Activation and apoptosis requirements in human T lymphocytes

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ACTIVATION AND APOPTOSIS REQUIREMENTS IN HUMAN T LYMPHOCYTES

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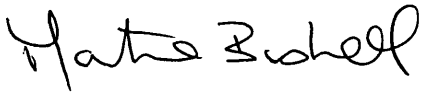

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This dissertation is dedicated to my parents
Bernadette and William Boshell
for their love and support.

Abstract.

T cell activation may result in productive outcomes such as proliferation or IL-2 production. Alternatively, anergy or apoptosis may result from TCR engagement. One of the main objectives of this study is the investigation of what determines which of these outcomes occurs.

In order to examine T cell activation requirements, anti-CD3 monoclonal antibodies, the mitogen PHA and the superantigen SEB have all been utilised. The ability of human peripheral T lymphocytes to respond to these stimuli was determined by measuring proliferation and IL-2 responses.

The role of costimulation was addressed using transfectants expressing the LFA3 or B7 cell surface molecules. T lymphocytes were found to require APC-dependent signals in addition to TCR engagement. B7 was demonstrated to be a potent costimulatory molecule capable costimulating resting and activated T lymphocytes and of providing third party costimulation for SEB responses. LFA3 appeared to predominantly function as an adhesion molecule although it costimulated PHA responses in resting T cells.

In addition to productive activation, the requirements for apoptosis of T lymphocytes was also examined. PHAwas demonstrated to induce time and dose dependent apoptosis in Jurkat T cells via a Fas-independent pathway. In contrast SEB-induced apoptosis in T lymphocytes was shown to occur via a Fas-dependent pathway. Fas-L up-regulation was found to be intimately linked to SEB-induced apoptosis. Additionally Fas-resistant cell lines which were also resistant to SEB-induced apoptosis were also observed. Interestingly, Fas ligation was also shown to costimulate anti-CD3 induced proliferation.

One of the main conclusions presented is that more than one signal is required both for productive T cell activation to result in proliferation and IL-2 production and for apoptosis of human peripheral T lymphocytes to occur.

ACKNOWLEDGEMENTS.

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A very special thanks to Dr Julie McLeod who constantly cheered me and took time to offer words of support and encouragement. I will remember this always and appreciate our friendship. Your happiness is guaranteed by the contribution you make to other people's lives.

To my parents and family, thanks for your investment in me and my future, my success reflects the love and support you have shown me. All things become possible when you truly believe in them. To EEIFTM and to MMAI, thank you.

Finally for the future I will carry this thought with me

"Be not the slave of your own past,
plunge into sublime seas,
dive deep and swim far,
So you shall come back with self respect,
With new power, with an advanced experience,
That shall explain and overlook the old."

Ralph Waldo Emerson.

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ABBREVIATIONS

AICD	Activation induced cell death
AP-1	Activating protein-1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CHO cells	Chinese hamster ovary cells
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytolytic T lymphocyte associated antigen
DAG	1,2, diacylglycerol
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagles medium
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
Fas-L	Fas ligand
FAST	Fas activated serine/threonine kinase
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosohate dehydrogenase
gld	generalised lymphoproliferative disease
H_2O_2	Hydrogen peroxide
ICAM-1	Intracellular adhesion molecule-1
ICE	Interleukin-1b converting enzyme
IFN	Interferon
Ig (G,M,A)	Immunoglobulin (G,M,A)
IkB	Inhibitor kB (binds to NF-kB)
IL-	Interleukin

IP3	Inositol 1,4,5-triphosphate
JAK	Janus kinase
lpr	lymphoproliferative
LPS	Lipopolysaccharide (endotoxin)
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MFI	Mean fluorescence intensity
Mg^{2+}	Magnesium ions
MHC	Major histocompatability complex
Mls	Minor lymphocyte stimulating
mRNA	Messenger ribonucleic acid
Na^{+}	Sodium ions
NF-kB	Nuclear factor-kB
NIBSC	National Institute for Biological Standards and Controls.
PARP	poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PCD	Programmed cell death
PDGF	Platelet derived growth factor
PHA	Phytohaemagglutanin
PIP2	Phosphatidyl inositol 4,5, bisphosphate
PKC	Protein kinase C
PLC'	Phospholipase C
PMA	Phorbol myristic acid
PTK	Protein tyrosine kinase
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
SAgs	Superantigens
SDS	Sodium dodecyl sulphate
SE (A, B etc)	Staphylococcal enterotoxin (A, B etc)
SH	Sulphydryl group
SLE	Systemic lupus erythematosus
SM	Sphingomyelin
SSC	Side scatter (FACS analysis)
SSC	Saturated sodium citrate (Southern blotting)
SSPE	Saturated sodium phosphate with EDTA chelator

TCR	T cell receptor
TE	Tris-EDTA
Th	T helper
TINUR	Transcritionally inducible nuclear receptor
TNF	Tumour necrosis factor

CHAPTER ONE.

INTRODUCTION.

1.1 T lymphocytes in the immune response.

The eukaryotic immune system is a complex network of multiple cellular and soluble mediators which protect the host from disease. The cells involved include T and B lymphocytes; T lymphocytes play a pivotal role in the efficient functioning of the immune response providing both help for B lymphocytes as well as direct cytotoxic functions. The soluble mediators produced by activated T cells attract and activate B lymphocytes and macrophages promoting the elimination of pathogens. T cells are critical for the development of cytotoxic responses to virus-infected cells, malignant cells and the response to foreign antigens.

In order to mount an immune response a T lymphocyte must first proceed from a resting to an activated state progressing through the cell cycle to proliferate and expand. Activated T cells are then capable of the lymphokine secretion and cell lysis required for their effector role.

T cells are broadly divided into two classes based on their mutually exclusive expression of the cell surface glycoproteins CD4 and CD8. The function of $CD8^+$ cytotoxic T cells is to eliminate virally infected cells or tumour cells while $CD4^+$ cells are known as T helper (Th) cells because they provide help in the form of cytokines to B cells to assist in the production of antibodies. There is a level of overlap however in that $CD4^+$ T cells can also be cytotoxic. Murine $CD4^+$ Th cells can be divided into two distinct subsets, Th1 and Th2 cells based on their pattern of cytokine production (Mossman and Coffman 1989). Th1 clones secrete predominantly interleukin (IL)-2 and interferon (IFN)γ and promote cellular immunity whereas Th2 clones secrete IL-4, IL-5 and IL-10, providing help for B cell differentiation and humoral responses in the form of antibody production.

One of the most prominent features of the T cell system is the generation of diversity by random somatic gene rearrangement of the T cell receptor (TCR) for antigen. This diversity supplies the variety of receptors needed to recognise an almost infinite array of foreign antigens. However, the process of gene rearrangement results in T cells which also have the capacity to react to self antigens. It is therefore critical to regulate this process, by having control points during development in order to eliminate potentially harmful self-reactive T cells.

The central question of self-tolerance in the immune system is how to maintain a repertoire of cells which have the ability to recognise a myriad of foreign antigens while at the same time ensure that cells which are self-reactive are either removed or prevented from being activated. The consequences of such inappropriate activation could lead to autoimmune damage of the host as is thought to occur in a number of diseases. These include rheumatoid arthritis, multiple sclerosis and insulin-dependent diabetes melitus (IDDM). The increasing awareness of autoimmune pathology of such diseases highlights the necessity to fully characterise the way in which a normal immune system operates. The importance of efficient functioning of the immune system is underscored by the observation that immunomodulatory intervention is seen as the way forward for the treatment of a large number of diseases.

Unrestricted tumour growth and metastasis may occur if cytotoxic T cells do not recognise and eliminate cancer cells. It will be interesting to elucidate the molecular basis of immune recognition and the manner in which a T cell interacts with tumour cells. The differences that occur between a cell which is recognised as foreign or virally infected by the immune system and eliminated and a cancerous cell which avoids detection may provide strategies for targeting and destroying tumour cells in the treatment of cancer.

When the immune system is confronted by an antigen it must either recognise the antigen as foreign and destroy it , or recognise at as self and ignore it. The aim in the immunoregulation of transplant rejection is to change the aggression of alloreactive T cells into passive bystanders which do not attack the transplanted organ. The mechanisms that control T cell recognition and rejection of foreign antigens are of prime importance in strategies for suppression of the immune system to control graft rejection. Therefore an understanding of how the immune system remains tolerant to self antigens will aid transplant technology.

The underlying immunopathology in patients who are HIV+ has become increasingly obvious recently with the discovery that CD4 T cells are being rapidly deleted. This has been proposed to be due to dysregulated apoptosis (Ameisen and Capron 1991) resulting in immunocompromised patients and eventually leading to death. It will be interesting to determine the role played by apoptosis in T cell homeostasis. An understanding of the factors that regulate T cell function will facilitate the study of human disease.

It has thus become apparent that T lymphocyte activation needs to be tightly orchestrated to protect against development of autoimmunity. Each T cell clone produced needs to be tested for self reactivity and autoreactive T cells inactivated or eliminated. The requirement for clonal expansion of antigen-specific cells provides the immune response with a crucial control point at which to address the problem of self-nonself discrimination. The control of T cell activation is the central subject of this thesis and the essential features which influence this process will now be outlined.

1.2 Antigen Recognition - the Antigen Presenting Cell.

T cells recognise antigen displayed on the Major Histocompatibility Complex (MHC) of Antigen Presenting Cells (APCs), which include dendritic cells, macrophages and activated B cells. The MHC molecules are a group of highly polymorphic cell surface glycoproteins that present antigen, which has been processed into peptides, for recognition by T cells. In general CD4+ T cells recognise antigen associated with MHC class II whereas CD8+ T cells recognise MHC Class I bound antigen, although exceptions to this have been identified. The crystal structure of the MHC class I molecule HLA-A2 revealed a peptide binding groove formed by two α helices lying on a β pleated sheet (Bjorkman et al 1987). A similar structure has been identified for class II molecules (Brown et al 1993).

It has been demonstrated that peptide plays a critical role in stabilising Class I structure (Townsend et al 1989). The peptides bound by class I molecules are usually 8-10 amino acids in length and in most cases are derived from cytosolic or nuclear proteins (Townsend and Bodmer 1989, Yewdell and Bennick 1992) possibly through the action of proteasomes, macromolecular multicatalytic proteases abundant in the cytosol and nucleus (Monaco 1992). These peptides are delivered to the secretory pathway via the transporter associated with antigen processing (Townsend and Trowsdale 1993), which is formed by two MHC encoded gene products.

Class II MHC differs from class I MHC in that it binds peptides more promiscuously, and the bound peptides are longer, usually 15-20 amino acids. Peptides are important in regulating surface expression of class II molecules (Germain and Hendrix 1991)

but are unnecessary for stable association with the α and β chains. Instead, Class II α and β chains assemble as oligomeric complexes with a third polypeptide the Invariant chain, a non-polymorphic non-MHC -encoded protein, that is essential for proper assembly and transport of class II dimers (Peterson and Miller 1990, Anderson and Miller 1992, Lamb et al 1991, Lotteau et al 1990, Bakke and Dobberstein 1990, Neefjes and Ploegh 1992 , Guagliardi et al 1990). An invariant chain peptide is responsible for this stable association (Romaglioni and Germain 1994). Another MHC-encoded Class II protein DM has also been demonstrated to play a vital role in processing of Class II antigens, by enhancing peptide binding to Class II (Sherman et al 1995).

Thus, a complex network of protein associations ensures that antigens are processed and presented on the surface of APCs and are available for recognition by the T cell receptor on T lymphocytes.

1.3 Antigen recognition - the T lymphocyte.

The T cell receptor (TCR) is a multi-subunit receptor for antigen recognition composed of an antigen binding heterodimer (α/β) with structural diversity, complexed with the CD3γδε chains and the ζ family of hetero- and homo-dimers (TCRζ–ζ,ζ–η and ζ–γ) (Marrack and Kappler 1987). The α and β chains determine the antigen specificity of the receptor, binding to a complex formed between antigenically derived peptides and the cell surface proteins encoded by the MHC genes, whereas the ζ and CD3 chains mediate signalling.

T cells are characterised by the expression of various lineage-specific cell surface molecules. In addition to CD3 other molecules such as CD2, CD4 and CD8 which are all members of the immunoglobulin superfamily, play a role in T cell activation. The two co-receptors CD4 and CD8 are modulated on the surface of thymocytes and expression is mutually exclusive on mature T cells as a result of thymic selection. CD4 and CD8 bind to non-polymorphic regions of MHC class II and MHC class I antigens respectively.

1.4 Signalling through the TCR.

Signalling through the TCR/CD3 complex has been proposed to take place through an apparatus composed of one signal-transduction module consisting of a ζζ dimer or another involving the CD3ε chain (Howard et al 1992). Cross-linking of chimaeric proteins that contain the cytoplasmic domains of ε or ζ induces increased tyrosine phosphorylation of distinct groups of proteins, consistent with the existence of multiple signalling pathways. Anti-CD3 antibodies can signal through either signal transduction module whereas anti-CD2 appears to signal exclusively through a module involving ζ .

Triggering of the TCR/CD3 complex initiates multiple signal transduction pathways (Figure1.1 page 7), which include generation of intracellular second messengers, activation of protein kinases, synthesis or phosphorylation of nuclear factors, functional activation of nuclear factors and stimulation of gene transcription (Berridge and Irvine 1994, Klausner and Samelson 1991, Rudd et al 1994).

The major TCR- linked signalling pathway appears to be activation of intracellular protein tyrosine kinases (PTKs) which is essential for all subsequent activation events (Klausner and Samelson 1991). The TCR itself however has no intrinsic TK activity but T cells express three src-related PTKs; $p56^{lck}$, $p59^{fyn}$ and $p60^{yes}$ (Rudd et al 1984). These PTKs possess a characteristic structure that includes a unique N-terminal region, src homology regions 2 and 3 (SH2 and SH3 domains), a catalytic tyrosine kinase domain (SH1 domain) and a regulatory C-terminal motif. The CD4 and CD8 molecules were initially found to associate with $p56^{lck}$, and subsequently $p59^{fyn}$ was found to interact with the TCRζ chain and the CD3γδε chains through its N-terminal region. TCR crosslinking has been shown to lead to the rapid activation of c-fyn. In addition a fourth PTK, Zap-70, which is a member of the syk family of TKs is also implicated in TCR signal transduction (Samelson and Klausner 1992, Rudd et al 1994). Therefore it has become apparent that a variety of PTKs are crucially involved in T cell signalling.

The kinase activity coupled to TCRζ/CD3 -CD4 leads to phosphorylation of downstream targets such as phospholipase C γ (PLCγ) and possibly Mitogen Activated Protein (MAP)-2 kinase (Park et al 1991, Rudd et al 1995).

Phosphorylation of PLC γ by an as yet unidentified PTK leads to the hydrolysis of phosphatidyl inositol 4,5, bisphosphate (PIP2) and the formation of inositol 1,4,5 triphosphate (IP3) and 1,2-diacylglycerol (DAG) which act as second messengers in the signalling pathway (Perlmutter et al 1993, Berridge and Irvine 1984, Imboden and Stobo 1985).

IP3 causes release of calcium (Ca2+) from intracellular stores leading to a transient increase in intracellular Ca2+ concentration followed by extracellular Ca2+ influx which can be mimicked by ionophores such as ionomycin (Weiss et al 1984, Weiss and Imboden 1987). Calcium ions then bind to a small protein - calmodulin which acts as a regulatory subunit for other enzymes essential for T cell activation such as the threonine/serine phosphatase - calcineurin. The importance of calcium signalling is evidenced by the inhibition of T cell activation using cyclosporin A (CsA see Figure 1.1 page 7), CsA blocks T cell activation by binding to cyclophilin which inhibits the ability of calcineurin to dephosphorylate its substrate (Mueller et al 1989, Fruman et al 1992). The calcineurin pathway is known to control activation of the transcription factors NF-AT and NFkB. These events in association with a second signal lead to the production of cytokines that mediate T cell effector function (Wolff et al 1992).

Cytokines play essential roles in the regulation of the growth, differentiation and functional activities of immune cells. The pleiotropic effects of cytokines are mediated through binding to specific high affinity receptors on the cell surface. One of the most crucial cytokines produced by T lymphocytes is IL-2 (Fraser et al 1993).

Phosphorylation and dephosphorylation appear to be integrally involved in T cell activation. PKC phosphorylates the CD3γ chain of the CD3/TCR complex on serine (Cantrell et al 1985) which appears to play an essential role in the induction of the IL-2 gene expresssion by the CD3/TCR complex (Valge et al 1988). Protein tyrosine phosphorylation is also a proximal and obligatory cellular response to cytokine receptor occupancy. Phosphorylation and dephosphorylation of NFkB or NF-AT induces or represses target genes such as IL-2. The signal transduction of most if not all cytokine receptors is critically dependent on asssociation with the Janus Kinase (JAK) family of PTKs (Rudd et al 1995).

Active PKC can stimulate the cellular proto-oncogenes $p74^{raf}$ and $p21^{ras}$, second messengers common to multiple different signal transduction pathways

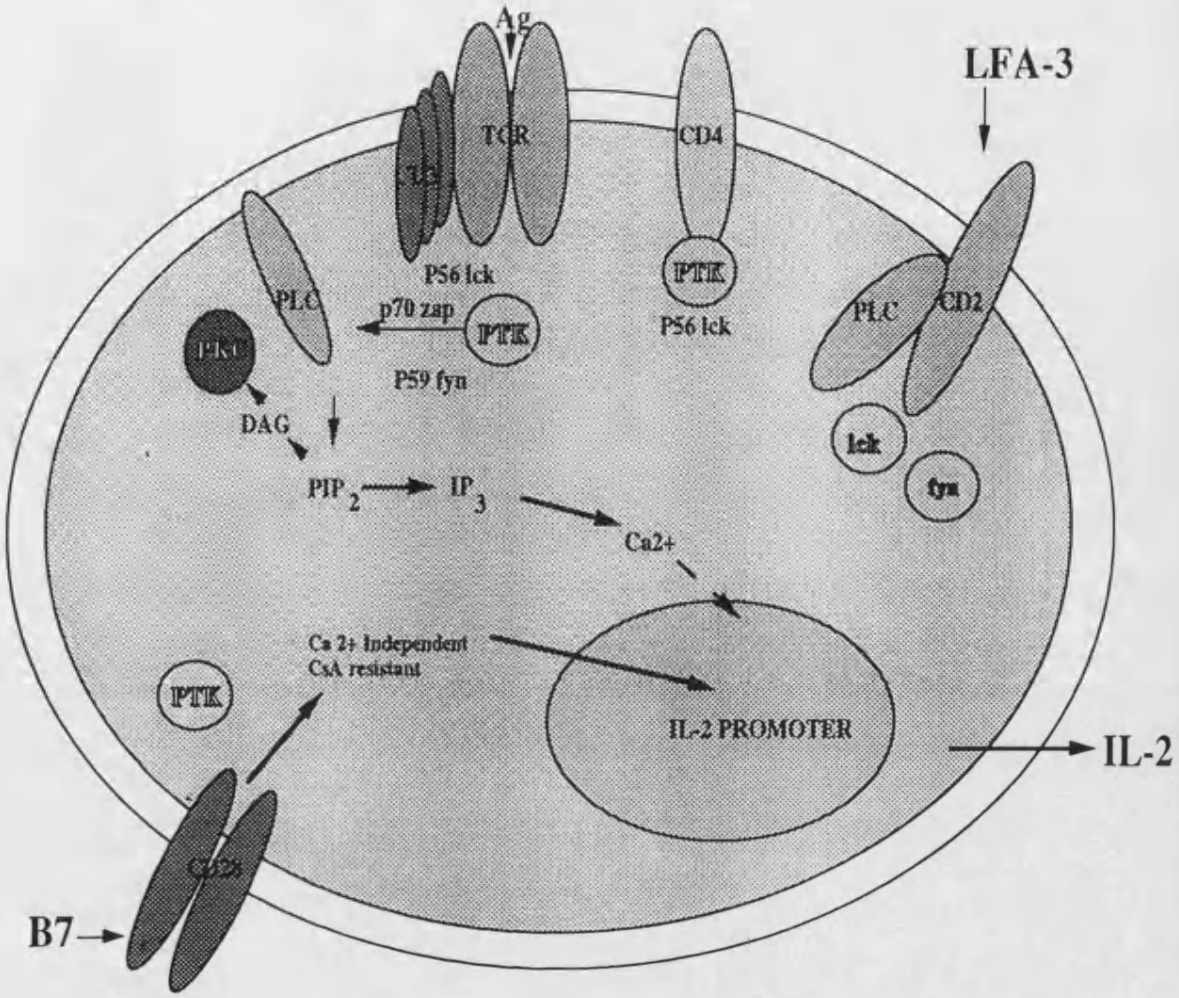


Figure 1.1 Schematic diagram of signalling pathways in a T lymphocyte.
Some of the pathways involved in T cell signalling leading to production of IL-2 from CD4+ T lymphocytes are shown. TCR signalling leads to activation of a number of tyrosine kinases (PTK) such as p56lck, p59fyn and p70 zap. CD4 and CD8 (not shown) interact with p56lck Phosphorylation of PLC leads to the hydrolysis of PIP2 and the formation of DAG and IP3. TCR signalling appears to be calcium dependent as CsA inhibits activation whereas CD28 signalling appears to be Ca independent and resistant to CsA.
Ag = peptide antigen presented on MHC class II molecules, PTK = protein tyrosine kinase, PLC = phospholipase C, DAG = diacylglycerol, CsA = cyclosporin A, PIP2 = phosphatidyl inositol 4,5 bisphosphate, PIP3 = inositol 1,4,5 triphosphate , PKC = protein kinase.

(Downward et al 1992). $p21^{ras}$ activation can regulate cell cycle progresssion (Downward et al 1992) triggering a serine threonine kinase mediated signal amplification cascade culminating in the activation of MAP-kinase (Karnitz and Abraham 1995) which regulates the expression of mitogen responsive genes by phosphorylating specific transcription factors. Many cytokines trigger rapid increase of activated Ras which is evidence supporting a crucial role of this molecule in T cell signalling.

Activation of the two src-related PTKs, $p56^{lck}$ and $p59^{fyn}$ is regulated by the protein tyrosine phosphatase CD45 and TCR mediated signalling crucially requires the phosphatase activity of this molecule (Mustelin et al 1989, Mustelin et al 1992). CD45 is expressed on the cell surface of all haematopoietic cells and their precursors with the exception of platelets and erythrocytes.

It can therefore be seen that rather than one pathway of TCR signal transduction there exists in T cells a complex web of coupling and signalling molecules and of kinases and phosphatases.

1.5 T cell interaction with APCs.

It has become apparent that in addition to the TCR/MHC there are multiple receptor-ligand interactions that occur between the T cell and the APC. Many are adhesive such as that between LFA1(CD11a/CD18) and ICAM-1-3, or between LFA3 and CD2, which reinforce the contact between the two cells (Springer 1987, Springer 1990) while others serve to transduce additional signals to the T cell (Bierer and Burkoff 1991). It is difficult to distinguish between an adhesive compared to a stimulatory signal, for example CD2 ligation may mediate or enhance T cell activation, suggesting that the signals from the CD2 adhesive interaction with LFA3 are integrated with TCR signals (Springer et al 1990). One of the difficulties in the study of T cell activation has been resolving whether an interaction is merely adhesive or provides a function which contibutes to activation signals. Interactions which provide additional signals independent of those produced via TCR engagement are termed costimulatory.

1.6 Costimulation of T cell activation.

The interaction which takes place between the T lymphocyte and an APC is increasingly being seen as crucial for T cell effector function. Moreover, following the discovery that ligation of the TCR in the absence of a functional APC lead to functional inactivation of a T cell clone (Quill et al 1987, Jenkins et al 1987) it was hypothesised, that in addition to MHC/peptide interaction with the TCR, other accessory cell dependent signals were required to costimulate T cell activation (Mueller et al 1989). These experiments suggested that signals from accessory cells were required in addition to TCR engagement to facilitate T cell effector function in agreement with a two signal model of T cell activation first proposed by Bretscher and Cohn (1970). Signal 1 is via the TCR while signal 2 (the costimulatory signal) is thought to be dependent on accessory cells.

Attempts to elucidate the nature of the costimulatory signal has led to the conclusion that the interaction between the CD28 molecule expressed on T lymphocytes and its ligand B7-1 generates such a signal.

1.7 The CD28 family of receptors.

The first ligand demonstrated for B7-1 was the CD28 molecule which is a 44Kd homodimeric glycoprotein conserved between human and mouse and which belongs to the immunoglobulin superfamily. This molecule was first identified in studies with anti-T cell mAbs as a differentiation antigen expressed on $CD4^+$ and on some $CD8^+$ T cells (Hansen et al 1980). CD28 is also present at detectable but at low levels on $CD4^+CD8^+$ thymocytes and expression is enhanced by activation in vitro (Turka et al 1990).

A second receptor for B7-1 has also been identified, CTLA-4 (cytolytic T lymphocyte associated antigen 4), which was cloned from a subtraction library derived from cytotoxic T cells (Brunet et al 1988). CTLA-4 is induced transiently at

low levels on activated CD4+ and CD8+ T cells and its presence is restricted to the memory population of these cells (Linsley et al 1992, Freeman et al 1992). There is a high homology between the mouse and human genes for CTLA-4, in addition CTLA-4 has homology with the CD28 gene and maps to the same chromosome (Harper et al 1991, Dariavach et al 1988 and Lafage-Pochitaloff et al 1990). Surprisingly, despite low level expression, CTLA-4 binds to B7-1 with 20 times higher affinity than CD28 (Linsley et al 1991a), which suggested that this molecule may play a vital role in T cell function and may influence the interaction between CD28 and B7-1. In addition knock-out mice lacking CTLA-4 develop lymphoadenopathy and massive tissue destruction and die by 3-4 weeks, supporting a critical role for CTLA-4 in T cell activation (Tivol et al 1995, Waterhouse et al 1995).

1.8 The B7 family

The B7-1 molecule, originally identified on the surface of activated B cells using the monoclonal antibody (mAb) BB1(Yokochi et al 1982), is a 44-54 kD glycoprotein also known as CD80. Much evidence now exists that B7-1 binds to CD28 providing costimulation to T cells promoting activation resulting in proliferation and IL-2 production (Koulova et al 1991, Gimmi et al 1991).

It was first postulated that there might be a CD28/CTLA-4 ligand in addition to B7-1 when it was demonstrated that the functional interaction of CD28 with its ligand was less efficiently blocked by B7-1 antibodies than by CTLA-4- Ig, a chimaeric construct (Boussiotis et al 1993, Lenschow et al 1993, Wu et al 1993A). With the discovery that B7-1 deficient mice had very few obvious immune defects and that despite lacking B7-1, activated B cells still bound CTLA-4 came the search for a second ligand for CD28 (Freeman et al 1993). Simultaneously two groups reported the cloning of B7-2 (CD86), an additional ligand for CD28 (Freeman et al 1993, Azuma et al 1993a). When the human B7-1 and B7-2 cDNA sequences were examined only 25% sequence identity in the extracellular region was found, (Azuma et al 1993, Freeman et al 1993a and b) although there are indications of structural similarities (Freeman et al 1993b). B7-2 was shown to bind CD28 and induce the CD28 specific signal (Lenschow et al 1993, Engel et al 1994, Freeman et al 1993b). Therefore there is an additional complexity in the CD28/B7 interaction and ligation of CD28 by either B7-1 or B7-2 results in CD28 signalling.

Studies on the expression of B7-1 and B7-2 have revealed that both are co-expressed on professional APCs, dendritic cells, activated B cells and macrophages but not on resting T or B cells or non-haematopoietic cells, B7-1 has also been shown to be found on some activated T cells (Sansom and Hall 1993, Azuma et al 1993b). Although the stimuli that induce B7-1 and B7-2 expression are similar, B7-2 is induced more quickly than B7-1, and is usually expressed at much higher levels (Linsley et al 1994) suggesting perhaps that these molecules serve different functions. The expression of both molecules is induced by LPS, IL-2, IL-4 or signals through surface immunoglobulin, CD40 or Class II MHC (Nabavi et al 1992, Hathcock et al 1994). In contrast, B7-1 and B7-2 have been demonstrated to have different binding determinants for CTLA-4 and to have differential kinetics for CTLA-4-Ig binding (Linsley et al 1994).

However, to date a distinct role for B7-1 compared to B7-2 is controversial, although differential roles for B7-1 and B7-2 have been suggested by the demonstration that administration of anti-B7-1 antibody resulted in reduced disease severity in EAE whereas the use of B7-2 antibody increased disease severity (Kuchroo et al 1995). Additionally recent reports have shown that B7-1 preferentially acts as a inducer of Th1 cells whereas B7-2 induces Th2 cells (Freemann et al 1995, Kuchroo et al 1995). These data suggest a role for B7-1 in determining which group of cytokines is produced in an immune reaction by influencing the development of either Th1 cells or Th2 cells from a Th0 precursor. Interestingly in autoimmune diabetes in the nonobese diabetic (NOD) mouse model the opposite was seen (Lenschow et al 1995). Mice treated with anti-B7-2 mAb did not develop the disease whereas anti-B7-1 administration accelerated the development of the disease. These studies demonstrated that B7-1 and B7-2 have distinct regulatory roles during the development of autoimmune diseases and therefore may play different roles in costimulation of T cell activation. The conflicting results in these two diseases may reflect the stage at which the antibodies interact with the disease process and the involvements of different patterns of cytokine production. Clearly, an understanding of how these interactions regulate the disease process will contribute to our understanding not only of the disease itself but also autoimmunity and the process of T cell activation.

Recent evidence has shown that B7-1 molecules are capable of inhibiting T cell activation (Sethna et al 1994). Constitutive expression of B7-1 on resting B cells in

transgenic mice resulted in a defect in T cell-dependent antibody production and treatment with anti-B7-1 restored the capacity of these mice to respond, demonstrating that the deficient antibody responses are directly attributable to the expression of B7 (Sethna et al 1994). These results suggest that in addition to costimulating productive outcome of TCR engagement B7-1 may also have negative effects on T cell activation. The B7 family thus plays an important role in regulation of T cell function while modulation of B7 expression may influence the outcome of T cell activation.

1.9 Function of the CD28/B7 interaction.

The functional outcome of CD28 interaction with B7 has been the subject of intense study. Anti-CD28 mAbs were found to augment the expression of the IL-2 receptor (CD25) α chain and to enhance production of cytokines from T lymphocytes including IL-2, IFNγ, TNFα and IL-4 (Thompson et al 1989, Lindsten et al 1989). Costimulation appears to occur at the transcriptional level of the IL-2 promoter, causing an increase in IL-2 via accumulation and stabilisation of IL-2 mRNA and direct upregulation of the IL-2 enhancer (Linsley et al 1991b). Thus T lymphocyte function is critically dependent on the interaction between B7 and CD28.

Antigen and accessory cell-dependent priming for IL-4 and IFNγ production was shown to be critically dependent on CD28 costimulation as assessed using inhibition by CTLA-4-Ig, thus suggesting that CD28 costimulation pathway can also play a role in the development of Th cells (Seder et al 1994). Th1 cells predominantly produce IL-2 and IFNγ whereas Th2 cells secrete IL-4, IL-5 and IL-10. The factors that govern the Th subset differentiation are not completely understood, however a critical role for IL-4 in induction and expansion of the Th2 response and for IFNγ in promoting a Th1 profile has been shown (Swain et al 1990, Maggi et al 1992). Since IL-4 and IFNγ are produced by activated and partially differentiated T cells the initial factors that favour IL-4 or IFNγ secretion remain uncertain. However it has recently been demonstrated that CD28 may play an important role in the differentiation of the Th2 subset in humans (King et al 1995). This places CD28 as an important molecule in the development of specific T cell responses resulting in either the predominance of a Th1 or Th2 response leading to production of a particular group of cytokines. This is crucial to the host defence mechanism in diseases such as Leishmaniasis

where regulation of a favourable versus deleterious outcome is mediated by the initial group of cytokines produced.

The two signal model for T cell activation can therefore be viewed as the ability of CD28 to deliver biochemical signals that synergise with TCR derived signals to mediate T cell responses.

1.10 Intracellular responses to CD28

In agreement with the two signal model of T cell activation, the signalling pathway coupled to CD28 appears to be different to the TCR/CD3 signalling pathway. The major pathway by which CD28 signals is resistant to CsA and consequently independent of calcium signals (June et al 1987).

T lymphocyte proliferation and IL-2 production was blocked using herbimycin A, a PTK inhibitor (Vandenburghe et al 1992) which suggested that PTKs and tyrosine phosphorylation are essential for CD28 induced responses. CD28 activates a signalling pathway that results in the appearance of a nuclear protein complex. This complex in turn interacts with a CD28-specific response element in lymphokine gene regulatory regions corresponding to their transcriptional activation (Fraser et al 1991, 1992).

Phosphorylation of a specific tyrosine residue in the intracellular domain of CD28 creates a binding domain for PI-3 kinase (Page et al 1994). Formation of products of PI3 kinase and association of active PI-3 kinase with CD28 have been reported by several groups (Ward et al 1993, Stein et al 1994, Ueda et al 1995). In addition blocking of proliferation and IL-2 production dependent on CD28 costimulation was demonstrated using wortmannin, a specific inhibitor of PI3 kinase. These results however are controversial and Lu and colleagues (1995) have shown no effect of this inhibitor which may indicate the involvement of other signalling pathways in CD28 costimulation. It appears that the state of activation of the T cell may account for this discrepancy as CD28 has the capacity to couple to multiple signalling pathways depending on the overall state of T cell activation (Sansom et al 1995).

In addition to PI-3 kinase signalling CD28 costimulation has also been found to induce the sphingomyelin (SM) signalling pathway. T cell proliferation is under the strong influence of this pathway which has similarities to the phosphoinositide signal transduction pathway (Kolesnick et al 1994). The SM pathway is initiated by the enzymatic hydrolysis of the phosphodiester bond of SM by a specific SM-directed phospholipase C, generating ceramide and phosphocholine. Two forms of sphingomyelinase (SMase) have been identified, a neutral SMase is distinguished from an acidic isozyme by Mg2+ dependence and DAG independence. Ceramide serves as the second messenger of the SM pathway and is known to induce proliferation, differentiation, or both in some cells (Olivera et al 1992). Recently, CD28 has been demonstrated to trigger A-SMase activation in resting as well as in activated T cells (Boucher et al 1995). CD3 triggering did not elicit ceramide production by A-SMase indicating that this lipid messenger system is a distinct costimulatory pathway. Additionally over-expression of recombinant A-SMase was shown to substitute for CD28 in the NFκB pathway (Boucher et al 1995). Therefore the SMase signalling pathways appears to be critically important in transducing signals received through CD28.

Several immediate targets for ceramide action have been identified. A serine/ threonine kinase termed Ceramide-activated Protein Kinase has been characterised (Mathias et al 1991) which has been shown to phosphorylate and activate recombinant human Raf. Other targets include a cytosolic serine /threonine phosphatase and the ζ isoform of PKC (Lozano et al 1994).

Another immediate consequence of CD28 ligation is phosphorylation of the Protein Tyrosine Kinase Itk (August et al 1994). CD28 signalling has also been shown to cause a sustained down-regulation of IkBα, an inhibitor of the c-rel family of transcriptional factors, which allows the nuclear translocation of c-rel (Lai and Tan 1994). This is suggested to cause sustained upregulation of IL-2 gene expression supporting the hypothesis that CD28 plays a crucial role in IL-2 production and T cell activation.

A recent report demonstrated that TCR and CD28 signals are both required for full activation of two mitogen activated protein kinases, JNK1 and JNK2 which are involved in phosphorylation of the c-jun activation domain and activation of the transcription factor AP1 (Su et al 1994). Studies from several laboratories have revealed a crucial role for AP-1 in T cell activation. Thus it appears that TCR and

CD28 signalling may be integrated at the level of JNK rather than by eliciting different transcriptional factors that integrate at the level of the promoter.

These data add considerable weight to the ability of CD28 signals to act as a prime costimulator of T cell activation. Co-operation between the CD3 and CD28 pathways is essential in order to mount an effective T cell response. Co-ordination of signals may be one way in which the immune response regulates T cell activation.

1.11 Function and signalling capcacity of CTLA-4.

The function of CTLA-4 on T cells is still under investigation, but unlike CD28, CTLA-4 expression is not constitutive and only occurs after T cell activation by TCR/CD3 and CD28 ligation (Guinan et al 1994, June et al 1994, Linsley and Ledbetter 1993). It has been shown that CTLA-4 co-operates with CD28 to enhance T cell proliferation (Linsley et al 1991a, Linsley et al 1992, Damle et al 1994). However, others have suggested that CTLA-4 might inhibit CD28-induced functions either by competing for B7-1 or B7-2 or by generating a negative regulatory signal (Walunas et al 1994, Jenkins 1994). Studies with CD28 deficient mice have demonstrated much reduced proliferative responses (Green et al 1994a), CTLA-4$^+$ T cells from these mice do not benefit from costimulation by B7-1$^+$ accessory cells in the presence of phorbol esters suggesting that this interaction does not promote proliferation. In addition, CTLA-4 knockout mice have recently been generated and CTLA-4 demonstrated to be critically important for down-regulating T cell activation and maintaining lymphocyte homeostasis (Tivol et al 1995, Waterhouse et al 1995)

On balance the CTLA-4/B7 interaction does not appear to transduce a positive costimulatory signal and may in fact interfere with CD28 signals. It thus appears that ligation of CD28 may costimulate T cell activation while engagement of CTLA-4 may be involved in regulation of T cell effector function. CTLA-4 may therefore play a crucial yet still unclear role in T cell signalling. In support of a differential function of CD28 versus CTLA-4 it has been shown that CD28 engagement by B7-1 downregulates CD28 synthesis and function at approximately the same time as CTLA-4 expression increases (Linsley et al 1993). Furthermore, it was demonstrated that crosslinking of CTLA-4 together with the TCR and CD28 results in profound inhibition of IL-2 production and proliferation (Krummel et al 1995). It is thus suggested that crosslinking of CTLA-4 in the absence of CD28-mediated

costimulation can induce deletion of previously activated T cells, indeed a mAb to CTLA-4 has recently been generated which induces apoptosis in previously activated T cells (Gribben et al 1995). It can be hypothesised from these studies that CD28 and CTLA-4 do not carry out redundant functions and that CTLA-4 may modulate CD28 dependent responses.

The signalling capacities of CTLA-4 are relatively unknown although an association between CTLA-4 and PI3 kinase has been observed (Schneider et al 1995). However no such association was observed by other investigators (Stein et al 1994).

Integration of costimulatory signals from CD28 and inhibitory signals from CTLA-4 may therefore determine the outcome of TCR engagement. The role of CTLA-4 thus might be to regulate T cell responses by terminating IL-2 production and thereby promoting the generation of memory T cells in order to mount a more effective secondary response to infection. These studies warrant fresh investigation of the signals induced by engagement of the CTLA-4 molecule perhaps looking at the SM pathway and ceramide production.

The temporal patterns of expression of B7/CD28/CTLA-4 suggest that the biological activities of B7 - CD28/CTLA-4 costimulation are modulated during the development of immune responses. Perhaps therefore there is an additional complexity to the idea of the two signal hypothesis in that regulation of the expression by T cells of CD28 and CTLA-4 may modulate T cell responses. In addition regulation of expression of B7-1 and B7-2 , by the APC and also due to local environment, may add to the complexity of immune response generated by the T lymphocyte. Together these data suggest that CD28/CTLA-4 is the major , if not the only receptor family of APC-derived signals that costimulate TCR signals and control proliferation. The interaction of the CD28/B7 families is therefore a prime mediator of the outcome of T cell activation and regulation of this interaction may influence the development of the immune response.

1.12 Mechanisms of self tolerance

The establishment of a T cell repertoire which reacts to and eliminates foreign pathogens but remains unreactive or tolerant to self-antigens is essential in order to maintain a functioning immune system. Two distinct mechanisms of T cell tolerance have been defined and studied. First, clonal deletion is considered the principal mechanism of selection occurring during maturation of thymocytes in the thymus (Kappler and Marrack 1987). In contrast hyporesponsiveness (anergy) and Activation Induced Cell Death (AICD) are considered to be the mechanisms responsible for tolerance induction of mature peripheral T lymphocytes. Both functional inactivation and deletional mechanisms are now thought to contribute to central and peripheral tolerance (Cohen et al 1992).

1.12.1 Central tolerance: Elimination in the Thymus.

Mechanisms that regulate cell death and survival are essential for normal development and maintenance of homeostasis. The major mechanism for elimination of self-reactive thymocytes is by programmed cell death (PCD) in the thymus (Jenkinson et al 1989, Kappler and Marrack 1987). The most common morphological expression of such programmed cell death is apoptosis. The characteristics of apoptosis include ; cell shrinkage, blebbing of the plasma membrane and nuclear collapse. Fragmentation of the nuclear chromatin at internucleosomal sites occurs via activation of an endogenous nuclease (Kerr 1971, Wyllie 1994). During the apoptotic process, the cytoplasm of affected cells condenses, the plasma membrane becomes convoluted and condensation of the nucleus occurs. At the final stage of apoptosis, the cells themselves are fragmented (apoptotic bodies) and are phagocytosed by neighbouring macrophages and granulocytes (Raff 1992, Wyllie 1980). This ensures that the apoptotic cell contents do not remain in the immune system which may lead to inflammation and tissue damage. This contrasts with necrosis which can lead to inflammation (Barr and Tomei 1994).

PCD occurs when a cell activates an internal programme of suicide as a result of either external or internal signals, apoptosis is therefore often termed death from the inside out. External signals include an array of pathological conditions such as

irradiation, heat shock and toxic substances. PCD has been observed in most if not all multicellular organisms and it occurs in a variety of processes from embryonic development to neurological homeostasis and lymphocyte maturation. Apoptotic death has also been shown to be highly conserved throughout evolution from plants, Caenorhabditis elegans and Drosophila to mice and humans. Just as cells induced to proliferate activate a programmed series of biochemical events leading to cell division, cells induced to die activate a distinct program of biochemical events leading to apoptosis.

During thymocyte ontogeny the genes of the TCR undergo rearrangement, thereby generating a large number of clonally diverse cells (Kappler and marack 1987, von Boehmer 1992). PCD is induced in immature T cells which have failed to express a functional TCR (selection by default) and T cells which express a high affinity TCR with potential self reactivity (negative selection) (von Boehmer 1992, Schwartz 1989). These cells are then eliminated from the thymus by engulfment by macrophages.

Concurrent with gene rearrangements, developing thymocytes also modulate the expression of a number of molecules on their cell surface, one of the first molecules newly expressed on human thymocytes is CD2 (Fox et al 1985). These $CD2^+$ $CD3^-$ $CD4^-$ $CD8^-CD28^-$ cells expand, rearrange their TCR genes and progress to an intermediate stage of development where they express low levels of TCR and CD3. The vast majority of thymocytes use the TCR α and β chains, acquiring other accessory molecules, including CD4, CD8, and CD28 at this stage (Lanier et al 1986). Thymocytes which express a TCR with low affinity for self-MHC/peptide complexes survive (positive selection) and migrate from the thymus to begin life as mature peripheral T cells. Both positive and negative selection are thought to occur at this stage after which surviving thymocytes increase expression of CD3 and CD28, but lose either CD4 or CD8 before export into the periphery.

