual experiments.

2 The Effects of Mycophenolic Acid on Cellular Proliferation

Previous studies have already shown that MPA inhibits the proliferation of various cell types (Allison and Eugui, 1994) but preliminary experiments were performed to determine the effect of MPA in our system, using the cell lines that would be used for future experiments.

2.1 Mycophenolic Acid and CEM-C7 Proliferation

To determine the dose response of MPA on CEM-C7 T cell proliferation, cellular proliferation was investigated by measuring ^{3}H-thymidine incorporation (Figure 9A). Proliferation of Con A stimulated CEM-C7 cells was inhibited in a dose dependent manner with similar kinetics to previous reports. Proliferation was significantly inhibited at doses of $1x10^{-6}$M and $1x10^{-4}$M but was similar to control levels at $1x10^{-8}$M where proliferation was unaffected. Replacement of exogenous guanosine in MPA treated cultures (Figure 9B) reversed the antiproliferative effect of MPA at guanosine concentrations up to 20μM, but failed to completely restore proliferation. When the guanosine concentration was increased further, proliferation became inhibited once again suggesting that at higher concentrations, guanosine itself was becoming toxic.

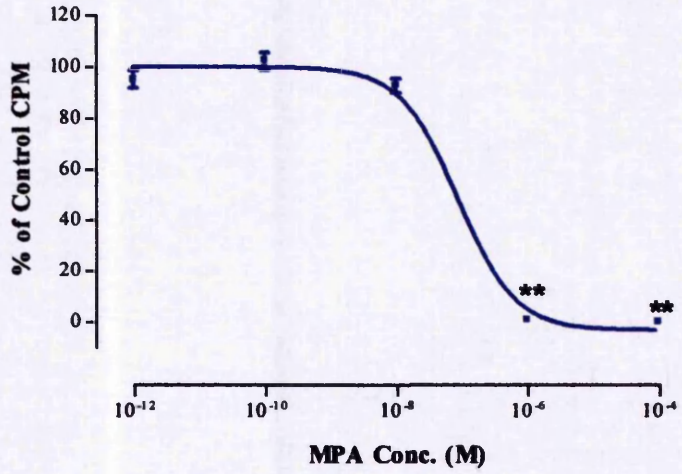
2.2 Mycophenolic Acid and Endothelial Cell Proliferation

An endothelial cell line (ECV304) isolated from human umbilical vein endothelial cells was also utilised to investigate the susceptibility of cells of non-lymphoid origin to MPA. Proliferation was again significantly inhibited at concentrations down to $1x10^{-6}$M, although not to the same extent as was seen with CEM-C7 cells, and there was no inhibition at $1x10^{-8}$M (Figure 10A). To determine if the effect seen was due to depletion of guanosine nucleotides, increasing concentrations of exogenous guanosine were added to the cultures along with MPA and the effect on proliferation measured (Figure 10B). The addition of exogenous guanosine reversed the antiproliferative

effect of MPA at concentrations of 10μM and caused an actual increase over control proliferation at higher concentrations (50μM).

In Summary. MPA is able to inhibit the proliferation of both a T cell line and an endothelial cell line. However it is a more potent inhibitor of T cell proliferation than endothelial cell proliferation. Proliferation in T cells is inhibited almost completely at 10^{-6}M MPA in T cells but only to about 50% of controls in endothelial cells. In addition, the replacement of exogenous guanosine was able to reverse the inhibition by MPA completely in endothelial cells but only to 60-70% of controls in T cells.

A.



B.

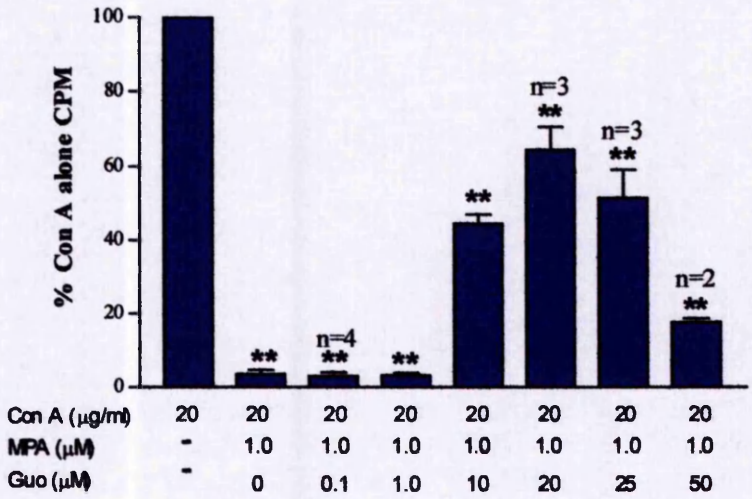


Figure 9. The effect of MPA on Con A stimulated CEM-C7a proliferation. Cells were cultured with both Con A and different concentrations of MPA (Top figure A) or cultured with exogenous guanosine as well (Bottom figure B). ^{3}-H thymidine incorporation was determined as a measure of proliferation. Data shown is the mean of triplicate wells from 6 individual experiments (Figure A) or 7 experiments (Figure B) (except where indicated) ± s.e.m. Data is plotted as % of the counts per minute (cpm) from Con A stimulated cells alone. Mean Con A stimulated control values were 290204 cpm (352374 unstim.) and 170000 cpm for A and B respectively. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts multiple comparisons test.

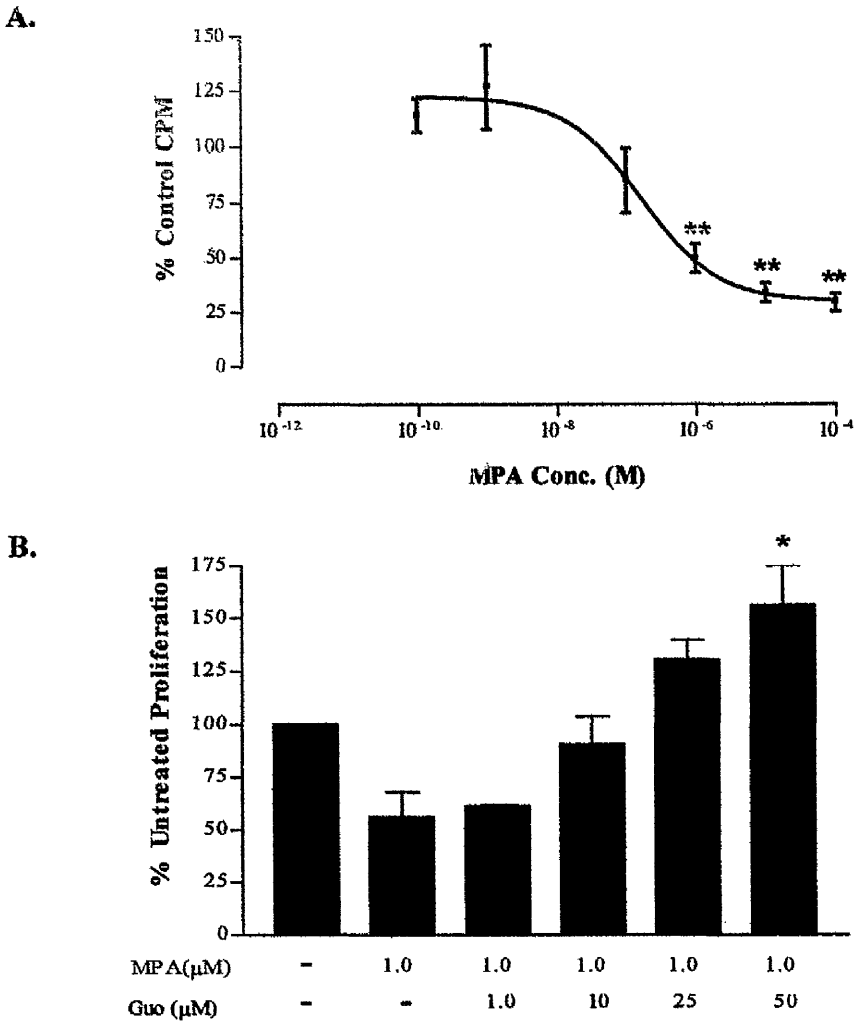


Figure 10. The effect of MPA on ECV304 proliferation. ECV304 cells were cultured for 72hr in the presence of different concentrations of MPA (Top figure A) or with added exogenous guanosine as well (Bottom figure B). ^{3}-H thymidine incorporation was determined as a measure of proliferation. Data shown is the mean of triplicate wells from 3 individual experiments, ± s.e.m, and is plotted as % of the counts per minute (cpm) from untreated cells alone. Mean control values were 31410.21 cpm and 44052.61 cpm for A and B respectively. * and ** denotes $P<0.05$ and $P<0.01$ respectively one-way ANOVA followed by Dunnettes multiple comparison test.

3 Investigation of the Effect of MPA on Protein Glycosylation

The transfer of fucose and mannose residues to proteins requires the presence of GDP to act as a carrier molecule. To determine the effect of MPA on this process, cell surface expression of fucose and mannose containing glycoproteins was investigated by flow cytometry using fluorescently labelled lectins specific for fucose and mannose residues linked to cell surface proteins

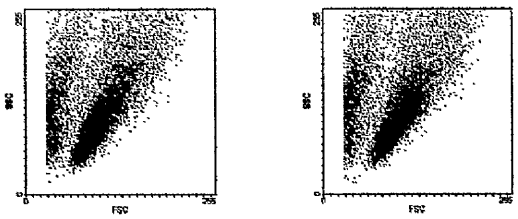
3.1 MPA and Cell-Surface Expression of Mannose and Fucose Residues on CEM-C7 Cells

CEM-C7 cells were cultured alone or stimulated with Con A for 24hr, 48hr and 72hr and then stained with either Lotus tetragonolobus (LTA) a fucose-specific lectin, or Vicia faba (VFA) a mannose-specific lectin. The forward scatter/side scatter profiles of MPA treated CEM-C7 cells show the effect of MPA on cell size (FSC) and granularity (SSC) (figures 11 and 12). MPA causes a change in both cellular granularity and size after 48hr and 72hr in unstimulated cells (figure 11), which becomes more apparent after stimulation with Con A (Figure 12). Identical populations were then gated on the dot-plots from the different cultures and the mean fluorescence compared between treatment groups.

3.1.1 Cell-Surface Expression of Fucose Residues on CEM-C7 Cells

The mean fluorescence of cells treated with MPA alone for 24, 48, and 72hr shows no differences from untreated control groups (Figure 13 and 14). However when a known inhibitor of protein glycosylation was included, tunicamycin, fluorescence intensity was decreased significantly showing that it is possible to detect changes in fluorescence. When Con A was also added to the cultures, again there were no significant effects seen on fluorescence intensity between MPA-treated and control cultures except for tunicamycin (figures 15 and 16). These results therefore suggest that MPA has no effect on the cell surface fucose expression in CEM-C7 cells.

1.



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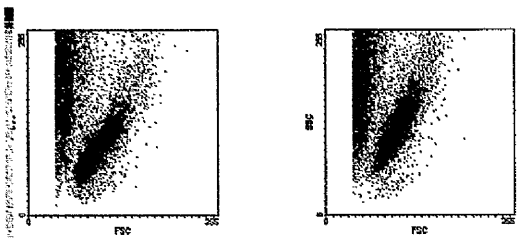


3.

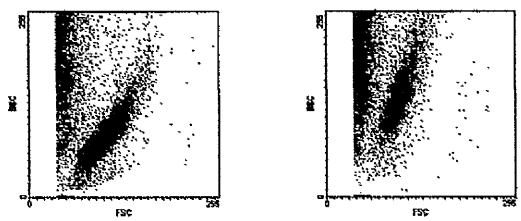


Figure 11. The Effect of MPA on CEM-C7 Cells. CEM-C7 cells were cultured for 24hrs (1), 48hrs (2) or 72hrs (3) either alone (A) or in the presence of MPA 10^{-6}M (B), other concentrations not shown. Cultures were then harvested after the appropriate length of time for FACs staining with appropriate lectins and gated around the distinct population for analyses to determine the mean fluorescence.

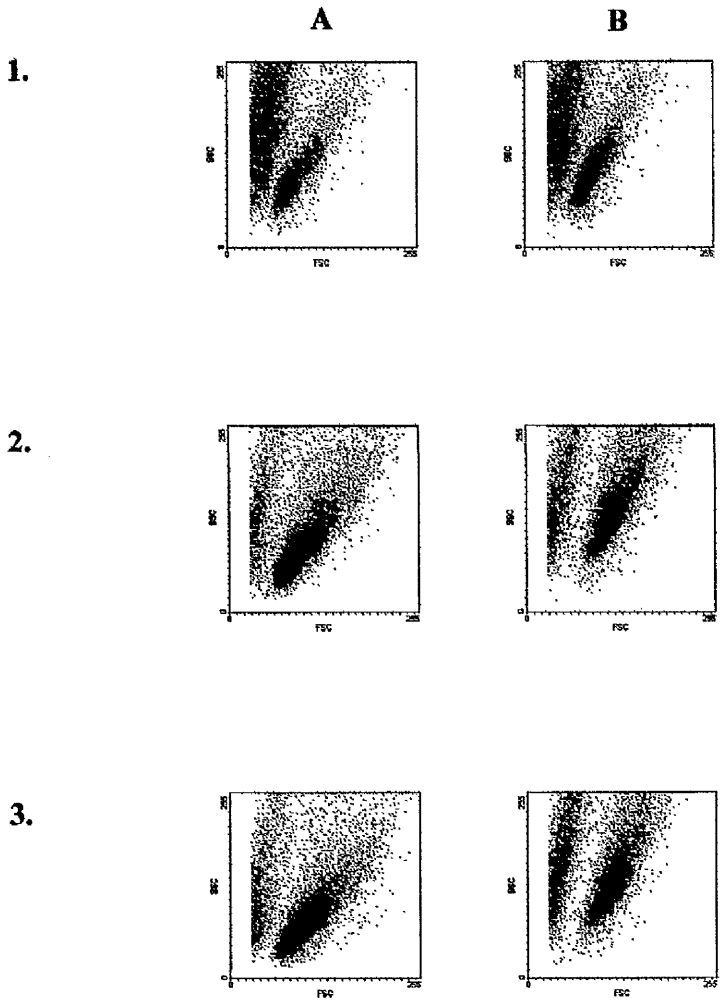
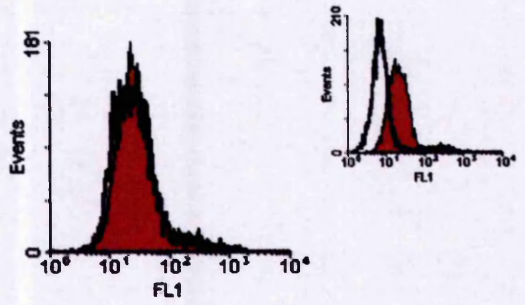


Figure 12. The Effect of MPA on Con A stimulated CEM-C7 Cells. CEM-C7 cells were stimulated with Con A (10ug/ml) in culture for 24hrs (1), 48hrs (2) or 72hrs (3) either alone (A) or in the presence of MPA 10^{-6}M (B) other concentrations not shown. Cultures were then harvested after the particular length of time for FACs staining with appropriate lectins and gated around the distinct population for analyses to determine the mean fluorescence.

A.



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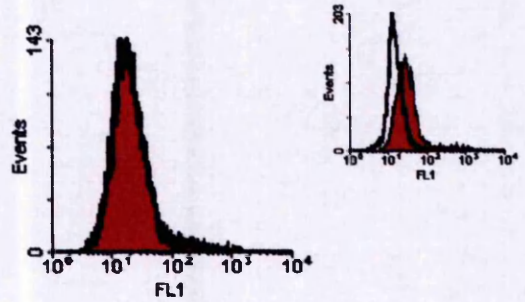

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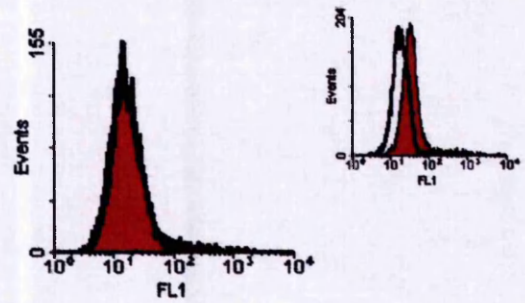
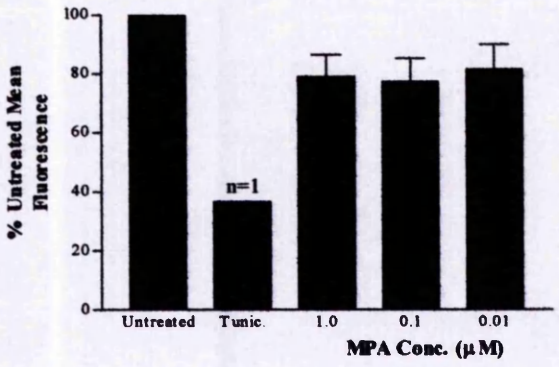


Figure 13. The effect of MPA on binding of the fluorescently labelled lectin *Lotus tetragonolobus*. CEM-C7 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) in the presence of different concentrations of MPA. The figures show the effect of MPA $1x10^{-6}$M (Solid black line) compared with untreated cells (filled red trace). The figure inset shows the effect of tunicamycin (10ug/ml, solid black line) a known inhibitor of glycosylation

A.



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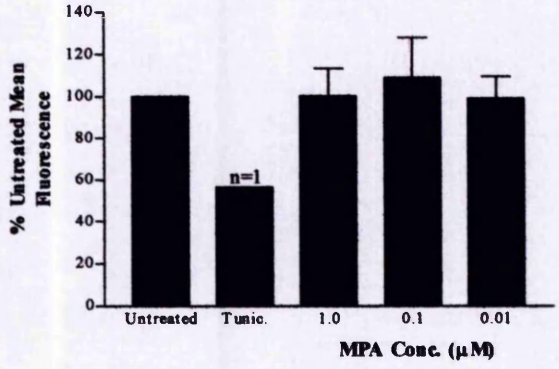


C.

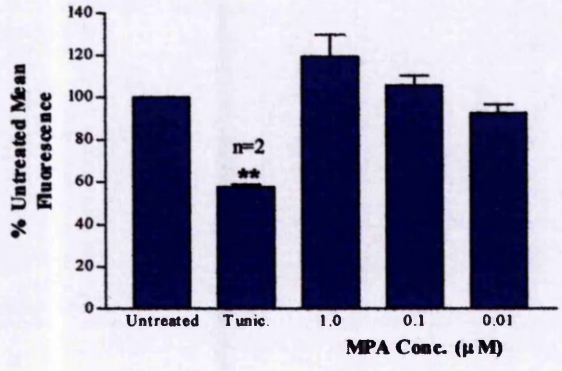


Figure 14. The effect of MPA on mean fluorescence intensity of unstimulated CEM-C7 cells stained with FITC-labelled *Lotus tetragonolobus*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) with different concentrations of MPA then stained with FITC-labelled LTA and fluorescence measured by flow cytometry. The mean fluorescence intensity for control cells was 21.6, 22.0, and 20.1 for 24hr, 48hr, and 72hr respectively Results shown are the means of three separate experiments ± s.e.m except where indicated. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts multiple comparisons test.

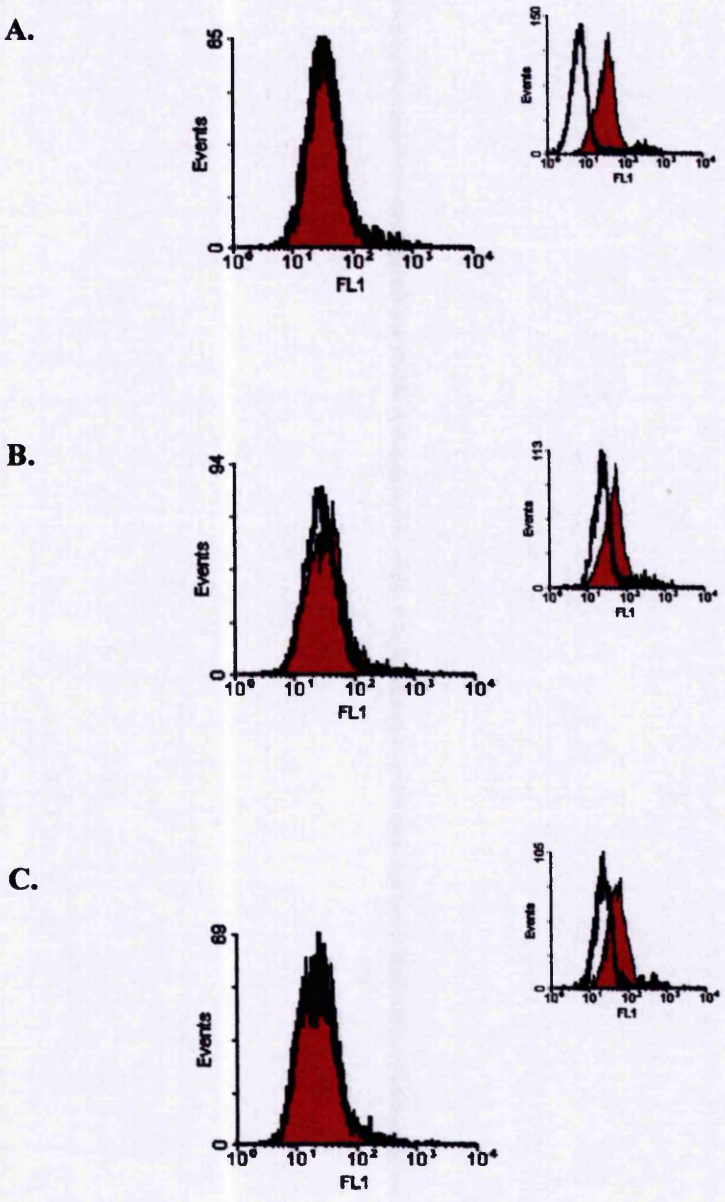


Figure 15. The effect of MPA on binding of the fluorescently labelled lectin *Lotus tetragonolobus* to Con A stimulated CEM-C7 cells. CEM-C7 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) with Con A (10μg/ml) and in the presence different concentrations of MPA. The figures show the effect of MPA $1x10^{-6}$M (solid black line) compared with untreated cells (filled red trace). The figure inset shows the effect of tunicamycin (10μg/ml, solid black line) a known inhibitor of glycosylation

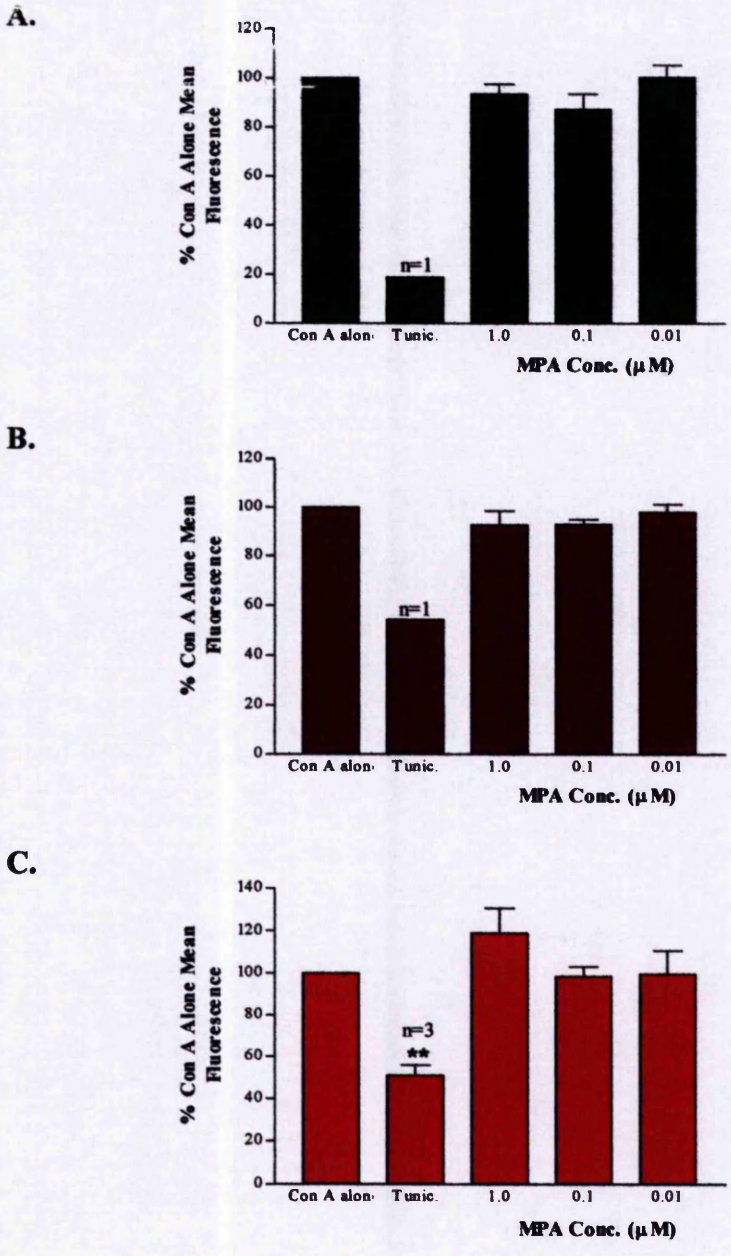


Figure 16. The effect of MPA on mean fluorescence intensity of Con A stimulated CEM-C7 cells stained with FITC-labelled *Lotus tetragonolobus*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) with Con A (10ug/ml) and different concentrations of MPA then stained with FITC-labelled LTA and fluorescence measured by flow cytometry. The mean fluorescence intensity for Con A stimulated control cells was 44.7, 47.9, and 39.3 for 24hr, 48hr, and 72hr respectively. Results shown are the means of three separate experiments ± s.e.m except where indicated. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts multiple comparisons test.

