PHD

The role of Fas in human SLE

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Award date:
1998

Awarding institution:
University of Bath

THE ROLE OF FAS

IN HUMAN SLE.

Submitted by Yusuf Ismail Patel

for the degree of PhD at the University of Bath.

1998

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This thesis is dedicated to

Nigar Sultana

Suhail

Aadil

and

Eesa

For all your patience and perseverance.

Acknowledgements

A very special thank you to my supervisor, colleague and friend, David Sansom for all his help and guidance, without whom this work would not have been possible.

Thank you to all at BIRD and particularly in the Immunology group (Anne Wilson, Martina Boshell, Christine Edmead, Julie McLeod, Lucy Walker, George Boulougouris and Claire Ellwood) for practical advice and for putting up with a "medic" in the lab. I must also include Jackie Russell, Maureen Jacobs and Fiona Davies for a special thank you for making it so easy and pleasant to work at the old Bath Institute for Rheumatic Diseases.

I should thank Peter Maddison, for first accepting me as a visiting research fellow in Bath, and for his constant encouragement and enthusiasm ever since. No doubt, I cannot thank Neil McHugh enough for his support, encouragement and friendship from my first days in Bath. As for Jon Beresford, much of this thesis would not have been possible if he did not lend a hand to the computer systems at BIRD and also my own computer at home.

This acknowledgement would not be complete if I didn't thank the person who first instilled in me the passion for academic medicine. Thank you Joe (Prof Y Veriawa), for all your advice and help over the years!

I cannot thank my family enough for putting up with my long journey of study. Thank you Mum and Dad for all your help which has been forthcoming from the very beginning and still doesn't cease; and thank you Nigar and "the boys" for your patience and perseverance with all the challenges of living in a foreign land.

Finally, thank you to the MRC (both in South Africa and the UK) and the Wellcome Trust without whose funding I would not have been able to conclude this work.

CONTENTS

SUMMARY

Apoptosis is an important homeostatic mechanism for the regulation of cellular differentiation and proliferation. Within the immune system in particular, a failure of apoptotic regulation leads to lymphoproliferative disease and / or autoimmunity. A number of molecules have been identified with an important role in regulating apoptosis, including both pro-apoptotic and anti-apoptotic proteins. One important pro-apoptotic receptor, Fas (CD95 / Apo-1) was identified as responsible for the autoimmunity and lymphoproliferative disease in the MRL-*lpr/lpr* mouse. The autoimmune disease in this mouse has a striking resemblance to human Systemic Lupus Erythematosus (SLE). As a result, the Fas gene was an important candidate for analysis in human SLE.

However, to study Fas biology in human disease more information was required about Fas function in normal human physiology. Therefore, a major objective was to establish useful reagents to enable further analysis of the role of Fas in the maintenance of lymphocyte homeostasis. With these reagents, a putative ligand (FasL) for the Fas receptor was identified on activated T cells, thus establishing a basis for further analysis of Fas-FasL interactions. As a result, a soluble FasL was also created in vitro, which contributed further to our understanding of Fas mediated apoptosis. The ability of a soluble ligand to induce apoptosis has also been confirmed by recent studies with naturally produced soluble FasL.

Finally, an analysis of the role of Fas was carried out in patients with SLE using genetic and functional approaches. PCR-SSCP analysis of the Fas gene cDNA revealed one patient with a mutation in the cytoplasmic region of Fas, suggesting that no major defect of Fas exists in the SLE population as a whole. However, functional data suggested enhanced in vitro apoptosis following Fas engagement on SLE T cells. The clinical significance of this with regard to the pathogenesis of the autoimmune disorder needs to be defined.

Abbreviations

Ab	Antibody
AICD	Activation Induced Cell Death
ALPS	Autoimmune Lymphoproliferative Syndrome
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
Ca^{2+}	Calcium ions
CAPK	Ceramide Activated Protein Kinase
CAPP	Ceramide Activated Protein Phosphatase
CD	Cluster of Differentiation
cDNA	complementary Deoxyribonucleic Acid
ced genes	*Caenorrhabditis elegans* death genes
CHO cells	Chinese Hamster Ovary cells
CO_2	Carbon Dioxide
CPM	Counts Per Minute
CQ	chloroquine
^{51}Cr	radioactive chromium
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic Lymphocyte Associated Antigen 4
ddH_2O	double distilled H_2O
DED	Death Effector Domain
DEPC	Diethylpyrocarbonate
DISC	Death Inducing Signalling Complex
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic Acid
DR-3/4	Death Receptor 3/4
EAE	Experimental Autoimmune Encephalomyelitis

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FADD	Fas Associated Death Domain containing protein
FAP1	Fas Associated Phosphatase 1
FasL	Fas ligand
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FL1	Fluoresence intensity (channel 1)
FLICE	FADD-like ICE
FLIP	FLICE Inhibitory Protein
FSC	Forward Light Scatter
GST	glutathione - s - transferase
h	Hour(s)
^{3}H-thymidine	Tritiated thymidine
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICE	Interleukin-1 β Converting Enzyme
IFN γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IU	International Units
JNK	c-Jun N-terminal Kinase
Kda	Kilodaltons
LFA	Lymphocyte Function Associated Antigen
mAb	monoclonal antibody
MAPK	Mitogen Activated Protein Kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex

min	minute (s)
mRNA	messenger Ribonucleic Acid
ms	milliseconds
NFκB	Nuclear Factor κB
NGFR	Nerve Growth Factor Receptor
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	Poly (ADP-Ribose) Polymerase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PMA	Phorbol Myristate Acetate
PS	Phosphatidylserine
RNA	ribonucleic acid
RNAse	Ribonuclease
RT-PCR	reverse transcription PCR
SDS	Sodium Dodecyl Sulphate
SEB	Staphylococcal Enterotoxin B
SEM	Standard Error of the Mean
sFasL	soluble Fas ligand
ssFasL	signal sequence soluble Fas ligand
SLE	Systemic Lupus Erythematosus
SLICC	Systemic Lupus International Cooperating Clinics
SSC	Saturated Sodium Chloride
TAE	Tris - acetate - EDTA buffer
TBE	Tris - borate - EDTA buffer

TE	Tris - EDTA buffer
TCR	T Cell Receptor
TNF	Tumour Necrosis Factor
TNF-R	Tumour Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis Inducing Ligand
UV	ultraviolet light
v/v	volume / volume
w/v	weight / volume
z-VAD-FMK	Cbz-Val-Ala-Asp(OMe)-fluoromethyl ketone

CHAPTER 1

INTRODUCTION

1.1) APOPTOSIS

1.1.1) General

Homeostasis refers to the essential process of check and balance that is operative within the human body, and in fact in all living systems. Cell numbers are essentially maintained by a balance between proliferation and death, both of which occur simultaneously and continuously within the normal human body. Examples of homeostasis can be seen in the cellular turnover of the skin, the gastointestinal tract, and particularly in the immune system.

Cell death is known to take two forms; namely necrosis and apoptosis (Kerr et al, 1972). Apoptosis is frequently also referred to as programmed cell death. Cell death by apoptosis is different from necrosis mainly in the highly organised nature of the process (reviewed by Cohen, 1993). Whereas necrotic cells undergo early swelling and enlargement followed by membrane rupture and spillage of the intracellular contents, apoptotic cells develop characteristic cytoplasmic shrinkage and membrane blebbing followed by nuclear condensation and fragmentation.

An important functional correlate of apoptosis is that this form of cell death is not usually associated with an inflammatory response because there is no extrusion of intracellular contents as occurs with necrosis **(Figure 1.1)**.

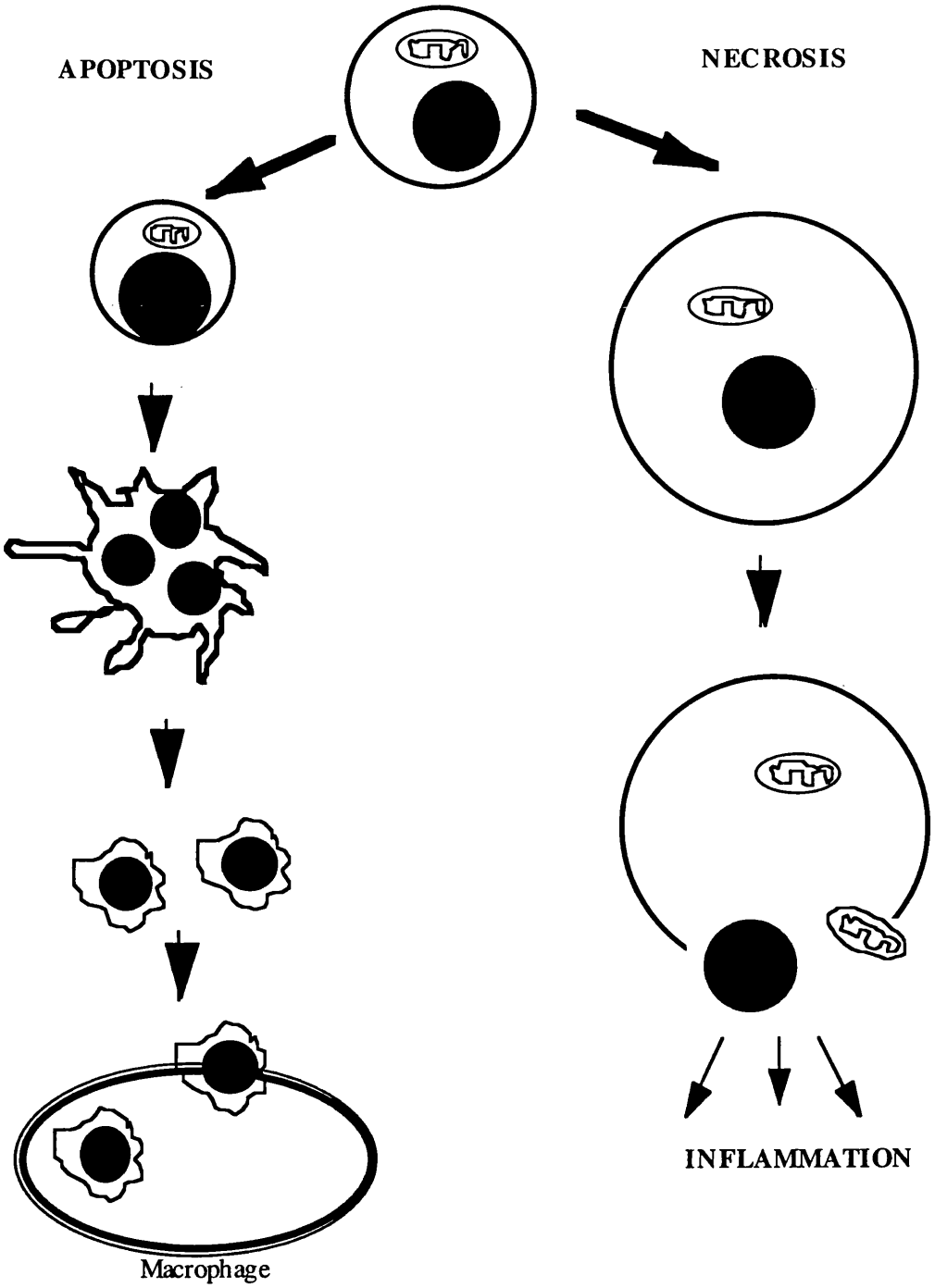


Figure 1.1: Morphologic cellular changes in Apoptosis and Necrosis.

Cells undergoing death by necrosis usually swell and rupture to release intracellular contents which induce an inflammatory response. In contrast apoptotic death is characterised by a shrinking cytoplasm, membrane blebbing and nuclear condensation. Membrane-bound apoptotic blebs are rapidly phagocytosed thus preventing the non-specific induction of an inflammatory reaction.

Rapid phagocytosis of the apoptotic membrane-bound cellular contents by macrophages prevents the induction of an inflammatory cascade. Macrophages and other phagocytes have highly specific and sensitive receptors for apoptotic particles (reviewed by Savill et al, 1993) and it may be the rapid nature of the mopping up process which has led to the failure to detect apoptosis as a ubiquitous process in standard histochemical sections in the past. As a result, the vigilant observations of Wyllie and Kerr only really induced a greater interest in apoptosis amongst the scientific community approximately twenty years after its first description (Kerr et al, 1972). Subsequently the scale of interest in apoptosis both in biology and in the context of disease has led to an escalation in the pace of research such that a number of improved techniques have become available for the detection of apoptosis (Studzinski, 1995).

Apoptosis can be induced by a variety of stimuli, including ultraviolet irradiation, toxins, viral infections, drugs (particularly cytotoxic anti-cancer agents), growth factor withdrawal as well as specific apoptosis-inducing proteins like TNFα and CD95/Fas. Most of these factors usually involve the stimulation of a cascade of intracellular events leading to the morphologic manifestations described. A number of surface and intracellular molecules have been identified with important functions in the apoptotic process. These may take part in induction or inhibition of apoptosis either directly or through a network of complex intracellular interactions with other proteins or genes. This is a rapidly advancing field and could not justifiably be reviewed completely in detail here, but relevant areas will be discussed in relation to Fas-mediated apoptosis later.

1.1.2) Apoptosis in Lymphocytes

Control of cell number and cellular differentiation is an example of homeostasis within the immune system. Immune cells such as T cells and B cells undergo continuous modification from the time of development within the thymus and bone marrow, respectively, until they are no longer required for function within the body. Large numbers of immune effector cells are destroyed or inactivated during development within the thymus and bone marrow, and the evidence suggests that this process of negative selection occurs through apoptosis (Kappler et al, 1987).

In addition to central tolerance, there is also a need to control activated T and B cells once they are circulating in the periphery, particularly when they have carried out their primary function in the immune response. If this peripheral control fails then homeostasis can be dangerously upset in favour of autoimmune disease states or malignancy. Apoptosis is therefore considered to play a significant role in the control of peripheral activated immune cells (Singer and Abbas, 1994). However, apoptosis is by no means the only homeostatic control and other regulatory mechanisms such as anergy and clonal ignorance are also thought to operate in the maintenance of peripheral tolerance (Abbas et al, 1997).

Apart from the regulation of central and peripheral tolerance, apoptosis of lymphocytes also serves important functions in the regulation of cytotoxic lymphocyte function, NK cell function and acitvation induced cell death (reviewed by Cohen and Duke, 1992).

1.1.3) Apoptosis in Disease

Apoptosis in disease states may be either increased or decreased **(Figure 1.2)**. Examples of reduced apoptosis include lymphoid malignancies and autoimmune diseases (Krammer and Debatin, 1992), whilst enhanced lymphocyte apoptosis has been associated with conditions like AIDS (Meyaard et al, 1992). Many anti-cancer drugs and glucocorticoids mediate their effects through enhancing apoptosis of lymphocytes and other cell types (Kerr et al, 1994). Similar patterns may be found in other disease states not involving lymphocytes. For example decreased apoptosis is seen in carcinomas associated with p53 mutations, hormone-dependent breast and prostatic tumours, and viral infections with Herpesvirus, Adenovirus or Poxviruses, whilst increased apoptosis has been associated with neurodegenerative diseases like Alzheimer's disease, Amyotrophic lateral sclerosis, Cerebellar degeneration, and reperfusion ischaemic injury following myocardial or cerebral infarction.

INCREASED APOPTOSIS	DECREASED APOPTOSIS
AIDS	Malignancy
Hashimoto's Thyroiditis	Viral infections: Herpes virus Adenovirus Pox virus
Neurodegenerative disease: Alzheimer's disease Cerebellar degeneration Amyotrophic sclerosis	Autoimmunity
Reperfusion injury: Myocardial infarction Cerebral infarction	
Insulin Dependent Diabetes	
Anti cancer drugs: Adriamycin Methotrexate Bleomycin	

Figure 1.2: Examples of diseases associated with altered apoptosis.

1.1.4) Signalling pathways in Apoptosis

1.1.4.1) Caspases

The signalling mechanisms for apoptosis are still being deciphered but current evidence suggests a common committed pathway of intracellular events leading to the cytoplasmic and nuclear changes characteristic of apoptosis. This involves a series of proteases called Caspases (cysteine proteases that cleave after an aspartate residue), which act in a cascade type reaction to ultimately lead to the nuclear and morphologic changes characterising apoptosis (Martin and Green, 1995). Caspases are currently grouped into three families based on their amino acid sequence recognition but it is as yet unclear if they always function by sequential activation in a cascade-like reaction. However in the case of Fas it is now believed that sequential activation occurs of Caspase 8 (FLICE), Caspase 1 (ICE), and Caspase 3 (PRICE) (Los et al, 1995; Armstrong et al, 1996; Duan et al, 1996; Enari et al, 1996; Muzio et al, 1996; Anel et al, 1997).

Caspases have been shown to mediate apoptosis following a variety of stimuli (Sarin et al, 1996). The essential nature of this signalling function is highlighted by the ability of soluble inhibitors of Caspases, like Z-VAD-FMK (Cbz-Val-Ala-Asp(OMe)-fluoromethylketone), to completely block apoptosis (Sarin et al, 1996). A stimulus-dependant and hierarchical response is indicated by the Caspase-1-deficient mice, where a defect is seen in CD95-mediated apoptosis but dexamethasone and γ-irradiation maintain the ability to induce cell death by apoptosis (Cleveland and Ihle, 1995).

The Caspases mediate cleavage of various cellular substrates including fodrin, PARP, topoisomerase and various others (Labeznik et al, 1994; Martin and Green, 1995). The exact position of the Caspases within the signalling pathway to death in relation to surface stimuli is still being mapped out.

1.1.4.2) Mitochondrial function in apoptosis

The inner mitochondrial membrane has an asymmetric distribution of ions resulting in the inside being negatively charged. This transmembrane potential is disrupted at an early stage of cellular commitment to apoptosis, and can be detected using various cationic lipophilic fluorochromes (Idziorek et al, 1995). The process of disruption of the membrane potential is known as permeability transition (PT), where mitochondrial proteins leak out into the cytoplasm. PT can be induced by a variety of apoptotic stimuli, including CD95 (Castedo et al, 1996). Included amongst the mitochondrial proteins which leak out during PT are cytochrome c and the pro-apoptotic protein, AIF (apoptosis inducing factor) (Liu et al, 1996a ; Zamzami et al, 1996). It is interesting that AIF can proteolytically activate caspase 3 (Susin et al, 1997). The exact role of mitochondria and hierarchical interactions with the caspase cascade remains to be fully defined, but it is intriguing that most of the Bcl-2 gene family are localised to the outer mitochondrial membrane where they may regulate mitochondrial function (reviewed by Cory 1995).

1.1.4.3) Bcl-2 as a negative regulator of apoptosis

The Bcl-2 gene was first identified from follicular B cell lymphomas (Bakhshi et al, 1985), and has been regarded as a prototypic cell death repressor gene responsible for the prolonged cell survival in these lymphomas. During the course of the last five years a large number of family members belonging to the Bcl-2 gene family have been isolated including both anti-apoptotic (Bcl-X_L and BAG-1) and pro-apoptotic molecules (BAX, BAK, Bcl-X_S, and BAD) (Boise et al, 1993; Takayama et al, 1995; Oltvai et al, 1993; Simonian et al, 1997; Gajewski and Thompson, 1996). Homo- or hetero-dimerisation of the various family members can take place and the combination of molecules within this dimeric structure appear to determine the cellular outcome

(Cory, 1995). The exact mechanisms by which Bcl-2 family members mediate their functions is currently being deciphered, but it appears that three major mechanisms may be operative. These include influence on the Caspase pathway via interactions with CED-4 (Spector et al, 1997), regulation of the release of cytochrome c from mitochondria (Yang et al, 1997a), and regulation of the Bcl-2 proteins by phosphorylation (Chang et al, 1997).

An understanding of the molecular interactions which determine the ultimate outcome with respect to apoptosis and identification of key regulators of the process should allow more targeted intervention in an attempt to control some of the diseases mentioned above. One pro-apoptotic player in this cellular process of death is Fas (CD 95), which will be outlined in more detail now.

1.2) FAS BIOLOGY

1.2.1) Identification and characterisation of Fas

Of the large number of molecules now identified to play a role in apoptosis, CD95/Fas is thought to have an important pro-apoptotic function. Fas was named as such by Yonehara et al in 1989 when a monoclonal antibody was raised with reactivity to "fibroblast associated surface" antigen derived from a human diploid fibroblast cell line FS-7 (Yonehara et al, 1989). An independent group in Germany simultaneously published another monoclonal antibody called anti-Apo-1 which was developed following immunisation of mice with a human B lymphoma cell line (Trauth et al, 1989). Subsequent cloning and characterisation of Fas and Apo-1 revealed identical sequences (Oehm et al, 1992) and CD95 became the assigned cluster of differentiation (CD) number for Fas / Apo-1 at the Human Leucocyte Differentiation Workshop in Boston in 1993 (Schlossman et al, 1995). Except where Apo-1 is specifically referred

to, the term Fas will be used in this dissertation as interchangeable and synonymous with CD95.

Anti-Fas antibody (CH 11) was developed by immunising mice with human diploid fibroblast cells FS-7 (Yonehara et al, 1989). Cell lines were stained with the antibody and lymphoma cells (KT-3) were found to expresss large amounts of Fas. Using cDNA libraries from KT-3 cells, Itoh et al in 1991 cloned and identified the cDNA for human Fas which consisted of 2534bp with a polyadenylation signal (ATTAAA) at the 3' end. A long open reading frame (1005 nucleotides) from the initiation codon at nucleotide positions 195-197 to the termination codon TAG at position 1200-1202 was found to code for a protein of 335 amino acids. Hydropathy analysis indicated the presence of a signal sequence at the N-terminal end, and predictions from analysis of typical peptide cleavage sites suggested that the mature Fas protein had approximately 319 amino acids with a calculated Mr of 36000. Amino acid analysis predicted that Fas contained an extracellular domain of 157 amino acids, a membrane-spanning domain of 17 amino acids, and a cytoplasmic domain of 145 amino acids. The extracellular domain is rich in cysteine residues (18 of 153 amino acids) and the cytoplasimc domain is relatively rich in charged amino acids (24 basic and 19 acidic out of 143 amino acids). Western blotting analysis confirmed a specific band of apparent Mr 43000, where the difference from predicted was explained by glycosylation at two sites in the extracellular domain (Yonehara et al, 1989).

1.2.2) Fas and the TNF receptor superfamily.

Comparison of the Fas sequence revealed similarity to the TNF-receptor (TNF-R) family of proteins with overall identity to other family members being 24 -30 % (Itoh et al, 1991). The TNF-R family consists of a number of type 1 membrane proteins including the TNF receptors type 1 and 2, CD40, CD30, CD27, OX 40, 4-1BB and

including the TNF receptors type 1 and 2, CD40, CD30, CD27, OX 40, 4-1BB and the NGF-R. The family as a whole is characterised by cysteine rich extracellular domains with their homologous regions confined to the extracellular region (reviewed by Smith et al, 1994). There was no evidence to suggest that anti-Fas or TNF could transmit their effects by binding to each others receptors (Owen-Schaub et al, 1992). **Figure 1.2.2** shows some of the features of the TNF-R family of proteins.

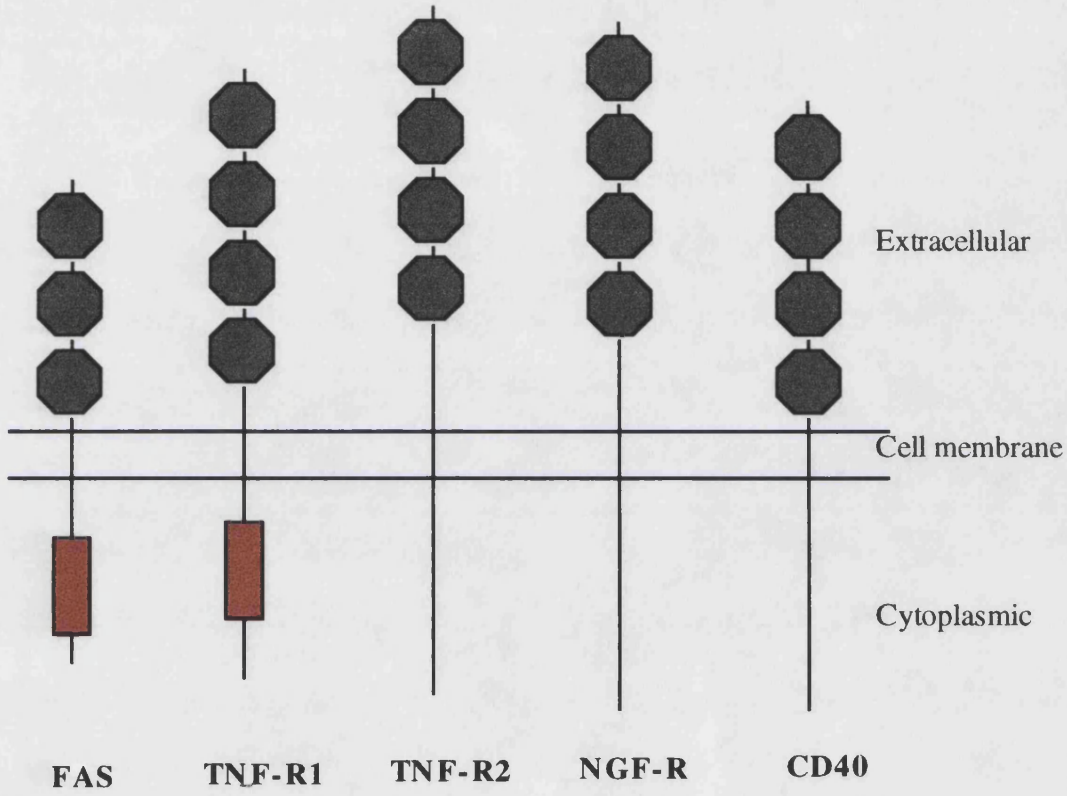


Figure 1.2.2: The TNF-NGF receptor family of proteins.

A schematic diagram is shown with only some representative members of the family of TNF-R like proteins. Amongst a growing family these include CD27, CD30, OX40, and 4-1BB. They all have cysteine rich extracellular domains as indicated but Fas and TNF-R1 differ from the others in the highly homologous death domain (approximately 80 amino acids in length and indicated by the red boxes) situated in the cytoplasmic region of the molecule. Despite the homologous extracellular and death signalling regions of Fas and TNF-R1they do not cross-transduce apoptotic responses.

1.2.3) The early evidence for apoptosis following Fas engagement.

Anti-Fas antibody was shown to induce apoptosis by DNA laddering patterns and ultrastructural electron microscopic changes which first became visible by 3 hours after exposure to monoclonal CH11 (Itoh, 1991). CH 11 being an IgM antibody did not require cross linking in order to mediate apoptotic changes unlike the Apo-1 antibody (Dhein et al, 1992). As mentioned above a German group working on anti-Apo-1 identified an antibody with reactivity to activated lymphocytes and they subsequently cloned the gene (Oehm et al, 1992) which was found to be identical to the Fas sequence. The original monoclonal anti-Apo-1 antibody showed restricted expression on malignant lymphocytes (Trauth et al,1989). Anti-Apo1 also induced apoptosis and tumour regression in mouse models. The issue of resistance or insensitivity to Fas-mediated apoptosis was briefly mentioned in this paper but no attention was dedicated to the phenomenon of resistance to apoptotic cell death at that stage. Apo-1 expression was also found on resting lymphocytes but upregulation occurred on activation with phorbol ester. It was interesting that Apo-1 expression was found on freshly isolated lymphocyte subpopulations from leukaemic patients probably reflecting their in vivo activation in this disease (Trauth et al, 1989).