Positive selection in the thymus is thought to involve engagement of the TCR on immature $CD3^{lo+}$ $CD8^+$ $CD4^+$ thymocytes with self MHC products expressed on the surface of thymic epithelial cells. It has been suggested recently that the overall avidity of the TCR complex and co-receptor molecules such as CD4 and CD8 determine whether a T cell is positively selected or undergoes PCD due to default and negative selection (Penninger and Mak 1994). This ensures that only those thymocytes expressing TCRs with sufficient but not too great an affinity for self

MHC are given the signal for maturation and proceed to leave the thymus and seed the peripheral lymphoid organs (Kisielow et al 1988, Sha et al 1988). These cells are then capable of recognising foreign peptide presented by self-MHC on an APC. T lymphocytes which fail to express functional TCR or those with too high an affinity for self-MHC die by PCD and are removed from the thymus.

Successful engagement of the TCR with MHC class I molecules leads to differentiation into $CD8^+$ $CD4^-$ T cells which are most often cytotoxic T cells, while engagement with class II leads to differentiation into $CD4^+CD8^-$ T helper cells (Teh et al 1988). Signals mediated by CD4 and CD8 have also been shown to influence the specificity of the emerging thymocyte: upon MHC recognition by the developing thymocyte, TCR/CD3 mediated signalling is enhanced by co-ligation with CD4 or CD8 (Turka et al 1991). It has been suggested that the state of activation and differentiation could render thymocytes susceptible to positive or negative signals (Russell et al 1991). Therefore thymic development depends on the expression of molecules other than TCR for selection of T cells capable of responding to antigen.

It is well established that thymocytes are highly susceptible to PCD and most of the death of unwanted T cells seen in thymic deletion has been shown to occur via apoptosis. Triggering of cell surface receptors such as CD3/TCR with anti-CD3 mAbs has been shown to lead to PCD in immature T cells (Smith et al 1989). This provides evidence for a dual outcome of TCR engagement resulting in a proliferative response when costimulated or in death when costimulation signals are not provided. Ionomycin alone strongly induced death via apoptosis in immature thymocytes (Smith et al 1989) which suggested that an increase in intracellular calcium alone was sufficient to induce the cell suicide response. Further evidence that clonal deletion of autoreactive cells involves apoptosis was reported in the neonatal mouse thymus (MacDonald and Lees 1990). A significant population of $CD4^+$ T cells bearing autoreactive TCR were found in these mice and when placed in culture a large proportion die. Death was prevented by inhibitors of macromolecular (RNA and protein) synthesis implying that this was an active metabolic process. T cell death in the thymus therefore may occur via TCR engagement without costimulation via a calcium dependent metabolically active pathway.

1.12.2 The role of costimulation in thymic deletion.

The role of CD28 as a costimulus in the thymus remains controversial. CD28 is expressed on mouse and human thymocytes and B7-1$^+$ T cells have been observed in sections of human thymus (Yang et al 1988, Gross et al 1992). A potential role for CD28 signal transduction was suggested by the demonstration that B7-1 is expressed on thymic stromal cells (Turka et al 1990, Vandenberghe et al 1993). A possible model of thymic selection was proposed in which CD4 or CD8 enhance TCR/CD3 signalling upon co-ligation by an MHC molecule. If CD28 is ligated by a B7-1 expressing stromal cell, this could lead to proliferation and positive selection. In the absence of CD28 signalling the enhanced TCR/CD3 signals might lead to apoptosis and negative selection. However in vitro studies using CTLA-4-Ig to study thymic negative selection in mice have not supported a role for B7-1/CD28 (Jones et al 1992, Page et al 1993). In addition negative selection as assessed by deletion of self-reactive T cells bearing particular Minor lymphocyte stimulating (Mls) antigen determinants proceeded normally in CD28 deficient mice (Shahinian et al 1993). However in vivo studies indicated that, consistent with the medullary location of B7-1 expression, CD28 may indeed have a role in negative selection (Punt et al 1994). IL-7, which is abundantly expressed in thymus where it drives T cell gene rearrangement, has been shown to induce T cell B7-1 expression (Yssel et al 1993), which might support the idea of CD28 costimulation in the thymus.

The different consequences of TCR signalling in CD4$^+$CD8$^+$ thymocytes depending upon the presence or absence of costimulatory signals, provides another model for the basis for the difference between positive and negative selection processes. Positive selection may be initiated when the TCR is engaged in the absence of costimulation, whereas negative selection may occur as a result of a high affinity TCR interaction in the presence of a costimulatory signal (Punt et al 1994). The nature of the cell presenting antigen to an immature thymocyte may also determine whether positive or negative selection takes place as not all cells which express class II MHC in the thymus are also capable of costimulation. This is similar to the requirement for costimulation in order for T cell proliferation to occur in the periphery. Costimulatory signals and cell to cell interactions are therefore important throughout T cell development. T cells are thus programmed with the information they need to make the decision to live or die depending on their surroundings.

1.12.3 Peripheral Tolerance: Anergy

The existence of a multitude of diseases with an autoimmune pathology lays testimony to the prevalence of autoreactive T cells in the periphery. The functioning of a normal immune system depends on silencing these autoreactive T cells and preventing their expansion. The ability to prevent activation of autoreactive T cells may be a routine function of a normal immune system, maintained by limiting exposure of professional APCs to self antigen or by presenting self antigen on inefficient APCs in a way that functionally inactivates these T cells.

An in vitro model of T cell tolerance was first described in which IL-2 driven proliferation of $CD4^+$ T cell clones, responding to peptide antigen and syngeneic APCs, was blocked if the APCs were pretreated with a chemical fixative (Jenkins and Schwartz 1987). Instead of stimulating proliferation in the presence of specific antigen , these fixed APCs induced the T cells into a state of proliferative unresponsiveness termed anergy. This led to the suggestion that the APCs lacked the capacity to provide costimulation to drive a proliferative response, supporting the two signalling model of T cell activation first proposed by Bretscher and Cohen (1970), who hypothesised that T cells, in addition to signal 1 (TCR engagement), also required signal 2 (costimulation) in order that T cell activation takes place.

Peripheral tolerance in vivo was first observed by demonstrating unresponsiveness to the Mls antigen (Ramensee et al 1989). Most murine T cells that express the Vβ6 TCR gene segment are reactive against Mls-1a antigens, therefore by using an anti Vβ6 mAb the fate of Mls-1a specific T cells were able to be monitored in Mls-1b mice. These mice were made specifically unresponsive to Mls-1a because the antigen was expressed on resting B cells, which are not professional APCs. The induced unresponsiveness in this system was shown to be due to clonal inactivation or anergy and not deletion since Vβ6 cells were still present in the periphery. The anergic cells in Mls-1b mice expressed IL-2 receptors but did not produce IL-2, in contrast to Vβ6 cells from naive mice. In agreement with this model, presentation of antigen by MHC expressed on non-lymphoid cells which do not function as professional APCs has been shown to result in the functional inactivation of T cell clones (Lo et al 1990, Burkly et al 1990). These cells do not express B7-1 or B7-2 , thus they cannot trigger CD28 signalling. Therefore tolerance in the

periphery may be mediated by presentation of antigen on non-professional APCs which are incapable of providing costimulation via CD28.

A number of models of T cell anergy have now been described (Hewitt et al 1992, Lamb et al 1983), including the induction of T cell unresponsiveness by modulating surface CD3 with an anti-CD3 mAb in the absence of costimulatory signals (Davis et al 1989). This generated an initial increase in Ca2+ concentration which failed to induce T cell activation. This again indicates the potential for multiple outcomes of TCR engagement depending on the presence or absence of costimulation. Therefore there is considerable evidence which shows that in the absence of costimulation, engagement of the TCR results in a state of unresponsiveness in the T cell, characterised by failure to activate the IL-2 gene and proliferate in response to further antigenic challenge. Anergy can thus be considered to be an important mechanism in maintaining self tolerance and in regulating the magnitude of responses to foreign antigen.

It has also been proposed that differential tolerance induction occurs in naive and memory T cells, the latter being shown to be much more susceptible to anergy induction than naive T cells (Davis and Lipsky 1993). T cell clones which are further differentiated than memory cells were also shown to be particularly sensitive to inactivation by tolerising signals. The sensitivity to tolerance induction may play a crucial role in limiting the capacity of memory cells to respond to self antigens after presentation by non-professional APCs. This suggests that there are multiple pathways of anergy induction, each acting at different stages of T cell development providing the immune system with fail-safe mechanisms to prevent self-reactivity.

1.12.4 The involvement of CD28 in peripheral tolerance.

Ectopic expression of MHC molecules on non-lymphoid cells, which do not express B7-1 or B7-2, can induce anergy in T cell clones (Lo et al 1990, Burkly et al 1990) which lead to the suggestion that lack of costimulation via CD28 may lead to tolerance induction in the periphery. Furthermore, the involvement of CD28-mediated signalling in prevention of anergy has been demonstrated using a mAb to murine CD28 (Harding et al 1992). Evidence from studies on human T cells has shown that blocking the interaction between CD28 and B7-1 when T cells are presented with alloantigen in a mixed leukocyte culture, leads to hyporesponsiveness

to the alloantigen (Tan et al 1993). Blocking was carried out using either anti-CD28 mAb fragment or CTLA-4-Ig, a chimaeric immunoglobulin fusion protein containing the extracellular domain of CTLA4. These experiments demonstrated the importance of the CD28/B7 interaction in T cell proliferative responses.

Interestingly it has been shown that T cells receiving a tolerising signal from the superantigen Staphylococcal enterotoxin B (SEB) downregulate expression of CD28 (O'Hehir et al 1990). Ligation of CD28 as has been discussed earlier results in the upregulation of IL-2 (Thompson et al 1989, Fraser et al 1991). Thus the loss of CD28 will render this costimulatory pathway inactive, therefore emphasising the role played by CD28 in prevention of functional inactivation of T lymphocytes. Expression of B7 and CD28 may therefore be critically involved in regulating the response to autoantigens.

It has been recently demonstrated that hyporesponsiveness resulting from TCR engagement stems from a selective defect in the early signal transduction initiated by TCR crosslinking (Dubois et al 1994). Furthermore it was shown that tyrosine phosphorylation and calcium mobilisation were both deficient in anergic cells while diminished inositol phosphate hydrolysis has also been shown in these cells (Dubois et al 1994). The AP-1 complex may also be a unique target for T cell clonal anergy (Kang et al 1992). CD28 signalling results in activation of AP-1 and it can therefore be concluded that CD28 costimulation is intimately involved with the prevention of anergy.

The importance of the B7-1-CD28/CTLA-4 pathway for peripheral tolerance has also been demonstrated in vivo. Blocking CD28-B7-1 interactions inhibits rejection of human pancreatic islet grafts in mice (Lenschow et al 1992) and CTLA-4-Ig treatment resulted in prolonged donor specific unresponsiveness to the xenogeneic islets. This suggests that blocking the CD28/CTLA-4-B7-1 interaction may provide an approach to the suppression of graft rejection in humans. These results emphasise the importance of CD28/B7-1 interactions in preventing the induction of anergy and in delivering a costimulus to drive productive outcome of TCR triggering resulting in proliferation of antigen-specific T cells and effector function. Therefore CD28 costimulation is intimately linked with the maintenance of tolerance in the immune system preventing the harmful activation of self-reactive T cells which could result in autoimmunity and disease.

1.12.5 Peripheral Tolerance : Activation Induced Cell Death (AICD)

Dysregulated apoptosis has been proposed to be involved in the pathology of a multitude of diseases including cancer, heart disease, neurological disorders such as Alzheimers and AIDS. The uncontrolled cell growth observed in tumours may be caused by cell populations increasing their rate of proliferation, or decreasing their rate of apoptotic cell death or both. Therefore maintenance of a balanced immune response in the periphery is critically dependent on apoptosis.

1.12.5.1. Apoptosis in T lymphocytes

A number of murine T cell hybridomas undergo apoptosis within a few hours of activation by specific antigens, mitogens, mAbs against the TCR or a combination of phorbol ester and calcium ionophore (Shi et al 1989, Ucker et al 1989, Shi et al 1990). The mechanisms of death seen involved de novo synthesis of RNA and proteins which suggested that the apoptosis proceeds via an active metabolic pathway. Activation induced apoptotic death of T cells in the periphery has been demonstrated (Liu and Janeway 1990. Newell et al 1990). In addition, activated murine and human T cells expressing either α/β or γ/δ TCR undergo apoptosis when they are incubated with anti-CD3 mAb or PHA (Russell et al 1991, Janssen et al 1991, Owen-Schaub et al 1992). Thus, evidence has accumulated that AICD via apoptosis is not limited to immature thymocytes and transformed T cells (hybridomas) but can be similarly triggered in mature peripheral T cells.

During primary activation both human and murine mature T cells change from an AICD resistant to AICD sensitive phenotype over a period of several days (Wesselborg et al 1993, Russell et al 1991). In the thymus however, elimination of T cells occurs within hours of first exposure to the deleting epitope (Murphy et al 1990). A characteristic of peripheral clonal deletion of mature T cells is that a transient phase of proliferation precedes cell deletion and ligand-specific hyporeactivity of persisting cells. Regulating the time needed to convert from a resistant to a sensitive phenotype may be one way in which T cells can undergo a primary activation without risking elimination. It is only during the second encounter with antigen that responding T cells are at risk of deletion through AICD.

Consequently, AICD provides a mechanism for clonal deletion in the peripheral immune system and is therefore important in the maintenance of cell tolerance.

1.12.5.2 Susceptibility to apoptosis: activated versus resting cells

Data from numerous experimental systems have suggested that previously activated T cells are predisposed to the induction of apoptosis when given a second signal through the TCR. $CD8^+$ $CD57^+$ T cells can be induced to die via AICD following ligation of CD2 by certain combinations of anti-CD2 mAbs (Rouleau et al 1993). Signals mediated through CD3 allow these cells to enter cell division whereas signals via CD2 result in proliferation or apoptosis depending on the conformational changes imposed on the CD2 molecules and on the activation state of the cells. It appeared from these studies that once the cells have entered the cell cycle they become extremely sensitive to apoptosis. It can therefore be hypothesised that T cells are predisposed to apoptosis while undergoing cell division, perhaps by being driven to proliferate by the addition of growth factors such as IL-2. Additionally, it has also been shown that pre-exposure of T cells to IL-2 renders these cell more susceptible to the induction of apoptotic death (Lenardo 1991). Interestingly, crosslinking of the TCR where IL-2 gene expression is prevented programs T cells at different stages of development for apoptosis in response to IFNγ (Groux et al 1993). This is different to the death induced by cytokine deprivation as engagement of the TCR is necessary to induce death as well as inhibition of cytokines. This indicated that IL-2 production may be necessary for prevention of apoptosis. These results emphasise the complex nature of the T cell signalling response and that it is influenced by many factors. At some stages IL-2 may protect the cell from induction of apoptosis whereas at others IL-2 production may drive the T cell towards apoptotic death.

HIV-infected cells are more sensitive to apoptosis than are uninfected cells (Kobayashi et al 1990). An apoptotic mechanism of cell death after HIV infection has been suggested (Ameisen and Capron 1991) and it has also been demonstrated that mouse splenic T cells pretreated with anti-CD4 antibodies die by apoptosis. $CD4^+$ T cells from HIV-infected individuals are primed in vivo for suicide via apoptosis, upon TCR activation by both superantigen and MHC II-restricted antigen (Groux et al 1992). Crosslinking of gp120 on human CD4+ T cells followed by signalling through the TCR results in AICD (Banda et al 1992). The relevance of apoptosis to

the aetiology of HIV infection has been substantiated by the demonstration of elevated levels of Fas/APO-1 on peripheral blood T cells of HIV infected individuals (Katsikis et al 1995). This results in the more rapid induction of apoptosis on these T cells.The role of Fas in mediating apoptosis of T lymphocytes is becoming increasingly more apparent.

1.13 The Fas Receptor.

The Fas antigen was first identified using a mAb which recognised myeloid cells, T lymphoblastoid cells or diploid fibroblasts and induced a cytolytic activity in cells expressing this antigen (Yonehara et al 1989). Another antibody termed APO-1 was also described which recognised activated or malignant T lymphocytes (Trauth et al 1989) and lead to death of tumour cells. Derived amino acid analysis of Fas cDNA indicated that it is identical to APO-1 and that both are members of the NGF/TNF superfamily which includes TNFRs, NGFR and CD40 (Itoh et al 1991, Oehm et al 1992).

The mature Fas antigen is a 36 kD glycoprotein, comprised of 319 amino acids whose N-terminal 157 amino acids is extracellular while the C- terminal 145 intracellular region is cysteine rich. Additional features include a membrane spanning region of 17 amino acids which place Fas as a Type I membrane protein. Staining of mouse thymocytes with an anti-Fas antibody indicated that these cells expressed the Fas antigen and were rapidly killed upon intraperitoneal administration of the anti-Fas antibody (Ogasawara et al 1993). Therefore Fas was demonstrated to be involved in cell death in vivo. Furthermore, expression of the cDNA for Fas in murine T cell lymphomas or fibroblasts resulted in susceptibility to apoptosis when ligated by a mouse anti-Fas antibody (Itoh et al 1991). Death was mediated by an endonuclease that preferentially digests internucleosomal DNA (Itoh et al 1991), thus the peptide encoded by the Fas cDNA transduces the apoptotic death signal into cells. Together these results led to the hypothesis that Fas is intimately associated with apoptotic cell death in T lymphocytes.

The death signal is mediated by a segment at the carboxyl terminal of the Fas molecule that exhibits high homology with that of human TNFR1 (Itoh et al1991, Itoh et al1993) which can also transduce an apoptotic signal into cells (Tartaglia et al

1991). Specific mutational analysis (Itoh and Nagata 1993) defined a critical 68 amino acid portion of Fas required for apoptotic signal transduction. A point mutation within this region was sufficient to abolish the ability of Fas to mediate apoptosis. This region has been called the death domain and has been demonstrated to be homologous to a molecule discovered in Drosophila termed reaper (Golstein et al 1995). Reaper is a 65 amino acid peptide, the expression of which is both necessary and sufficient for developmental cell death in Drosophila. The homology between reaper and the death domain of Fas suggested a common mechanism of PCD from drosophila to mammals indicating the importance of this process.

The Fas antigen has been shown to be encoded by the gene for the lymphoproliferative (*lpr*) mutation in MRL mice(Watanabe-Fukunaga et al 1992). This is an autosomal recessive mutation which results in a phenotype which includes formation of multiple autoantibodies and accumulation of large numbers of non-malignant CD4 $^-$ CD8 $^-$ T lymphocytes in the lymph nodes and spleen of affected mice. All strains of mice that bear the *lpr/lpr* gene are predisposed to the development of this lymphoproliferative autoimmune disorder (Cohen and Eisenberg 1991). It is thus possible that Fas is involved in the development of autoimmunity seen in these mice. A comparison of the Fas gene from *lpr* mice with that of wild type mice indicated an insertion of an early transposable element into intron 2 of the Fas gene (Adachi et al 1993). In addition two other mutations have been identified, lpr^{cg} and *gld* which cause similar complex disorders of the immune system. In all three cases an unusual type of T cell accumulates in the periphery which expresses the Thy-1 antigen and polyclonal α and β TCRs but lacks both CD4 and CD8, and expresses a number of cell surface molecules more often found on B cells such as the B220 antigen. The lpr^{cg} mutation results in a Fas antigen which is unable to transduce the apoptotic signal inside the cell. The mutated site of lpr^{cg} has been shown to occur in the cytoplasmic death domain of Fas. Furthermore it has been shown that *lpr* mice are resistant to AICD (Russell et al 1993, Bossu et al 1993). This suggested the involvement of Fas in autoimmune disease perhaps due to a loss of tolerance in the periphery. Consequently it is suggested that the Fas antigen plays a role in the development of peripheral tolerance mediated perhaps by its intrinsic involvement in T lymphocyte apoptosis. A defect in the Fas antigen may therefore result in aberrant apoptosis leading to autoimmunity and disease.

SLE is a human autoimmune disease which has characteristics very similar to those seen in MRL *lpr/lpr* mice. It has been postulated that due to accelerated apoptosis in

SLE increased amounts of nucleosomes are released into the extracellular milieu. (Emlen et al 1994). This may provide a continuous source of nuclear antigens to drive the autoantibody response in SLE hence contributing to disease aetiology. Additionally in both *lpr* mice and SLE patients apoptosis of T lymphocytes is accelerated in vitro (Van Houten and Budd 1992) which suggests that they may have been primed in vivo for apoptosis but due to a defect in their ability to undergo Fas-induced apoptosis, they are prevented from dying in vivo. It is hypothesised that failure to undergo apoptosis in response to Fas ligation generates a population of cells that are predisposed to undergo apoptosis when the environmental conditions are altered such as in vitro culture. Any deviation from the balance between lymphogenesis and lymphocyte death may endanger an individual by causing hypo- or hyper-immune responses, as evidenced by the consequences of altered lymphocyte death in SLE and *lpr* mice.

1.14 Fas Ligand (Fas-L).

It has previously been suggested that *lpr* and *gld* are mutations encoding an interacting pair of molecules involved in T cell development (Allen et al 1990). Using a CTL line which had cytotoxic activity against wild type but not *lpr* mice Rouvier et al (1993) suggested the presence of a ligand for Fas on the surface of these cells. A cDNA for the Fas ligand (Fas-L) was isolated using these cells and structural analysis carried out (Suda et al 1994) which revealed that the Fas-L was a type II membrane protein homologous to members of the TNF family. This includes TNFα, TNFβ, LT and the ligands for CD40, CD27 and CD30. The murine gene for Fas-L has been mapped to Chromosome 1 (Takahashi et al 1994) where the *gld* mutation is localised and the gld mutation shown to abolish the ability of Fas-L to bind Fas. This indicated that *gld* mutation lies within the Fas-L gene and this mutation and *lpr* encode a ligand and receptor involved in mediating cell death. When recombinant Fas-L was expressed in COS cells apoptosis was induced in Fas-expressing target cells (Suda et al 1994). Northern blot analysis of rat tissues indicated that Fas-L mRNA is expressed abundantly in the testis, moderately in the small intestine and weakly in the lungs. The limited expression of Fas-L RNA suggested perhaps that expression of this gene is tightly regulated in order to control Fas-L mediated apoptosis. These results indicate the importance of the Fas/Fas-L interaction in T cell cytotoxicity.

A direct role for Fas-L in T cell mediated cytotoxicity is supported by two studies suggesting that Fas is the major target for CD4+ mediated cytolysis (Stalder et al 1994) and that a component of CD8+ cytotoxic T lymphocyte (CTL) killing involves Fas-L (Rouvier et al 1993). The importance of Fas engagement in CD4+ CTL mediated cytotoxicity is further substantiated by the failure of both cloned and normal CD4+ CTL to lyse B cell blasts from *lpr* mice (Stalder et al 1994). The direct involvement of Fas in the apoptotic pathway induced by CD4+ Th1 and CD8+ cytotoxic T lymphocytes has also been demonstrated (Ju et al 1995). Additionally, Fas-L is expressed on freshly isolated natural killer cells (Arase et al 1994), which exhibit Fas-mediated cytotoxicity against Fas-expressing target cells. These studies emphasise the importance of the Fas/Fas-L interaction in mediation of cytotoxic killing and that, together with perforin-mediated cytotoxicity this is a major mechanism of T cell death.

1.15 The function of the Fas/Fas-L interaction.

There is conflicting evidence as to whether negative selection of autoreactive T cells is impaired in *lpr* mice and whether the presence or absence of negative selection of T cells in the thymus determines whether lymphoproliferation occurs in the lymph nodes. The current consensus is that negative and positive selection is normal in *lpr* and *gld* mice (Sidman et al 1992). In contrast it has been demonstrated that AICD of mature T cells does not occur in *lpr* or *gld* mice in vitro (Russell *et al* 1993, Russell and Wang 1993). Furthermore, when SEB is injected into wild type mice, mature T cells expressing SEB-reactive Vβ8 TCR chain initially proliferate, then die by apoptosis. This process could not be seen or was severely retarded in *lpr* mice (Scott et al 1993) supporting the hypothesis that Fas expression is intimately linked with the apoptotic process.

A model for the involvement of the Fas system in the clonal deletion of autoreactive T cells has been proposed (Suda and Nagata 1995). APCs express the autoreactive peptide as a complex with MHC on their surface. The peptide/MHC complex interacts with the TCR on autoreactive T cells, which activates the cells and induces the expression of Fas and Fas-L. Such autoreactive T cells then kill each other through Fas-Fas-L interactions. The defect in Fas in *lpr* mice results in the failure of T cells to undergo AICD leading to a build up of T cells specific for self antigen. This

in turn leads to an accumulation of aberrant T cells in the mutant mice resulting in an enlarged spleen and large lymph nodes.

In conclusion it has been established that Fas-L binds to Fas and induces apoptosis. *Lpr* and *gld* mutations result in the development of lymphadenopathy and are loss of function mutations in Fas and Fas-L respectively. Thus it appears that the Fas/Fas-L interaction is intimately involved in the apoptotic process that occurs during the maturation of T cells and hence the maintenance of T cell tolerance in the periphery. Recently it has been demonstrated that stimulation of Fas receptor is essential for TCR-activation death induced in T cell hybridomas, Jurkat T leukaemia cells and previously activated T cells (Dhein et al 1995, Ju et al 1995, Brunner et al 1995). TCR stimulation in all these cells was shown to induce expression of both Fas and Fas-L. Death in these systems has been shown to be mediated via Fas/Fas ligand interactions. Additionally, elimination of self-reactive B cells has also been shown to be dependent on Fas (Rathmell et al 1995). Together these studies established Fas as an important mediator of death in T cells at various activation states and also suggested its involvement in tolerance of both T and B lymphocytes. Fas-L is thus the prime mediator of the peripheral deletion of T cells and maintenance of peripheral tolerance. Therefore the interaction between Fas and Fas-L is critical in the development of T cell repertoire and immune cell homeostasis.

1.16 Fas signalling.

Despite an explosive interest in apoptosis and its importance in the maintenance of cellular homeostasis in many organisms, relatively little is known about the biochemical basis of this process. However in the last few years pieces of the jigsaw are being located although a complete picture is still a long way off.

Similar to TCR stimulation, Fas-initiated transmembrane signalling in Jurkat T cells has been demonstrated to involve the rapid activation of PTK resulting in tyrosine phosphorylation of cellular proteins (Eischen et al 1994). Blocking this protein kinase activation using inhibitors such as herbimycin A, genistein or staurosporine inhibited Fas -induced DNA fragmentation. Fas and TNFR share a 70 amono acid intracellular death domain that transduces the signal for cell death (Tartaglia et al 1993, Itoh and Nagata 1993). This conserved sequence does not contain any intrinsic kinase or

phosphatase domains and it is therefore assumed to be coupled to separate signalling elements. Recently, three groups reported the isolation of genes encoding proteins that associate with the cytoplasmic domains of Fas or TNFR1, which were termed TRADD, FADD, and RIP (Hsu et al 1995, Chinnaiyan et al 1995, Stanger et al 1995). These proteins when transiently over-expressed in cells, all induce apoptosis, supporting a functional relationship to cell death. Apart fom their associating domains these proteins are unrelated and only one of the proteins RIP has been found to contain a kinase domain. Their exact role in Fas-dependent cell death has yet to be elucidated. Recently, a Fas activated serine threonine kinase (FAST) has been identified (Tian et al 1995) which is rapidly activated during Fas-mediated apoptosis.The activation of this kinase has been postulated to be involved in triggering the downstream events involved in apoptosis and to be one of the molecules activated during ceramide production.

The signal generated by Fas is transduced by a SM-ceramide turnover-mediated signalling pathway in a manner similar to CD28 and TNFα (Cifone et al 1993). Direct measurement of enzyme activity in Fas-stimulated U937 promyelocytic cells as well as other tumour cell lines and Fas transfected murine cells showed SMase activity. All Fas sensitive cells tested could be induced to undergo apoptosis after exposure to ceramide. Additionally Fas-ligation generated ceramide in Jurkat T cells and in a mastocytoma cell line transfected with Fas (Gulbins et al 1995). In these cells Fas ligation also activated Ras which was found to constitute a critical component of the apoptotic pathway. Ras is activated upon TCR ligation (Downward et al 1990) and has been demonstrated to lead to apoptosis in immature thymocytes (Green and Scott 1994). This therefore demonstrates the involvement of similar second messenger pathways in proliferation and apoptosis and indicates the interlinked nature of cell survival and cell death.

The involvement of calcium signalling in apoptosis and generation of the death signal is contentious. TCR/CD3 stimulation of mixed lymphocyte cultures or hybridoma T cells was able to induce Fas-based cytotoxicity (Vignaux et al 1995). The induction of Fas-mediated cytotoxicity was shown to be Ca2+ dependent whereas execution of cytotoxicity was not. The induction was sensitive to macromolecular synthesis inhibition in line with the demonstration of increased Fas-L expression (Vignaux et al 1995). The CD28 pathway and the Fas death pathway are therefore similar in their downstream signalling via the SM pathway and minimal dependency on calcium signalling.

Fas also contains a negative regulatory element, a "salvation" domain at the carboxy terminus that structurally is not present in TNFR1 (Itoh and Nagara 1993). A recent report documented evidence that the 15 amino acid carboxyl terminus of Fas associates with a protein phosphatase that had previously been identified in basophils (Sato et al 1995). Cells that are resistant to Fas-L killing expressed this phosphatase whereas susceptible cells did not and resistance could be conferred by introducing the phosphatase into sensitive cells. This demonstrates the involvement of phosphorylation in mediating not only TCR signalling but also apoptosis once again demonstrating the similarity between the cell death and cell survival pathways.

The Fas/Fas-L pathway thus plays a vital role in apoptosis of T lymphocytes and the maintenance of a tolerant T cell repertoire. The signalling pathways mediating Fas-induced apoptosis remain the focus of intense study as does that role the Fas/Fas-L interaction might play in functions other than death.

1.17 Superantigens and the T cell response.

One of the major obstacles to studying clonal expansion and regulation of normal T cell function is the low frequency of antigen specific naive cells. Much has been learnt about the development of the T cell repertoire and immune effector functions by the study of superantigens (SAgs). These are a group of molecules which stimulate T cells at a higher frequency than normal peptide antigen. T cell receptors are composed of five variable elements, Vα, Jα, Vβ, Dβ Jβ all of which contribute to the specific interaction of T lymphocytes with conventional peptide antigens presented in the context of MHC molecules (Herman et al 1991). There are potentially billions of possible combinations of these variable elements, and so the frequency of responding T cells to a given conventional antigen is very low. SAgs stimulate such large numbers of T cells because they stimulate virtually all T cells bearing particular Vβ elements and are therefore a useful tool in the investigation of T cell function.

MHC class II proteins are the specific receptors for Staphylococccal enterotoxins (SEs) and other foreign SAgs (Buxser et al 1981). The requirement for class II MHC products for SEB binding has been demonstrated (White et 1989). Unlike

conventional antigens , SEs do not require processing prior to their presentation by class II MHC. Metabolically inactive cells are able to present SEs to T cells while unable to present other intact protein molecules, these antigens are therefore useful for studying the role of accessory cell molecules in T cell activation.

SEB administration to neonatal mice has been shown to lead to the elimination of virtually all T cells bearing Vβ3, 8.1, 8.2, and 8.3. while in vitro, it has been demonstrated that T cells bearing these Vβs were stimulated by SEB (Callahan et al 1989). Thus SEB can stimulate a dual outcome in T lymphocytes resulting in either proliferation or death. Upon challenge with SEB, a powerful immune response occurs in vivo in mice, within 1-2 hours high serum levels of IL-2 and TNF are released. Apoptosis and/or anergy induction has been proposed to reflect exhaustive differentiation , a consequence of vigorous T cell responses in vivo (Webb and Sprent 1990). It has been shown that both $CD4^+$ and $CD8^+$ Vβ8+ T cells proliferate vigorously in vivo in response to SEB (MacDonald et al 1991). Lymph nodes from Balb C mice previously injected with SEB failed to respond to subsequent challenge by SEB in vitro as assessed by IL-2 production. These data lend weight to the observation that a differential outcome of TCR signalling by the same trigger may occur at different stages in an immune reponse depending on the maturation state of the T cell and its environment.

Interestingly, SEB induced anergy was not inhibited by anti MHC class II monoclonal antibodies (Hewitt et al 1992). The binding site for SEB lies on the side of the TCR remote from the peptide/MHC binding site. This study showed that a TCR of defined antigen specificity is able to interact with SEB in the absence of MHC class II antigens. Mutational and structural analysis of SEB suggests that the TCR and the MHC are brought into close proximity during SAg engagement (Kappler et al 1992, Swaminathan et al 1992). It has also been demonstrated that mutations within the β chain of class II MHC but not the α chain result in a unique pattern of SEB recognition by Vβ8+T cells (Deckhut et al 1994). This supports the idea that there is a functional interaction between the α chain of the TCR and the β chain of the MHC, providing further evidence for the formation of a tertiary complex between the TCR and peptide held in the groove of the MHC.

It has been proposed that TCR-CD3 down-regulation is involved in the induction of anergy (Hewitt et al 1992). Consistent with this, treatment of cells with SEB reduced TCR levels in a dose dependent manner. The capacity of the cells to increase

cytosolic free Ca2+ concentration in response to subsequent exposure to SEB was proportional to CD3 levels (Hewitt et al 1992). This proposal has been substantiated recently by the demonstration that SEB induces TCR downregulation by increasing internalisation of the TCR into endocytic vesicles (Niedergang et al 1995). The result of TCR downregulation may be the uncoupling of the TCR from the signal transduction machinery or may result in lower levels of signalling to the cell. This may be why tolerised cells can be rescued by giving them a higher threshold of second signal or by the addition of IL-2. This however does not appear to be the universal mechanism of anergy induction but may serve as a model for the investigation of downstream signalling defects in maintenance of tolerance.

SAg mediated T cell anergy may be due to the differentiation of SEB reactive virgin T cells into anergic memory cells and/or the activation and subsequent deletion of virgin T cells. Naive and memory T cells are thought to be differentially stimulated by SAgs. Memory T cells are deficient in their ability to produce IL-2 after stimulation with SEB. The activation requirements of memory T cells are more stringent than those of naive T lymphocytes. This may reflect downregulated TCR level, however other factors such as expression of CD45 isoforms, CD28 regulation and the expression of Fas/Fas-L may influence memory versus naive T cell responses.

Neonatal tolerance induction with SEB results in non-responsiveness to subsequent challenge in vivo or in vitro in wild-type MRL +/+ mice but loss of tolerance induction has been observed in *lpr/lpr* mice resulting in a proliferative response of T cells when challenged in vitro (Zhou et al 1994). The primary defect in these mice appears to be loss of tolerance rather than clonal deletion. This substantiates the hypothesis that Fas plays a role in peripheral T cell tolerance and highlights the the importance of apoptosis in maintaining immune homeostasis.

Therefore, studies with SEB have allowed more precise dissection of the fate of antigen-responsive T cells by virtue of the magnitude of the immune response induced. The need for proliferation in order for death to occur in the periphery has been observed with SEB and other antigens and is reinforced by the observation that treatment with IL-2 enhances apoptosis (Leonardo et al 1991). The interactions of both CD28 and Fas with their respective ligands are vitally important in the immune system via T cell activation and the maintenance of a fully functioning T cell response which recognises and eliminates deleterious antigens but does not respond

to and damage self antigens. It has also become apparent that life and death of a T cell are intimately associated as are the molecules that promote growth and survival responses.

1.18 Genetics of the cell survival programme.

The consistency of the morphological and biochemical pattern of apoptosis within different cell types and species during normal development and as a response to external stimuli, suggests a common cause of cellular mortality. This idea is supported by the concept of an endogenous program responsible for death and the presence of gene products that are positive and negative regulators of apoptosis.

1.18.1 Bcl-2 family : modulation of the death signal

Bcl-2 provides the strongest evidence for a shared mammalian pathway of cell death by its ability to block a wide variety of cell death models. The bcl-2 gene was discovered as a result of its translocation to the H chain gene locus in most human follicular B cell lymphomas (Tsujimoto and Croce 1986). The survival of quiescent lymphocytes in the mouse is dependent on the expression of the bcl-2 gene (Nakayama et al 1993). Animals deficient in the bcl-2 gene have T cells with an increased susceptibility to undergo PCD when placed in culture. Bcl-2 expression is topographically restricted to the tissues characterised by apoptotic cell death and has been shown to prevent apoptosis via an antioxidant pathway at sites of oxygen free radical generation (Hockenberry et al 1990,1993). Excess production of reactive oxygen intermediates results in DNA strand breakage and membrane blebbing which match some of the hallmarks of apoptosis.

Bcl-2 exists as part of a higher molecular weight complex associating with a 21kd protein partner bax (Oltvai et al 1993). Bax shows extensive homology with bcl-2 forming homodimers and heterodimers with bcl-2 in vivo. When bax is in excess cell death is accelerated and the death repressor activity of bcl-2 is countered, but when bcl-2 is in excess cell death is repressed. Bax is encoded by six exons and demonstrates a complex pattern of alternative RNA splicing that predicts a 21kd

membrane and two forms of cytosolic proteins. The complexity of the RNA splicing pattern regulates bax activity and localisation.

Another bcl-2 related gene, bcl-x has also been identified (Boise et al 1993). This gene makes protein products that either prevent or cause cell death depending on how the mRNA is processed. A larger transcript (bcl_{xl}) encodes a bcl-2 related protein product that can inhibit cell death induced by growth factor withdrawal when over-expressed in an IL-3 dependent cell line. Alternative splicing of bcl-x transcripts can generate a smaller transcript bcl-xs, that encodes an internal truncated version of Bcl-x protein that prevents overexpression of bcl-2 from inducing resistance to apoptosis. Thus a clear picture of a family of bcl-2 like genes is emerging. These genes are likely to be sequential members of a single death/survival pathway or regulators of parallel pathways.

The dynamic bcl-2 -bax pattern is reminiscent of that observed with the Myc family of proteins. The c-myc protein is known to associate with several intracellular proteins including Max (Blackwood and Eisenman 1991). The c-myc proto-oncogene product has been implicated in cell cycle progression (Green et al 1994b) and has also been shown to promote apoptotic death (Evan et al 1992). Ligation of the TCR on T cell hybridomas resulting in apoptosis is inhibited by the addition of anti-sense oligodeoxynucleotides which inhibit c-myc expression. (Green et al 1994b) In these proteins varied dimerisations between the partners determine the final activity of the myc-max complex. Importantly it has been shown that a mutant interfering with the function of the myc-max heterodimer inhibits the induction of apoptosis by TCR ligation. (Green et al 1994b) This similarity between bcl-2 bax and myc-max indicates that competing dimerisation among similar proteins may be a common mechanism for regulating cellular survival and growth.

With regard to Fas, transfectants expressing this antigen alone were killed by anti-Fas antibody, while overexpression of bcl-2 resulted in a partial inhibition of the Fas-mediated death, suggesting that the regulated expression of bcl-2 and Fas may control the PCD (Itoh et al 1993b).In addition bcl-xl has been demonstrated to repress cell death and functions with bcl-2 in a common pathway to regulate cell death (Chao et al 1995). In summary, it appears that the bcl-2 family of genes plays an important role in the regulation of T cell survival during an immune response and the interplay of death promoting and death enhancing factors may control the outcome of T cell activation.

1.18.2 p53 regulation of cell survival

Cell death can be developmentally controlled by the expression of novel genes that induce the death signal at a specific stage of differentiation in response to defined physiological stimuli. Gene products may exert their effect through either repression of apoptosis or failure to correctly induce the cell death program. Wild type p53 appears to function as an oncosuppressor by leading to inhibition of G1 cyclin-dependent kinases, thereby halting cell cycle progression. Under conditions where DNA repair is necessary , the cell is blocked in the G1 phase allowing time for repair to reach completion prior to cell division. If irreparable DNA damage exists, the cell becomes committed to the apoptosis pathway and is deleted from the system. Mutant p53 is an anti-apoptotic oncoprotein which may allow escape from this surveillance mechanism and generation of a malignant phenotype.

1.18.3 The involvement of the transcription factor nur 77 in apoptosis

Elucidation of some of the genetic elements involved in T cell function has been aided by the use of CsA, a potent immunosuppressive agent used clinically to control allograft rejection, graft versus host disease and some autoimmune diseases. CsA is known to block a Ca^{2+} mediated T cell activation pathway and has also been demonstrated to inhibit anergy. The prinicipal inhibitory reaction of CsA is the result of impaired lymphokine production by T cells, especially IL-2. Anti-CD3-induced PCD in the thymus has been blocked by CsA (Shi et al 1989) while recent data suggest that CsA may enhance apoptosis in mature antigen-reactive T cells (Vanier and Prud'homme 1992). This suggests that there are differential requirements for calcium signalling in the thymus compared to the periphery. Low doses of CsA inhibit anergy induction while higher doses were needed to enhance T cell deletion in mice injected with SEB (Vanier and Prud'homme 1992). The mechanism by which CsA can interfere with anergy is not clear, however it is known that CsA blocks T cell activation by binding to cyclophilin which inhibits the ability of calcineurin to dephosphorylate its substrate, NF-AT.

CsA blocks apoptosis by inhibiting the TCR- mediated activation of nur 77 transcription factor in T cells. This effect is mediated by blocking the DNA binding activity of the nur 77 protein rather than its de novo synthesis (Yazdankakhsh 1995). Nur 77 is a member of the steroid/thyroid receptor superfamily and has been shown to be necessary for the induction of apoptosis in T cell hybridomas (Woronicz et al 1994). Apoptotic signals delivered through the TCR in immature T cells and a T cell hybridoma also require the expression of nur 77 (Liu et al 1994).

Interestingly nur 77 knockout mice have been generated and apoptosis been demonstrated to be normal in these mice (Lee et al 1995). Recently a new gene has been cloned from an apoptotic cell line (Okabe et al 1995). This gene is called Transcriptionally inducible nuclear receptor (TINUR). and expression was induced by cross-linking the TCR complex. TINUR has the characteristics of an immediate early gene,was found to be a member of the NGF1-B/nur 77 family and to bind the same DNA sequence as nur 77. Consequently, defects in this gene rather than in nur 77 may influence apoptotic death and the existence of this gene may explain the normal apoptosis found in nur 77 knock out mice.

Therefore factors which are involved in transcription may also plays a role in T cell apoptosis leading to the conclusion that T cell activation and apoptosis are intimately linked at the nuclear level resulting in the intrinsic capability of a cell to decide between life and death.