3.1.2 Cell-Surface Expression of Mannose Residues on CEM-C7 Cells

To look at the effect of MPA on cell surface expression of mannose residues on glycoproteins an alternative lectin, Vicia faba (VFA) was utilised. The fluorescence intensity of cells cultured in the presence of different concentrations of MPA was determined and compared with untreated controls, along with tunicamycin as a positive control (Figure 17 and 18). No significant effects were seen with MPA treated cells compared to controls except again for tunicamycin treated cells. Tunicamycin treatment caused a dramatic decrease in fluorescence intensity. Following incubation of cells with Con A in combination with MPA (Figure 19 and 20), again we could detect a significant decrease in fluorescence intensity with tunicamycin but no effect was seen by MPA at any concentration. There was therefore no effect by MPA on cell-surface expression of mannose residues in CEM-C7 cells.

3.2 The Effect of MPA on Mannose and Fucose Expression in ECV304 Endothelial Cells

ECV304 cells were utilised to investigate the effects of MPA on the expression of mannose and fucose residues on the surface of endothelial cells. The cells were stained with identical lectins to those used with CEM-C7 cells, Lotus tetragonolobus (LTA) a fucose-specific lectin or Vicia faba (VFA) a mannose-specific lectin, and again acquired by flow cytometry. Identical gates were then placed around the dot-plots to compare the effects of MPA on lectin binding between samples. MPA appears to have no effect on the general morphology of the cells with the FSC and SSC profiles appearing to be identical between controls and MPA treated groups (Figure 21 and 22).

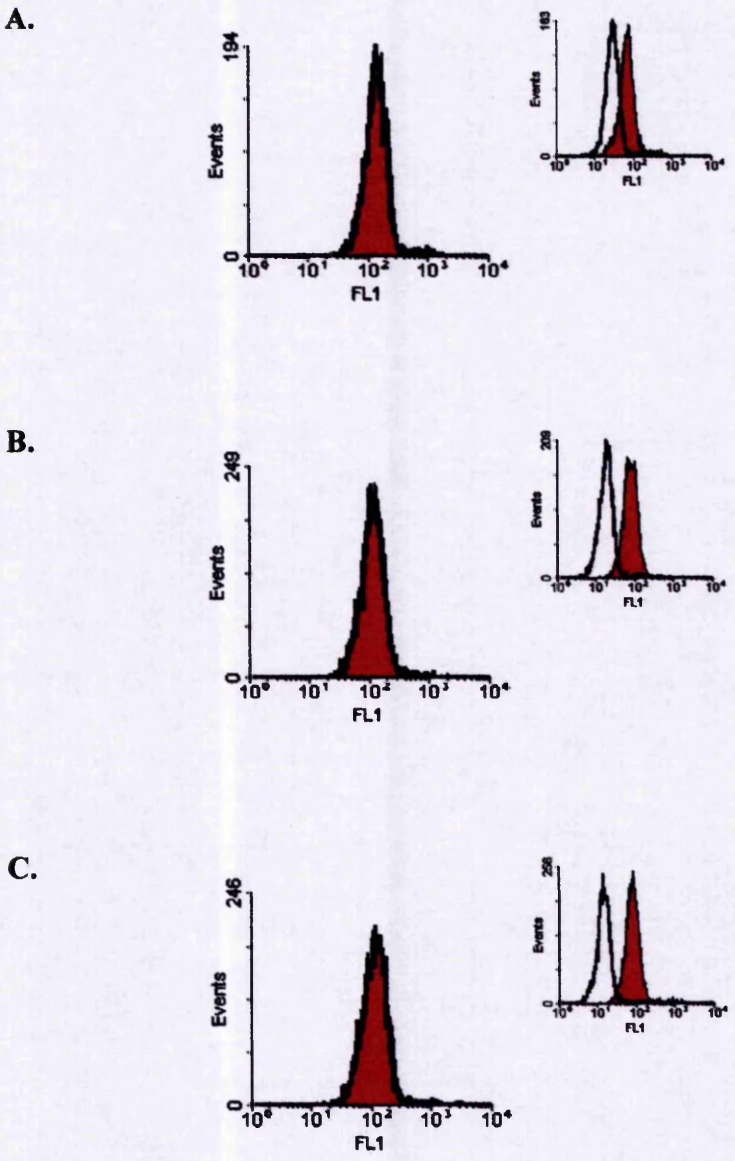


Figure 17. The effect of MPA on binding of the fluorescently labelled lectin *Vicia faba*. CEM-C7 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) in the presence different concentrations of MPA. The figures show the effect of MPA $1x10^{-6}$M (Solid black line) compared with untreated cells (filled red trace). The figure inset shows the effect of tunicamycin (10ug/ml, solid black line) a known inhibitor of glycosylation

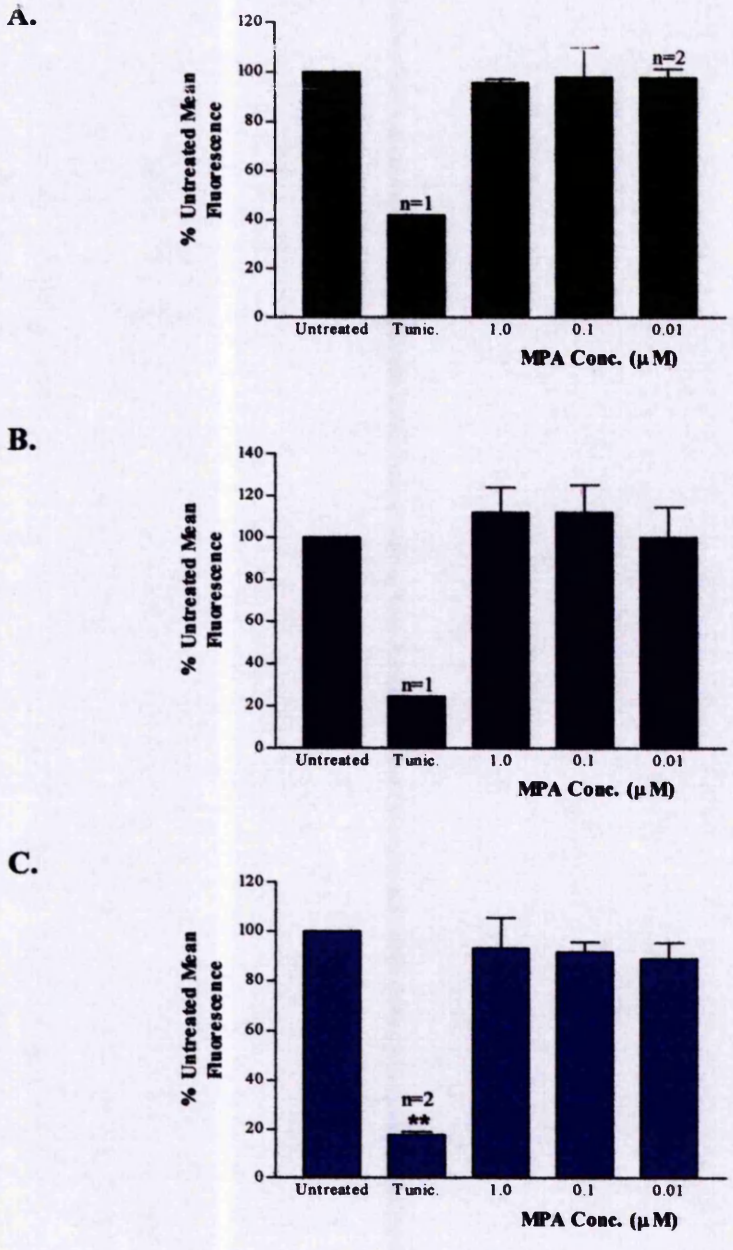


Figure 18. The effect of MPA on mean fluorescence intensity of unstimulated CEM-C7 cells stained with FITC-labelled *Vicia faba*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) with different concentrations of MPA then stained with FITC-labelled VFA and fluorescence measured by flow cytometry. The mean fluorescence intensity for control cells was 105.3, 107.7, and 101.9 for 24hr, 48hr, and 72hr respectively. Results shown are the means of three separate experiments ± s.e.m, except where indicated. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts multiple comparisons test.

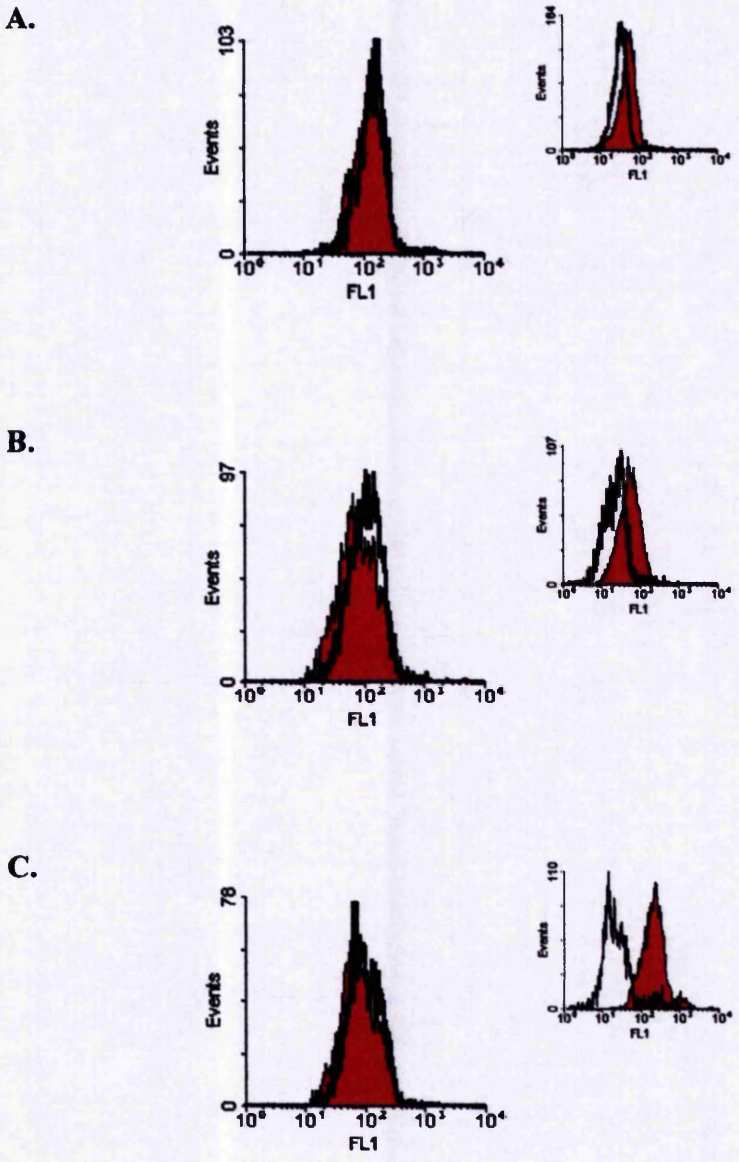


Figure 19. The effect of MPA on binding of the fluorescently labelled lectin *Vicia faba* to Con A stimulated CEM-C7 cells. CEM-C7 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) with Con A (10μg/ml) and in the presence different concentrations of MPA. The figures shows the effect of MPA $1x10^{-6}$M (Solid black line) compared with untreated cells (filled red trace). The figure inset shows the effect of tunicamycin (10μg/ml- solid black line) a known inhibitor of glycosylation

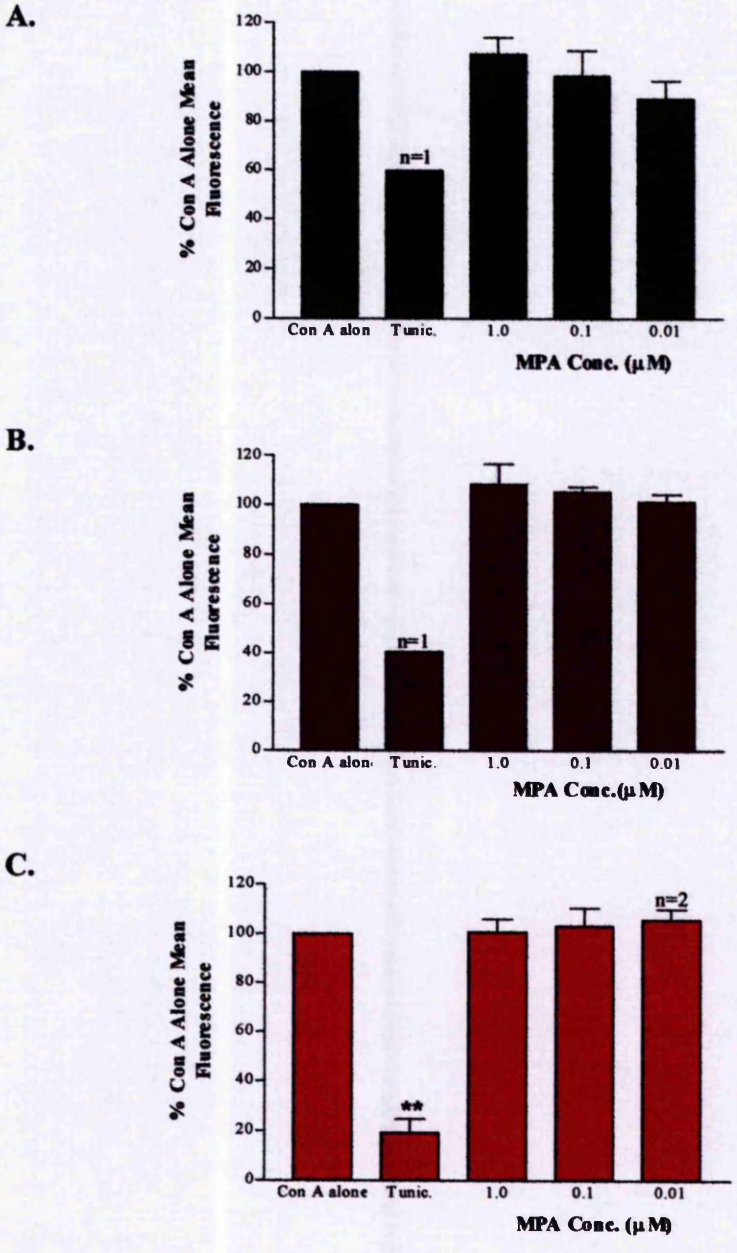


Figure 20. The effect of MPA on mean fluorescence intensity of Con A stimulated CEM-C7 cells stained with FITC-labelled *Vicia faba*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) with Con A (10ug/ml) and different concentrations of MPA then stained with FITC-labelled VFA and fluorescence measured by flow cytometry. The mean fluorescence intensity for Con A stimulated control cells was 88.3, 83.4, and 139.8 for 24hr, 48hr, and 72hr respectively. Results shown are the means of three separate experiments ± s.e.m, except where indicated. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts multiple comparisons test.

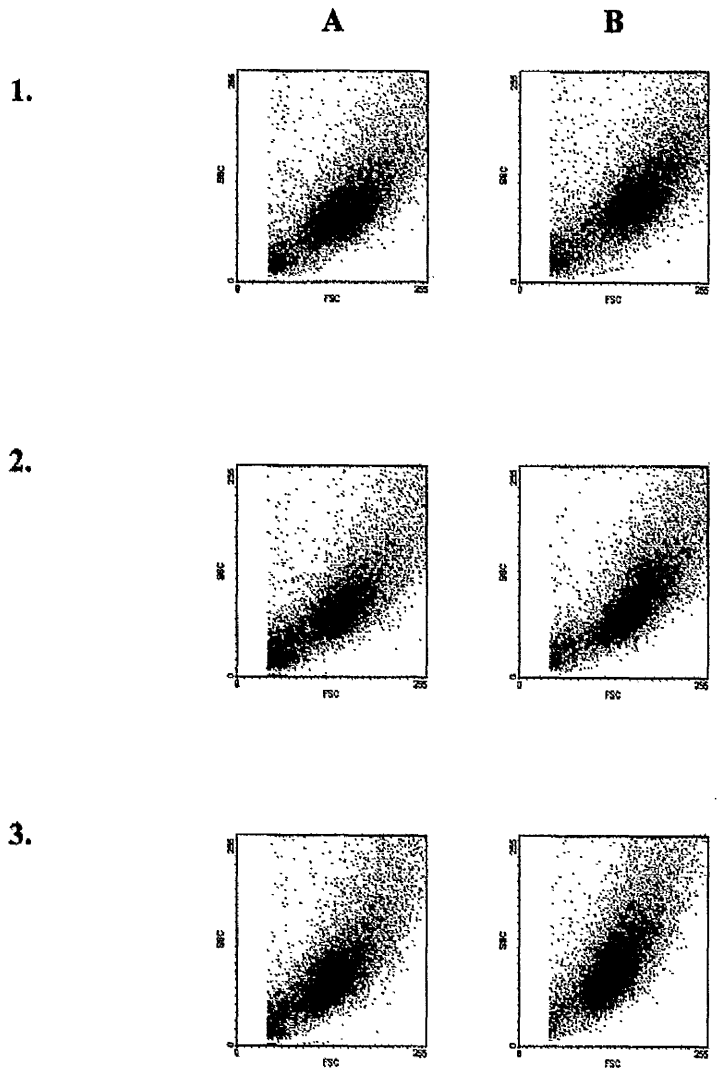


Figure 21. The Effect of MPA on ECV304 Cells. ECV304 cells were cultured for 24hrs (1), 48hrs (2) or 72hrs (3) either alone (A) or in the presence of MPA 10^{-5}M (B). Dot-plots for other MPA concentrations not shown. ECV304 cells cultured in the presence of the different treatments were removed after the appropriate length of time for FACs staining and gated around the distinct population to determine the mean fluorescence.

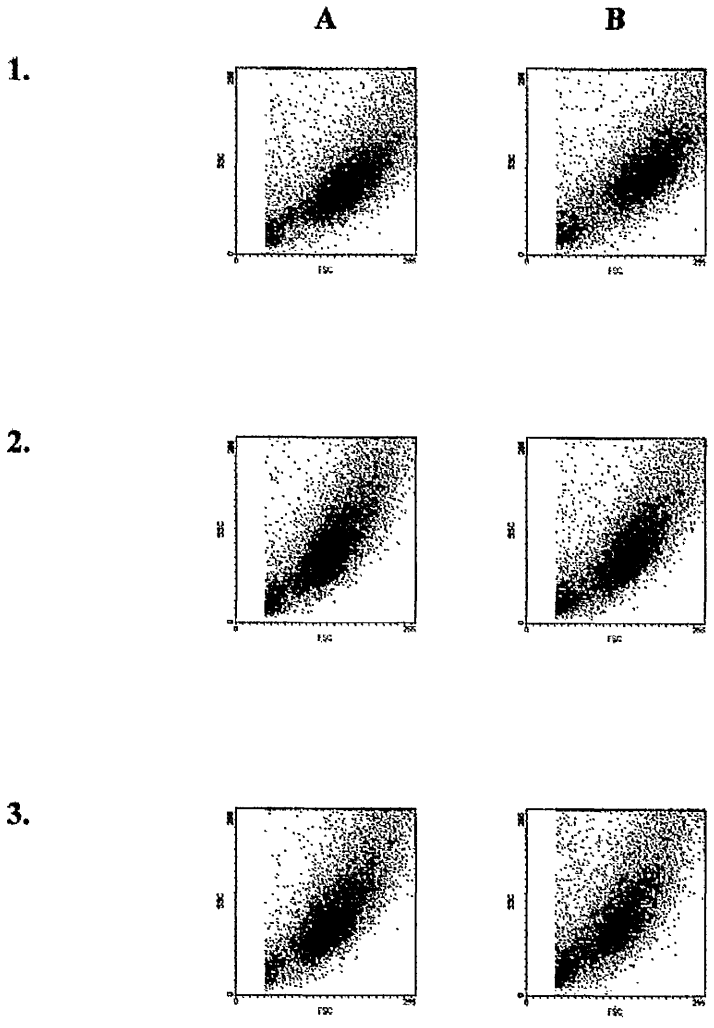


Figure 22. The Effect of MPA on TNFα stimulated ECV304 Cells. ECV304 cells were stimulated with TNFα (200U/ml) and cultured for 24hrs (1), 48hrs (2) or 72hrs (3) either alone (A) or in the presence of MPA 10^{-5}M (B) dot-plots for other concentrations not shown. ECV304 cells cultured in the presence of the different treatments were removed after the appropriate length of time for FACs staining and gated around the distinct population to determine the mean fluorescence.

3.2.1 Cell Surface Expression of Fucose Residues on ECV304 cells

The fluorescence intensity of cells incubated with MPA and stained with LTA shows a shift to left compared with control samples (Figure 23). MPA caused a significant decrease in intensity at doses of $1x10^{-5}$ M and $1x10^{-6}$M. The effect seen was similar to that seen with tunicamycin (figure 24). The effect of MPA was also increased as the incubation time increased, being greatest at 72hrs. When the endothelial cells were activated by co-incubating with TNFα as well, MPA was again shown to produce a shift in fluorescence with a significant decrease compared to controls at $1x10^{-5}$ M and $1x10^{-6}$ M (Figures 25 and 26). MPA therefore causes a decrease in cell-surface fucose expression on both resting and TNFα stimulated ECV304 cells.

3.2.2 Cell Surface Expression of Mannose Residues on ECV304 cells

ECV304 cells were stained with VFA to determine the effect of MPA on the cell-surface expression of mannose residues. Resting endothelial cells incubated with MPA show a slight decrease in fluorescence intensity, which was particularly evident at 48hr, but this failed to reach significance (figures 27 and 28). Experiments with tunicamycin also showed a decrease in fluorescence that became greater as the time increased. MPA produced a greater effect on VFA binding with TNFα stimulated ECV304 cells, again most evident at 48hrs although not significant (Figures 29 and 30). After 72hr of treatment with MPA, there was a significant decrease in VFA binding at the highest concentration of $1x10^{-6}$M MPA.

In Summary. The effect of MPA on the binding of mannose and fucose specific lectins to both T cells and endothelial cells was investigated. It was demonstrated that MPA has no effect on the binding of either the fucose specific lectin LTA or the mannose specific lectin VFA to CEM-C7 T cells either alone, or following stimulation with Con A. This was in contrast to being able to detect decreases in the expression of both fucose and mannose caused by tunicamycin, a known inhibitor of glycosylation. However, when ECV304 cells were cultured in the presence of MPA, a significant

decrease in the binding of LTA to fucose residues in both resting and TNFα stimulated cells was detected. MPA also produced a decrease in fluorescence caused by decreased VFA binding compared with controls but it was not as great as that seen with LTA and did not reach significance.