1.2.4) Fas expression on human cells

Using the monoclonal CH 11 anti-Fas antibody, Fas expression was first seen by FACS analysis of human T and B cells but not on NK cells (Miyawaki et al, 1992). In this study no Fas expression could be seen on T cells, B cells or NK cells of neonates but Fas expression did increase with age. Fas expression was also upregulated on activation of T cells (PHA) and B cells (*S.aureus* Cowan 1 + IL-2) but it must be noted that these experiments were conducted using neonatal blood which does not show any Fas expression at baseline, hence an apparently marked maximal expression

occurring on day 5 after activation. It was noted however that Fas expression on mature T cells were largely seen on activated memory $CD45Ro^+$ cells and not on naive $CD45Ro^-$ cells.

A similar pattern of staining for Fas occurred in B cells displaying activation or memory phenotypes where surface expression of sIgD is lost (naive B cells express sIgD and sIgM and lose expression of sIgD on activation). Cell lines originally derived from leukaemia and lymphoma patients (Jurkat and KT-3, respectively) were also tested for Fas expression and found to have very high levels of constitutive Fas on their surface. Whilst Jurkat and KT-3 cell lines underwent apoptosis (measured by propidium iodide staining), freshly isolated memory T cells and B cells from adults did not show Fas sensitivity, nor did 5 day activated neonatal T and B cells (Miyawaki et al, 1992).

The Fas death pathway has now been shown to be active in many cell types other than lymphocytes, including neutrophils (Liles et al, 1996), eosinophils (Tsuyuki et al, 1995), monocytes (Shinohara et al, 1995), hepatocytes (Galle et al, 1995), muscle cells (Seino et al, 1996), a variety of brain cells (Dowling et al, 1996; Dsouza et al, 1996), synoviocytes (Kawakami et al, 1996; Matsumoto et al, 1996), keratinocytes (Sayama et al, 1994), and thyroid cells (Giordano et al, 1997). In fact, Fas-mediated apoptosis appears to occur in virtually any cell type that expresses Fas under the appropriate conditions, hence placing Fas as a molecule whose role extends beyond just the maintenance of lymphocyte homeostasis.

1.2.5) The signalling cytoplasmic region of Fas

1.2.5.1) Identification of the critical cytoplasmic domain

A further advance in our understanding of the apoptosis mediated by Fas came with the work of Itoh et al in 1993 where they described cytoplasmic deletion mutants and demonstrated the importance of a cytoplasmic death domain in the human Fas gene. Mature human Fas has 319 amino acids with 157 extracellular and 145 cytoplasmic amino acids.

The cytoplasmic region was shown to contain a cell killing domain of 68 amino acids closely conserved with TNF-RI (p55, ≈24% identity). In the case of Fas the 130 amino acid stretch between 175 - 304 includes this conserved region and deletion mutants generated in this region resulted in impaired apoptosis. Deletion of the C-terminal 15 amino acids resulted in enhanced apoptosis implying a regulatory role for this domain **(Figure 1.2.5.1).** The C-terminal domain in other proteins have also been shown to play a role as regulators of function, for example tyrosine kinase receptors like PDGF receptors and G-protein coupled receptors like adrenergic receptors. Itoh et al (1993) also generated a human Fas mutant resembling the *lpr* ^{cg} mouse model (Ile-225 to Asn-225) but in the case of human Fas the equivalent amino acid Val-238 was mutated to Asn-238 and shown to have a major defect in Fas-mediated apoptosis just like the *lpr* ^{cg} mouse (discussed later).

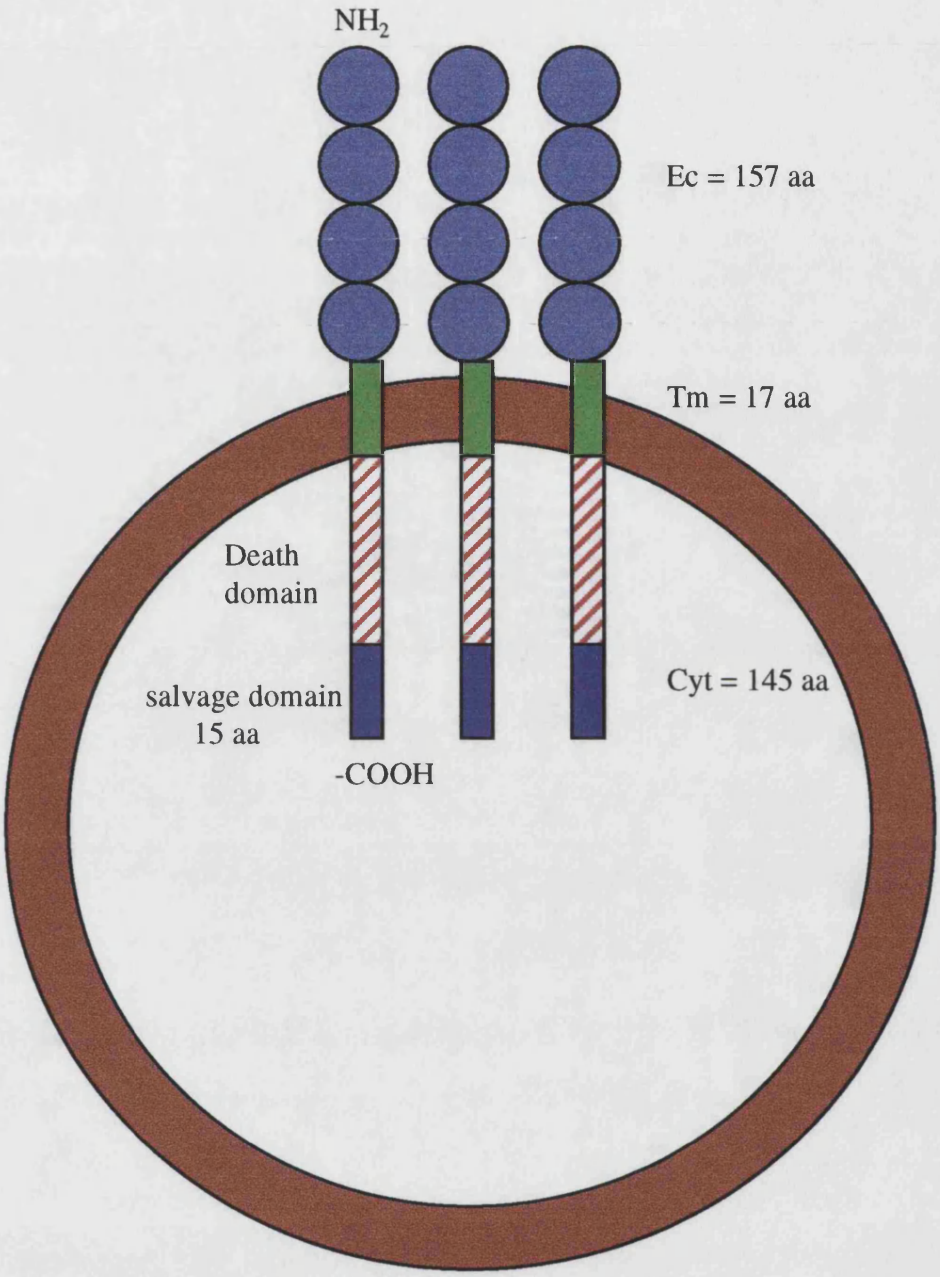


Figure 1.2.5.1 : Schematic representation of the Fas protein

The 319 amino acid (aa) Fas transmembrane protein is depicted in the diagram with the extracellular (Ec), transmembrane (Tm) and cytoplasmic (Cyt) regions as shown. Within the cytoplasmic signalling region is a 130 aa death inducing domain (red hatched) essential for apoptosis and which has a 68 aa homology with the death domain of the type 1 TNF-R. The terminal 15 aa of the Fas cytoplasmic tail (blue boxes) plays a regulatory role in preventing uncontrolled apoptosis

1.2.5.2) Factors that associate with the Fas death domain.

In an attempt to trace the signalling events following Fas engagement, many groups have been isolating a variety of proteins that interact directly or indirectly with the Fas cytoplasmic domain. Current evidence suggests that FADD (Fas-associated death domain binding protein), binds to the death domain of Fas by means of an homologous region (Chinnaiyan et al, 1995; Muzio et al, 1996). Following the FADD - Fas interaction, another protein, FLICE is recruited to the death domain. FLICE has Caspase activity and may either directly activate a downstream cascade of Caspases or may depend on other Caspases for its activation (Enari et al, 1996; Muzio et al, 1996).

More recently death domain associating proteins have been identified which influence the cellular path to death. Daxx (Yang et al, 1997b) appears to bind to the death domain at a site different from FADD, and promotes apoptosis just like FADD but in addition also stimulates JNK signalling. FLIP is a protein with homology to a viral FLIP previously identified and has been shown to block apoptosis by binding to either FLICE or FADD thereby preventing Caspase activation at a very early point in the Caspase death pathway (Irmler et al, 1997). Independantly cloned molecules have been found to be identical to FADD (MORT-1) (Boldin et al, 1995) and FLICE (MACH) (Boldin et al, 1996). Other family members like the TNF-R also use similar death domain associated proteins to transduce their effects. TNF-R signalling at this stage appears to be more complex with divergence of signalling pathways for death (TRADD (Hsu et al, 1995), FADD, FLICE, Caspases) and survival (TRAF, NF-kB) (Liu et al, 1996b).

A number of other death proteins have been identified including DR3/Wsl (Chinnaiyan et al, 1996), TRAIL (Wiley et al, 1995), TRAMP (Bodmer et al, 1997), RIP (Stanger et al, 1995), REAPER (Golstein and Depraetere, 1995) and Apo-2 ligand (Marsters et al, 1996) but how these interact with Fas is not clear as yet. The latter proteins may be alternative mechanisms for apoptosis rather than factors that directly influence Fas-

mediated death. Bcl-2 and Bcl-xL have been shown in many in vitro and in vivo experiments to inhibit Fas-mediated apoptosis (Memon et al, 1995; Reap et al, 1995; Martin et al, 1995). The exact mechanism by which the inhibition occurs is not entirely clear but based on the effects of the homologous Ced9 inhibiton it is most likely at the level of the mitochondria (Kroemer et al, 1997) **(Figure 1.2.5.2).**

1.2.5.3) Other signalling mechanisms implicated following Fas engagement.

Many other signalling mechanisms have been implicated following Fas stimulation, including protein tyrosine kinases (Eischen et al, 1994), reactive oxygen intermediates (Hug et al, 1994), calcium (Oshimi and Miyazaki, 1995; Vito et al, 1996), Fas-associated serine-threonine kinases(Tian et al, 1995), phosphatases (FAP (Cleveland and Ihle, 1995) and HCP (Su et al, 1995), Ras (Gulbins et al, 1995), Lyn and p59fyn kinase (Wang et al, 1996), sentrin (Okura et al, 1996), and JNK (Verheij et al, 1996; Chen et al, 1996; Nishina et al, 1997) and ceramide generated from sphingomyelinase activity (Cifone et al, 1994; Gulbins et al, 1995).

Exactly how these fit into the puzzle is not entirely clear, and it may be that some of these are epiphenomena related to phosphorylation changes induced in intracellular proteins following Fas receptor stimulation and Caspase activation. The experimental data presented for many of these signalling molecules suggest an ability to influence apoptotic outcome in the models studied but it is difficult to allocate a hierarchical importance to the various molecules at present.

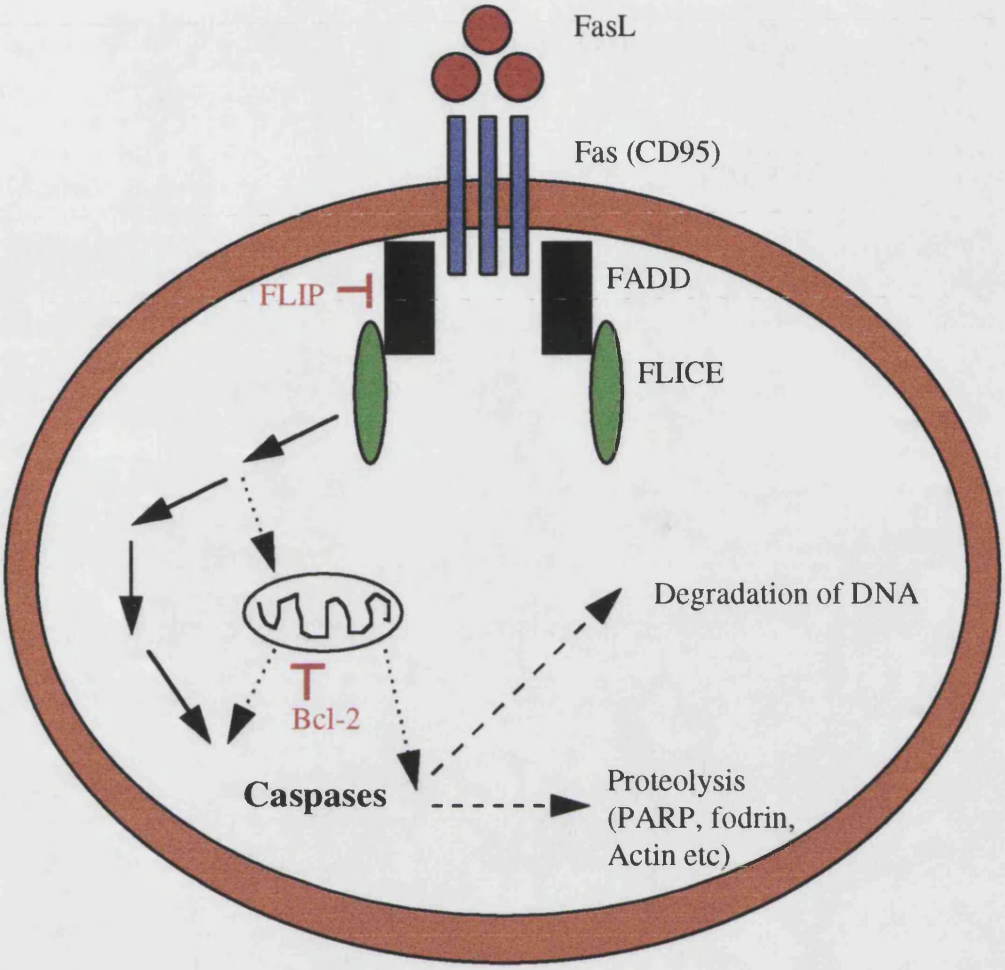


Figure 1.2.5.2 : Some signalling mechanisms induced by Fas.

Diagram showing some of the intracellular signalling mechanisms mediating apoptosis. Following Fas engagement by ligand , trimerisation occurs which is followed by binding of FADD to the death effector domain of Fas. The formation of this complex then attracts binding of intracellular FLICE (caspase 8) in order to generate a death inducing signalling complex (DISC). The signalling pathways that follow are not clearly defined but a series of caspases are activated possibly in a cascade like reaction as depicted by the arrows. Caspases are thought to mediate the final morphological changes which characterise apoptosis through their proteolytic activity, and may infact function in close association with mitochondrial factors. Bcl-2 and other family members are thought to influence apoptosis by their action on the mitochondrial function (inhibitory activity of Bcl-2 is indicated here by the red T-shaped bar). Other inhibitory molecules also play an important role and FLIP (FLICE inhibitory protein) is shown in the figure to block the interaction of FADD and FLICE and hence the DISC complex. Other complicated interactions are being defined as new molecules are identified and their functions characterised.

1.2.5.4) Fas modulation by IL-2

Studies by Owen-Schaub et al (1992) showed that IL-2 in culture resulted in upregulation of Fas expression over 7 days and also increased the percentage lymphocytes expressing Fas on the surface (similar to other stimuli for T cell activation like CD3, PHA and superantigen). In contrast, lymphocytes cultured in vitro without any stimulation lost Fas expression progressively. Anti-Fas-induced apoptosis could be restored in insensitive lymphocytes with IL-2 exposure (usually required for 4 days prior to anti-Fas exposure) (Owen-Schaub et al, 1992).

Other factors have also been identified which alter sensitivity to Fas-mediated apoptosis. It was previously documented that CD3 stimulation of T cells (thymocytes, peripheral CD4 and CD8 cells, and T cell clones) could result in apoptosis (Russell et al, 1992). IFN-γ did not have the same enhancing effect on CD3-mediated apoptosis of T cells as it had on the colon cancer cell line HT-29 which displayed upregulation of Fas on the surface on stimulation with IFN-γ (Itoh et al, 1991).

1.2.5.5) Fas function modulated by Bcl-2.

The interaction of anti-apoptotic proteins like Bcl-2 was investigated in relation to Fas (Itoh et al, 1993). Using a transfectant model with double transfectants (Fas and Bcl-2), Fas-induced apoptosis (measured by trypan blue and DNA fragmentation) was shown to be reduced in the presence of Bcl-2. Similar results were obtained in a transfected T cell line WR19L (murine). Further independent evidence for an interaction between Bcl-2 and Fas was found in a human B cell chronic lymphocytic leukaemia line where malignant lymphoblasts with upregulated Fas as well as Bcl-2 were protected from Fas-mediated apoptosis in vitro (Mapara et al, 1993). Other

studies have, however, suggested that Fas/CD95 signalling is not regulated by Bcl-2 (Moreno et al, 1996; Chiu et al, 1995; Strasser et al, 1995). Thus at present there is no final consensus about the influence of Bcl-2 and its family members on the outcome following Fas engagement.

1.2.6) Fas function and lymphocyte homeostasis.

1.2.6.1) Central and peripheral tolerance

The consensus appears to be that Fas plays a greater role in peripheral than in central mechanisms of lymphocyte homeostasis as relates to both B and T cells (Daniel and Krammer, 1994; Gilette-Ferguson and Sidman, 1994; Rothstein et al, 1995). Peripheral deletion of T cells depends on the activation state of these cells but may also depend on other factors including the cellular environment (Akbar and Salmon, 1997), and factors like Bcl-2 (Lacronique et al, 1996), Bcl-xL (Van Parijs et al, 1996), TGF-B (Cerwenka et al, 1996; Kawakami et al, 1996), IL-2 (Fournel et al, 1996; Van Parijs et al, 1996), as well as a growing range of death domain associated proteins (Cascino et al, 1996; Chinnaiyan et al, 1995 and Muzio et al, 1996). It has been suggested that this process of "activation-induced cell death" may play an important role in eliminating T cells that have been activated to mount a natural immune response to a variety of foreign antigens (Nagata and Golstein, 1995).

In addition to peripheral tolerance of T cells Fas has been shown to be important in the elimination of activated B cells, where appropriate CD40 ligation is an important determinant of outcome (Rothstein et al, 1995; Rathmell et al, 1995; Cleary et al, 1995; Garrone et al, 1995; Schattner et al, 1995; Rathmell et al, 1996).

1.2.6.2) Fas-mediated cytotoxicity

Fas was not only shown to play a role in death of activated B and T cells (both CD4+ and CD8+) but also in cytotoxicity reactions (Rouvier et al, 1993). Cytotoxicity usually has a Ca^{2+} -dependent and a Ca^{2+} -independant component. Fas-mediated cytotoxicity was shown to constitute the Ca^{2+} -independent component (10-20%). In fact most of the latter was attributable to Fas, supported by the finding that the cytotoxic effect could not be reproduced on *lpr* thymocytes defective in Fas but was normal on *gld* thymocytes (Walsh et al, 1994a).

Further research has confirmed the role of Fas in cytotoxicity mechansims (Stalder et al, 1994; Ju et al, 1994; Hanabuchi et al, 1994; Anel et al, 1994), and as yet there is no evidence of any cytotoxicity pathways other than the perforin-granzyme and Fas pathways. Cytotoxicity experiments done in perforin or Fas knockout mice support the latter argument (Walsh et al, 1994b; Kataoka et al, 1996). It has been suggested that the two pathways complement each other in that the perforin-granzyme pathway may be important in cytotoxic cell death of viral infected or tumour cells where no target cell cooperation is required, whilst the Fas-mediated cytotoxicity via either CD4+ or CD8+ cytotoxic lymphocytes may be more important in maintaining homeostatic control of activated lymphocytes (Carter and Dutton, 1995; Podack, 1995). In the latter case more target cell cooperation is required, and may be more influenced by cytokine modulation.

1.2.6.3) Fas and co-stimulation of T cells.

In contrast to apoptotic effects of anti-Fas antibody reports were emerging of costimulatory effects of certain new monoclonal anti-Fas antibodies (Alderson et al, 1993) like M38 immobilised on plastic plates with CD3. A similar B cell proliferative response was seen in one patient with a B cell lymphocytic leukaemia (BCLL) on exposure to anti-Apo-1 following stimulation in vitro with SAC and IL-2 (normally this stimulation would make the BCLLs more susceptible to apoptosis) (Mapara et al,

1993).The suggestion that Fas could act as a costimulator of cellular responses could not be confirmed by other investigators (Van Parijs et al, 1996) (including ourselves). One paper did show NF-κB activation following Fas engagement (Ponton et al, 1996) but this does not necessarily confirm a proliferative effect and the whole area of Fas costimulation needs further clarification.

1.2.7) Diseases associated with dysregulation of Fas.

Whilst research work progressed in vitro, Fas-mediated apoptosis was being demonstrated in vivo in the mouse where anti Fas (Jo-2 antibody) injected into mice (Balb/c, Mrl +/+, C3H/HeJ) intraperitoneally resulted in rapid and fulminant hepatic cell death (Ogasawara et al, 1993). This suggested not only a role for Fas in fulminant hepatitis but also provided a cautionary note against the premature use of anti-Fas reagents in the manipulation of immune responses.

Although many papers have suggested a role for Fas in disease pathogenesis, this has often been based on surface expression patterns in these conditions and no clear proof exists for a direct role for Fas. Very few genetic studies, if any, have been reported in diseases thought to be related to Fas dysfunction. However, the evidence appears much stronger in the case of diseases like Hashimoto's thyroiditis (Giordano et al, 1997), Hepatitis (Kondo et al, 1997), lymphoproliferative disease (Kondo et al, 1994), and a recently identified childhood equivalent of the murine *lpr* model, known either as the Canale-Smith-syndrome or Autoimmune lymphoproliferative disorder (Fisher et al, 1995; Rieux-Laucat et al, 1995; Le Deist et al, 1996; Drappa et al, 1996).

In addition there are good experimental models showing a role for Fas in "immune privilege" (Bellgrau et al, 1995; Griffith et al, 1995). This phenomenon describes the ability of some tissues such as the eye, testis, and brain to tolerate foreign grafts

without the problems of graft rejection. A similar type of "immune evasion" may protect some tumour cells from destruction by lymphocytes (both immune privilege and immune evasion will be discussed later in the section on Fas ligand). Also much work is ongoing in the haematology field where Fas is being studied not only in the context of drug resistant tumours but also as a possible additional tool in the fight against cancer (Friesen et al, 1996).

1.2.7.1) Soluble Fas

A soluble form of Fas resulting from splice variants was reported by Kimura et al (1994) in the rat liver. Soon thereafter a report appeared describing soluble Fas in humans (Cheng et al, 1994), suggesting that increased levels of soluble Fas may inhibit Fas-mediated apoptosis. Murine soluble Fas variants have also been reported (Hughes et al, 1995), but not all research groups working on soluble Fas have been able to reproduce the results and the controversy continues as to whether soluble Fas plays a major role in altering cellular outcome following Fas engagement. A very recent suggestion has been that it may not be the serum levels of soluble Fas that are important but rather the shift toward production of splice variants following activation (Kovacs et al, 1997). The controversy surrounding soluble Fas in SLE will be discussed further in chapter 5.

1.2.8) The lpr mouse and Fas

A major milestone in Fas biology which heralded the huge proliferation of research into Fas was the identification by Watanabe-Fukunaga et al that the *lpr* mutation in mice corresponded to a defective Fas gene (Watanabe-Fukunaga et al, 1992). It would therefore be prudent to provide some background about the *lpr* mouse model in order to appreciate the importance of the identification of the *lpr* - Fas defect.

lpr refers to "lymphoproliferation" and describes the phenotypic changes of lymphadenopathy occurring in mice of various strains (MRL/Mp, C3H/HeJ, BALB/cBy, AKR/J, C57BL/6 and SJL/J) that carry the homozygous *lpr* gene. The *lpr* gene usually has autosomal recessive expression but heterozygotes also manifest some lymphoproliferation and autoimmune features (Morse et al, 1985). The autoimmune features of *lpr* mice will be discussed in a later section on SLE. The lymphoproliferation in the MRL-*lpr* /*lpr* mouse is profound with a two hundred fold increase in cell number compared to wild type MRL +/+ mice (Cohen and Eisenberg, 1991). Lymphoid infiltrates are seen in all lymph node groups and can also be found in the spleen and other organs including salivary and lacrimal glands.

The lymphoid infiltrates consist of an increase in the total number of CD4+ and CD8+ cells but there is also a disproportionate increase in the very curious double negative (DN) T cells which characterises the *lpr* phenotype. DN T cells in these *lpr* mice are CD4- CD8- Thy 1+ and thus appear to bear a superficial resemblance to developing DN T cells in the thymus. Further analysis however revealed the presence of surface markers that are not usually present on developing thymic T cells, including a number of B cell antigens such as B220. The DN T cells do not simply reflect defective apoptosis and subsequent leakage into the peripheral lymphoid circulation. These DN T cells have been reported to have quite a variety of defects amongst which are included abnormal cellular responses, T cell receptor rearrangements, T cell receptor coupling and signalling defects, and abnormal oncogene expression (Cohen and Eisenberg, 1991).

The source of the DN T cells has proved elusive and there is no indication of active proliferation of these DN T cells in peripheral lymph nodes. Two favoured hypotheses include the generation of DN T cells in the liver (Ohteki et al, 1990) and the differentiation into DN cells from previously expanded single positive CD4+ or CD8+ cells (Cohen and Eisenberg, 1991). Defective cellular function in *lpr* mice is not limited

to the DN T cell population. Abnormal function has also been demonstrated in B cells, CD4+ T cells and macrophages derived from *lpr* mice and there is also evidence for deranged cytokine activity.

The biological consequences of the *lpr* mutation are clearly quite multiple and varied, and may reflect some influence of background genes on the *lpr* mutation, but excitement in the *lpr* model mounted following the identification by Watanabe-Fukunaga (Watanbe-Fukunaga et al, 1992) that *lpr* encodes the structural gene for murine Fas. The excitement centred around the potential benefits for cancer and autoimmunity research given the striking resemblance of the *lpr* lymphoproliferation and autoimmune features to human lymphoma and SLE, respectively.

1.2.8.1) Retroviral insertion in the Fas gene of the lpr mouse

Adachi et al from the same Japanese research group subsequently demonstrated that the *lpr* Fas gene defect was due to an insertion of a retroviral transposable element into intron 2 which gave rise to aberrant mRNA splicing and premature termination before exon 3 (Adachi et al, 1993). The poly(A) site of the early transposable element (Etn) was thought to be responsible for the premature gene termination. Wu et al (1993) confirmed similar findings but also showed that when the abnormal Fas gene is placed under the control of the CD2 promoter in transgenic mice then the Etn expression is reduced in thymic cells and Fas expression is increased. The dynamic interplay of retroviral Etn expression with other gene promoters or enhancers was previously reported (Keshet et al, 1991) and (Watson et al, 1992).

Any doubt about the role of retroviral influence on Fas gene expression in the *lpr* mouse was laid to rest when a third group independently identified a further Etn insertion (Chu et al, 1993) with a similar retrotransposon to Wu et al (1993). The former paper also showed quantitative differences in Fas expression in *lpr* compared to

wild type mice with 10 fold lower expression in *lpr*. It is interesting to note that heterozygous *lpr* defects are also associated with some decrease in Fas mRNA and autoimmune features (Carlsten et al, 1990).