1.19 The involvement of cysteine proteases in PCD.

One of the approaches that has provided the most insight into the mechanism of cell death has been the analysis of the nematode Caenorhabditis elegans. During normal development, certain cells of this worm are predestined to die, of the 1090 somatic cells formed during the development of this organism 131 undergo PCD (Wang et al 1994). Two genes ced-3 and ced-4 are required for this PCD. Ced-3 shares functional and sequence similarity with a mammalian cysteine protease, IL-1b- converting enzyme (ICE) (Yuan et al 1993). Both ICE and ced-3 contain the conserved pentapeptide sequence QACRG surrounding a cysteine residue that is known to be essential for ICE function. ICE is a cysteine protease that converts the IL-1b precursor to the active protein (Yuan et al 1993, Thornberry et al 1992). Transfection

of the gene for ICE or ced-3 into rat fibroblasts has been shown to induce PCD (Miura et al 1993).

Another member of the ICE family was isolated recently (Wang et al 1994). This gene was originally identified from a cDNA expressed mostly during early mouse embryonic development and down-regulated in adult brain and called NEDD-2 (Kumar et al 1992). It was renamed Ice and ced-3 homologue (Ich-1) and mRNA for this gene was found to be alternatively spliced into two different forms. One form Ich-1L contains sequence homology to ICE and ced-3, whereas the other species named Ich-1s, terminates 21 amino acids after the pentapeptide QARCG. Expression of these two species was found to have opposite effects on cell death. Overexpression of Ich-1L induces PCD while overexpression of Ich-1S suppressed cell death induced by serum deprivation. These results suggested that Ich-1 plays an important role in positive and negative regulation of PCD in vertebrates. The control of RNA splicing seen here is reminiscent of that seen in the bcl-x and myc systems, this may reflect the use of alternative gene splicing as a general mechanism for regulation of PCD.

Ced-9, a negative regulator of ced-3 and ced-4, has also been found, which is structurally and functionally homologous to mammalian bcl-2. Human bcl-2 is able to function in cells from worms and insects as well as mammals. The fate of eukaryotic cells may therefore reside in the balance between the opposing pro-apoptotic effects of an ICE/CED-3 protease and an upstream regulating bcl-2 and its homologues.

One of the substrates for an ICE/CED-3 protease during apoptosis is poly (ADP-ribose) polymerase (PARP), an enzyme that appears to be involved in DNA repair, genome surveillance and integrity. The protease (apopain) that is responsible for cleavage of PARP has been identified and shown to be derived from a proenzyme, identified as CPP.32 (Nicholson et al 1995). This proenzyme is related to ICE and CED-3. The Ca2+ endonuclease that is implicated in the characteristic internucleosomal cleavage of apoptosis has been shown to be regulated by poly ADP-ribosylation. Inhibition of apopain mediated PARP cleavage attenuates apoptosis in vitro demonstrating the importance of this protease in apoptosis. Sequence relationship between CPP-32 and CED-3 suggests that CPP-32 may be the human equivalent of CED-3. Cowpox virus encodes a gene crm A that encodes a serine protease inhibitor that can bind to and inactivate ICE (Ray et al 1992).A similar protease has also been identified (Tewari et al 1995), Yama/CPP-32 , which shows

far greater sensitivity to crm A than apopain. Expression of crmA can prevent apoptosis induced by ICE (Miura et al 1993). Expression of a baculovirus gene p35 prevents PCD in C. elegans, mammals and Drosophila (Hengartner 1994). This again suggests the universality of a death program and hints at the evolutionary development of a cell survival program in order for multi-cellular organisms to maintain homeostasis.

1.20 Aim of this project.

It has become apparent that TCR interactions alone are not sufficient to allow T cell proliferation , therefore an understanding of the molecular interactions generated during a T cell response is of considerable interest. Consequently, one aim of this project was to establish some of the costimulation requirements of T cell activation. Previous work has indicated that cell-cell interactions may be critical to T cell function, therefore to address this issue the relative roles of the interactions of LFA3 and B7-1 with their respective ligands CD2 and CD28 was examined. Much use has been made of antibodies to CD28 and CD2 to investigate the roles played by these molecules in costimulating an immune response. A much more physiological approach and,one which will be used here is the use of specific ligands such as LFA3 and B7-1 to trigger CD2 and CD28 respectively. Transfectants expressing either LFA3 or B7-1 have been established which will be used to study the costimulation requirements of T lymphocytes. In addition to proliferation , the costimulation required for IL-2 production will also be examined, again using LFA3 and B7-1 transfectants in order to establish the interactions necessary for production of this cytokine.

Previously, the exact function of APCs in T cell stimulation has not been fully elucidated and so it was interesting to examine the role of presentation in T cell activation using the SAg SEB, presented by transfectants expressing MHC class II HLA DR4 molecules. HLA-DRtransfectants co-expressing MHC class II and either B7-1 or LFA3 were utilised to study the requirement for T cell costimulation. One of the criteria proposed to test the ability of a molecule to costimulate T cell responses is its capability to costimulate from a cell distinct from the one providing the TCR stimulus (third party costimulation). Both B7-1 and LFA3 were tested for this ability using SEB as the TCR stimulus.

The factors which influence the outcome of TCR engagement are poorly understood, in addition to proliferation , engagement of the TCR may result in anergy and /or apoptosis. In order to investigate the effects of negative encounters with antigen, T cell apoptosis was studied. In addition the signals required to turn a potentially apoptotic stimulus into a signal to proliferate were examined as was the role played by APCs.

It is known that Fas ligation results in apoptotic death of T lymphocytes. However, the expression of Fas-L during the death process has not been examined. Hence, one question to be addressed is the regulation of Fas-L expression during AICD and what factors modulate this expression. The significance of the role played by Fas-L expression was also an attractive focus of study.

In summary the main questions to be addressed are , the role of costimulation during T cell encounters with antigen, the function of APCs during T cell activation, what determines negative outcomes of T cell ancounters with antigen, such as apoptosis, and the role of Fas/Fas-L expression during T cell apoptosis.

The underlying hypothesis is that engagement of the TCR results in different outcomes and the question to be addressed is what controls these outcomes. The results of triggering the TCR was the central subject of this thesis. The fate of a cell upon engagement of the TCR was investigated in order to elucidate what determines the decision of life and death of a T lymphocyte.

CHAPTER TWO.

MATERIALS AND METHODS.

2.1 MATERIALS

2.1.1 Preparation of solutions and tissue culture reagents

Glu-Free medium for CHO transfectants

To 400ml of autoclaved distilled deionised H_2O, the following were added;

DMEM (10X, Gibco BRL 12501-011)	55ml
FCS	50ml
Penicillin /streptomycin (Gibco BRL 15140-114) (10000IU/ml)/10000µg/ml	5ml
Sodium bicarbonate (Gibco BRL 25080-060)	28ml
Sodium pyruvate (Gibco BRL 31870-025)	5ml
Nucleosides (100X)	5ml

RPMI/10% FCS.

The following were added to 400ml of RPMI (Gibco BRL 31870-025),

FCS	50ml
Penicillin/Streptomycin	5ml
Glutamine (200mM, Gibco BRL 25030-024)	5ml

Nucleosides (all from Sigma)

0.34mg/ml thymidine	(T-1895)
0.7mg/ml guanosine	(G-6264)
0.7mg/ml adenosine	(A-4036)
0.7mg/ml cytidine	(C-4654)

The nucleosides listed were made up to 50 mls using filter-sterilised H_2O and used at 1:100 dilution.

L-Methionine sulfoxamine (Sigma M-5379)

Stock 18mg/ml was made in Glu-Free medium and used at 1:100 dilution.

Hygromycin (Sigma H-7772)
stock at 25mg/ml in PBS and used at 1:100 dilution.

Phosphate buffered saline (PBS, Oxoid).
5 tablet s were added to 500ml distilled deionised H_2O and autoclaved.

Glutaraldehyde (Sigma G-6257)
25% aqueous solution
used at 1:1000 = 0.025%.

G418, Geneticin G418-sulphate (Gibco-BRL 1181-031)
Stock 50mg/ml in PBS, used at 1:100 dilution.

Tris acetate EDTA (TAE 10X)

Tris base	24.22g (0.4M)
Sodium acetate,	2.05g (0.05M)
NA_2 EDTA	1.86 g (0.01M)

these reagents were made up to 500ml using deionised distilled H_2O,

Diethyl Pyrocarbonate (DEPC) -treated H_2O,
500µl of DEPC was added to 500ml dH_2O, mixed and left at room temperature overnight, followed by autoclaving.

Solution D (RNA extraction buffer)

Guanidinium thiocyanate (Sigma G-6639)	250g
0.75M Sodium Citrate pH7.0	17.6ml
10% (w/v) N- laurylsarcosine(sodium salt)	26.4ml

The above were dissolved in 293 ml sterile DEPC-treated water at 65°C and stored at 4°C. For use, solution D was prepared by adding 72µl ß mercaptoethanol to 10ml stock solution and stored for up to a week at 4°C.

Prehybridisation solution

20X SSPE	25ml (5X)
50X Denhardts	2.5 ml (5X)
10%(w/v) SDS	1.25ml (0.5%)
10mg/ml Salmon sperm DNA	0.5ml 20μg/ml
Distilled H_2O	14.5ml

SSPE was made using NaCl (175.3g), NaH_2PO_4 (27.6g) and Na_2 EDTA (7.4g) and making volume up to 1000ml using distilled H_2O.

Salmon sperm DNA was prepared in distilled H_2O by shearing several times through a 19-gauge needle, followed by denaturation by boiling for 10 minutes. The solution was cooled on ice for 2-3 minutes and could be stored at -20°C until required for use.

50X Denhardt's solution was prepared by addition of 1%w/v ficoll, 1% w/v PVP and 1%w/v BSA to distilled deionised H_2O,followed by filter sterilisation and storage in aliquots at -20C until required (see Maniatis).

SSC (20X)

The following were mixed together,

NaCl	175.3
Na Citrate	88.2
H_2O	800ml

pH was adjusted to 7.0 using HCl and final volume made up to 1000ml using H_2O.

2.2 Suppliers of Reagents

Most laboratory reagents were supplied by Sigma (Poole, Dorset) , however a list of other suppliers is given below.

2.2.1 Suppliers.

Tissue Culture Reagents

The following reagents were all purchased form Gibco BRL (Paisley, UK).

RPMI 1640,
Dulbecco's Minimal Essential Medium (DMEM),
Iscove's medium,
AIM-V serum free medium,
Heat -inactivated foetal calf serum (FCS),
penicillin-streptomycin,
L-glutamine,
trypsin-EDTA

Phosphate-buffered saline tablets Oxoid (Bristol, UK)

Lymphoprep Nycomed Ltd. (Birmingham, UK)

Heparin (Monoparin) CP Pharmaceuticals Ltd. (Wrexham, UK).

Hygromycin and ionomycin Calbiochem (Nottingham U.K).

rIL-2 Glaxo.

CsA was a generous gift from Sandoz.

Mycoplasma ELISA kit Boehringer Mannheim (Lewes, U.K.).

SEB Sigma (S-4881)

Molecular biology reagents

M-MLV reverse transcriptase GIBCO (Paisley UK)

Taq DNA polymerase NBL, Promega Ltd (Southampton UK).

pd(T)12-18, RNAguard RNase inhibitor and an Ultrapure dNTP set Pharmacia Biosystems Ltd.

λ HIII DNA markers Gibco BRL
pX174 DNA markers NBL

Water-saturated phenol (AquaPhenol) Appligene (Co. Durham, UK).

Biodyne B was supplied by Pall (UK)

DE81 discs and chromatography paper (3MM) Whatman Scientific (Maidstone, UK).

Gene Clean II Kit Bio 101 (Stratech)

Prime-a-gene kit. from Promega

Radiochemicals

[3H] thymidine and [51 Cr] Amersham International plc (Amersham UK).

[α-32P] dATP ICN Flow (High Wycombe, UK).

Optiscint HiSafe from Wallac Ltd. (Milton Keynes, UK).

2.2.2 Suppliers: contact numbers.

The following list is not comprehensive but gives telephone numbers of suppliers most often used for laboratory reagents.

Amersham International plc (Aylesbury, UK)	0800 515 313
Boehringer Mannheim UK (Lewes UK)	0800 521 578
Calbiochem Novabiochem UK Ltd(Nottingham, UK)	0800 622 935
Dynal UK Ltd (Bromborough UK)	0151 346 1234
Gibco BRL Life Technologies LTd (Paisley, UK)	0141 814 6100
Hybaid Ltd (Middlesax, UK)	0181 614 1000
NEN Dupont (Stevenage, UK)	01438 734 865
NBL Northumbria Biologics Ltd (Northumberland UK)	01670 739 297
Nycomed UK Ltd (Birmingham, UK)	0121 742 2444
Pharmacia Biotech Ltd (St Albans, UK)	01727 814 000
Promega Ltd (Southampton, UK)	01703 760 225
Sigma (Poole, UK)	01202 733114
Stratagene Ltd (Cambridge, UK)	0800 585 370
Whatman Labsales Ltd (Maidstone, UK)	01622 692022

2.3 Methods.

2.3.1 Monoclonal antibodies used.

The following antibodies were used as either staining reagents or blocking reagents at 10μg/ml.
OKT11 (anti-CD2), OKT3 (anti-CD3), L243 (anti HLA-DR), TS2/9 (anti-CD58), HB8784 (anti-CD25), all from the ATCC (Rockville, MD, U.S.A.); 9.3 (anti-CD28) obtained from Dr P Linsley (Bristol-Myers Squibb, WA, U.S.A.); UCHT1 (anti-CD3) and UCHM1(anti-CD14) received from Dr P Beverly (ICRF, London , U.K.); BB1 (anti- B7-1) obtained from Dr E. Clark (University of Washington, WA, U.S.A.); 15.2 (anti CD54) and 38 (anti-LFA1) received from Dr N. Hogg (ICRF, London, U.K.); BU12 (anti-CD19) obtained from Dr I. McLennon (University of Birmingham, U.K.); CH11 (anti-Fas, TCS Biologicals, U.K.); M3 (anti-Fas) received from Dr D.Lynch (Immunex, WA U.S.A.) was used at 3μg/ml.

2.3.2 Maintenance of cells.

All cells were cultured at 37°C and 5% CO_2 in a humidified incubator while manipulations were carried out in a sterile environment using a laminar flow hood.

Transfectants

Chinese hamster ovary (CHO) K1 cells were transfected with human cDNAs encoding LFA3, B7-1 and HLA DRA and HLA DRB1 DR4/DR B1 * 0401 as described previously (Sansom et al 1993). Double transfectants were also made co-expressing HLA-DR and B7-1(DR4/B7-1) and triple transfectants expressing DR , B7-1 and LFA3 (DR4/LFA3/B7-1).

Cells were routinely screened and sorted for expression of cell surface molecules by FACS analysis and were tested for mycoplasma using a commercially available ELISA detection system (Boehringer Mannheim). Transfectants were comparable for levels of expression of surface molecules.

Transfectants were routinely grown in Glu-Free medium. Selection of LFA3 was based on G418 at 500ng/ml. DR4/B7-1 and DR4/LFA3/B7-1 were grown under selection with hygromycin at 250µg/ml. L-methionine sulfoxamine was used to select for expression of Class II on DR positive transfectants.

Removal of CHOs from plastic culture flasks was carried out as follows: medium was aspirated from flasks and cells washed with PBS. Having aspirated the PBS, trypsin-EDTA was added (Gibco BRL 45300-027, 2ml to a 75cm^2 flask or 3ml to a 125cm^2 flask) and incubated at 37^{o}C for 5 minutes. When the cells were detached medium was added to a final volume of 10ml and excess cells removed from the flask. Fresh medium and appropriate selection were added. Transfectants were normally passaged every two to three days or when cells became confluent.

T cell cultures.

Jurkat cells were grown in a 1:1 mixture of RPMI:Iscoves (10% FCS, Glutamine, Pen/Strep) and were passaged every two to three days. To ensure optimal growth cells were kept below 1 X10 ^{6} /ml.

PBMCs and purified T cells were cultured in RPMI containing 10% FCS . T cell lines were cultured in above medium or AIM V -serum free medium and supplemented with recombinant IL-2 (rIL-2 at 20 International units (IU) /ml) every 2-3 days as necessary.

2.3.3 Purification of PBMCs and T cells from blood.

All blood samples were taken from healthy volunteers. Heparinised blood was separated on a Lymphoprep (1.077) density gradient by centrifugation at 420g for 30 minutes. The layer of mononuclear cells recovered from the gradient, which constitutes Peripheral Blood Mononuclear Cells (PBMCs), was washed three times in RPMI/10% FCS.

To purify T lymphocytes from PBMCs, cells were allowed to adhere to plastic petri-dishes for 1 hour at 37^{o}C in RPMI/10% FCS. Non-adherent cells which include T cells were pipetted off into a fresh tube. These cells were incubated with mAbs to monocytes (UCHM1, anti-CD14, 500µl 10µg/ml), B cells (BU12, anti-CD19, 1:10

dilution of ascites) and activated T cells (L243, anti-HLA-DR, 500µl 10µg/ml) for 1 hour at 4°C on a rotator. Having been washed with RPMI/10% FCS, the labelled cells were incubated with sheep anti-mouse IgG coated magnetic beads for 1 hour at 4°C and then removed using a magnet (Dynal). The resulting T cell population was greater than 95% CD3 positive as assessed by FACS analysis.

2.3.4 Production of SEB responsive T cell lines.

T cell lines were established by incubating PBMCs with 1-5µg/ml SEB for 4 days followed by addition of rIL-2 (20IU/ml). In order to remove dead cells and debris, cells were layered onto a lymphoprep gradient, centrifuged and live cells recovered. These were restimulated every 12-14 days using SEB pulsed DR4/B7-1 transfectants as APCs (see below) added at a ratio of 3:1 (T cells :transfectants). Cell lines were maintained for 8-10 weeks. The specificity of these cells was tested by determining reponses to SEB using a proliferation assay.

2.3.5. Preparation of transfectants for use in proliferation assays

Transfectants were trypsinised, counted and washed twice with PBS to remove traces of FCS which interferes with the fixative. Cells were fixed for 2 minutes with 0.025% (v/v) glutaraldehyde (Sigma G-6257) in PBS. This was followed by washing twice in FCS containing medium to remove excess glutaraldehyde. MHC class II expressing cells to be used as APCs were trypsinised and counted. These cells were incubated with SEB at appropriate concentration in medium at 37°C for 3-4 hours followed by washing with PBS as above and fixation with glutaraldehyde.

2.3.6 Proliferation assay

2.3.6.1 Principle of assay.

As a cell divides it progresses through the cell cycle, duplicates its DNA and divides by mitosis. Addition of a radiolabelled nucleotide to the culture which is then incorporated into the cells as the DNA is replicated, can indicate whether the cells are dividing or not. Having washed away unincorporated radiolabel, these cultures are

then harvested on to glass-fibre filter-mats where the radiolabelled DNA is trapped. Incorporation of radioactivity is measured using a β counter. Proliferation was therefore evaluated by measuring tritiated thymidine incorporation of dividing cells.

2.3.6.2 Assay.

Cell counting was carried out using a haemocytometer. T cells (5 $X10^4$) and APCs (2.5 $X10^4$) were dispensed into flat bottomed 96 well microtitre plates in a final volume of 100µl. Plates were incubated at 37°C usually for 3 days or as indicated for individual experiments. Tritiated thymidine incorporation was measured at indicated times by adding 1µCi of tritiated thymidine(5 Ci/mmol specific activity in RPMI/10% FCS) for the last 8-18 hours of culture followed by harvesting onto filtermats (Skatron) using a semi-automatic Skatron Cell harvester and counted by liquid scintillation counting using a β counter. The results are expressed as mean counts per minute (cpm) for triplicate cultures.

2.3.7 Cryogenic storage of cell stocks.

In order to maintain stocks all cells used were routinely frozen. Having been passaged, cells were washed in medium and resuspended in 20% FCS, 10% DMSO, which acts as a cell preservative. Cells were aliquoted into freezing vials (Nunc) at ($1x10^6$/ml). Vials were placed at -80°C, which allows cells to freeze slowly (at approximately 1°C per minute) followed by storage at -190°C in liquid nitrogen.

To recover from frozen, cells were brought to 37°C by immersing under warm running water. This must be done as quickly as possible to prevent the formation of ice crystals in the cells which could lead to cell lysis and death. Cells were washed in FCS containing medium, resuspended in the appropriate medium and placed into culture.

2.3.8 Bioassay for IL-2 using CTLL-2.

2.3.8.1 Principle of assay

The ability of T cells to produce IL-2 was measured by taking advantage of a murine cytotoxic T cell line, CTLL-2, which depends on IL-2 for its survival. This cell line can only proliferate in response to exogenous IL-2. Measurement of the proliferation responses of CTLLs using tritiated thymidine incorporation was used to evaluate the amount of IL-2 produced in a given set of culture conditions. Supernatants were harvested from T cell cultures and used as sources of IL-2 in this CTLL-2 assay. In order to determine the amount of IL-2 in the supernatants a standard curve of titrated IL-2 concentration was determined and IL-2 measurements of sample supernatants were extrapolated from this.

2.3.8.2 Assay

CTLL-2 cells were routinely cultured in RPMI/10% FCS supplemented with human rIL-2 (10-15 IU/ml) in upright 75cm^2 flasks. Cells used for the bioassay were taken three days after feeding and washed twice with complete medium by centrifuging the cells at 420g for 5 minutes. The bioassay was only performed using cells which were > 80% viable. Viability was determined by staining cells with the exclusion dye Trypan Blue which is only taken up by dead cells. Live cells exclude it and can then be counted. Percentage viability was calculated as follows

$$\frac{\text{Number of cells excluding dye}}{\text{Total number of cells}} \times 100 = \% \text{ viability.}$$

Cells were resuspended in RPMI/10%FCS to a final concentration of 1 X10^5 cells/ml and aliquoted at 5 X 10^3 /well in 50 µl volume. A titration of IL-2 standard (NIBSC) was carried out using serial twofold dilutions starting at 20IU/ml down to 0.019 IU/ml. Test samples were also diluted (usually by two or ten fold) in triplicate. The negative control was culture medium without exogenous IL-2. Each of the controls and samples were aliquoted in triplicate (50µl) in 96 well microtitre round bottomed plates. Assay was incubated at 37^{o}C for 18 hours. Proliferation was measured by adding 0.5µCi of tritiated thymidine to each well for 4 hours. The cells were harvested on to glass fibre filtermats and counted by liquid scintillation counting in a ß counter.

2.3.9 IL-2 ELISA.

A solid phase Enzyme Immunoassay (Medgenix) was used to quantitate IL-2 in culture supernatants from proliferation assays. Several standards are provided from which a standard curve of concentration of IL-2 versus O.D. 450/490 was generated. These standards in IU/ml are as follows: 0, 0.9, 2.3, 7.5, 15, 30.

The assay was carried out according to manufacturer's instruction in duplicate wells, 100µl of each standard or sample being dispensed in the appropriate well of a microtitre plate coated with IL-2 antibody. The absorbance at 450nm and at 490nm was read and a standard plot generated from which amounts of IL-2 in sample supernatants were extrapolated.

2.3.10 ^{51}Cr release cytotoxicity assay ; bioassay for determining Fas-L expression.

2.3.10.1 Principle of assay

This assay is based on the principle that live cells incorporate ^{51}Cr labelled sodium chromate, which are used as target cells. The chromate becomes protein bound and converts to dichromate. ^{51}Cr release reflects protein leakage from dying cells and released dichromate cannot be retaken up by surviving live cells. When cytotoxic killing occurs the target cells are lysed and release the radiolabel into their culture medium which can be harvested and counted. This gives a measurement of % cytotoxicity when compared to cells induced to release all their label which is set at 100% cytotoxicity.

2.3.10.2 Method

Fas sensitive Jurkat target cells were counted (5 X10^6), washed, resuspended in FCS containing medium and labelled with 100µl of 1mCi Na^{51} Cr in 500µl for 2 hrs at 37^{o}C. Cells were washed three times in medium followed by incubation for 30 mins in medium at 37^{o}C to allow any further chromium leach out into medium which was then washed off. Labelled cells were used at 5x10^4 /well in a 96 well round bottomed plate. Effector T cells (either unstimulated or stimulated with PMA and ionomycin or with SEB) were incubated with the labelled Jurkats for 16-18 hours at 37^{o}c. M3, anti-

Fas blocking antibody was used at 3μg/ml and the Fas-Fc was used at 1/10 dilution. The ^{51}Cr release results are for triplicate cultures.

The % specific release was calculated as follows:

$$\% \text{ killing} = \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

Maximum release was determined by measuring the radioactivity released by adding NP40 (final concentration 1% in a final volume of 200μl) to the labelled cells. Spontaneous release was cpm measured from target cells alone in the absence of effectors.

2.3.11 FACS analysis.

2.3.11.1 Principle

The principle of flow cytometry is that a single cell suspension passes through a light beam emitted by a laser source. This light excites the fluorescent dyes used to label the cells leading to light amplification. Scattered or fluorescent light is collected. List mode data was collected for Forward Scatter (FSC), Side Scatter (SSC), fluorescence (FL)1 and (FL)2. Prior to data collection the nozzle head through which the labelled cells pass was aligned to maximise the detection of fluorescent events using a mixture of two latex beads coated with FITC of different intensities (Polysciences).

Analysis was carried out on a Becton Dickinson FACStar-plus equipped with a 100mW 488nm air cooled laser and a Consort 32 computer. In addition for Hoechst staining a Becton Dickinson FACs Vantage equipped with a 50mW UV was used.

2.3.12 Analysis of expression of cell surface molecules.

Transfectants were trypsinised, washed and resuspended in primary antibody (10μg/ml, 50μl/1x10^6 cells). PBMCs and T cells were washed in complete medium

and resuspended in antibody as above. Cells were incubated with the primary antibody at 4°C for 45 minutes, washed with an excess of PBS, centrifuged at 420g and resuspended in secondary antibody, fluorescein isothiocyanate (FITC) conjugated anti-mouse polyvalent immunoglobulin, 1/50 dilution, (Sigma F-1010, 100µl). After 45 minutes incubation at 4°C the cells were washed as above and resuspended in PBS, then analysed. Alternatively, cells were fixed with 1% paraformadehyde/1% BSA and stored at 4 °C for up to one week prior to flow cytometry.

2.3.13 Analysis of DNA content for determination of apoptotic cells.

2.3.13.1 Principle

Morphological and biochemical analyses of apoptotic cell death have demonstrated a delayed loss of membrane integrity, accompanied by extensive degradation of DNA. In addition to loss of DNA, chromatin condensation occurs both of which can be used as a measure of cell death by analysing the DNA content of cells.

A quiescent cell which is not involved in cell division is often referred to as being in the Go state. If division is triggered, the cell first enters the G1 phase of the cell cycle, then the cell starts to synthesise new DNA during the (S) phase of the cell cycle until the DNA content has doubled. At this point DNA synthesis ceases and the cell is in G2 phase. Finally, the cell will enter mitosis (M) and divide. Cells in G2 and M phases of the cell cycle have double the DNA content of those in G0 and G1. Cells in S phase will have a DNA content lying between these extremes. Decreased uptake of the dye Propidium Iodide (PI), when used on fixed cells is an indication of apoptotic death when analysis of the cell cycle is determined. Measurement of the number of cells with reduced PI staining in the sub-G0 region will represent the % of apoptotic cells induced by a particular treatment. If the DNA of 10,000 cells is measured and a histogram of number of cells against DNA content is plotted, this will reflect the state of the cell cycle (Figure 2.1). The appearance of cells with DNA content lower than that of G1 cells (subGo/G1 peak) in cultures treated to induce death, has been considered to be a marker of cell death by apoptosis. This reduced stainability represents both an decrease in DNA content (as a result of activation of the apoptosis specific endogenous endonuclease) and a change in conformation and accessibility to the dye (due to nuclear chromatin condensation).

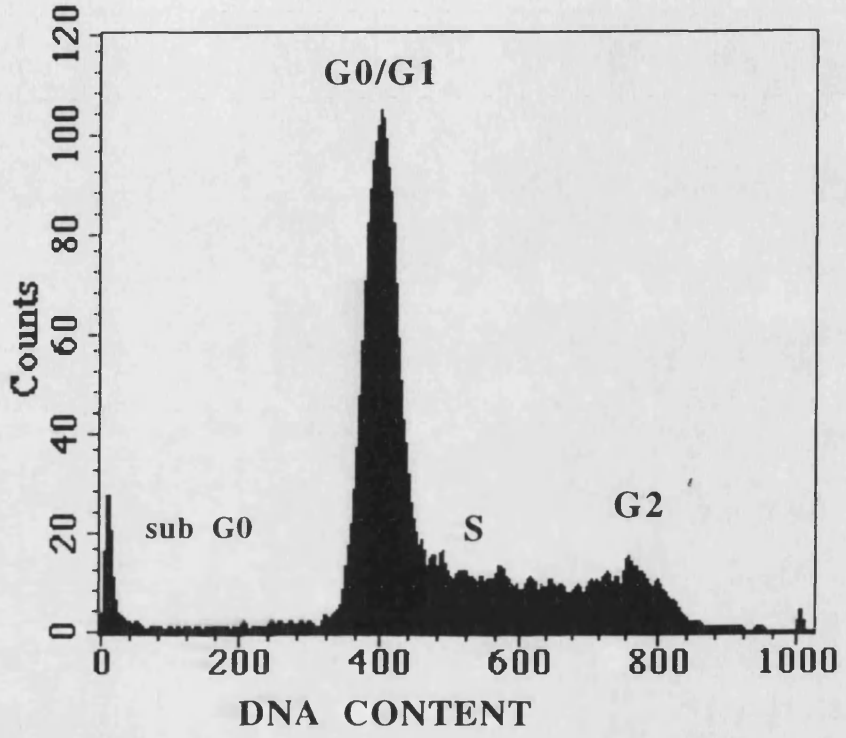


Figure 2.1 Diagram illustrating DNA histogram of the cell cycle.
The DNA content of cells can be determined by using a dye such as propidium iodide which binds to nucleic acid and whose fluorescence is enhanced on binding. Cells in the G0/G1 region are resting or have just been triggered to enter the cycle and begin cell division, while cells in S or DNA synthetic phase are making new DNA. During this part of the cell cycle the DNA content of the cell increases until it has doubled, when this happens synthesis ends and the cell is in G2 phase. Cells in G2 therefore have double the DNA content of those in G1 or G0 phase. When the DNA content of a given number of cells is measured by FACS analysis and a histogram of cell number against DNA content is plotted, this reflects the state of the cell cycle. Cells in the subG0 phase contain fragmented and condensed DNA due to apoptosis, thus binding reduced amounts of the DNA binding dye. Therefore, a measure of apoptotic cell death can be achieved by determining the number of cells in this region using FACS analysis.

2.3.13.2 Assay

Briefly, apoptosis treatment was carried out by incubating cells with PHA (generally, 5μg/ml) or SEB (generally, 1μg/ml) or anti-Fas antibody (CH11 0.5μg/ml) for appropriate time. Cells were washed in PBS, centrifuged at 420g and fixed in 80% EtOH in PBS at -20°C for times indicated. Cells were washed as before, resuspended in PBS (1ml) to which RNase A was added (final concentration 20μg/ml) and incubated at 37°C for 30 minutes in an incubator. Propidium iodide was added to cells (to final conc 10μg/ml) and incubated at room temperature for 15 minutes. Cells were given a final wash in PBS and stored in PBS at 4°C for up to one week prior to analysis.

2.3.14 Analysis of apoptotic cells using Hoechst 33342.(Dive et al 1992)

In addition to propidium iodide staining apoptotic cell death was determined by staining cells using the fluorochrome Hoechst 33342 (Ho33342). This dye is increasingly taken up as the amount of apoptosis increases therefore an increase in dye absorbance reflects increasing death.

A stock solution of Ho33342 (1mM in distilled water) was freshly prepared for each experiment. HO33342 and propidium iodide were added to single cell suspensions such that their respective final concentrations were 10μM and 32μM. Ho33342 was added immediately prior to propidium iodide. Propidium iodide was used to discriminate between necrotic and apoptotic cell death. 10,000 cells were analysed using a FACS with UV laser excitation (100mW). Blue fluoresence (FL4 Ho33342 staining) and red fluoresence (FL2, propidium iodide staining) were measured for each cell analysed with a flow rate of about 500 cells / second. Cell debris was excluded by gating.

2.3.15 Agarose Gel Electrophoresis.

The standard method used to separate, identify and purify DNA fragments is electrophoresis through agarose gels. Horizontal submarine gel electrophoresis was used in this study for the analysis of both DNA and RNA.

Agarose gels are made using TAE (section 2.1), heated in a microwave until melted and allowed to cool to approximately 60°C, when ethidium bromide was added to give a final concentration of 0.5µg/ml., after which gel was poured into mold. Ethidium bromide contains a planar group which intercalates betweeen stacked bases of single- or double-stranded DNA or RNA causing the fluorescent intensity of the dye to increase, allowing visualisation of the DNA or RNA under a UV light source.The gel was then run in 1X TAE buffer, DNA samples loaded in DNA loading dye (15% w/v Ficoll, 0.25% w/v xylene cyanol, 0.25% w/v bromophenol blue. The samples were electrophoresed at 100V, until the bromophenol blue dye was half/three-quarters of the way down the gel. The DNA was visualised using a UV transilluminator. Appropriate markers were used to allow sizing of DNA fragments. λ HIII DNA cut with EcoR1 (Gibco BRL 15612-013) or ϕX174 DNA digested with Hae III (NBL 031204). The molecular weights of marker in base pairs are as follows:
ϕX174 1,353 1,078, 872, 603, 310, 281, 271, 234, 194, 118, 72.
λ HIII fragments are : 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, 125.

2.3.16 RNA extraction (Chomczynski and Sacchi 1987).

Cells were dissolved in Solution D (section 2.1.) (100µl/1x 10^6 cells) in a microfuge tube. The following were added sequentially and mixed by inverting, sodium acetate (2M, pH4) 50µl , phenol (Water saturated AquaPhenol) 500µl, chloroform: isoamyl alcohol (49:1 vol:vol) 100µl. Cells were vortexed for 10 seconds and cooled on ice for 15 minutes after which they were centrifuged at 13,000 rpm for 10 minutes at room temperature. The top aqueous layer which contained the RNA was removed using a pipette, placed into a clean microfuge tube and an equal volume of chloroform: isoamyl alcohol was added. Having vortexed for 10 seconds, cells were centrifuged for 5 minutes as above and aqueous layer extracted once more with chloroform:isoamyl alcohol. The final aqueous layer was precipitated with an equal volume of isopropanol at -20°C overnight. RNA was centrifuged as above for 10 minutes, pellet dissolved in Solution D(150µl) and precipitated with equal volume of isopropanol overnight at -20°C. RNA was centrifuged and pellet washed twice with 70% (vol) ethanol and once with absolute ethanol. Final pellet was dried under vacuum for 15 minutes and pellet redissolved in DEPC treated water (50µl) and allowed to dissolve overnight at 4°C.

RNA was quantified using Optical Density measurement at 260nm and 280nm. Integrity of 18s and 28s ribosomal RNA bands were checked by electrophoresis on a 1.5% agarose gel. RNA was stained with ethidium bromide at 0.5µg/ml and visualised using a U.V. source.

RNA is very easily degraded due to the presence of RNase on hands and in solutions, therefore gloves were worn during all manipulations involving RNA and reagents used were dedicated to RNA work only. When electrophoresis was to be carried out, gel tanks were washed with H_2O_2 and DEPC-treated water.

2.3.17 Reverse transcription.

RNA was reverse transcribed to form cDNA prior to amplification in the Polymerase Chain Reaction (PCR). In order to prevent cDNA formed from sticking, reactions were carried out in silanised eppendorf tubes. Approximately 500ng of total RNA was mixed with 50mM Tris-HCl (pH8.3), 75mM KCl, 3mM $MgCl_2$, 10mM dithiothreitol, 0.5mM each of dATP, dCTP, dGTP, dTTP, 1.5µM pd(T)12-18, 30 units RNAguard RNase inhibitor and 400 units M-MLV reverse transcriptase. The volume was made up to 30µl using DEPC treated H_2O and the tube placed in a water bath at 37°C for 60 minutes. The reaction was stopped by incubation at 95°C for 10 minutes and put on to ice. PCR could be set up directly from this. Alternatively the cDNA could be stored at -20°C until required followed by a 5 minute incubation at 95°C and then onto ice.

2.3.18 PCR.

2.3.18.1 Principle

The principle of the Polymerase Chain Reaction is that specific DNA sequences can be amplified by the simultaneous primer extension of complementary strands of DNA. Amounts as small as 30-50ng of cDNA can be used as the template to exponentially produce as much as 1mg of DNA. The reaction takes place in three steps , the first is a denaturation step at temperature of 90-95°C, this allows double stranded DNA to denature forming a template for priming. The enzyme used is the DNA polymerase Thermus aquaticus (Taq) DNA polymerase which extends the

primer sequence by using a complementary strand as a template. This takes place at the annealing temperature determined from the base sequence of the primers used. This is followed by a final incubation step at the extension temperature resulting in fully double stranded molecules . The result is the production of large amounts of the required fragment.

2.3.18.2 Method.

PCR was carried out in a final volume of 50µl containing 2µl of the cDNA reaction mix,

200µM each of dATP, dCTP, dGTP, dTTP,
1µM 5' and 3' primers,
2.5 units Taq,
1.5mM $MgCl_2$
1X buffer (supplied with the Taq polymerase).

In order to minimise evaporation the reaction was overlaid with 40µl of mineral oil and amplification carried out in the Perkin-Elmer Cetus DNA Thermal Cycler. Thirty cycles were carried out as follows;

1minute at 94°C,
1.5 minutes at 55°C
1.5 minutes at 72°C.

This was followed by 10 minutes at 72°C . Samples were stored at 4°C until ready to be visualised. PCR products were loaded onto a 1% agarose gel containing ethidium bromide and visualised using a uv transilluminator.

2..3.19 Primer Pairs

The following amino acid sequences are for 5' and 3' primers respectively.

Fas-L

GTTTGCTGGGGCTGGCCTGACT and GGAAAGAATCCCAAAGTGCTCC

GAPDH

GGTGAA GGTCGGAGTCAACGG and GGT CATGAGTCCTTCCACGAT

Bax

TCC GGG GAGCAGCCCAGG GGCGGG and GTCCCAAAGTAGGAGAGGAG

2.3.20 Generation of Fas-L cDNA probe.

Fas-L cDNA was expressed in the pcDNA3 eukaryotic expression vector (Invitrogen). In order to prepare a Fas-L probe a fragment of Fas-L specific DNA was first removed from the expression vector by restriction digest. 10µg of Fas-L pcDNA clone 9 was digested with EcoR1. 30 units of enzyme was used and reaction carried out in a 30µl reaction volume at 37°C overnight as follows:

Reagents	volume(µl)	stock concentration
DNA	10	1mg/ml
EcoR1	3	12U/ml
Buffer 10X	3	
H_2O	14	

The fragment produced by restriction endonuclease digestion was electrophoresed on a 1% agarose gel and the relevant DNA fragment excised with a clean scalpel blade. The DNA fragment was purified according to manufacturer's instructions using a Geneclean II kit (Bio 101), designed to purify DNA from 10base pairs to 1kb in length. The DNA was resuspended at 25µg/ml

Probe labelling

In order to probe DNA for presence of Fas- L or bax sequences a radiolabelled probe was first made using the appropriate cDNA .The "prime a gene" labelling system (Promega),based on the method developed by Feinberg and Vogelstein (1983) was

used. The following reagents were added together in a 50µl reaction volume in an eppendorf tube:

10µl 5X labelling buffer,
2µl non-labelled dNTPs (final concentation 20µM each)
1µl denatured DNA template (25ng) (500ng/ml final),
2µl nuclease free BSA (400µg/ml)
5µl (α-^{32}P) dNTP
5 units Klenow enzyme (100u/ml).

Reagents were mixed gently and incubated at room temperature for 1hr.

Incorporation of radioactive label into the DNA was measured as follows: 1µl of the reaction mix was spotted on to the centre of each of two 2.3cm discs of Whatman DE81 ion exchange paper. This paper is ionically charged so that DNA will bind to it but unincorporated nucleotides will not. One disc was washed three times, for 5 minutes each wash, with 0.5M Na_2HPO_4, rinsed in water, followed by two washes in 70% ethanol and finally in absolute ethanol. Once dry, an accurate measurement of the percentage incorporation of label was made by counting the filters in a LKB 1209 Rackbeta liquid scintillation counter, using OptiScint HiSafe scintillant. Percent incorporation was calculated using the formula

$$\frac{\text{cpm washed disc}}{\text{cpm unwashed disc}} \times 100 = \%\ \text{incorporation},$$

The probe is then ready to use for hybridisation.

2.3.21 Southern blotting

2.3.21.1 Principle

Localisation of particular sequences within DNA fragments or genomic DNA is usually accomplished by the transfer techniques originally described by Southern (1979). Briefly, DNA is separated on an agarose gel followed by denaturation and transfer to a solid matrix such as a filter membrane. The relative positions of the DNA fragments remain unchanged during their transfer to the filter. The DNA

attached to the filter is hybridised to a radiolabelled probe and autoradiography used to locate the positions of the band complementary to the probe.

2.3.21.2 Blotting

In order to probe for Fas-L or bax sequences, PCR product DNA samples were first electrophoresed on a 1.5% agarose gel . The gel was then Southern blotted onto a Biodyne B membrane. Blotting was set up at room temperature for 3 hours using 0.4 M NaOH as the transfer buffer (Figure 2.2). The blot was washed twice in 2XSSC and air-dried for 30 minutes. This can be stored wrapped in saran wrap at 4^{o}C until ready to carry out hybridisation.

Filter was hybridised in prehybridisation solution (25ml) in a hybridisation bottle. The labelled probe was added to prehybridisation mix and the blot hybridised overnight at 65^{o}C. The hybridisation mix was removed and the filter was washed twice in 2X SSPE, 0.1% SDS (25ml) at room temp for 10 minutes. This was followed by washing with 1X SSPE, O.1% SDS for 15 minutes at 65^{o}C, then 0.1SSPE, 0.1% SDS twice for 10 minutes. This is a high stringency wash used when cDNA probes were utilised. The blot was removed from bottle, wrapped in Saran-wrap and autoradiographed by exposing to X-ray film with an intensifying screen for times indicated.

2.4 Statistical analyses.

Statistical analysis was carried out using the Students unpaired t test for Figures 3.6 and 3.8.

* = probability (p), where $p<0.005$.