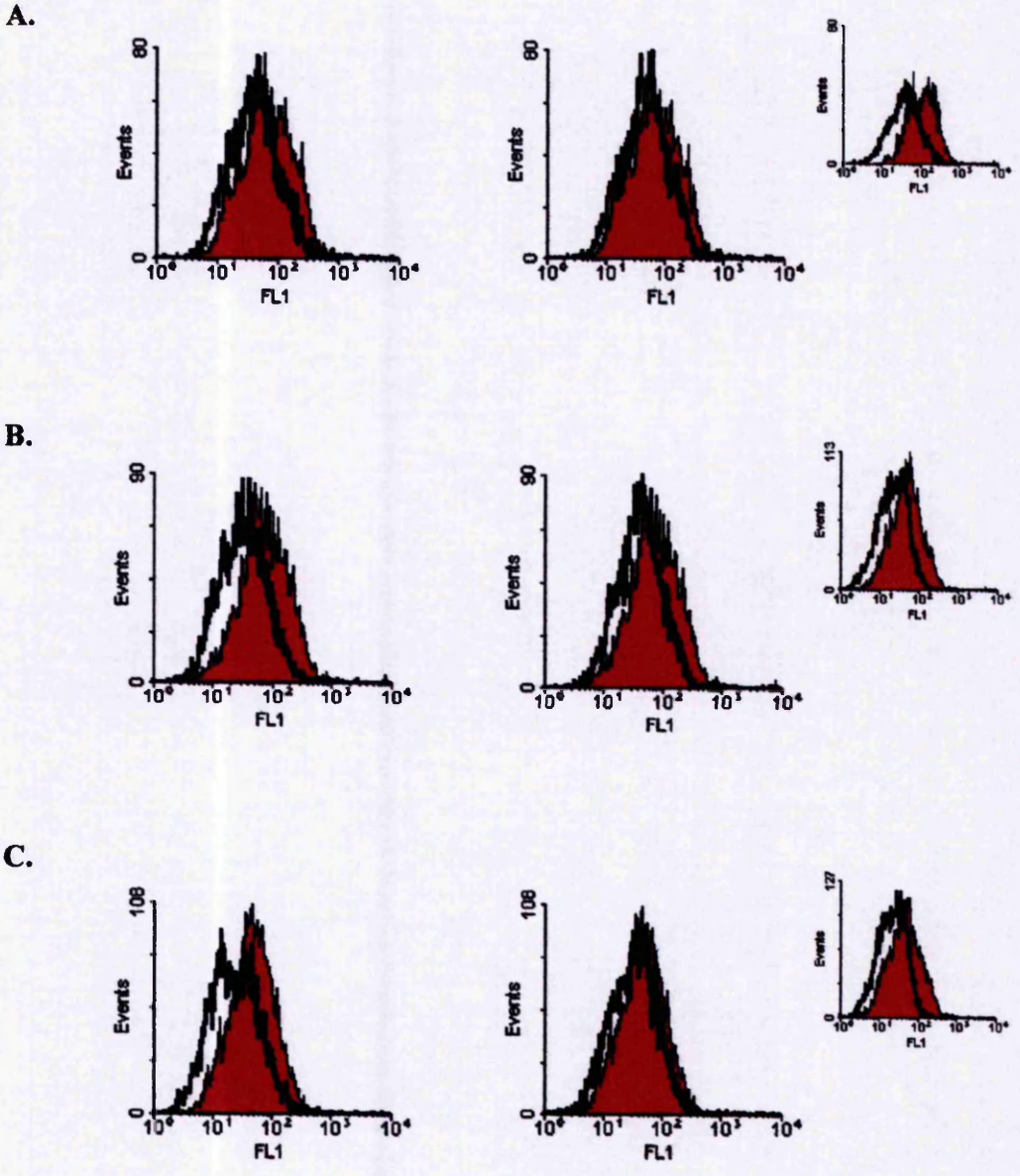


Figure 23. The effect of MPA on binding of the fluorescently labelled lectin *lotus tetragonolobus*. ECV304 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) in the presence of different concentrations of MPA (black line): $1x10^{-5}$M (left figure) and $1x10^{-6}$M (right figure). Control cells are shown by the filled red trace. The figure inset shows the effect of tunicamycin (10μg/ml) a known inhibitor of glycosylation

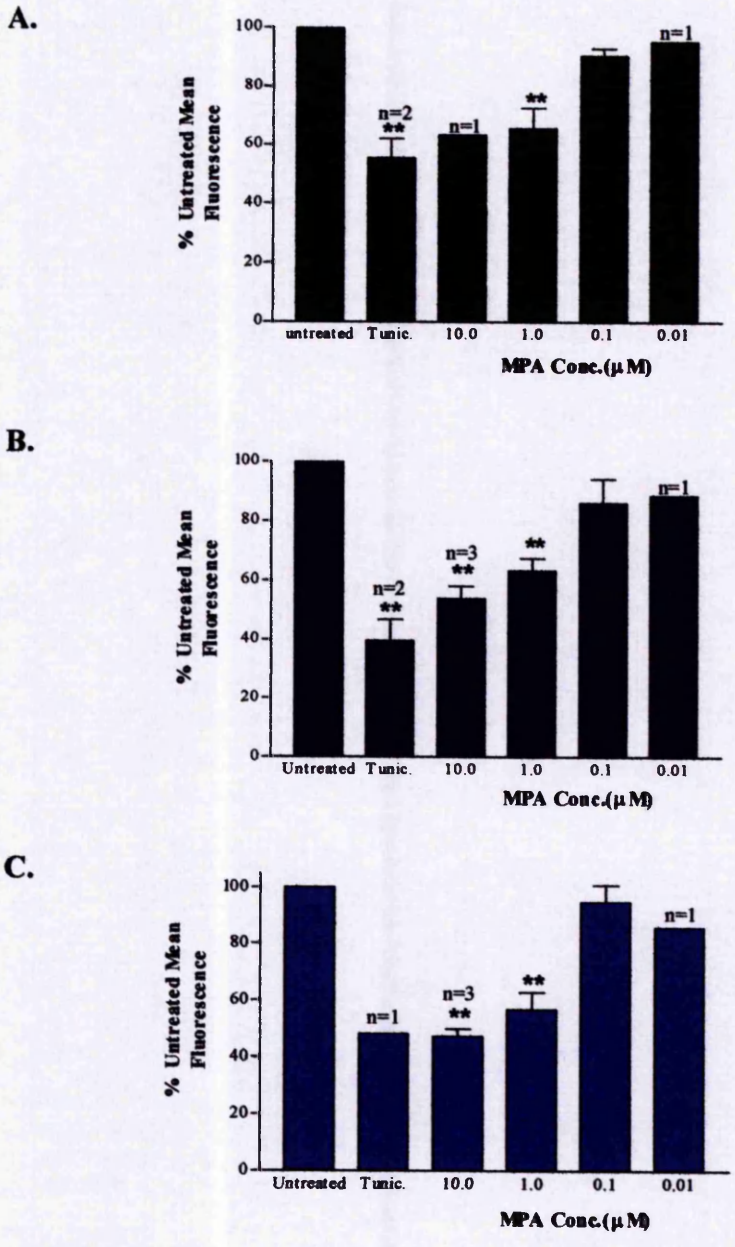


Figure 24. The effect of MPA on mean fluorescence intensity of ECV304 cells stained with FITC-labelled *Lotus tetragonolobus*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) in the presence of different concentrations of MPA then stained with FITC-labelled LTA and fluorescence measured by flow cytometry. The mean fluorescence intensity for control cells was 65.76, 44.31, and 44.76 for 24hr, 48hr, and 72hr respectively. Results shown are the means of two or four separate experiments for 24hr and 48hr/72hr respectively (except where indicated) ± s.e.m. ** Denotes $P<0.01$ One-way ANOVA followed by Dunnetts multiple comparisons test.

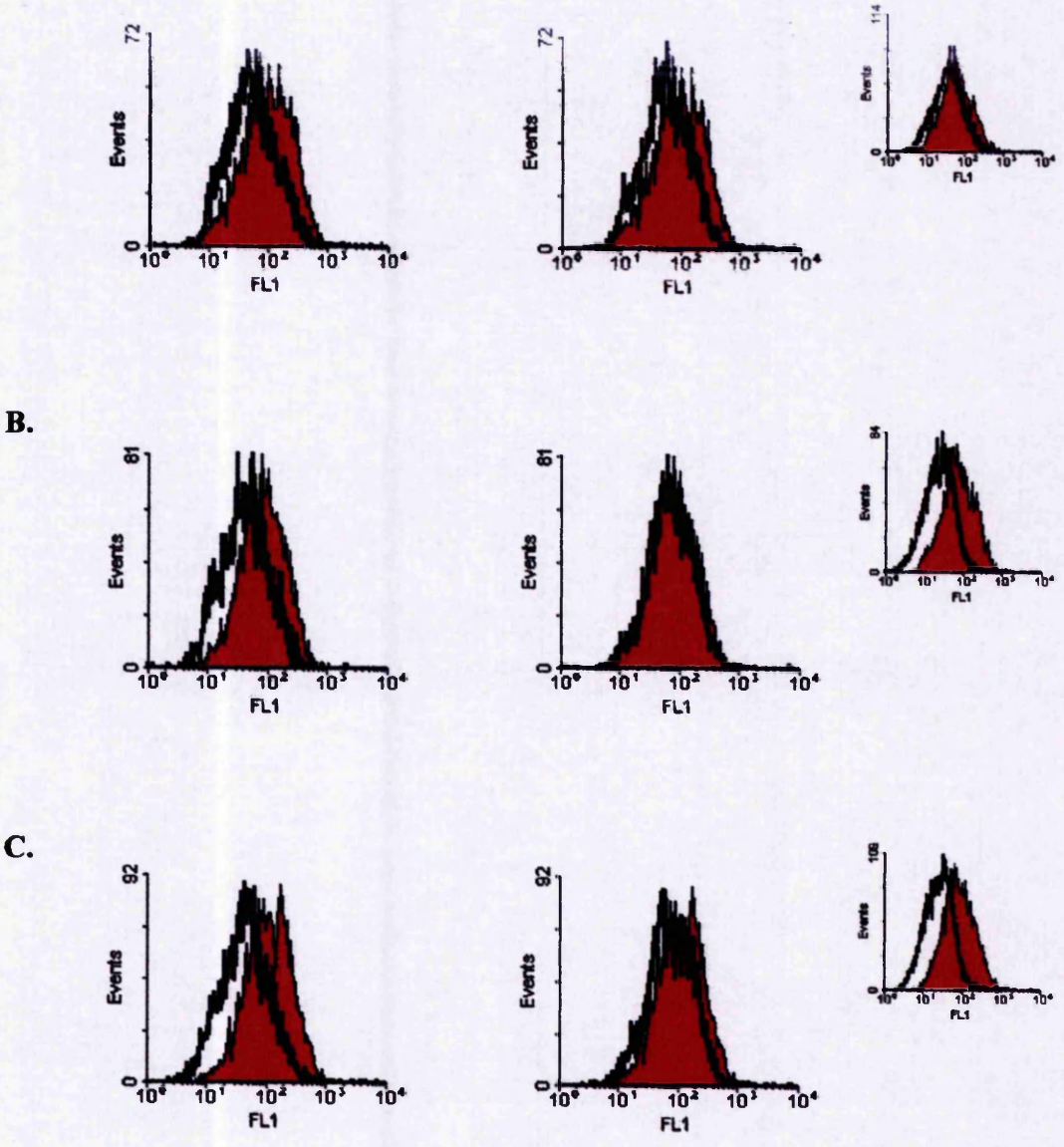


Figure 25. The effect of MPA on binding of the fluorescently labelled lectin *lotus tetragonolobus* to TNFα stimulated ECV304 cells. ECV304 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) in the presence of TNFα (200U/ml) and different concentrations of MPA (black line): $1x10^{-5}$M (left figure) and $1x10^{-6}$M (right figure). Control cells are shown by the filled red trace. The figure inset shows the effect of tunicamycin (10μg/ml, black line) a known inhibitor of glycosylation

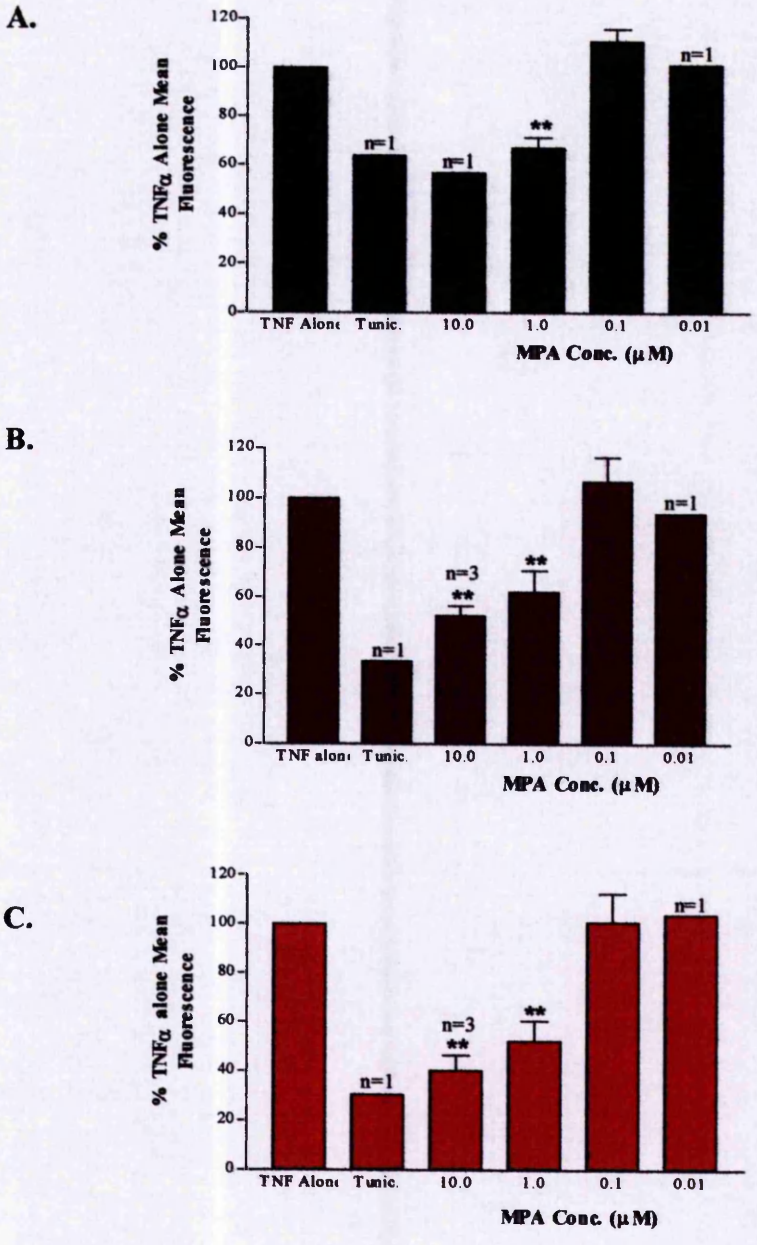


Figure 26. The effect of MPA on mean fluorescence intensity of TNFα stimulated ECV304 cells stained with FITC-labelled *Lotus tetragonolobus*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) with TNFα and in the presence of different concentrations of MPA then stained with FITC-labelled LTA and fluorescence measured by flow cytometry. The mean fluorescence intensity for TNFα stimulated control cells was 75.2, 61.8, and 84.9 for 24hr, 48hr, and 72hr respectively. Results shown are the means of two or four separate experiments for 24hr and 48/72hrs respectively (except where indicated) ± s.e.m. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts multiple comparisons test.

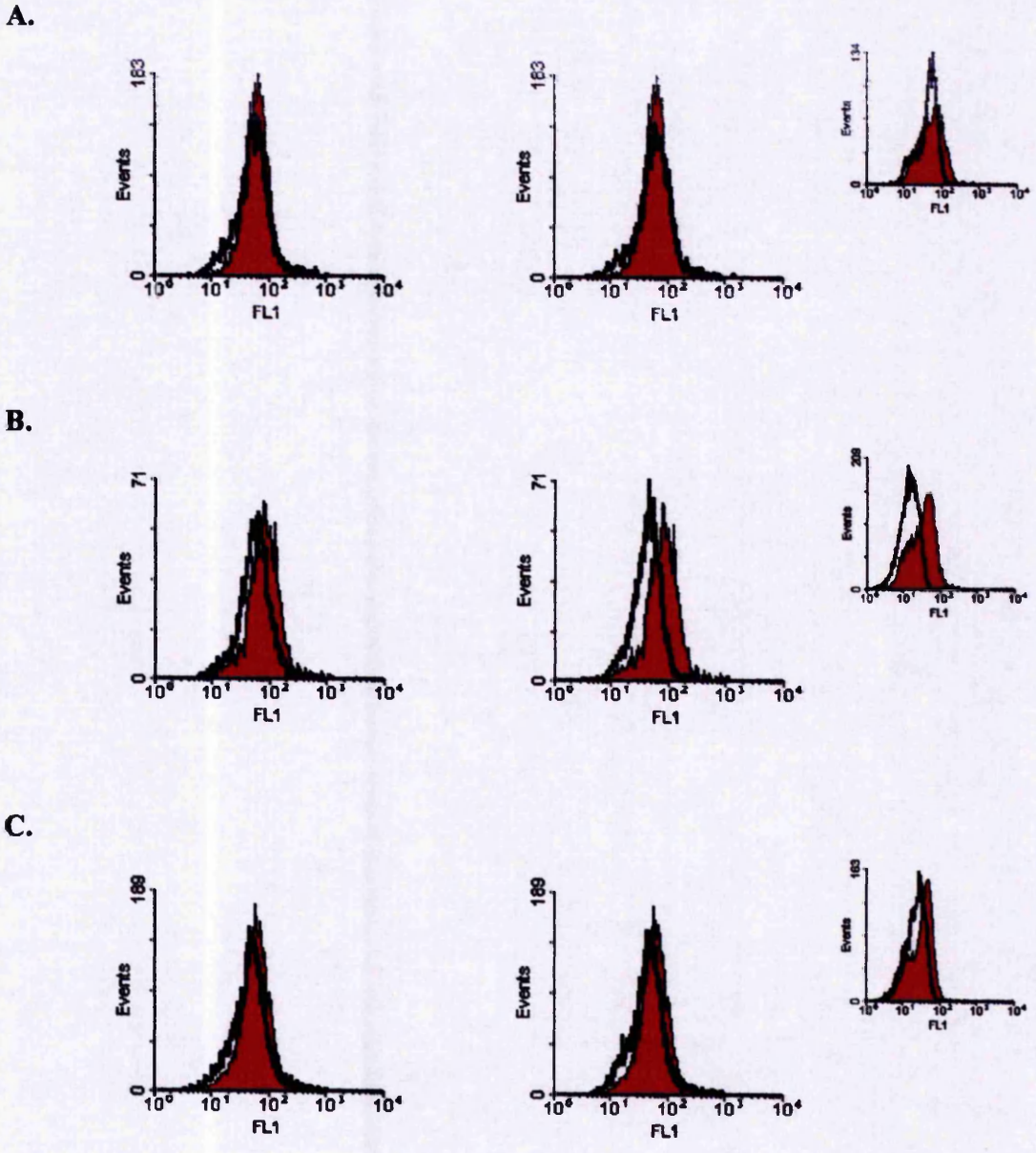
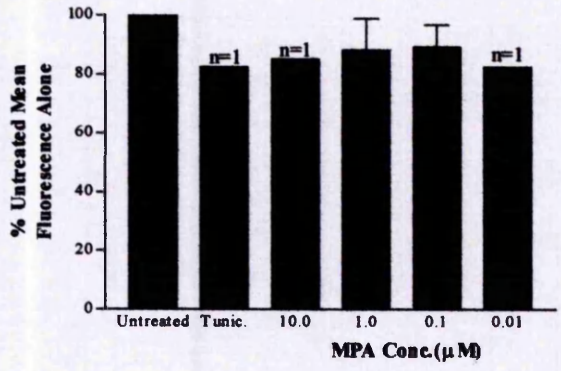
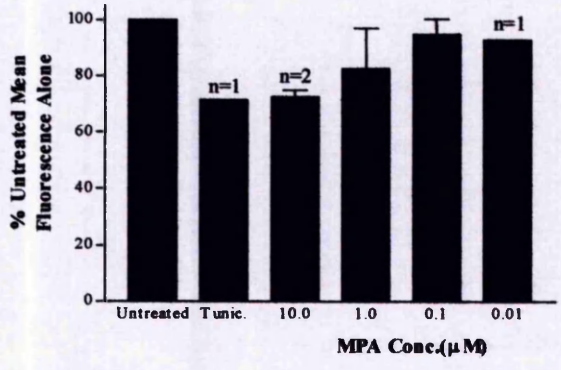


Figure 27. The effect of MPA on binding of the fluorescently labelled lectin *Vicia faba*. ECV304 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) in the presence of different concentrations of MPA (black line): $1x10^{-5}$M (left figure) and $1x10^{-6}$M (right figure). Control cells are shown by the filled red trace. The figure inset shows the effect of tunicamycin (10μg/ml, black line) a known inhibitor of glycosylation

A.



B.



C.

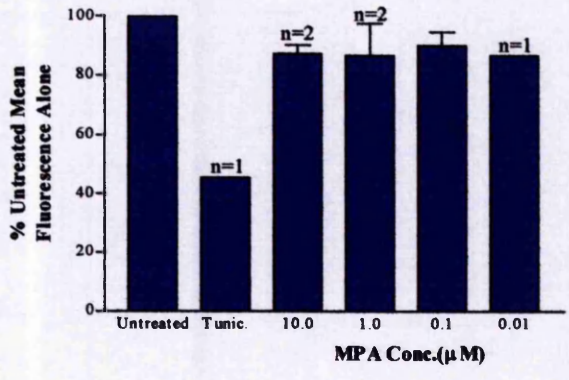


Figure 28. The effect of MPA on mean fluorescence intensity of ECV304 cells stained with FITC-labelled *Vicia faba*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) with different concentrations of MPA then stained with FITC-labelled VFA and fluorescence measured by flow cytometry. The mean fluorescence intensity for control cells was 58.0, 70.9, and 69.0 for 24hr, 48hr, and 72hr respectively. Results shown are from the means of two or three separate experiments for 24hr and 48hr/72hr respectively (except where indicated) ± s.e.m,

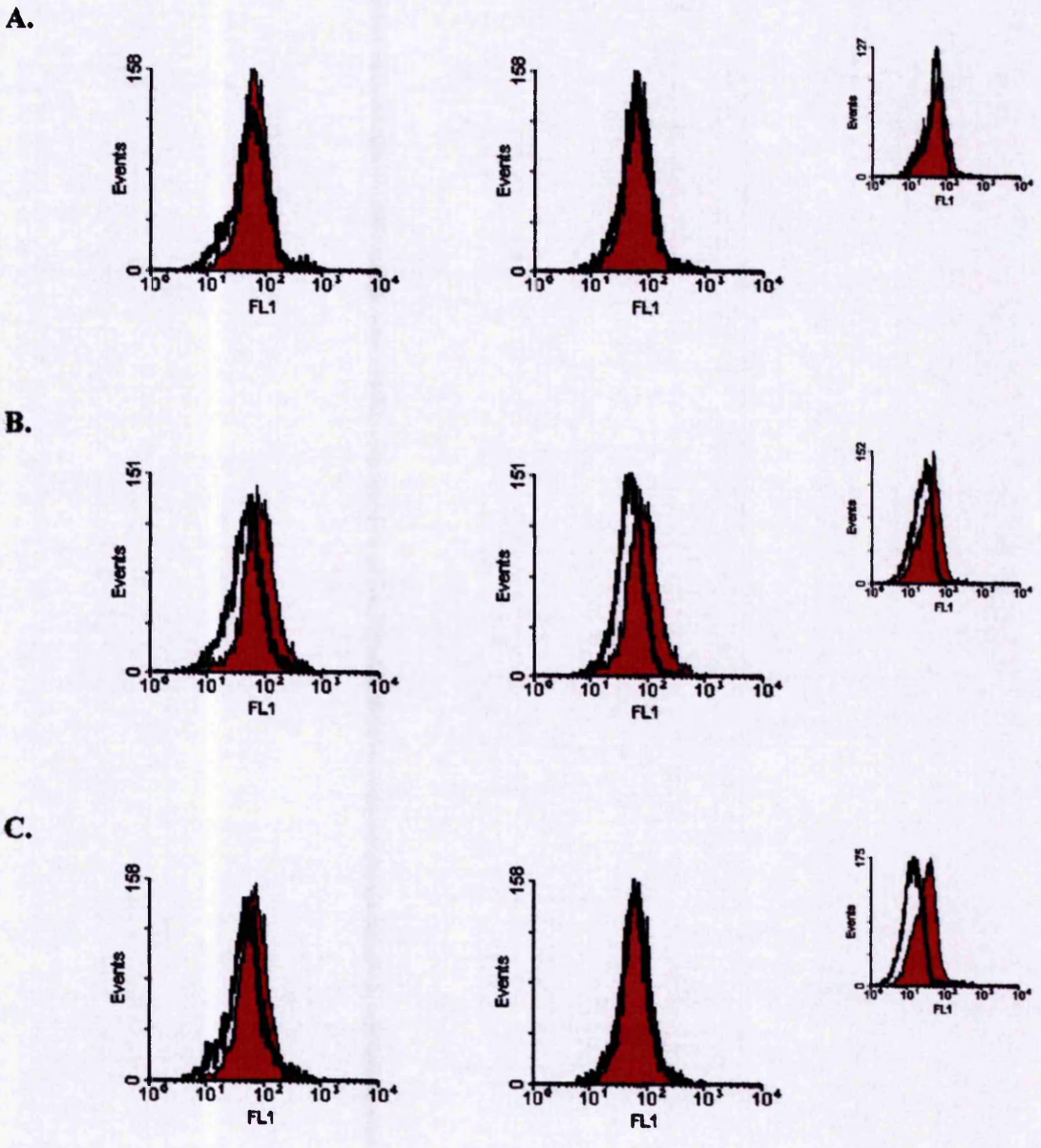


Figure 29. The effect of MPA on binding of the fluorescently labelled lectin *Vicia faba* to TNFα stimulated ECV304 cells. ECV304 cells were incubated for 24hr (A), 48hr (B) and 72hr (C) in the presence of TNFα (200U/ml) and different concentrations of MPA (black line): $1x10^{-5}$M (left figure) and $1x10^{-6}$M (right figure). Control cell are shown by the filled red trace. The figure inset shows the effect of tunicamycin (10μg/ml, black line) a known inhibitor of glycosylation

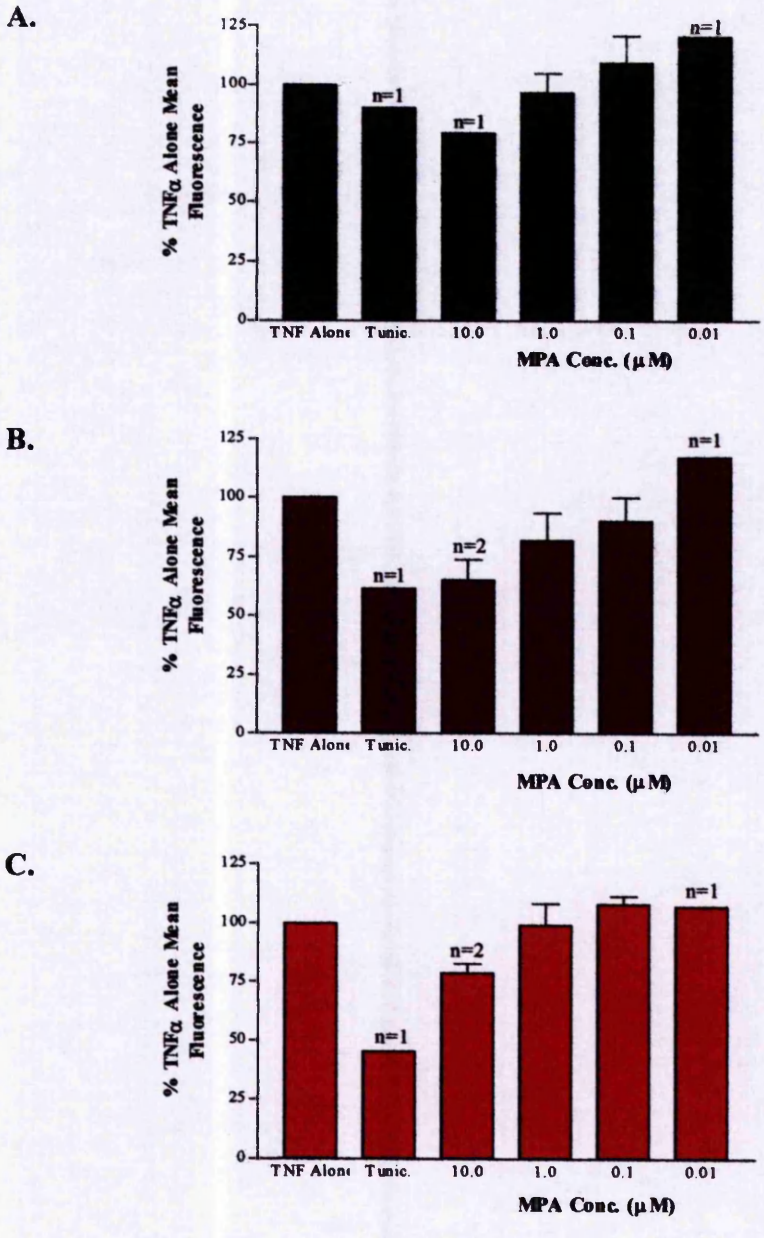


Figure 30. The effect of MPA on mean fluorescence intensity of TNFα stimulated ECV304 cells stained with FITC-labelled *Vicia faba*. Cells were incubated for 24hr (A.), 48hr (B.), or 72hr (C.) with TNFα and different concentrations of MPA then stained with FITC-labelled VFA and fluorescence measured by flow cytometry. The mean fluorescence intensity for TNFα stimulated control cells was 59.9, 87.1, and 78.3 for 24hr, 48hr, and 72hr respectively. Results shown are from the means of two or three separate experiments, 24hr and 48hr/72hr respectively (except where indicated), ± s.e.m.