1.2.8.2) The lpr ^{cg} allelic variant

An allelic variant of the *lpr* gene was known for some time and was called *lpr* ^{cg} because it complemented the defect found in another genetically different but phenotypically similar *gld* mouse (cg being "complementary to *gld*"). Following the cloning and identification of the Fas gene it was rapidly discovered that the *lpr*^{cg} phenotype was due to a point mutation in the murine Fas gene within the cytoplasmic domain so critical for Fas-mediated cytolysis (Watanabe-Fukunaga et al, 1992). A point mutation at position 786 with a nucleotide transition from T to A results in an amino acid change from isoleucine to asparagine and subsequent failure to respond to anti-Fas antibody. The *gld* mouse model will be discussed in the section on Fas-ligand. **Figure 1.2.1** shows the sites of genetic alteration within the Fas gene in the *lpr* and *lpr* ^{cg} mouse.

1.2.9) Fas biology in relation to this thesis

When our research into Fas began, it was clear that whilst the human Fas gene was cloned and identified at the outset, most of the research that followed concentrated on the *lpr* mouse model. We therefore aimed to study the role of the Fas gene in human Fas biology using new molecular techniques for the generation of appropriate reagents. This will be outlined in more detail in chapter 3.

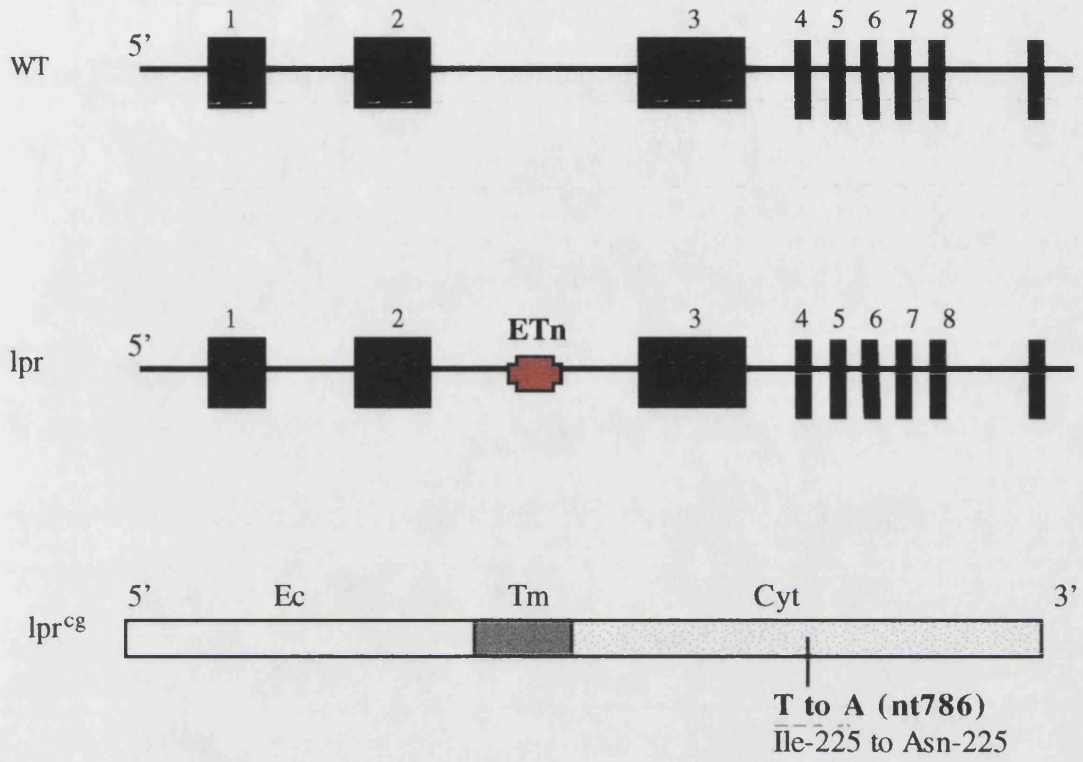


Figure 1.2.1: Changes within the Fas gene of the *lpr*

The genomic outline is shown for the wild type (WT) and *lpr* Fas genes with exons indicated as black boxes. The *lpr* mouse has a ±5.3Kb retroviral transposon inserted (ETn indicated by red box) between exons 2 and 3 which results in premature termination of the Fas transcripts and impaired function.
A very similar functional defect of Fas is found with a single point mutation within the cytoplasmic region of the coding sequence of Fas in the *lpr*^{cg} mouse. The cDNA for Fas is shown with the site of the point mutation (nucleotide 786) and the resultant amino acid transition is indicated.
Abbreviations: Ec = extracellular coding region, Tm = transmembrane region, Cyt = cytoplasmic coding region, Ile = isoleucine, Asn = asparagine.

1.3) FAS LIGAND BIOLOGY

A Historical perspective

1.3.1) The gld mouse model

The natural ligand for Fas was not identified for some years after the Fas gene was first cloned. It was however, well known that the *gld* mouse had the phenotypic picture of the *lpr* mouse (lymphoproliferation and autoimmune disease) but the *gld* gene was not allelic to *lpr* (Matusuzawa et al, 1990). The *gld* gene was also found to be an autosomal recessive gene like *lpr* but it was localised to chromosome 1 whilst *lpr* localised to chromosome 19 (Roths et al, 1984 and Watanabe-Fukunaga et al, 1992). It was thus suggested that *lpr* and *gld* represented two different enzymes that probably acted in a common metabolic pathway of T cell differentiation and function (Davidson et al, 1985).

1.3.2) Cellular expression of Fas Ligand inferred from the gld model

Both mature *gld* T cells as well as wild type B6 derived T cells displayed normal proliferation and cytokine responses to CD3 stimulation but the *gld* cells did not undergo apoptosis on exposure to antigen (CD3, TCR, APC presented or SEA) (Russell and Wang, 1993). This suggested that within the *gld* phenotype there was uncoupling of the normal relationship between the TCR and a *gld* derived T cell molecule responsible for inducing apoptosis after antigen exposure. Russell et al (1992) also demonstrated that the *gld* factor responsible for the death pathway was present on T cells rather than APC's. These findings led to much speculation at the time that *gld* probably represented a defect of Fas ligand.

Further ideas about the ligand for Fas came from the cytotoxicity work of Rouvier et al (1993) where they used cytotoxic clones derived from a rat - mouse hybridoma (PC60-d10s) as effector cells to induce apoptosis in Fas positive target thymocytes. They found that apoptosis of Fas expressing target cells could also be achieved with other stimulated effector T cells (stimulated with PMA / ionomycin) or antigen specific stimulated effector cells. This work hence suggested the presence of Fas ligand on these activated T cells and on the d10s cytotoxic hybridoma cells. The idea that a Fas ligand may have been expressed on activated T cells was supported by the data showing that no cytotoxic effect was inducable on *lpr* thymocytes which lacked functional Fas receptors. CD8+ lymphocyes have also been known to undergo cell death on exposure to high doses of cognate peptide (Su et al, 1993). It was interesting to note in this paper that cell death was prevented if cell to cell contact was inhibited in agarose gel microdroplets hence indicating a need for a cell surface ligand to mediate cell death. It would have been interesting to postulate a role for Fas ligand in this system.

The evidence thus pointed to a pattern of cell surface expression of the putative ligand, with a suggestion that T cells were implicated. Given that T cells are regarded as important regulators of the immune response in general, it was not unreasonable to postulate that a T cell may express a ligand for Fas in order to kill Fas positive target cells by a cell-cell interaction on completion of an immune response. Could activated T cells expressing ligand therefore be able to kill not only Fas expressing B cells and monocytes recruited to sites of immune responses but also other activated T cells expressing Fas? Such downregulation of the immune response by a Fas-FasL interaction may explain how peripheral deletion of activated lymphocytes occurred. In a project aimed at studying the role of Fas in SLE, it was clearly important to understand the natural ligand for the Fas receptor, hence as part of this project we aimed to identify the natural human ligand for Fas using a molecular approach outlined

in chapter 3. However, during the course of this work other researchers succeeded in cloning the FasL gene and some of these data will be discussed.

1.3.3) Cloning of the rat FasL gene

Following compelling evidence from the d10s cytotoxicity model of Rouvier et al discussed above, Suda et al (1993) in fact used the same d10s cell line after repeated sorting on FACS to clone a rat FasL. They used a recombinant murine Fas-Fc which was used to identify the "ligand expressing" cells. Expression cloning of a cDNA library from the "high" expressing d10s cells eventually revealed the first clone of Fas ligand, which turned out to be a rat ligand derived from the rat component of the genome in the hybridoma cell line. Gene analysis of the cloned rat ligand for Fas revealed a gene of 1623 nucleotides encoding a protein of 278 amino acids with a calculated Mr of 31,138.

The features of the protein suggested a type II transmembrane structure, lacking a signal sequence at the N-terminus and containing a proline rich region,a transmembrane anchor of 22 hydrophobic amino acids, and a 179 amino acid carboxyl region defining the extracellular domain. There were four potential N-glycosylation sites in the extracellular region. Biochemical characterisation of immunoprecipitated Fas ligand revealed a Mr of 38-42 Kd, and it was interesting to note that they found differences in the Mr of Fas ligand precipitated from d10s cells and transfected COS cells, which were explained on the basis of glycosylation differences. Although Fas ligand expression was limited to the surface of the d10s cells it was found that transfection of COS cells with cDNA for Fas ligand resulted in both surface expression and secretion of soluble Fas ligand into the culture medium, perhaps by cleavage from the cell surface.

1.3.4) FasL is a member of the TNF family

Amino acid alignment analysis showed Fas ligand to be a member of the TNF / NGF family, with homologous regions being restricted to the carboxyl terminus which encodes the extracellular receptor-interacting domain of the proteins. Homology was highest with TNFα and TNFβ (27.8% and 28.7%) rather than with other family members like CD40L, CD27L and CD30L (**figure 1.3**). Despite the homology with TNF, Fas ligand could not bind the TNF-receptor p55 nor activate mouse TNF receptors in WR19L cells.

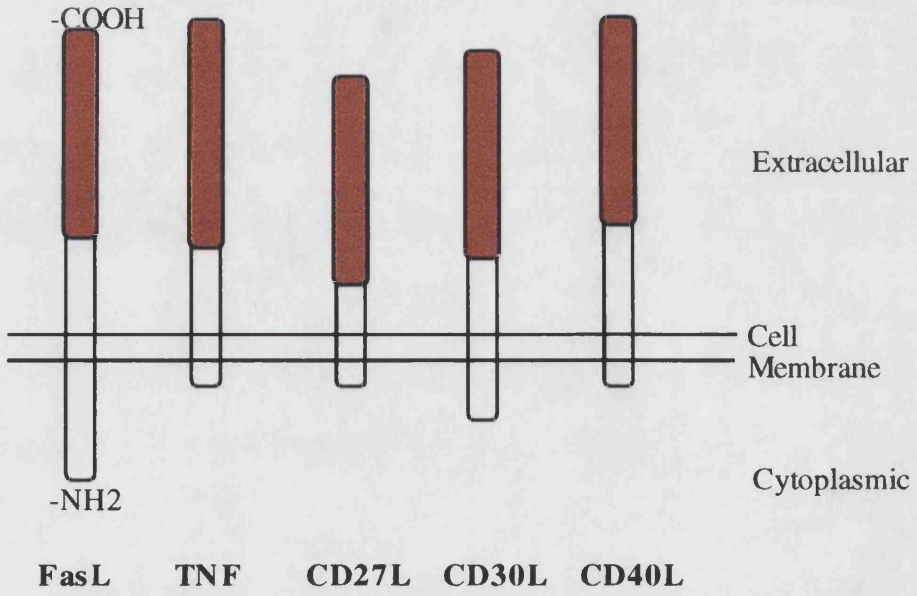


Figure 1.3: The TNF family of proteins

FasL was identified to be a type II transmembrane protein with 25-30% homology to the other TNF family members (homologous regions shown as shaded area in the extracellular region). Not all family members are represented here.

1.3.5) Murine Fas ligand gene and the gld mutation

Subsequently, the FasL genes for mouse and human were also cloned using expression cloning techniques (Takahashi et al, 1994b and Lynch et al, 1994). The mouse ligand was localised to chromosome 1 and corresponded to the *gld* chromosomal localisation (Suda et al, 1993). Independant observations confirmed the *gld* mutation to be a single base change at position 847 (amino acid transition T to C resulting in leucine replacing phenylalanine at position 273) (Takahashi et al, 1994a and Lynch et al, 1994). A variety of experiments confirmed that the *gld* mutations resulted in impaired apoptosis of Fas positive target cells (Ramsdell et al, 1994 ; Takahashi et al, 1994a and Lynch et al, 1994), and the defect could be corrected in chimeric marrow transplant experiments (Sobel et al, 1995 and MacDonald et al, 1995).

The data presented on FasL can therefore also be seen to originate largely from experimental mouse models. Whilst human diseases often teach us much about the biology of newly discovered molecules and animal models provide useful information about their in vivo relevance, it remains important to establish the "normal biology" of these molecules as much as possible within the human context. With this in mind our strategy in generating reagents that could be used to study the biology of FasL in human peripheral blood will be outlined in chapter 4.

Fas Ligand - more recent developments

1.3.6) Soluble Fas ligand

The *gld* model provided data on cytotoxicity and cell-cell interactions between Fas and FasL (Matusuzawa et al, 1990). Given that the prototype family member, TNF, was

mainly secreted following cleavage from the cell surface, the same was considered to be true for FasL. In vitro expression of FasL in Cos cells suggested that FasL was also secreted into the culture medium (Suda et al, 1994). This was confirmed by other groups in Jurkat and hybridoma experiments (Brunner et al, 1995; Ju et al, 1995; Dhein et al, 1995; Martinez-Lorenzo et al, 1996), and it is thought that release from the cell surface occurs by similar metalloproteinase activity (Kayagaki et al, 1995) as is the case with TNF. The identification of soluble FasL led to the suggestion that Fas-FasL interactions were important not only in the context of cell-cell interactions as mentioned above but also in autocrine and paracrine cell death (Brunner et al, 1995; Ju et al, 1995; Dhein et al, 1995). Also further studies have shown that high levels of soluble FasL could be found in some haematological malignancies like LGL leukaemia and NK cell lymphoma and may be implicated in their pathogenesis (Tanaka et al, 1996).

1.3.7) Diseases associated with altered FasL expression

Upregulated surface expression of FasL has also been implicated in Lyme arthritis (Vincent et al, 1996), HIV (Westerndorp et al, 1995) and immune evasion by tumour cells. Tumour cell expression of FasL appears to allow them to kill Fas positive lymphocytes that would normally play a role in tumour surveillance, and this has been shown for hepatocellular carcinoma (Strand et al, 1996), melanoma (Hahne et al, 1996), colon carcinoma cell lines (O'connell et al, 1996), as well as the NK cell lympoma and LGL leukaemia (Tanaka et al, 1996).

1.3.8) Immune privilege

The expression of FasL by immune privileged tissue has been shown in experimental models (Bellgrau et al, 1995; Griffith et al, 1995). Immune privilege refers to the areas where transplanted foreign cells are protected from attack by immunocytes such as

cytotoxic lymphocytes or NK cells. The eye and the testicle have been known for some time to be protected from such immune attack, as has the brain. Whilst the latter site has immune privilege largely conferred by the impermeable blood-brain barrier, the experimental evidence clearly suggests a major role for FasL-mediated destruction of Fas positive host lymphocytes in the case of the eye and testicular tissue. In the eye FasL expression has also been suggested to play a role in dampening inflammatory cell responses after infections so as to minimise the risk of collateral damage to ocular tissue from non-specific inflammatory mediators (Griffith et al, 1995).

Will FasL prove to be the magical molecule in transplantation? A warning against extending the "immune privilege"-FasL hypothesis for generalised use in transplantation comes from two papers. Yagita et al (1996) showed that an attempt to express FasL on grafted tissue resulted in a severe inflammatory reaction, thought to be mediated by FasL-induced death of Fas-positive host neutrophils with a resultant release of inflammatory mediators.

The other lesson was learnt when an attempt was made to prolong islet cell survival by creating FasL transgenic NOD mice in the hope that FasL expression by the islet cells would protect them from lymphocyte-mediated destruction. The islet B cells in this case upregulated Fas expression and thereby became even more susceptible to FasL killing from both the transgenic FasL expressing cells as well as cytotoxic T cells (Chervonsky et al, 1997). Clearly we have a lot more to learn about the regulation of the Fas-FasL molecules before they can be exploited for therapeutic purposes.

1.4) SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

1.4.1) Autoimmunity

The immune system has the ability to discriminate between self and non-self antigens. Autoimmunity refers to an immune response to self antigens which results in varying degrees of organ damage. Examples of autoimmune diseases include diabetes, Grave's disease, Hashimoto's thyroiditis, myasthenia gravis, autoimmune thrombocytopaenia, haemolytic anaemia, and a number of rheumatic diseases including rheumatoid arthritis, scleroderma and SLE. Autoimmune diseases are usually characterised by the presence of autoantibodies and self reactivity of T cells (Eisenberg and Horsfall, 1993). However, multiple interacting factors may contribute to the development of autoimmunity including genetic factors (both MHC and non-MHC), gender, microbial infections, and a variety of functional defects of antigen presenting cells and lymphocytes (reviewed by Abbas et al, 1997).

1.4.2) Central and peripheral control of immune responses

The immune system has a number of mechanisms for controlling a response to self antigens (**figure 1.4**). This includes central and peripheral mechanisms of control of T cells. Central tolerance occurs by thymic deletion of T cells responding to self antigens presented by MHC (Ashton-Rickardt et al, 1994). Peripheral control becomes important in the elimination of autoreactive cells which may have slipped through the thymic net but is also particularly important following immune responses to foreign antigen (Newell et al, 1990). A large number of activated T cells must be removed after an immune response to foreign antigens and this peripheral deletion is known to occur through apoptosis. The exact mechanisms are still being determined but both Fas and

TNF have been implicated in this "activation-induced cell death" (Shi et al, 1990). Other mechanisms of peripheral tolerance apart from apoptosis include anergy and clonal ignorance (Jones et al, 1990 and Schwartz 1990) **(Figure 1.4).** Anergy differs from apoptosis in that peripheral T cells are not killed but remain in a state of unresponsiveness which can often be rescued by IL-2 in culture. The in vivo relevance of anergy is as yet uncertain but it has been suggested that a molecule called CTLA-4 (CD152) may play an important role in this regard (Bluestone 1997). The in vivo significance of clonal ignorance is even more uncertain and is based largely on experimental murine work. It is thought that a breakdown in peripheral tolerance mechanisms plays an important part in the pathogenesis of SLE and other autoimmune diseases.

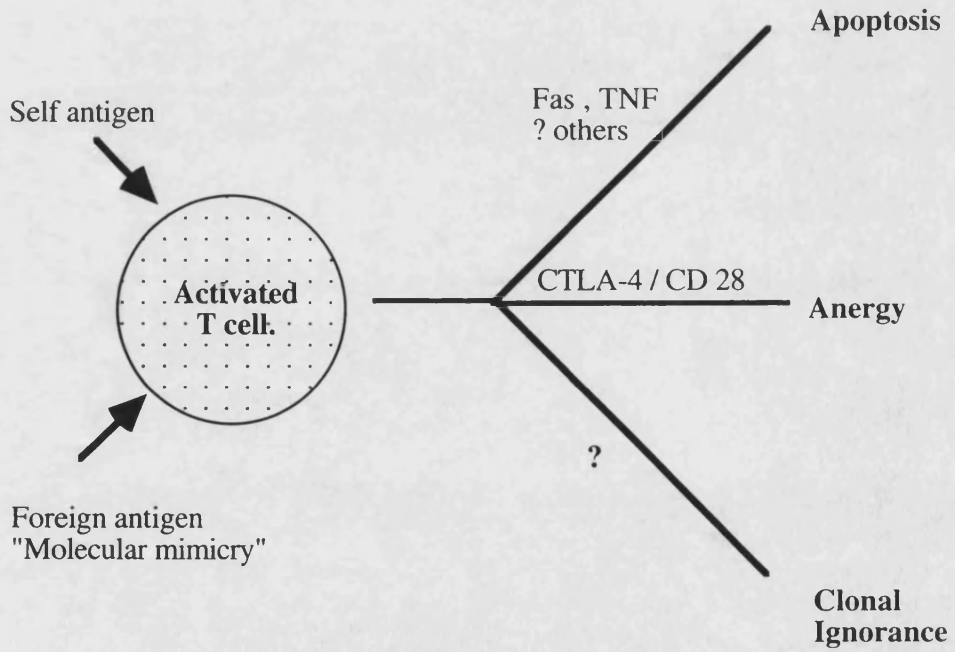


Figure 1.4 : Mechanisms of Peripheral Tolerance

T cells activated on exposure to self antigen may be regulated by a number of mechanisms as outlined. Apoptosis of activated cells is thought to occur mainly via Fas and TNF receptors , whilst the regulation of Anergy has largely been ascribed to CD28 and CTLA-4 expressed on T cells. The mechanisms underlying clonal ignorance are as yet undefined.

1.4.3) The lpr mouse, Fas, and peripheral tolerance

From the *lpr* mouse model it appears that Fas plays an important role in peripheral tolerance. It is very interesting that a single gene defect represented by the *lpr* mouse has such a dominant effect on the autoimmune manifestations (Wu et al, 1994). In fact transgenic correction of the Fas defect in T cells of the *lpr* mouse resulted in complete correction of the autoimmune features including the glomerulonephritis, arthritis, autoantibody production and also the lymphoproliferative disorder The latter model supports the argument that T cells play a central role in the autoimmune response because correction of only T cell Fas defects under a CD2 promoter also resulted in correction of B cell function in that autoantibody and immunoglobulin levels were normalised (Wu et al, 1994).

1.4.4) Clinical Features of SLE

SLE is a multisystem disease often quoted as a prototypic autoimmune disorder. The most common sites affected include the skin, joints and serosal surfaces but the disease can be associated with devastating complications when it affects the kidneys, lung, heart, and brain. Amongst a variety of haematological features, lymphopaenia and thrombocytopaenia occur commonly and have largely been ascribed to antibody mediated cellular destruction (Eisenberg and Horsfall, 1993). SLE has been associated with both MHC (DR2 and DR3) and non-MHC genes (Complement C2 and C4) and occurs more commonly in females (ratio of 10:1).

SLE is characterised by autoantibodies to double stranded DNA (dsDNA) but it is often associated with a variety of autoantibodies directed at functional nuclear or cytoplasmic antigens **(figure 1.4.2)** (Galperin et al, 1996). These include anti-nuclear antibodies, anti-Sm, anti-Ro / La, anti-cardiolipin, anti-phosphatidylserine,

anti-platelet, anti-red cell, anti-neutrophil, anti-histone, and anti-u1RNP antibodies amongst many others. Whilst it is clear that immune complexes resulting from the interaction between autoantigens and autoantibodies in SLE account for much of the tissue damage, recent research has suggested that direct autoantibody penetration of cells may play a role too (Elkon 1994 and Tsai et al, 1993). Autoantibody production is associated with altered B cell function but this is in turn subject to regulation by helper T cells. It is known that B cell differentiation and antibody production is dependent on CD40 function which is tightly coupled to the ligand of CD40 expressed on T cells (Grewal et al, 1996 and Van Essen et al, 1996). Similarly, T cells express Fas ligand which can regulate Fas-mediated apoptosis of activated B cells. We therefore hypothesised that a breakdown in Fas-mediated apoptosis could account for some of the pathology seen in SLE.

Autoantigen	**Cellular Function**
Sm	Splicing of pre-mRNA
U1 snRNP	Splicing of pre-mRNA
SS-B / La	Termination of RNA polymerase III transcription
SS-A / Ro	Unknown
Ribosomal RNP's (P proteins)	Involved in protein biosynthesis by ribosomes

Figure 1.4.2 : Functions of common autoantigens in SLE

1.4.5) The role of Apoptosis in SLE

Evidence from mouse models such as the MRL-*lpr /lpr* mouse suggested that decreased apoptosis accounted for the phenotypic changes including the lymphoproliferation and autoimmune features. At the time of commencement of our project no human data existed on the role of apoptosis in SLE. More recently experimental evidence from one group suggested that a soluble Fas molecule may be increased in SLE which could result in reduced Fas-mediated apoptosis (Cheng et al, 1994). This has not been reproducible experimentally by independant researchers (Knipping et al, 1995 and Goel et al, 1995), and the validity of an ELISA based on a mutant splice variant of Fas brings into question the issue of what the measurement actually reflected as regards normal Fas biology.

Thus it was clear at the outset that the role of apoptosis in the pathogenesis of human SLE was poorly defined. Whilst the MRL-*lpr/lpr* mouse provided useful insights about defective apoptosis and autoimmunity there was no indication of any defective Fas function in humans. Also the Fas gene had not been studied in human SLE except for the splice variant described above. Genetic and functional studies on Fas and apoptosis in human SLE have subsequently been completed by a number of investigators and will be discussed in relation to our results in chapter 5.

AIMS AND OBJECTIVES OF THE PROJECT

The *lpr* mouse model of SLE was identified to have a single gene defect, i.e. the Fas gene. Correction of the Fas gene defect in T cells of these mice abrogated most of the autoimmune and lymphoproliferative features, but there was no indication if the Fas gene played such a dominant role in the equivalent human disease. In addition the emphasis of research into Fas was focused on murine biology. Given this scenario a number of objectives were determined for this project:

1) To generate Fas reagents for the study of human Fas biology.
2) To identify a human ligand for Fas.
3) To generate FasL reagents in order to study its role in human disease.
4) To study the Fas gene in human SLE.
5) To investigate for functional defects of Fas-mediated apoptosis in human SLE.

In order to meet the objectives a molecular approach was used to generate the necessary reagents and various apoptosis assays as well as SSCP polymorphism analysis had to be established. The results chapters that follow integrate the components of reagent generation and experimental assays.

CHAPTER 2

MATERIALS and METHODS

REAGENTS

Reagents were purchased from a variety of suppliers as indicated with each method but the majority of standard chemical reagents were obtained from Sigma Chemical Co. A list of all suppliers with telephone numbers appears in appendix 1 of the thesis.

2.1) CELL CULTURE

A variety of cell types were used in this project with some differences in the culture medium requirements (culture medium from Life Technologies). CHO transfectants were all grown in Glutamine - free DMEM (methods from Sansom et al, 1993) ; COS-7 cells and lymphocytes including the transformed cell lines Jurkat and H9 were all grown in RPMI medium, and various supplements were added into the medium as shown in table 2.1. All cell culture work was carried out under sterile conditions in a Class II Biological Safety cabinet and cells were grown in incubators at 37°C with 5% CO_2 in a humidified atmosphere.

2.1.1) Recipes for culture media:

1). Glutamine - free DMEM.

To 400 ml of autoclaved distilled deionised water the following were added:

Dulbecco's MEM (10 X)	55 ml
Foetal calf serum (FCS)	50 ml
Penicillin / Streptomycin (10 000 u/ml, 10 000 ug/ml)	5 ml
Sodium bicarbonate (7.5 %)	28 ml
Sodium pyruvate (100mM)	5 ml
Nucleosides (100 X) (see below)	5 ml

Nucleosides (100 X) was made up as follows:

0.24 mg/ml	thymidine
0.70 mg/ml	guanosine
0.70 mg/ml	adenosine
0.70 mg/ml	cytidine
0.70 mg/ml	uridine

The nucleosides were added to 50 ml of distilled deionised water, filter sterilised, and stored at 4°C.

2). RPMI medium.