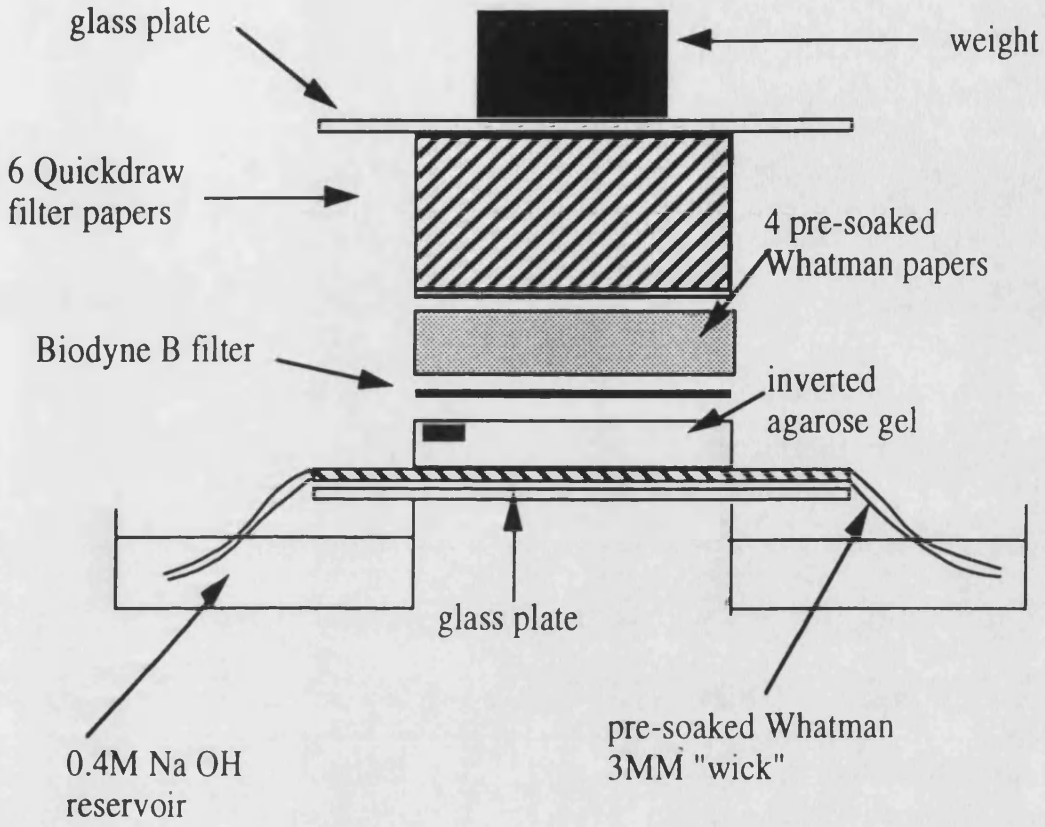


Figure 2.2 Southern Blotting Apparatus.

Transfer of DNA from agarose gel to Biodyne B filter. Gel was placed upside down on top of a pre-wetted 3MM wick sitting on a clean glass plate. In order to prevent leakage the gel was surrounded by parafilm. A piece of Biodyne B membrane cut to size was placed on the gel followed by Whatman 3MM and 6 sheets of Quickdraw papers .A glass plate topped with a small weight was put on top to secure the apparatus. The gel was blotted at room temperature for 3 hours.

CHAPTER THREE.

RESULTS I.

ACTIVATION REQUIREMENTS OF T LYMPHOCYTES

3.1 Proliferation requirements of human T lymphocytes.

T lymphocytes play a crucial role in the efficient functioning of the immune system. In order to carry out their effector function, T cells must first become activated and proceed through the cell cycle to expand and produce cytokines. In this chapter T cell responses to various stimuli including anti-CD3, PHA and the superantigen SEB are explored. The ability of these reagents to stimulate human peripheral T lymphocytes will be examined. Also, the necessity for signals in addition to engagement of the TCR will be investigated to determine the role costimulation plays in T cell activation. In order to examine the activation requirements of human peripheral T lymphocytes, measurements of the outcome of productive activation were defined as proliferation and the secretion of T cell associated cytokines such as IL-2.

3.1.1 TCR engagement by anti-CD3.

Engagement of the TCR by antigen/MHC class II complexes can be mimicked by aggregation of the TCR using immobilised anti-CD3 antibody. In order to define a useful dose, range the ability of PBMCs to respond to engagement of the TCR/CD3 complex by cross-linked anti-CD3 mAb was examined initially. It can be seen (Figure 3.1) that PBMCs responded vigorously to anti-CD3 antibody stimulation, proliferation increasing as the concentration of anti-CD3 increased. Therefore, proliferation of PBMCs in response to engagement of the TCR by anti-CD3 mAb occurred in a dose dependent manner. However, when T cells were purified from PBMCs no proliferation was seen in response to anti-CD3 stimulation (Figure 3.1). The removal of T cells from other cells in the PBMC population led to abrogation of the ability of the T cells to respond to anti-CD3 stimulation. This therefore suggested that endogenous accessory cells were present in addition to T lymphocytes in the PBMC population. One possibility is that these cells provided signals which were necessary for T cells to proliferate in response to TCR signalling. Therefore an accessory cell dependent costimulatory signal was required in addition to the signal provided by anti-CD3 engagement of the TCR in order for T lymphocytes to proliferate .

In an attempt to reconstitute the accessory cell-dependent factors required to costimulate TCR signalling the effects of two accessory molecules, LFA3 and B7-1, on costimulation of anti-CD3 signals were determined. Chinese hamster ovary (CHO) cells transfected with

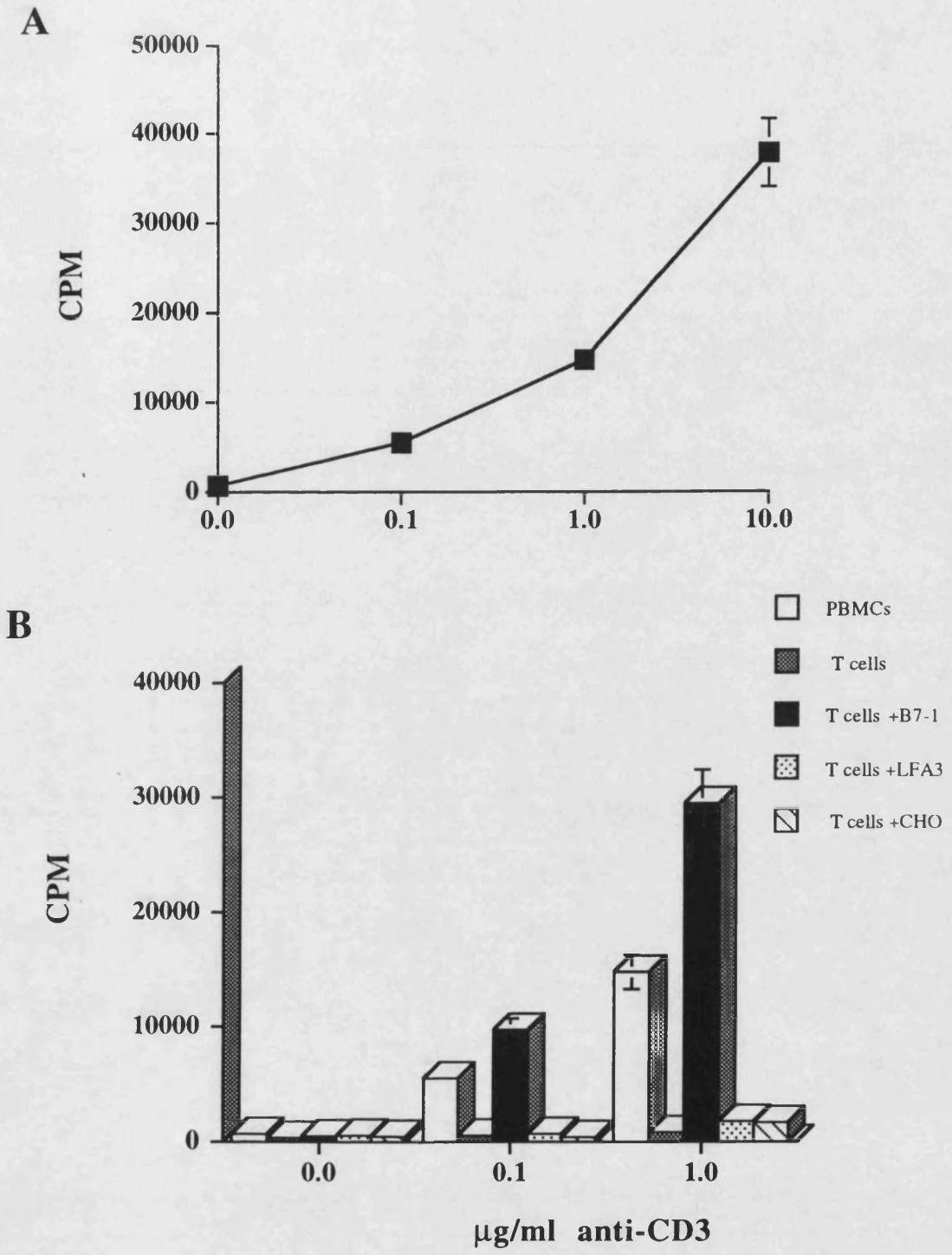


Figure 3.1 Anti-CD3 stimulation of PBMCs and purified T cells.

(A) freshly isolated PBMCs (1 x 10^5) were stimulated with various concentrations of anti-CD3 for 3 days and proliferation measured.

(B) PBMCs (1 x 10^5) or T lymphocytes (5 x 10^4) were stimulated with various concentrations of anti-CD3 for 3 days. The ability of LFA3 or B7-1 to costimulate proliferation was determined by addition of transfectants expressing these molecules (2.5 x 10^4) to anti-CD3 stimulated T cells. Untransfected CHO cells (2.5 x 10^4) were added as a negative control. Proliferation was assessed by ^{3}H-thymidine addition (1µCi/well). Data are representative of 3 experiments.

cDNAs for either LFA3 or B7-1 which expressed high levels of these molecules were used in these experiments (Figure 3.2). Anti-CD3 induced proliferation of purified T cells was not costimulated by the addition of LFA3 transfectants or by untransfected CHO cells (Figure 3.1). Binding of LFA3 to its ligand CD2 on T cells therefore was not capable of providing costimulation for TCR induced signals. In contrast, addition of B7-1 expressing cells to anti-CD3 stimulated T cells resulted in a proliferative reponse which increased as the dose of anti-CD3 increased from 0.1 to 1.0 μg/ml (Figure 3.1B). Therefore ligation of CD28 by B7-1 results in costimulation of anti-CD3 triggered signals leading to T cell proliferation. In addition B7-1 costimulated responses were more potent than those obtained from unseparated PBMCs. This demonstrated that engagement of the CD28 receptor on T cells by its ligand B7-1 resulted in an important costimulating signal which synergises with TCR signalling. B7-1 therefore replaced the costimulation that was removed when the PBMCs were depleted of accessory cells. The B7-1/CD28 interaction thus was necessary and sufficient to synergise with TCR signalling resulting in proliferation of T lymphocytes.

The interaction of LFA3 with CD2 on T cells was however unable to costimulate TCR signalling. This interaction is therefore predicted to act merely via adhesion and does not function as a costimulus of TCR signalling during anti-CD3 stimulation of T lymphocytes. ,

These results demonstrated the need for more than one signal for productive T cell activation resulting in proliferation. Such a signal can be provided by the interaction of B7-1 with CD28 resulting in T lymphocyte proliferation in response to anti-CD3 triggering. It was thus concluded that CD28 signalling can synergise with TCR signalling costimulating proliferation of T lymphocytes and can replace the accessory cell dependent signals in PBMCs signals.

3.2 Stimulation of T lymphocytes with the lectin PHA.

3.2.1 Proliferation requirements.

T cell activation requirements were also defined by the use of the mitogenic lectin PHA, which is thought to be capable of stimulating T cell proliferation by directly binding to the TCR. PHA is a polyclonal stimulator of T lymphocytes and therefore the magnitude of this response would be expected to be much greater than that seen with peptide responses, so

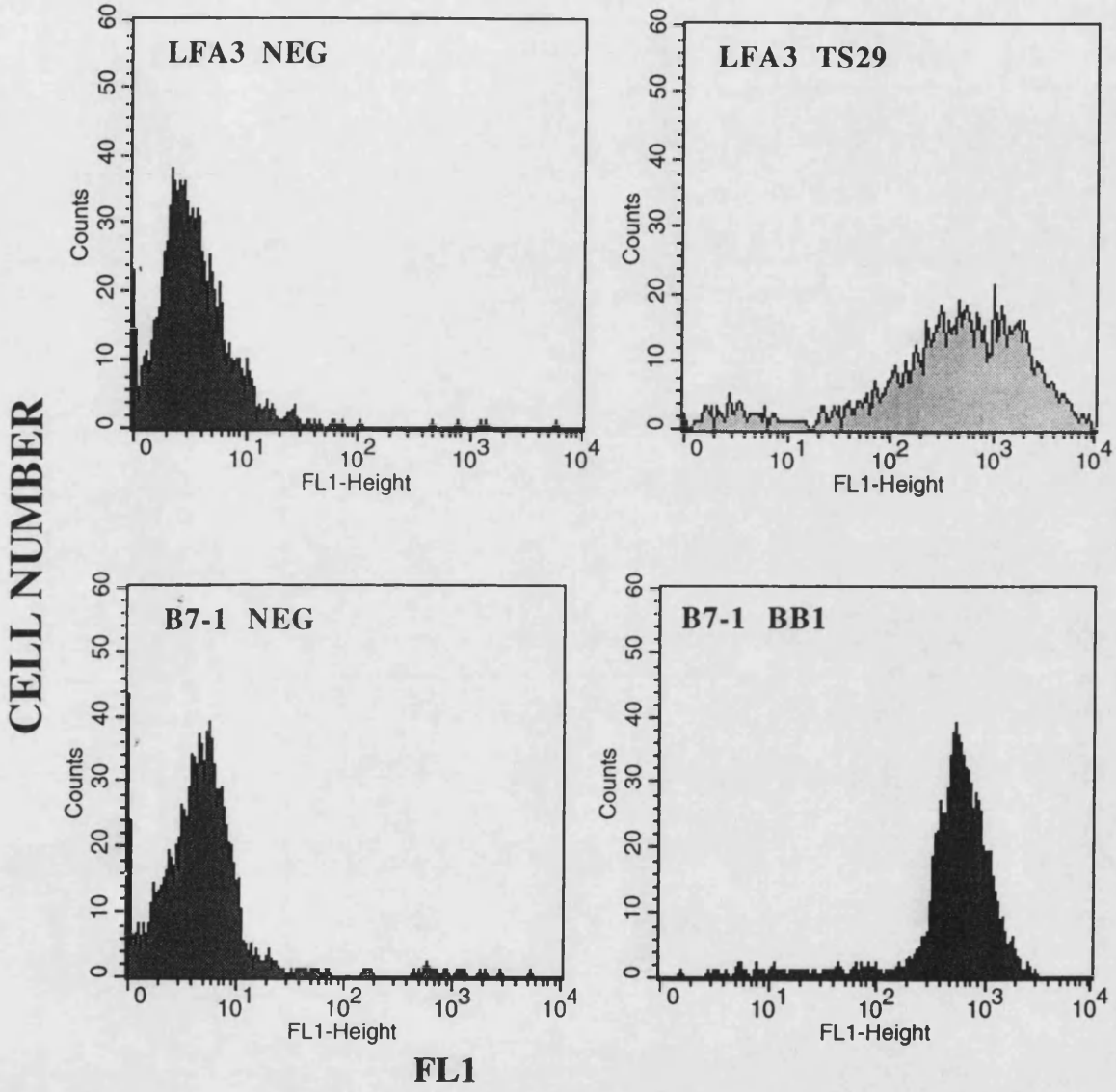


Figure 3.2 Phenotypic analysis of LFA3 and B7-1 transfectants.
Cells were stained with TS29 (anti-LFA3) and BB1 (anti-B7-1) and analysed by FACS using a Becton Dickinson FACStar Plus. 5,000 events were analysed. Antibodies were used at 10µg/ml.

this molecule is useful for in vitro investigation of T cell proliferation. Consequently, in order to further assess the costimulation requirements of T cell proliferation, responses to PHA were examined.

Initially a useful dose range for PHA stimulation was established by examining PBMC responses to concentrations of PHA from 0.1 to 10μg/ml. Similar to anti-CD3 stimulation, it was seen that PBMCs were capable of proliferating in response to PHA in a dose dependent manner (Figure 3.3). Next the ability of T lymphocytes removed from accessory cells to proliferate was examined. Purified T cells proliferated weakly in response to 5μg/ml PHA whereas T cells in the PBMC population responded vigorously at this dose (Figure 3.3), consistent with the presence of endogenous APCs in this population. Similar to anti-CD3 stimulated responses, PHA responses therefore were also dependent on accessory cell factors. To define the interactions necessary to costimulate PHA responses, LFA3 and B7-1 transfectants were used once again . These transfectants were added to PHA stimulated T lymphocytes. However, in contrast to anti-CD3, addition of either B7-1 or LFA3 transfectants restored the proliferation of purified T cells in response to PHA (Figure 3.3). Both LFA3 and B7-1 were thus capable of costimulating PHA proliferation in T lymphocytes and substituting for accessory cell dependent functions.

These experiments once again indicated the requirement for signals in addition to that transduced via TCR engagement in order for productive T cell activation resulting in proliferation to occur. In addition the ability of endogenous APCs to costimulate T cell responses was again shown. These results also demonstrated that costimulation requirements differed between anti-CD3 driven and PHA-driven proliferation, both LFA3 and B7-1 being capable of stimulating PHA-derived signals to the TCR whereas B7-1 but not LFA3 costimulated anti-CD3 driven responses.

Preliminary results also showed the ability of the B7-2 molecule to costimulate PHA induced responses (Figure 3.4). Therefore both B7-1 and B7-2 were capable of costimulating PHA-induced proliferation and replacing endogenous APCs. These results also add weight to the concept that B7 molecules costimulate T cell signalling emphasising the importance of the CD28/B7 interaction in T cell proliferative responses.

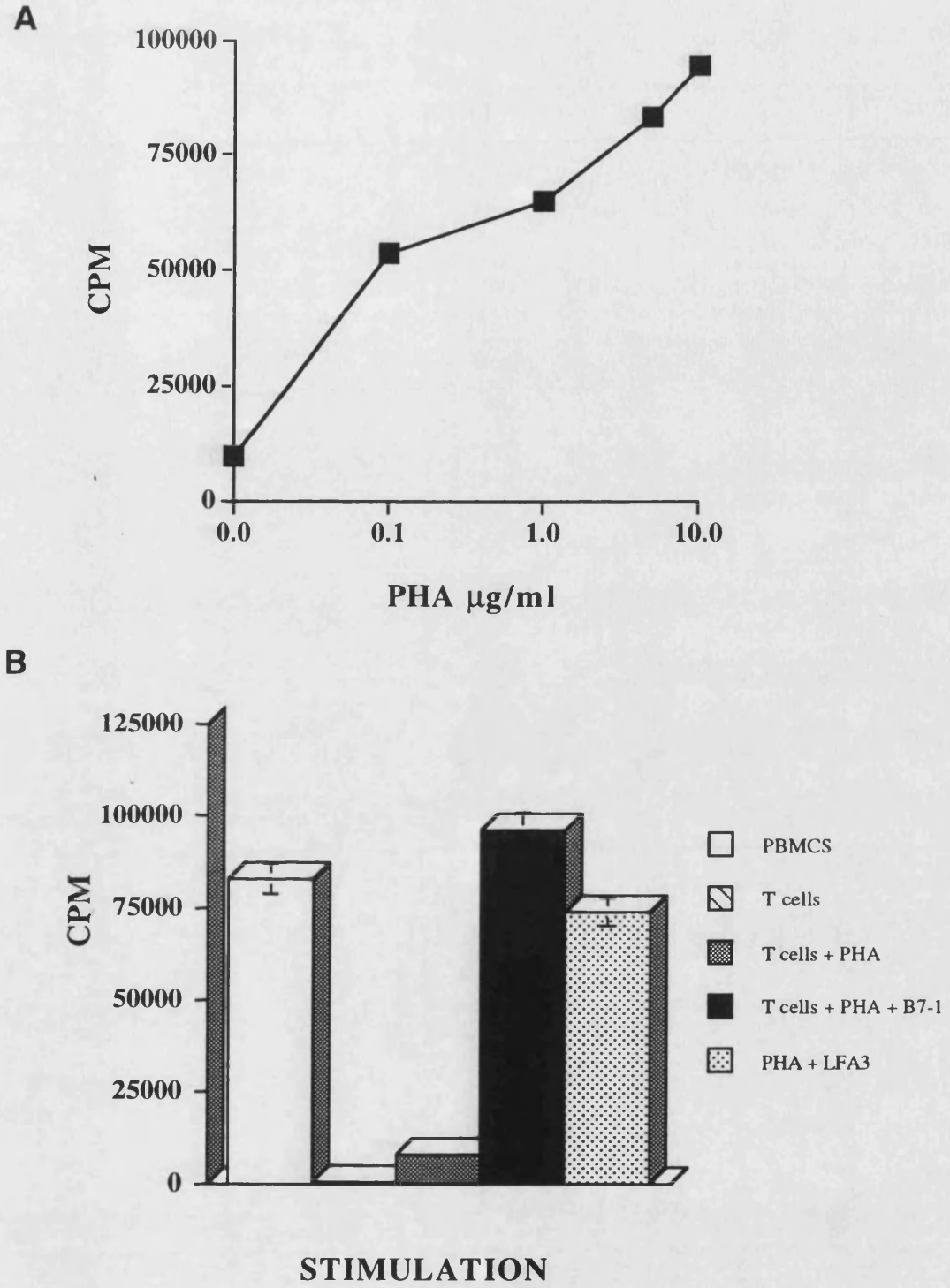


Figure 3.3 Proliferation responses to PHA in PBMCs and T lymphocytes
(**A**) Dose response of PBMCs (1 x 10^5) to PHA (**B**) PHA (5µg/ml) was added to PBMCs (1 x 10^5) or purified T cells (5 x 10^4). The effect of addition of LFA3 (2.5 x 10^4) and B7-1 (2.5 x 10^4) to PHA stimulated T cells was also measured. Background proliferation is shown in T cells without stimulation . Proliferation was measured by tritiated thymidine incorporation. Data are representative of 4 experiments.

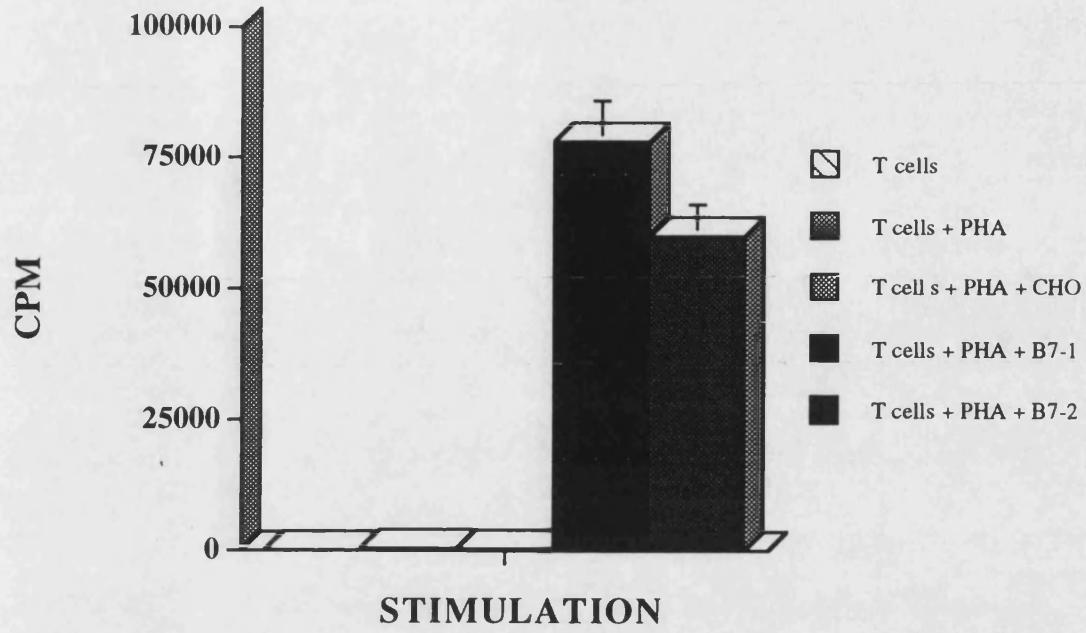


Figure 3.4 B7-1 and B7-2 costimulation of PHA-driven proliferation in T lymphocytes

PHA (5μg/ml) was added to purified T cells (5 x 10^4). The effect of addition of B7-1 or B7-2 cells(2.5 x 10^4) to PHA stimulated T cells was measured. Background proliferation is shown in T cells without stimulation and with untransfected CHO cells . Proliferation was measured by tritiated thymidine incorporation. Data are representative of 2 experiments.

3.2.2 IL-2 responses in PHA stimulated T cells.

In addition to proliferation, production of IL-2 was also measured in PHA stimulated T cells.The amount of IL-2 produced in PHA-treated T cells was determined using an ELISA method. No IL-2 was produced in unstimulated or PHA-treated T cells, whereas both LFA3 and B7-1 were capable of costimulating IL-2 production from PHA-stimulated T cells. It was seen also that B7-1 was much more efficient than LFA3 at costimulating IL-2 response (Figure 3.5). Therefore in addition to costimulating PHA proliferation, LFA3 and B7-1 were also capable of costimulating IL-2 production from T lymphocytes. These data support the hypothesis that TCR engagement is insufficient for T cell activation and that other costimulatory factors are required for PHA-driven responses which may be mediated by LFA3 or B7-1 interactions with their respective ligands on T lymphocytes.

These observations led to the hypothesis that costimulation requirements differ between PHA-induced and anti-CD3 -induced T cell proliferation. This may reflect differential initial signalling triggered by these molecules. In addition to signals transduced through CD3, PHA may result in signalling via the CD2 molecule which requires a further engagement provided by the LFA3 transfectant. It is also hypothesised that B7-1 may be capable of providing a signal that is independent of TCR-transduced signals. This adds weight to the importance of CD28 signalling acting to costimulate TCR activation. The interactions provided by accessory cells can therefore be replaced by either LFA3 or B7-1 interactions with their respective ligands in PHA-stimulated proliferation and IL-2 production. In contrast, B7-1 but not LFA3 costimulated CD3-driven responses and was also a more potent costimulator of IL-2 production during PHA responses. These results highlight the crucial role played by costimulation in T cell responses and the potency of the CD28 costimulation pathway.

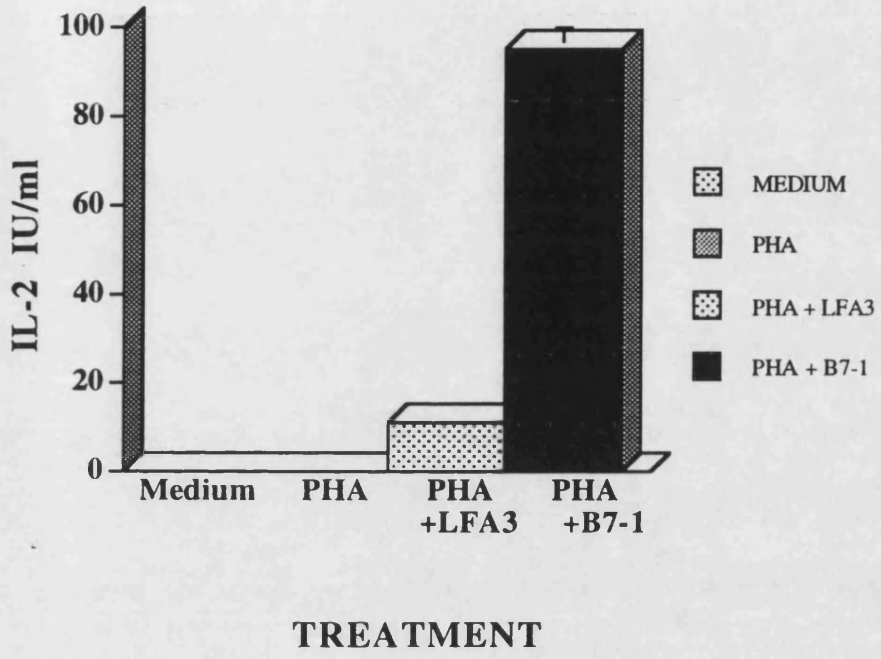


Figure 3.5 IL-2 production in PHA stimulated T cells.
PHA (5μg/ml) was added to T lymphocytes (5 x 10^4) and the effect of addition of LFA3 or B7-1 transfectants (2.5 x 10^4) on IL-2 secretion was determined. IL-2 production was measured in International units (IU)/ml using an ELISA. Unstimulated T cells were cultured with medium alone. Data are representative of 3 experiments.

3.3.Stimulation requirements for superantigen driven proliferation.

The stimuli used thus far to determine costimulation requirements of T lymphocytes were not dependent on the TCR interaction with MHC molecules on an APC. One group of antigens which are believed to depend on presentation by MHC molecules are superantigens such as SEB. It was therefore useful to examine this physiologically relevant MHC-dependent T cell response.

In addition, SAg responses are a function of their interaction with families of TCR Vβ chains , generating a polyclonal response of much greater magnitude than for most nominal peptide antigens. This enables the study of a greater number of T cells than would be possible with peptide antigens.

Superantigens also do not require processing in order to be presented to T cells as they interact directly with MHC class II molecules and thus metabolically inactivated presenting cells are still capable of presenting SAgs to T cells. These cells do not divide and therefore do not incorporate radioisotope making them ideal for use in proliferation assays. Fixed CHO transfectants expressing class II molecules were thus used as APCs to present SEB in order to analyse the requirements of the T cell proliferative response to this antigen.

3.3.1 Proliferation responses to SAgs

Initially it was decided to look at the response of PBMCs to the SAg SEB and to define a useful working range of concentrations. It was found that PBMCs proliferated in response to SEB, proliferation increasing as the concentration was raised from 0.0001μg/ml to 1.0μg/ml (Figure 3.6). Even at doses as low as 0.001 μg/ml SEB, PBMCs were capable of proliferative responses. Therefore PBMCs proliferated vigorously at concentrations of SEB from 0.0001 to 1 μg/ml. These cells thus had the capacity to respond to this superantigen. The ability of purified T cells to respond to SEB was also examined. It can be seen (Figure 3.6) that purified T cells alone do not respond to SEB by proliferating. The removal of T cells from other cells in the PBMC population therefore abrogated the ability of these cells to respond to superantigen.

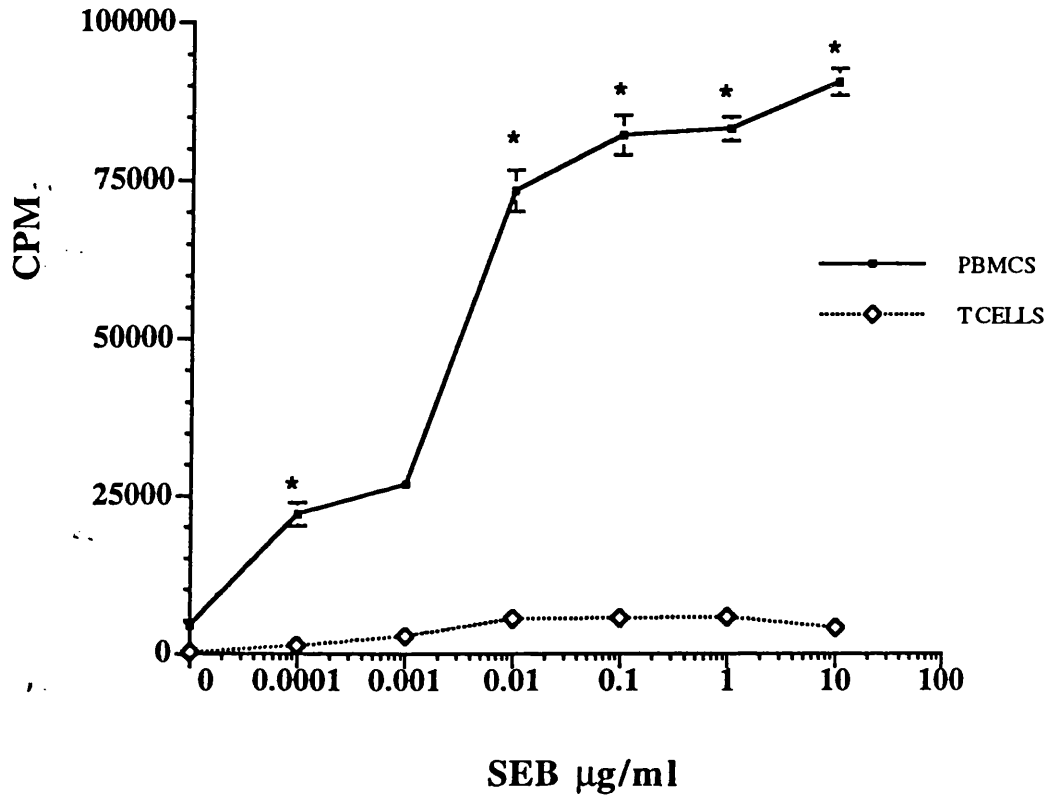


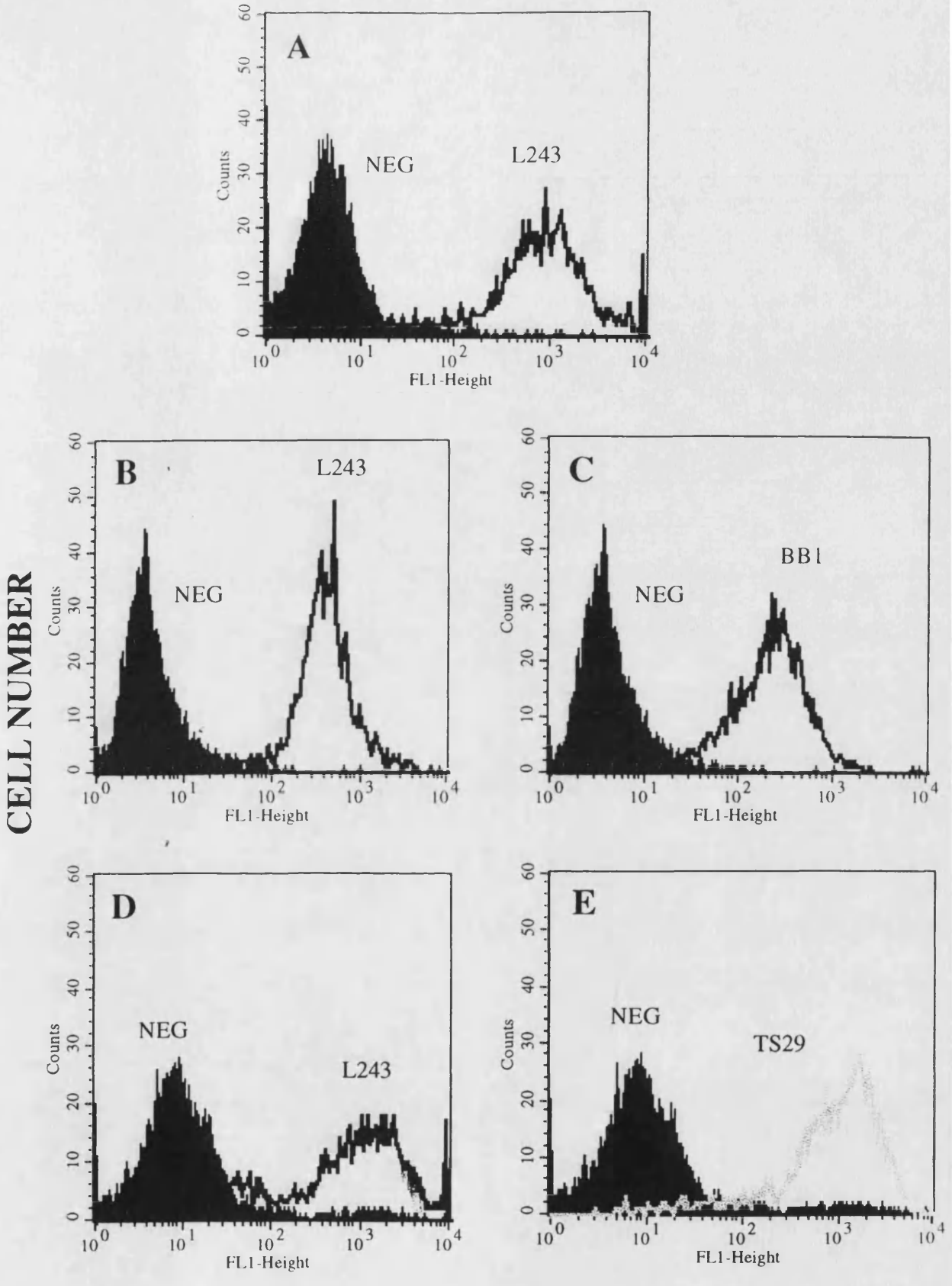
Figure 3.6 SEB stimulation of PBMCs and purified T lymphocytes.

Freshly isolated PBMCs ($1x10^5$) or T lymphocytes ($5x10^4$) were stimulated with SEB at indicated concentration for 5 days. Proliferation was measured by 3H thymidine incorporation. Data are representative of 3 experiments. Points shown are mean +/- standard deviation. * = $p < 0.005$, when compared to proliferation in the absence of SEB.

This was similar to the responses demonstrated for anti-CD3 and PHA, again emphasing the importance of accessory cell dependent costimulation in T lymphocyte proliferation. The T lymphocytes used in these experiments had been depleted of cells expressing class II MHC molecules, therefore autopresentation by the T cells could not have taken place. Class II MHC positive transfectants (DR4) (Figure 3.7) were used to examine whether presentation of SEB on MHC molecules resulted in proliferation. SEB was pulsed onto these cells, which were used as APCs to present SEB directly to T cells, resulting in a very small proliferative response (Figure 3.8). This confirmed that SEB proliferative responses were dependent on MHC presentation by APCs. Thus, SEB responses were MHC-dependent and more physiologically relevant than the stimuli previously used.

3.3.2 Costimulation requirements of SEB responses.

The proliferative responses resulting from class II MHC presentation of SEB were far less potent than the T cell response to SEB seen in PBMCs, which suggested that additional signals provided by accessory cells were required. Consequently, it was decided to attempt to reconstitute these signals using ligands for CD28 or CD2 as before. In addition to class II expressing CHOs, transfectants co-expressing class II and either B7-1 or LFA3 were also available (Figure 3.7). This enabled examination of the ability of the LFA3 and B7-1 molecules to costimulate SEB responses. The addition of SEB pulsed DR4/LFA3 or DR4/B7-1 cells to purified T cells resulted in significantly increased proliferation when compared to SEB pulsed DR4 cells alone (Figure 3.8). It was also seen that DR4/B7-1 costimulated responses were greater than DR4/LFA3 costimulated SEB reponses. Therefore LFA3 and B7-1 were capable of providing costimulation to SEB presented on an APC. This was in agreement with results demonstrated for PHA responses but differs from that seen with anti-CD3 in that LFA3 did not costimulate this response. Once again B7-1 costimulation appeared to be more potent than LFA3 costimulation confirming the importance of the interaction of B7-1 with its ligand CD28 on the T cell .SEB responses therefore required additional signal to TCR engagement to induce T cell proliferation. These signals could be provided by either LFA3 or B7-1, the latter being the more potent costimulatory molecule. It therefore appears essential that for productive T cell activation to occur signals in addition to TCR engagement are required.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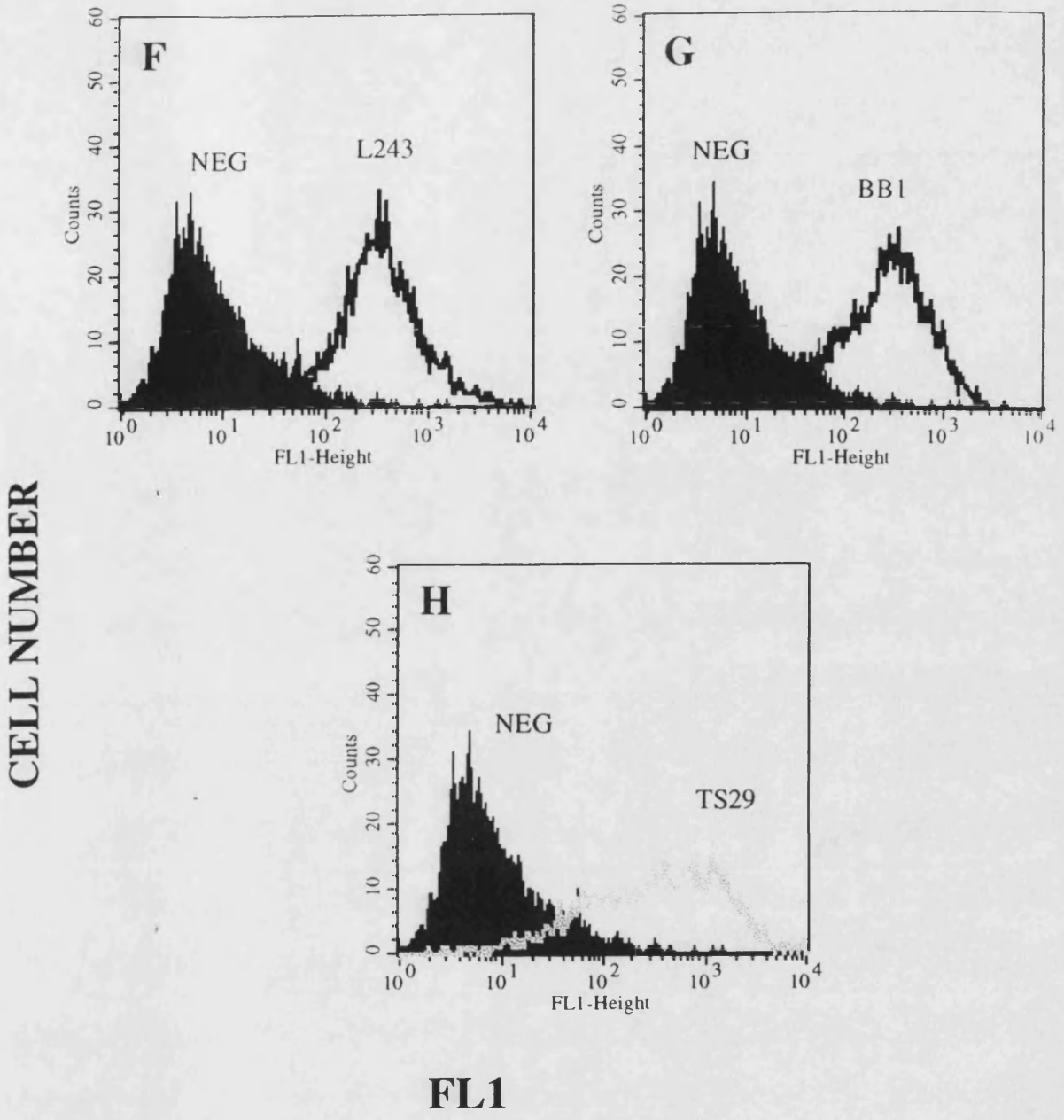


Figure 3.7 Expression of Class II and B7-1 and LFA3 on transfectants.
Cells stained with L243 (anti-MHC Class II), or BB1 (anti-B7-1) or TS29 (anti-LFA3) and analysed by FACS using a Becton Dickinson FACStar Plus. Panels are as follows: **A** DR4 cells, **B and C** DR4/B7-1, **D and E** DR4/LFA3, **F, G and H** DR4/LFA3/B7-1. Antibodies were used at 10 μg/ml.