4 The effect of MPA on Transcription Factor Activity

The effect of MPA on NF-κB and AP-1 transcription factor activity was investigated using a reporter gene approach. Initial experiments were performed in transiently transfected Jurkat T cells using the different reporter genes. There are disadvantages to this technique because the length of time for which we could incubate the transfected cells with the treatments would be limited. This is due to dilution of the plasmid because it is lost from the cells as they go through cycles of replication. Another disadvantage is that the plasmid DNA is not stably integrated into the cellular genome and therefore not subjected to the processes involved in the control of normal cellular gene expression. Figure 31 shows the result of a preliminary experiment using Jurkat T cells transfected with an AP-1 reporter gene construct. After 24 hours of culture with varying concentrations of PMA and MPA there is no effect on the activity of AP-1. It was also attempted to determine the effect of MPA on NF-κB activity using a similar approach, but using a variety of different stimuli it was not possible to induce any NF-κB activity even in control cells (data not shown). This was therefore another limitation to the use of this method.

We then chose to use stably transfected CEM-C7 T cells, which had been transfected with the same AP-1 reporter gene or NF-κB reporter gene used in the previous transient transfections. Stably transfected cells not only allow longer incubation times to be tested because the plasmid is replicated as the cells replicate but also, because the DNA is stably integrated into the cellular genome its expression is therefore under the normal physiological controls. Attempts to set up stably transfected cell lines ourselves were unsuccessful so we had to return to the use of transiently transfected cells for experiments to investigate the effects of MPA on AP-1 and NF-κB activity in ECV304 endothelial cells.

Using these approaches, it was therefore possible to investigate the effect of MPA treatment on the signalling pathways leading to transcription factor activation

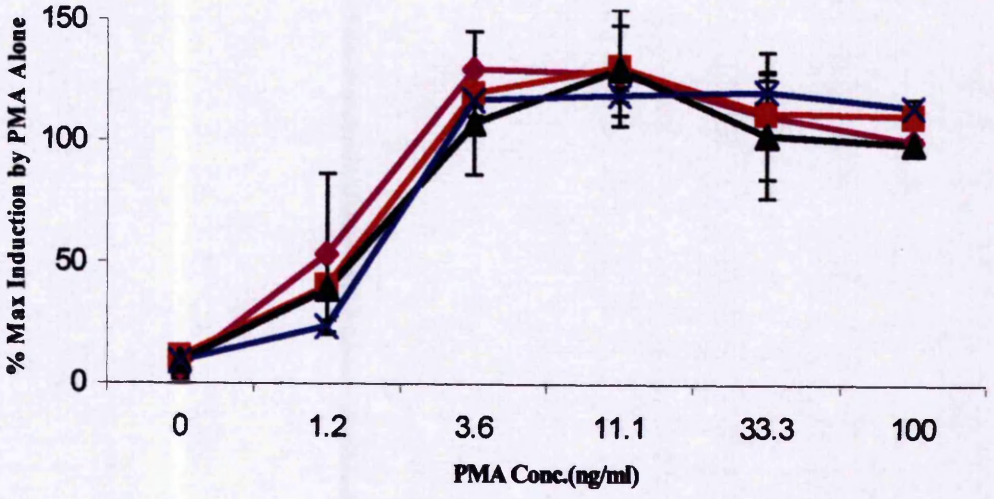


Figure 31. The Effect of MPA on PMA stimulated AP-1 activity in transiently transfected Jurkat cells. Cells were treated for 24hr with increasing concentrations of PMA either alone (◆)or in the presence of different concentrations of MPA $1x10^{-6}$M (■), $1x10^{-8}$M (▲), and $1x10^{-10}$ M (x) before assaying for reporter gene activity. Data plotted is the mean of two individual experiments with triplicate samples for each (± one standard deviation) and represent the percentage optical density (570nm) of that seen with the maximum dose of PMA alone (100ng/ml). PMA (100ng/ml) resulted in a 15-fold induction over unstimulated O.D. values (0.49 compared with 0.034).

4.1 MPA and NF-κB Activity in CEM-C7 Cells

CEM-C7 cells that had been stably transfected with an NF-κB reporter gene construct were stimulated with increasing concentrations of TNFα to induce NF-κB activity (table 1). Cells were cultured for 24hr, 48hr, and 72hr with TNFα alone or in combination with MPA. Increasing doses of TNFα increased NF-κB activity but when MPA was included as well there was an inhibition of NF-κB activity particularly at $1x10^{-6}$M which increased as time progressed. To more clearly define the effect of MPA on NF-κB activity, cells were stimulated with a single dose of TNFα (200U/ml) and increasing doses of MPA (Figure 32). MPA produced a significant inhibition of NF-κB activity at concentrations from 100μM to 0.5μM at both 48 and 72hrs. To determine if the effect seen was due to the depletion of guanosine nucleotides by MPA, exogenous guanosine was also added to the cultures along with TNFα (200U/ml) and MPA ($1x10^{-6}$M) (Figure 33). Cells incubated with TNFα alone and MPA had decreased NF-κB activity however this returned to normal as the concentration of exogenous guanosine added increased.

MPA inhibits CEM-C7 cell proliferation therefore, it was thought that the reason for the decrease in NF-κB activity might be due to a decrease in the number of cells/protein concentration in MPA treated wells. Protein assays were therefore performed on identical cultures to determine the effect of MPA on protein levels (figure 34). Figure 35 shows that MPA does in fact cause a decrease in protein concentration as incubation time increases. MPA therefore seems to inhibit TNFα mediated NF-κB activity in stably transfected CEM-C7 cells. But the effect appears to be due to the antiproliferative effect of MPA on CEM-C7 cell proliferation decreasing the protein concentration, which is confirmed by protein assays on the cultures.

24hr

MPA (μM)	TNFα Conc. (U/ml) 0	7.4	22.22	66.67	200	600
0	6.54 ±4.1	44.16 ±21.6	70.37 24.7±	89.82 ±17.4	102.85 ±5.8	100.00 ±0
0.0001	11.85 ±5.8	47.90 ±12.9	77.59 ±9.7	98.64 ±11.2	101.07 ±10.0	92.43 ±4.6
0.01	15.44 ±7.0	54.54 ±8.0	87.24 ±11.7	101.3 ±6.8	103.56 ±6.1	104.31 ±12.6
1.0	9.07 ±1.3	38.42 ±15.7	58.22 ±17.0	76.10 ±13.6	81.89 ±5.0	85.07 ±10.0

48hr

MPA (μM)	TNFα Conc. (U/ml) 0	7.4	22.22	66.67	200	600
0	5.67 ±1.9	20.31 ±10.0	39.58 ±16.6	72.47 ±20.7	100.22 ±10.4	100.00 ±0
0.0001	10.87 ±9.3	19.66 ±10.4	37.72 ±17.7	66.52 ±22.3	98.36 ±18.7	94.86 ±17.1
0.01	9.32 ±5.6	20.34 ±9.8	38.92 ±14.9	72.53 ±14.3	103.04 ±12.3	93.70 ±5.0
1.0	4.04 ±1.7	15.70 ±6.4	27.88 ±7.8	44.59 ±8.0	63.15 ±10.6	62.36 ±6.2

72hr

MPA (μM)	TNFα Conc. (U/ml) 0	7.4	22.22	66.67	200	600
0	6.90 ±0.7	10.62 ±4.1	17.69 ±7.0	48.25 ±17.5	84.89 ±24.8	100.00 ±0
0.0001	6.74 ±0.6	9.73 ±4.0	17.91 ±7.5	43.55 ±18.9	77.10 ±29.8	80.42 ±13.1
0.01	7.00 ±2.3	9.23 ±3.6	18.13 ±8.0	47.24 ±16.5	84.31 ±25.4	92.80 ±14.2
1.0	3.91 ±3.0	8.18 ±4.2	15.88 ±4.3	31.00 ±7.4	46.78 ±12.9	44.32 ±3.0

Table 1. The Effect of MPA on TNFα stimulated NF-kB activity in stably transfected CEM-C7 cells. Cells were treated for 24hr, 48hr, or 72hr with increasing concentrations of TNFα either alone or in the presence of different concentrations of MPA (as indicated) before assaying for reporter gene activity. The tables show the mean of triplicate samples from three individual experiments and represent the percentage optical density of that seen with the maximum dose of TNFα alone (200U/ml) at each time point. The O.D. values for TNFα controls (200U/ml) were 0.96 (0.06), 1.02 (0.05), and 1.13 (0.08) for 24, 48, and 72hr, numbers in brackets are unstimulated O.D's.

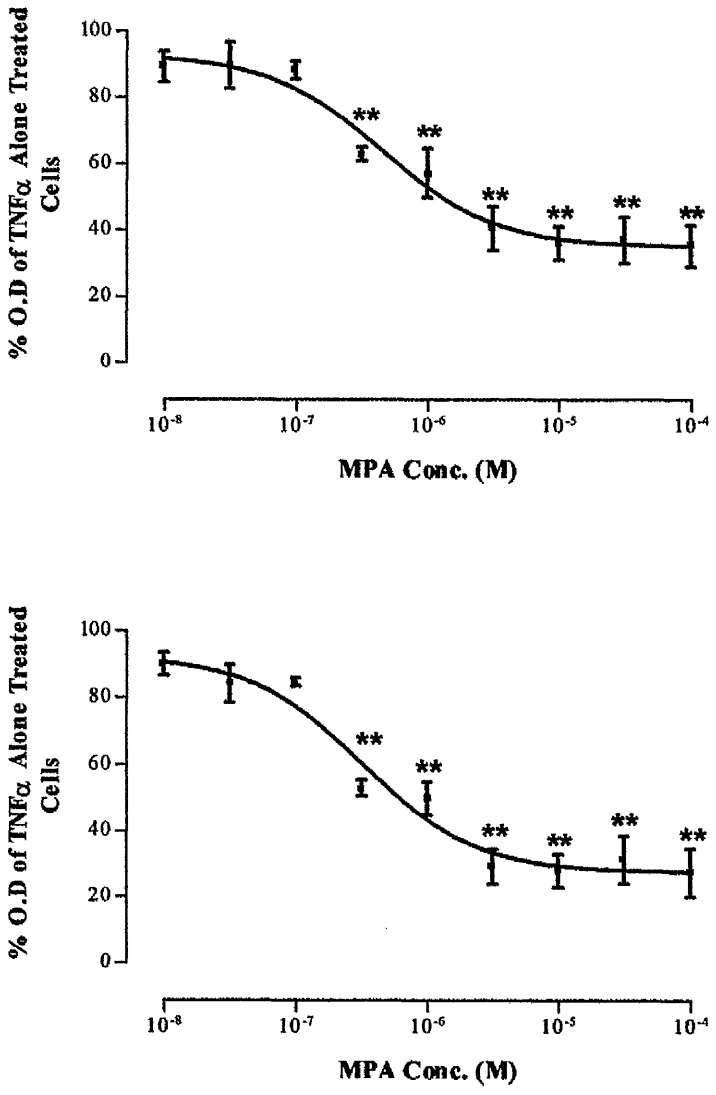


Figure 32. The effect of MPA concentration on TNFα stimulated NF-κB activity in stably transfected CEM-C7 cells. CEM-C7 cells were stimulated with TNFα (200U/ml) in the presence of different concentrations of MPA. Cultures were incubated for 48hr (top) or 72hr (bottom) before assaying for reporter gene activity. Data shown is the mean of triplicate samples from 4 individual experiments ± sem and represents the percentage optical density compared with TNFα treated cultures alone. Mean TNFα alone control O.D. values were 1.3 (0.08) and 1.01 (0.12) units for 48hr and 72 hr, numbers in brackets show the respective background O.D. values. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts multiple comparisons test.

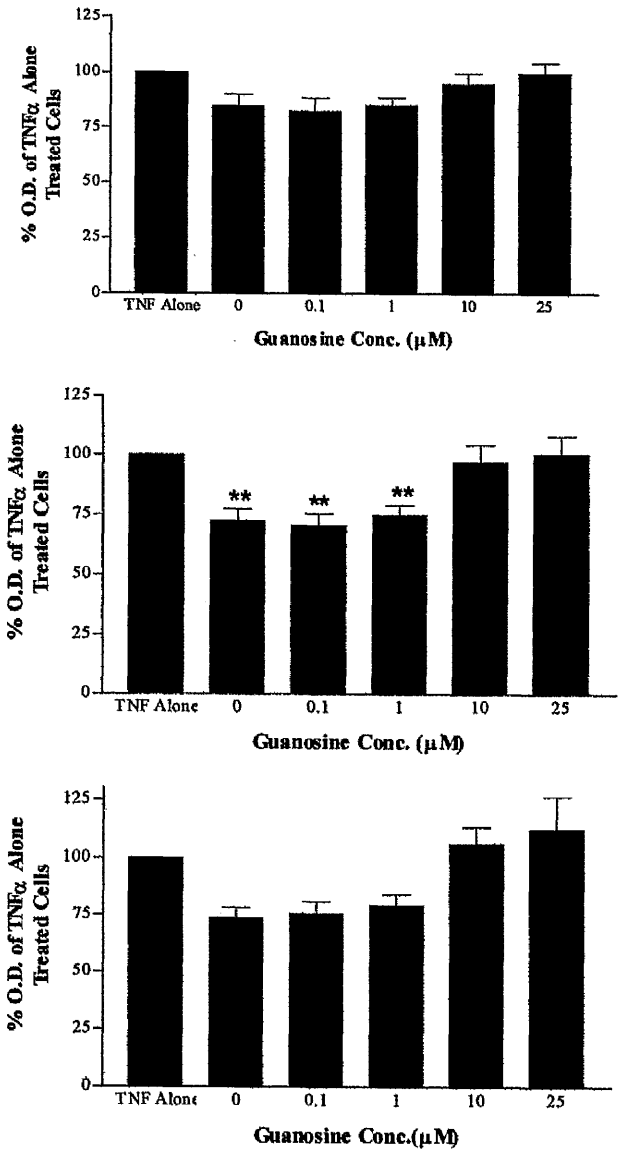


Figure 33. The effect of exogenous guanosine on TNFα stimulated NF-κB activity in MPA treated stably transfected CEM-C7 cells. CEM-C7 cells were stimulated with TNFα (200U/ml) alone or in the presence of MPA ($1x10^{-6}$ M) with the addition of exogenous guanosine. Cultures were incubated for 24hr (top), 48hr (middle) or 72hr (bottom) before assaying for reporter gene activity. Data shown is the mean of triplicate samples from 6 experiments ± sem, and represents the percentage optical density compared with TNFα treated cultures alone. Mean TNFα alone control O.D. values were 1.04 (0.1), 1.01 (0.1) and 1.03 (0.11) for 24hr, 48hr and 72hr, numbers in brackets are the respective background O.D. values. ** Denotes $P<0.01$ one-way ANOVA followed by Dunnetts Multiple Comparisons test.

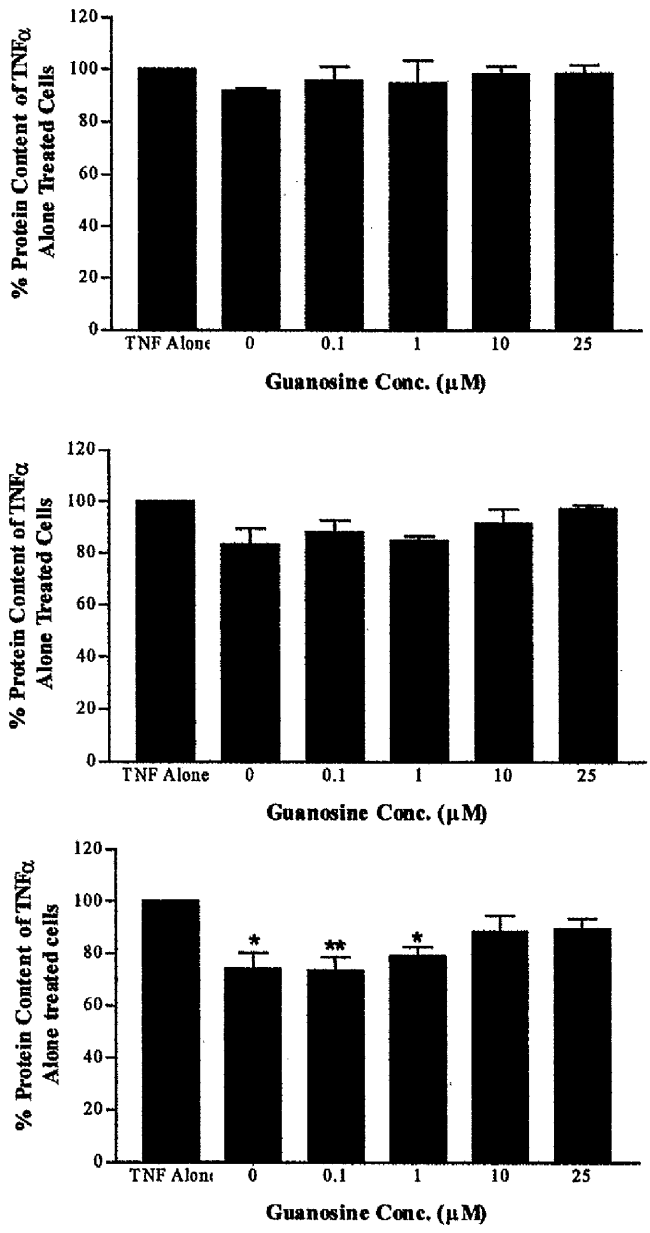


Figure 34. The effect of MPA on protein concentration in NF-κB reporter gene assays. Cells were incubated for 24hr (top), 48hr (middle) and 72hr (bottom) in exactly the same way as for reporter gene assays and then assayed for protein concentration. Data shown is the mean of duplicate samples from two or three individual experiments (24hr and 48/72hrs respectively) and is plotted as the percentage of the protein concentration contained in control TNFα alone treated samples. Mean TNFα alone control protein concentrations were 79.1, 91.1, and 103.43μg/ml for 24, 48 and 72 hr respectively. * and ** denotes $P<0.05$ and <0.01 respectively one-way ANOVA followed by Dunnetts multiple comparisons test.

4.2 MPA and AP-1 Activity in CEM-C7 Cells

We then chose to look at the effect of MPA on AP-1 activity using CEM-C7 cells stably transfected with an AP-1 reporter gene construct. Cells were stimulated with increasing concentrations of PMA to activate AP-1 either alone or in the presence of MPA (table 2). PMA increased AP-1 activity in a dose dependent manner at 24, 48, and 72hr however when MPA (1μM) was also included, AP-1 activity was potentiated above untreated cells an effect that increased with incubation time. To define the response to MPA further, cells were activated with one concentration of PMA (5ng/ml) and different doses of MPA (Figure 35). AP-1 activity was increased above controls at doses as low as 0.1μM with a significant increase seen at 0.1μM and 0.5μM (48hr) and 0.5μM and 1.0μM (72hrs). To determine whether the effect being observed was due to guanosine nucleotide depletion by MPA, exogenous guanosine was added into the cultures along with PMA (5ng/ml) and MPA ($5x10^{-7}$M) (Figure 36). At 24hrs there was no effect by either MPA or the addition of exogenous guanosine on AP-1 activity. But after 48hrs and 72hrs MPA significantly increased AP-1 activity, an effect which was reversed by the addition of exogenous guanosine (0.1μM). Protein assays were also performed on cultures to determine if we were seeing any effect of MPA on the protein concentration in the cultures (figure 37). MPA caused a slight decrease in protein concentration after 72 hours this therefore indicates that MPA is directly affecting AP-1 activity and not indirectly by influencing the level of protein in the cultures.

	PMA Conc. (ng/ml)					
	MPA (μM)	0	0.62	1.85	5.55	16.67	50
24hr	0	0.3 ±0.6	109.5 ±22.9	140.4 ±23.5	126.4 ±8.3	114.8 ±1.8	100.0 ±0
	0.0001	1.5 ±1.3	115.8 ±31.0	143.7 ±33.3	129.5 ±16.8	118.4 ±11.7	100.8 ±11.1
	0.01	1.7 ±2.9	117.4 ±27.7	146.0 ±20.0	138.2 ±15.2	119.9 ±6.6	106.7 ±6.4
	1.0	6.7 ±11.6	148.6 ±46.8	165.3 ±19.9	146.1 ±5.3	131.1 7.8±	111.5 ±5.8
	PMA Conc. (ng/ml)						
	MPA (μM)	0	0.62	1.85	5.55	16.67	50
48hr	0	0.6 ±0.8	165.9 ±41.8	182.6 ±13.9	155.7 ±5.7	127.6 ±5.5	100.0 ±0
	0.0001	0.8 ±0.9	195.2 ±30.4	210.2 ±1.8	176.2 ±15.8	136.8 ±7.7	115.4 ±8.3
	0.01	1.3 ±1.3	189.1 ±51.1	222.8 ±8.4	171.3 ±3.5	139.9 ±8.2	110.6 ±6.3
	1.0	1.2 ±1.5	518.8*** ±6.0	482.9*** ±58.5	370.5*** ±21.6	311.1*** ±20.0	256.3*** ±19.5
	PMA Conc. (ng/ml)						
	MPA (μM)	0	0.62	1.85	5.55	16.67	50
72hr	0	5.1 ±5.6	219.7 ±55.3	201.1 ±13.6	157.5 ±12.6	118.6 ±1.0	100.0 ±0
	0.0001	5.8 ±5.4	233.1 ±68.7	209.5 ±28.2	156.5 ±11.5	128.3 ±15.2	97.5 ±10.1
	0.01	5.9 ±6.2	249.7 ±72.1	237.9 ±39.4	173.7 ±11.5	134.3 ±13.9	105.1 ±7.5
	1.0	1.3 ±1.2	890.3* ±301.0	749.9* ±216.4	584.5** ±154.4	462.2** ±127.0	347.8* ±108.8

Table 2. The Effect of MPA on PMA stimulated AP-1 activity in stably transfected CEM-C7 cells. Cells were treated for 24hr, 48hr, or 72hr with increasing concentrations of PMA either alone or with different concentrations of MPA (as indicated). The tables show the mean of triplicate samples from three individual experiments and represent the percentage optical density of that seen with max. dose of PMA alone (50ng/ml) at each time point. The O.D. values for PMA alone controls (50ng/ml) were 0.71 (0.002), 0.24 (0.001) and 0.14 (0.01) for 24, 48, and 72hr; numbers in brackets are the respective unstimulated O.D. values. *, **, *** $P<0.05$, <0.01, <0.001 respectively denote significant differences between PMA alone and MPA ($1x10^{-6}$M) treated, by two-way ANOVA followed by unpaired students T-tests.