RPMI 1640	400 ml
FCS	50 ml
Penicillin / Streptomycin (10 000 u/ml ; 10 000 ug/ml)	5 ml
L-Glutamine (200 mM)	5 ml

2.1.2) Cell culture technique

2.1.2.1) Culture of CHO transfectants / COS-7 cell transfectants

Both cell lines were adherent lines requiring regular (3-4 days) subculture in the relevant medium. For this the culture medium was removed from the tissue culture flask, and 5 ml of PBS was added to the flask for a washing step of approximately two minutes (to ensure removal of medium before the addition of trypsin). Trypsin-EDTA (1:250) (Life Technologies Ltd) was added to the culture flask in a volume of 2.5 - 3 ml and incubated at 37°C for 3 minutes to remove adherent cells from the flask surface. Trypsin was immediately neutralised with 10 ml of appropriate medium, and cells passaged from the cell suspension for future use at 2:10. Remaining cells were used for relevant experiments, frozen for storage in liquid nitrogen, or discarded after chlorination.

Table 2.1: Medium requirements of cells used.

	CELL TYPE	
	CHO Transfectants	Cos cells, Lymphocytes, Jurkat cells , H9 cells.
Medium	DMEM without L-glutamine.	RPMI 1640
Foetal calf serum	10%	10%
Sodium bicarbonate (7.5% w/v)	0.4%	0.25%
L-Glutamine	nil	2mM
Sodium Pyruvate	1mM	nil
Penicillin	100 units/ml	100 units/ml
Streptomycin	100 ug/ml	100 ug/ml
Nucleosides	26uM adenosine 28uM cytidine 29uM uridine 25uM guanosine 9uM thymidine	nil
Additional Reagents	500 ng/ml Hygromycin for Fas transfectant selection.	500 ng/ml Neomycin for Fas-L transfectant selection. 10 Units/ml IL-2 for lymphocyte culture.

Media and supplements from Life Technologies Ltd
Nucleosides from Sigma Chemical Co.
PBS (phosphate buffered saline) tablets from Unipath Ltd.
All tissue culture plastics from Falcon Ltd.

2.1.2.2) Culture of Jurkat cells, JLW cells and H9 T cells

Jurkat cells, JLW cells and H9 T cells are transformed T cell lines which are non-adherent and were cultured in RPMI medium supplemented as shown. Cells were passaged every week by removal of three quarters of the culture medium containing the

cells in suspension and replenishment with fresh RPMI medium. Cells removed from the culture were either used in apoptosis experiments, signalling experiments and RNA extraction or disposed of following chlorination if not required at the time. Cells were grown at densities of 1 X 10^6 / ml.

2.1.2.3) Cryogenic storage of cells

To ensure a continued supply of cell lines for future experimental use a regular store of each cell line including the new transfectants was created in liquid nitrogen. Cells were washed after passage in medium and centrifuged at 350 g for 5 minutes and the medium removed. Cells were resuspended in 2 ml of 40% FCS in RPMI 1640 and placed on ice. An equal volume of 20% DMSO (Sigma) in RPMI medium was added to the cell suspension drop by drop with gentle mixing and the cells were then subdivided into cryogenic storage vials at 1x10^6 cells/vial. Vials were then cooled in an insulated cooling box at -80°C for 24-48 hours before being placed in long term liquid nitrogen storage.

To recover cells from cryogenic storage, the cells were brought to 37°C rapidly and the contents transferred in aseptically to a tube containing 10 ml of pre-warmed medium (37°C). Cells were centrifuged at 350g for 5 minutes and the supernatant was discarded. Cells were then resuspended in their appropriate pre-warmed medium and placed in culture at 37°C with 5% CO_2 .

2.1.3) Purification of peripheral blood mononuclear cells (PBMC) and T cells from peripheral blood.

2.1.3.1) Peripheral blood mononuclear cells (PBMC)

Peripheral blood samples were drawn from randomly selected SLE patients attending our Connective Tissue Diseases clinic at the Royal National Hospital for Rheumatic Diseases in Bath. On the same day a healthy normal volunteer from amongst staff at the hospital or the Rheumatic Diseases Institute was asked to donate a blood sample for concurrent separation and analysis so as to minimise any variation in the results secondary to differences in sample handling . Heparinised blood samples were diluted 1:1 (volume:volume) in sterile PBS before being layered onto 15 ml of Ficoll (Heparin from CP Pharmaceuticals Ltd and Ficoll from Nycomed UK Ltd). Following centrifugation at 420 g for 30 minutes the layer of mononuclear cells (PBMCs) was recovered from the gradient (containing PBMC 1-2 X 10^6 / ml of blood). The PBMCs were washed twice in PBS to remove any residual Ficoll and then a third time in complete RPMI medium. PBMCs were then either used directly for various experiments or separated further into purified populations of T cells as described below.

2.1.3.2) T cell purification

Monocytes were removed by adherence to tissue culture plastic petri dishes by culturing the PBMCs in 10 ml of RPMI medium supplemented with 10% FCS at 37°C for 1 hour. All non-adherent cells (which included B cells, T cells and some residual monocytes) were recovered by gentle washing in RPMI. Monocytes adherent to the plate were gently scraped off the surface of the petri dish for FACS analysis for FasL expression. To purify the remaining lymphocytes, the cell suspension was incubated with a number of antibodies in order to deplete non-T cells. Antibodies used were anti-

monocyte (anti-CD14, 500ul of 10ug/ml UCHM1 hybridoma supernatant), anti-B cell antibodies (anti-CD19 1:10 dilution of ascites from BU12), and antibody to L243 (anti-HLA DR) at 500 ul of a 10 ug/ml hybridoma supernatant (to remove activated T cells, B cells, monocytes and macrophages). The cell suspension with added antibodies was incubated for 1 hour at 4°C on a spiramix rotating platform (Jencons Scientific Ltd), then washed in RPMI to remove unbound antibody and incubated in a 2ml suspension containing 50 ul of sheep anti-mouse-IgG-coated magnetic beads (Dynal ; diluted 1:10 in medium) at 4°C on a rotator for a further hour. Cells were then washed in RPMI and adhered to a magnetic particle concentrator applied to the outside of the tube (repeated 2-3 times). Cells remaining in suspension were pooled as the purified T cell population which were 95% pure by FACS analysis for the CD2 marker.

2.1.3.3) Generation of T cell blasts

Either PBMCs or purified T cells were used depending on the experiment in question. Cells were suspended in RPMI at 1 x 10^6 cells/ml and incubated with PMA (10ng/ml ; Sigma) + ionomycin (1uM ; Sigma) or SEB (10ug/ml ; Sigma) for 5 days. Fresh medium containing IL-2 (gift from Glaxo-Wellcome) at 10 Iu/ml was added at this time and cells cultured for a further period to increase cell numbers as required for the relevant experiment. IL-2 was added every two or three days with fresh medium depending on cellular requirements as judged by the change in colour of the medium.

2.1.4) Preparation of cells for FACS analysis.

Cells were resuspended at 1-2x10^5 cells / tube and washed in medium (Omerod, 1990). Where Fc receptor blocking was required human IgG was used at 50ug/ml and

cells were incubated for half an hour at 4°C. Cells were washed in 2ml of medium and resuspended in 50ul of the relevant antibody (at 10ug/ml in medium) for further incubation at 4°C for an hour. Any unbound primary antibody was washed away in PBS and cells were resuspended in a FITC-conjugated secondary antibody at 10ug/ml in a volume of 50ul (either IgG or IgM depending on primary antibody used) for a 30 minute incubation at 4°C. Cells were then washed in PBS and analysed in FACS sheath fluid (Becton Dickinson) on a FacStar Plus analyser (Becton Dickinson). Information about cellular forward scatter (FSC), side scatter and the fluorescence intensity of labelling (FL1) was obtained using a LYSIS II programme.

2.1.5) Transfection of CHO or COS-7 cells by Electroporation.

A number of cell transfectants were generated in this project. CHO cells were used for the surface molecules like Fas and CD28 and COS-7 cells for the soluble Fas-Fc and Fas ligand expression. As both cell lines were immortal adherent lines the conditions for transfection were the same (Chu et al 1987). Cells were prepared in culture by growing to 50-75% confluence in a large 175 cm^2 Falcon flask. Following trypsinisation as described in 2.1.2.1 cells were washed in HeBs saline (recipe below) and resuspended in HeBs saline at $5x10^6$ / ml. Salmon sperm DNA was added (20 ul of 10mg/ml stock added to 1ml HeBs) to the HeBs cell suspension as carrier DNA. Plasmid containing the DNA for transfection was added at 50 ug/ml of DNA (prepared by Promega column purification). A final volume of 500 ul of cell suspension in HeBs containing salmon sperm DNA and plasmid DNA was pipetted into a 0.4cm BioRad electroporation cuvette. A BioRad electroporator (BioRad Gene Pulser$^{Tm)}$ was used at a capacitance of 250uF and voltage of 300V to provide an electrical discharge to the cells with a time constant of between 3-5 ms. Cells were allowed to recover in 1ml of medium at room temperature for 10 minutes before being seeded into petri dishes with

15 ml of appropriate culture medium and incubated at 37°C with 5% CO_2. Cells were analysed by FACS at 24-48 hours for transient expression and cultured in selection medium (hygromycin 500ug/ml for Fas transfectants and neomycin 500ug/ml for FasL transfectant selection) before further sorting by magnetic beads and/or FACS sorting.

Recipe for HeBs saline:

20 mM Hepes pH 7.05	(Life Technologies)
137 mM NaCl	(Sigma)
5 mM KCl	(Sigma)
0.7 mM Na_2HPO_4	(BDH)
6 mM Dextrose	(Sigma)

made up in sterile MilliQ water to 500ml.

2.1.6) Chromium release assay

Fas positive target cells (Jurkat cells) were washed in RPMI medium and resuspended at $5x10^6$ cells/ml in medium. One hundred ul of isotonic ^{51}Cr (Amersham) was added to the cell suspension and incubated at 37°C for 2 hours. The cells were washed in RPMI medium three times and then allowed to rest at 37°C for 1hour before pipetting into a 96 well round bottomed plate at $2x10^4$ cells per well (triplicates for each stimulation). Stimulations were carried out as described in the results (section 3.2.3.4) for 18 hours before harvesting and counting on a γ-counter (Wallac) programmed for the measurement of ^{51}Cr. NP-40 (Sigma) was used to induce maximal release of ^{51}Cr from the Jurkat cells, and PMA and ionomycin (Sigma) was used at 40ng/ml and 1uM, respectively, to stimulate FasL upregulation on the effector Jurkat cells.

2.2) DNA and RNA manipulation

All the molecular techniques except RNA extraction have been based on the Laboratory Manual of Molecular Cloning edited by Sambrook et al (1989). Enzymes were handled on ice and added as the last step of any reaction described.

2.2.1) RNA extraction

(Chomczynski and Sacchi, 1987)

Reagents:

Denaturing Solution (solution D):	4 M	guanidinium thiocyanate	(Sigma)
	25mM	sodium citrate, pH 7	(Sigma)
	0.5%	sarcosyl	(Sigma)
	0.1 M	2-mercaptoethanol	(BDH)

To minimise handling of guanidinium thiocyanate a stock solution was prepared as follows :

250 g guanidinium thiocyanate
293 ml water (DEPC treated).
17.6ml 0.75M sodium citrate pH7
26.4ml 10% Sarcosyl

The solution D was dissolved in the manufacturer's bottle at 65°C and stored in the fridge at 4°C. 2-mercaptoethanol was added to an aliquot of the stock solution prior to use (72ul 2-ME to 10ml stock solution).

Other reagents used were:

Phenol (water saturated) (Appligene)

2M Sodium acetate pH 4 (Sigma)

Chloroform - isoamyl alcohol (49:1) (Sigma)

Isopropanol (Sigma)

Protocol for RNA extraction :

RNA is very sensitive to degradation by RNAse enzymes, hence there was a need to observe strict procedures with bench cleansing using alcohol, separate RNA pipettes, equipment, and solutions and regular change of gloves. All pipette tips and eppendorf tubes were autoclaved before use, and as far as possible no DNA work was conducted simultaneously. The steps involved in the RNA extraction from cells which were washed in PBS were as follows:

1) Cells were resuspended and disrupted in fresh solution D (between 1-2 x 10^6 cells / 100 ul solution D) in a final volume of 500 ul in a 1.5ml microfuge tube.

2) The following were sequentially added with mixing by inversion between each:

50 ul 2M sodium acetate pH4

500 ul phenol (water saturated)

100 ul chloroform-isoamyl alcohol (49:1)

3) Samples were shaken vigorously for 10 seconds (vortexing) and chilled on ice for 15 minutes.

4) Tubes were spun at 13000g in a microcentrifuge for 10 minutes.

5) The top aqueous layer containing RNA was removed gently by pipetting and transferred to a fresh tube where an equal volume of chloroform-isoamyl alcohol was added. Samples were vortexed briefly and spun for 5 minutes at 13000g.

6) One more extraction was performed with chloroform-isoamyl alcohol as above before the final aqueous layer was precipitated with an equal volume of isopropanol (samples were then stored at -20°C for 1-24 hours).

7) Samples were spun at 13000g for 10 minutes and the supernatant removed.

8) The pellet was redissolved in solution D (150 ul) and 150 ul isopropanol was added before samples were again stored at -20°C between 1-24 hours.

9) The samples were spun at high speed for 10 minutes and the supernatant discarded.

10) The RNA pellet was washed once with 70% ethanol and once in absolute alcohol before drying down under vacuum for 15 minutes (GyroVap vacuum dryer, HOWE).

11) The RNA pellet was dissolved in 50 ul DEPC water and the RNA quantitated by spectrophotometry (Ultrospec II, LKB) at 260nm using the equation:

A_{260} x 40 x dilution = ug/ml DNA,

where A = absorbance, the dilution of RNA was 1/100, and 40 represents a constant for ssRNA. An OD_{260} : OD_{280} of 1.8 - 1.9 indicated good purity RNA.

12) RNA was routinely electrophoresed on agarose to check for degradation before use in RT-PCR reactions.

2.2.2) Reverse transcription (RT) and cDNA production.

To generate cDNA total RNA was reverse transribed for 1 hour at 37°C . The reactions were carried out in autoclaved siliconised eppendorf tubes (Sigma) to minimise adherence of cDNA to the tube. The reverse transcription reactions were set up as follows:

Total RNA 350 ng		5 ul
M-MLV RT buffer (5X)	(Life Technologies)	6 ul
M-MLV RT enzyme	(Life Technologies)	2 ul
DTT (0.1 M)	(Sigma)	3 ul
dNTP mix (10mM)	(Pharmacia)	1.5 ul
Oligo-dT (45 uM)	(Pharmacia)	1 ul
RNAse inhibitor	(Pharmacia)	1.5 ul
DEPC water	(Sigma)	make up to 30 ul.

After incubation at 37°C for 1 hour the reaction was terminated by heating to 95°C for 10 minutes and the tubes then placed on ice. cDNA generated by the latter reaction was either used immediately for PCR reactions or stored at -20°C.

2.2.3) PCR (polymerase chain reaction)

PCR was either carried out with template cDNA as generated above or with plasmid containing the DNA sequence of interest (as outlined in the various results sections). Reactions were set up as follows in 0.65 ml eppendorf tubes:

cDNA (or plasmid DNA diluted to 10ng/ul)		2 ul
Taq polymerase buffer (10X)	(Promega)	10 ul
$MgCl_2$ (25 mM stock)	(Promega)	6 ul
Taq polymerase enzyme	(Promega)	0.5 ul
dNTP (2 mM stock)	(Pharmacia)	10 ul
Oligonucleotide primers 5' and 3'		
(from Pharmacia; 1uM final concentration)		1 ul each
water (milliQ)		upto 100 ul.

All volumes were scaled down appropriately if reactions were carried out in 25 - 50 ul final volume for "test runs" on new reactions.

PCR reactions were all carried out on a Perkin Elmer Cetus thermal cycler and a typical programme would have 25 - 30 cycles of :

95°C for 1 minute (denaturing)

55°C for 1 minute (annealing) (varied according to Tm of oligonucleotides)

72°C for 1 minute (extension)

72°C for 10 minutes at the end of all the cycles followed by 4°C until the samples were retrieved for analysis.

In order to ensure complete strand separation and oligonucleotide denaturation, the PCR samples were heated to 95°C for 3 minutes before the Taq polymerase enzyme was added. Samples were then layered with mineral oil (Sigma) before PCR reactions were commenced. For radiolabelling of PCR fragments either for probe generation or SSCP analysis 2 ul of a 1:10 dilution of α^{32}P-dCTP (10uCi/ml)(Amersham) was included in the PCR reaction and all samples were then handled behind perspex shields.

2.2.4) Restriction digests

Restriction digests were carried out frequently for a variety of reasons including the preparation of DNA for ligation, screening of recombinant plasmid clones, restriction mapping of cloned and subcloned DNA fragments, and excision of cDNA fragments for radiolabelled probe generation for southern blotting. In general enzyme choices were determined by the requirements of the experiment in question and much of this has been outlined with the relevant results. Restriction digests were carried out in autoclaved eppendorf tubes in a volume of 20 ul unless larger amounts of DNA were required for gel excision for further DNA manipulation when reactions were carried out in 100-500 ul volumes. A typical restriction digest was as follows:

Plasmid DNA (0.5 ug/ul)	5 ul
buffer (10X) (for relevant enzyme)	2 ul
restriction enzyme (3-5 u / ug DNA)	1 ul
water	to volume of 20 ul.

Where double digests were required the appropriate buffer was used or two separate digests carried out if no single appropriate buffer was available. Enzyme reactions were conducted in a water bath heated to the appropriate temperature for the enzyme in use, and most restriction enzymes were purchased from New England Biolabs or Promega. Samples were electrophoresed on 1% (w/v) agarose gels for analysis on a UV transilluminator.

2.2.5) Ligation reactions

DNA ligation was necessary for generation of recombinant plasmid DNA in the variety of cloning experiments undertaken in this project. This was achieved using the following type of reaction:

Plasmid DNA (with appropriate restriction sites) (50 ng/ul)	1 ul
cDNA or restriction fragment with compatible restriction sites (50 ng/ul see calculation formula below)	1 ul
ligase buffer (10X) (New England Biolabs ; NEB)	1 ul
Ligase enzyme (T4 DNA ligase 400,000 u/ml) (NEB)	1 ul
water (milliQ)	6 ul
	10 ul

Ligation reactions were carried out in eppendorf tubes at 16°C for 4-16 hours depending on the nature of the ligated ends. Cohesive end ligations are more efficient and require shorter incubation periods whilst blunt end ligations were left for 16 hours. To facilitate the cloning of blunt Fas fragments into pEe6, polyethylene glycol (PEG-8000 15% w/v; Sigma) was added to the ligation reactions.

To achieve optimal ligation a vector : insert ratio of 1:3 or 1:5 molar ends of DNA was used. With information of the size of vector and size of insert available the amount of insert DNA needed for a given amount of plasmid DNA in the ligation reaction (50 - 100 ng) was calculated as follows:

$$\text{ng of insert} = \frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \text{ X molar ratio of } \frac{\text{insert}}{\text{vector}}$$

Ligations were terminated by heating to 70°C for 10 minutes (to inactivate T4 DNA ligase) before DNA was either used for transformation of bacteria or stored at -20°C. Ligation controls were always carried out to help with decisions about transformation efficiency of recombinant clones. The ligation controls used were, vector religated to itself, unligated vector, and ligation reactions containing only the insert of interest.

2.2.6) Transformation of bacteria (Electroporation).

In order to identify recombinant clones of insert and vector DNA, bacteria were transformed by electroporation (Sambrook et al, 1989) and colonies grown on agar plates. Transformation by electroporation is much more efficient than methods like heat shock. A BioRad Gene Pulser^{Tm} was used for all the transformation of bacteria with DNA. All work was done on ice with a mix of 40 ul of electrocompetent *E.coli* and 1-2 ul of ligation reactions (and controls as described above) in 0.2cm electroporation cuvettes (BioRad). Bacterial electroporation was carried out using the pulse controller with a resistance of 200 Ohm, capacitance 25 uF, and voltage of 2.5 Kv. Time constants were between 3.5 - 4.5 ms. Nine hundred and fifty ul of SOC medium (Bio 101 Inc via Stratech Ltd) (at room temperature) was immediately placed onto the transformed bacterial mix which was then incubated at 37°C (slow rotation in a bacterial Incubator Shaker, Model G25 from New Brunswick Scientific Co. Inc) for thirty minutes before plating (aliquots of 50-100 ul per plate) onto agar containing ampicillin.

Reagents required for bacterial transformation:

1) Electrocompetent bacteria.

Whilst commercial sources are available, electrocompetent *E.coli* are relatively easy to produce and remain competent for a considerable time if appropriately stored in liquid nitrogen. A variety of host strains of *E.coli* were prepared for the project because of the variety of plasmids used, but essentially all electrocompetent bacteria were produced as follows:

a) 10 - 12 ml of bacterial culture was grown in CircleGrow medium (Bio 101 Inc) overnight from an inoculate of stock *E.coli* stored in liquid nitrogen.

b) Sterile autoclaved 400ml CircleGrow medium was seeded with 1ml of the overnight culture and placed into the Incubator Shaker at 37°C, 250 - 300 rpm.

c) The optical density (OD) was checked at baseline and then hourly until an OD_{600} of 0.55 - 0.6 (takes 2-3 hours and culture becomes cloudy indicating log phase growth).

d) The culture was placed on ice and transferred to cooled 50 ml Falcon tubes for centrifugation at 4000rpm for 10 minutes at 4°C (Beckman benchtop GPR centrifuge).

e) Supernatant was disposed following chlorination and the bacterial pellet was resuspended in 20ml ice cold sterile Milli-Q water, pooled and centrifuged at 4000rpm at 4°C for 10 minutes.

f) The bacterial pellet was resuspended in 20 ml cold sterile Milli-Q water and spun as before.

g) The pellet was resuspended in 2 ml cold sterile Milli-Q water containing 10% glycerol (Sigma) and aliquoted into freezing vials for rapid storage in liquid nitrogen.

2) Growth Media:

Circle-Grow bacterial growth medium (Bio 101 Inc via Stratech Ltd) : dissolved 40g in 500 ml milliQ water and autoclave for 20 minutes. Allow to cool before adding antibiotics.

SOB medium (Bio 101 Inc via Stratech Ltd): Dissolved one capsule per 20 ml water and autoclaved for 20 minutes.

SOC medium : SOB medium with sterile 1M glucose (Sigma) added at 400mM final concentration.

Agar plates : 1% agar w/v (Sigma) was added to CircleGrow medium and autoclaved, cooled in a water bath to 50°C before ampicillin (purchased from local Hospital Pharmacy) was added at 50 ug/ml. The agar was poured in a standard manner onto bacterial grade petri dishes.

2.2.7) Selection of transformant colonies

Antibiotics added to the agar plates help selection of plasmid-encoded antibiotic-resistant colonies. Typical concentrations of antibiotics used for selection included ampicillin 50 ug/ml, tetracycline 15 ug/ml, and kanamycin 50 ug/ml. In addition colour selection using blue/white screening was done with vectors capable of alpha-complementation (lacZ gene) in conjunction with the appropriate host bacterial strain. Agar plates were spread with 50 ul of 50 mg/ml X-Gal (NBL) and 100 ul of 0.1M IPTG (NBL) and left to absorb for 30 minutes at 37°C before plating of transformants. Recombinant colonies were white in colour due to disruption of the lacZ gene of the plasmid but some pale blue colonies also contained inserts. Leaving the agar plates at 4°C for a few hours helped enhance the blue/white colour segregation.

2.2.8) Plasmid minipreps

Colonies chosen either by blue/white colour selection or at random where no colour selection was available were picked off the agar plates (flame sterilised loop) and placed into 5 ml of CircleGrow medium in 30 ml universal plastic tubes (Fahrenheit) for shaking in an Incubator Shaker at 37°C overnight. 1.5 ml of the overnight growth was transferred to eppendorf tubes for centrifugation at 6 500 rpm in a benchtop micro-centrifuge for 5 minutes.The supernatant was suitably disposed of following chlorination and the bacterial pellet was handled as follows to extract the plasmid DNA:

1) Add 100 ul resuspension solution (solution I) and mix by vortexing.

2) Add 200 ul lysis solution (solution II) and mix by inversion. The solution clears with this step.

3) Add 150 ul neutralising solution (solution III) and mix by vortexing. The solution again becomes cloudy with white "floccular" material.

4) Centrifuge at 13 000 rpm for 5 minutes and remove supernatant to fresh eppendorf tube.

5) Add 500 ul of a 1:1 mix of chloroform : phenol (ie, 250 ul of each mixed in a glass bottle and added to the tubes in step 4). Tubes were mixed by vortexing and spun in a centrifuge at 13 000 rpm for 3-5 minutes.

6) The aqueous (upper) layer was removed carefully from each tube and placed in fresh eppendorf tubes. The remaining phenol / chloroform was all pooled for appropriate disposal at a later stage.

7) An equal volume of chloroform was added to the aqueous phase in step 6 and mixed by vortexing before centrifugation at 13 000 rpm for 5 minutes.

8) The aqueous phase (upper layer) was again carefully removed by pipette into fresh eppendorf tubes. Absolute ethanol was added at twice the volume of the aqueous layer and mixed by inversion. This would reveal clouding within the solution as the DNA became precipitated by the ethanol. Samples were stored at -20°C for half an hour to allow further precipitation to occur before centrifugation at 13 000 rpm for 10 minutes.

9) The supernatant was discarded and the DNA pellet washed in 70 % ethanol before drying under vacuum for 10 - 15 minutes.

10) Pellets were resuspended in 25 - 30 ul of TE buffer containing RNAse (20ug/ml). The RNAse prevented small molecular weight DNA bands being obscured by RNA during agarose gel electrophoresis. Plasmid DNA was then subjected to restriction digests with appropriate enzymes as described above (3-5 ul DNA per reaction) for screening of recombinant clones.

Reagents used : (all chemicals from Sigma)

Solution I: 50 mM glucose
25 mM Tris-Cl pH 8
10 mM EDTA pH 8
made up in milli-Q water.

Solution II: 0.2 N NaOH
1 % SDS
made from stock 10M NaOH and 10% SDS before use.

Solution III:	5M Potassium acetate	60	ml
	Glacial Acetic acid	11.5	ml
	water	28.5	ml
	The resulting solution was 3M for potassium and 5M for acetate		

TE buffer: 10 mM Tris-Cl pH 8

1 mM EDTA

made up with milli-Q water.

2.2.9) Plasmid maxipreps

Following identification of cloned plasmid DNA by restriction mapping of miniprep DNA, larger quantities of purified DNA were obtained for transfection and expression. Bacterial cultures were set up in 2L Pyrex flasks containing 500ml of autoclaved CircleGrow medium with ampicillin 50 ug/ml. Larger flasks allowed better agitation of the culture in the incubator shaker and was carried out at 37°C overnight from a seeding of 1 ml of a relevant miniprep bacterial culture. The turbid overnight bacterial growth was handled as follows:

1) 150-200 ml bacterial growth was poured into 250 ml Nunc bottles for centrifugation in a Beckman J2-21 centrifuge with a fixed angle rotor (JA 14) at 10 000 rpm, 4°C for 10 minutes.

2) Supernatants were appropriately disposed of following disinfection, and the bacterial pellet was resuspended in 100ml STE solution by gentle pipetting. Centrifugation was again carried out as above and the pellet resuspended in 20 ml cell resuspension buffer (solution I) by gentle pipetting.

3) 40 ml of freshly prepared Solution II (lysis solution) was added and the contents mixed by inversion. This resulted in a clearing of the mixture which sometimes required 20 - 30 minutes on the bench (facilitated in a 40° water bath).

4) 20 ml of solution III was added and mixed by shaking and inversion to give rise to a turbid "floccular" solution, which was then centrifuged at 10000 rpm, 4°C for 30 minutes without the brake on (so as not to disturb the pellet).