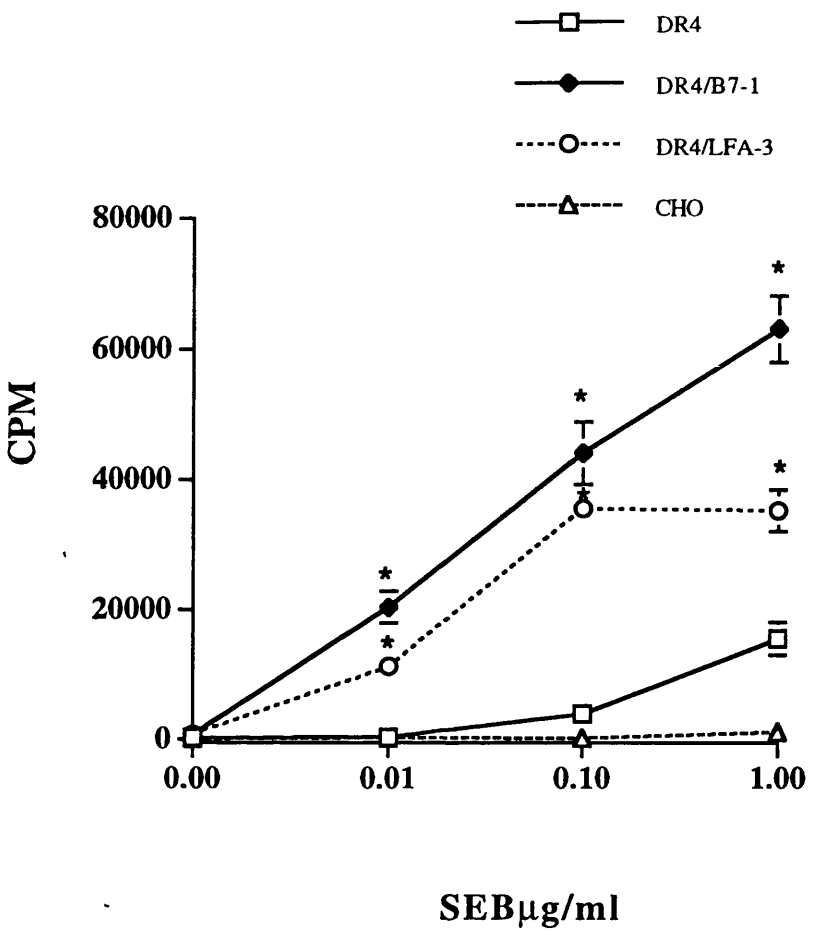


Figure 3.8 LFA3 and B7-1 costimulation of SEB responses in resting T lymphocytes. Class II MHC positive transfectants (DR4) (2.5 x 10^4) were used to present SEB at various concentrations to purified T cells. The effect of LFA3 or B7-1 was measured by presenting SEB on class II MHC transfectants co-expressing either LFA3 (DR4/LFA3) or B7-1 (DR4/B7-1) (2.5 x 10^4). Prolferation values for T lymphocytes with SEB and CHO cells are also shown. Proliferation was measured by ^{3}H thymidine incorporation as before and values shown are cpm for triplicate cultures. Data are representative of 3 experiments. Points shown are mean +/- standard deviation. *= $p < 0.005$ when compared to T cell proliferation in response to DR4 pulsed with SEB at appropriate concentration.

In order to distinguish between the ability of LFA3 or B7-1 to function either as adhesion molecules promoting cell to cell contact or to provide costimulation , the ability of these molecules to costimulate SEB responses from a cell distinct from the APC was examined (see Figure 3.9). When SEB was presented by a separate class II MHC positive APC to trigger the TCR and LFA3 added on a separate cell it was seen that this molecule was not capable of enhancing proliferation of resting T cells in response to SEB (Figure 3.10). In contrast, the addition of B7-1 transfectants resulted in costimulation of the response elicited by SEB on class II MHC transfectants. Even at B7-1 cell numbers as low as 10^3 a substantial enhancement in proliferation was seen demonstrating the potency of the CD28 signal. As the number of B7-1 cells added to the culture was increased the proliferation increased demonstrating that enhanced costimulation was occurring as the number of B7-1/CD28 interactions taking place increased. Therefore B7-1 was capable of costimulation from a separate cell to the one stimulating the TCR and thus provided third party costimulation of SEB responses. This molecule thus acts to costimulate T cell responses even when it is not located on the same cell surface as the MHC derived signal.

It has been demonstrated therefore that B7-1 costimulated PHA and anti-CD3 responses in T cells removed from endogenous APCs. Additionally these results showed that LFA3 acted as a costimulatory molecule in the T cell responses induced by PHA, functioned as an adhesion molecule in SEB responses but did not costimulate CD3 responses or provide third party costimulation for SEB proliferation. In contrast, the ability of the B7-1 molecule to costimulate SEB responses from a cell distinct from the cell providing the antigen specific signal which engages the TCR has been clearly demonstrated. These results lend considerable weight to the ability of the B7-1/CD28 interaction to act as a costimulatory signal in T cell responses placing this interaction as the principle costimulatory encounter in productive T cell signalling. In addition, these results suggest that CD28 may not require co-association with the TCR and is likely to provide a distinct signal involved in T cell activation. CD28 signalling thus provided essential costimulation for T cell activation resulting in proliferation and IL-2 production.

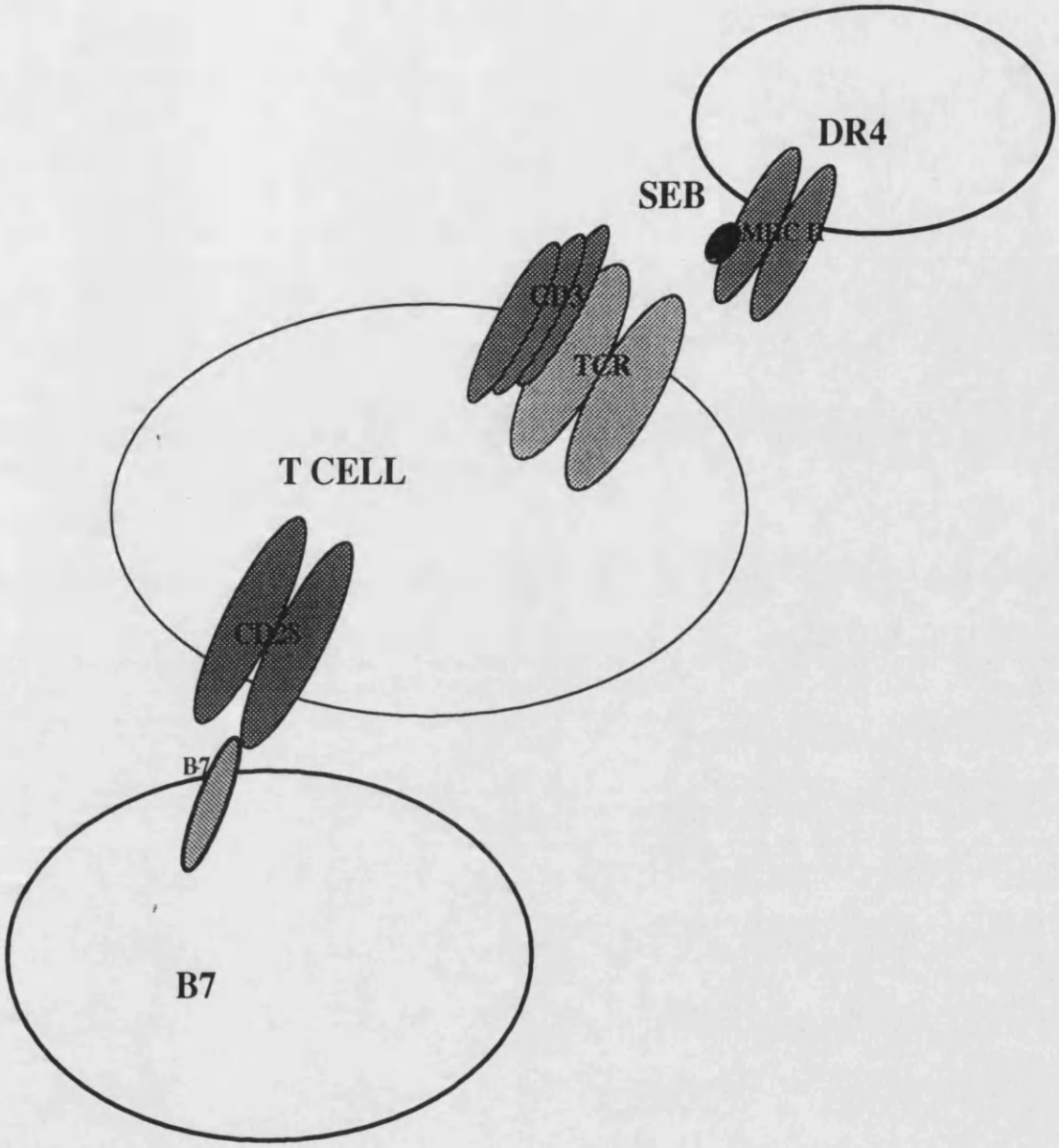


Figure 3. 9 Schematic diagram of third party costimulation of T cell activation.
T cells were stimulated using SEB presented by MHC class II positive transfectants (DR4) and the ability of transfectants expressing either B7 (shown here) or LFA3 (not shown) to costimulate T cell activation was determined by measuring proliferative responses.

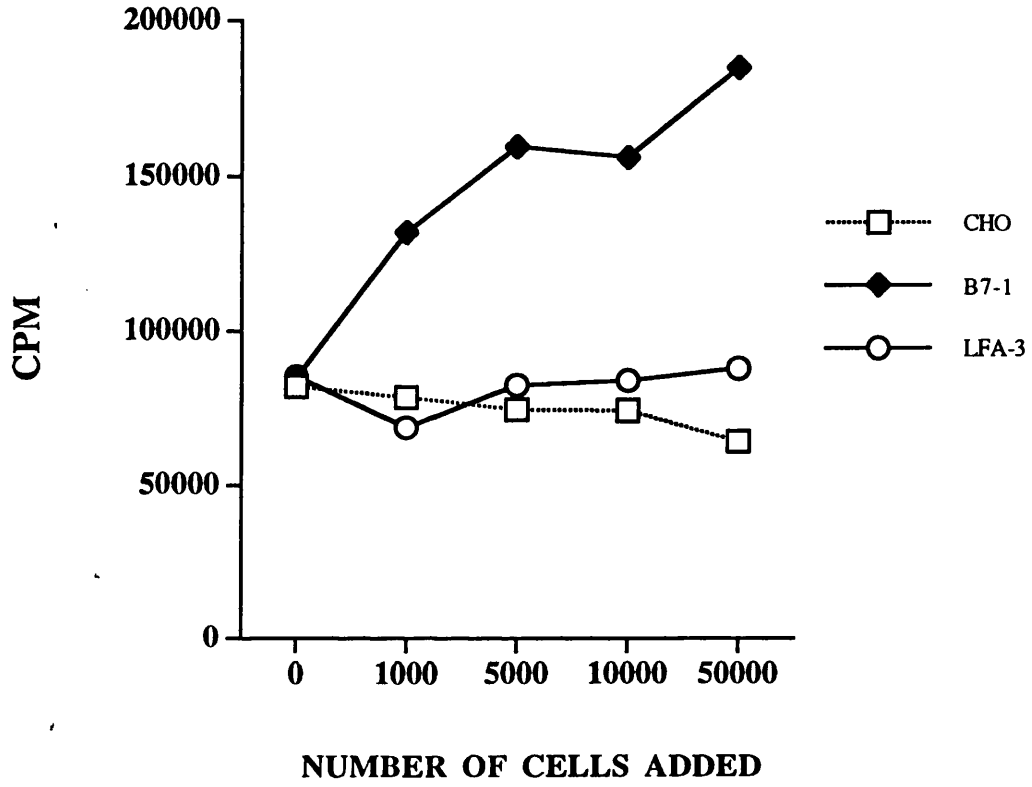


Figure 3.10 Third party costimulation of SEB proliferation in resting T lymphocytes.
Class II positive transfectants (DR4, 2.5 x 10^4) were pulsed with 1 μg/ml SEB and used to stimulate freshly purified T cells. The effects of addition of untransfected CHO cells or either LFA3 or B7-1 on a cell separate to the one engaging the TCR was determined. Increasing numbers of costimulator cells were added to the cultures. Proliferation was measured 18 hours after tritiated thymidine addition. Data are representative of 3 experiments.

3.4 SEB responses in SAg-specific T cell lines.

In order to determine whether there were differences between engagement of the TCR on resting versus activated T cells, SEB specific T cell lines were developed. These cells were phenotyped by FACS analysis and the profile of expression of a number of cell surface molecules on three representative SEB specific T cell lines is shown in Table 3.1. Class II MHC expression was observed on these cells, consequently, SEB specific T cells were potentially capable of autopresentation of antigens. Having established some of the requirements for SEB-induced proliferation in resting T cells which do not express MHC it was interesting to examine the effect of SEB on previously activated T cells.

3.4.1 Proliferation requirements of SEB T cell lines.

To investigate the proliferation requirements of SEB T cell lines, SEB was added to the cells and proliferation responses measured. In contrast to PBMCs, addition of SEB alone did not stimulate proliferation in these cell lines (Figure 3.11). SEB T cells were thus incapable of proliferating in response to SEB alone, this population of cells had been depleted of professional APCs and consisted of T cells which expressed class II MHC but did not act as professional APCs. It was therefore interesting to investigate whether class II MHC presentation of SEB on APCs could result in a proliferative response of these T cells. Presentation of SEB by class II MHC expressing transfectants resulted in a proliferative response to SEB indicating that these cells were both SEB-specific and capable of a proliferative response to antigen (Figure 3.11). The responses to DR4/SEB were low compared to the responses from SEB stimulated PBMCs. It was therefore necessary to determine if augmented responses could be achieved by costimulation .

The ability of LFA3 and B7-1 to costimulate SEB responses was evaluated using DR4/LFA3 or DR4/B7-1 transfectants pulsed with SEB. The responses seen with DR4/LFA3 were not much greater than with DR4 alone (Figure 3.12). However it was shown that SEB presented by DR4/B7-1 transfectants induced a much greater proliferative response to SEB (Figure3.12). The response increased to 10 times that seen in DR4/SEB driven proliferation. B7-1 was therefore capable of potently costimulating SEB driven responses in previously activated T cells as well as in resting T lymphocytes. LFA3 was much less potent at costimulating SEB responses in

	Line 1		Line 2		Line 3	
Antibody	**MFI**	**% cells**	**MFI**	**% cells**	**MFI**	**% cells**
Negative	2.3	92.3	3.3	97.3	3.7	91.1
CD2	42.4	99.8	112.9	99.4	98.5	98.8
CD3	56.9	99.6	36.6	97.0	100.8	99.2
CD4	24.7	89.3	35.6	88.9	30.2	85.0
CD25	20.1	27.0	101.2	97.2	96.4	86.3
CD28	12.0	73.9	15.5	46.9	37.1	28.8
HLA-DR	26.3	37.0	63.7	71.0	93.2	37.0
CD95	25.9	97.6	28.3	98.1	35.7	96.5

Table 3.1.

Phenotypic analysis of T cells from 3 representative SEB-specific T cell lines. Cells ($2x10^5$/antibody) were removed from culture and washed in PBS. Cells were resuspended in antibodies shown for 45 minutes at 0^oC, washed in PBS and detected with anti-mouse FITC conjugated Ig. Stained cells were analysed using a Becton Dickinson FACStar Plus for Mean fluorescence intensity (MFI) and percentage (%) positivity above a region set at the 95 percentile of the negative control.

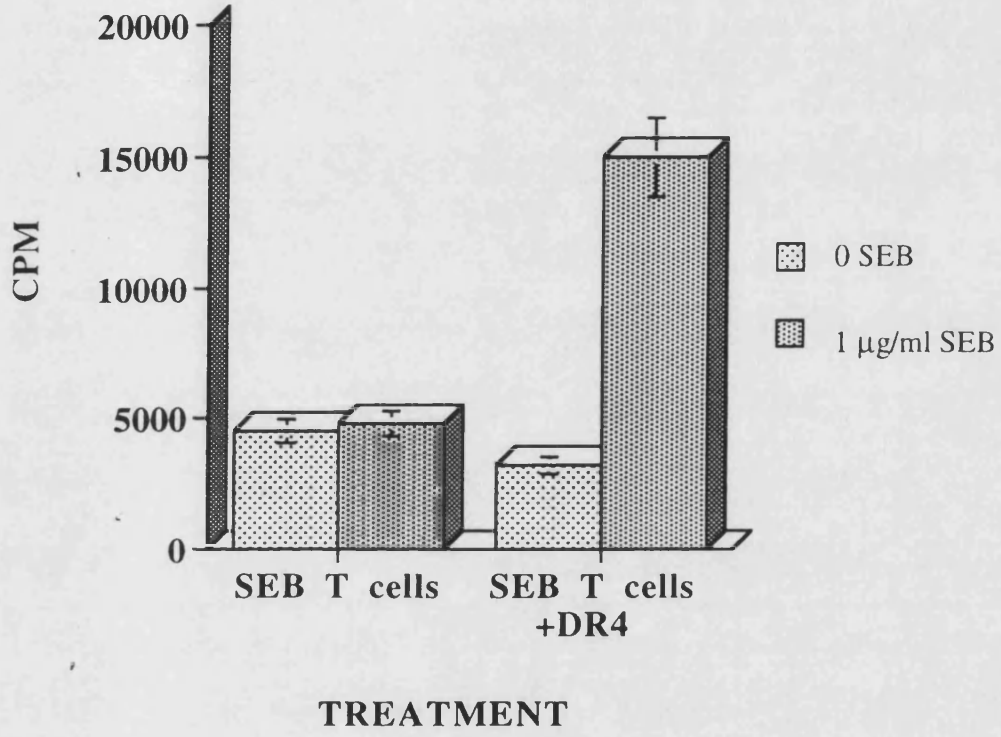


Figure 3.11 SEB stimulation of antigen specific T cells. SEB responsive T cells($1x10^5$) were cultures with or without SEB (1μg/ml) alone or pulsed onto DR4 transfectants for 3 days. Proliferation was measured using tritiated thymidine incorporation. Data are representative of 4 experiments.

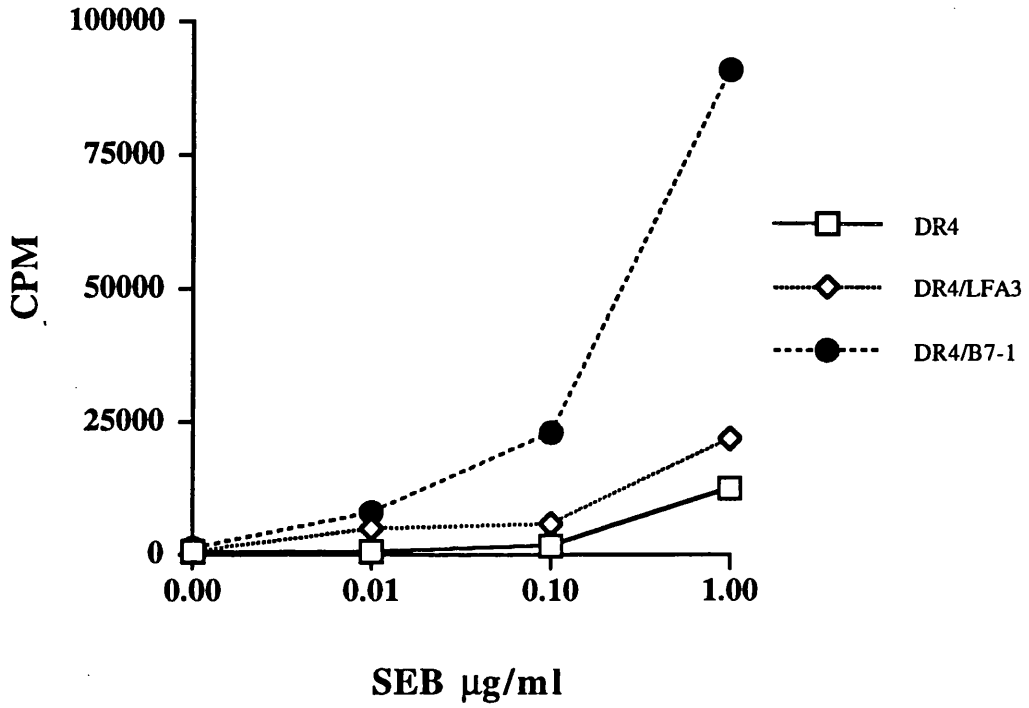


Figure 3.12 Costimulation of SEB proliferation in superantigen specific T cells. Superantigen at various concentrations was pulsed onto transfectants expressing Class II (DR4 2.5 x 10^4) or co-expressing class II MHC and either LFA3 (DR4/LFA3) or B7-1 (DR4/B7-1 2.5 x 10^4) . These cells were added to SEB specific T cells (5 x 10^4) and cultured for 3 days. Proliferation was measured 18 hours after ^3H thymidine addition. Data are representative of 3 experiments.

activated T cells, in contrast to its ability to enhance SEB responses in resting T cells by acting as an adhesion molecule.

Transfectants co-expressing DR4 and both B7-1 and LFA3 (Figure 3.7) were also used to examine costimulation of SEB proliferation in SEB responsive T cells. When DR4/LFA3/B7-1 cells were added to SEB stimulated T cells an increased response compared to responses demonstrated previously with DR4 or DR4/B7-1 was seen (Figure 3.13). The proliferation response was equivalent to proliferation induced by SEB in PBMCs. This demonstrated that DR4/LFA3/B7-1 transfectants provided the T cell lines with the necessary costimulation requirements to respond to SEB. This suggested that both adhesion and costimulation were necessary in order to produce optimum proliferation in response to SEB in activated T cells. Increasing the adhesive interactions may also facilitate enhanced B7-1/CD28 interactions. Proliferation increased as concentration of SEB increased, therefore these antigen specific T cells were capable of proliferating to SEB in a dose dependent manner similar to PBMCs (Figure3.13).

The production of IL-2 was also evaluated using CTLL-2 bioassay. Addition of SEB directly to the T cell lines did not result in IL-2 production (Figure 3.13) which was consistent with the lack of proliferation induced by SEB in these cells. In contrast, when DR4/LFA3/B7-1 was added to these cultures, IL-2 was produced, which increased as the concentration of SEB increased from 0.1 to 1.0 μg/ml. Therefore SEB-specific T cells produced IL-2 in response to costimulated SEB signals in a dose dependent fashion which correlated with proliferation responses. These cells express IL-2 receptor (Table 3.1) and were therefore capable of proliferating in response to IL-2 produced. The signal generated by SEB alone when added to previously activated T cells was insufficient to generate proliferation or IL-2 production in these cells whereas the signals generated by presenting SEB using DR4/LFA3/B7-1 transfectants was sufficient to allow proliferation of these cells and to enable production of IL-2.

It was also observed (Figure 3.13) that addition of SEB to some of the T cell lines resulted in proliferation while increasing the dose of SEB resulted in a decrease in proliferative responses. This suggested that engagement of the TCR by SEB alone in these cells may be resulting in an outcome other than proliferation, perhaps inducing anergy or apoptosis. Interestingly, addition of B7-1 or B7-1-2 transfectants to activated T cells resulted in a proliferative response which was inhibited by addition

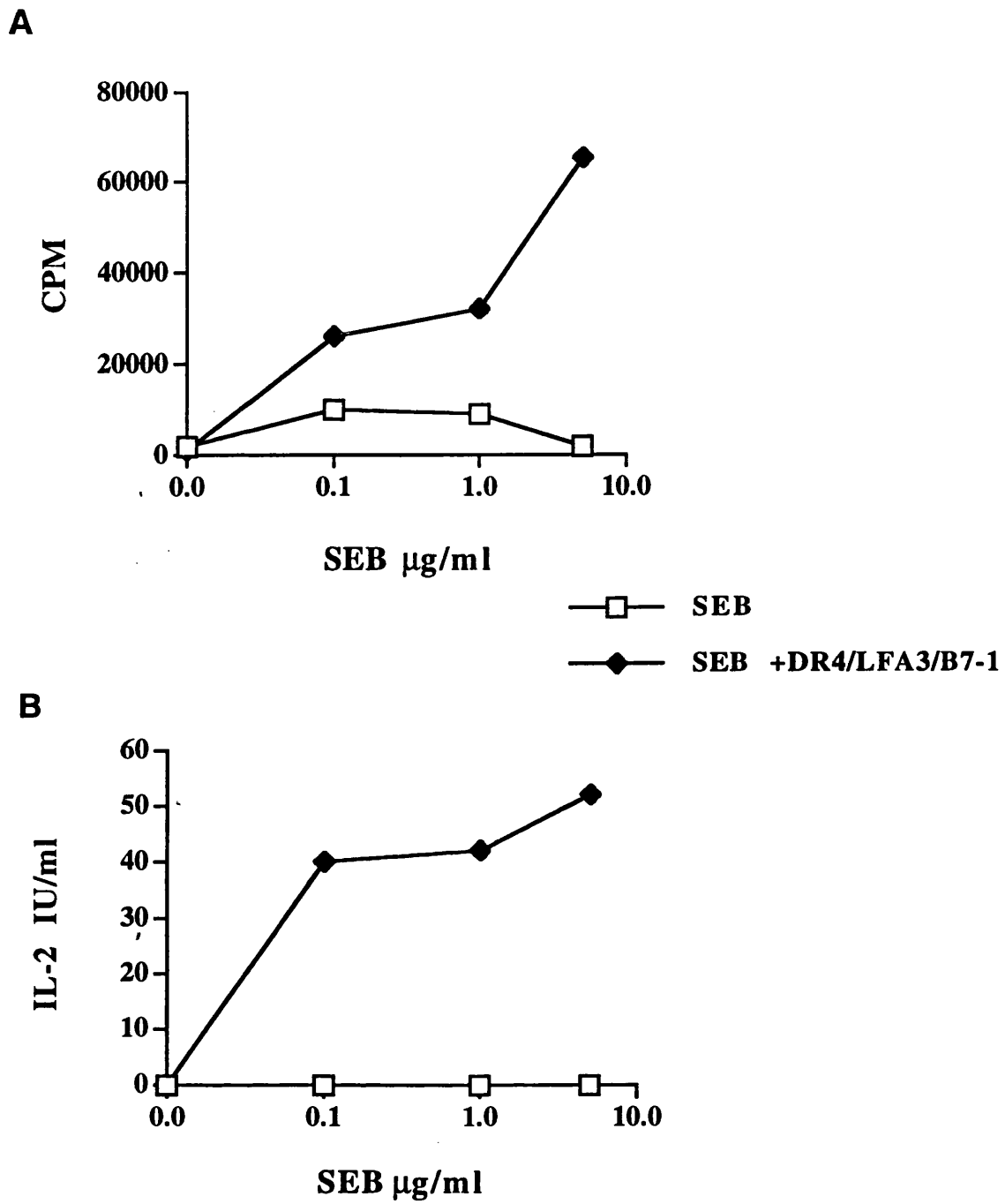


Figure 3.13. Costimulation of SEB driven proliferation in SEB specific T cells.
SEB specific T cells (5 x 10^4) were stimulated for 3 days by the addition of SEB at various concentrations either alone or pulsed onto DR4/LFA3/B7-1 transfectants (2.5 x 10^4). Proliferation (A) was measured using thymidine incorporation while IL-2 production (B) was measured using a CTLL-2 bioassay. Data are representative of 4 experments.

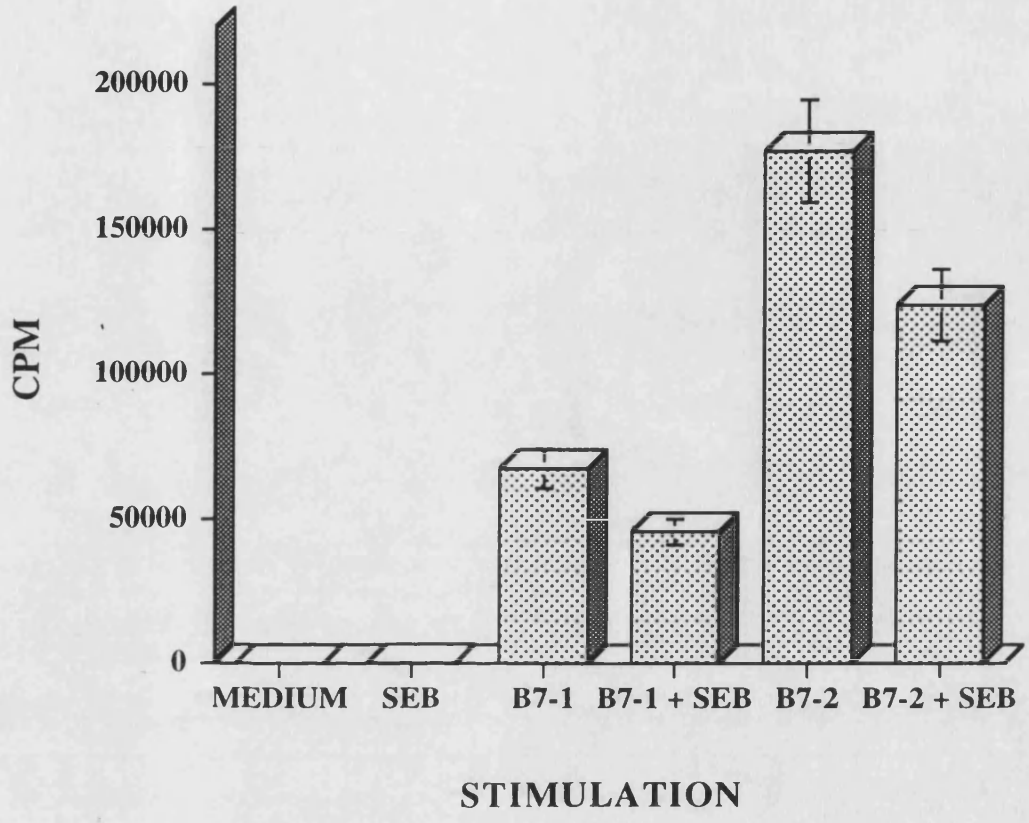


Figure 3.14 SEB effect on B7-1 and B7-2 stimulated proliferation of antigen specific T cells.

B7-1 or B7-2 transfectants (2.5×10^4) were used to stimulate SEB specific T cells (5×10^4) for 3 days. The effect of addition of SEB (1μg/ml) on B7-1- or B7-2- stimulated proliferation was determined. Proliferation was measured 18 hours after ^3H thymidine addition. Data are representative of 2 experiments.

of SEB (Figure 3.14). It was therefore suggested that SEB either by binding directly to the TCR or by autopresentation on Class II molecules may be inducing death of antigen-specific T cells .

3.5 Discussion.

The proliferation requirements of human T cells in response to a variety of stimuli have been explored here. The removal of T cells from PBMCs resulted in the loss of the T cell proliferative response to anti-CD3, PHA and SEB demonstrating the requirement for accessory cell dependent factors to costimulate T cell activation signals. It was therefore concluded that T cell proliferation required costimulation in addition to engagement of the TCR, in agreement with the two signal model of T cell activation first proposed by Bretscher and Cohn (1970).

It has been shown that signals received by a T cell when the TCR is triggered with anti-CD3 antibodies required costimulation which was provided by accessory cell dependent molecules. B7-1 costimulated anti-CD3 proliferative responses equivalent to that seen in PBMCs. Therefore signal one was provided by triggering of the TCR by anti-CD3 antibody while signal two was provided by B7-1 ligation of CD28. CD28 is thus capable of providing a signal distinct from that mediated through the TCR. Consequently,,the B7-1/CD28 interaction plays a critical role in anti-CD3 stimulated T cell proliferation.

The interaction of LFA3 with CD2 on T cells was however unable to costimulate TCR signalling. Certain combinations of anti-CD2 mAbs can activate T cells alone (Moingeon et al 1989) which suggested that there were differences between ligand induced signals and those induced by antibodies. Mimicking CD2 ligation using LFA3 expressed on cell surface is a more physiologically relevant response, therefore implying that the natural ligand LFA3 is unable to costimulate TCR signalling. The interaction between LFA3 and its ligand CD2 on T cells is therefore predicted to act merely as a adhesive interaction and does not function as a costimulus of TCR signalling during anti-CD3 stimulation of T lymphocytes.

The use of anti-CD3 required a costimulus to induce proliferation in these cells which may also reflect the ability of anti-CD3 to induce anergy in these cells, although induction of anergy in peripheral blood T cells was not directly demonstrated. The ability of B7-1 to provide this costimulus is consistent with a role of this molecule in

the prevention of T cell anergy. The induction of hyporesponsiveness by MHC presentation of antigen on non-lymphoid cells which do not express either B7-1 or B7-2 (Burkly et al 1990, Lo et al 1989) is in agreement with CD28 signalling abrogating T cell tolerance. Moreover, Harding and colleagues (Harding et al 1992) have elegantly demonstrated the prevention of unresponsiveness using anti-CD28 monoclonal antibody. This is in agreement with a direct role for CD28 in the prevention of tolerance. These data further emphasise the role the CD28/B7 interaction plays in T cell effector function.

The costimulation requirements of PHA driven responses differ from anti-CD3 induced responses in that either LFA3 or B7-1 can provide the costimulation necessary for T cell proliferation and IL-2 production. One possibility is that PHA, in addition to signalling via the TCR may also bind to the CD2 molecule on T cells. Once this ligation takes place engagement of CD2 by LFA3 may result in sufficient signalling to facilitate T cell activation. In addition PHA may also bind to other cell surface molecules to strengthen the adhesive interaction as it was observed microscopically that PHA addition to T cell cultures resulted in cellular aggregation. The fact that pairs of antibodies are required to costimulate through CD2 (Moingeon et al 1989) is consistent with this observation. The ability of LFA3 to costimulate PHA proliferative responses differed from that seen with anti-CD3 stimulated cells which may reflect the ability of PHA to signal through the CD2 complex. One interpretation was that concommitant complexing of two determinants on the CD2 molecule, the first by PHA and the second by LFA3 together resulted in a productive CD2 signal leading to proliferation.

B7-1 was capable of providing costimulation for PHA responses, moreover this molecule was a more potent inducer of both proliferation and production of IL-2, which further emphasised the importance of the B7/CD28 interaction in facilitating T cell effector function. Therefore during PHA-mediated responses both LFA3 and B7-1 may provide the additional signals necessary for T lymphocytes to proliferate and produce IL-2. Once again the results of these experiments highlighted the importance of costimulation in T cell activation.

In order to examine MHC-dependent responses, the SAg SEB was used and as expected it was shown that T lymphocytes respond to this antigen in an MHC-dependent manner. This is a more physiological stimulus since peptide antigens are presented on MHC molecules on the surface of APCs, therefore the costimulation

requirements for SEB proliferation were investigated. It was demonstrated that either LFA3 or B7-1 co-expression on a class II MHC positive APC was capable of costimulating SEB responses. Similar to responses seen in PHA-driven activation, B7-1 was a much more potent costimulatory molecule than LFA3. Therefore SEB responses were also dependent on costimulation which could be provided by LFA3 or B7-1 on the same cell on which antigen was presented. This is in agreement with data published by Damle and colleagues (1992) who demonstrated that SAg-primed T cells can proliferate efficiently by engaging B7-1. In contrast it has been demonstrated that SAg-induced T cell responses are not critically dependent on the interaction of CD28 with B7-1 (Damle et al 1993A). However, the discrepancy in these results may reflect the use of SEA rather than SEB in these studies. Additionally, CTLA-4-Ig was used to block proliferation whereas the direct use of B7-1 ligand for CD28 was utilised here which is a more physiological test of the ability of the CD28/B7-1 interaction to costimulate responses. One possibility is that the B7-1/CD28 interaction can costimulate SAg responses but when this is blocked other interactions such as LFA3/CD2 or as yet unidentified interactions compensate to allow proliferation to occur. CD28 costimulation may therefore be the primary interaction but other pathways may compensate if required. Indeed a monocyte derived costimulatory factor has recently been identified which synergises with B7-1-dependent costimulation (Johnson and Jenkins 1994).

It has been argued that one essential feature of a costimulatory molecule is its ability to provide signal two (the costimulus) from a cell separate from the one engaging the TCR and providing signal 1 (Jenkins et al 1988). To determine whether either LFA3 or B7-1 meet these requirements SEB was presented on a cell distinct from the cell providing accessory molecule stimulation. B7-1 but not LFA3 was capable of costimulating from a separate cell from the one triggering the TCR. The LFA3 molecule therefore appears to function as an adhesion molecule in SEB responses whereas B7-1 is suggested to provide a signal distinct from that induced via the TCR. Consequently, the CD28/B7-1 interaction provides signals which synergise with TCR-mediated signals to enable T cell activation to proceed. CD28 signalling may therefore be considered as being truly costimulatory and acting as signal 2 in the two signal model of T cell activation (Bretscher and Cohn 1970).

Recently, the ability of B7-1 to provide third-party costimulation has been looked at using a mouse system (Liu and Janeway 1992). Interestingly, the authors concluded that delivery of two signals from separate cells was substantially less efficient than

their combined delivery by one cell. There may be several reasons for the difference between these results and the results demonstrated here, first, in the experiments carried out by Liu and Janeway, they used anti-CD3 to stimulate the TCR, while SEB was used here. Second, the levels of B7-1 expressed on the costimulator cells may differ between the two experimental systems thereby affecting the ability of the third party cells to costimulate. Third, the mouse system may differ from humans in its ability to receive third party costimulation. Interestingly, mouse T cells do not express class II MHC when activated in contrast to human T cells which upregulate class II MHC on activation. However some of the data shown are consistent with that of Liu and Janeway in that expression of both ligands on the same cell is a more potent stimulus for proliferation .

In order to determine whether proliferation requirements differed between resting and activated T cells, SEB responsive T cell lines were developed. B7-1 costimulated SEB- driven signals in previously activated T cells, while LFA3 did not costimulate SEB responses. These data add weight to the hypothesis that LFA3 does not function as a costimulatory molecule but merely acts to enhance adhesive interactions. LFA3 and B7-1 together provided SEB-specific T cells with the interactions necessary to produce a proliferative response equivalent to that demonstrated for PBMCs. LFA3 might act to strengthen adhesion between the T cell and the APC and enhance B7-1/CD28 interaction. Productive engagement of the TCR resulted in IL-2 production by SEB specific T cells which was dependent on B7-1 and LFA3 costimulation. IL-2 production was not elicited in these cells by SEB alone, thus costimulation signals were required in addition to TCR stimulation to lead to T cell effector function.

The importance of the B7-1/CD28 interaction in T cell effector function has been highlighted by these results and the potency of this reaction leads to the suggestion that understanding the molecular and biochemial pathways involved in CD28 is crucial to understanding the role played by T lymphocytes in immune regulation.

Interestingly, the addition of SEB alone to antigen-specific T cells resulted either in no or very poor proliferative responses which decreased as the dose of SEB increased. B7-1 and B7-10 alone stimulated proliferation responses in antigen-specific T cells which is in agreement with data from Liu and Janeway 1992. Therefore B7-1 was capable of directly stimulating activation , hence emphasising the potency of this interaction with CD28. Additionally SEB was demonstrated to have an inhibitory effect on B7-1 or B7 -2 stimulated T cell responses. This led to

the suggestion that SEB was inducing anergy or apoptotic death in activated T lymphocytes. Consequently, having examined the requirements for costimulation of proliferation in resting and activated T lymphocytes to SEB it was next decided to look at the ability of SEB to induce apoptosis in T cells.

CHAPTER FOUR.

RESULTS II.

APOPTOSIS REQUIREMENTS OF T LYMPHOCYTES

4.1 Apoptosis in T lymphocytes:

Previously, it has been demonstrated that TCR signalling in the absence of APCs resulted in a poor proliferative response in resting and activated T cells. The proliferation requirements of resting and activated T cells have been examined and it is hypothesised that in addition to proliferation , PHA and SEB may also induce a death response in these cells. The most common morphological form of T cell death in the periphery appears to occur via apoptosis, therefore the ability of SEB and PHA to induce apoptotic death in resting and activated T cells was examined.

4.1.1 PHA-induced apoptosis in resting T lymphocytes.

Previously it was shown that PBMCs proliferated vigorously in response to PHA. In contrast the addition of PHA to purified T cells did not result in a proliferative response, demonstrating the requirement for accessory cell dependent factors for PHA responses. Proliferation and IL-2 production was restored in these cells by B7 or LFA3 costimulation indicating that additional signals were required to fully activate PHA stimulated T lymphocytes. Having examined the effect of PHA on T cell proliferation it was next decided to determine whether this stimulus induces death in these cells. Therefore, PHA (5μg/ml) treated T lymphocytes were examined for evidence of apoptosis by staining DNA with propidium iodide, followed by FACS analysis. Apoptosis was measured by the appearance of a sub G_0 peak representing condensed and fragmented apoptotic nuclear DNA. However, PHA treatment did not result in the apppearance of an apoptotic sub G_0 peak in resting T lymphocytes (Figure 4.1). Interestingly almost all the treated cells were in the G_0 (or resting) phase of the cell cycle with very few in S phase during which cells replicate their DNA prior to division. This demonstrated that PHA did not induce a proliferative response in resting T lymphocytes, consistent with what has been previously demonstrated. One possibility was that these cells were refractory to PHA-induced apoptosis because they were not proliferating and were therefore not progressing through the cell cycle.