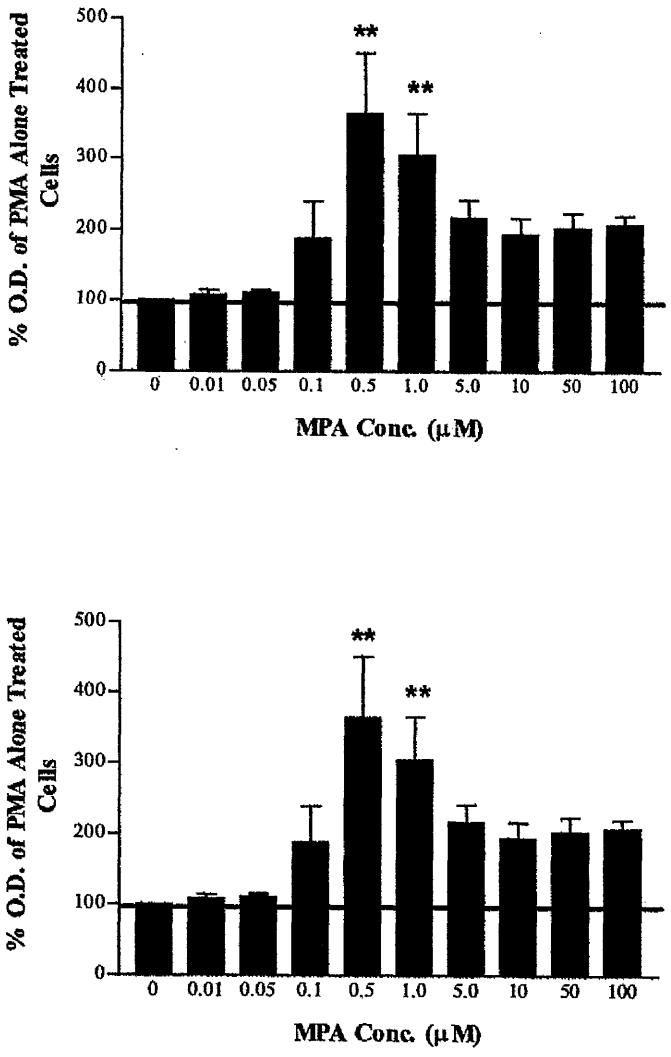


Figure 35. The effect of MPA concentration on PMA stimulated AP-1 activity in stably transfected CEM-C7 cells. CEM-C7 cells were stimulated with PMA (5ng/ml) in the presence of different concentrations of MPA. Cultures were incubated for 48hr (top) or 72hr (bottom) before assaying for reporter gene activity. Data shown is the mean of triplicate samples from 6 or 7 experiments (48hr and 72hr respectively) ± sem and represent the percentage optical density compared with PMA treated cultures alone. Mean PMA alone control O.D. values were 0.34 (0.098) and 0.26 (0.12) for 48 and 72hr, numbers in brackets show the respective background O.D. values. * and ** denotes $P<0.05$ and <0.01 respectively one-way ANOVA followed by Dunnetts multiple comparisons test

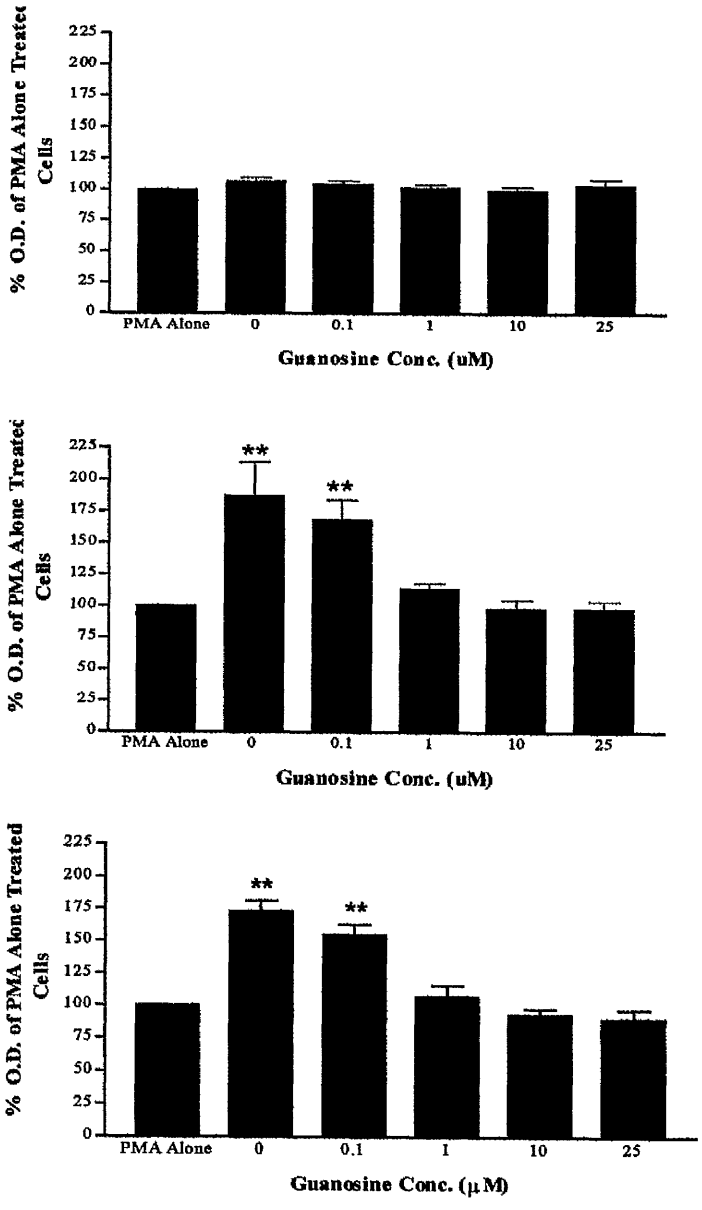


Figure 36. The effect of exogenous guanosine on PMA stimulated AP-1 activity in MPA treated stably transfected CEM-C7 cells. CEM-C7 cells were stimulated with PMA (5ng/ml) in the presence of MPA ($5x10^{-7}$ M). Exogenous guanosine was also added and cultures were incubated for 24hr (top), 48hr (middle) or 72hr (bottom) before assaying for reporter gene activity. Data shown is the mean of triplicate samples from 6 experiments ± sem and represent the percentage optical density compared with PMA treated cultures alone. Mean PMA alone control O.D. values were 0.5 (0.21), 0.55 (0.22), and 0.517 (0.22) for 24, 48 and 72hr, numbers in brackets are the respective background O.D. values. * and ** denotes $P<0.05$ and 0.01 respectively one-way ANOVA followed by Dunnetts multiple comparisons test.

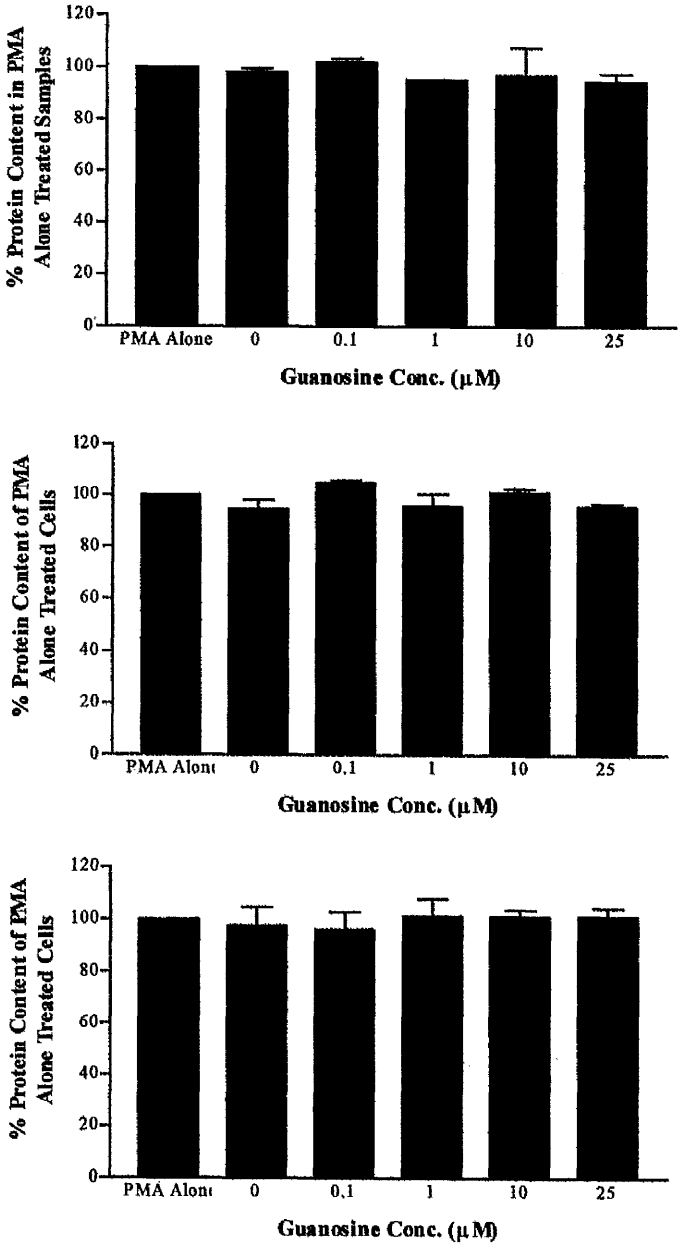


Figure 37. The effect of MPA on protein concentration in AP-1 reporter gene assays. Cells were incubated for 24hr (top), 48hr (middle) and 72hr (bottom) in exactly the same way as for reporter gene assays and then assayed for protein concentration. Data shown is the mean of duplicate samples from two or three individual experiments (24 hr and 48,72hrs respectively) ± s.e.m and is plotted as the percentage of the protein concentration contained in control PMA alone treated samples. Mean PMA alone control protein concentrations were 66.8, 67.9, and 65.6μg/ml for 24, 48, and 72hr respectively.

4.3 MPA and NF-κB Activity in ECV304 Cells.

Transiently transfected ECV304 cells were used to investigate the effect of MPA on NF-κB activity in endothelial cells (Figure 38). Cells were transfected with an NF-κB reporter gene construct and stimulated for 24, 48 and 72 hrs with TNFα (200U/ml) in the presence of increasing concentrations of MPA. There was no significant effect on reporter gene activity by MPA at any of the concentrations used even the highest concentration ($5x10^{-5}M$) was ineffective. The same effect was seen at all the time points observed

4.4 MPA and AP-1 Activity in ECV304 Cells

The effect of MPA on AP-1 activity was also measured in ECV304 cells (figure 39). Cells were transfected with an AP-1 reporter gene construct and stimulated with PMA (5ng/ml) in the presence of different concentrations of MPA. At 24hrs there was no effect by MPA at any concentration used but at 48 hrs there was a slight decrease in AP-1 activity although it was not significant. At 72hrs, again there was a decrease in reporter gene activity at all concentrations used but these decreases in activity may have been due to plasmid loss from the cells as they replicate.

4.5 MPA Effects on the IL-2 promoter

We ultimately intended to investigate the effect of MPA on the activity of a physiological promoter by cloning the human IL-2 promoter into a reporter gene construct. After several attempts, which involved solving various different problems, the IL-2 promoter (-361 to -48bp) was successfully cloned into a polyGal reporter gene plasmid. The construct was sequenced and found to contain the correct insert. But initial experiments with the construct failed to stimulate any activity even in control cells. This was despite using a variety of different stimuli that had previously been shown to stimulate IL-2 reporter gene constructs containing similar regions.

In summary. Investigating the effect of MPA on AP-1 and NF-κB activity in CEM-C7 T cells has shown that MPA causes an increase in AP-1 activity after 48hrs and 72hrs while causing a decrease in NF-κB activity at the same time points. These activities were also shown to be due to guanosine nucleotide depletion because the addition of exogenous guanosine was able to reverse both of the effects. However the decrease seen caused by MPA on NF-κB activity was most probably due to an indirect effect of MPA on cellular proliferation causing a decrease in protein concentration. When we looked at endothelial cells to see if MPA would have a similar action, we found that MPA had no effect on either AP-1 or NF-κB activity at any of the time points.

We have therefore shown that MPA is able to specifically cause an increase in AP-1 activity in CEM-C7 T cells stimulated with PMA.

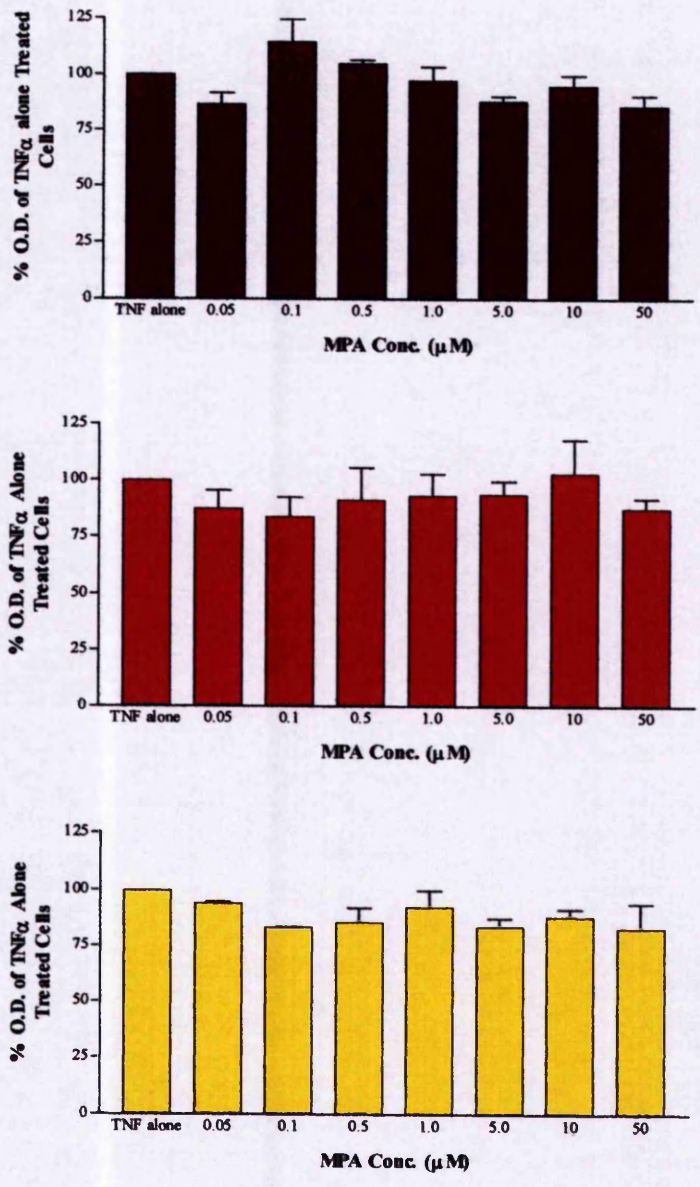


Figure 38. The effect of MPA on ECV304 cells transiently transfected with an NF-κB reporter gene construct. Transfected ECV304 cells were stimulated with TNFα (200U/ml) in the presence of MPA for either 24hr (top), 48hr (middle) or 72hr (bottom) then assayed for reporter gene activity. Data shown is the mean of triplicate wells from 3 individual experiments (except 72hr where there were only 2 experiments) ± s.e.m and represents the percentage optical density of MPA treated samples compared to TNFα treatment alone. Mean TNFα alone control O.D. values were 0.7 (0.19), 0.65 (0.20) and 0.64 (0.21) for 24, 48, and 72hr, numbers in brackets show the respective background O.D. values.

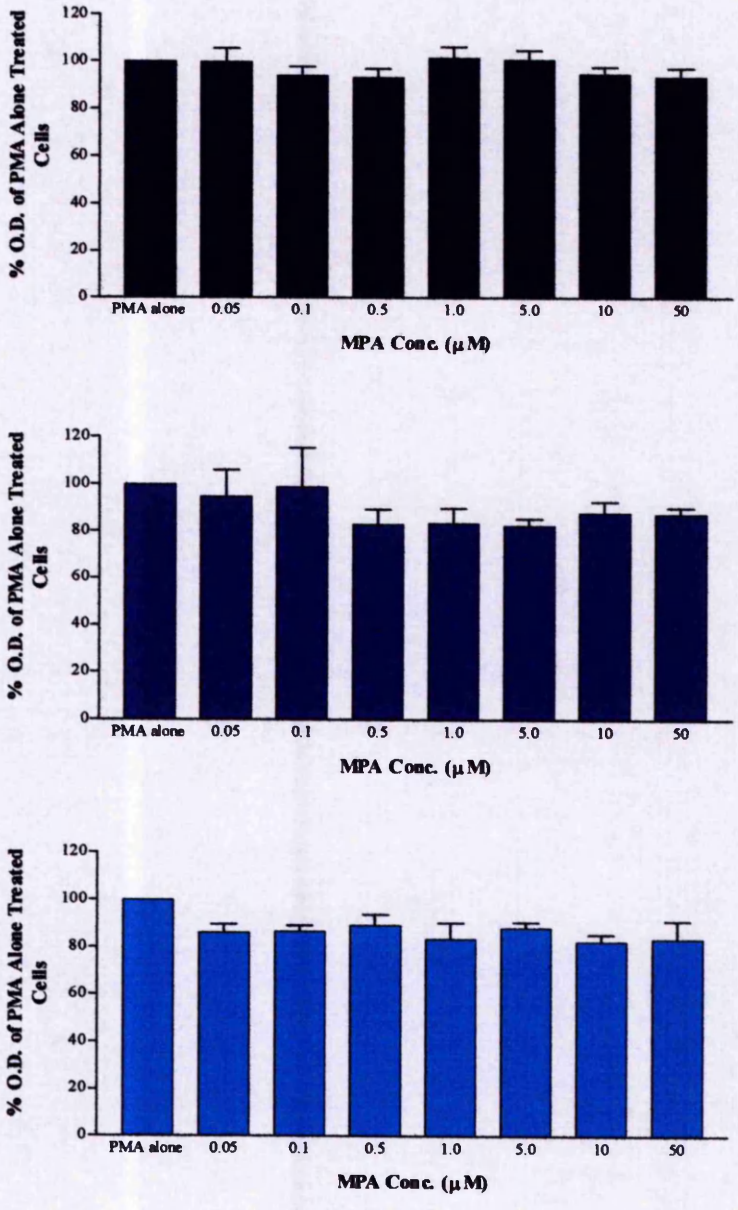


Figure 39. The effect of MPA on ECV304 cells transiently transfected with an AP-1 reporter gene construct. Transiently transfected ECV304 cells were stimulated with PMA (5ng/ml) in the presence of MPA for either 24hr (top), 48hr (middle) or 72hr (bottom) then assayed for reporter gene activity. Data shown is the mean of triplicate wells from 3 individual experiments ± s.e.m and represents the percentage optical density of the MPA treated samples compared to PMA treatment alone. Mean PMA alone control O.D. values were 0.86 (0.09), 1.24 (0.08) and 1.2 (0.07) for 24, 48, and 72hr, the numbers in brackets show the respective background O.D. values.

5 The Effect of MPA on Transcription Factor-DNA Binding

Using radiolabelled consensus oligonucleotides specific for NF-κB, AP-1 and CREB we were able to investigate the effect of MPA on the ability of these transcription factors to bind DNA following culture of CEM-C7 cells and ECV304 cells in the presence of MPA. Figure 40 shows the control reactions, using HeLa extract, which were included for each experiment. The competition reactions (lanes 2 and 3), using cold probes to compete out the radiolabelled probe, were always included to ensure that the probes we were using were specific for that particular transcription factor

5.1 Transcription Factor Binding in CEM-C7 Cells

CEM-C7 cells were cultured either alone, or in the presence of the T cell mitogen Con A, and with different concentrations of MPA for 24hr, 48hr, and 72hr. The effect of MPA on transcription factor-DNA binding was then determined by gel shift assay (EMSA).

5.1.1 CREB

Following culture of CEM-C7 T cells in the presence of MPA, at 24 hr there were 2 CREB bands close together the intensity of which were decreased slightly at the highest MPA concentration (10^{-6}M) (Figure 41A). After 48 hrs a band of larger size became apparent, which in $1x10^{-6}$M treated MPA samples, was completely absent accompanied with a decrease in intensity of the remaining bands. The bands seen in cells treated with $1x10^{-7}$M MPA were the same as controls. A similar pattern as that seen at 48 hr was also observed at 72hr with similar effects on band density observed following treatment with MPA.

CEM-C7 T cells were then stimulated with Con A (Figure 41B) and three bands close together were present, the intensities of the bands were decreased following treatment with MPA ($1x10^{-6}$M). Following treatment with MPA $1x10^{-6}$M for 48hrs the two upper bands were again diminished in intensity but the lower band had increased in

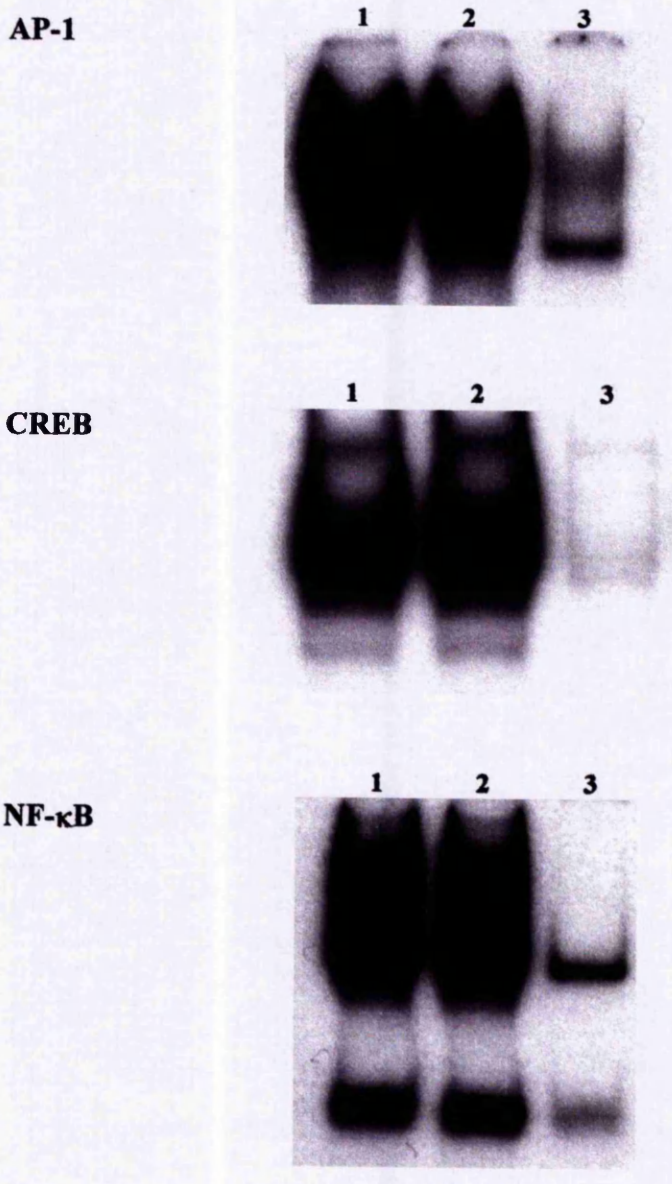


Figure 40. Control reactions for gel shift assays. The figure shows a representation of the control reactions set up for each transcription factor probe. Control reactions were performed with HeLa extracts (5.5μg per lane) incubated with radioactive probe for the particular transcription factor either alone as a positive control (lane 1), with a cold unlabelled non-specific probe (lane 2); or with a cold unlabelled probe specific for the particular transcription factor (lane 3)

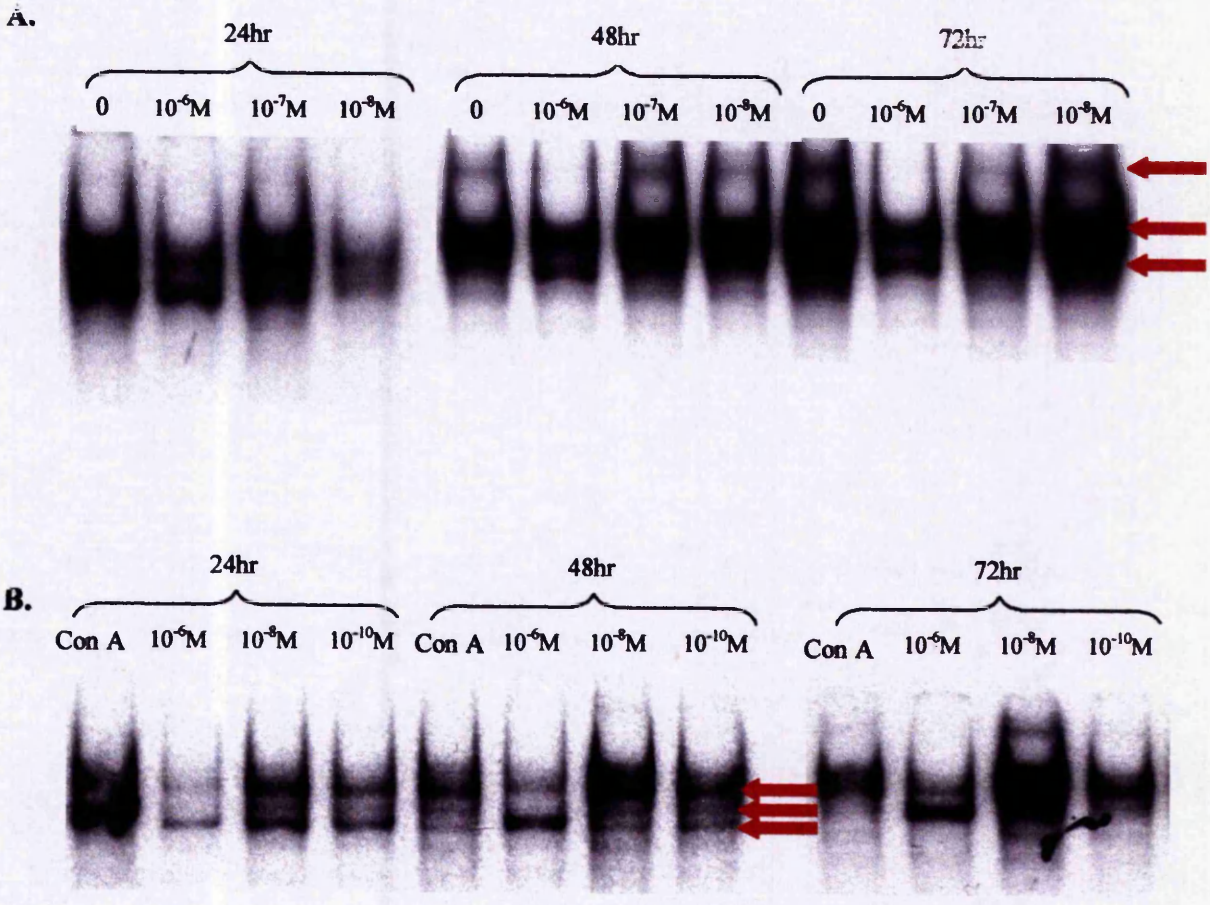


Figure 41. The effect of MPA on the DNA binding ability of CREB in CEM-C7 cells. CEM-C7 cells were incubated for 24hr, 48hr, and 72hr either alone (A) or with Con A (10μg/ml) (B) and in the presence of different concentrations of MPA as indicated above each picture.

intensity above controls. A similar pattern was also observed at 72hrs with $1x10^{-6}$M MPA again decreasing the intensity of the two upper bands with the lower band intensity being unaffected. After 72hrs, at a concentration of $1x10^{-8}$M the intensity of all the bands had increased above controls with a pattern similar to unstimulated cells therefore this was probably not a real effect but experimental error, as it was not seen in a separate experiment (data not shown).