5) The plasmid DNA in the supernatant was carefully removed by pipetting to minimise transfer of any bacterial debris and transferred into 30 ml Corex tubes.

6) An equal volume of isopropanol was added at room temperature to precipitate the plasmid DNA (left on the bench for 15 minutes). Samples were centrifuged at room temperature at 10 000 rpm for 10 minutes and the supernatant drained. Pellets were washed in 70 % ethanol and dried at room temperature before resuspension in TE buffer in a volume of 3 - 5 ml for purification.

7) Plasmid purification was carried out by the method supplied with the Promega Wizard purification kit (Promega TB139, Wizard™ plus Maxipreps DNA purification system). Purified DNA was quantitated by spectrophotometry using a quartz glass cuvette and reading the optical density (OD) at wavelengths of 280 and 260 nm.

A ratio of OD_{260} : OD_{280} = 1.7 - 1.8 indicated sufficiently pure quality DNA for transfection.

STE solution: 0.1 M NaCl
10 mM Tris-Cl pH 8
1 mM EDTA pH 8
made up in milli-Q water.

2.2.10) Colony screening by hybridisation

Where no colour selection was available and a random selection of 12-24 colonies did not reveal any recombinant clones, selection by colony lifting and hybridisation was conducted. Agar plates containing approximately 100 - 150 colonies reasonably spaced apart were covered by Hybond N membranes (Amersham) until the latter was evenly wet (within one minute). Identification points were marked using ink with a sterile needle to leave an imprint in both the agar and the membrane for alignment at a later stage. Membranes were then evenly lifted off the agar plates and handled as follows:

(agar plates were incubated at 37°C for 6 hours to allow colonies to regrow before storage at 4°C)

1) Membranes were placed colony side up onto Whattman filter paper pre-soaked with denaturing solution for 7 minutes.

2) Membranes transferred onto Whattman filter paper pre-soaked with neutralising solution for 3 minutes ; then placed onto fresh filter paper with neutralising solution for another 3 minutes.

3) Membrane filters were then washed in 2X SSC solution for a minute and drained on a dry piece of Whattman filter paper for a few minutes.

4) Membranes were wrapped in Saran wrap and placed face down on a UV transilluminator for 2 - 3 minutes to fix the DNA by cross-linking.

5) Membranes were incubated in 30ml pre-hybridisation buffer in Hybaid bottles at 65°C for 3-6 hours in a Hybaid hybridisation oven (rotated continuously).

6) A radiolabelled probe (50ul) was added to the 30ml pre-hybridisation mix for further incubation with the membranes overnight at 65°C.

7) The radioactive probe was appropriately stored at -20°C for upto one week for re-use, and the membranes were washed of non-specific binding as follows:

8) Membranes were washed at 65°C for 15 minutes in a solution containing 2X SSC and 0.5% SDS, then for another 15 minutes at 65°C in solution containing 0.2X SSC and 0.5% SDS. The membrane was tested for radioactive signals by a hand held

counter (Mini-Monitor series 900) to determine the duration of the final stringent wash in solution containing 0.1X SSC and 0.5% SDS. This final wash was done for 5 minutes if background radioactivity on monitoring was < 20 counts per second or for 10 -15 minutes for higher levels of radioactivity.

9) Membranes were wrapped in Saran wrap and fixed onto autoradiography casettes for exposure to Blue Sensitive Xray film (Genetic Research Instruments Ltd).

10) Autoradiographs were developed in the Xray department of the Royal National Hospital for Rheumatic Diseases, and aligned onto the previous postions marked on the membranes. Appropriate identification marks were made on the autoradiographs for alignment on the agar plates previously stored at 4°C, and the colonies which most closely aligned to the "hot" spots on the autoradiograph were chosen for further miniprep analysis.

Solutions used in this procedure:

SSC: made up as 20X stock as follows:

3 M NaCl (Sigma)

0.3 M Sodium citrate (Sigma)

SDS: made up as 10% solution w/v of lauryl sulphate (Sigma) in distilled water.

Denaturing solution: 1.5 M NaCl

0.5 M NaOH

Neutralising solution: 1.5 M NaCl

0.5 M Tris-HCl pH 7.2

0.001 M EDTA pH 8

Pre-hybridisation solution: made up as follows:

Stock solution	Final vol.	Amount
20 X SSPE	5 X	4 ml
Denhardt,s 50 X	5 X	3 ml
10 % SDS	0.5 %	1.5 ml
ss DNA	30 ug/ml	100ul
distilled water		22 ml

20 X SSPE : 3.6 M NaCl

0.2 M sodium phosphate

0.02 M EDTA pH 7.7

50 X Denhardt's solution:

2 % w/v BSA

2 % w/v Ficoll

2 % w/v PVP (polyvinylpyrolidine)

Radiolabelling of probes was done using a Promega Prime-a-Gene kit as outlined in the following reaction (various components added in sequence as shown):

Nuclease free water to achieve final volume 50 ul	x ul
5 X labelling buffer	10 ul
dNTP mix (500 uM of each unlabeled dNTP)	2 ul
DNA (25 ng/ul denatured)	1 ul
Nuclease free BSA (10 mg/ml)	2 ul
α-^{32}P-dCTP (50 uCi, 3000 Ci/mmol)	5 ul
Klenow fragment of DNA polymerase I (5 U)	1 ul
Total volume	50 ul

2.2.11) Agarose gel electrophoresis

Agarose gels were made in 1 X TAE buffer as a 1% or 2% solution (w/v agarose from Sigma), boiled in a conical flask in a microwave oven until the agarose was completely in solution and then cooled on the bench (± 50°C) for addition of ethidium bromide (5 ul of 10mg/ml stock solution to 100 ml of agarose). The gel solution was poured into a gel tray and allowed to set at room temperature (low melting temperature gels were set in the fridge). All DNA electrophoresis was conducted in 1X TAE buffer (containing Ethidium bromide) using a standard horizontal electrophoresis tank attached to a power source (at 70 - 100 volts).

Gels were analysed over UV light and photographed with a hand-held camera.

TAE buffer: made as 50 X stock as follows:

242 g Trizma Base (Sigma)

57.1 ml Glacial acetic acid (Sigma)

100 ml 0.5 M EDTA pH 8

made up to 1 litre with milli Q water.

2.2.12) TA cloning

This was achieved using a pT7Blue T-Vector Kit (Novagen, catalogue number 69836-1). TA cloning differs from standard cloning techniques only in the use of compatible T and A nucleotide overhangs to facilitate ligation of PCR products (Marchuk et al, 1990). The pT7Blue T-vector supplied with the kit was prepared by EcoRV digestion and addition of single 3' dT residues at each end. Standard PCR was carried out with Taq DNA polymerase (Promega) (section 2.2.3) which leaves single 3'A-nucleotide overhangs on the reaction products (Clark, 1988). Ligation reactions were carried out using components of the kit (as per ligation reaction outlined in section 2.2.5). Subsequent transformation of bacteria and plasmid miniprep analysis was conducted as described in the methods above (sections 2.2.6, 2.2.7, 2.2.8).

2.3) BIOCHEMISTRY / IMMUNOCHEMISTRY

2.3.1) Fas-Fc protein purification from culture supernatants

2.3.1.1) Concentration of supernatants

Fas-Fc transfectant supernatants were pooled and stored at 4°C until approximately 500 - 1000 ml of supernatant was available. The latter was placed in a large glass beaker and placed on a magnetic stirrer. An equal volume of saturated ammonium sulphate solution was added to the beaker by dripping through a column (a syringe barrel attached to a 20 gauge needle). The mixture was left at 4°C for 6 - 12 hours and centrifuged at 3000 g for 30 minutes (4°C). The pellet was resuspended in PBS at 0.1-0.3 X the original start volume of supernatant, transferred to dialysis tubing and dialysed against PBS at 4°C (three exchanges of PBS at 4-6 hourly intervals). The dialysed concentrate was then removed from the dialysis tubing for affinity chromatography.

Saturated Ammonium sulphate (Sigma):

761 g in 1 litre distilled water (at room temperature = 4.1M).

PBS: prepared by dissolving 1 tablet per 100 ml distilled water (CP pharmaceuticals Ltd).

Preparation of dialysis tubing by boiling:

1) Cut tubing (Sigma) to appropriate lengths.

2) Place tubing in large volume of 5mM EDTA, 200mM sodium bicarbonate and boil for 5 minutes.

3) Rinse tubing briefly in distilled water and add a large volume of solution as in (2) for a further boiling for 5 minutes. Decant the solution and rinse in distilled water.

4) Cover tubing in large volume of distilled water and cover with foil for autoclaving (10 minutes).

5) Dialysis tubing stored at 4°C with 0.02% sodium azide.

6) Tubing rinsed before use (inside and outside) with distilled water.

2.3.1.2) Affinity chromatography with Protein G sepharose

Dialysed concentrated protein from the pooled cell culture supernatants could be purified by affininty chromatography because of the immunoglobulin Fc region designed into the Fas-Fc molecule. Essentially a Protein G-Sepharose-4-FastFlow column from Pharmacia was set up in a cold cabinet as per the guidelines of Pharmacia (Technical help catalogue 18-1022-29).The column was monitored by paper recording (1mm/min) throughout the procedure. The dialysate was diluted 1:1 in start buffer and passed throught the column at 1ml/min (ensuring that the column did not run dry at any time). The column was then washed with the start buffer until a stable baseline trace was recorded, when elution buffer (pH 2.7) was passed through at 1ml/min until the tracing again returned to baseline. One ml aliquots of eluates were collected in eppendorf tubes during the elution peak of the tracing and the eluate in each eppendorf tube was immediately neutralised by having a pre-added 20 ul of neutralising buffer (1M Tris pH 9.0) within the eppendorfs. Protein concentrations were checked in each eppendorf using an OD280 reading on the spectrophotometer, and the appropriate protein eluates were pooled for further biochemical characterisation on SDS-PAGE gels and immunoblotting.

Start / Wash buffer:	0.02M Sodium phosphate pH 7 made up as follows:
	Na_2HPO_4 : 1.42 g in 500 ml dH_2O
	NaH_2PO_4 : 1.38 g in 500 ml dH_2O
	Mixed in equal volume to give pH7 start buffer.
Elution buffer:	0.1M glycine-HCl pH 2.7
	(3.75g in 500 ml dH_2O)

2.3.2) SDS PAGE electrophoresis

Standard vertical electrophoresis apparatus (Hoefer) was used for SDS-PAGE resolution of proteins and in general was attached to a power source at constant current of 20 - 25 mA over 2-4 hours depending on the size of protein being resolved and the percentage acrylamide within the gel. The percentage polyacrylamide in gels varied according to requirements but a typical 1mm thick 10% gel was made up as follows:

Running gel:	Acrylamide / Bisacrylamide 30% (37.5:1) (NBL)	10 ml
	4X gel electrophoresis buffer	7.5 ml
	10% SDS	0.3 ml
	ddH_2O	12.1 ml
	10% ammonium persulphate (w/v) (NBL)	150 ul
	TEMED (Sigma)	10 ul

After polymerisation at room temperature (layered with water saturated butanol) the stacking gel was poured with a comb in situ:

Stacking gel:	Acrylamide / Bisacrylamide 30% (37.5:1)	1.33 ml
	4X stacking gel buffer	2.5 ml
	10% SDS	0.1 ml
	ddH_2O	6.0 ml
	10% ammonium persulphate	50 ul
	TEMED	5 ul

Before electrophoresis samples were mixed with an equal volume of 2X SDS sample buffer and heated at 95-100 ^oC for 5 minutes, then placed on ice and dispensed by pipette into the wells under running buffer.

Buffers for gel electrophoresis:

Running gel buffer: 1.5 M Tris-HCl pH 8.8 (to 800ml dH_2O add 182g Trizma base and bring pH to 8.8 with HCl, make upto 1L with dH_2O).

Stacking gel buffer: 0.5 M Tris-HCl pH 6.8 (to 400ml dH_2O add 30g Trizma base and pH to 6.8 with HCl, make upto 500ml with dH_2O).

Electrophoresis running buffer (made up as 10X stock as follows):

250mM Tris-HCl

1.9 M glycine

1% SDS

To 800 ml dH_2O add 30.3g Trizma base and 144g glycine and 10g SDS. Dissolve and make up to 1L with dH_2O and pH to 8.3 with HCl.

10% SDS: made up by dissolving 10 g SDS (Sigma) in dH_2O in a final volume of 100ml.

2X SDS sample buffer:

Tris-Cl pH 6.8 (62.5mM)	= 312.5 ul of 1M stock
SDS 2%	= 1000 ul of 10% stock
Glycerol 10%	= 500 ul of 100% stock
DTT 50 mM	= 250 ul of 1M stock
Bromophenol blue 0.1%	= 50 ul of 10% solution.

made up with dH_2O (2887.5ml) to a final volume of 5 ml and stored at room temperature.

Formula for calculating the percentage acrylamide in a gel:

$$\text{Acrylamide volume (ml)} = \frac{\text{vol of gel} \quad \text{x} \quad \% \text{ gel}}{\% \text{ acrylamide}}$$

2.3.3) Coomassie staining

Gels were submerged in Coomassie Brilliant blue stain for 30 - 45 minutes with gentle agitation at room temperature followed by destaining solution for the same period. The destain solution was replenished once to improve resolution of protein bands above background over a 1-2 hour period. Gels were then transferred to a layer of Whatman paper and covered in Saran wrap before drying on a vacuum gel dryer (RAPIDRY, Genetic Research Instruments Ltd).

Coomassie blue staining solution:	50% w/v methanol
	10% w/v acetic acid
	0.25% w/v Coomassie blue R-250.
Destaining solution:	10% w/v methanol
	5% w/v acetic acid

2.3.4) Western Blotting

Proteins from SDS-PAGE gels were transferred to either nitrocellulose or PVDF membranes (BioRad) by electrophoresis on a semi-dry blotting apparatus (Sigma). PVDF membranes are hydrophobic and required an additional step of wetting in 100% methanol before being placed in transfer buffer. All membranes and Whattman filter paper were incubated in transfer buffer for at least ten minutes before the blotting transfer was set up. Gloves were used at all times to avoid background staining of membranes. Four layers of pre-soaked filter paper were placed onto the transfer unit, followed by the wet blotting membrane. Air bubbles were removed by rolling a pipette over the membrane and the SDS-PAGE gel was placed onto the transfer membrane. Air bubbles were again removed by rolling followed by layering another 4 pieces of

wet filter paper (one at a time with removal of air bubbles at each stage). The sandwich was placed with the membrane on the side of the positive electrode, the semi-dry blotting apparatus was assembled and connected to a power source at 1mA / cm^2 of membrane (80mA for an 8 X 10 cm membrane). Electrophoretic transfer took place over 1 hour unless a higher percentage gel or higher molecular weight proteins were being transferred in which case the transfer took between 90 and 120 minutes.

Transfer buffer:	Tris-HCl pH 8	20 mM
	Glycine	150 mM
	Methanol	20% v/v
	SDS	0.1% w/v
	made up in deionised water.	

A 10X stock of transfer buffer without methanol and SDS could be stored and fresh 1X buffer made up as required.

Following western blot transfer the membrane was subjected to immunoblotting as follows. The transfer membrane was incubated with blocking buffer at room temperature for one hour with gentle agitation. Blocking buffer was poured off and the membrane was incubated with relevant primary antibody (dilution 1:1000 but titrated according to background upto 1:10 000) for one hour at room temperature with gentle agitation. Membranes were washed in PBS-tween for 15-30 minutes with three exchanges of fresh wash buffer before the secondary peroxidase conjugated antibody was added at a dilution of 1: 10 000. In the case of Fas-Fc immunodetection direct detection of the immunoglobulin tail was possible by using an anti-mouse IgG peroxidase conjugate followed by the detection stage. After a 1h incubation the secondary antibody was also extensively washed in three exchanges of PBS-Tween over 30 minutes. The membranes were then incubated with substrate solution containing DAB tablets (3,3'-Diaminobenzidine)(Sigma) for colour detection of immunoreactive proteins. Later in the project immunoblotting experiments were

conducted with ECL detection using an Amersham ECL detection system and autoradiography.

Blocking solution:	PBS with 0.1% (v/v) Tween-20 and 5% (w/v) milk powder.
Antibody dilution buffer:	PBS with 0.1% Tween-20 (Sigma)
DAB substrate solution:	1 DAB tablet (3,3'-Diaminobenzidine) (Sigma) in 15 ml of Tris-Cl pH 8 and 15ul of 30% hydrogen peroxide solution.

2.3.5) Dot blots

For the detection of native undenatured protein dot blot analysis was carried out as follows. Antigen was spotted onto marked pieces of nitrocellulose paper (circles 2cm in diameter). The membrane spotted with antigen was air dried and soaked in blocking buffer as for western blots for 30 minutes. Primary antibody was then incubated with the blots for one hour followed by three exchanges of wash buffer (PBS + Tween 0.1%). Secondary antibody was incubated for an hour followed by three washes and then incubation with substrate solution (Tris-Cl pH 8 15ml + DAB tablet + H_2O_2 15 ul). Colour visualisation of protein was evident within minutes of incubation with substrate solution.

2.3.6) Protein quantitation by ELISA (BioRad Protein Assay)

Protein quantitation was either carried out by spectrophotometry (OD_{280}) or by a Bio-Rad protein quantitation assay as described here. Bovine serum albumin was used as a standard and diluted from a stock solution of 25 mg/ml as follows:

1/16 = 1.5 mg/ml

1/20 = 1.2 mg/ml

1/28 = 0.9 mg/ml

1/50 = 0.5 mg/ml

1/125 = 0.2 mg/ml

All standards and samples were dispensed in 5 ul aliquots (duplicate) into a 96 well ELISA plate (Maxi Sorp, Nunc, Denmark). Twenty ul of reagent S was added to 1ml of reagent A and mixed by inversion before dispensing 25 ul per well, followed by 200 ul of reagent B per well. Plates were left at room temperature for 15 minutes (stable for upto 1 hour) and read on a Dynatech MR 5000 plate reader at an absorbance of 750nm. The plate reader was programmed to plot a linear curve of Abs (750nm) from the standards using the known concentrations and measure protein concentrations of the samples from the resulting curve. Results were plotted as absorbance and concentration values in mg/ml.

2.3.7) Immunoprecipitation

Whole cell lysates were used for immunoprecipitation experiments. Cultured cells were washed in PBS to get rid of any residual medium before resuspension in lysis buffer (between $5x10^6$ and $40x10^6$ cells per ml of lysis buffer). The 1ml lysates were transferred to 1.5ml eppendorf tubes and placed on ice for 10 minutes, vortexed briefly and left on ice for a total of 30 minutes. Tubes were spun at 13 000 rpm in a cooled

benchtop microcentrifuge (4°C) (BioFuge Fresco, Heraeus) for 10 minutes to separate nuclear debris and membranous material before the lysate was transferred to a fresh eppendorf tube for protein quantitation by one of the methods mentioned above (Bio-Rad quantitation by ELISA). Equal quantities of protein were then transferred to eppendorf tubes for addition of 30 ul of protein G-sepharose beads (Pharmacia) and incubation on a rotating platform at 4°C for 1 hour. Samples were spun at 13000 rpm for 2 minutes and the pre-cleared lysate removed by pipette into fresh eppendorf tubes for incubation with the primary antibody on ice for 1 hour. Fas-Fc was regarded as a primary antibody in the immunoprecipitation of FasL in chapter 3. After incubation on ice for one hour 30 ul of protein G sepharose was added to the lysate-antibody mix and rotated at 4°C for 2 hours. Antibodies of most species bind to protein G sepharose beads so that a gentle centrifugation of the lysate at this stage produced a pellet of beads with antibody-bound proteins from the lysate which could be resolved on SDS-PAGE electrophoresis. To remove non-specifically bound proteins the sepharose beads were washed in the lysis buffer 3 times with gentle centrifugation (short pulse on a microcentrifuge) before mixing with an equal volume of SDS sample buffer for heat denaturation and loading onto a gel.

Lysis buffer: (all made up fresh from stock solutions, chemicals all from Sigma):

1) General lysates:	NaCl	150 mM
	NP-40	1%
	Tris-Cl pH 8	50 mM
	DTT	1 mM
	PMSF	1 mM
	leupeptin	1 ug/ml
	pepstatin	1 ug/ml
	made up in milli-Q water.	

2) Lysis buffer for Kinase / signalling experiments:

Hepes buffer pH 7.4	20 mM
EGTA	2 mM
B-glycerophosphate	50 mM
DTT	1 mM
Orthovanadate (Na_3VO_4)	1 mM
Triton X - 100	1 %
Glycerol	10 %
leupeptin	2 uM
soybean trypsin inhibitor	10 ug/ml
PMSF	400 uM

made up in milli-Q water.

2.3.8) In vitro Jun kinase (JNK) assay

In vitro kinase reactions for Jun kinase were established using the method of Kyriakis et al (1994). Lysates from stimulated cell cultures were made and quantitated for protein content by ELISA as described. Each lysate was incubated with 10 ul (2.5 ug) GST-cJun (coupled to sepharose beads) on a rotating platform at 4°C for 4-6 hours before being washed with lysis buffer three times. Between each wash the beads were gently pelleted in a cooled benchtop microcentrifuge at low speed (10 second pulse only). The beads were drained of lysis buffer by a 19 gauge needle which does not allow the beads to be aspirated (with insulin syringe) and then added to an in vitro kinase reaction as follows:

Lysate (GST-cJun bound beads)	10 ul
10 x kinase buffer	4 ul
gamma-^{32}P ATP (1 uCi / ul)	2 ul
milli-Q water	24 ul
Total volume	40 ul

Gamma ^{32}P-ATP was from Amersham and diluted 1:10 from a 10 uCi / ul stock in milli-Q water before use.

The reaction mix was incubated at 37°C for 30 minutes before adding an equal volume of SDS sample buffer and heat denaturation of the proteins at 95°C for 5 minutes. Samples were held on ice before loading onto an SDS PAGE gel (12.5 %). Gels were dried after washing in milli-Q water for 5 minutes and autoradiographs were developed and read on image analysis software (Imagedok and Phoretics).

Kinase reaction buffer 10X:

Hepes pH 7.5	250 mM	(Life technologies)
Magnesium acetate	100 mM	(Sigma)
Mg-ATP	500 uM	(Sigma)

made up in milli-Q water.

Preparation of GST-cJUN:

GST-cJun protein (1-173) was prepared from a plasmid kindly donated by Dr S Ward. *E.coli* were transformed with the plasmid by electroporation and a colony was chosen for a miniprep culture which was grown over 6 hours at 37°C. Ten ml of the miniprep culture was seeded into a 2 litre flask containing 400 ml of CircleGrow culture medium containing ampicillin 50 ug/ml and grown to an OD_{600} of 0.6 - 0.8 (maximum 0.9). Expression of the recombinant GST-cJun was induced by IPTG (Sigma) added to the culture at this point to a final concentration of 0.4mM (160 ul of a 1M stock added to a

400 ml culture). The bacterial culture with IPTG was incubated in the incubator shaker for a further 3-5 hours at 37°C and the culture was then transferred to centrifugation bottles and spun at 4°C, 4000 rpm for 20 minutes (Beckman J2-21 centrifuge, JA14 rotor). The bacterial pellet was resuspended in 10 ml lysis buffer containing :

Triton X-100	1%
EDTA	2 mM
PMSF	1 mM
SP1 (small peptide inhibitor)	1 ug/ml

made up in PBS.

Resuspension was facilitated by pipetting the lysate up and down and then transferred to 50 ml Falcon tubes for overnight storage at -20°C. Samples were next sonicated four times for 40 seconds with one minute intervals on ice between sonication (power setting 50%). Sonicated lysates were transferred to 30 ml corex tubes and centrifuged at 10 000 rpm, 4°C for 10 minutes (Beckman J2-21 centrifuge). Supernatants were transferred to 15 ml Falcon tubes and incubated with 1 ml of 50% glutathione agarose beads (Pharmacia) per 10 ml at 4°C, on a spiramix rotating platform for 1-2 hours (Note that the glutathione beads were washed in PBS prior to their use in order to remove any alcohol from the storage mixture). Incubated samples were spun in a 4°C centrifuge by gentle pulsing only and allowed to settle on ice before doing a wash in lysis buffer once, followed by three washes in PBS containing 2mM EDTA. A final wash in PBS was done before mixing with storage buffer containing:

Hepes pH 7.4	50 mM
NaCl	50 mM
Glycerol	50 %

For the last wash in storage buffer samples were spun at 2 000 rpm for 1 minute and left to settle on ice for at least ten minutes before removing the supernatant. Beads were then transferred to eppendorf tubes and stored in 50 % storage buffer at -20°C ready for use. GST cJun protein coupled to the agarose beads were checked and quantitated on SDS PAGE gels before use in immunoprecipitations and in vitro kinase assays.

2.3.9) Sphingomyelinase assay

Cells stimulated as required were washed in cold PBS before incubation with lysis buffer (as shown in 2.3.7) on ice for 30 minutes. All stimulations were carried out in a 37°C water bath and rapidly terminated by immersion in liquid nitrogen. Samples were spun at 13000 rpm, 4°C for 10 minutes and the supernatants quantitated for protein by the Bio-Rad ELISA assay (section 2.3.6). Fifty ug of lysate from each stimulation was incubated in an in vitro sphingomyelinase assay (Wiegmann et al, 1994) as follows:

50 ug	Lysate
250 mM	Sodium acetate (Sigma)
1 mM	EDTA pH 5 (Sigma)
1.1 uCi/ml	^{14}C sphingomyelin (Amersham)

made up to a final volume of 50 ul in milli-Q water.

The samples were incubated at 37°C for 2 hours before a phase extraction with 250 ul water and 800 ul of a 2:1 mix of chloroform:methanol. The upper aqueous layer was used with 5 ml aqueous scintillation fluid (Optiphase Hisafe 3, Wallac) in miniature polyethylene vials for liquid scintillation counting (Packard Instrument Co) and radioactivity measured on a Liquid Scintillation Counter (1209 Rackbeta, LKB Wallac). Results were expressed as a percentage change from baseline unstimulated cells at time point zero (chapter 3).

2.4) Single Stranded Conformational Polymorphism (SSCP)

Blood was taken from patients and controls as described in 2.1.3 and stimulated with PMA / ionomycin to upregulate Fas expression (see results 5.2.1). RNA was extracted as described (section 2.2.1) and RT PCR conducted using the oligonucleotide primers shown in figure 5.1.2. Radiolabelled PCR (Orita et al, 1989) was carried out for 30 cycles as described in 2.2.3 above and the PCR samples were handled as follows. Five ul of the PCR mix was removed and mixed with 5 ul of SSCP stop solution/ loading buffer comprising:

Formamide	95% (Sigma)
EDTA	20 mM (Sigma)
NaOH	10 mM (Sigma)
Bromophenol blue	0.05 % (Sigma)
xylene cyanol	0.05 % (Sigma)

The PCR + SSCP stop solution was heat denatured at 95°C for 5 minutes and kept on ice for loading onto a non-denaturing polyacrylamide gel. TBE-PAGE gels were poured as 20 x 40 x 0.05 cm, 6% acrylamide gels made up in TBE buffer using Appligene electrophoresis apparatus. The gel plates were attached to a Haake K15 cooling unit (Fisons) when polymerised and denatured samples were loaded in 2 ul aliquots per well (remainder stored at -20°C). Electrophoresis was carried out at 12 W constant power for 20 hours in 1 x TBE buffer, following which gels were carefully removed and dried under vacuum before being subjected to autoradiography.