4.1.2 PHA-induced apoptosis in Jurkat T cells

In order to examine this hypothesis further, advantage was taken of the Jurkat T cell line which proliferates continuously in culture without additional growth factors. Jurkat T cells

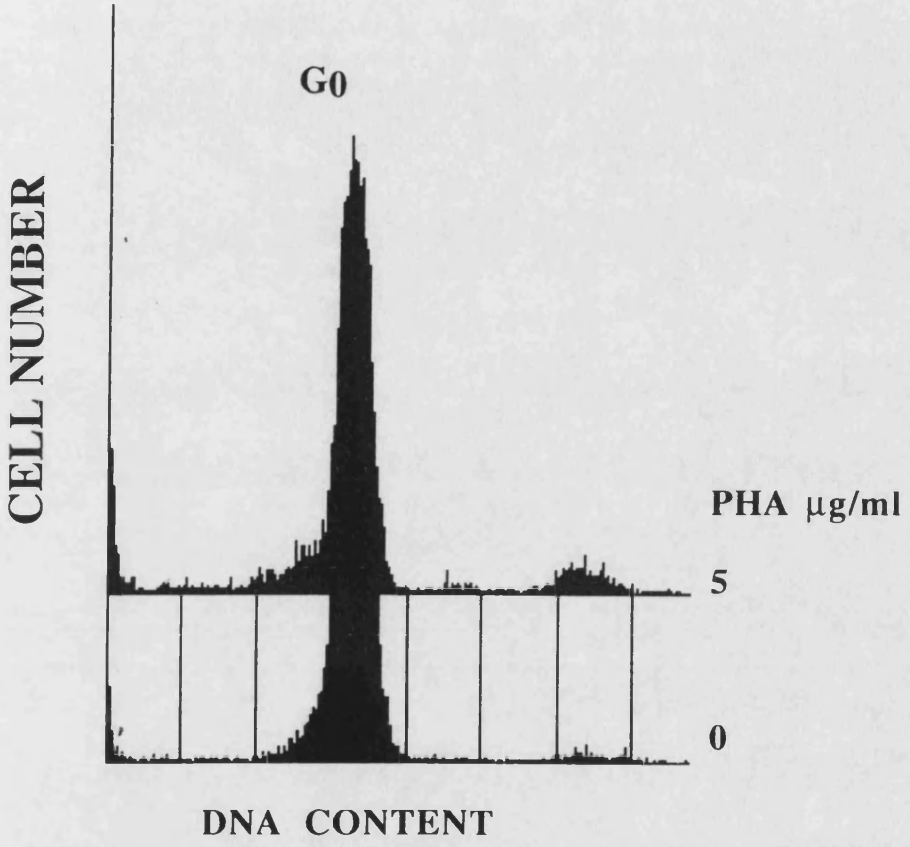


Figure 4.1 PHA-induced apoptosis in resting T lymphocytes.
Freshly isolated T lymphocytes (5x 10^4) were cultured for 72 hours in medium with or without the addition of PHA (5µg/ml). Cells were fixed with EtOH at 0°C, stained with propidium iodide and examined by FACS analysis using a Becton Dickinson FACStar Plus for evidence of cells demonstrating sub G_0 DNA which have undergone apoptosis

may therefore respond differently to PHA stimulation compared to resting T lymphocytes and may be susceptible to PHA-induced apoptosis. Proliferation was measured in unstimulated and PHA treated Jurkats and it was seen (Figure 4.2) that PHA totally inhibited normal proliferation in these cells. It was possible that PHA was inducing apoptosis in these cells. Thus PHA-treated cells were examined for evidence of apoptotic cell death by FACS analysis. The appearance of a sub G_0 peak in PHA-treated cells was demonstrated (Figure 4.2) which suggested that PHA inhibits proliferation of Jurkat T cells by inducing apoptosis. This indicated that the direct outcome of stimulation by PHA in these T cells is apoptotic death. This was in contrast to the inability of PHA to induce death in resting T lymphocytes adding weight to the hypothesis that resting cells were resistant to PHA-induced apoptosis because they were not dividing. Additionally, there must be some cell cycle arrest since not all DNA showed apoptotic features. There are check points in the cell cycle during which DNA is synthesised where cells may become sensitive to induction of the death pathway. Consequently, continuously cycling cells such as Jurkat T cells may be more sensitive to apoptosis than resting T lymphocytes which are not progressing through the cell cycle.

Therefore, PHA was useful as a model to begin to study apoptosis in T lymphocytes. One possibility was that PHA-induced apoptosis in Jurkat cells may be the result of lack of costimulation, hence it was attempted to provide costimulation using LFA3 and B7-1 transfectants. Both LFA3 and B7-1 had previously been demonstrated to costimulate PHA-dependent proliferation in resting T lymphocytes. The ability of B7-1 and LFA3 costimulation to rescue proliferation in PHA treated cells was therefore examined. However, in contrast to the costimulation seen in resting T lymphocytes, proliferation was not restored by the addition of these factors (Figure 4.3). The predominant response of Jurkat cells to PHA stimulation was thus apoptotic death, which may reflect the fact that these are transformed cells and may exist in a preapoptotic state already primed for the induction of apoptosis.

In addition to costimulating T cell proliferation, B7-1 and LFA3 have also been shown to be capable of costimulating IL-2 production in resting T cells. It was therefore decided to ascertain if costimulation of PHA responses in Jurkats resulted in IL-2 production. Interestingly, IL-2 production was increased on costimulation (Figure 4.3), the response to B7-1 being more potent than for LFA3. This was consistent with data previously shown demonstrating that B7-1 costimulation of resting T lymphocytes resulted in greater proliferation and IL-2 production than LFA3 costimulation (Chapter 3). The inability of

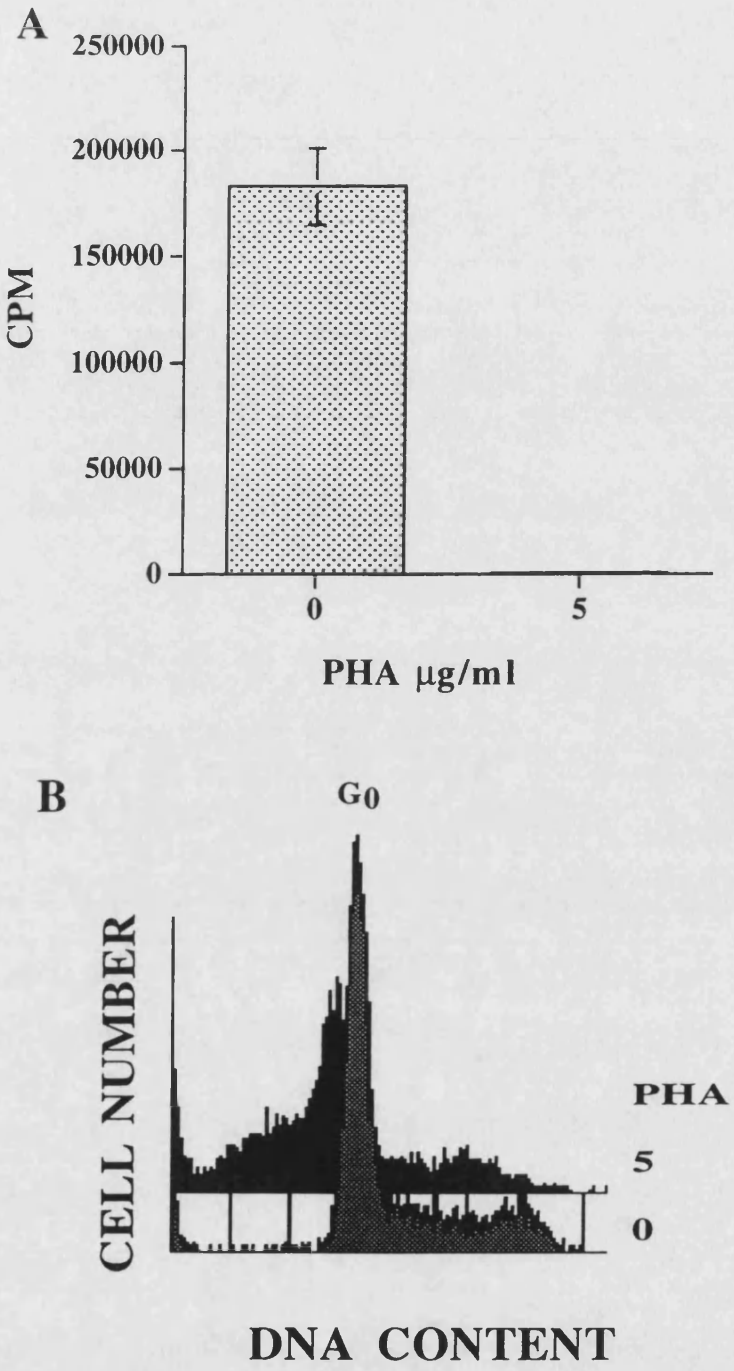


Figure 4.2. PHA effect on Jurkat T cells.
Jurkat cells were cultured in medium for 18 hours with or without the addition of PHA (5µg/ml) and proliferation (**A**) or apoptosis measured (**B**). Cell numbers for proliferation were $5x10^4$ and for apoptosis $1x10^6$. Proliferation was measured by ^3H thymidine incorporation while apoptosis was measured by staining cells with propidium iodide followed by examining DNA by FACS analysis using a Becton Dickinson FACStar Plus. A and B: representative of 4 experiments

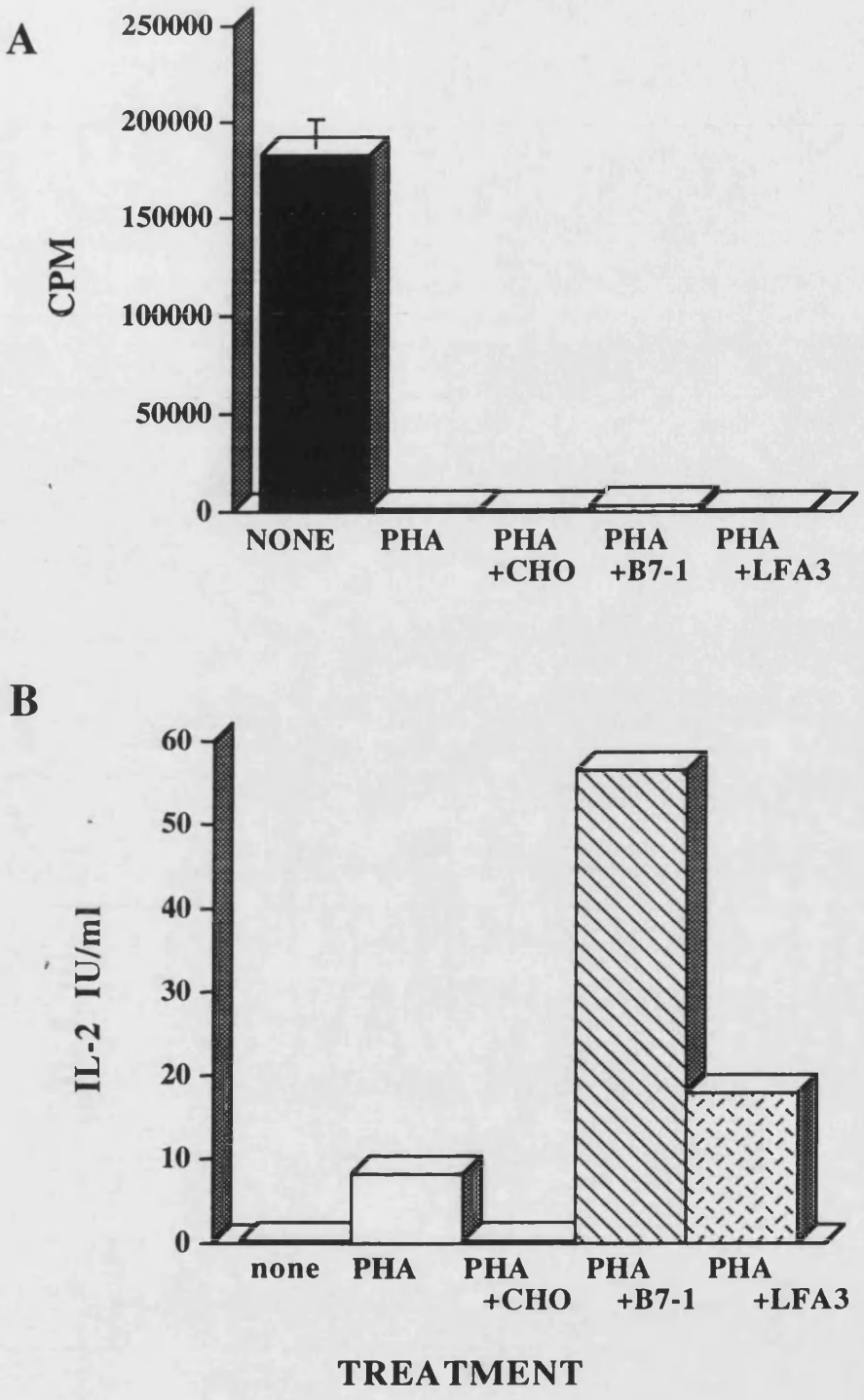


Figure 4.3 Costimulation of PHA-induced responses in Jurkat cells .
Jurkat cells (5 x 10^4) were cultured for 18 hours in medium with or without (none) PHA at 5µg/ml. CHO, LFA3 or B7-1 cells (2.5 x 10^4) were added to PHA stimulated cells. and proliferation (**A**) or IL-2 responses (**B**) determined. Proliferation was measured by ^3H thymidine incorporation while IL-2 prodction was measured using a CTLL-2 bioassay. Data are representative of 4 experiments.

costimulation of IL-2 responses to rescue proliferation was interpreted by the fact that Jurkat T cells do not express IL-2 receptors. and were thus incapable of responding to the IL-2 produced.

PHA therefore induced apoptosis in Jurkat T cells while proliferation was inhibited and could not be restored by LFA3 or B7-1 costimulation. B7-1 was demonstrated to be a more potent costimulator of IL-2 responses which was consistent with the importance of CD28 signalling in IL-2 production and the effector function of T lymphocytes.

4.2. Dose reponse of Jurkats to PHA-induced apoptosis.

In order to look at the requirements for PHA-induced apoptosis, death in Jurkat T cells was examined in greater detail. Jurkat T cells treated with various concentrations of PHA were examined using FACS analysis, the cells being stained with both propidium iodide and the fluorochrome Hoechst 33342 (Figure 4.4 and 4.5) to establish a useful dose range of PHA. It can be seen by FACS analysis of propidium iodide-stained cells (Figure 4.4) that even at doses of PHA as low as 0.5μg/ml there was a proportion of cells induced to die by apoptosis, which increased as the concentration increased. Further evidence of the dose dependency of PHA-induced apoptotic death was investigated using the fluorochrome Hoechst 33342 (Figure 4.5), with increasing amounts of this dye being taken up as the dose of PHA was increased.The advantages of using this dye are that live cells can be used and the staining procedure is faster than for propidium iodide. Conversely, a FACS machine equipped with a uv laser must be used to analyse results which is slightly disadvantageous.

Together these results demonstrated that PHA induced dose dependent apoptosis in T lymphocytes. From these experiments the working concentration of PHA to be used was chosen to be 5μg/ml unless stated differently.

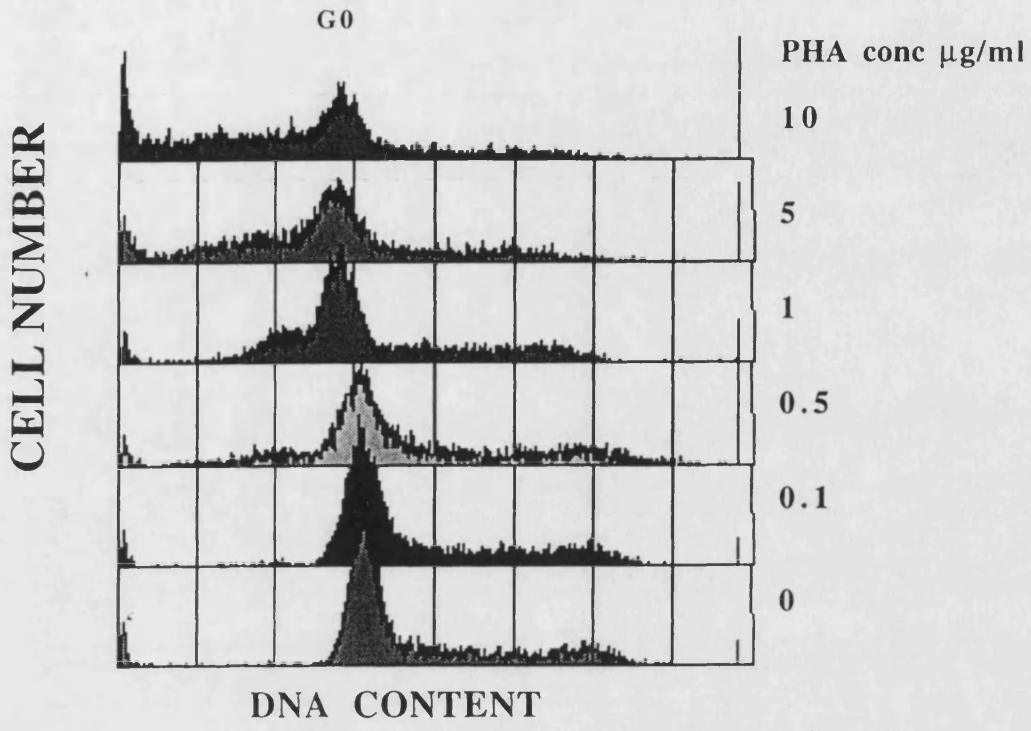


Figure 4.4 PHA-induced apoptosis : dose response in Jurkats
Jurkat cells ($1x10^6$) were treated for 18 hours with PHA at indicated concentrations. Cells were fixed at 0°C using EtOH, stained with propidium iodide and DNA content examined by FACS analysis using a Becton Dickinson FACStar Plus. Data are representative of 3 experiments

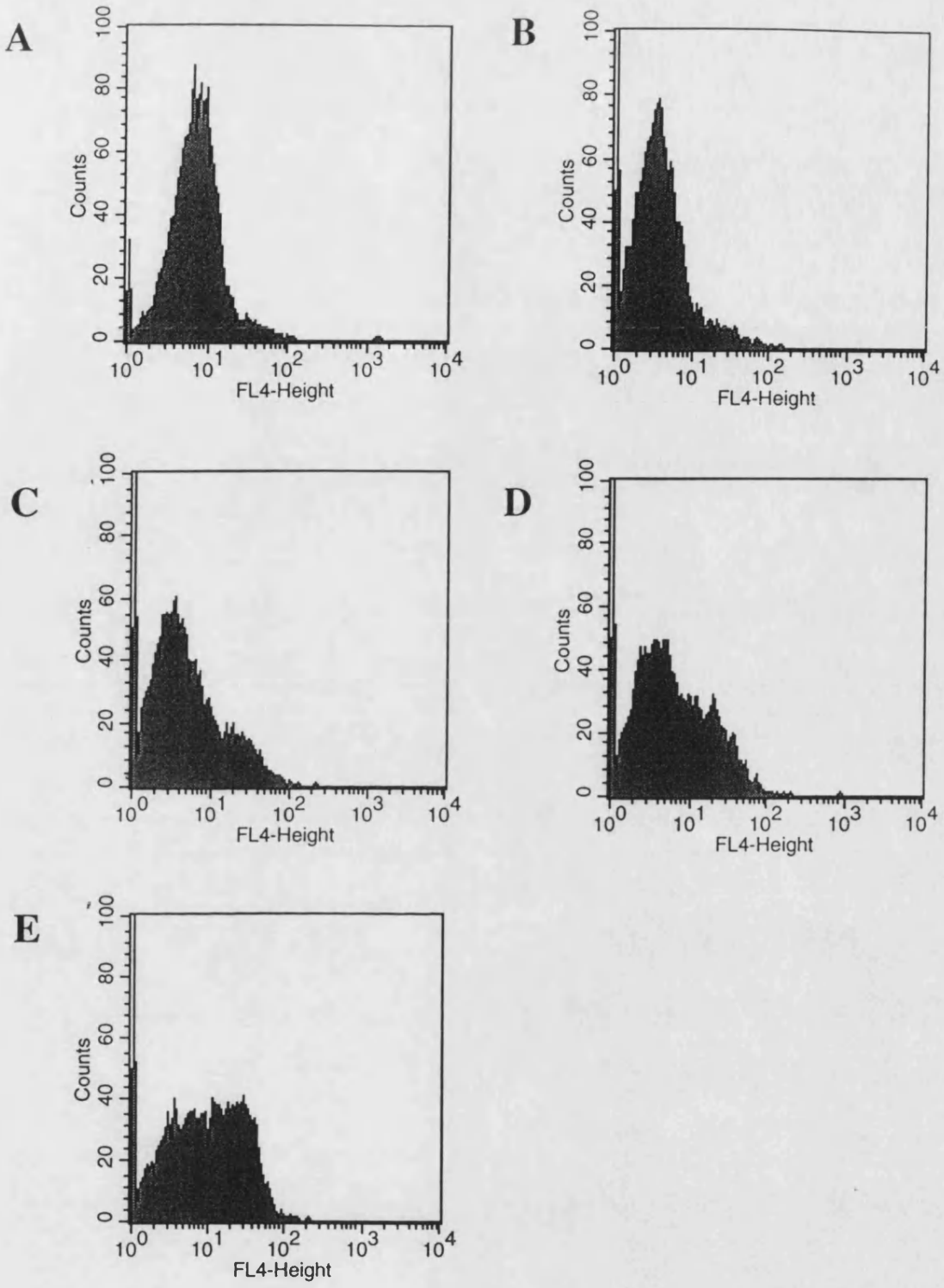


Figure 4.5 PHA-induced apoptosis in Jurkats : dose response measured using Hoechst staining.

Jurkat cells ($1x10^6$) were treated for 18 hours with PHA at various concentrations (A = 0, B = 0.5, C = 1, D = 5, E = 10 µg/ml). Cells were stained with the fluorochrome Hoechst 33342 and examined by FACS analysis using a FACS Vantage equipped with a 50mW uv laser. Necrotic cells were eliminated by simultaneously staining cells with propidum iodide and gating using FL-2. Data are representative of 2 experiments

4.3 Fas expression and apoptosis in Jurkat T cells.

It was next decided to examine the mechanism involved in PHA-induced apoptosis. One pathway by which apoptosis of T cells occurs is via ligation of the Fas receptor. Jurkat T cells express Fas (Figure 4.6), consequently, one possibility was that PHA-induced death might be mediated via a Fas-dependent pathway. In order to examine whether these cells were susceptible to Fas-induced apoptosis induction of apoptotic death using an anti-Fas mAb (CH11) was examined. Jurkats were treated with CH11, stained with propidium iodide and examined by FACS analysis. A substantial increase in the number of cells appearing in the sub G_0 region was observed indicating loss of DNA stainability with propidium iodide due to condensation of chromatin and DNA degradation (Figure 4.6D). Jurkat T cells thus underwent apoptotic death in response to ligation of Fas receptor. Interestingly however, apoptosis seen in these cells was of much greater magnitude than that seen in PHA-induced death. This suggested either that the antibody treatment was much more potent than PHA treatment or alternatively that PHA-induced death may be Fas-independent

4.4 Kinetics of PHA-induced apoptosis.

In order to further dissect PHA-induced death of Jurkats the kinetics of induction of apoptosis was determined. Cells were taken at various time points after the addition of PHA and their DNA examined for apoptosis. Surprisingly, very little death was in evidence up to 8 hours while significant amounts of apoptotic death only occured after 18 hours (Figure 4.7), PHA therefore induced slow time-dependent apoptotic death in Jurkat T cells. In order to determine whether PHA death could be mediated via ligation of Fas the kinetics of Fas- induced apoptosis in Jurkats was also examined (Figure 4.7). A large number of apoptotic cells were seen as early as 2 hours post addition of CH11 followed by a rapid rise in apoptotic cell numbers increasing to almost 70% by 18 hours. The kinetics of these systems were therefore remarkably different and it was predicted that PHA did not induce apoptosis via a Fas-dependent pathway.

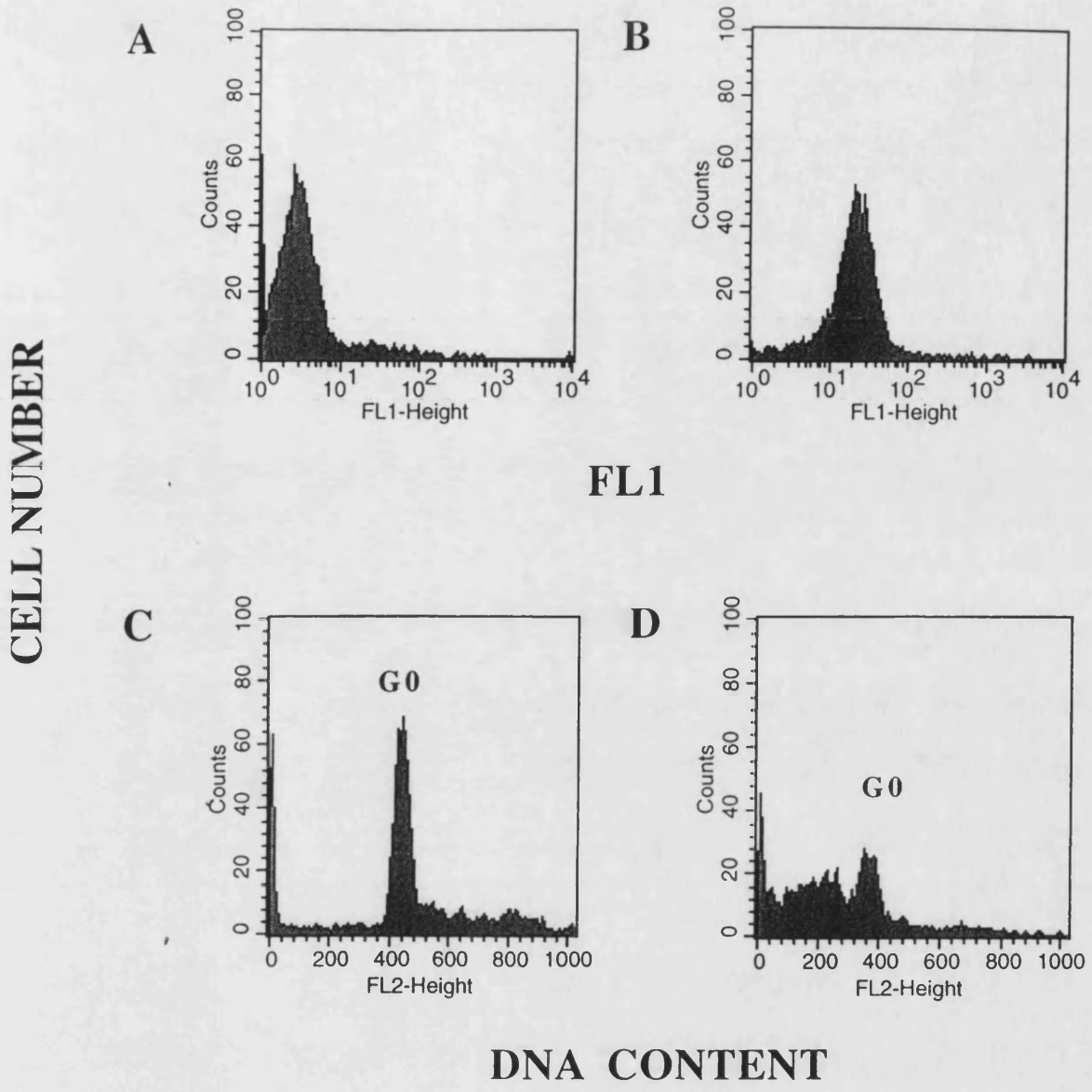


Figure 4.6 Fas expression on Jurkat cells and Fas-induced apoptosis.
Jurkat cells ($5x10^5$) were stained for 45 minutes at 0°C with a control Ig (**A**) or an anti-Fas mAb M3 (1μg/ml) (**B**) followed by 45 minutes at 0°C with anti-mouse FITC conjugated Ig and examined by FACS analysis. In order to look at Fas-induced apoptosis, Jurkat cells ($1x10^6$) were treated for 18 hours with control Ig (**C**) or anti-Fas mAb CH11 (0.1μg/ml) **(D),** fixed with EtOH at 0°C and stained with propidium iodide. DNA was then examined by FACS analysis for evidence of sub G_0 apoptotic DNA using a Becton Dickinson FACStar Plus. Data are representative of 4 experiments.

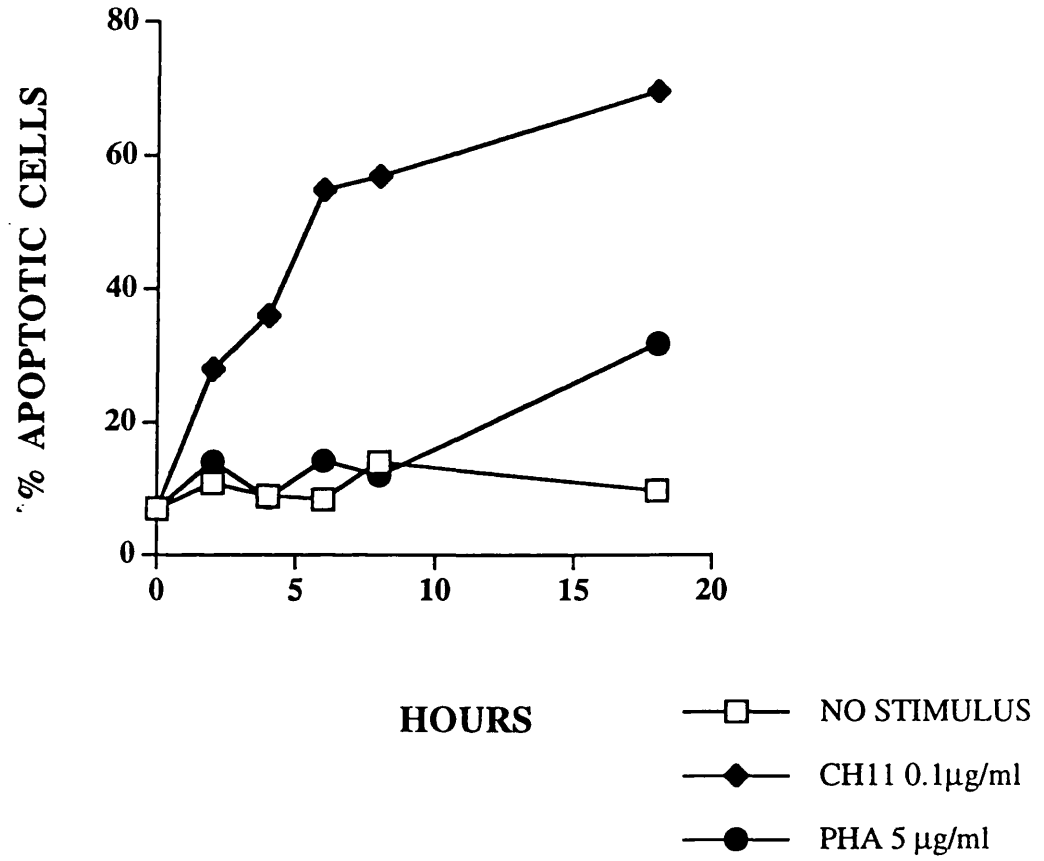


Figure 4.7 Kinetics of PHA and Fas- induced apoptosis.
PHA (5µg/ml), anti-Fas mAb CH11(0.1µg/ml) or medium alone was added to Jurkat cells ($1x10^6$) and cells incubated at 37°C. At time points indicated cells were fixed, DNA stained with propidium iodide and% apoptotic cells determined by measuring cells with DNA in the subG0 region by FACS analysis using a Becton Dickinson FACStar Plus. Data are representative of 4 experments.

4.5 Modulation of PHA induced apoptosis.

The signals involved in apoptosis have yet to be completely elucidated although there are indications that a calcium-dependent endonuclease is involved in DNA fragmentation. In order to determine whether the mechanism of death induced by PHA involved a calcium signal, cyclosporin A(CsA) was utilised in an attempt to inhibit apoptosis. CsA appears to modulate T cell functions by blocking Ca -dependent signalling via the calcineurin dependent second messenger pathway, therefore the effect of CsA on PHA induced death was examined. A small amount of inhibition of PHA-induced apoptotic death was seen using CsA even at doses of PHA as high as 10μg/ml (Figure 4.8) although inhibition was not complete. This suggested that although calcium signalling was involved in PHA-induced apoptosis it may not be a critical factor.

To examine whether other receptor-ligand interactions were involved in PHA-induced apoptosis, antibody blocking experiments were performed in an attempt to prevent death. None of the antibodies tested substantially inhibited the apoptotic death induced in Jurkats by PHA (Figure 4.9). Binding of these antibodies to the appropriate cell surface receptor would occur before onset of apoptosis as suggested by the kinetics of PHA-induce death consequently, the antibodies would have access to their ligand. Interestingly, the antibody which had some effect on PHA- driven apoptosis was an anti-CD28 antibody, (9.3) indicating that the CD28-B7-1 interaction may be involved in the induction of apoptosis. This was in contrast to the inability of B7-1 molecules to rescue the proliferative response in PHA-treated Jurkats but reflected the ability of B7-1 to costimulate IL-2 production in PHA-treated cells.

Together these results suggested that there was minimal requirement for cell contact in PHA-induced apoptosis. However as expected from the definition of PHA as a lectin, microscopic examination PHA treated T cells appeared clustered, therefore one of the characteristics of PHA was to cause aggregation of T cells in culture. This may not however be a necessary event for the induction of apoptosis.

4.6 Involvement of Fas in PHA-induced apoptosis of Jurkats.

The different kinetics of PHA-induced death compared to Fas-induced apoptosis suggested that PHA-induced apoptosis proceeded via a Fas-independent pathway. In order to

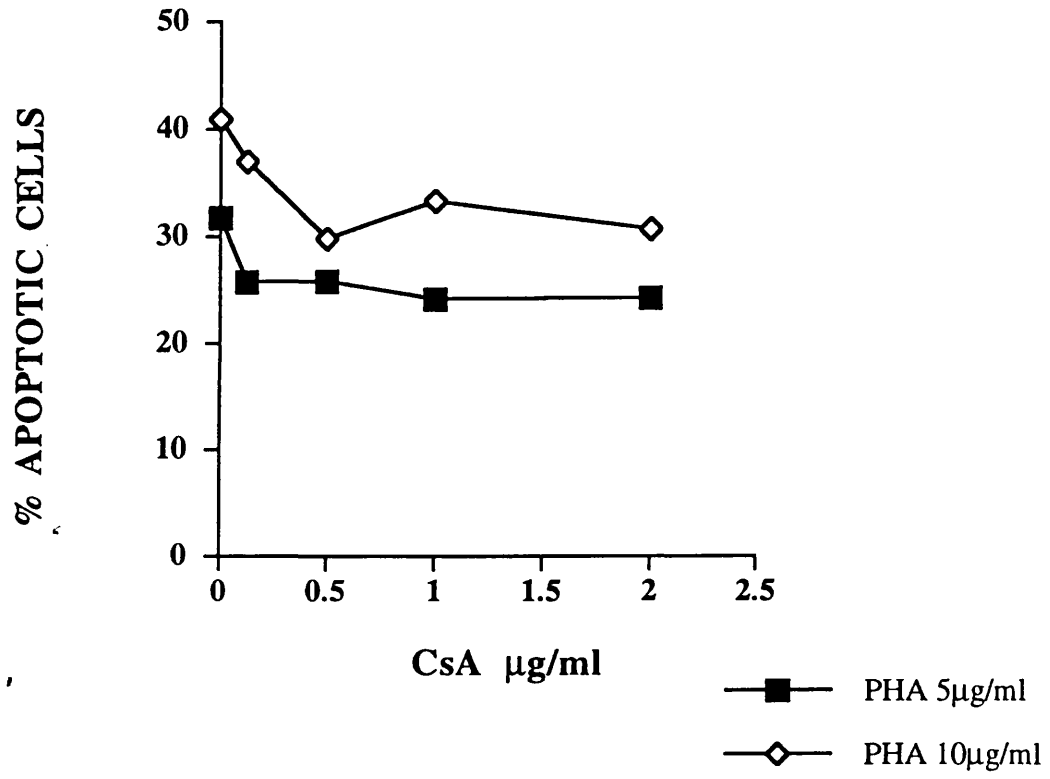


Figure 4.8 Effect of cyclosporin A (CsA) on PHA-induced apoptosis.
Jurkat T cells (1x 10^6) were treated with PHA at concentrations indicated and the effect of varying concentrations of CsA on PHA-induced apoptosis was determined by measuring the % of apoptotic cells using FACS analysis of fixed propidium iodide stained cells . Data are representative of 3 experiments.

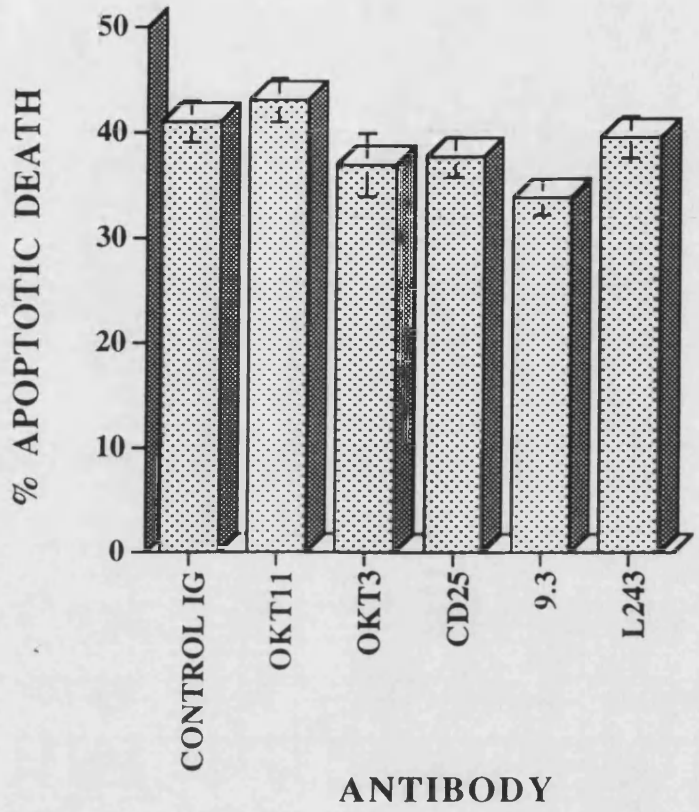


Figure 4.9 Modulation of PHA-induced apoptosis.
The effect of antibodies to various cell surface molecules on PHA-induced apoptosis in Jurkat cells (1x 10^6) was determined by measuring % apoptotic cells by propidium iodide staining and FACS analysis. Antibodies used were to CD2 (OKT11), CD3 (OKT3), CD25 (CD25), CD28 (9.3) class II MHC (L243) or control antibody (control Ig). Data are representative of 3 experiment

definitively investigate whether PHA induced death occured independently of Fas receptor engagement, an anti-Fas blocking antibody (M3) was used in an attempt to block apoptosis. Very little inhibition of PHA-induced apoptosis was seen (Figure 4.10) indicating that PHA-induced apoptosis did not depend on Fas engagement. Overall, these finding suggested that Fas was not involved in PHA-induced apoptosis in Jurkats. There are many other possibilities for the death pathway induced by PHA in these cells including the involvement of TNF, the activation of an ICE-like cysteine protease or engagement of as yet unidentified receptors. Further work is needed to determine the pathway utilised in PHA-mediated death of Jurkat T cells.

4.7 Summary.

T lymphocytes have been examined for evidence of PHA-induced apoptotic death, and it was observed that resting T cells were resistant to death. Lack of proliferation in these cells was hypothesised to be one factor determining resistance to death. Evidence for this hypothesis was provided by the demonstration that PHA induced apoptosis in continuously proliferating Jurkat cells. Jurkat cells are transformed and may therefore be in a preapoptotic state expressing altered levels of bcl-2 or c-myc. Much evidence has accumulated to support the hypothesis that activated T cells are more susceptible to apoptosis than resting cells. It has been shown that IL-2 treatment can render cells sensitive to apoptosis by driving them into a proliferative state (Leonardo 1991) during which the cells synthesise DNA and may become more susceptible to death.

PHA induced dose- and time- dependent apoptosis in Jurkat T cells, however the kinetics of death were very different to that seen during Fas-dependent death. Consequently, it was predicted that Fas-mediated apoptosis was not involved in the death induced in Jurkats by addition of PHA. More importantly, PHA-induced death was not inhibited by an anti-Fas blocking antibody which lead to the conclusion that PHA-induced death was independent of Fas.

Interestingly, an antibody to CD28 had some inhibitory effect on PHA-induced death, indicating the involvement of the CD28 signalling pathway in the prevention of PHA death. In contrast, the addition of B7-1 transfectants did not restore proliferation in

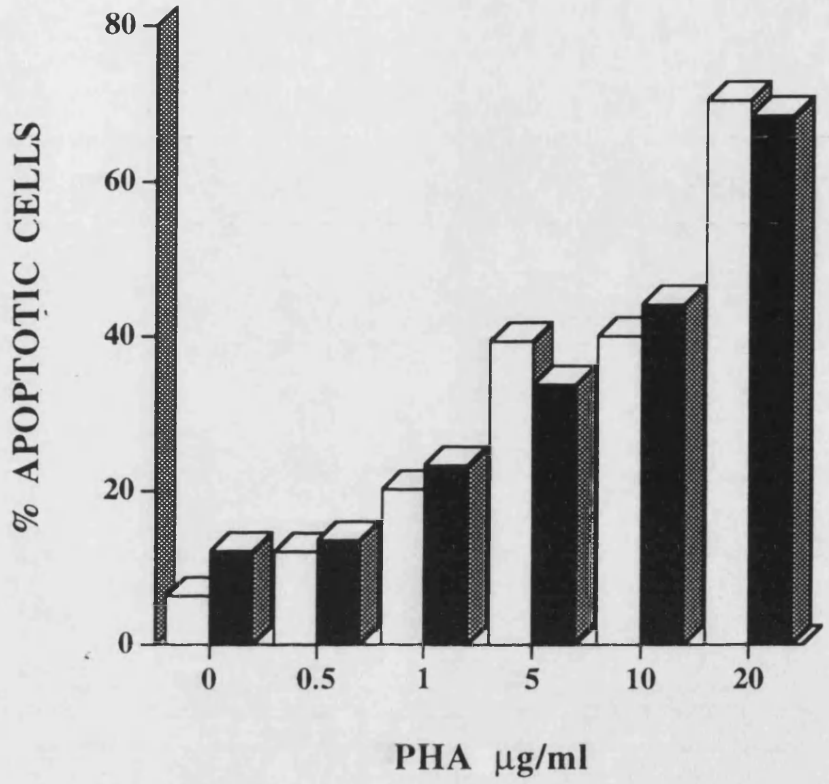


Figure 4.10 Effect of anti-Fas blocking antibody on PHA induced apoptosis of Jurkat T cells.

Jurkat T cells (1x 10^6) were treated with PHA at indicated concentrations with (black) or without (white) the addition of M3 (5μg/ml) anti-Fas blocking antibody. Apoptosis was evaluated by FACS analysis of fixed, propidium iodide stained cells by measuring the number of cells displaying DNA as a sub Go peak. Data are representative of 3 experiments.

these cells, which indicated that costimulation did not restore a proliferative response in these cells. However, IL-2 production was costimulated by the addition of B7-1 transfectants. It will be interesting to examine the effect of B7-2 transfectants on this response.

Apoptosis induced by PHA was sensitive to CsA indicating the involvement of calcium in cell death. In addition the inability of anti-MHC antibody to block PHA-induced death was demonstrated. Previously MHC-dependent responses were investigated using the superantigen SEB, therefore induction of apoptosis using this MHC-dependent antigen was investigated.