5.1.2 AP-1

Two AP-1 bands were seen with untreated CEM-C7 T cells (Figure 42A) but MPA had no real effect on their density at any concentration either at 24hr or 48hrs. But after 72hr of culture with MPA, at a concentration of $1x10^{-6}$M, both bands were decreased but with the upper band being decreased to a greater extent than the lower band.

The CEM-C7 cells were then stimulated with a combination of both Con A and MPA (Figure 42B). After 24hrs MPA ($1x10^{-6}$M) caused a slight increase the intensity of the two bands detected. Then after 48hrs MPA ($1x10^{-6}$M) treatment decreased the intensity of all the bands seen but the upper band was decreased to a greater extent than the lower band with a similar effect seen again at 72hrs as well.

To determine if the effect we were seeing in the reporter gene assays was due to differences in AP-1 binding caused by MPA following PMA stimulation. Cells were stimulated for the same period, but this time with PMA and in the presence of MPA, before gel shift assays were performed (Figure 43). Figure 45 shows the result from a single experiment and therefore it needs confirmation, but it can be seen that after 24hr and 48hr of culture with MPA there was no effect on AP-1 binding. But following 72hrs of culture with the two agents, a concentration of MPA of $1x10^{-6}$M caused a decrease in AP-1 binding with respect to controls but there did appear to be another smaller complex present (red arrow)

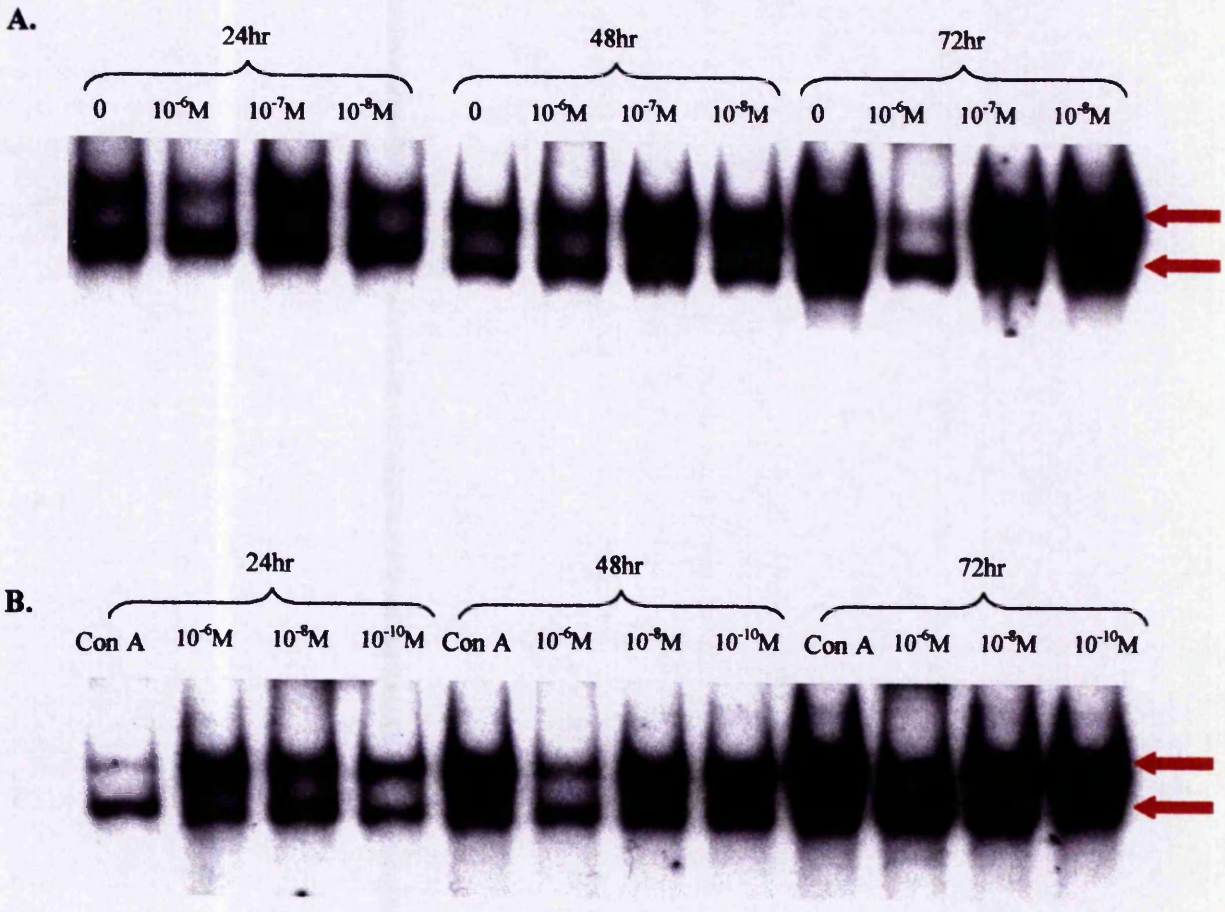


Figure 42. The effect of MPA on the DNA binding ability of AP-1 in CEM-C7 cells. CEM-C7 cells were incubated for 24hr, 48hr, and 72hr either alone (A) or with Con A (10μg/ml) (B) and in the presence of different concentrations of MPA as indicated above each picture.

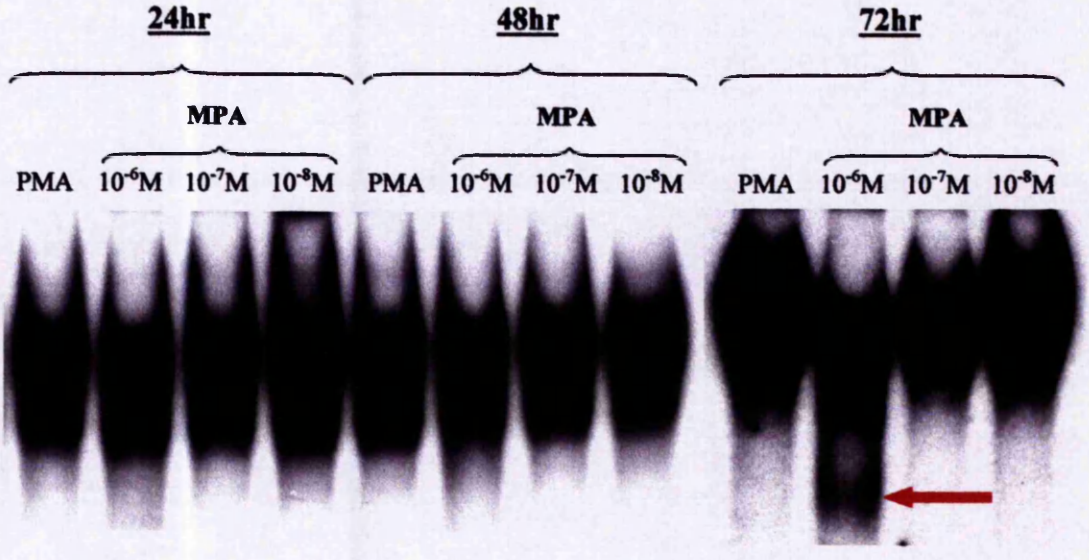


Figure 43. The effect of MPA on PMA stimulated AP-1 DNA binding in CEM-C7 Cells. CEM-C7 cells were stimulated with PMA (5ng/ml) for 24hr, 48hr and 72hr, in the presence of different concentrations of MPA as indicated above each picture.

5.1.3 NF-κB

After 24hrs of culture of CEM-C7 cells with MPA, two bands were present but there was no effect on NF-κB binding (Figure 44A). After 48hrs a different pattern was seen, there were two larger complexes present and one band caused by a smaller complex in the control samples. Following treatment with MPA at $1x10^{-6}$M, the largest complex was inhibited and so was the lower complex, both of which were visible again at $1x10^{-7}$M. The same effect was also seen at 72hr but the decrease in intensity was greater.

The cells were then stimulated with Con A in the presence of MPA (Figure 44B). After 24hrs, two bands were present which were not affected by MPA treatment at any concentration. The same bands were seen at 48hr but the intensity of the smaller complex band was decreased by MPA $1x10^{-6}$M. At 72hr there was a third band present due to a larger complex with a pattern similar to that seen with unstimulated cells at 48 and 72hr. MPA ($1x10^{-6}$M) inhibited the presence of the largest and the smallest complexes so that only one band remained, but the bands returned similar to controls at $1x10^{-7}$M.

The effects on NF-κB activity seen with the TNFα stimulation in reporter gene assays were also investigated by looking at NF-κB binding. Cells were stimulated with TNFα in the presence of MPA for the same period of time as in the reporter gene assays (Figure 45). Following TNFα stimulation three larger complexes were present and there was also a smaller complex seen, all four of these bands were unaffected by MPA at 24hrs. After 48hrs the three bands caused by the larger complexes were still unaffected by MPA but the smaller complex was inhibited at $1x10^{-6}$M. Then after 72hrs of incubation with MPA $1x10^{-6}$M, the largest complex was also inhibited as well as the smallest complex.

5.2 Transcription Factor Binding in ECV304 Cells

Transcription factor binding was also investigated in ECV304 cells. Cells were either unstimulated or stimulated with TNFα, in the presence of different concentrations of MPA for either 24hr or 72hr and then gel shift assays performed on whole cell extracts

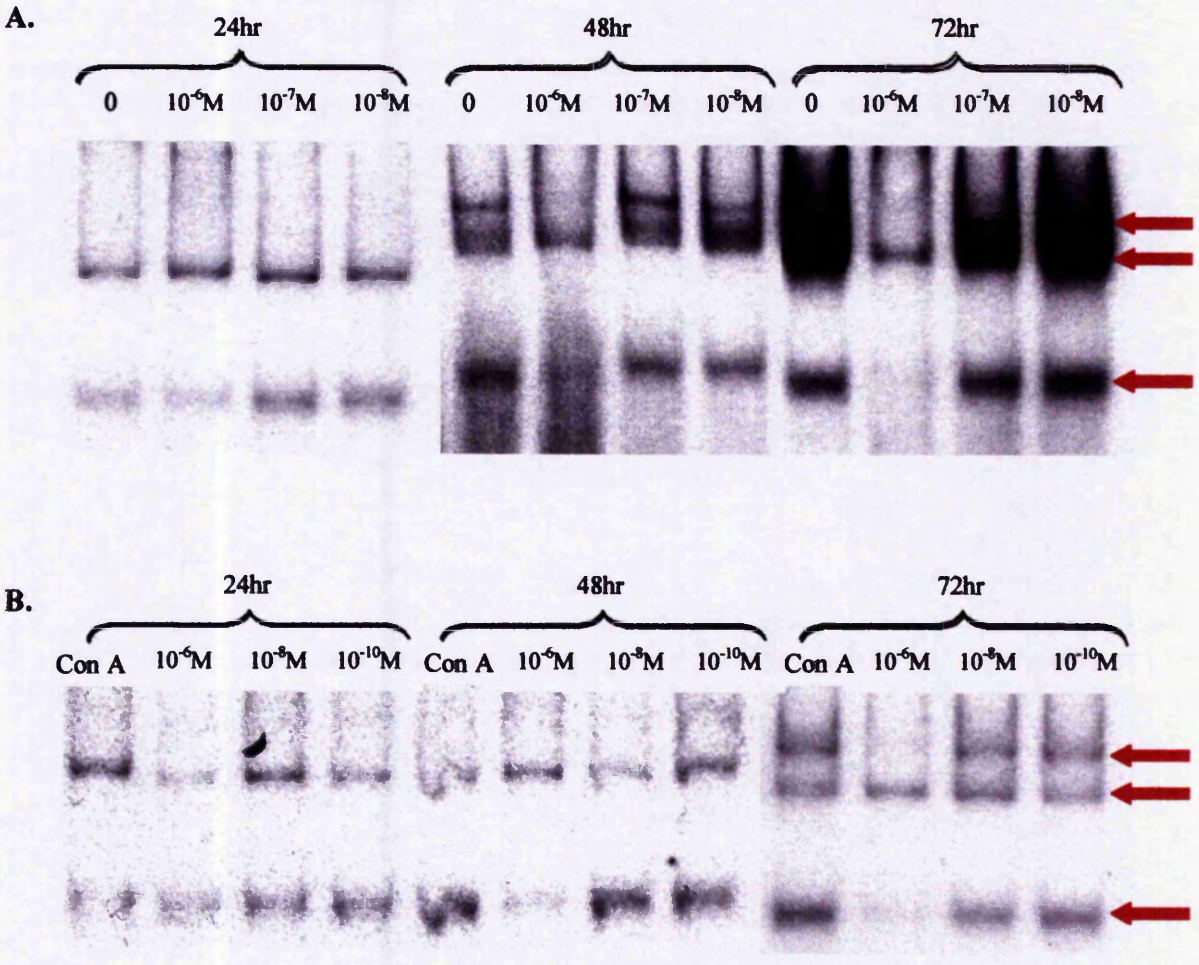


Figure 44. The effect of MPA on the DNA binding ability of NF-κB in CEM-C7 cells. CEM-C7 cells were incubated for 24hr, 48hr, and 72hr either alone (A) or with Con A (10μg/ml) (B) and in the presence of different concentrations of MPA as indicated above each picture.

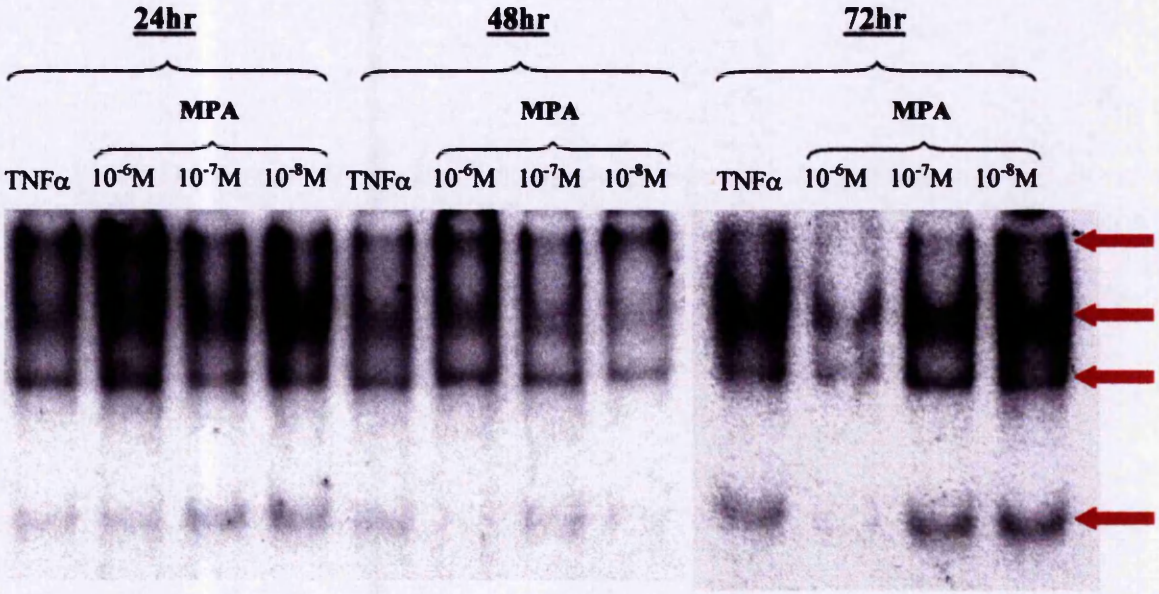


Figure 45. The effect of MPA on TNFα stimulated NF-κB-DNA binding in CEM-C7 Cells. TNFα stimulated (200U/ml) CEM-C7 cells were incubated for 24hr, 48hr and 72hr, in the presence of different concentrations of MPA and transcription factor binding determined by gel shift assay.

The data shown is from preliminary experiments and therefore requires further confirmation.

5.2.1 CREB

Unstimulated ECV304 cells had two prominent bands at 24hr which remained unaffected by TNFα stimulation and also TNFα in combination with MPA treatment (Figure 46A). Following 72hrs of culture, TNFα appeared to induce the appearance of a third band. Again all the bands remained unaffected in the MPA treated samples (Figure 46B)

5.2.2 AP-1

Analysis of AP-1 binding in unstimulated ECV304 cultures showed the presence of two bands which were increased in intensity following TNFα stimulation for 24hr (Figure 47A). Treatment with MPA failed to show any effect on AP-1 binding at any of the concentrations used. A similar pattern of bands was seen at 72hr as well but it was difficult to interpret as the film was very dark however MPA treatment appeared not to affect AP-1 binding (figure 47B).

5.2.3 NF-κB

After 24hrs of culture, TNFα stimulation produced an increase in the intensity of the bands present such that four were visible (figure 48A). MPA had no effect on the intensity or number of bands seen at 24hrs. After 72hrs of culture a similar pattern of bands was again seen (Figure 48B), but there was no effect by MPA on DNA binding by NF-κB at any concentration.

In summary. In CEM-C7 T cells, MPA produces a general decrease in the DNA binding of the different transcription factors although certain bands are decreased in intensity more than others. MPA inhibits the appearance of specific bands seen using a CREB consensus oligonucleotide in MPA treated unstimulated cultures. When cells were stimulated with Con A in the presence of MPA, MPA caused a decrease in intensity of one band while increasing the intensity of another. The effect of MPA on AP-1 was to decrease the intensity of both bands seen with unstimulated and Con A stimulated samples, but causing one to decrease in intensity more than the other. An interesting observation was made following PMA stimulation for 72hr in the presence of MPA ($1x10^{-6}M$), with the appearance of an additional band not seen with control samples. Following incubation of extracts with NF-κB after culturing with MPA at its highest concentration specific bands were inhibited at 48hr and 72hr in both unstimulated and Con A stimulated samples. A similar effect was also seen following TNFα stimulation of cells in combination with MPA. However an additional larger complex was seen following TNFα stimulation that was not observed in untreated or Con A stimulated samples. The appearance of this band was inhibited by MPA ($1x10^{-6}M$) in the presence of TNFα after 72hr of culture

In contrast, MPA was found to have no effect on the DNA binding ability of any of the transcription factors examined in TNFα stimulated ECV304 cells cultured in the presence of MPA for either 24hr or 72hr.

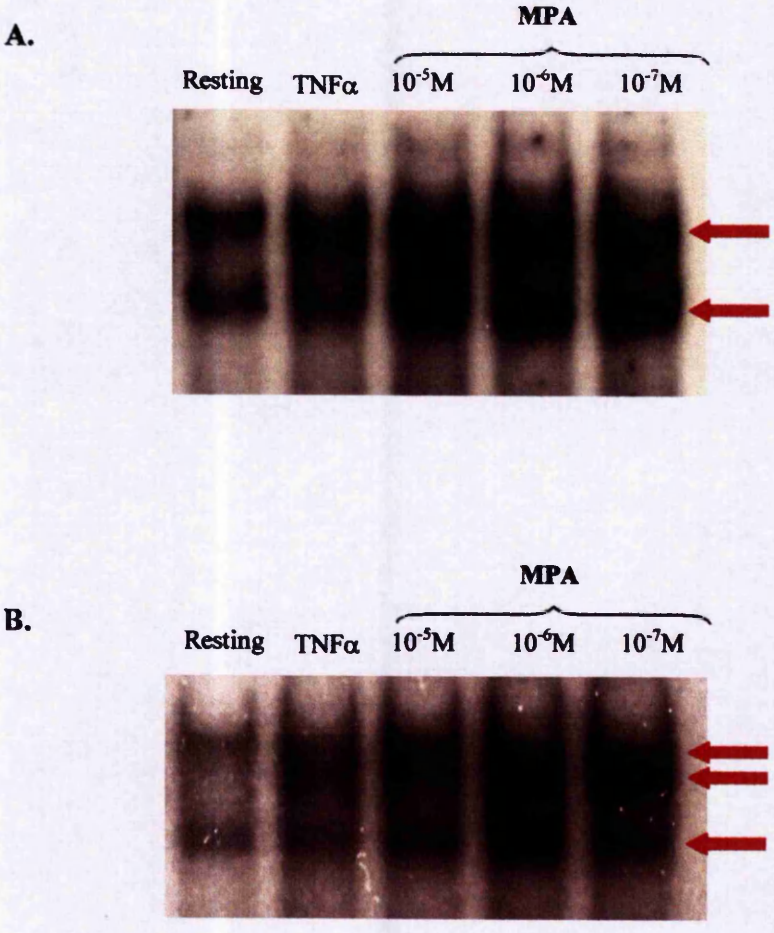


Figure 46. The effect of MPA on the DNA binding of CREB in ECV304 cells. TNFα stimulated ECV304 cells were incubated for 24hr (A.) or 72hr (B.) in the presence of different concentrations of MPA and transcription factor binding determined by gel shift assay.

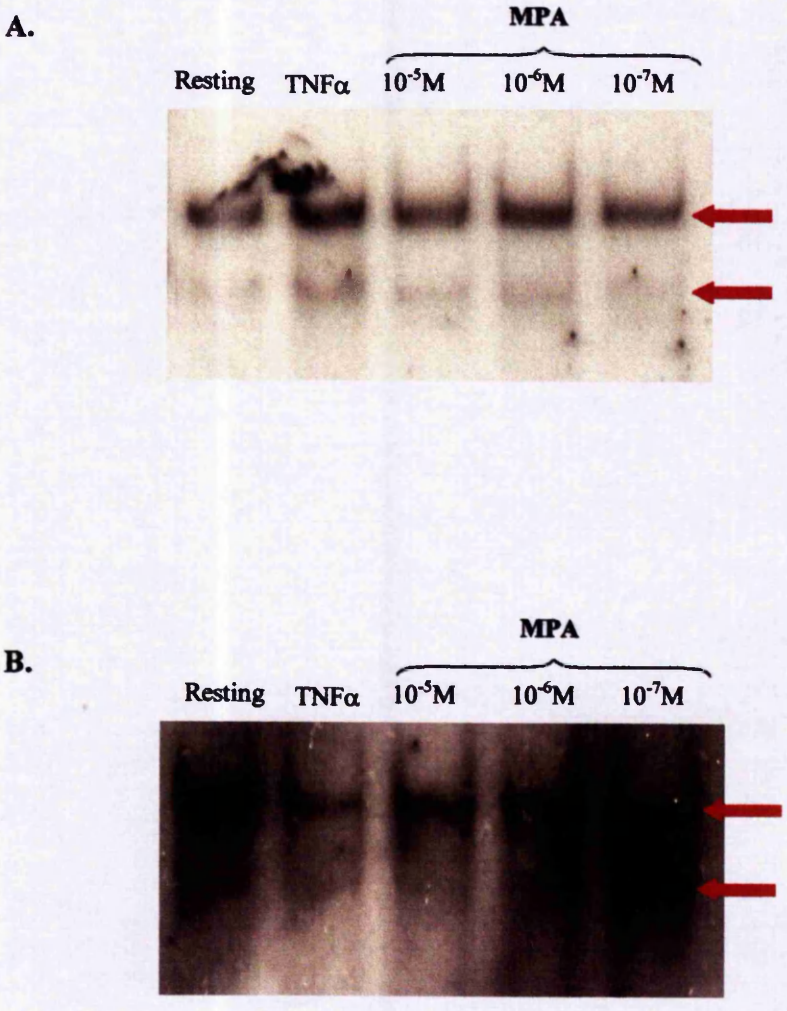


Figure 47. The effect of MPA on the DNA binding of AP-1 in ECV304 cells. TNFα stimulated ECV304 cells were incubated for 24hr (A.) or 72hr (B.) in the presence of different concentrations of MPA and transcription factor binding determined by gel shift assay.