Buffers:	TBE 1X:	90 mM Tris-borate pH 8.3	(Sigma)
		4 mM EDTA pH 8	(Sigma)

A 6% acrylamide gel comprised of:

5 x TBE buffer	10	ml
milli-Q water	29.5	ml
Acrylamide (30%)	10	ml
TEMED	50	ul
Ammonium persulphate	500	ul

Gel conditions were optimised using mutant PCR products. Gels run at room temperature required the addition of glycerol 10% to the gels for resolution of bands but the best differentiation of polymorphic bands was found at 4°C with no added glycerol. Autoradiographs were visualised on a light box for polymorphic bands and any polymorphism was confirmed by independent PCR and SSCP gels. Sequence analysis was conducted for the polymorphic samples by automated cycle sequencing at the Blood Transfusion Service in Bristol but all primer and template material was provided in a ready-to-go PCR tube as for a standard PCR reaction.

2.5) APOPTOSIS ASSAY

A variety of in vitro assays of apoptosis were established and the methods are outlined below.

2.5.1) JAM assay

This is a method based on DNA labelling with ^{3}H-thymidine (Matzinger, 1991) where apoptotic fragmented DNA is reflected by decreased thymidine retention compared to non-apoptotic DNA. Target cells were labelled with ^{3}H-thymidine (ICN) by co-culture overnight at 37°C in a CO_2 incubator with 1.5 uCi/ml of ^{3}H-thymidine (from 20 uCi /

ml stock made up in culture medium). Labelled cells were washed in medium and left in an incubator for 6-8 hours before being plated out in a u-bottomed 96 well plate at 1×10^5 cells per well (in triplicate). Stimulations were carried out in a final volume of 200 ul per well for between 6 - 20 hours depending on the experiment in question and cells were then harvested on filter mats using a Skatron semi-automatic cell harvester. The filter mat was dried in a microwave oven for 2 minutes between hand paper towels and individual filters were punched out into scintillation polypropylene vials. Five hundred ul of scintillation fluid (Optiscint Hisafe, Wallac) was added to each vial before analysis on a Rackbeta scintillation counter programmed for reading triplicate samples.

2.5.2) Annexin V-FITC assay

The Annexin assay (Martin et al, 1995) depends on detection of exposed phosphatidyl-serine on the surface of apoptotic cells. A kit (Annexin V-FITC from Nexins Research) was used for the detection of phosphatidyl-serine on apoptotic cells. Cells were stimulated as required with anti-Fas antibody or sFasL and washed in ice-cold PBS at the end of the stimulation. Cells were then suspended in ice-cold diluted binding buffer at 10^5-10^6 cells / ml (in a volume of ± 445 ul). Five ul of freshly diluted Annexin V-FITC (1:10 dilution in binding buffer) and 50 ul propidium iodide (100 ug/ml in culture medium) was added to the 445 ul cell suspension and the tube incubated on ice (in the dark) for 10 minutes before FACS analysis on a Becton Dickinson flow cytometer. All the components were provided in kit form (Nexins Research B.V.) except the propidium iodide which was purchased from Sigma.

2.5.3) Detection of apoptotic DNA by TdT-mediated dUTP nick end labelling (TUNEL).

This was carried out by means of the TUNEL kit from Boehringer Mannheim.
TUNEL provided an additional method for detecting the DNA strand breaks of apoptosis by measuring the 3'-OH labelling of strand breaks in apoptotic DNA using an enzymatic reaction catalysed by terminal deoxynucleotidyl transferase (TdT). The fluorescein labelled dUTP incorporated into the strand breaks is detectable on FACS (Negoescu et al, 1996). Cells (approximately 1 x 10^6 per stimulation) were stimulated as usual and washed in PBS / 1% BSA at 4°C. Suspensions of 100 ul in FACS tubes had 100 ul of 4% paraformaldehyde / PBS added and incubated at room temperature for 30 minutes. Cells were washed in PBS / 1% BSA and resuspended in 100 ul permeabilisation solution (0.1 % Triton X-100 in 0.1 % sodium citrate) and incubated on ice for two minutes. Cells were washed twice in PBS / 1% BSA and resuspended in 50 ul TUNEL reaction mixture. Cells were incubated in the dark in a humidified atmosphere at 37°C for one hour, and then washed twice in PBS before analysis by FACS in PBS solution.

2.5.4) Cytospin analysis for nuclear morphology in apoptosis

Cells stimulated via the Fas receptor and control samples were spun onto slides at a density of 5 x 10^4 cells per slide on a Cytospin 3 apparatus (Shandon). Slides were air dried and fixed in acetone solution for 5 minutes before air drying again. Propidium iodide was added to the slides at 50 ug/ml and incubated at room temperature for 10 - 15 minutes before washing in PBS and analysing the slides on a fluorescent microscope (Laborlux S, Leitz) at high power. Photographs were taken at 40 x magnification.

2.6) STATISTICAL ANALYSIS.

All statistical analysis was conducted on a Statview programme (Abacus, Macintosh version) using data imported from Microsoft Excel version 5.0. Analysis included calculation of descriptive statistics like mean and standard deviations and comparison of groups was done by a Mann-Whitney U test for non-parametric data. Correlations were done by Spearman's rank correlation coefficient. Data from the JAM assays are displayed as the mean of triplicate wells and the standard error of the mean (SEM) is shown.

CHAPTER 3

FAS / CD95

A molecular approach to reagent generation

INTRODUCTION.

As stated earlier, research into murine Fas biology accelerated after the discovery that the *lpr* defect in mice was due to abnormalities of the Fas gene. The autoimmune features found in the MRL-*lpr / lpr* mouse had a striking resemblance to some of the manifestations of human SLE (Cohen and Eisenberg 1991), which is generally regarded as a prototypic non-organ specific autoimmune disease. With this in mind we embarked on this study to look at the role of Fas in human biology, with specific reference to human SLE. The project commenced when very little was known about Fas function in normal human biology, so that in order to study Fas we had to generate the necessary reagents. A molecular approach was taken with the aim of generating reagents that would be useful in guiding genetic studies on Fas as well as providing tools for use in functional studies of apoptosis. Given that the natural ligand for Fas was not identified at the time, we also used a molecular approach to construct a probe in order to search for a cell surface ligand for Fas.

RESULTS.

3.1) Cloning and expression of Human Fas cDNA

3.1.1) Generation of Fas cDNA

In order to identify a source of Fas RNA for reverse transcription, various cell lines available in the laboratory were screened for surface expression of Fas by FACS analysis. Using a monoclonal antibody to human Fas (CH11) cell surface expression of Fas was found on the transformed T cell lines, H9 and Jurkat as well as the transformed B cell line, Priess. **Figure 3.1.1** shows the high levels of Fas expressed

on H9 cells which were subsequently chosen for total RNA extraction. Purified total RNA was therefore used from the Fas expressing H9 cells in a reverse transcription PCR (RT-PCR) reaction to generate Fas cDNA.

To achieve this primers were designed from the published sequence data (genbank accession no. : M67454) in order to generate the coding region of human Fas. **Figure 3.1.2** shows the published Fas sequence in relation to the oligonucleotide primers designed in this experiment to generate a gel fragment of approximately 1000 base pairs representing the coding region without the untranslated 5' and 3' sequences. Oligonucleotide primers 5' (CGGAGGATTGCTCAACAACCATGCTG, nt 177-202) and 3' (CACTCTAGACCAAGCTTTGGATTTC, nt 1210-1185) were used in the RT-PCR reaction. A fragment of predicted size (±1000 bp) was generated representing Fas cDNA and is shown in **figure 3.1.3** as an agarose gel band. No PCR fragments were evident in the negative control reactions devoid of template DNA. Thus a specific Fas cDNA was generated with the oligonucleotide primers designed from the published Fas sequence.

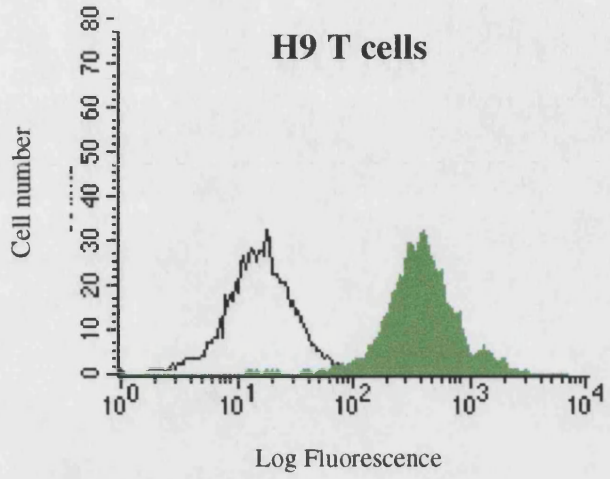


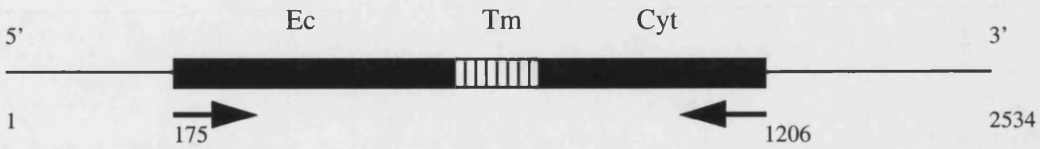
Figure 3.1.1: Fas Expression on H9 T cells

Surface expression of Fas was seen on H9 T cells (shaded histogram) without any activation stimuli. Cells were stained with monoclonal anti-Fas antibody (CH 11) at 1ug/ml and detected by anti-mouse IgM FITC. Control antibody (mouse IgM 1ug/ ml) staining is shown (unshaded histogram).

```
gacgcttctg gggagtgagg gaagcggttt acgagtgact tggctggagc      50
ctcaggggcg ggcactggca cggaacacac cctgaggcca gccctggctg     100
cccaggcgga gctgcctctt ctcccgcggg ttggtggacc cgctcagtac     150
ggagttgggg aagctctttc acttcggagg attgctcaac aaccATGCTG     200
GGCATCTGGA CCCTCCTACC TCTGGTTCTT ACGTCTGTTG CTAGATTATC     250
GTCCAAAAGT GTTAATGCCC AAGTGACTGA CATCAACTCC AAGGGATTGG     300
AATTGAGGAA GACTGTTACT ACAGTTGAGA CTCAGAACTT GGAAGGCCTG     350
CATCATGATG GCCAATTCTG CCATAAGCCC TGTCCTCCAG GTGAAAGGAA     400
AGCTAGGGAC TGCACAGTCA ATGGGGATGA ACCAGACTGC GTGCCCTGCC     450
AAGAAGGGAA GGAGTACACA GACAAAGCCC ATTTTTCTTC CAAATGCAGA     500
AGATGTAGAT TGTGTGATGA AGGACATGGC TTAGAAGTGG AAATAAACTG     550
CACCCGGACC CAGAATACCA AGTGCAGATG TAAACCAAAC TTTTTTTGTA     600
ACTCTACTGT ATGTGAACAC TGTGACCCTT GCACCAAATG TGAACATGGA     650
ATCATCAAGG AATGCACACT CACCAGCAAC ACCAAGTGCA AAGAGGAAGG     700
ATCCAGATCT AACTTGGGGT GGCTTTGTCT TCTTCTTTTG CCAATTCCAC     750
TAATTGTTTG GGTGAAGAGA AAGGAAGTAC AGAAAACATG CAGAAAGCAC     800
AGAAAGGAAA ACCAAGGTTC TCATGAATCT CCAACCTTAA ATCCTGAAAC     850
AGTGGCAATA AATTTATCTG ATGTTGACTT GAGTAAATAT ATCACCACTA     900
TTGCTGGAGT CATGACACTA AGTCAAGTTA AAGGCTTTGT TCGAAAGAAT     950
GGTGTCAATG AAGCCAAAAT AGATGAGATC AAGAATGACA ATGTCCAAGA    1000
CACAGCAGAA CAGAAAGTTC AACTGCTTCG TAATTGGCAT CAACTTCATG    1050
GAAAGAAAGA AGCGTATGAC ACATTGATTA AAGATCTCAA AAAAGCCAAT    1100
CTTTGTACTC TTGCAGAGAA AATTCAGACT ATCATCCTCA AGGACATTAC    1150
TAGTGACTCA GAAAATTCAA ACTTCAGAAA TGAAATCCAA AGCTTGGTCT    1200
agagtgaaaa acaacaaatt cagttctgag tatatgcaat tagtgtttga    1250
aaagattctt aatagctggc tgtaaatact gcttggtttt ttactgggta    1300
cattttatca tttattagcg ctgaagagcc aacatatttg tagattttta    1350
atatctcatg attctgcctc caaggatgtt taaaatctag ttgggaaaac    1400
aaacttcatc aagagtaaat gcagtggcat gctaagtacc caaataggag    1450
tgtatgcaga ggatgaaaga ttaagattat gctctggcat ctaacatatg    1500
attctgtagt atgaatgtaa tcagtgtatg ttagtacaaa tgtctatcca    1550
caggctaacc ccactctatg aatcaataga agaagctatg acctttgct     1600
gaaatatcag ttactgaaca ggcaggccac tttgcctcta aattacctct    1650
gataattcta gagattttac catatttcta aactttgttt ataactctga    1700
gaagatcata tttatgtaaa gtatatgtat ttgagtgcag aatttaaata    1750
aggctctacc tcaaagacct ttgcacagtt tattggtgtc atattataca    1800
atatttcaat tgtgaattca catagaaaac attaaattat aatgtttgac    1850
tattatatat gtgtatgcat tttactggct caaaactacc tacttctttc    1900
tcaggcatca aaagcatttt gagcaggaga gtattactag agctttgcca    1950
cctctccatt tttgccttgg tgctcatctt aatggcctaa tgcaccccca    2000
aacatggaaa tatcaccaaa aaatacttaa tagtccacca aaaggcaaga    2050
ctgcccttag aaattctagc ctggtttgga gatactaact gctctcagag    2100
aaagtagctt tgtgacatgt catgaaccca tgtttgcaat caaagatgat    2150
aaaatagatt cttatttttc ccccaccccc gaaaatgttc aataatgtcc    2200
catgtaaaac ctgctacaaa tggcagctta tacatagcaa tggtaaaatc    2250
atcatctgga tttaggaatt gctcttgtca taccctcaag tttctaagat    2300
ttaagattct ccttactact atcctacgtt taaatatctt tgaaagtttg    2350
tattaaatgt gaattttaag aaataatatt tatatttctg taaatgtaaa    2400
ctgtgaagat agttataaac tgaagcagat acctggaacc acctaaagaa    2450
cttccattta tggaggattt ttttgcccct tgtgtttgga attataaaat    2500
ataggtaaaa gtacgtaatt aaataatgtt tttg                     2534
```

Figure 3.1.2: Human Fas Gene Sequence.

The full gene sequence for Human Fas is shown (GenBank no. M67454) with the coding region (nucleotides 195 - 1200) outlined in upper case and the transmembrane encoding domain shown in the boxed area. Oligonucleotide primers were designed (underlined sequences) to amplify the coding region of human Fas as a PCR fragment of ±1005 bases.



Primers used for PCR (compare figure 3.1.2):
5': CGGAGGATTGCTCAACAACCATGCTG (nt 175-200) and
3': CACTCTAGACCAAGCTTTGGATTTC (nt 1205-1182)

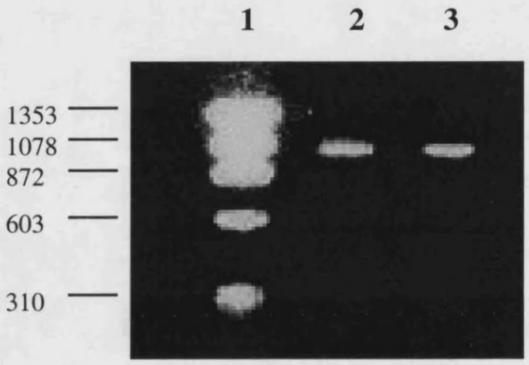


Figure 3.1.3 : PCR of the Fas coding region.

A line drawing above shows the Fas coding region amplified by PCR using oligonucleotide primers underlined in figure 3.1.2 (sequence of oligonucleotides shown below the drawing). An agarose gel is shown below where the Fas PCR product of ± 1031 bases was amplified from H9 T cells (lane 2) and Priess B cells (lane 3). Molecular weight markers are shown in lane 1 (ϕ-X 174 *Hae* III digest).

Ec = Extracellular coding region
Tm = Transmembrane coding region
Cyt = Cytoplasmic coding region

3.1.2) Cloning Fas cDNA into pT7

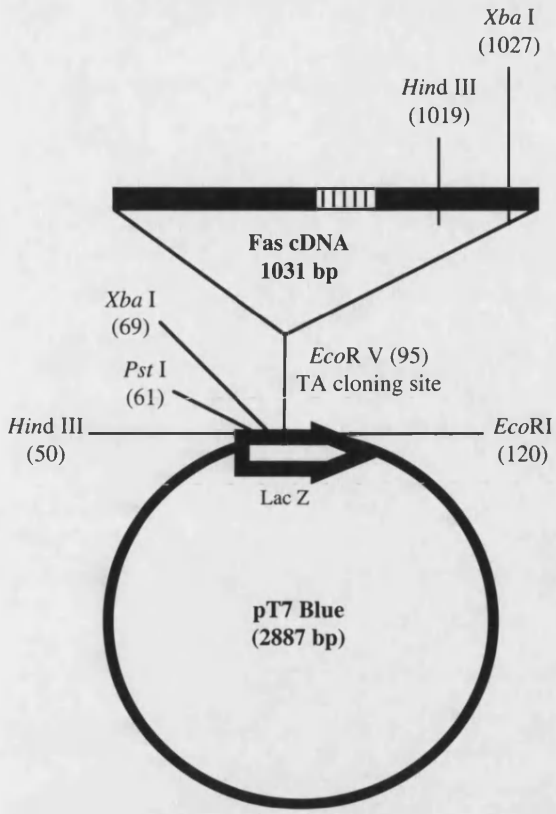
In order to test if the amplified PCR product was in fact Fas cDNA, it was necessary to clone the PCR product into a plasmid vector. This would enable the preparation of larger quantities of Fas DNA for analysis by restriction mapping and sequencing if necessary.

To achieve this we employed a method of TA cloning (chapter 2.2.12). Ligated PCR products of Fas cDNA were transformed into *E.coli* and grown on agar plates. White bacterial colonies were chosen for plasmid minipreps. In all thirty six clones were selected for miniprep analysis of which six were found to have an appropriate sized insert. **Figure 3.1.4** shows a representative agarose gel where a restriction digest with EcoR1-*Pst*1 removed an insert of the correct size (± 1090bp) from the vector (gel A lane 8). Lane 4 of the same gel shows an example of a clone with no DNA insert removable by the *Eco* R1-*Pst* 1 digest. The relatively high frequency of non-recombinant clones such as these on a given agar plate makes it imperative to screen miniprep colonies for an insert before proceeding to restriction mapping of minipreps with a larger selection of restriction enzymes. Miniprep screening by restriction analysis with a combination of *Eco* R1 + *Pst* 1 in this case therefore revealed six clones with inserts which could be further analysed by restriction mapping.

In order to characterise the recombinant clones as human Fas cDNA, analysis by restriction mapping was conducted. Plasmid clones containing inserts as shown in **figure 3.1.4** were analysed by restriction mapping using a range of restriction enzymes. The predicted fragments from a mapping experiment are shown in the same figure (3.1.4). Restriction mapping in this case confirmed the presence of a number of clones of Fas cDNA PCR products in the pT7 vector (Fas-pT7 clones) in a variety of orientations. Amongst the 6 clones analysed by this method one was found to be in the

appropriate orientation (ie, the 5' end proximal to the T7 promoter and referred to here as sense clones) (lanes 2 and 3 of gel B) and two clones were in the opposite orientation (lanes 5, 6, 8, and 9 in gel B referred to here as anti-sense clones). The orientation of the Fas cDNA within the pT7 vector was not important from the viewpoint of cellular expression (because the pT7 vector is not a mammalian cell expression vector) but it was useful to know the orientation for the purpose of sub-cloning into mammalian expression vectors as will be seen later.

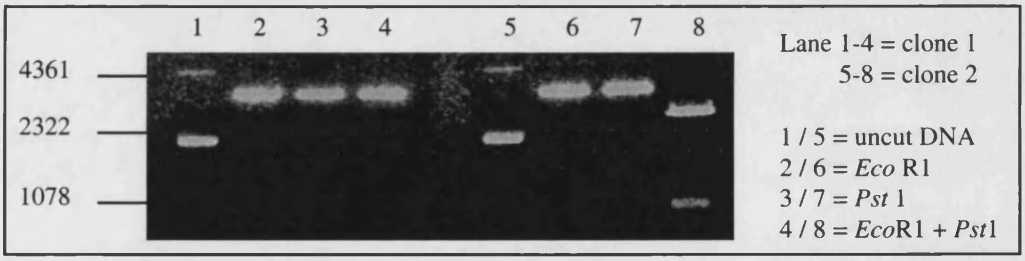
Therefore it was evident that the Fas cDNA was successfully amplified by PCR, the gene was subcloned into pT7, and that restriction mapping data were consistent with the correct sequence.



Predicted DNA patterns with restriction digests:

Pst I / *Eco*RI	: 1090 + 2828 bp
Hind III (sense)	: 1064 + 2854 bp
(anti-s)	: 57 + 3861 bp
Xba I (sense)	: 1053 + 2865 bp
(anti-s)	: 30 + 3888 bp

GEL A



GEL B

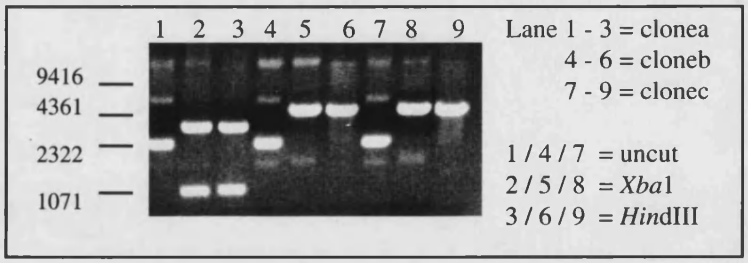


Figure 3.1.4: Cloning of Fas cDNA into pT7 Blue.

A cloning diagram is shown above with selected restriction sites and predicted fragment sizes for the chosen restriction enzymes. Agarose gels are shown below with Gel A showing screening of miniprep colonies and Gel B showing restriction mapping of three recombinant clones. In Gel A lane 8 shows a positive clone from which a fragment of ± 1090 bp was removed by a *Pst* 1 / *Eco*R1 digest. Lanes 2 and 3 of Gel B show the excision of ±1050bp fragments by *Xba* 1 and *Hind*III, respectively, to represent a 5' to 3' sense orientation of clone a, whilst clones b and c were anti-sense clones.

3.1.3) Cloning Fas into pEe6 expression vector

In order to generate transfectants, Fas cDNA was introduced into a eukaryotic expression vector which contained the necessary promoter regions for driving transcription. The pT7 vector in which Fas was initially cloned provided a useful means to TA clone directly from PCR and amplify and map the human Fas cDNA but it lacked the genetic promoter machinery necessary for expression in eukaryotic cells.

It was therefore necessary to subclone the Fas cDNA into an appropriate vector. A plasmid which was already available within the laboratory was chosen for this purpose (pEe6). pEe6 uses a CMV promoter to drive expression of the inserted gene in eukaryotic cells (Cockett et al, 1991). This requires the gene of interest to be correctly cloned into the vector in relation to the promoter (5' - 3') (referred to as sense direction in this thesis). Sub-cloning the Fas cDNA from the pT7 vector into pEe6 in the correct orientation could not be achieved readily since no suitable combination of restriction enzymes could excise a full Fas cDNA such that it could be religated to the equivalent sites on pEe6.

It was thus decided to use an approach of blunt end cloning. **Figure 3.1.5** shows the strategy where a *Pst* 1 - *Eco* R1 fragment from Fas-pT7 was removed and blunt ended by a Klenow reaction and cloned into *Sma* 1 cut pEe6. The ligated Fas-pEe6 was transformed into *E.coli* by electroporation. Colony screening following "colony lifts" and hybridisation with radiolabelled Fas cDNA as a probe is shown in figure 3.1.5. Miniprep screening of selected "hot colonies" revealed some clones with larger DNA bands thus suggesting the presence of an inserted DNA sequence into the pEe6 vector (lanes 6, 7, 8, 9, and 10 in the agarose gel of figure 3.1.5).

To identify which clones contained the appropriate Fas insert further restriction mapping was carried out as shown in **figure 3.1.5 a**. As seen in the figure the

fragment sizes help predict the orientation of Fas within the vector. In the case of*Xba* 1 and *Hin* d III a clear difference in size is evident so that the ± 1050 bp fragments generated by both these enzymes (lanes 4 and 5 respectively of figure 3.1.5 a) suggested that the clone was in a sense orientation within the vector (ie 5' end is proximal to the promoter within the vector). An antisense clone would have given fragments of ± 25-40 bp which would have been very easy to differentiate on an agarose gel. Whilst *Bam* H1 cuts twice in the Fas sequence and once in the pEe6 sequence one of the three fragments generated by this enzyme helped in orientation of the clone (sense = 260 bp versus antisense = 797 bp). Lane 6 in figure 3.1.5a shows a ± 260 bp fragment to support the findings of a sense clone of Fas-pEe6 with the*Xba* 1 and *Hin* d III digests above.

Thus this experiment shows that Fas was subcloned into pEe6 from pT7 using a blunt cloning technique. Miniprep screening was carried out using radioactive hybridisation and the restriction mapping confirmed a sense clone of Fas in pEe6 (Fas-pEe6) which could be tested for expression in mammalian cells.