4.8 Induction of apoptosis by SEB

The superantigen SEB has been shown to have pleiotropic effects on specific Vb8 expressing T cells when administered in vivo to mice (Callahan et al 1989). In addition it has been established in this study that PBMCs respond to SEB by proliferating, however concomitant with proliferation other events such as anergy and/or apoptosis may be taking place. It was therefore decided to examine human peripheral T cells for evidence of SEB induced apoptosis.

4.8.1 SEB induced apoptosis in PBMCs

Initially it was decided to investigate SEB-treated PBMCs for evidence of apoptosis. SEB was added to freshly isolated PBMCs over a period of 7 days during which cells were taken, their DNA stained with propidium iodide and examined for evidence of apoptotic death by FACS analysis (Figure 4.11 day 5). As early as day 1 post SEB addition, apoptosis as evidenced by the appearance of DNA with a sub G0 peak was demonstrated (Figure 4.11) which was followed by an increase in amounts of apoptotic cell numbers peaking at day 5 and decreasing from day 7. Therefore in addition to eliciting a proliferative response SEB also induced death in PBMCs. This emphasised the ability of this antigen to bring about both proliferation and apoptosis, underscoring the dual nature of SEB stimulation. TCR engagement may therefore result in more than one outcome in human peripheral T lymphocytes.

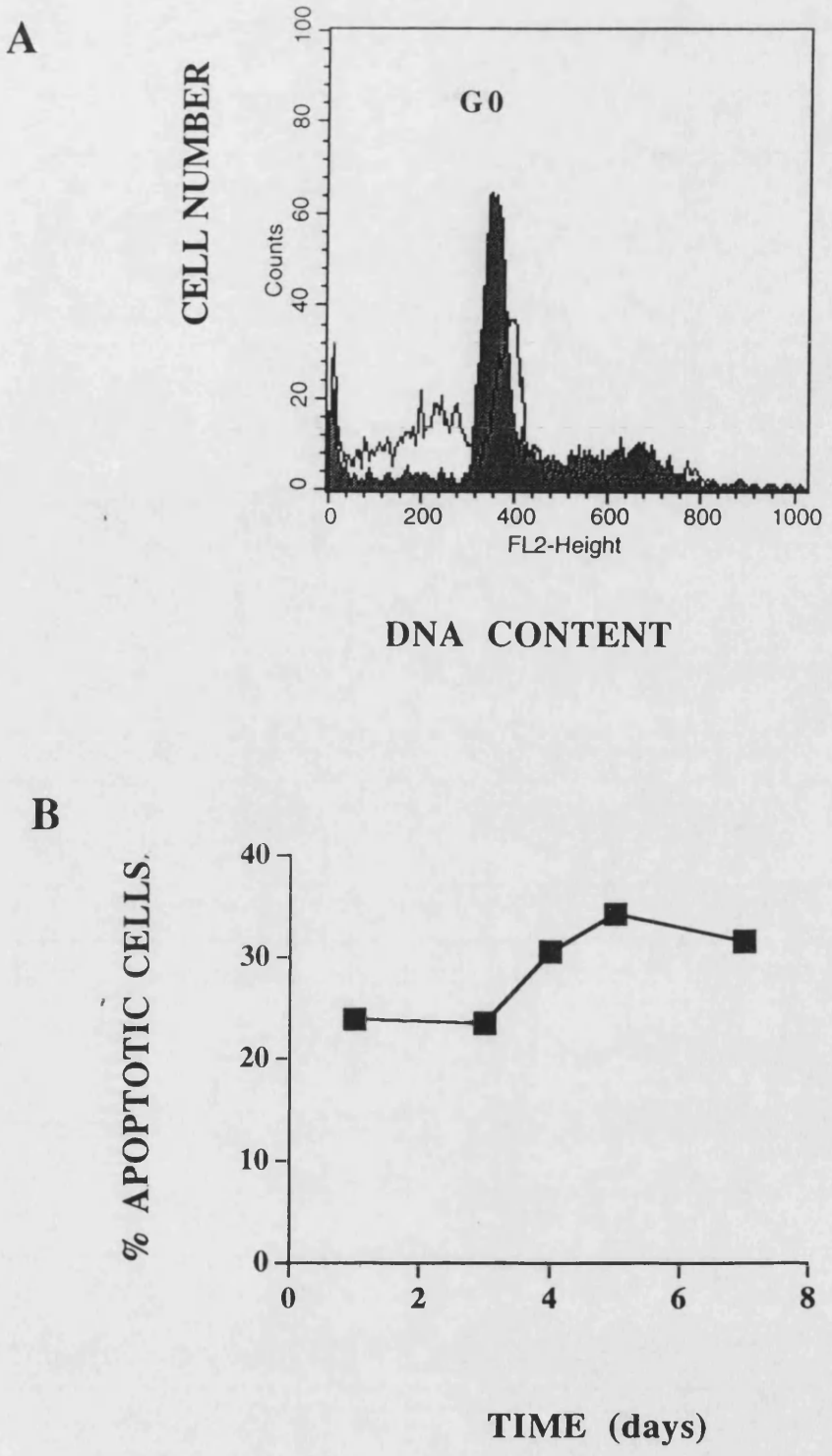


Figure 4.11 Time course of SEB induced apoptosis in PBMCs.
SEB (1μg/ml) was added to freshly isolated PBMCs (1x 10^6) which were taken at day 5 (**A**) or at time points indicated (**B**), fixed with EtOH, stained with propidium iodide and DNA analysed by FACS for evidence of apoptosis. % apoptotic death was determined by measuring the number of cells whose DNA appeared as a sub G_0 peak. Data are representative of 4 experiments.

4.8.2 SEB-induced death in antigen specific T cells

In order to examine apoptosis in activated T cells SEB specific T cells were used. These were generated by stimulating PBMCs with SEB followed by rIL-2 addition and restimulation using SEB presented on DR4/LFA3/B7-1 transfetcants. These cells were demonstrated to express MHC class II in contrast to resting cells which do not express class II and have upregulated CD28 expression. Previously it was shown that SEB in the absence of APCs did not induce proliferation of activated T lymphocytes and inhibited B7-1- and B7-10- induced proliferation in antigen specific T cells . One possibility is that SEB alone may be inducing death in these cells, consequently it was decided to study SEB specific T cells for evidence of SEB-induced apoptosis.

Superantigen specific T cells were treated with SEB, their DNA stained with propidium iodide and examined by FACS analysis for evidence of apoptosis. A striking increase in cells whose nuclei showed signs of apoptosis as a result of the addition of SEB was observed (Figure 4.12). Apoptotic features such as accumulation of DNA staining in the sub-G0 region of the DNA histogram, reflecting loss of DNA stainability due to condensation and fragmentation of the nuclear DNA were seen. Hence SEB induced apoptosis in antigen specific T cells whereas it did not produce a significant proliferative response in these cells. The predominant outcome demonstrated upon SEB addition to antigen-specific cells thus appears to be apoptosis in the absence of APCs.

4.9 Kinetics of SEB-induced apoptosis

It was next decided to determine the requirements for SEB-induced death. The kinetics of SEB-induced apoptosis in antigen specific T cells was examined initially. Cells were incubated with SEB over a period of 24 hours and at various time points were examined for evidence of apoptotic death by FACS analysis. The kinetics of this response revealed that nuclear changes were apparent as early as 1 hour following addition of SEB reaching almost maximal response after as little as 4 hours (Figure 4.13). Therefore a rapid response resulting in apoptosis of previously activated T cells was produced by SEB addition. These kinetics are very similar to those measured when Fas-mediated death was induced in Jurkats, leading to the suggestion that SEB may induce Fas-L expression and thus Fas/Fas-L interaction may be involved in SEB-induced death.

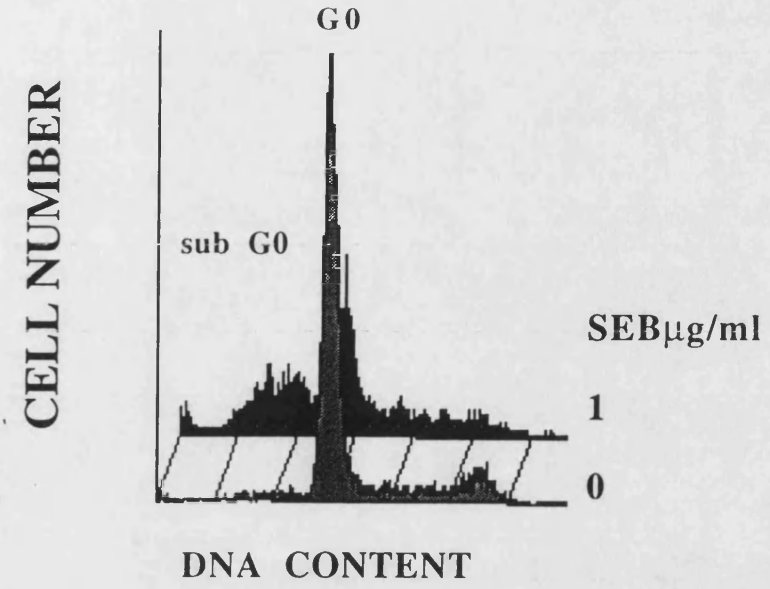


Figure 4.12 SEB-induced apoptosis in antigen specific T cells.
SEB (0 or 1μg/ml) was added to SEB-specific T cells ($1x10^6$) for 8 hours at 37oC Cells were examined for evidence of apoptotic death by propidium iodide staining followed by FACS analysis of DNA content. Data are representative of 4 experiments

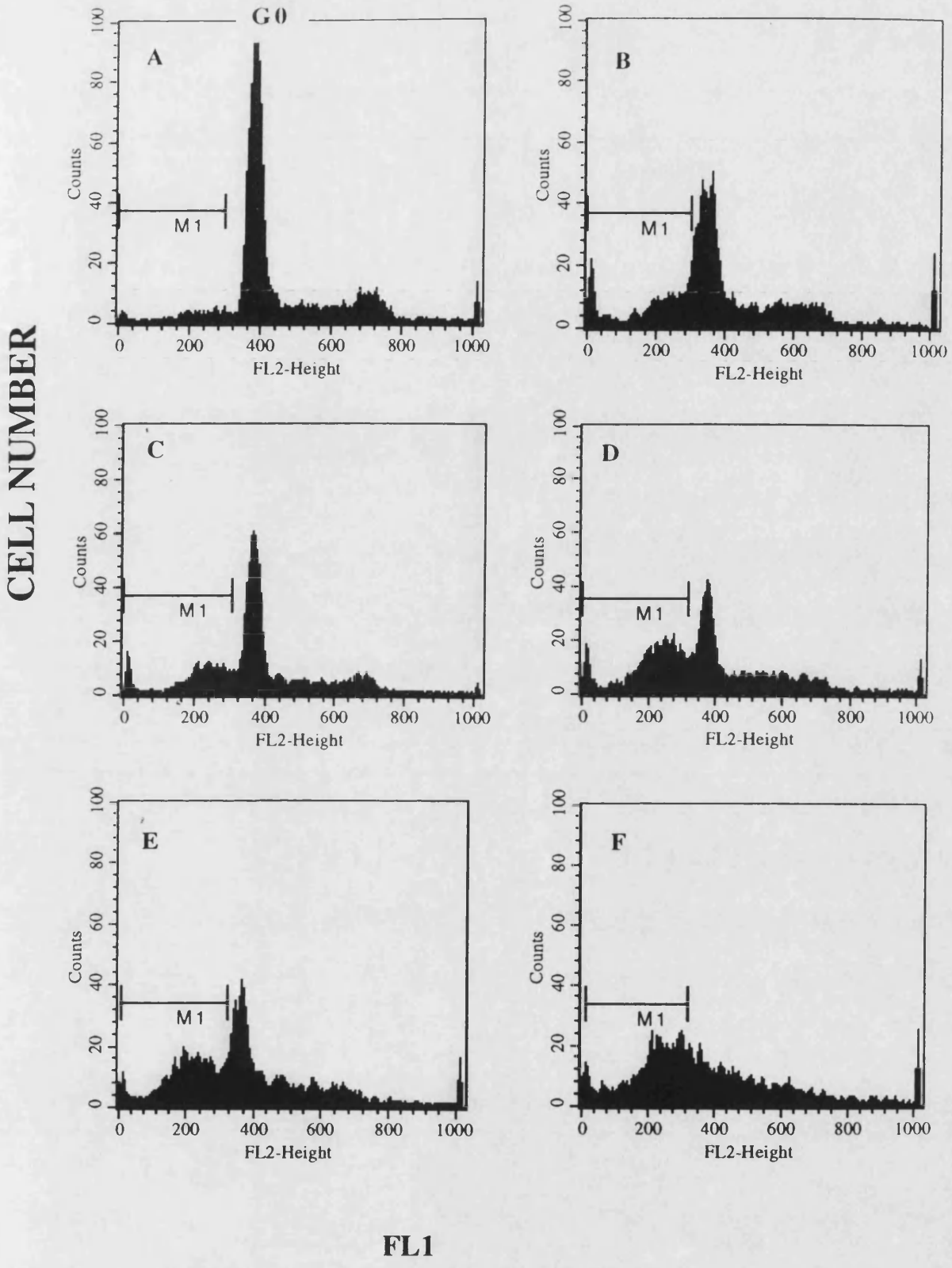


Figure 4.13 Kinetics of SEB-induced apoptosis in antigen-specific T cells.
SEB (1µg/ml) was added to antigen specific T cells(1x 10^6) and DNA examined for apoptosis at various time points following addition. Percentage apoptosis was measured as accumulation of DNA in the sub G0 region (M1). A=0 hour, B = 1hour, C= 2hour, D= 4 hour, E= 8 hour and F= 24 hours.. FL-2 = PI staining, counts + relative cell number. Data are representative of 4 experiments.

4.10 SEB-induced apoptosis: dose response of SEB specific T cells

In order to determine the effective dose of SEB at which apoptosis was induced the response of these SEB T cell lines at various doses of antigen was also measured. Increasing amounts of apoptotic death were seen as the dose of SEB increased, leading to apoptosis of 40-50% of the T cells at 1μg/ml (Figure 4.14). These features were highly consistent with an apoptotic response and it was therefore concluded that the addition of SEB to these T cell lines caused the rapid induction of apoptosis. One possibility is that presentation of SEB by DR4/B7-1 transfectants prevented induction of apoptosis so that in the presence of an APC SEB may induce proliferation.

The death induced by SEB may reflect the involvement of Fas, as a similar high percentage of cells were induced to die on engagement of Fas in Jurkat cells. SEB-induced death in these cells was therefore time and dose dependent in a manner similar to that seen in Fas-induced apoptosis in Jurkats.

4.11 The death signalling pathway induced by SEB

Whilst SEB is known to bind to MHC class II it has also been suggested that SEB may bind directly to the TCR and induce activation signals via engagement of this receptor (Hewitt et al 1992). It was therefore decided to establish whether apoptosis induced by SEB in these T cells was occurring via this pathway. CsA was used to investigate the calcium dependence of SEB- induced apoptotic death. Results are shown as percentage inhibition of apoptotic death as measured by FACS analysis. It was clearly seen (Figure 4.15) that SEB-induced apoptosis was almost completely abolished by the addition of increasing concentrations of CsA. This indicated an important role for a calcium/calcineurin- mediated signal in SEB-induced apoptosis which may support the involvement of TCR signalling in the death process. In addition this was consistent with a role for Fas-dependent responses in SEB-mediated apoptosis as Fas-L expression has also been demonstrated to be inhibited by CsA (Vignaux et al 1995). Inhibition of Fas-L expression might thus be a possible cause of inhibition of SEB-mediated death by CsA if the interaction of this ligand with Fas plays a role in SEB-induced apoptosis.

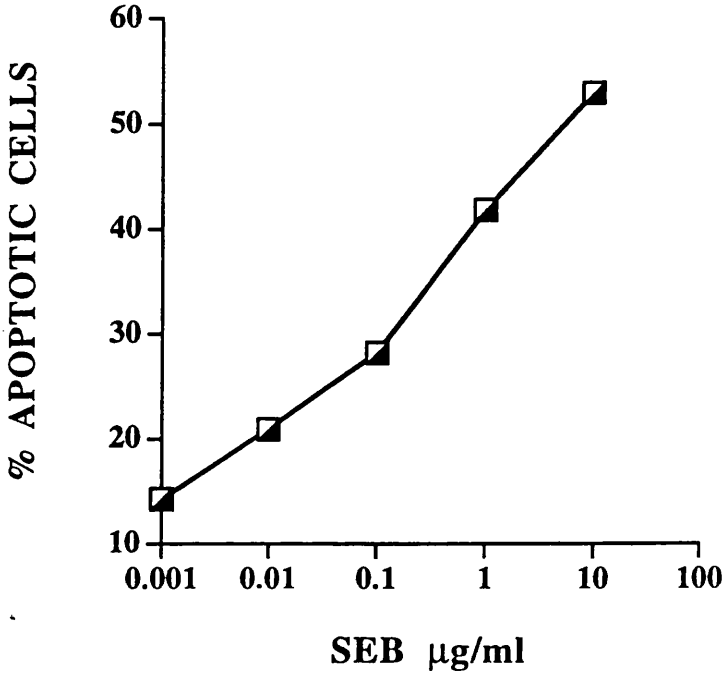


Figure 4.14 Dose response of SEB-induced apoptosis in specific T cells to SEB.
SEB specific T cells were incubated for 16 hours with indicated concentrations of SEB. Apoptosis was measured by staining cells with propidium iodide followed by examining DNA content by FACS analysis measuring the amount of cells displaying DNA in the subG0 region. Data are representative of 4 experiments

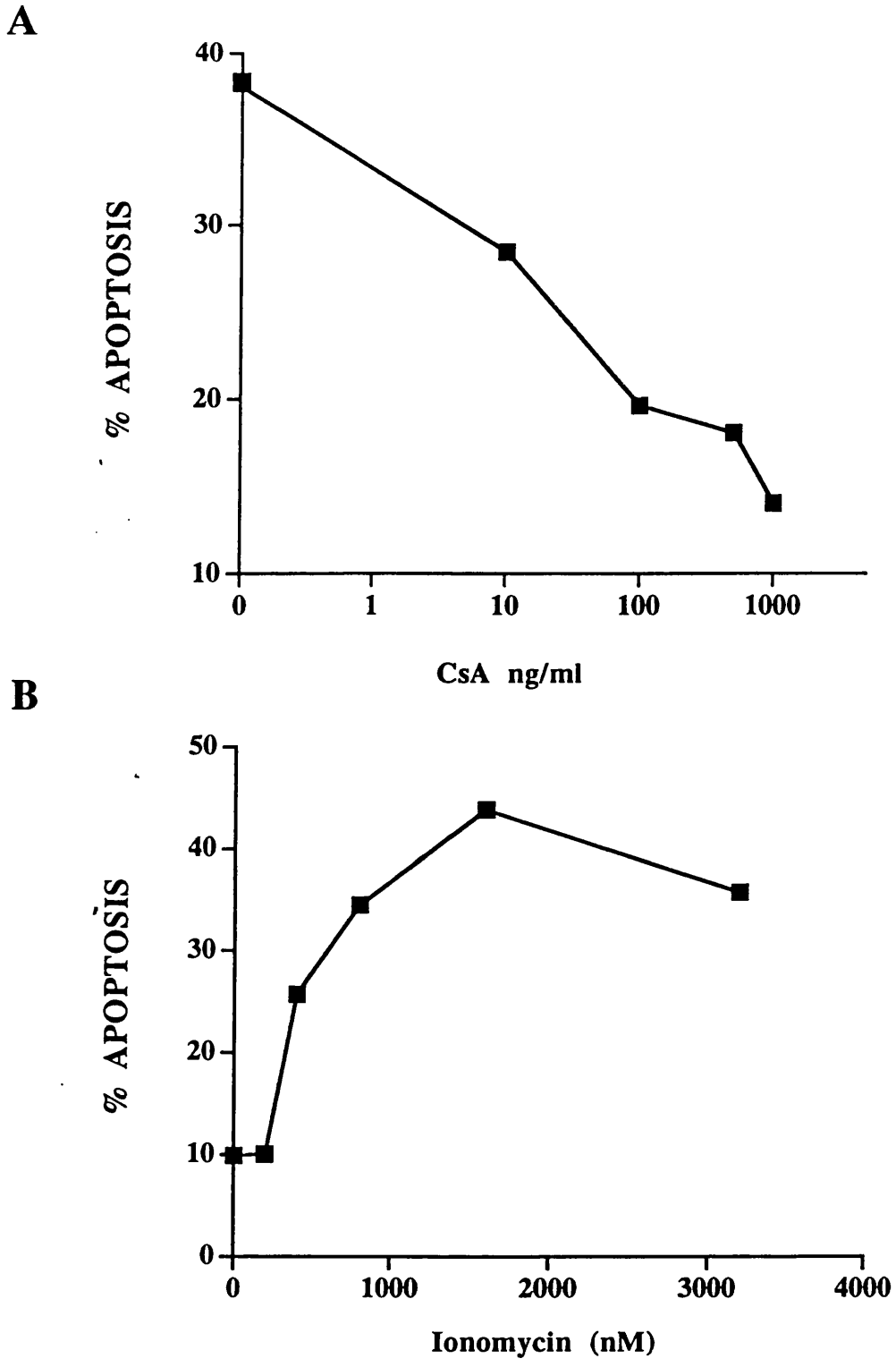


Figure 4.15 (A) Effect of cyclosporin A on SEB induced apoptosis.
CsA was added to SEB specific T cells ($1x10^6$) 30 minutes prior to SEB (1μg/ml) and cells incubated for 18 hours.
(B) Effect of ionomycin on the induction of T cell apoptosis.
Ionomycin at indicated concentrations was added to SEB specific T cells ($1x10^6$) and cells incubated for 18 hours.
Apoptotic DNA was measured by FACS analysis of fixed propidium iodide stained cells and % apoptotic cells determined. Data are representative of 3 experiments.

In order to further investigate the ability of calcium signalling to mediate an apoptotic response in SEB specific T cells the calcium ionophore ionomycin was used. A dose dependent apoptotic response was elicited in SEB T cell lines with ionomycin (Figure 4.15). Therefore SEB-induced apoptosis could be mimicked by the addition of the calcium ionophore to the SEB T cell lines. These experiments were consistent with the involvement of an intracellular calcium signal in SEB-induced apoptosis of activated T cells. In addition these results correlated well with the hypothesis that direct binding of the TCR by SEB occurred during the apoptotic death induced by this antigen.

Antibody blocking experiments were carried out to determine if SEB-induced apoptosis was dependent on T-T cell contact. None of the antibodies used were particularly effective in inhibiting cell death (Figure 4.16). The most effective were those against the adhesion molecules LFA1 (38.1) and LFA-3 (TS2/9) indicating only the involvement of cell to cell contact in the induction of apoptosis in this system. An antibody against the HLA-DR molecules was also ineffective in blocking the death response, indicating that MHC presentation of SEB is not required to induce apoptosis. This suggested that MHC presentation of SEB to the T cells was not necessary and that activation of the T cells via direct interaction of SEB with the TCR resulted in a calcium signal which is intimately involved in the apoptotic death process induced by SEB.

4.12 The role of the Fas/Fas-L interaction in SEB induced apoptosis.

SEB presentation occurs primarily via MHC class II although it can costimulate the TCR in the absence of class II (Hewitt et al 1992) whilst death induced via the TCR has been shown to be dependent on the Fas pathway (Vignaux et al 1995). Also apoptosis induced in antigen-specific T cells by SEB and by Fas engagement of Jurkats has been demonstrated to display similar kinetics. These data indicated that Fas-mediated death pathway may be responsible for apoptosis induced by SEB. Consequently it was decided to investigate the role played by Fas in SEB induced apoptosis.

The expression of Fas-L on the surface of activated T cells as a result of antigen stimulation was determined in order to investigate the role of Fas and Fas-L in SEB

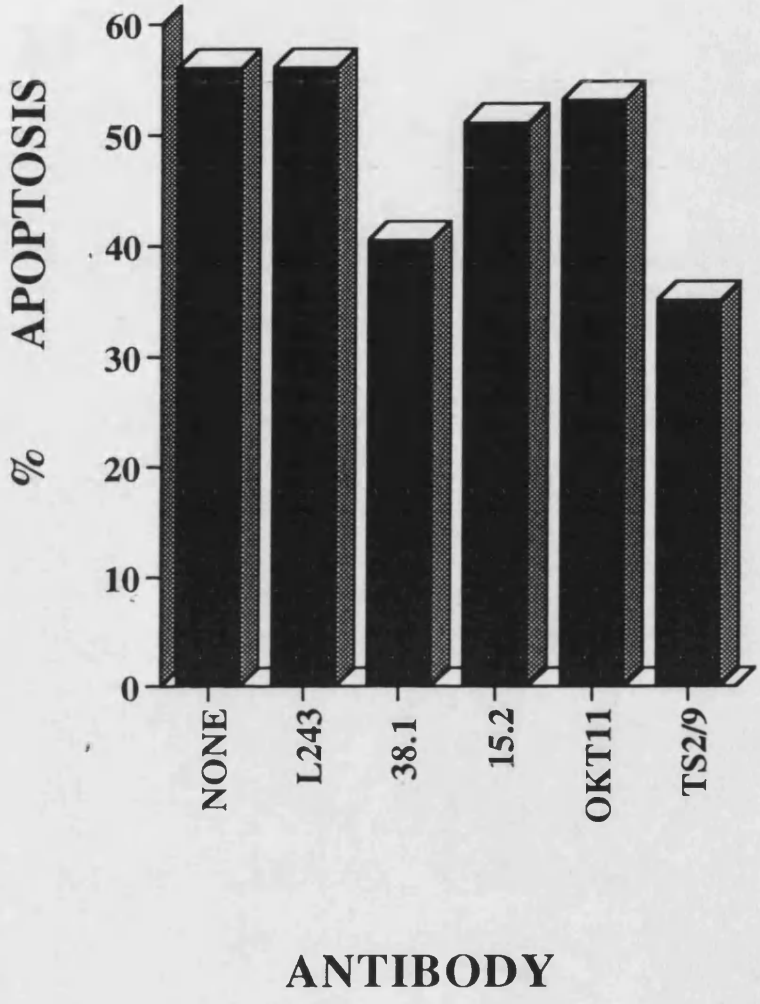


Figure 4.16 Effect of antibody blocking on SEB-induced apoptosis.
Monoclonal antibodies (10μg/ml) to the following cell surface molecules : HLA-DR (L243), LFA-1 (38.1), ICAM1 (15.2), CD2 (OKT11), LFA-3 (TS29) were added to SEB-specific T cells ($1x10^6$) at the start of the experiment and incubated for 18 hours in the presence of SEB (1μg/ml). Apoptosis was measured by propidium iodide staining followed by FACS analysis and determination of % apoptotic cells displaying DNA in subG0 region of the histogram. Data are representative of 2 experiments.

induced death. It has previously been established that Jurkat T cells express Fas on theirsurface and that ligation of Fas by an anti-Fas mAb leads to apoptosis in these cells. Consequently, it was decided to use these cells as targets for killing in a sensitive bioassay for Fas-L expression. In order for killing of Fas sensitive targets to occur, the cytotoxic effector cells must express Fas-L which may be induced by activating T cells using PMA and ionomycin. Therefore killing of Jurkats by unstimulated and PMA/ionomycin activated SEB T cells was initially examined using a sensitive ^{51}Cr release assay. As expected no killing was detectable by unstimulated cells as evidenced by ^{51}Cr release (Figure 4.17). In contrast, activating T cells by the addition of PMA and ionomycin resulted in the death of the Fas sensitive Jurkat cells, which released ^{51}Cr into the culture medium. Therefore activation may have induced Fas-L expression on SEB specific effector T cells, followed by ligation of the Fas receptor on the surface of Jurkat T cells leading to the death of Jurkat cells . To prove that this death was due to the interaction of Fas-L with Fas receptor, a blocking anti-Fas antibody (M3) was used. Cytotoxic killing of Fas sensitive cells by Fas-L expressing SEB T cells was inhibited by this antibody (Figure 4.17), thereby confirming that death in this sytem is due to engagement of the Fas receptor on the Jurkat T cells by activation induced Fas-L on SEB specific T cells.

These experiments demonstrated indirectly that expression of Fas-L was up-regulated on SEB T cells by activation. These Fas-L-expressing cells were then capable of killing Fas sensitive targets. It was next determined whether SEB stimulation was also capable of increasing Fas-L expression and inducing cytotoxic killing. When SEB was used to induce Fas-L expression, killing of the Jurkat T cells was also demonstrated (Figure 4.18). Furthermore, the M3 anti-Fas blocking antibody and in addition a recombinant Fas-Fc protein clearly inhibited SEB induced death of the Fas-sensitive target cells . This Fas-Fc fusion molecule acts as a receptor decoy for Fas and therefore blocked the interaction of Fas-L with Fas expressed on Jurkat cells thus preventing induction of killing. This confirmed that cytotoxicity was due to engagement of Fas by SEB induced Fas-L expressed on antigen specific T cells. Therefore SEB induced T cell death occurred via interaction of the Fas receptor with Fas-L expressed on antigen specific effector cells. SEB-induced apoptosis was therefore dependent on the Fas mediated death pathway. These experiments demonstrate that Fas is intimately linked with SEB-induced apoptosis of antigen specific T lymphocytes.

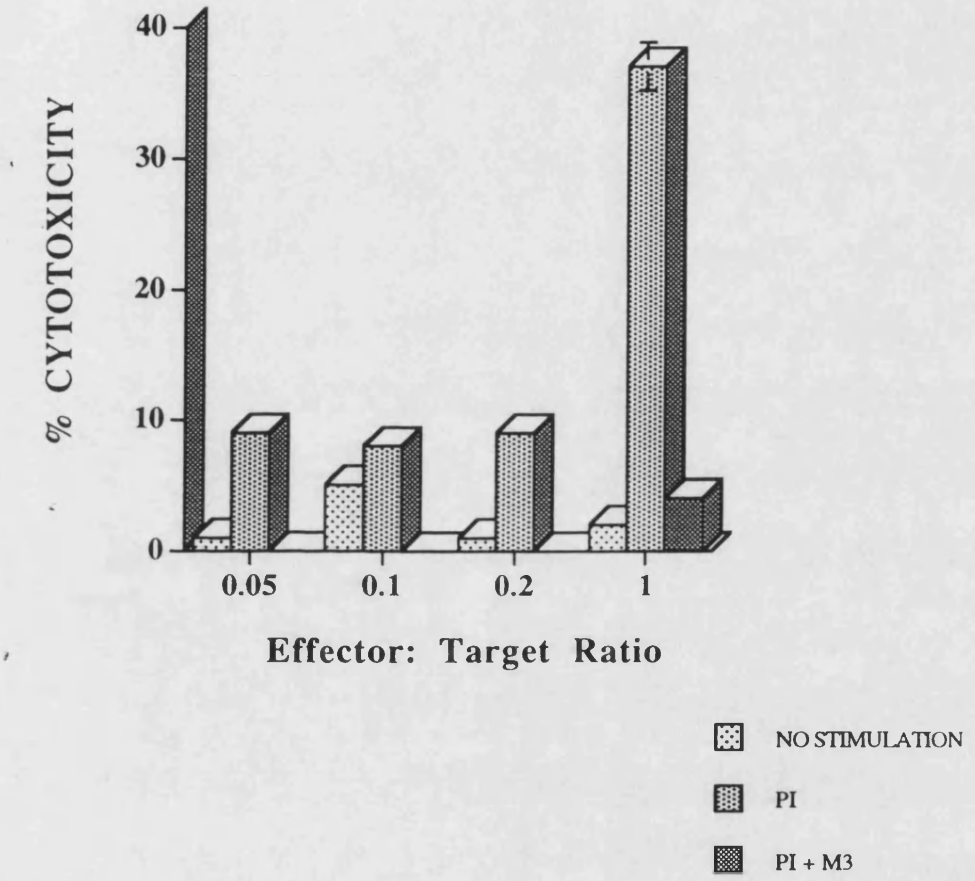


Figure 4.17 Effect of PMA and ionomycin on Fas-L expression in SEB-specific T cells.

SEB T cells were assessed for their ability to induce cytotoxicity via apoptosis in ^{51}Cr labelled Fas sensitive Jurkat cells at various effector to target ratios. Labelled Jurkats were co-incubated with T cells in the presence or absence of PMA and ionomycin. The blocking effect of 3μg/ml of an anti-Fas antibody, M3 on cell death was measured. Data are representative of 3 experiments

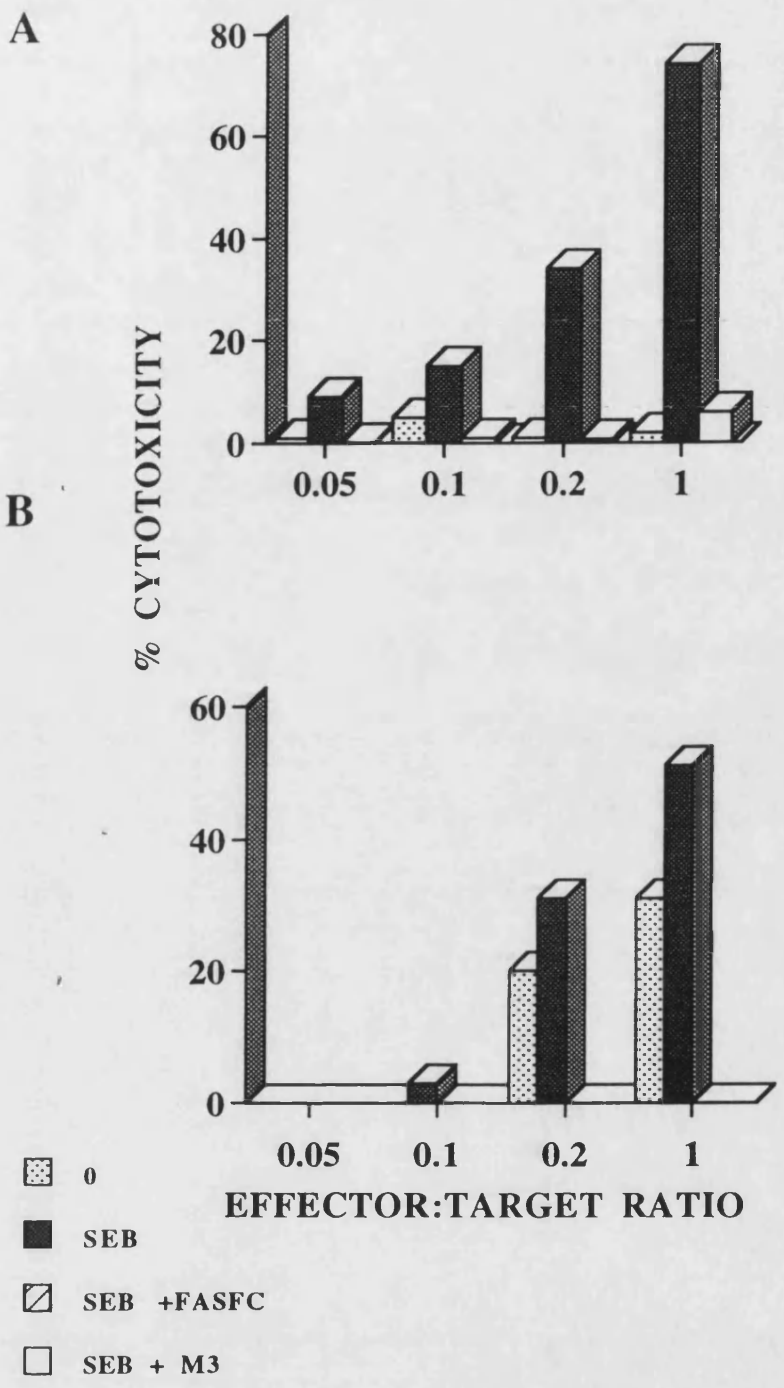


Figure 4. 18 Fas-L expression on SEB specific T cells.
Two independent T cell lines (panels **A** and **B**) were assessed for their ability to induce apoptosis in ^{51}Cr labelled Fas sensitive Jurkat cells at various effector to target ratios. Labelled Jurkat cells were co-incubated with T cells in the presence or absence of SEB. The blocking effects of 3µg/ml of an anti-Fas antibody, M3 or 1/10 dilution of a recombinant Fas-Fc protein (panel B only) on induction of death were measured. Data are representative of 3 experiments

An independent SEB-specific T cell line was investigated and interestingly it was seen that there was a basal expression of Fas-L in this line as well as upregulation of expression induced by SEB (Figure 4.18). Both death induced by unstimulated T cells expressing basal Fas-L and SEB stimulated T cells with upregulated Fas-L was inhibited by blocking the Fas/Fas-L interaction using both M3 and the Fas-Fc fusion molecule. It was therefore concluded that SEB activation resulted in Fas-L up-regulation on SEB T cells which induced cell apoptosis in Fas positive target cells. This supported the hypothesis that the Fas/Fas-L interaction is intimately involved in SEB mediated T cell death. This was in contrast to the death induced by PHA in Jurkat T cells demonstrated earlier.

The involvement of Fas-mediated death in SEB-induced apoptosis was further examined using FACS analysis of SEB-specific T cells which underwent apoptosis in response to SEB. It can be seen (Figure 4.19) that the appearance of SEB-induced apoptotic DNA was inhibited in these cells by M3 confirming that SEB-induced death occurred via a Fas-dependent pathway. SEB death and Fas dependent death were therefore hypothesised to be intimately linked. This led to the prediction that activation would result in the up-regulation of Fas-L mRNA expression in SEB specific T cells.

4.13 Kinetics of Fas-L expression

The kinetics of SEB stimulated regulation of Fas-L mRNA was examined using RT PCR. SEB specific T cells were stimulated with SEB (1μg/ml) and mRNA analysed at various times following stimulation by using primers specific for Fas-L (Figure 4.20). Fas-L mRNA was not detected in unstimulated cells. However, following activation with SEB, mRNA for Fas-L was detectable by 1 hour and expression increased up to 4 hours. Levels of mRNA were compared by using a GAPDH control. These data confirmed the ability of SEB T cells to rapidly upregulate Fas-L mRNA in a manner consistent with the expression of this molecule in T cells undergoing apoptosis.

Previously SEB was demonstrated to induce apoptosis in PBMCs over a period of 7 days. Therefore Fas-L expression on SEB treated PBMCs was measured during this time period. RNA isolated from PBMCs incubated with or without SEB at various time points was subjected to RT PCR for Fas-L and the PCR product probed for expression of Fas-L by Southern blotting (Figure 4.21). Expression levels were quantitated by comparing with

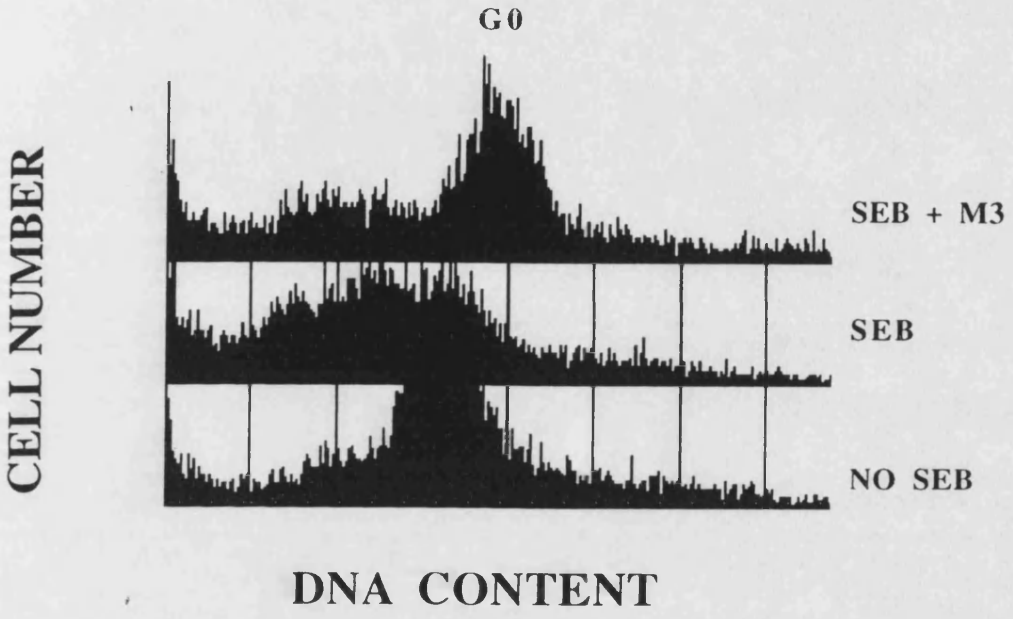


Figure 4.19 Involvement of Fas/Fas-L interactions in SEB-induced apoptosis.
SEB-specific T cells ($1x10^6$) were incubated with SEB(1μg/ml) for 18 hours and the effect of addition of an anti-Fas blocking antibody (M3 at 3μg/ml) on apoptosis was determined. Cells were stained with propidium iodide and examined by FACS analysis. Data are representative of 3 experiments

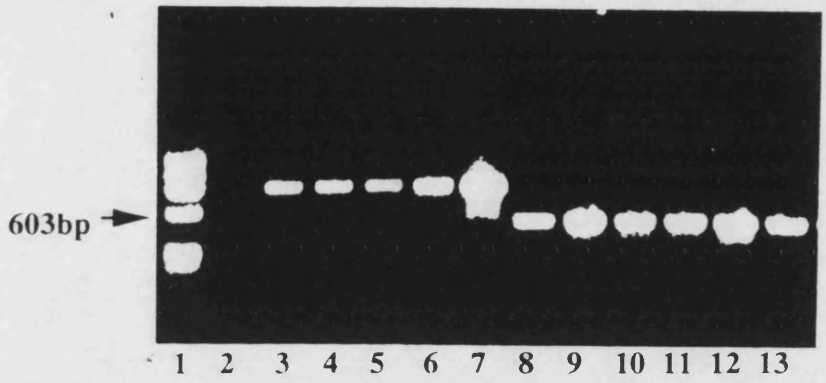


Figure 4.20 Induction of Fas-L mRNA expression in SEB specific T cells.
T cells were stimulated with SEB (1μg/ml) for 0 hr (lanes 2 and 8), 1 hr (lanes 3 and 9), 2hr (lanes 4 and 10), 3 hr (lanes 5 and 11) and 4hr lanes 6 and 12). Lane 7 contains a PCR amplified Fas-L cDNA control while lane 13 contains a GAPDH cDNA control. Samples were subjected to reverse transcriptase PCR for Fas-L (lanes 2-7) or GAPDH (lanes 8-13). Lane 1 contains ϕX174 /Hae III markers.