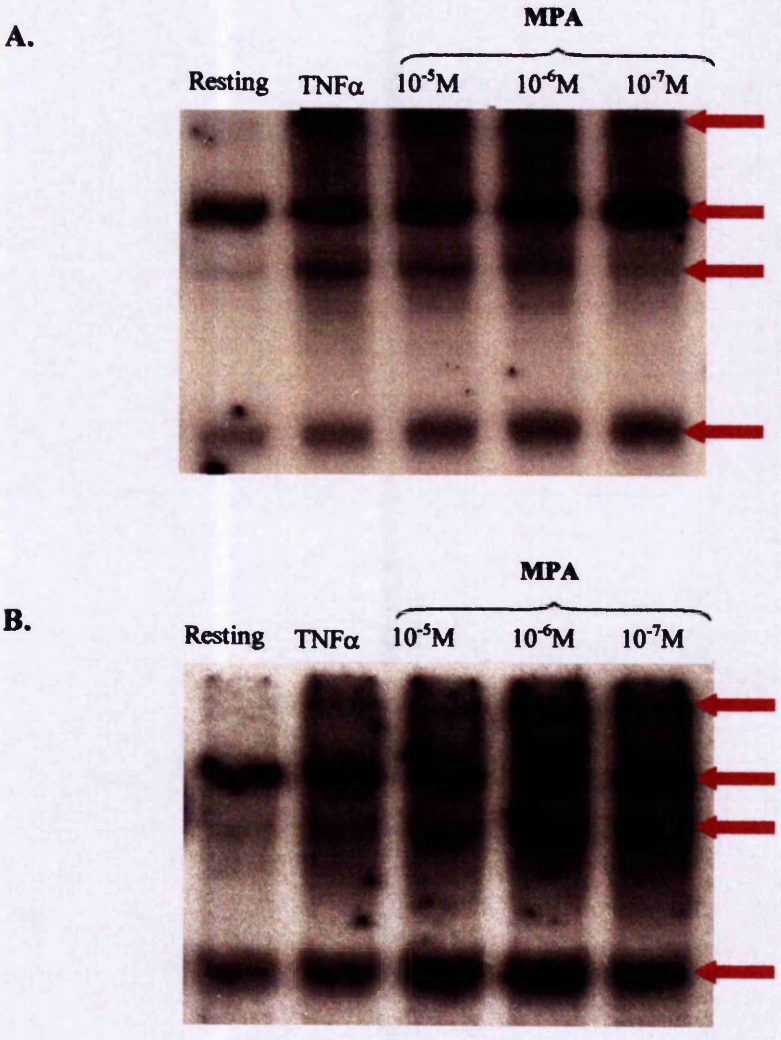


Figure 48. The effect of MPA on the DNA binding NF-κB in ECV304 cells. TNFα stimulated ECV304 cells were incubated for 24hr (A.) or 72hr (B.) in the presence of different concentrations of MPA and transcription factor binding determined by gel shift assay.

Discussion

1 Proliferative Responses in the Presence of MPA

Proliferation assays using CEM-C7 T cells and ECV304 endothelial cells has shown that CEM-C7 cells are more susceptible to the inhibitory effects of MPA than ECV304 cells. This result was not surprising because cells of non-lymphoid origin have efficient salvage pathway function and, unlike T cells, they are able to overcome the inhibition of IMPDH by MPA and produce guanosine nucleotides for DNA synthesis via this alternative pathway. The salvage pathway of T cells is unable to supply sufficient levels of guanosine nucleotides for DNA synthesis and so proliferation is inhibited to a greater extent. The restoration of proliferation with exogenous guanosine supports the fact that MPA exerts its effect by guanosine nucleotide depletion, as a result of IMPDH inhibition, although it was only possible to restore proliferation to approximately 70% of that seen in control CEM-C7 cultures. However, at higher concentrations of guanosine (50μM) CEM-C7 proliferation was inhibited. High levels of guanosine nucleotides have been shown to lead to growth inhibition in T lymphoid cell lines due to the accumulation of GTP, which inhibits ribonucleotide reductase and depletes other deoxynucleoside triphosphates (Sidi and Mitchell, 1984). The effect of exogenous guanosine treatment on CEM-C7 cell proliferation was in contrast to that on ECV304 cells where proliferation was completely restored by addition of exogenous guanosine. Furthermore, exogenous guanosine did not have a toxic effect even at concentrations up to 50μM. The failure to completely restore proliferation in CEM-C7 cells with exogenous guanosine is most likely due to the guanosine being rapidly utilised by the cells. Therefore repeated administration of guanosine is probably required to maintain the necessary levels of guanosine nucleotides required by T cells (Stet *et al*, 1994).

Con A stimulation of CEM-C7 cells did not increase cellular proliferation when the cells were pulsed with ^{3}H-thymidine for 24hrs. However, it has since been shown that if cells are pulsed for a shorter period of time (1-4hrs) then Con A does stimulate an increase in proliferation. Therefore CEM-C7 cells are not unresponsive to Con A, we are just not detecting it when pulsing for 24hrs. It is possible that DNA repair mechanisms remove the incorporated thymidine residues, which results in an apparent decrease in proliferation after 24hrs.

2 MPA and Protein Glycosylation

The effect of MPA on the binding of mannose and fucose specific lectins was also found to be different in T cells and endothelial cells. We demonstrated that MPA has no effect on the binding of mannose- or fucose-specific lectins to CEM-C7 cells. However, in endothelial cells MPA caused a decrease in the binding of both mannose- and fucose-specific lectins although the effect on fucose expression was more significant.

These results conflict with previous reports that suggest MPA interferes with protein glycosylation in T cells. Sokoloski and Sartorelli demonstrated that MPA treatment of sarcoma 180 cells resulted in a decrease in the rate of incorporation of mannose and fucose, although this may not directly reflect cell surface expression (Sokoloski and Sartorelli, 1985). Experiments by Allison *et al* suggested that MPA inhibited the expression of mannose containing oligosaccharides on Con A stimulated human peripheral blood lymphocytes using a mannose specific lectin (Allison *et al*, 1993b). Experiments *in vivo* showed mycophenolate mofetil (MMF) treated rats to have a decreased cellular infiltrate in kidney allografts, resulting in better graft preservation (Heeman *et al*, 1996). The authors attributed this to an effect of MMF on lymphocyte adhesion molecules and possible interactions with macrophages. In addition, MPA was shown to cause a decrease in mannosylation of membrane glycoproteins in human monocytes and decrease the adhesiveness of these cells to human umbilical vein endothelial cells (HUVECs) and laminin (Laurent *et al*, 1996). The decreased adhesion of these cells to HUVEC was greater with IL-1 stimulated HUVEC. We did not look at the effect of MPA on monocytes and therefore cannot rule out the possibility of there being different effects on cells from different lineages.

In contrast to these reports, other studies show that MPA increases the level of ICAM-1 expression after 48hr of incubation with HUVECs (Bertalaffiny *et al*, 1998). In a separate report, treatment of HUVECs with MPA and TNFα caused these cells to exhibit increased binding of U937 monocytes and also show increased VCAM-1 and E-selectin expression (Hauser *et al*, 1997). However, the authors were unable to reverse the increased VCAM-1 and E-selectin expression with exogenous guanosine implying that the effect is not caused by MPA depletion of guanosine.

The failure of MPA to affect fucose and mannose expression on CEM-C7 cells in our experiments could be due to differences between the use of cell lines in our studies and primary cells used in the studies of others. Cell lines are good models for events occurring in primary cells and are therefore an obvious starting point for an investigation. However in our original experiments, to repeat previously published work, we did use primary cells from both human PBMCs and rat LNCs, and we were still unable to show any difference between control and MPA treated groups. In addition the effect of MPA on cellular proliferation was still very similar in CEM-C7 and primary cells with respect to the effective concentration of MPA and extent of proliferative inhibition. Therefore the differences in action of MPA on glycosylation observed between our work and that of others is unlikely to be due to the cell lines used.

It may also be argued that our methodology for detecting lectin binding was not sensitive enough to detect changes in the binding of the different lectins to fucose and mannose residues. However, we were able to detect a significant decrease in lectin binding to cells cultured in the presence of tunicamycin, a known inhibitor of glycosylation. Tunicamycin inhibits the first reaction in the lipid-saccharide pathway, the formation of Dol-PP-*N*-acetylglucosamine from UDP-*N*-acetylglucosamine and dolicholphosphate (Speake, Hemming and White, 1980; Schwarz and Datema, 1980, 1982). Therefore it was possible to detect changes in the cell surface expression of mannose and fucose with the use of tunicamycin in both a T cell line and an endothelial cell line. However when we cultured cells with MPA we were unable to detect any differences in lectin binding to CEM-C7 cells but we did see significant differences in binding to ECV304 cells. Our results not only contradict previous reports, but they also demonstrate a cell-type specific difference. In summary, MPA was able to inhibit the cell-surface expression of both fucose and mannose residues on endothelial cells, but had no effect on their expression on T cells.

2.1 Glycosylation in CEM-C7 cells

To attempt to repeat previous work, we followed the methodology exactly and were still unable to repeat the findings. This involved the use of Con A as a T cell stimulus,

however Con A is a lectin that also shows specificity for mannose residues and may therefore be an inappropriate stimulus to use in these experiments. Con A will therefore compete with VFA for mannose binding which will decrease the sensitivity of the assay. This is probably why there was a decrease in staining intensity with VFA following stimulation of CEM-C7 cells with Con A. There was no effect by MPA on VFA binding in CEM-C7 cells in the absence of Con A and also using fucose specific lectins. Therefore it is unlikely that the reason for not observing any difference in VFA binding is because we are unable to detect an effect caused by MPA due to interference by Con A. However to rule out completely an effect by MPA on VFA binding to mannose, experiments stimulating the cells with anti TCR antibodies or PMA and ionomycin would be more appropriate.

Con A was used to stimulate the cells, by mimicking T cell activation via the cell surface and TCR, in order to activate intracellular signalling pathways. However, the CEM-C7 T cell line already has the phenotype of a lymphoblastoid cell, which is an activated cell and therefore this may affect the response to Con A. Again this highlights differences between the use of cell lines, which are already spontaneously proliferating, in comparison to primary cells that require specific activation to initiate proliferation. Cell lines are therefore good model systems but primary cells should always be used to confirm the results found in cell lines.

We have shown that treatment of CEM-C7 cells with MPA produced no effect on lectin binding to these cells, which may be because CEM-C7 cells have a slower turnover of cell surface glycoproteins than endothelial cells and therefore have a slower rate of glycoprotein synthesis. However it has been shown that following mitogen stimulation of HuPBLs, there is a 10-fold increase in the rate on N-glycosylation (Kumar, Heinemann and Ozols, 1998). The effect on O-glycosylation was less clear, because following mitogen stimulation of murine T cells there was an increase in O-glycosylation of nuclear proteins but a decrease in glycosylation of cytosolic proteins (Kearse and Hart, 1991). CEM-C7 cells have the phenotype of T-lymphoblast cells, which are activated cells (Foley *et al*, 1965). Furthermore they were stimulated with mitogen as well and so would be expected to have a high rate of glycosylation. We were also able to detect differences in glycosylation on CEM-C7 cells following tunicamycin treatment suggesting that the cell surface glycoproteins are turning over in these cells.

2.2 Compartmentalisation of Nucleotides

Intracellular nucleotides may not be homogeneously distributed within a cell and it has been suggested that compartmentalisation of intracellular nucleotide pools does occur (Moyer and Henderson, 1985). For example the nucleotide pools in mitochondria form a separate compartment from the cytoplasmic pool of nucleotides, although it is likely that some exchange does occur with the cytoplasm. Platelets have also been found to contain pools of ATP and ADP within granules. In lymphocytes, it has been suggested that there are two pools of dTTPs with one of them being fed by *de novo* synthesis and used preferentially for DNA synthesis. From experiments using MOLT-4 cells, an acute lymphoblastic leukaemia cell line, it was suggested that a discrete compartment of thymine nucleotides exists serving exclusively as precursors for DNA synthesis (Taheri, Wickremasinghe and Hoffbrand, 1982). This suggests that another pool of dTTP exists which is not available for DNA synthesis. Similar evidence has been found for CTP pools, with one pool being preferentially available for DNA synthesis (Reichard, 1987).

The effect of compartmentalisation of guanine ribonucleotides has also been studied (Nguyen and Sadée, 1986). It was shown in S-49 lymphoma cells, that DNA synthesis reduction caused by inhibition of IMPDH is associated with depletion of guanine ribonucleotide pools rather than dGTP pools – the latter were found not to be available for DNA synthesis. This indicated that a discrete guanine ribonucleotide precursor pool might play a crucial role in limiting substrate supply for DNA replication under conditions of purine starvation. It was proposed that GMP might play a role in this process, as it is the smallest of all the ribonucleotide pools. In support of this hypothesis, following pulse labelling of cells with ^{14}C, uptake of ^{14}C into DNA ceased after 15-30mins and GMP was the only guanine nucleotide pool that lost a large fraction of ^{14}C label within 10mins. It was also shown that following MPA treatment, that the GMP pool of nucleotides was the only pool largely depleted when DNA synthesis ceased. These data confirm that the immediate and dramatic inhibition of DNA synthesis following MPA treatment of cells occurs before guanine nucleotides other than GMP are exhausted.

Therefore it is possible that following treatment of CEM-C7 cells with MPA, GMP pools utilised for DNA synthesis and cellular proliferation are depleted leading to

inhibition of these processes, but there are still sufficient levels of GDP remaining within a separate cellular pool to allow protein glycosylation to continue normally.

2.3 Glycosylation in Endothelial Cells

MPA caused a decrease in the expression of both mannose and fucose residues on endothelial cell surface glycoproteins, with a more significant effect on fucose expression than mannose. The ability of MPA to decrease glycoprotein expression on endothelial cells but not T cells suggests that endothelial cells might not have the same kind of cellular compartmentalisation of nucleotides and/or that their cell surface expression of glycoproteins might be higher than seen in T cells. Therefore endothelial cells require higher levels of GDP for protein glycosylation and consequently may be more susceptible to the effects of MPA.

It should be highlighted that certain experimental details should be taken into consideration for the design of future experiments with ECV304 cells. For example, the use of trypsin/EDTA to harvest the adherent monolayers may decrease the sensitivity of the assays. This is because trypsin may cleave some cell surface glycoproteins that may otherwise bind the lectins so we may be losing potential recognition sites. Therefore it may be more appropriate to use a non-enzymatic method for disrupting the cells such as EDTA alone. This could be used as a control experiment to compare the fluorescence intensity of stained cells following harvesting with or without trypsinisation, to determine if trypsin is actually affecting the staining we observe. The use of a different method such as growing the cells directly onto slides, without the need for further trypsinisation, and then staining with fluorescent lectins for example and analysing by fluorescent microscopy would be an alternative experiment to investigate the effect of MPA on endothelial cell glycosylation.

In addition, ECV304 cells show contact inhibition with a doubling time of 33hr (Takahashi *et al* 1990) and therefore this should be considered when performing time course experiments. When the cells proliferate and become confluent they come into contact with each other which causes them to stop proliferating and down-regulate some cellular processes. This may affect the expression of cell surface molecules and thus the results obtained. In our experiments, the antiproliferative effect of MPA

prevented MPA treated ECV304 cells from becoming confluent, however in untreated cultures they were not confluent until 48-72hrs of culture. However, we still detected differences as early as 24hrs. Therefore at early time-points, contact inhibition was unlikely to be having an effect but it may be possible that at later time points it could influence the results and contribute to experimental variation.

2.3.1 Mannose Expression on Endothelial Cells

Cell surface expression of mannose was inhibited by MPA in endothelial cells but to a lesser extent than that of fucose expression. A possible explanation for the reduced effect of MPA on mannose expression is that the residues are masked by other residues on the cell surface of endothelial cells, and are therefore unable to bind the lectin. Because tunicamycin inhibits the first step in glycoprotein synthesis it is possible that there are no other residues available to mask the mannose residues, which suggests a mechanism by which tunicamycin has a more potent effect on lectin binding than MPA. However, we observed strong positive staining with the mannose-binding lectin, VFA, in both drug treated and control samples suggesting that this explanation is unlikely.

More interestingly, there have been reports that some cell types are able to utilize exogenous GDP-mannose for the synthesis of mannose containing glycoproteins. Using hen oviduct cells, it was shown that exogenous GDP-mannose could serve as an effective mannose donor for the synthesis of mannosyl lipids, oligosaccharide-lipids and glycoproteins (Struck and Lennarz, 1976). Mouse fibroblasts have also been shown to be able to incorporate exogenous mannose into glycoproteins using exogenous GDP-mannose (Patt and Grimes, 1976). Similar observations were also made with rat-spleen lymphocytes (Cacan, Hoflack and Verbert, 1980). It may therefore be possible that endothelial cells are able to utilise exogenous GDP-mannose from the culture medium as a source of mannose that they can then incorporate into glycoproteins. These observations help explain the decreased sensitivity of mannose containing glycoproteins to the effects of guanosine nucleotide depletion.

2.3.2 Fucose Expression on Endothelial Cells

MPA treatment of endothelial cells produced a greater inhibition of cell-surface fucose expression than mannose expression. The greater inhibition of surface expression of fucose might be because GDP-fucose can be produced directly from fucose, and also by the reduction of GDP-mannose (Hughes, 1983). Therefore, if GDP-mannose formation were inhibited, the amount of GDP-mannose available for reduction to GDP-fucose would also be reduced. Therefore MPA inhibits fucose expression by decreasing the direct and indirect formation of GDP-fucose.

Endothelial cells may also express higher levels of fucosylated glycoproteins on their surface and therefore require an increased rate of fucosylation. Indeed endothelial cells express a variety of adhesion molecules that are glycosylated, such as those belonging to the selectin family and their ligands (Varki, 1994). For example, E-selectin, P-selectin, and ligands for L-selectin which include GlyCAM-1, CD34, MadCAM-1 and Sgp200. Selectins bind with high affinity to carbohydrates ligands displayed on glycoproteins from leukocytes and endothelial cells (McEver, Moore and Cummings, 1995). These ligands contain many ser/thr residues that are potential sites for attachment of O-linked glycans, particularly those containing sialic acid and fucose residues, which comprise the recognition site for L-selectin (Hemmerich *et al*, 1994). Glycosylation of L-selectin ligands with fucose is essential for their activity as L-selectin ligands (Maly *et al*, 1996). It was shown that inhibition of fucosylation of these ligands lead to deficient high endothelial venule (HEV) L-selectin ligand activity and leukocyte adhesion deficiency. It therefore appears that fucosylation of glycoproteins is an important process for endothelial cell functions. An increased requirement of endothelial cells for fucose transfer to glycoproteins may therefore account for the greater decrease in surface expression of fucose observed following MPA treatment compared with mannose.

2.4 Implications for the Inhibition of Glycosylation

Our results indicate that the ability of MPA treatment to cause a decrease in fucose and mannose expression on endothelial cells is very important. Endothelial cells play

... role in the inflammatory response (Pober and Cotran, 1990). Endothelial cell-leucocyte interactions are essential for both leucocyte activation (Briscoe, Alexander and Lichtman, 1998) and leukocyte extravasation into inflammatory sites due to their expression of adhesion molecules and costimulatory molecules. Therefore, if leucocytes are unable to adhere to the endothelium, they will not remain in contact with the endothelium for long enough to receive the necessary signals they require for activation and/or migration.

Another important consequence of the effect of MPA on endothelial cells relates to potential side effects seen following MPA treatment of transplant patients. One of the most commonly reported side effects is gastrointestinal (GIT) disturbances with symptoms that include diarrhoea, abdominal cramps, frequent stools and vomiting (Silverman Kitchin *et al*, 1997).

Cells lining the GIT secrete large quantities of mucins that are O-glycosylated with different sugar residues particularly fucose (Hughes, 1983). In the GIT, this thick layer of mucus plays a role as a lubricant and also protects the underlying tissue against mechanical and chemical damage. Glycosylation of a protein has important consequences that include effects on protein conformation, prevention of degradation, "routing" and secretion (Schwartz and Datema, 1982). Inhibition of glycosylation by MPA may therefore affect the production, secretion and/or function of glycoproteins produced from, or on the surface, of the cells lining the GIT. Consequently the risk of damage to the surface of the GIT may be increased resulting in the GIT disturbances seen in MPA treated patients.

Further experiments to investigate the effect of MPA on cells of the GIT could be performed using lectin staining of histological sections obtained from the GIT of long-term MPA treated rats or transplant patients. Fluorescently labelled lectins could be used to stain GIT sections and then analysed by fluorescent microscopy to determine if there are any changes in staining following treatment with MPA.

3 MPA Effects on Transcription Factor Activity

MPA treatment produced different effects on transcription factor activity, which were found to be cell-type specific. In CEM-C7 T cells MPA caused an increase in the transcriptional activity of AP-1 when cells were stimulated with PMA and a decrease in TNFα stimulated NF-κB activity after 48hr and 72hr of culture. However there was no effect on either NF-κB or AP-1 activity by MPA in ECV304 endothelial cells. The effect on transcription factor activity was due to the depletion of guanosine nucleotides by MPA because the addition of exogenous guanosine to the cultures reversed the changes in activity we observed. It appears therefore that depletion of guanosine nucleotides with MPA is producing intracellular changes in the signalling pathway(s) involved in AP-1 and NF-κB activation.

3.1 The effect of MPA on AP-1 Activity

Transcription factor activation and regulation is a complex process, therefore the effect of MPA on AP-1 activity requires the consideration of several different factors relating both to the stimulation of AP-1 activity by PMA and also the interaction between different components of the AP-1 complex and their regulation.

3.1.1 PMA and Protein Kinase C Stimulation

PMA stimulates protein kinase C (PKC) of which there are 3 different groups, classified depending on the activation signals they respond to (Mellor and Parker, 1998). The three families are, conventional PKC's (cPKC) - α, β1, β2, and γ; novel PKC's (nPKC) - ε, η, δ, and θ; and atypical PKC's (aPKC) - ι and ζ. Only members of the atypical family of PKC's are unresponsive to PMA. T cells express α, β1, δ, ε, η, θ and ζ, with multiple PKC isotypes being simultaneously activated by PMA treatment, and it was shown that PKCε was most efficient at activating an AP-1 reporter gene in Jurkat T cells (Genot *et al*, 1995). In a separate experiment using

COS-1 cells, PKCθ overexpression was more effective at increasing the activity of an AP-1-collagenase promoter than PKCα, β or ε (Baier-Bitterlich *et al*, 1996). Jurkat T cells express α, β, δ, ε, and ζ (Tsutsumi *et al*, 1993) so differences may exist in PKC isoform expression between different cell lines.

PMA stimulation of protein kinase C (PKC) produces a marked activation of ERK and only a small increase in JNK activity leading to Fos and Jun induction respectively, both of which are components of AP-1. Chronic PMA treatment leads to a downregulation of PKC due to an increased rate of proteolysis (Young *et al*, 1987; Hug and Sarre, 1993). Following PMA stimulation of Jurkat T cells there is usually a rapid downregulation of PKC activity and protein (Tsutsumi *et al*, 1993). This downregulation is also dependent on the isoform of PKC as well, as different isoforms were downregulated to different extents. In human PBL's, PMA stimulation leads to dramatic redistribution of nPKCδ, -θ, and -η from cytosol to membrane, changes not seen after αCD3 treatment (Keenan *et al*, 1997).

3.1.2 AP-1 Family of Transcription Factors

AP-1 transcription factors consist of heterodimers or homodimers of proteins belonging to the Jun and Fos families and the more recently described activating transcription factor (ATF) bZIP proteins (Karin, Liu and Zandi, 1997). Proteins belonging to the Jun family include; c-jun, v-jun, junB and JunD; Fos family members are; v-fos, c-fos, fosB, Fra1, Fra2. The ATF family consists of ATF2, ATF3/LRF1, and B-ATF with the list increasing. Jun proteins can form homodimers and heterodimers with Fos and ATF family members. ATF proteins can also homodimerize but Fos proteins are unable to. AP-1 activity is stimulated by extracellular stimuli that regulate its activity at two levels, by modulating both the abundance and activity of the AP-1 proteins. Both transcription of their genes and also modulation of their stability regulate the abundance of AP-1 proteins.