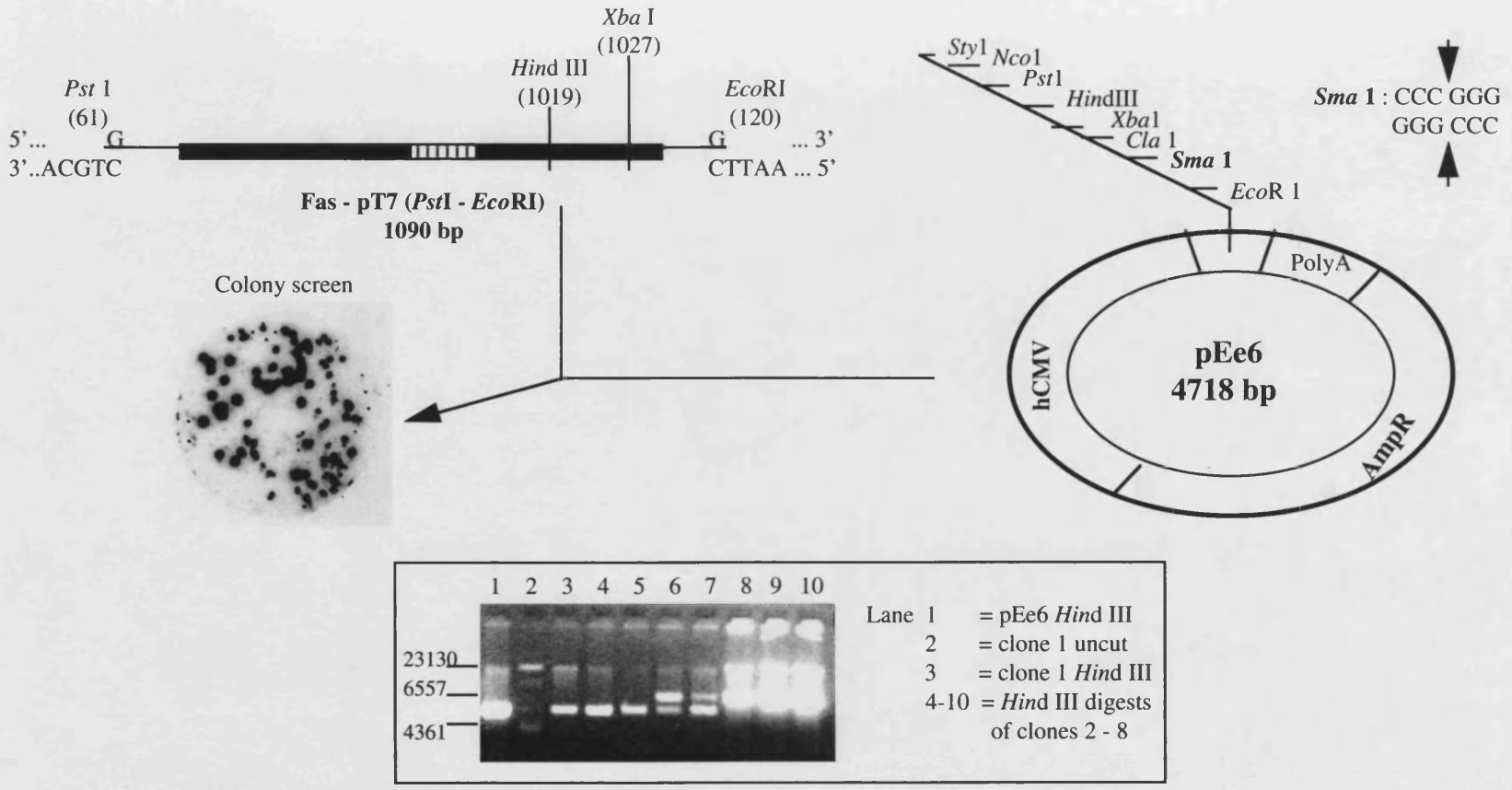
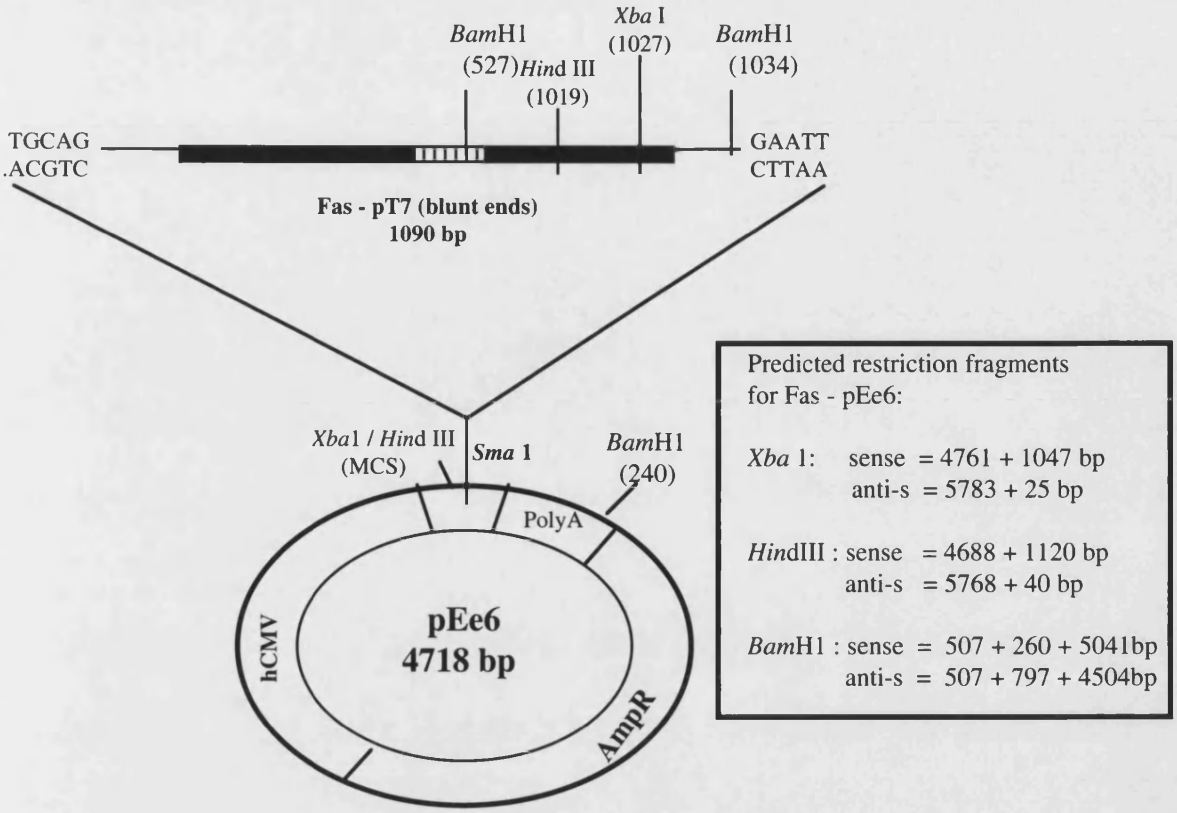


Figure 3.1.5: Sub-cloning Fas cDNA into pEe6.

The line drawings show the strategy for ligation of pEe6 cut with *Sma*1 to blunt-ended Fas cDNA excised from pT7. Recombinant clones were screened by colony hybridisation on Nylon membranes and radioactive colonies shown on the colony screen above represent pEe6 clones containing Fas cDNA. The agarose gel shows restriction enzyme screening with *Hin*dIII of eight "hot" clones from the colony screen above. Lanes 6, 7, 8, 9 and 10 show bands larger than the native pEe6 (lane 1) and represent recombinant clones that require further mapping (figure 3.1.5a). Clones without any inserts can be seen in lanes 3, 4, and 5 which show fragments equal in size to pEe6 (lane 1).



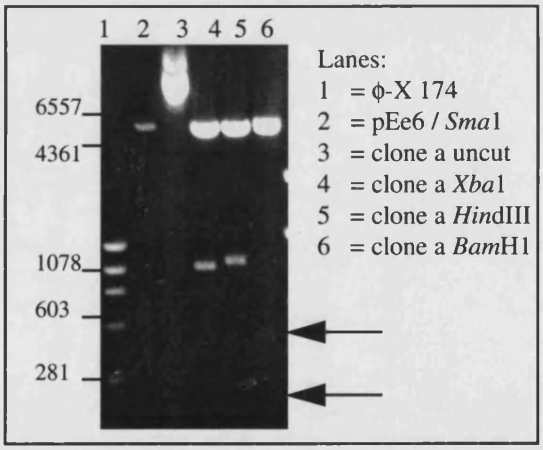


Figure 3.1.5a: Restriction Mapping Fas - pEe6.

A cloning diagram is shown for Fas cDNA in pEe6 with selected restriction enzyme sites and the predicted sizes for the restriction fragments. The agarose gel shows a restriction map for one recombinant clone. Lanes 4 and 5 show the excision of appropriate sized fragments with *Xba*1 and *Hind*III respectively. In lane 6 the vector size is larger than in lanes 4 and 5 and supports the suggestion of a sense orientation for this clone (the smaller *Bam*H1 fragments are not clearly visible on the image but their positions have been outlined by the arrows).

3.1.4) Expression of Fas-pEe6 in Chinese Hamster Ovary (CHO) cells

In order to express the cloned Fas-pEe6 in CHO cells, transfectants were generated using electroporation. Experience with transfecting other DNA into CHO cells in our laboratory had shown that transient expression varied depending on the electroporation conditions and the type of DNA used, but expression was usually in the order of 5 - 20% of cells transfected. CHO cells transfected with the Fas-pEe6 initially showed low levels of expression of surface Fas on FACS analysis at 24 hours **(figure 3.1.6a)**, however this confirmed the ability of CHO cells to express Fas in a recognisable form.

In an attempt to increase the levels of expression and to create stable expressing transfectant cell lines for Fas the cells that bound anti-Fas antibody were selected by magnetic beads coated with anti-mouse IgM. After cells were recovered and re-cultured, higher levels of Fas expression was achieved as shown in **Figure 3.1.6b**. FACS sorting was also subsequently used to enrich the Fas expressing CHO transfectants. The upper 10% of Fas expressing cells detected on FACS were sorted and propagated in culture. Continued growth of the sorted cells under selection with hygromycin resulted in a stable population of high Fas expressors (ie a relatively homogenous Fas expressing cell line **figure 3.1.6c**).

Thus the expression of Fas on the CHO cell line demonstrated that the cloned Fas cDNA derived from the previous cloning experiments was indeed functionally intact and recognisable by standard anti-Fas antibodies.

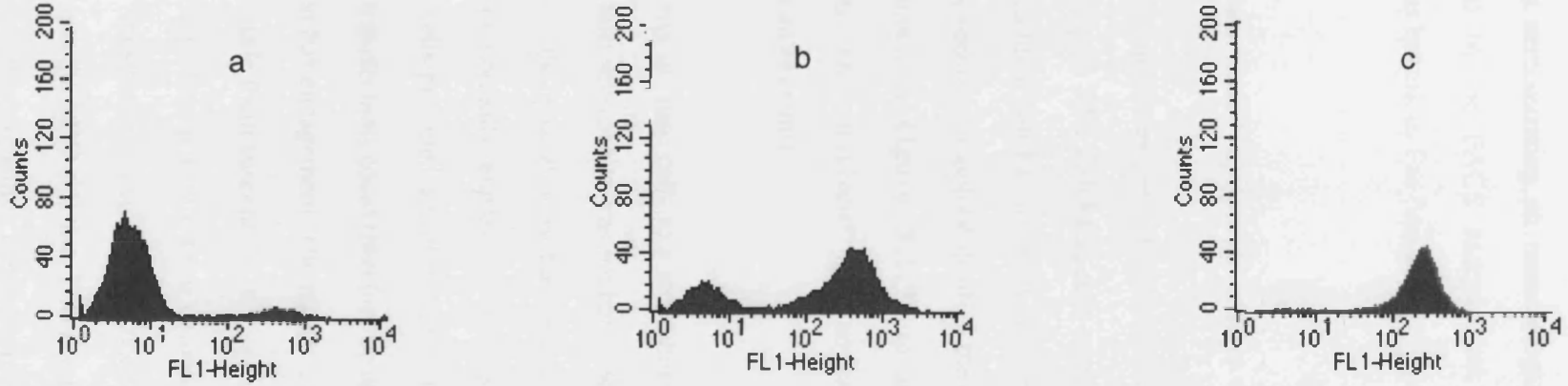


Fig 3.1.6 : FACS analysis of CHO-Fas transfectants at various stages of selection.

FACS histograms are shown for CHO cells transfected with Fas-pEe6 and stained with monoclonal anti-Fas antibody CH 11 (1ug/ml). Histogram **a** shows transfected cells 24 hours after electroporation whilst histogram **b** shows Fas staining after two rounds of magnetic bead selection of transfectants. Histogram **c** shows the homogeneous population of high Fas expressing CHO cells obtained after FACS sorting cells from b.

3.1.5) Characterisation of Fas function in CHO-Fas transfectants

Apart from demonstrating an immunologically intact Fas molecule on the CHO-Fas transfectants by the FACS analysis just described, we also tested the CHO-Fas transfectants in various Fas functional assays as follows:

3.1.5 a) *Functional apoptosis assays in CHO-Fas cells*

To test for apoptosis in general a number of assays were established in the laboratory (methods chapter 2.5). CHO-Fas cells were subjected to a JAM assay (section 2.5.1) in which monoclonal anti-Fas antibody was tested for the ability to induce apoptosis. This showed Fas-mediated apoptotic death in the CHO-Fas cells but not in untransfected CHO cells as shown in **Figure 3.1.7**. In addition there was no death of CHO-Fas transfectants when incubated with an isotype matched mouse IgM at equivalent concentrations (1ug/ml).

The sensitivity of these cells to a recombinant FasL molecule was also tested. CHO-Fas cells were also sensitive to Fas-mediated death by ligand as shown in figure 3.1.7 (the description of the generation of the soluble Fas ligand follows in chapter 4). As CHO cells did not normally express human Fas on their surface, untransfected or mock transfected cells provided a useful control in the study of Fas-mediated apoptosis. The CHO-Fas transfectants could therefore be used in functional assays because they undergo apoptosis on Fas engagement. The results thus suggested that the introduction of Fas into CHO cells made them susceptible to Fas-mediated death by apoptosis. The cloned Fas cDNA has therefore not only been demonstrated to have immunoreactivity on the cell surface of transfectants but is also functionally active in mediating cell death when introduced into the CHO cell line. This result suggested that the CHO-Fas system may be useful for studies of signalling mechanisms triggered following Fas receptor engagement.

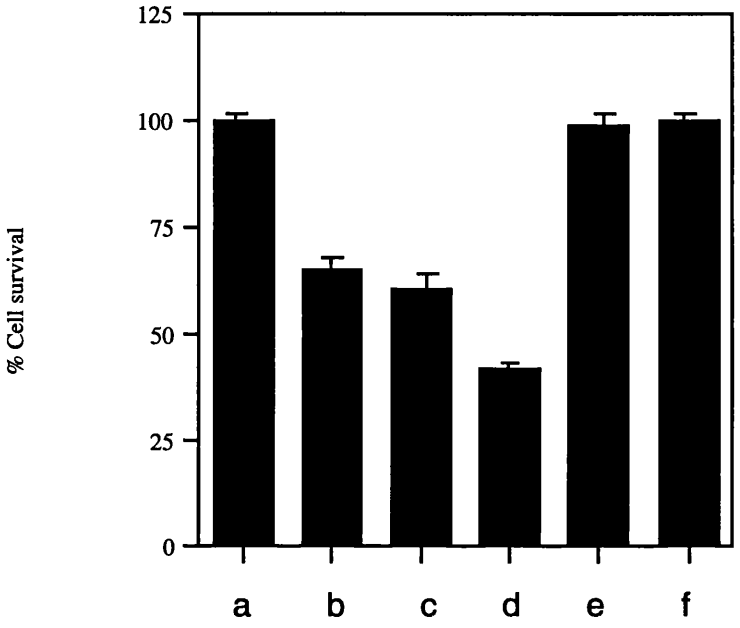


Figure 3.1.7 : Apoptosis of CHO-Fas transfectants.

A JAM assay is shown using transfected CHO-Fas cells. A reduction in ^{3}H-Thymidine incorporation (apoptotic DNA) is represented as a change in % cell survival which is evident in histograms **b** (CH11 antibody 1ug/ml), **c** (sFasL 1:10)*, and **d** (ssFasL 1:10)*. Controls are shown as unstimulated CHO-Fas transfectants (a), transfectants stimulated with istotype matched mouse IgM antibody 1ug/ml (e), and anti-Fas antibody with non-transfected CHO cells (f). All results represent triplicate samples.

* sFasL = soluble Fas ligand (generated in this project, section 4.1.2)
ssFasL = signal sequence Fas ligand (see section 4.1.5, ss representing the addition of a B7 signal sequence to the sFasL.

3.1.5 b) *Antibody screening and epitope mapping*

It was our intention to use the CHO-Fas transfectants for immunisation and generation of our own supply of anti-Fas antibodies. During the course of the project however, antibodies became commercially available so that there was no longer a need to spend limited time and resources on this. However, we had an opportunity during this project to test a soluble Fas ligand against the CHO-Fas transfectants which provided a useful tool for testing and validating the ligand (shown in figure 3.1.7 and details discussed in chapter 4). This in vitro model system could be useful in future to compare various anti-Fas reagents from the point of view of epitope mapping and Fas mutant generation.

3.1.5 c) *Signalling in the CHO-Fas cells*

3.1.5 c (1) Acidic Sphingomyelinase signalling

To assess mechanisms associated with Fas engagement in the CHO-Fas system where Fas can be easily introduced, a number of signalling assays were established. Reports were beginning to emerge of sphingolipid signalling following TNFR1 engagement (of which Fas is a family member). Thus we attempted to study if sphingolipid signalling occurred on Fas engagement.

In order to test sphingomyelinase signalling in the CHO-Fas transfectants the assay was first established in T cell blasts where CD28 was also known to activate the acidic sphingomyelinase signalling pathway (Boucher et al, 1995). **Figure 3.1.8a** shows the activation of acidic sphingomyelinase following stimulation of the CD28 receptor by its ligand, CD80 (B7). There was a rapid and transient activation of acidic sphingomyelinase following CD28 stimulation, and similar results were seen following Fas stimulation in these T cell blasts. Control antibody stimulation with anti-CD3 or mouse IgM did not activate the sphingomyelinase pathway. The CHO-Fas transfectants were tested in the

same assay and found to have intact sphingomyelinase signalling associated with Fas engagement as shown in **figure 3.1.8b**. As a positive control for CHO signalling a CHO-CD28 transfectant was used. In both the CHO-Fas and CHO-CD28 transfectants there was no activation of sphingomyelinase by control mouse antibody.

To test if the sphingomyelinase activity could be inhibited chloroquine was used in the assay. Chloroquine inhibited the Fas-mediated activation of sphingomyelinase as shown in **figure 3.1.8c**. Whilst it was well recognised that lysosomotrophic agents such as ammonium chloride, monensin and chloroquine inhibited acidic sphingomyelinase activity in general, it was nevertheless interesting to see the inhibition of Fas-induced sphingomyelinase activity by choloroquine especially because this drug is still widely used in the therapy of SLE, albeit in a chemically modified form (hydroxychoroquine). This could be an additional mechanism of action for hydroxychloroquine in SLE and will be discussed later.

Therefore the sphingomyelinase assay which was established on the background of a CD28 based system in the laboratory confirmed that Fas stimulation resulted in acidic sphingomyelinase activity both in the CHO-Fas transfectants as well as T cell blasts. This stimulation was rapid reaching a peak by one minute and returning to baseline by five to ten minutes.

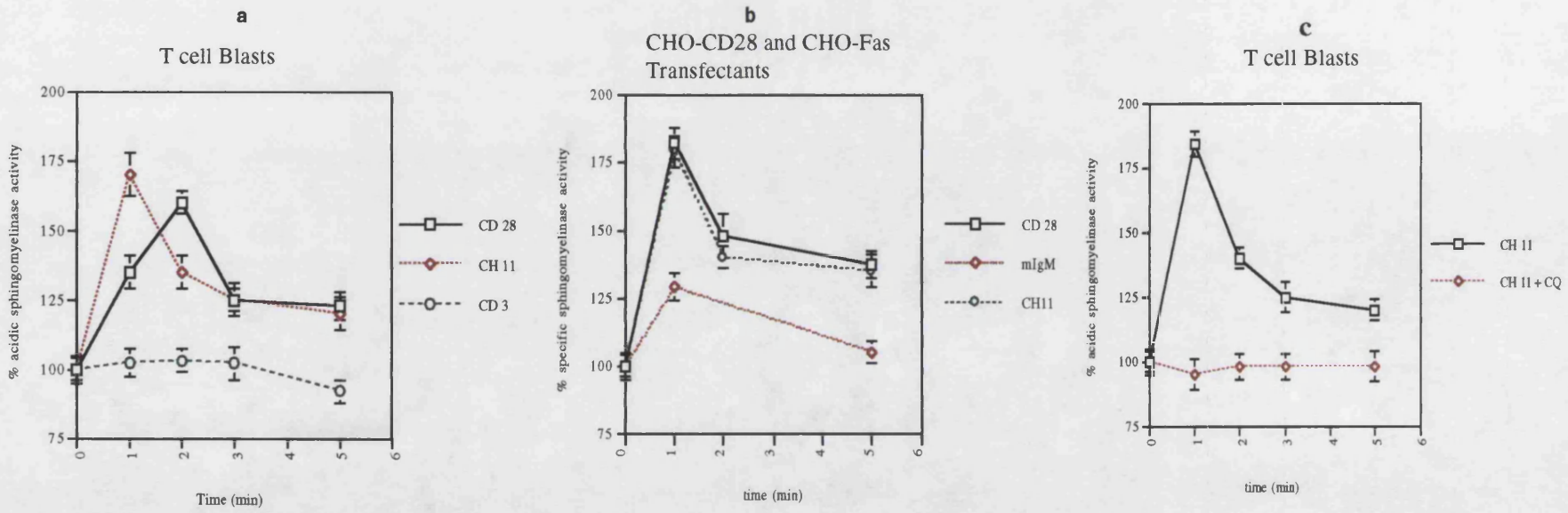


Figure 3.1.8: Acidic Sphingomyelinase signalling in CHO-Fas transfectants.

The percentage acidic sphingomyelinase activity above baseline unstimulated cells is shown over time (minutes) following cell stimulation. Rapid upregulation of acidic sphingomyelinase activity can be seen with both CD28 (9.3 antibody 1ug/ml) and Fas stimulation (CH 11 1ug/ml) both in T cell blasts (a) as well as CHO-Fas transfectants (b). Control antibody failed to stimulate acidic sphingomyelinase activity (anti-CD3 1ug/ml in {a} and Mouse IgM in {b}). Chloroquine(CQ) (100nM) inhibited the Fas-mediated acidic sphingomyelinase activation (c).

3.1.5 c (2) Jun Kinase signalling in CHO-Fas transfectants

One of the downstream consequences of acidic sphingomyelinase activation is the generation of ceramide. This in turn has been implicated in activation of c-Jun kinase (JNK) following stimuli such as ultraviolet irradiation and osmotic stress (Kyriakis et al, 1994).

To investigate whether JNK activity was induced in the CHO-Fas system, an in vitro kinase assay was established using a GST-cJun fusion protein as substrate. **Figure 3.1.9** shows autoradiographs from SDS-PAGE gels of in vitro kinase reactions from T cell blasts and CHO-Fas transfectants. As with sphingomyelinase above, both cell types showed JNK activation following Fas stimulation. The level of phosphorylation changes seen with CHO-Fas transfectants was however less than that seen in T cell blasts which may reflect cell specific differences in regulation of the pathway by phosphatases and other factors interacting with the JNK activity.

Activation of JNK may or may not play a critical role in Fas-mediated apoptosis (see later discussion with JLW cells in chapter 4) but these results demonstrated that JNK activation occurred following Fas engagement in the CHO-Fas transfectant system. With the development of specific pathway inhibitors and dominant negative inhibitors the CHO-Fas system may prove useful for introduction of individual genetic components in an attempt to dissect the various influences on the JNK pathway and to work out its relevance in the signalling cascade for apoptosis.

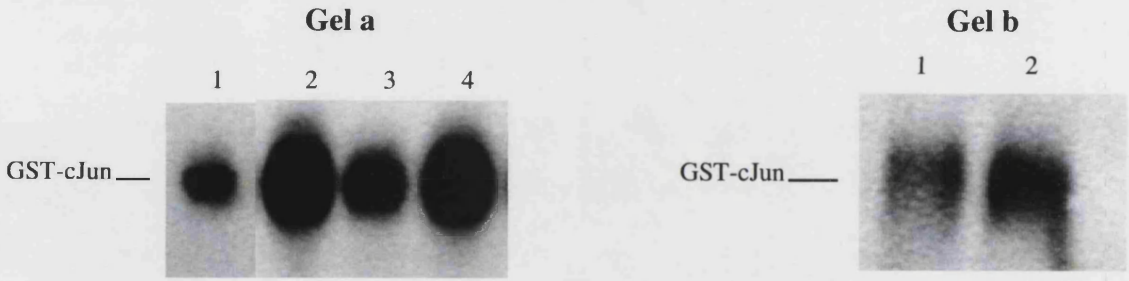


Figure 3.1.9: Jun kinase (JNK) signalling in CHO-Fas transfectants.

Autoradiographs of in vitro kinase reactions electrophoresed on SDS-PAGE gels. GST-cJun was used as phosphorylation substrate for measurement of JNK activity in whole cell lysates. The assay was established in T cell blasts (gel a) where activation of JNK can be seen with anti-Fas stimulation (CH 11 1ug/ml in lane 2 and sFasL 1:10 in lane 3) compared to unstimulated T cells (lane 1). Activation of JNK was also seen with ceramide in the T cell blasts (lane 4). Gel b represents the JNK assay in CHO-Fas transfectants which displayed elevated GST-cJun phosphorylation when activated by CH 11 (gel b lane2) as compared to mouse IgM (gel b lane 1).

Thus the first stage of this project was achieved with the cloning and expression of a human Fas cDNA. The transfection of human Fas into CHO cells resulted in expression of a functional protein and apoptosis was inducible in a Fas-specific manner in these transfectants. In addition the CHO-Fas transfectants were able to use some of the known signalling pathways following Fas activation. Successful cloning and expression of the human Fas cDNA therefore provided the confidence to proceed with further molecular manipulation of the Fas cDNA as described in the following section.

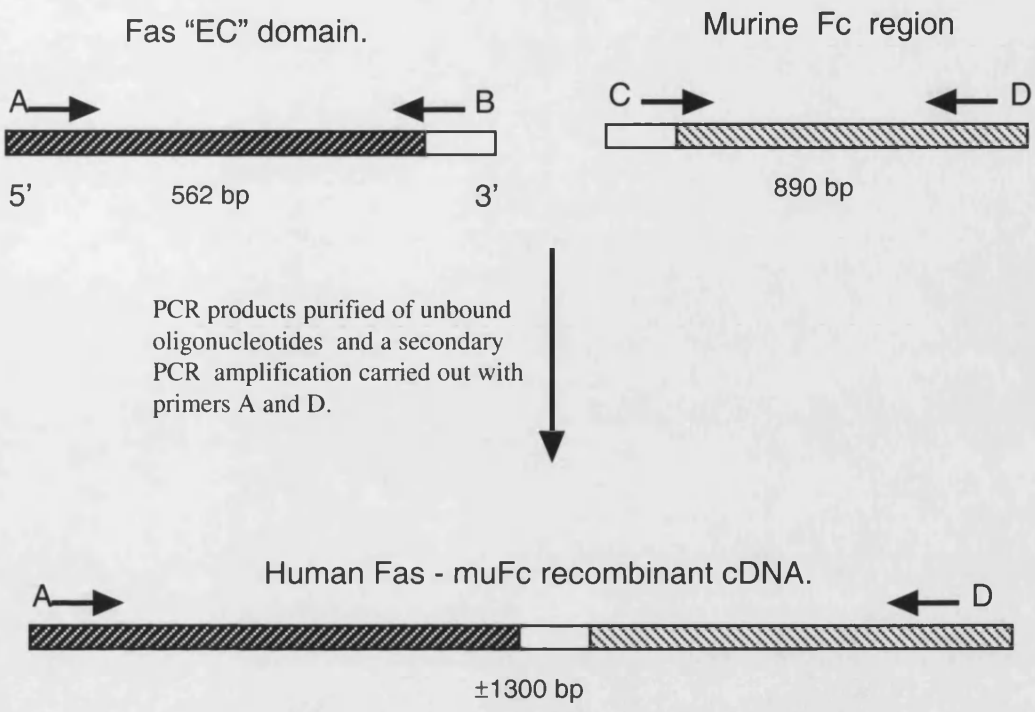
3.2) GENERATION OF RECOMBINANT FAS-Fc

Recombinant Fc fusion proteins provide useful tools for identification of surface counter-receptors and the soluble fusion protein could also serve a useful role as a receptor decoy in functional experiments, as was elegantly shown in the case of CTLA4-Ig (Linsley et al, 1991). In the same vein, a Fas-Fc protein would be potentially helpful in probing for Fas ligand and also serve as a blocking reagent in functional experiments. Fc fusion proteins comprise a hybrid of the protein of interest and the immunoglobulin constant region (Fc). The species from which the Fc domain can be chosen is wide but mouse and human Fc regions are useful because of the ready availability of secondary antibody reagents. We therefore employed this approach to generate a soluble Fas-Fc fusion protein comprising human Fas and mouse IgG. Mouse IgG was chosen because of the ready availability of secondary anti-mouse IgG reagents. We also reasoned that the use of a mouse immunoglobulin Fc region in such a Fas-Fc protein would facilitate identification of human Fas co-receptors without the problem of excessive non-specific Fc binding to human Fc receptors. In order to make a Fas-Fc fusion protein in vitro a cDNA comprising the appropriate genetic components was required.