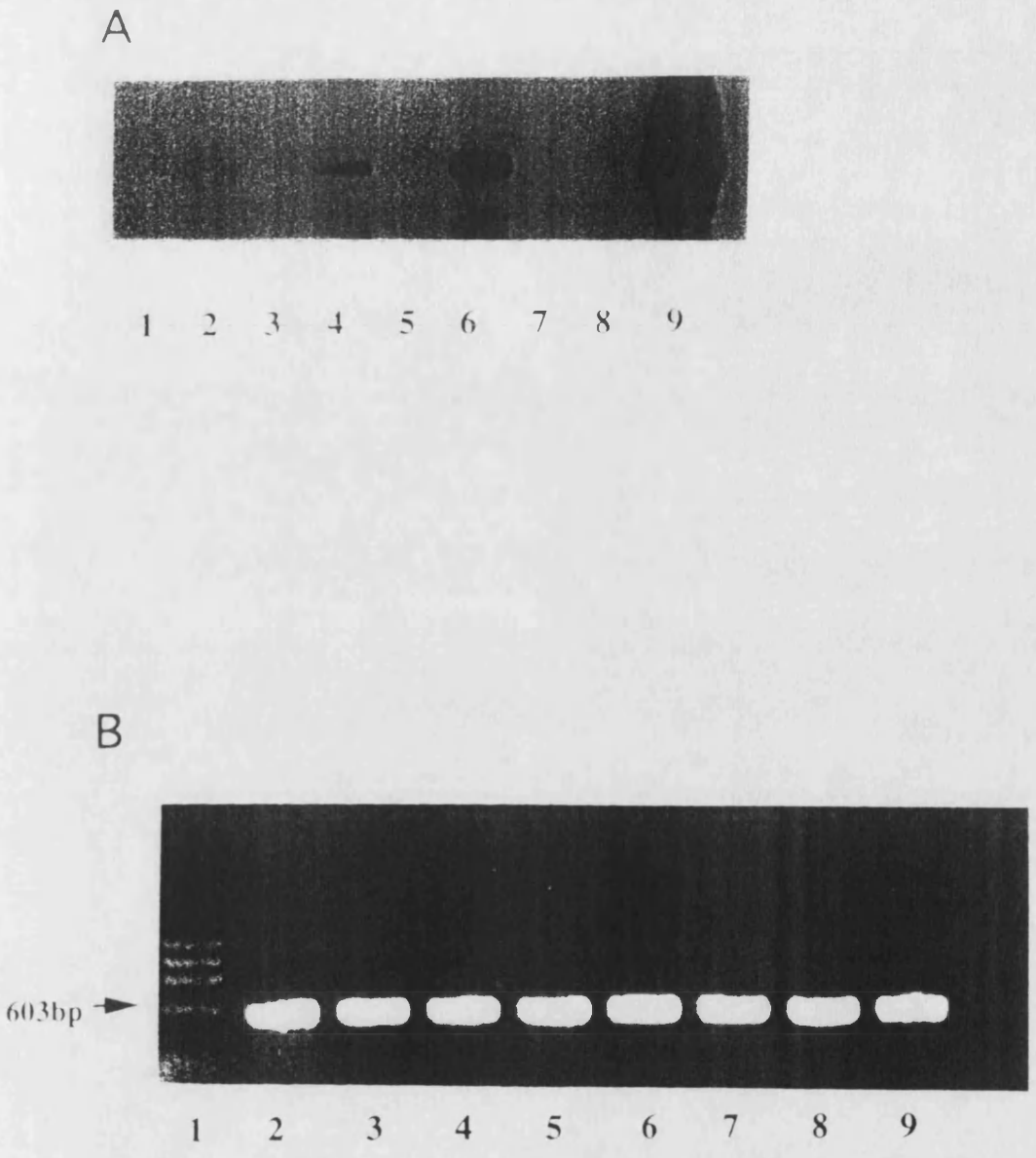


Figure 4.21 SEB induction of Fas-L mRNA expression in PBMCs.
Panel A Southern blot hybridised with Fas-L probe, **panel B** GAPDH controls. PBMCs were treated with (A lanes 2,4, 6 and 8, B lanes 3, 5, 7and 9) or without 1μg/ml SEB (A lanes 1, 3, 5 and 7, B lanes 2, 4, 6 and 8)) and RNA isolated at various time points from day 1(panel A lanes 1 and 2, panel B lanes 2 and 3), day 3 (panel A lanes 3 and 4, panel B lanes 4 and 5) , day 5 (panel A lanes 5 and 6, panel B lanes 6 and 7) and day 7 (panel A lanes 7 and 8, panel B lanes 8 and 9). Panel A lane 9 is Fas-L cDNA control , all samples were southern blotted and probed for Fas-L expression, autoradiograph was exposed for 2 hours. Panel B samples were subjected to reverse transcriptase PCR for GAPDH.Panel B lane 1 ϕX174 markers

GAPDH primed expression. Fas-L mRNA was rapidly induced by day 1 in SEB-treated PBMCs, which increased to a maximum at day 5 and rapidly declined at day 7. This expression correlated well with the kinetics of death induced by SEB in these cells, providing further evidence for the involvement of the Fas/Fas-L interaction in SEB induced apoptosis. The kinetics of Fas-L mRNA expression thus reflected the regulation of SEB-induced apoptosis demonstrated earlier in PBMCs. Consequently these results highlight the critical role played by the Fas/Fas-L interaction in SEB-mediated apoptosis of T lymphocytes.

These experiments led to the hypothesis that SEB can induce apoptosis in both resting and activated T lymphocytes and that Fas-L is upregulated by SEB stimulation. SEB-induced death can be blocked by inhibiting the Fas-Fas-L interaction which showed that SEB death is mediated via Fas/Fas-L interactions. Fas-L upregulation appears to be intimately associated with activation induced death of T cells.

4.14 Induction of apoptosis by ligation of Fas on activated T cells.

When a number of SEB-specific T cells were examined by FACS analysis, Fas expression was seen (Table 4.1) as would be expected on activated T cells. These T cell lines were examined for evidence of Fas-induced apoptosis. Surprisingly however, in no case was death of these cells demonstrated on ligation of Fas by CH11 (Table 4.1) . This was quite an unexpected finding as many of the SEB specific T cell lines had been repeatedly activated during restimulation and would thus be predicted to be sensitive to death upon engagement of Fas. Fas-dependent death may not occur in these cells for a number of reasons. One possibility to explain this finding was that signals in addition to Fas ligation were required to induce death in these cells. Death mediated by the Fas pathway may be due to trimerisation of this receptor on the cell surface which may not be occurring. Alternatively, the secretion of Fas-L by these cells which can bind to the antibody may inhibit death. Engagement of the TCR, which occurs during SEB-induced responses, may also be required in conjunction with engagement of Fas in order to induce apoptosis.

CELL LINE	FAS STAINING		% APOPTOTIC DEATH	
	MFI	% CELLS	ANTI-FAS (0.5 µg/ml)	SEB (1µg/ml)
LW.1	51.2	83.4	1.9	3.68
LW.2	26.8	95.8	1.33	2.35
SE.1	24.4	92.3	3.81	0.5
SE.2	56.5	81.3	5.5	4.08
MB.1	55.1	89.3	0.2	3.67
PI.1	83.9	98.0	0.6	N.D.

TABLE 4.1

6 independent SEB lines were assayed for Fas expression and susceptibility to anti-Fas induced or SEB-induced apoptosis. Cells (2x105/antibody) were removed from culture and washed with PBS, followed by suspension in M3 for 45 minutes at 0oC. Having washed with PBS antibody was detected using anti-mouse FITC conjugated Ig. Stained cells were analysed for Mean Fluorescence Intensity (MFI) and % positivity.To determine apoptotic death cells were cultured for 18 hours with or without an anti-Fas killing antibody (0.5µg/ml) or SEB (1µg/ml) and apoptotic cells determined by propidium iodide staining and examination of hypoploid DNA by FACS analysis.

As it has been demonstrated that SEB-induced death was dependent on Fas, it was also interesting to examine these Fas-resistant cell lines for SEB-induced apoptosis Interestingly all of the Fas-resistant SEB lines examined were also resistant to SEB-induced apoptosis(Table 4.1). It was therefore observed that various cell lines were resistant to Fas-mediated death using anti-Fas antibodies while a number of SEB lines were also refractory to SEB-induced death. This may reflect the inability to induce Fas-triggered death in these cells, as it is hypothesised that SEB death is intimately associated with triggering of the Fas-death signal. These cells may have an intrinsic defect in their Fas signalling pathway or may be deficient in the factors that contribute to Fas-induced death. It is possible that Fas-mediated death requires additional "costimulation" signals such as CTLA-4. Consequently the effect of Fas ligation on CD28 responses were examined. Interestingly, the anti-Fas antibody, CH11 was demonstrated to inhibit the proliferative response induced by CD28 costimulated SEB driven proliferation (Figure 4.22). CH11 also blocked proliferation in response to DR4/B7-1 transfectants . This suggested that in order for engagement of Fas to induce a death signal in T cell lines they must first receive activation signals to proliferate.

Alternatively, a feature of T cell lines may be regulation of factors which modulate apoptotic death. One of these death modulator-factors is the bcl-2 family which includes the bcl-2 and bax molecules. Therefore, the expression of bax was investigated as a preliminary step to determine whether resistance to apoptotic death was due to modulation of death influencing factors. An SEB specific T cell line which constitutively expressed Fas-L was examined for effect of SEB activation on bax expression. In addition to constitutive expression of Fas-L this cell line also expressed bax (Figure 4.23). However, the addition of SEB down regulated the expression of bax in this cell line (Figure 4.23). When bax is in excess, cell death is accelerated. Therefore, as SEB downregulates bax expression, these cells are suggested to increase their resistance to SEB/Fas induced apoptotic death. The balance between bcl-2/bax expression and Fas-L expression may determine the outcome of Fas receptor engagement in these cells. Therefore SEB-specific T cells may become resistant to death upon SEB addition by the down-regulation of death enhancing factors. The balance between expression of factors which influenc apoptosis may ultimately control life and death of a T lymphocyte.

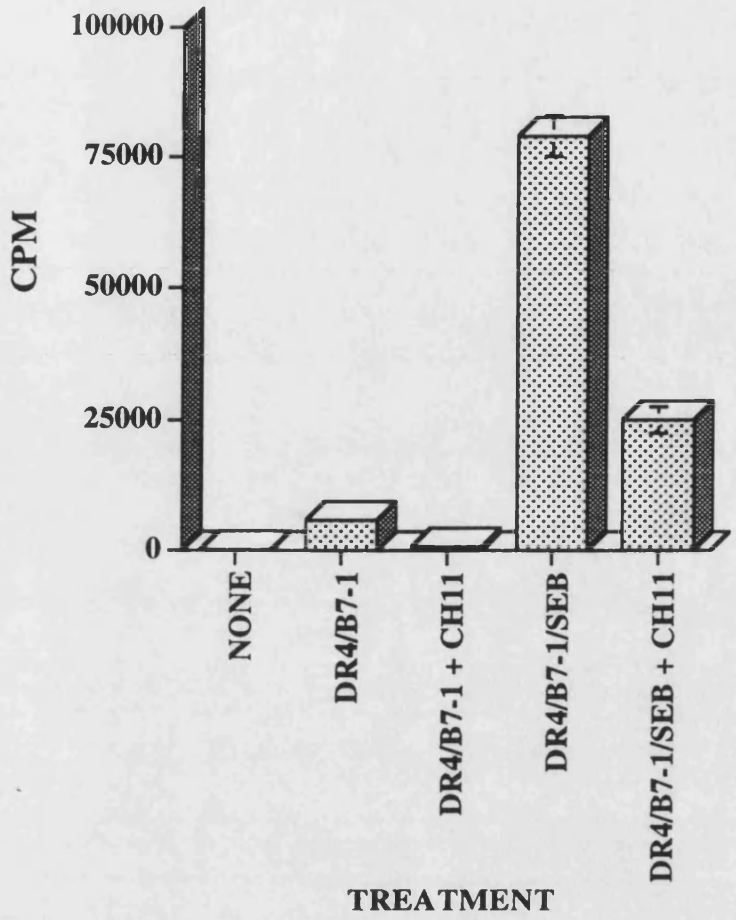
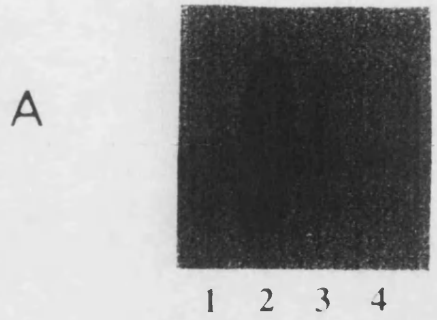


Figure 4.22 Effect of Fas ligation on CD28 costimulated SEB proliferation.
SEB specific T cells ($5x10^4$) were stimulated by presenting SEB on DR4/B7-1 cells ($2.5x10^4$) and the effect of addition of CH11 (0.1μg/ml), an anti-Fas antibody was determined by measuring proliferative responses. The response to DR4/B7-1 cells with and without CH11 is also shown. Proliferation was measured by ^3H thymidine incorporation. Data are representative of 2 experiments.



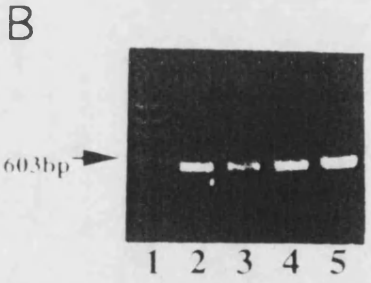


Figure 4.23 SEB effect on bax mRNA expression in SEB specific T cells.
Panel **A** southern blot hybridised with bax probe. **B** GAPDH controls.
Panel A SEB specific cells were treated with SEB and RNA isolated at 0 (lane 2), 4 hours (lane3) and 18 hours(lane 4), reverse transcribed, separated by agarose gel electrophoresis southern blotted and autoradiograph exposed for 24 hours. Lane 1 bax cDNA control probed with bax.
Panel B samples were subjected to reverse transcription PCR for GAPDH, 0 hrs (lane 2) 4 hrs (lane 3) 18 hrs (lane 4) GAPDH control cDNA (lane 5), lane 1 ϕX174 markers

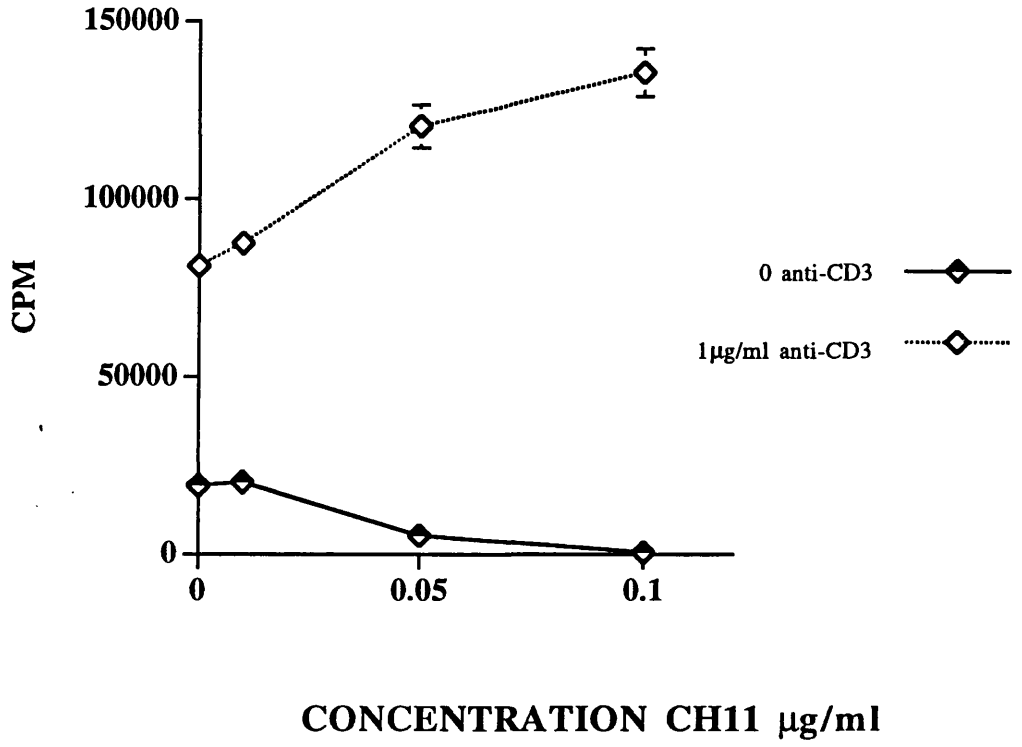


Figure 4.24 Effect of Fas ligation on CD3 stimulated proliferation.
Freshly isolated T lymphocytes ($5x10^4$) were stimulated for 3 days with or without anti-CD3 (1µg/ml). The effect of addition of various concentrations of CH11, an anti-Fas antibody on proliferation responses was determined. Proliferation was measured by ^3H thymidine incorporation. Data are representative of 2 experiments.

4.15 Fas-L ligation may result in costimulation in addition to inducing death in T cells.

Interestingly, it was demonstrated that Fas ligation in addition to signals induced upon TCR engagement using an anti-CD3 antibody resulted in enhanced proliferative responses in PBMCs (Figure 4.24). Therefore in addition to death signals Fas receptor engagement may also result in synergy with the signals that elicit a proliferative response. Consequently, a simple definition of Fas as a death inducing molecule needs some elaboration.

4.16 Discussion.

Having explored some of the factors necessary for productive T cell activation, it was decided to examine the possibility that apoptosis was also occurring in PHA or SEB-treated T cells. Resting T lymphocytes were found to be resistant to PHA-induced apoptosis, however when Jurkat T cells were examined for PHA-induced death a substantial apoptotic response was demonstrated. The different outcome of PHA treatment in Jurkat T cells may reflect the observation that they are continuously proliferating and thus may be constantly susceptible to apoptosis. The continuous progression of these cells through the cell cycle may lead to production of DNA strand breaks during mitosis resulting perhaps in irreparable DNA damage committing the cell to the apoptosis pathway. Alternatively, these T cells may be in a pre-apoptotic state because they are transformed and express altered levels of factors such as bcl-2 or c-myc or p53 which .may render the cell susceptible to the induction of death by PHA. It is suggested therefore that PHA triggers a death signal in previously activated (transformed) T cells which are highly susceptible to apoptosis when they are in a proliferative phase of the cell cycle. This is in agreement with data demonstrating that pretreatment of T cells with IL-2 which drives them towards proliferation renders these cells susceptible to the induction of apoptosis. (Lenardo 1990). Transformed cells and activated T cells appear to be much more susceptible to apoptosis than resting T lymphocytes (Nishioka et al 1994, Russell et al 1991, Salmon et al 1994) and it is suggested that together with an apoptotic signal T lymphocytes must also receive a further signal in order for death to occur. This may well involve similar signals to those which T cells receive in order to become activated or proliferate. The death pathway therefore appears to be more complicated

than requiring one signal and may have similar requirements to T cell activation in agreement with the two signal model first proposed by Bretscher and Cohn (1970).

Many mechanisms for the induction of cell death have been identified, one involving Fas/Fas-L interactions. This was not the mechanism induced by PHA stimulation as the kinetics of PHA-dependent apoptosis and death induced by ligation of the Fas receptor on Jurkats were remarkably different. More importantly, anti-Fas blocking antibody did not prevent PHA-induced death of Jurkats indicating that Fas was not involved in PHA-induced apoptosis. The apoptotic pathway induced by PHA remains unknown but may involve ligation of as yet undiscovered receptors. Alternatively, TNF-dependent cell death may be triggered by PHA in Jurkat cells which warrants investigation. Additionally activation of cysteine proteases such as ICE may be involved and the once surface molecules have been triggered to initiate apoptosis the intracellular pathway of PHA induced apoptosis and Fas-induced apoptosis may intersect.

The involvement of a calcium signal in PHA-induced apoptosis was demonstrated using CsA which partially blocked apoptosis. This may reflect the involvement of a Ca-dependent endonuclease and/or the importance of TCR mediated signals in PHA-induced death of Jurkat T cells. However, in this case triggering of the TCR did not lead to Fas mediated death as reported in Vignaux et al (1995). Alternatively, PHA may not signal through the TCR and other receptors such as CD2 may be incvolved in PHA-induced death.

In this study use was also made of the superantigen SEB to look at induction of apoptosis in human peripheral T cells. Previously it was demonstrated that SEB induced proliferation in PBMCs while SEB alone was insufficient to induce proliferation in antigen-specific T cells. However, when costimulation was provided by B7-1 or LFA3 these cells were capable of proliferating in response to SEB. Interestingly it was also found that increasing the dose of SEB in some antigen-specific T cells lead to a decrease in their proliferation . Additionally, proliferation induced in response to B7-1 or B7-1-2 stimulation was inhibited by the addition of SEB to these cultures. Consequently, it was predicted that not only was SEB capable of stimulating a proliferative response but was also capable of inducing apoptosis in T lymphocytes. SEB was indeed demonstrated to induce apoptotic death of PBMCs providing evidence for the dual outcome of TCR engagement . SEB stimulation of PBMCs can therefore result in either proliferation and IL-2 production or apoptosis.

Additionally it was demonstrated that antigen-specific T cells underwent apoptosis when treated with SEB. This is in direct agreement with data from Damle and his colleagues who showed SAg-induced death of antigen-specific T cells (Damle et al 1993). Therefore the predominant effect of SEB on activated T cells appeared to be death. It is intriguing however that in some cases low levels of proliferation were also seen, implying perhaps that activation signals were necessary in addition to apoptotic signals to induce death of these T cells.

SEB-induced apoptosis was completely inhibited by the addition of CsA indicating the involvement of calcium signalling in death. This death could be mimicked by the addition of the calcium ionophore ionomycin. Again these data are in aggreement with recently published data (Damle et al 1993a). This is in contrast to the enhancement of T cell deletion seen in mice using SEB and CsA (Vanier and Prud'homme 1992). CsA was demonstrated to block the the expansion phase of targeted T cells. The work of Shi et al (1989) demonstrated that CsA blocks anti-CD3 induced apoptosis in the thymus which support the hypothesis of calcium signalling playing a role in apoptosis and the direct involvement of TCR ligation in programmed cell death.

The failure of anti-Class II antibody to block SEB death led to the hypothesis that MHC presentation of this superantigen to the TCR was not necessary and death occurred via a direct interaction of SEB with the TCR. Anergy induced by SEB has also been demonstrated to occur via MHC-independent pathways (Hewitt et al 1992) by direct binding of SEB to the TCR which induces a calcium signal. These data are in direct agreement with the experiments seen here and it is therefore concluded that SEB interaction with the TCR on antigen-specific T cells results in apoptosis. T cells undergo apoptosis in response to SAg in mice but do not express MHC class II which supports the concept of SEB directly interacting with the TCR and causing a calcium signal.

Although not directly demonstrated here it may be suggested that in addition to apoptosis SEB may also be inducing anergy in antigen-specific T cells as in no case was 100% apoptosis shown. Additionally, there are many reports of SEB-induced anergy in the literature to support this hypothesis (Hewitt et al 1992). Interestingly most demonstrations of apoptosis occur in less than 100% of cells opening up the possibility that other events such as anergy and proliferation are occurring simultaneously.

The ability of an antibody to LFA1 (CD11a/CD18) to block SEB-induced apoptosis is consistent with recently published data (Damle et al 1993) which demonstrated that SAg-induced antigen specific death was inhibited by blocking the interaction of LFA1 with its ligands. Additionally, it has previously been demonstrated that proliferation of resting T cells with SAg is efficiently inhibited by anti-LFA1 antibody (Damle et al1992). One explanation for these results is that CD11a/CD18 plays an important role in the interaction of SAgs with T cells and the consequent results. Alternatively, the binding of antibodies to LFA1 may induce steric hindrance thus preventing SEB binding to the TCR. Another possibility for the role of LFA1-mediated cell adhesion is that it may facilitate the interaction of Fas with Fas-L thereby allowing this interaction to exert its death promoting activity. In contrast to data seen here these authors demonstrated that antibody to LFA3 had no such effect (Damle et al 1993) .The interaction of LFA3 with CD2 may transduce a signal which when blocked by anti-LFA3 antibody interferes with SEB-induced death implying perhaps that CD2 signalling may be involved in SEB-induced apoptosis.

SEB-induced death was shown to be rapid and occurred with similar kinetics to Fas-dependent death in Jurkats. More importantly, inhibition of SEB-induced killing of Fas-sensitive Jurkats was demonstrated using an anti-Fas blocking antibody. SEB up-regulation of Fas-L in this system lead to ligation of Fas receptor resulting in the death of the Jurkats. SEB induced apoptosis was therefore dependent on the interaction of Fas-L with its receptor. Fas-L mRNA expression was further demonstrated to be upregulated in SEB-specific T cells upon activation consistent with the involvement of this molecule in AICD. This is in complete agreement with recently published data demonstrating a role for the Fas/Fas-L in SEB induced T cell death (Ettinger et al 1995). It was demonstrated that SEB induced apoptosis was linked to the rapid induction of Fas-L expression in cells constitutively expressing high levels of Fas. In addition blocking of death was seen using a Fas-Fc fusion molecule which is consistent with data presented here.

The ability of SAgs to induce apoptosis via direct up-regulation of Fas-L has potential implication in the pathogenesis of AIDS. One of the symptoms of HIV+ patients is their susceptibility to a wide variety of infections during which they are exposed to an array of diverse micro-organisms including those expressing SAgs such as SEB. Direct exposure of antigen-specific T cells to SAg may lead to apoptosis via up-regulation of Fas-L and thus may contribute to the depletion of T

cells associated with AIDS pathogenesis. Therefore an understanding of the ability of SEB to influence Fas-L expression has implications for therapeutic intervention in AIDS. Moreover recently Fas induced T cell apoptosis in HIV-infected individuals has recently been demonstrated (Katsikis et al 1995). Anti-Fas induced apoptosis involved both CD4+ and CD8+ T cells and correlated with disease severity. These results emphasise the crucial role apoptosis plays in the maintenance of a fully functional immune system and the prevention of disease.

The inability of anti-Fas antibody and in some cases SEB to induce death in Fas positive T cells indicated that, while levels of Fas expression may be sufficient, there are other factors required to initiate the death outcome in response to Fas ligation in T cells. One possibility is that the differentiation state of the T cell influences its susceptibility to death via Fas engagement. It has been demonstrated (Miyawaki et al 1992), that viability of in vitro stimulated T cells was not significantly altered after treatment with anti-Fas antibody in contrast to Fas-expressing cultured cell lines, indicating that additional cellular conditions to Fas expression might be required for Fas-induced apoptosis. Additionally differential sensitivity of activated T cells to anti-Fas mediated apoptosis independent of Fas epitope density on activated T cells has also been demonstrated (Klas et al 1992). It may therefore be concluded that activation signals interfere with the Fas pathway thus preventing apoptosis.

Alternatively, Fas-mediated apoptosis may involve a delicate balance of receptor -ligand interaction and the possibility must be considered that this can be modulated by soluble proteins. Indeed, three human Fas variants have been identified and characterised which encode soluble Fas molecules which are capable of inhibiting antibody induced apoptosis (Cascino et al 1995). In addition elevated levels of soluble Fas observed in some patients with SLE may block apoptosis resulting in accumulation of self-reactive T and B lymphocytes, and perhaps accounting for the presence of increased mutations observed in the lymphocytes of SLE patients (Gmelig-Meyling et al 1992). The identification of such soluble forms of Fas implies that the simple model of Fas/Fas-L interaction as an apoptosis transducing complex needs some enrichment.

Engagement of receptors by specific ligands or crosslinking by specific mAbs causes receptor dimerisation or oligomerisation which promotes recruitment of mediators that trigger a signalling cascade. The apoptotic signal through Fas is induced by the binding of anti-Fas antibodies or the Fas-L to Fas. These tend to be IgM antibodies or

IgG3 antibodies which lead to aggregation of Fas. It has also been shown that the F(ab')2 fragment or other isotypes of the anti-APO-1 antibody hardly induce apoptosis (Dhein et al 1992). These results suggested that oligomerisation of at least three Fas molecules is a biologically relevant complex in generating an intracellular signal. The fact that Fas-L is a TNF-related molecule which exists as a trimer (Boldin et al 1995) agrees with this idea. Furthermore a functional soluble form of human Fas-L has been identified in the supernatant of activated lymphocytes and shown to have a trimeric structure (Tanaka et al 1995). Thus it is likely that Fas-L as a homotrimer binds to three Fas receptors and induces its trimerisation to transduce the apoptotic signal. The threshold of sensitivity to Fas-mediated cell death in partially resistant clones was reduced by the addition of cycloheximide (Tanaka et al 1995). This suggested that there are factors being produced in these clones which protect against Fas-induced death, and protein synthesis inhibition prevents these factors from being produced. Cytotoxic signalling is therefore subject to the inhibitory action of intracellular proteins. The efficacy of Fas cytotoxic signals may be dependent on a critical level of receptor surface expression. Optimal signal transduction presumably requires interaction between a critical fraction of receptors forming the ligand mediated clustering of cells and specific second messenger molecules.

The simple definition of Fas as a death inducer needs elaboration in light of the data presented here showing that Fas engagement can enhance CD3 stimulation which is in agreement with data recently published (Alderson et al 1993). These authors demonstrated that ligation of Fas on freshly isolated T cells by a mAb in conjunction with anti-CD3 stimulation lead to enhance proliferation, increased expression of the IL-2 receptor CD25 and the activation marker CD69 and also enhanced expression of cytokines including IL-2. Additionally similar results have also been obtained using recombinant Fas-L (Lynch et al 1995). It has been hypothesised that Fas might act as a costimulatory molecule during the early part of an immune response while as cells become activated Fas may then become important to suppress the immune response by eliciting apoptotic death of Fas positive activated T cells (Lynch et al 1995). In addition, antibodies have been raised to the Fas receptor which inhibit anti-Fas induced apoptosis and it is speculated that these antibodies bind to determinants on Fas which interfere with the formation of the trimolecular complex required to transduce the apoptotic signal (Alderson et al 1994). The idea of Fas as an immunomodulatory molecule rather than simply a death inducing molecule becomes more valid in light of these data. The immune system has developed an intricate way

of regulating life and death of T cells which is not yet fully understood but its complexity is no longer surprising.

It is possible that Fas-mediated signals may include both apoptosis and cellular activation depending on the activation state of the target cell. Thus conversion of Fas-mediated signals from activation to apoptotic may be an integral component of the transformation process and represents a safety mechanism by which the immune system can eliminate transformed cells from the host by stimulation through Fas. Freshly isolated T cells are not susceptible to Fas-mediated death signals and it takes multiple rounds of stimulation before a cell becomes susceptible to Fas-mediated apoptosis. This may reflect the use of antigenic stimulation as a mechanism to control lymphocyte homeostasis. The immune response may be regulated by the type and anatomical location of APC, which plays a role in determining whether the T cell responding to antigen remains capable of further proliferation or dies.

Induction of death by Fas-L requires the cell to have received more than one signal. Previous signals by antigen or signals occurring simultaneously may be required for the death signal to be triggered by Fas engagement. The signal induced by Fas has been intimately linked with ligation of the TCR (Vignaux et al 1995). It is suggested therefore that rather similar to CD3 Fas mediated death requires costimulation . Consequently, Fas is a neccesary component of the death pathway but is dependent on the integration of other stimuli for transduction of the death signal. This is supported by the recent isolation of death associated molecules (FADD, TRADD and RIP) (Hsu et al 1995, Stanger et al 1995, Chinnaiyan et al 1995). These results firmly place the Fas/Fas-L interaction as an important regulator of immune responses and suggest a role for expression of these molecules in harnessing T cell proliferative responses and terminating a T cell response once it has completed its function.

Ligation of Fas by a mAb was shown here to inhibit the proliferative response induced by CD28 costimulated SEB-driven stimulation. Additionally the proliferative response induced in activated T cells by class II MHC and CD28 stimulation was also inhibited by Fas ligation. These data demonstrate the ability of Fas signals to modulate CD28 induced costimulation. T cell activation, which is intimately dependent on CD28 costimulation, may therefore also involve Fas regulation. CD28 costimulation has been demonstrated to enhance the expression of the survival factor bcl-x_L (Boise et al 1995). Cells which express bcl-x_L at levels comparable to that of CD3/CD28 stimulated T cells were found to be resistant to the induction of cell death

by antibodies crosslinking Fas. Thus costimulation appears to prevent Fas-induced cell death. Together these data appear to imply that CD28 and Fas expression are co-ordinately regulated and the outcome may be determined by which signal predominates.

Down-regulation of bax mRNA expression due to SEB activation led to the hypothesis that in some cases addition of SEB actually protects the cells from apoptosis. Co-ordination of the expression of death repressor factors and death inducing factors may ultimately determine whether a stimulus results in proliferation, anergy or death of a T lymphocyte.

The involvement of B7-1-CD28 interactions in the inhibition of tolerance in the lpr/lpr mice has also been examined (Ettinger et al 1995). CD28 and CTLA4 mRNA were found to be overexpressed in the lymph nodes of lpr mice. Results from these studies suggested that the CD28/CTLA4 interaction with B7-1 is critical to the ability of lpr T cells to respond to SEB stimulation. By blocking the CD28 interaction it was demonstrated that TCR stimulation in the absence of costimulation reanergises the T cells and restores tolerance. This confirms that CD28 can block the induction of anergy . In addition these mice demonstrate a failure to undergo AICD in response to SAgs in vitro (Bossu et al 1992).This was demonstrated to be due to the defect in Fas expression and it has been shown that Fas-L mediated cytotoxicity is crucial in the apoptotic response to SEB (Ettinger et al 1995). The results in these mice suggested that the CD28/B7-1 interaction may be interlinked with the Fas/Fas-L interaction in mediating tolerance in the immune system and further strengthen the hypothesis that CD28 and Fas expression may be co-ordinately regulated.

In summary the Fas/Fas-L has been demonstrated to be intimately involved in the apoptotic death process induced by SEB in antigen-specific T cells. Additionally, the induction of apoptosis appears to require more than Fas ligation and elucidation of the factors which determine susceptibility to SEB- or Fas-induced apoptosis remain a matter of speculation and the focus of intense research interest.

CHAPTER FIVE.

DISCUSSION.

5.1 Discussion

A major focus of attention in this study has been to address the factors necessary for T cell activation . One initial finding was that TCR triggering alone did not stimulate proliferation in resting T lymphocytes and that additional APC-derived signals were required. CD28 costimulation using B7-1 or B7-2 was shown to synergise with TCR signals to induce T cell proliferation, highlighting the importance of the B7-1/CD28 interaction in T cell effector function. This agrees with the current consensus of opinion of the critical role played by CD28 signalling in T cell activation and production of IL-2 (Harlan et al 1995). Additionally, it has also been demonstrated that CD28 can provide costimulation for Th2 proliferation by increasing responsiveness to IL-4 and can costimulate CD8+ cytotoxic cells (McArthur et al 1993, Azuma et al 1993). Collectively, these results demonstrate that the CD28 pathway plays a central role in the activation and proliferation in both Th1 and Th2 classes of T lymphocytes.

It is intriguing to speculate that the B7-1/CD28 interaction might be a target for immune intervention. The demonstration that B7-1 transfection of tumour cells resulted in improved detection and elimination of these cells (Harlan et al 1995) and the ability of CD28 to costimulate CD8+ cytotoxicity (Harding and Allison 1993) indicates opportunities for tumour therapy. Additionally, CTLA-4-Ig induced long-term survival of pancreatic islet grafts (Lenschow et al 1992) suggesting that blocking the CD28/B7-1 interaction may have applications in human organ transplantation and provide an approach to improve transplant success. Interestingly, recent data has also showed that B7-1 induced Th1 cells whereas B7-2 induced Th2 cells (Freeman et al 1995, Kuchroo et al 1995). Another conclusion from these and other studies in NOD mice (Lenschow et al 1995) was that differential administration of anti-B7-1/B7-2 antibodies had opposing effects on disease progression. Collectively, these studies lead to the hypothesis that B7 regulation plays a vital role in T cell function and provides exciting possibilities for immunoregulation of autoimmune diseases.

Another stimulating finding was that LFA3 also costimulated PHA-driven proliferation and IL-2 production. Thus PHA and LFA3 together provided the T cell with all the necessary signals to become fully activated, produce cytokines and

expand. Knowing that LFA3 binds to its ligand CD2 on T lymphocytes, one possible interpretation is that PHA can also interact with this ligand. Both these interactions could then be said to mimic the effect of pairs of anti-CD2 mAbs which have been demonstrated to induce T cell proliferation (Moingeon et al 1992). CD2 has two binding moieties which may both need to be triggered in order that T cell activation occur. CD2 signalling may then be sufficient to induce proliferation and IL-2 production. PHA and CD28 signals synergise to result in T cell activation, thus it is also possible that LFA3 uses the same pathways as CD28, or in some way triggers CD28 signalling although this seems highly improbable. PHA signalling may however intersect the CD28 signalling pathway via PI3K for example which perhaps synergises with CD2 signalling. However, it has yet to be determined exactly what second messenger system is triggered in T cells by PHA.

It was also found that B7-1 or B7-2 alone was capable of inducing proliferation in activated T cells but not resting cells, leading to the hypothesis that resting cells and activated T cells differ in their requirements for proliferation. One attractive idea to explain this difference is that activated T cells have an active TCR signalling pathway which can synergise with CD28 signalling. Consequently, activated T cells are more receptive to B7-1 or B7-2 stimulation than resting cells. This correlates well with experiments showing that CD28 ligation in SAg specific T cells leads to induction of the SMase pathway minutes following addition of either B7-1 transfectants or anti-CD28 antibody (Dr Y. Patel personal communication), reflecting the idea that signal transduction occurs more readily in activated T cells.

Even more interesting was the observation that addition of free SEB to B7-1 or B7-10 stimulated T cells resulted in inhibition of the proliferative response. Evidence has been provided here that SEB in the absence of APCs induces apoptosis in activated T cells, which is in aggreement with work carried out by Damle and colleagues (1993a) which showed that SAg induced PCD in antigen specific T cells. Thus one possibility was that SEB was inducing apoptosis in these cells. Interestingly, proliferation was not totally inhibited which can be interpreted as the occurence of a number of events simultaneously. Therefore some cells may be triggered by B7-1 or B7-2 to proliferate and at the same time cells may be triggered by SEB to die. Alternatively,the majority of cells may receive CD28 signals to progress through the cell cycle where they become susceptible to SEB induced death and consequently die. The signals which result in AICD and proliferation may overlap and are still a stimulating focus of

research and so it is not surprising that there may be more than one interpretation of what may occur during the decision between life and death of T cells.

Progressing from the observation that SEB can inhibit T cell proliferation and induces apoptosis in activated T cells it was also found that not all activated T cells examined were induced to apoptose with SEB or with Fas. This begged the question; what are the factors which control cell death and why do some cells die while others survive and proliferate? One explanation could be that protective factors such as the bcl-2 family of proteins plays a role in determining survival. Bcl-2 plays a crucial role in determining survival of lymphocytes (Nakayama et al 1993) while expression of bcl-2 or bcl_{xl} may dictate whether a stimulus induces T cell death (Boise et al 1995, Chao et al 1995). Preliminary data demonstrated the involvement of bax in determining susceptibility to SEB-induced apoptosis, as SEB induced down-regulation of this death enhancing factor in activated T cells . Interestingly, some aspects of T cell survival appear to be linked to the state of activation of the T cell. It has recently been shown that costimulation through CD28 enhances the survival of T cells activated through the TCR not only by enhancing IL-2 production but also by regulating expression of bcl_{xl} (Boise et al 1995). By augmenting lymphokine production, CD28 costimulation is likely to effect the survival of T cells while bcl_{xl} transfected Jurkatts survived Fas-induced PCD, thus suggesting that bcl_{xl} is likely to play a role in the ability of CD28 to enhance T cell survival. These results reflect the importance of the activation state in regulating survival of T cells and the inter-relationship between factors that control proliferation and those that regulate death.

Another intriguing observation is that CD28 results in the generation of ceramides and induction of the SMase pathway, which has also been shown to be a signal for apoptotic death upon ligation of Fas or TNF. (Cifone et al 1993, Boucher et al 1995). How then does CD28 signalling result in costimulation rather than apoptosis? It can be proposed that the involvement of other signals such as PI3 kinase in the CD28 pathway may provide an answer to this question as it has also recently been found that PI3 kinase prevents apoptosis induced by NGF (Yao and Cooper 1995) .Consequently, the difference between life and death may reside in the interaction of the two pathways, and which predominates, which may in turn depend on the overall activation state of the T cell. The CTLA-4 signalling pathway has yet to be elucidated and it is intriguing that there is conflicting evidence as to whether PI3kinase is involved (Stein et al 1994, Schneider et al 1994). The finding that an antibody to CTLA-4 can induce apoptosis and that CTLA-4 knockout mice have

amino acid carboxyl terminus of Fas was shown to associate with a protein phosphatase that had previously been identified in basophils. Cells that were resistant to Fas-L killing expressed this phosphatase whereas susceptible cells did not. Resistance could be conferred by introducing the phosphatase into sensitive cells .It will be interesting to examine the SEB T cell lines produced here for expression of this phosphatase. Co-ordination of signals received from kinase and phosphatases may ultimately determine the outcome of Fas ligation in T lymphocytes.. This is reminiscent of the crucial role phosphorylation and dephosphorylation play in TCR-mediated signalling and substantiates the hypothesis of interaction of the pathways which determine life and death of T cells.

One final interesting observation was that the death domains of Fas and the recently isolated associated death molecules, FADD, TRADD and RIP revealed a significant homology to the family of ankyrins that mediate interaction between integral membrane components and cytoskeletal elements (Chinnaiyan et al 1995, Stanger et al 1995). Cytoplasmic molecules including IkBα and NFκB are ankyrin containing molecules which have been shown to be down-regulated by CD28 signalling leading to IL-2 gene expression . It is intriguing to infer from this that ankyrin molecules express a death domain which is important in suppressing IL-2 production which can lead to cell death. This demonstrates further similarities between the downstream events involved in CD28 signalling and the signalling involved in Fas-mediated apoptosis.

This work has examined two of the most exciting interactions in T lymphocyte immunology today, that of B7-1 and CD28 and Fas and its ligand Fas-L. The importance of these molecules in T cell function has been demonstrated and it has become obvious over the last year that the fate of a T lymphocyte is dependent on co-ordination of the signalling pathways involved in these interactions. The underlying message suggested by the results presented is that life and death of a T lymphocyte are complicated phenomenon.The requirements for T cell activation and apoptosis appear to be similar in that more than one signal is necessary for either to occur and that their signalling pathways may intersect or use similar second messenger systems .

An understanding of the molecular events which regulate T cell effector function is vital in order to facilitate prevention and cure of disease. The treatment of autoimmune diseases such as rheumatoid arthritis , insulin dependent diabetes

mellitus has improved enormously due to studies addressing the structure of the immune system. Equally significant has been the strides made in our understanding of cancer and relatively new diseases such as AIDS due to investigation of the mechanisms involved in expansion, regulation and deletion of immune effector cells. However, it has become increasingly apparent that the complexity of regulation of the immune system dictates that immunomodulatory intervention may not be as simple as once thought. It is hoped that this study has contributed to a further awareness of the role T cell activation and apoptosis plays in immune homeostasis .

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