The induction of both c-fos and c-jun by PMA occurs rapidly without the need for *de novo* protein synthesis (Angel and Karin, 1991).

3.1.3 Regulation of c-fos Expression

Expression of c-fos increases in response to various stimuli within minutes and without the need for *de novo* protein synthesis (Angel and Karin, 1991). A specific element within the c-fos promoter is required for its induction by serum/growth factors and phorbol esters. This element is known as the serum response element (Cahil, Janknecht and Nordheim, 1995) which binds a complex consisting of serum response factor (SRF) and a ternary complex factor (TCF) such as ELK-1. Most stimuli that cause induction of SRE-regulated genes function via activation of MAP kinase pathways leading to the phosphorylation and activation of TCF and SRF. Activation of Raf-1 by PKC or Ras leads to the phosphorylation and activation of MEK1 (MAPKK) which in turn phosphorylates ERK1 and ERK2 (MAPKs). ERK1 and ERK2 then enter the nucleus where they are able to phosphorylate ELK-1, which enables it to bind to the SRE and stimulate c-fos production (Karin, 1995)

The ERK signal transduction pathway mediates the effects of PKC since the response to PMA is only slightly decreased with a dominant negative form of MEK4, the kinase responsible for JNK and Jun activation (Whitmarsh *et al*, 1995).

The induction of c-fos is usually very transient with c-fos being rapidly degraded, but TCF dephosphorylation might also contribute to c-fos de-induction. Dephosphorylation of TCF and downregulation of c-fos transcription coincide, also the protein phosphatase inhibitor okadaic acid is able to block both these processes (Cahill, Janknecht and Nordheim, 1995). The phosphorylation status of TCFs may therefore be a balance between phosphorylation by MAPKs and dephosphorylation by phosphatases. The phosphatase MKP1 is responsible for dephosphorylation and inactivation of ERK, SAPK and p38 (Franklin and Kraft, 1997). MKP1 is also regulated at the transcriptional level and is induced in response to various extracellular stimuli suggesting a negative feedback loop is in operation.

3.1.4 Regulation of c-jun Expression

The induction of c-jun is also transient but lasts for a longer time than c-fos induction. C-jun is able to positively autoregulate its own transcription (Angel *et al*, 1988), which

occurs through the c-jun TRE that can be recognised by a c-jun-ATF-2 heterodimer. C-jun is activated by phosphorylation by the MAP kinases JNK and p38 while ATF-2, which is constitutively expressed, is phosphorylated only by p38 (Karin, Liu, and Zandi, 1997). JNK and p38 are activated by MAP kinase cascades in response to stresses such as heat shock, cytokines, DNA-damaging agents, UV irradiation and antioxidants etc (Robinson and Cobb, 1997).

The ability of c-jun to positively autoregulate itself suggests that it could become permanently expressed. However although PMA causes a 15-fold increase in c-jun mRNA, there is only a 3-4-fold increase in c-jun protein synthesis (Angel and Karin, 1991). Furthermore the SP-1 and CTF sites in the c-jun promoter decrease c-jun induction, if they are deleted the basal and induced activity of c-jun increases 10-fold. The AP-1 site in the c-jun promoter also binds junB and junD but they are less efficient activators and may therefore compete with c-jun for this site. Alternatively the transient nature of the c-fos protein might also decrease AP-1 activity by reducing the number of c-fos:c-jun heterodimers that form resulting in the formation of Jun:Jun homodimers which have a lower transactivating potential.

3.1.5 Coactivators

Jun proteins have been shown to interact with different coactivators, which modulate their activity. For example upon phosphorylation, c-jun has a higher affinity for CBP/p300. CBP/p300 is an essential co-activator for AP-1 proteins including both Fos and Jun (Glass, Rose and Rosenfeld, 1997). More recently another protein has been found to interact with c-jun (Claret *et al*, 1996). This protein called JAB1 interacts with the activation domain of c-jun, it has no effect on DNA binding by c-jun but increases the stability of the complexes formed with AP-1 sites. Little is known about the biological functions of JAB1 but it also potentiates the activation of junD but not junB, so its expression is likely to favour activation of c-jun and junD target genes over those regulated by JunB.

Gel shift experiments performed with whole cell extracts from CEM-C7 T cells stimulated with PMA in the presence of MPA had an additional band present at 72hr compared with untreated extracts. This result, although a preliminary observation,

suggests that we may be detecting changes caused by MPA on the binding of proteins to the TRE site that is causing the appearance of the additional band (figure 45).

3.1.6 PMA and Differentiation

AP-1 has been reported to be involved in cellular differentiation (Angel and Karin, 1991). Early experiments suggested that a sustained activation of MAP kinase cascades was required for cellular differentiation (Traverse *et al*, 1992). Both EGF and NGF, which cause an increase in MAP kinase activity, can stimulate rat PC12 neuronal cells. However it was found that NGF produced a sustained increase in this MAPK activity compared with EGF, and only NGF had the ability to induce neuronal differentiation in these cells. It was therefore suggested that a sustained activation of MAP kinase cascades was required for neuronal cell differentiation.

PMA has also been shown to induce changes associated with differentiation in myeloid cell lines (Nagasawa and Mak, 1980) and also in MOLT-3 and Jurkat T cell lines but not CEM-C7 cells (Nagasawa, Howatson and Mak, 1981). However in a separate study looking at different cellular markers, CEM-C7 T cells were also shown to be induced to differentiate in response to PMA and develop a surface antigenic pattern common to suppressor and cytotoxic T cells (Ryffel, Henning and Huberman, 1982). Therefore heterogeneity between different T cell lines does exist which may be a function of the specific stage of differentiation at which the cells were arrested.

In U937 cells, stimulation with PMA causes differentiation into macrophages and an increase in both Fos and Jun. In HL-60 leukaemic cells, PMA treatment causes macrophage differentiation associated with increased AP-1 DNA binding (Davis, Meighan-Mantha and Riegel, 1997). Fos and Jun proteins have also been implicated in regulation of bone growth and differentiation (McCabe *et al*, 1996). The levels of all Fos and Jun proteins are elevated during osteoblast proliferation. However, during osteoblast differentiation the protein and mRNA levels of all AP-1 members decline and Fra-2 and JunD become the predominant AP-1 proteins and are also principal members of AP-1 complexes. It was shown that co-expression of Fra-2 and JunD was the only combination of Jun and Fos proteins that was able to cause induction of an osteocalcin reporter gene construct that contained three AP-1 binding sites. Antisense

c-Fos oligonucleotides were also able impair the ability of normal bone marrow derived myeloblasts to be induced to differentiate in vitro (Lieberman, Gregory and Hoffman, 1998), confirming the importance of Fos proteins in the induction of differentiation.

The levels of Jun proteins were also shown to be important in adipocyte differentiation (Wang, Xie and Scott, 1996). CSV3-1 adipocyte cells exhibited an increase in AP-1 binding upon differentiation that was attributed primarily to an increase in the expression of JunD.

3.1.7 MPA and Differentiation

MPA induces differentiation in various cell lines such as HL-60 and U937 line (cells (Knight *et al*, 1987; Waters, Webster and Allison, 1993; Inai *et al*, 1998) and it has also been shown to induce differentiation in a CEM-2 leukaemic T cell (Kiguchi *et al*, 1990). MPA treatment was shown to induce cells to acquire reactivity with maturation specific monoclonal antibodies. Similar observations were also detected following PMA treatment of these cells, although differentiation was suggested to be induced by a different mechanism, as it was not reversed by exogenous guanosine.

A study using HL-60 and HL-525 cells, which are a HL-60 variant deficient in PKCβ and unresponsive to PMA, found the variant cell line to be more resistant to the growth arrest and induction of differentiation markers caused by MPA (Tsutani *et al*, 1998). This suggests that PKCβ-dependent myeloid cell differentiation is induced by MPA dependent decreases in the levels of guanosine nucleotides.

In our data, there also appear to be some morphological changes associated with MPA treatment of CEM-C7 T cells as indicated by the FACs profiles (Figures 11 and 12). These profiles demonstrate that MPA produces an increase in both forward and side scatter (fsc/ssc) suggesting there is an increase in both cell size and granularity. Previously CEM-C7 cells treated with PMA or MPA were shown to develop a surface antigenic pattern common to suppressor and cytotoxic T cells (Kiguchi *et al*, 1990). The increase in granularity may therefore be a feature of an altered cell phenotype and indicate that the cells are actually undergoing differentiation in response to MPA treatment.

3.2 Potentiation of AP-1 activity by MPA

The increase in PMA stimulated AP-1 activity we observed following MPA treatment of CEM-C7 cells could have several explanations. It is possible that depletion of guanine nucleotides by MPA is causing the induction of differentiation in CEM-C7 cells and this has been proposed to occur in other cell lines (Knight *et al*, 1987; Waters, Webster and Allison, 1993; Inai *et al*, 1998). The relationship between AP-1 and differentiation highlighted in previous reports suggests that differentiation will most probably involve the activation of specific AP-1 proteins. PMA induces differentiation and also the activation of Fos and Jun proteins as already described. Depletion of guanosine nucleotides in CEM-C7 cells by MPA increases AP-1 activity and also appears to induce differentiation. If MPA induces a PKC dependent increase in differentiation, the addition of PMA in combination with MPA may have a synergistic effect on the induction of differentiation via PKC so that AP-1 activation is potentiated.

Alternatively, PMA stimulated PKC can activate Raf-1 and Ras (Carroll and May, 1994; Izquierdo *et al*, 1992). However Raf-1 can also be activated directly by Ras. In cells stimulated with PMA alone, PKC will be activated and will be able to activate Raf-1 either directly or indirectly via Ras activation. PKC and Ras may therefore compete with each other for Raf-1 binding (Marquardt, Frith and Stabel, 1994). In addition, if Ras is less effective at activating Raf-1 than PKC it may limit the activation of the Raf pathway. GTP depletion following culture of cells with MPA may decrease the activation of Ras, which requires bound GTP to become active, and therefore Raf-1 will be activated almost exclusively by PKC. In addition if PKC is a more potent activator of Raf-1 then this may account for the increase in AP-1 activity detected following treatment of CEM-C7 cells with PMA and MPA.

MPA may also interfere with processes that lead to the degradation of either Jun, Fos or PKC. As already described, following PMA stimulation of PKC there is a downregulation of PKC activity caused by increased protein degradation. If MPA inhibited this process of degradation then higher levels of PKC would be maintained allowing it to be stimulated by PMA for longer. Furthermore if Jun or Fos downregulation were inhibited following MPA treatment this again would lead to a prolonged activation of AP-1.

Finally, depletion of guanosine nucleotides may affect the activation of other proteins that bind to AP-1 complexes, such as coactivators, and influence their transactivating potential. Gel shift assay performed with extracts from PMA stimulated CEM-C7 cells suggest that there may be different proteins bound to the AP-1 consensus site following MPA treatment that may be increasing the transactivation potential of AP-1. This may be due to activation and binding of different Fos and Jun proteins or different coactivators in the presence of MPA that causes a shift in the bands seen.
The scenarios outlined above are not mutually exclusive, and the increase in AP-1 activity observed may be a result of the accumulation of these individual effects. For example, MPA depletion of guanosine nucleotides leads to increased AP-1 activation via interference with either PKC/Ras activation or the downregulation of PKC, Fos or Jun. Interference with these pathway may lead to increased activation of MAPK pathways and alterations in the AP-1 complexes and coactivators that form at AP-1 binding sites, thereby producing alterations in gene expression that result in cellular differentiation.

3.3 MPA and NF-κB Activity

Our results initially indicated that MPA was inhibiting TNFα-stimulated NF-κB activation. However when the NF-κB regulated reporter gene activity was corrected for cellular protein concentration it was found that total cellular protein was decreased by MPA. Therefore the decrease in NF-κB-mediated reporter gene activity was probably not a specific effect of MPA on NF-κB but more likely due to a general decrease in cellular proliferation i.e. there were fewer cells containing the NF-κB reporter gene in the cultures. However, gel shift analysis of TNFα-stimulated CEM-C7 cells suggest that not all of the MPA affect on NF-κB activity is non-specific. Following TNFα stimulation, an additional band is observed in CEM-C7 extracts compared with unstimulated cells (figure 47 upper band). The DNA-binding of this factor is inhibited at 72 hours following MPA treatment suggesting it may having an effect on NF-κB transcriptional activity by. This band is also not observed in Con A treated samples and therefore appears specific to TNFα stimulation. Identification of

the proteins bound to the DNA, using gel supershift assays, following TNFα stimulation will provide more information on the effect, if any, of MPA on NF-κB activation and DNA binding.

The resistance of ECV304 cells transfected with either an AP-1 or NF-κB reporter gene to MPA suggests that either these pathways are not as active as they are in T cells and therefore less susceptible to guanosine nucleotide depletion. Alternatively because these cells have active salvage pathways they may be able to compensate and maintain efficient signalling pathways.

3.4 MPA and Transcription Factor Activity - Conclusions

The experiments were performed in cell lines and therefore it could be argued that it is not a true reflection of the effect of MPA on normal cells. Cell lines are generally good models for events occurring in primary cells and are a good starting point for molecular studies. Cell lines were therefore a convenient model for the reporter gene experiments because we already had stably transfected cells and also cell lines are easier to work with and transfect. Primary cell lines are more difficult to transfect and it would be impossible to generate stably transfected cells. However ultimately it would be interesting to see if similar effects of MPA on AP-1 and NF-κB activity are obtained in primary cells.

The data presented in the reporter gene assays did not account for the number of cells present in each well. MPA caused an increase in AP-1 activity and a decrease in NF-κB activity but protein assays showed there to be a decrease in actual protein levels in TNFα stimulated cultures while PMA stimulated cultures remained the same. Therefore, the reason for decreased activity in TNFα treated cultures appears to be due to a decrease in the level of protein in the wells. Not all cells contain the same amount of protein so in future experiments, it may be more appropriate to perform cell counts prior to assaying for reporter gene activity. Transcription factor activity can then be presented as O.D. units/cell which may give a more accurate reflection of transcription factor activity taking into account the influence of MPA on cell proliferation.

To further investigate the effect of MPA on PMA stimulated AP-1 induction, specific inhibitors of the MAPK pathways such as SB203580 (inhibits MKK1) or the p38 pathway inhibitor PD098059, could be added to the added to the reporter gene experiments. This would determine if one of the pathways is more involved than the other in the induction of AP-1 activity by MPA. It would also be interesting to utilise western blots with specific antibodies to the various Fos and Jun family members to determine if MPA affects the activation of different family members following PMA stimulation. A similar approach could also be used to determine the identity of the proteins induced following TNFα stimulation of NF-κB using specific antibodies for the various NF-κB components. Anti-sense techniques with the introduction of antisense mRNAs for the individual Fos and Jun family members could also be used to attempt to specifically block the increase we observed. The level of Ras-bound GTP would provide information on the effect of MPA on Ras activity, labelling studies using radiolabelled guanosine nucleotides would provide information on the amount of GTP-bound Ras following treatment of cells with MPA. If the level of GTP-Ras is influenced by guanosine nucleotide depletion, it may in turn influence the signalling pathways leading to AP-1 activation.

The effect of MPA on Raf activation could be investigated by western blotting with specific antibodies to Raf. MPA treated cultures could be immunoprecipitated with anti-Raf antibodies and then western blots performed using antibodies specific for phosphorylated forms of Raf. This would determine the amount of activated Raf in the cultures. In addition, investigation of the effect of MPA on PKC isotype expression by western blotting using specific antibodies might also be informative. The effect of MPA on the regulation of expression of different PKC isotypes would determine if the potentiation of AP-1 activity by MPA is caused by alterations in PKC isoform expression

3.5 The Effect of MPA at a Physiological Promoter

Considering the effects of MPA on the individual transcription factors, it would have been interesting to see what effect MPA mediates at a physiological promoter. However, because of the difficulties encountered with stimulation of the IL-2

promoter construct, this was unfortunately not possible within the time limit of the project. However the effect of MPA on individual transcription factors is intriguing and the effect of MPA on a complex physiological promoter represents the next logical step in this investigation. This would allow us to determine whether the effects seen with isolated transcription factors are mirrored in the activation of a complete physiological promoter.

4 MPA and DNA Binding Ability of Transcription Factors

The effect of MPA on the DNA binding ability of different transcription factors was also investigated to see if the differences seen in reporter gene assays were paralleled with changes in DNA binding. However, in unstimulated or Con A stimulated CEM-C7 cells, MPA treatment resulted in a general decrease in DNA binding of all the transcription factors examined. This may be due to the general inhibition of cell activation and proliferation in these cells as a result of MPA treatment. However the effect observed on all the transcription factors with MPA after Con A stimulation suggests that guanine nucleotide depletion by MPA is affecting transcription factor activation by disrupting guanosine nucleotide dependent signalling pathways. Con A binds to several cell surface receptors and therefore results in the activation of different pathways, which may involve GTP dependent protein for signal transduction. For example, Ras is important for the activation of signalling cascades including the activation of c-Raf-1 and MAP kinase cascades, and requires bound GTP for its activity. Therefore if GTP levels are decreased, its activity will also be decreased disrupting downstream signalling pathways and affecting transcription factor activation.

In support of this, MPA did not inhibit to the same degree AP-1 DNA-binding in PMA stimulated CEM-C7 cells compared to Con A stimulated cells. For instance AP-1 DNA-binding was unaffected until after 72hr of culture with MPA and PMA, and then the decrease observed with MPA was not as potent as in Con A stimulated cells. This is interesting because PMA bypasses signalling via cell surface receptors and GTP dependent proteins, entering the cells and directly activating PKC, which can then

directly activate c-raf-1. There is therefore no requirement for guanosine nucleotides in the signalling pathways activated by PMA. These data therefore present an explanation as to why there appears to be a non-specific inhibition of transcription factor-DNA binding of all three transcription factors by MPA following Con A stimulation.

Again resistance of TNFα-stimulated ECV304 cells suggests that either the pathways leading to DNA binding by these transcription factors following TNFα stimulation do not require the activity of G proteins, or if they do then the cells are able to compensate for the guanosine nucleotide depletion by production of guanosine nucleotides via the salvage pathway.

The effect of MPA on all the transcription factors examined in CEM-C7 cells suggests that the proliferative responses of these cells require the presence of different transcription factor family members. This is because the highest dose of MPA ($1x10^{-6}$M) was antiproliferative and decreased the DNA-binding of AP-1, NF-κB and CREB. However, the intensity of some bands was inhibited to a greater extent than others, suggesting that some components of the transcription factor complexes are more susceptible to the effects of guanosine nucleotide depletion than others. Future experiments utilising supershift assays, with specific antibodies for the individual transcription factor components would be extremely important in determining the constituents of these protein-DNA complexes before and after MPA treatment. Not only would this allow the identification of the individual proteins present but it would also identify if particular transcription factor family members are being specifically affected by MPA and indicate their importance in cellular functions.

There was no real change in transcription factor-DNA binding following Con A stimulation. This again suggests that because CEM-C7 cells are already a spontaneously proliferating cell line it may affect the response to stimulation with Con A. Therefore, the experiments should be repeated with primary cells to determine if Con A stimulation does actually increase transcription factor-DNA binding and also if MPA causes similar decreases in transcription factor binding.

5 Conclusions

In conclusion, in contrast to previous work performed with MPA (Allison *et al*, 1993b) we have failed to detect any effect on cell surface expression of fucose and mannose in CEM-C7 T cells. However we did detect significant inhibition of endothelial cell surface expression of fucose and an inhibition, although not significant, of mannose expression. From the data it appears therefore that MPA may affect the immune response to an allograft by not only interfering with cellular proliferation but also leucocyte-endothelial cell interactions, which are important for the activation of both cell types and also for leucocyte extravasation into the allograft. These results may also provide an insight into the mechanism causing the high frequency of GIT disturbances seen in MPA treated patients. Interference with protein glycosylation may compromise the protective layer of mucus that lines the GIT such that it is not formed properly. The surface of the GIT may therefore be more susceptible to insult in MPA treated patients.

The gel shift assays combined with the reporter gene assays suggests that MPA is having a specific effect on PKC stimulated AP-1 activity, because there was an increase in the activity of an AP-1 reporter gene and also different bands were seen following gel shifts of PMA treated CEM-C7 cells. This effect may be due to the fact that both PMA and MPA are able to induce cellular differentiation. AP-1 is important in cellular differentiation and may therefore be the common mediator. In this way, MPA may be inducing cellular differentiation by the induction of AP-1 activity in CEM-C7 cells.

Therefore, this work has implications for the use of MPA in transplant patients because of the ability of MPA to inhibit cell surface glycosylation in endothelial cells and therefore interfere with cell-cell interactions. The data also provides information on the effect of guanosine nucleotide depletion on cellular proliferation/differentiation and the role played by different transcription factors and their components in this process.

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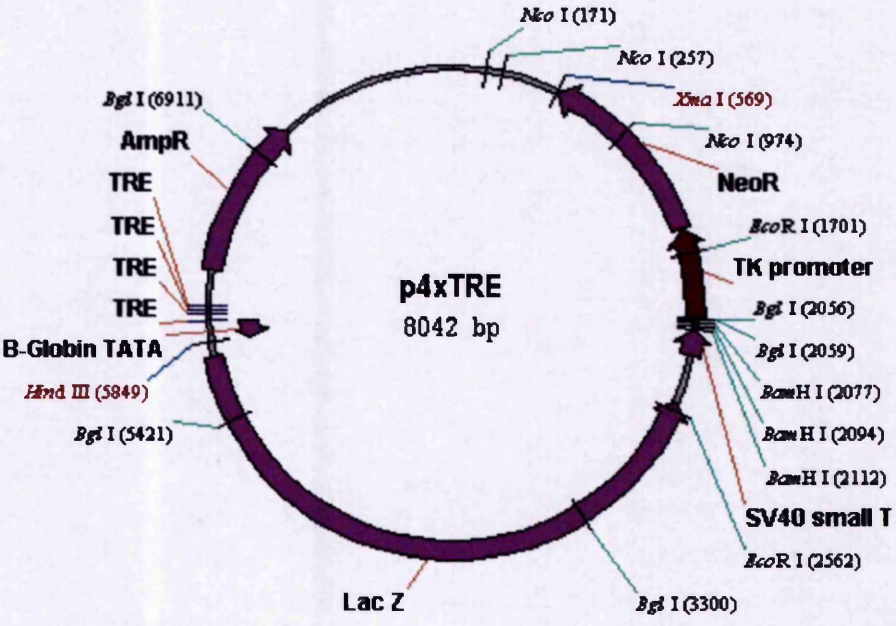
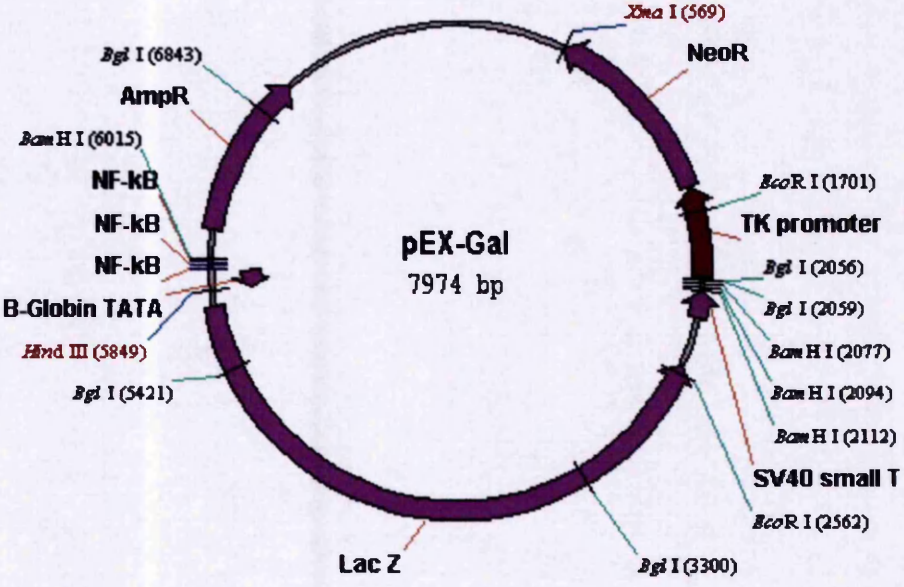
Plasmid Maps





Lectin Specificities

The major specificities of the lectins (All Sigma except GNA, TCS Biologicals UK) used were:

Lotus tetragonolobus (LTA)	Lfucα1,2Galβ1,4(Lfucα1,3)GlcNAc
Vicia faba (VFA)	Branched mannose residues
Ulex europaeus I (UEAI)	α-L-fucose
Galanthus nivalis (GNA)	mannose α(1,3) mannose

Transcription Factor Oligonucleotides

The sequences of the transcription factor oligonucleotides are shown below. Consensus transcription factor binding sites are highlighted in yellow (all Promega).

NF-κB-	5'-AGTTGAGGGGACTTTCCCAGGC- 3'
AP-1-	5'-CGCTTGATGAGTCAGCCGGAA- 3'
CREB	5'-AGAGATTGCCTGACGTCAGAGAGCTAG- 3'