3.2.1): Generation of Fas-Fc cDNA

3.2.1.1) Generation of cDNA by PCR

To create a fusion protein of Fas-Fc in vitro it was nesessary to generate a recombinant cDNA molecule comprising the extracellular domain of human Fas and the Fc region of mouse IgG. The successful cloning of a functional Fas cDNA described above provided the impetus to design new oligonucleotide primers from the Fas coding region to generate a truncated Fas molecule containing only the extracellular domain (EC). In addition primers were designed to generate a mouse immunoglobulin Fc cDNA. In order to facilitate annealing of the Fas extracellular cDNA and the mouse Fc cDNA the primer design incorporated an overlap sequence of twenty bases. **Figure 3.2.1.1** shows an outline of the strategy that was employed to generate the Fas-Fc cDNA using a recombinant PCR technique. Cell lines expressing Fas were previously identified for the Fas cDNA cloning as described and the same RNA template from H9 T cells was used in this experiment. An RNA template for the mouse immunoglobulin PCR reaction was obtained from a hybridoma cell line (UCHT4) which was used in our laboratory for production of antibody (UCHT4 hybridoma produces a mouse IgG2a subclass of anti-CD8 antibody). An initial PCR using the H9 and UCHT4 RNA was carried out using primers A and B in reaction 1 and primers C and D in reaction 2, in order to generate the human Fas extracellular encoding region and the mouse Fc region, respectively. The agarose gel in figure 3.2.1.1 shows the Fas EC domain cDNA (lanes 3, 4, and 5 ± 560bp product) and the mouse IgG Fc domain (lanes 6, 7, and 8 ± 890bp product) as well as the recombinant Fas-Fc cDNA (lane 10 ± 1300 bp product) which was obtained by amplification of a mix of the two cDNAs with the flanking primers A and D. Therefore the initial PCR products generated by the primer design above appeared to be of the correct predicted size and the overlap PCR reaction also produced the predicted ±1400 bp product representing a Fas-Fc hybrid cDNA.



Primer sequences:

5' A: CGGAGGATTGCTCAACAACCATGCTG 3'

B: AATGGGCCCGCTGGGCCCCAAGTTAGATCTGGATCCTTC

C: CCAGATCTAACTTGGGGCCCAGCGGGCCCATTTC

D: GGGTGCCAGTGTCTCTTAGG

Figure 3.2.1.1 : Strategy for generation of Fas-Fc cDNA.

Line drawings above outline the strategy employed to create a recombinant Fas-Fc cDNA by PCR. An overlapping primer sequence (21 nucleotides as underlined) facilitated the fusion of the human Fas extracellular (EC) domain to the mouse IgG Fc domain. The agarose gel shows the Fas (lanes 3, 4, and 5) and Fc (lanes 6, 7, and 8) PCR products as well as the recombinant Fas-Fc PCR product (lane 10). Molecular weight markers (ϕX-174 *Hae* III DNA) are shown in lanes 1 and 9.

3.2.1.2) Cloning the Fas-Fc cDNA into pT7

To validate the Fas-Fc PCR sequence a cloning procedure was undertaken. To achieve this an approach similar to the previous Fas cloning was adopted with the vector pT7 because of the advantages of TA cloning and colour selection of recombinant clones. Following selection, four Fas-Fc clones in pT7 were identified. Restriction mapping was carried out to confirm that the cloned fragment had all the predicted restriction enzyme sites (shown in **figure 3.2.1.2).** A *Kpn* 1-*Xba* 1 digest removed the insert of Fas-Fc from within the multiple cloning site of pT7 as shown by the ± 1300 bp fragment in lane 6 of the agarose gel. *Bam* H1 was chosen for its restriction sites in the Fas sequence and the multiple cloning site of pT7. The clear fragment of ± 523 bp rather than ±895 bp in lane 5 orientates the Fas-Fc product in an anti-sense direction (ie, in relation to the T7 promoter of the pT7 plasmid).

Similarly *Acc* 1 cuts in the Fc region and the multiple cloning site and the ± 530 bp fragment removed from the clone orientates it in an anti-sense direction within pT7 (lane 7). A *Bgl* II restriction site was unique to the overlap sequence of Fas-Fc so that a linear fragment of ± 4300bp as seen in lane 4 confirmed the presence of an appropriate Fas-Fc insert in the pT7 vector (the additional band seen in all lanes at around 2000bp represents partially digested DNA).

The presence of the appropriate sized restriction products on mapping thus confirmed the presence of a Fas-Fc sequence of cDNA within the pT7 vector, albeit in an anti-sense orientation. The orientation was not critical at this stage because expression of the protein was not possible in the pT7 vector. However the orientation within pT7 was exploited for subcloning as shown next.

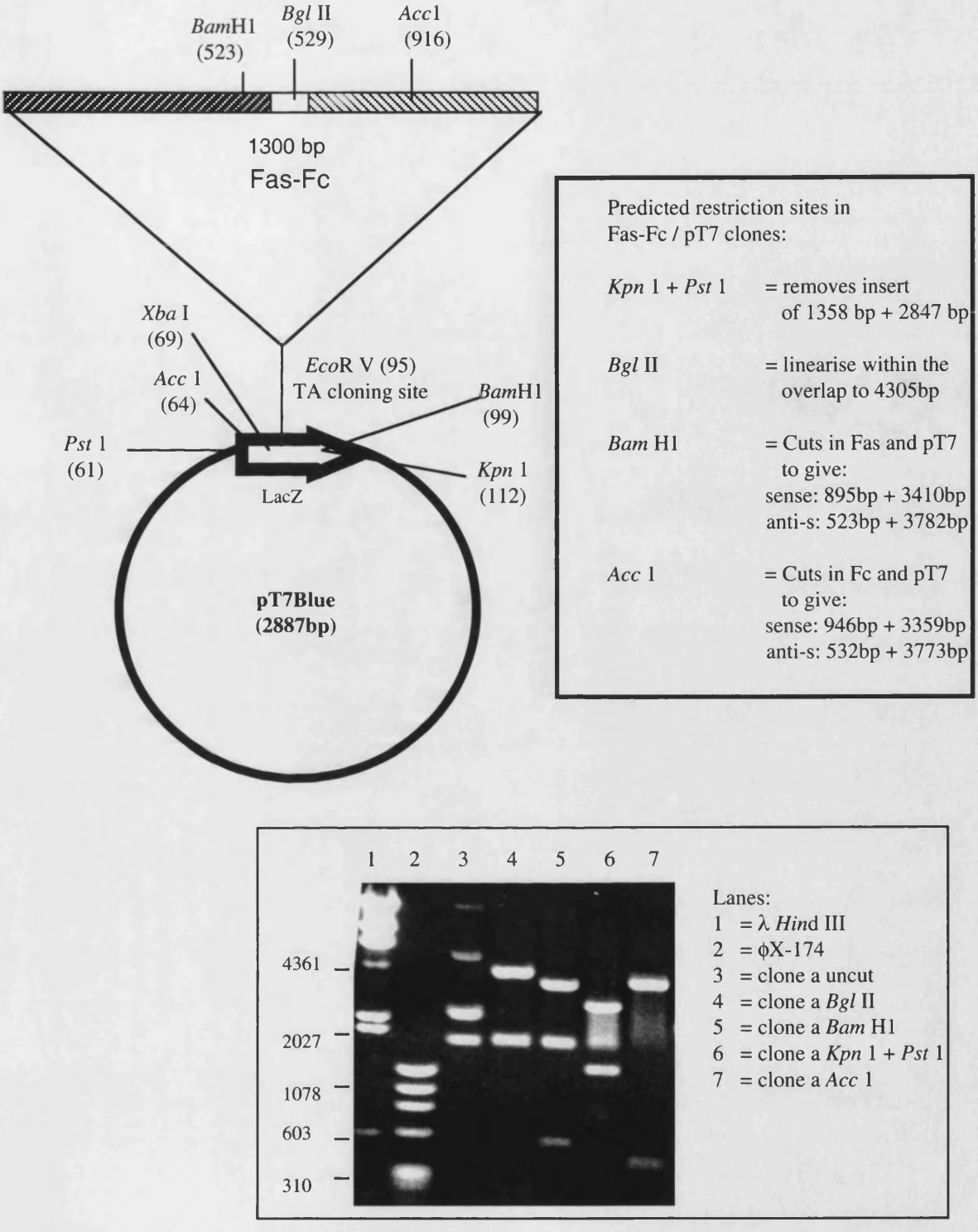


Figure 3.2.1.2 : Cloning Fas-Fc into pT7.

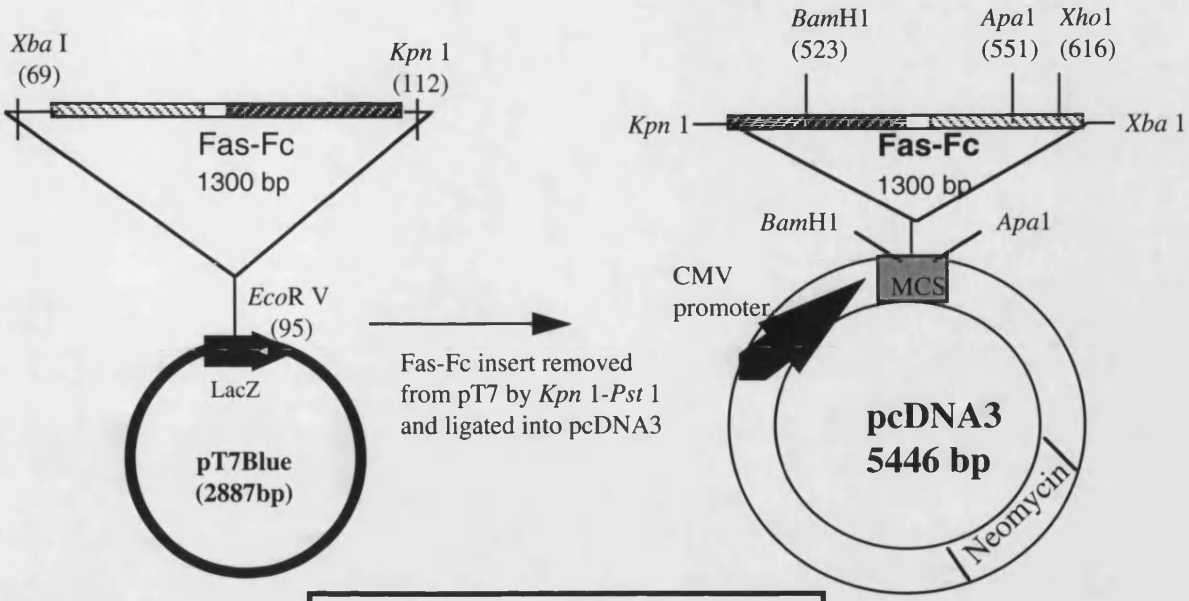
A cloning diagram of Fas-Fc in pT7 is shown with selected restriction sites and the predicted fragment sizes. The agarose gel shows a restriction map of a Fas-Fc / pT7 clone where the predicted ±4300 bp fragment of DNA can be seen for *Bgl* II (lane 4). A *Kpn* 1+ *Pst* 1 digest removes the ± 1300 bp insert (lane 6) and *Bam* H1 (lane 5) and *Acc* 1 (lane 7) remove ± 500bp fragments. Molecular weight markers are shown (lanes 1 and 2).

3.2.1.3) Cloning Fas-Fc into a mammalian cell expression vector (pcDNA3)

In order to express the recombinant protein in eukaryotic cell culture Fas-Fc was subcloned into an appropriate mammalian expression vector. The eukaryotic vector chosen for Fas-Fc cloning was pcDNA3 which allowed an advantage over pEe6 in that it had within its sequence a gene encoding resistance for neomycin. This allows selection pressure to grow transfected cells in culture medium containing neomycin hence enriching for cells expressing the gene of interest.

In order to subclone Fas-Fc in the correct orientation into pcDNA3 a *Kpn* 1-*Xba* 1 fragment was excised from Fas-Fc/pT7 and ligated into the complementary sites of the pcDNA3 as shown in **figure 3.2.1.3** . Miniprep analysis and restricion mapping was used again to confirm that the correct DNA was inserted into the mammalian expression vector pcDNA3. A *Kpn* 1-*Xba* 1 digest shown in lane 7 shows the removal of the ±1300 bp Fas-Fc insert from the pcDNA3 clone. *Bam* H1 (cutting in the Fas sequence and pcDNA3) and Apal (cutting in the Fc sequence and pcDNA3) gave the predicted restriction fragments of ±523 bp (lane 8) and ± 850bp (lane 6), respectively. A *Xho* 1 digest was used to linearise the cloned DNA within the Fc region to produce a ± 6864bp fragment (shown in lane 9 as a larger band compared to the adjoining lanes 6, 7, and 8).

Thus this demonstrated that the Fas-Fc cDNA was cloned in a sense orientation within the pcDNA3 plasmid and was therefore suitable for expression.



Predicted restriction sites for FasFc/pcDNA3 :

Kpn 1 + *Xba* 1 = insert removed ±1300bp

*Apa*1 = 850bp + 6014 bp
(cuts in Fc region)

*Bam*H1 = 523bp + 6341bp
(cuts in Fas)

*Xho*1 = linear 6864bp fragment
(cuts in Fc region)

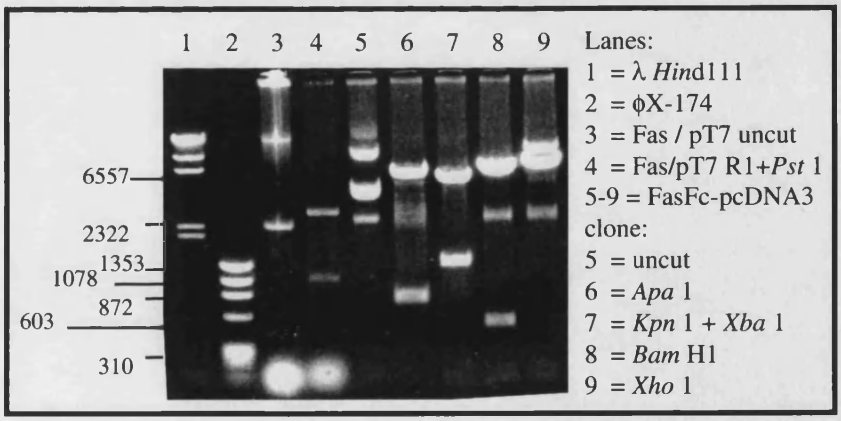


Figure 3.2.1.3: Subcloning Fas-Fc into pcDNA3.

The line drawings demonstrate the strategy of the sub-cloning of Fas-Fc. A *Kpn* 1- *Xba*1 insert was excised from pT7 and ligated to pcDNA3 prepared with a *Kpn* 1 - *Xba*1 digest. The agarose gel shows a restriction map of a clone of Fas-Fc / pcDNA3 (lanes 5-9). A *Kpn* 1-*Xba*1 digest removes the ± 1300 bp insert (lane 7). *Apa*1 cuts in the Fc region and pcDNA3 to yield a ± 850bp fragment (lane 6), whilst *Bam* H1 cuts in the Fas region and pcDNA3 to give a ± 530 bp fragment (lane 8). *Xho*1 linearises the clone within the Fc region to give a larger ± 6800 bp band (lane 9, additional band due to a partial digest).

3.2.2) Production of Fas-Fc fusion protein

3.2.2.1) Expression of Fas-Fc in COS-7 cell culture

In order to achieve in vitro expression of the Fas-Fc fusion protein the DNA clone above had to be introduced into a mammalian cell line. COS-7 cells were transfected with Fas-Fc / pCDNA3 by electroporation and cell culture supernatants were used for affinity purification of the secreted Fas-Fc protein.

To purify Fas-Fc protein from the pooled culture supernatants of the transfected cell lines, 500ml of the culture medium was concentrated using saturated ammonium sulphate and dialysed against PBS before affinity purification was carried out with protein G-sepharose columns. The eluted protein was electrophoresed on SDS-PAGE and a Coomassie Brilliant blue stain carried out. The gel revealed a 50 Kd protein which was purified by affinity chromatography as shown in **figure 3.2.2.1** lane 2. This was consistent with the predicted molecular weight and is shown in comparison to mouse IgG in lane 4. No protein could be purified from untransfected cell culture supernatants (lane 3).

Therefore this result reflected the successful expression of Fas-Fc cDNA in COS cells and purification of the protein from pooled culture supernatants by affinity chromatography. Further characterisation of the protein was now necessary to test for immune reactivity.

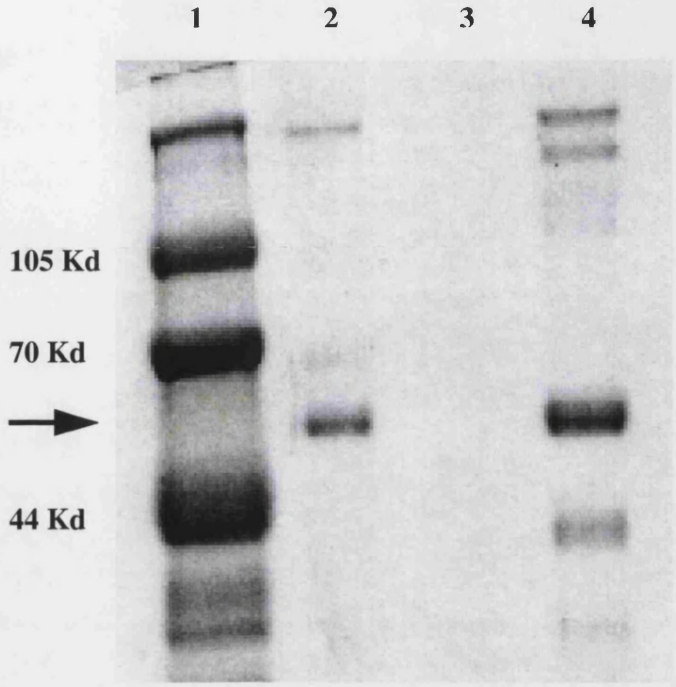


Figure 3.2.2.1: Coomassie stain of Fas-Fc protein.

A Coomassie stained SDS-PAGE gel demonstrates the affinity-purified Fas-Fc protein of 50 Kd (indicated by arrow) (lane 2). Mouse IgG derived from the original hybridoma used for the Fc component of Fas-Fc is shown in lane 4, and molecular weight markers are shown in lane 1. Controls are shown in lane 3 where no protein could be affinity purified from supernatants of untransfected cells. Affinity purification was carried out with Protein G-Sepharose using concentrated cell culture supernatants from Fas-Fc transfectants (lane2) and a UCHT4 hybridoma (lane 4).

3.2.2.2) Immunoreactivity of the purified Fas-Fc protein

To test the purified protein for immunoreactivity both dot blots and western blotting was carried out. The Fas-Fc protein was expected to have immunoreactivity to both anti-Fas and anti-mouse IgG antibodies because of the constituent parts of the fusion protein. This immunoreactivity was tested with Fas-Fc in native (dot blot) and denatured forms (western blot).

Figure 3.2.2.2 shows that both the individual Fas and immunoglobulin components of the fusion protein had specific antibody reactivity. In the native non-denatured form of the protein as tested by dot blotting both the Fas and mouse Fc components were immunoreactive (figure 3.2.2.2a). Control staining of IgM and mouse IgG, as well as concentrated supernatants from non-transfectants are shown.

The denatured form of Fas-Fc was tested by western blotting (figure 3.2.2.2b). Again both the Fas and Fc components demonstrated specific immunoreactivity with immunoblotting anti-Fas and anti-mouse IgG2a antibodies, respectively. These results therefore demonstrated that the Fas-Fc protein displayed immunoreactivity of its component parts in both the conformationally intact and denatured forms.

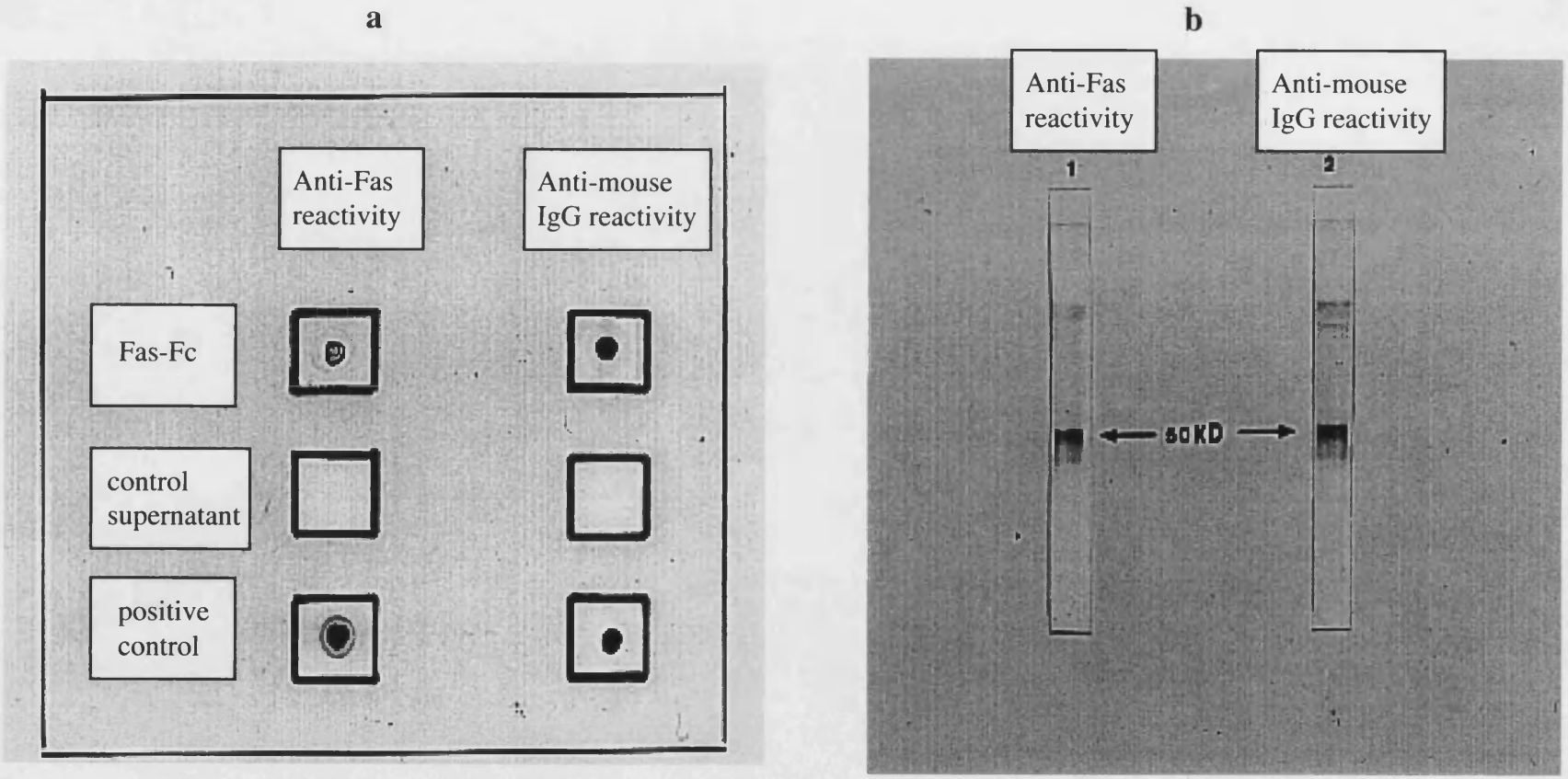


Figure 3.2.2.2: Immunoreactivity of Fas-Fc protein.

Immunoreactivity of the recombinant Fas-Fc protein is demonstrated on both dot blot (a) and Western blotting (b). The blots demonstrate immunoreactivity of both the Fas and the Immunoglobulin (Fc) components separately as detected by peroxidase conjugated anti-mouse IgG (Fc component) and peroxidase conjugated anti-mouse IgM (for Fas CH 11 antibody). Control positive antibodies are shown for each isotype and concentrated supernatants from untransfected cells was used as a negative control for the blots.

To further test for immunoreactivity of the Fas-Fc protein we devised a FACS-based assay. This was based on the use of the Fas-Fc to competetively bind anti-Fas antibody thereby preventing the binding of antibody to Fas positive cells. A shift on FACS staining indicated this competetive binding of anti-Fas antibody by Fas-Fc and **figure 3.2.2.3** demonstrates this result. H9 Fas-positive cells were used in the FACS immunoassay and stained with anti-Fas antibody which showed a log fluorescent shift to the right (shaded histogram in figure 3.2.2.3), but preincubation of the H9 cells with 50ug/ml Fas-Fc resulted in an inhibition of anti-Fas antibody binding as seen by a shift of the log fluorescence toward baseline control stained cells (dotted histogram in figure 3.2.2.3).

The above results thus provided further evidence that the 50 Kd protein purified from the transfected Cos cells was immunoreactive. That is, the human Fas component of Fas-Fc was binding anti-Fas antibody thereby preventing the antibody binding to Fas on the surface of H9 T cells. This FACS experiment also suggested that the Fas-Fc molecule was potentially capable of acting as a soluble receptor decoy to prevent Fas receptor engagement on cells expressing Fas. The functional use of Fas-Fc as a receptor decoy will be discussed later.

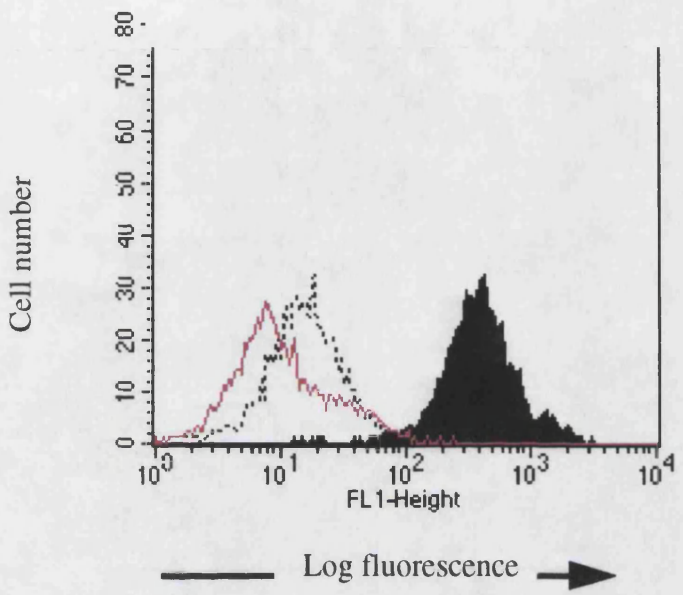


Figure 3.2.2.3 : Fas-Fc competetively inhibits binding of anti-Fas antibody.

A FACS analysis showing immunoreactivity of the Fas component of the Fas-Fc protein. Fas-expressing H9 cells bind anti-Fas antibody causing a log shift in fluorescence as shown by the shaded histogram. If the H9 cells are pre-incubated with 50 ug/ml Fas-Fc (dotted line) there is a marked reduction in the binding of anti-Fas antibody, indicating competition for binding of the anti-Fas antibody between the surface Fas on H9 cells and the Fas-Fc protein. Negative control staining of H9 cells with an IgM antibody is shown on the left (pink line).

3.2.3) Functional assays to test the Fas-Fc fusion protein

3.2.3.1) Detection of surface expression of human Fas-Ligand

3.2.3.1 a) Establishing FcR status and FACS staining with Fas-Fc

Clues from the published literature suggested that the natural ligand for murine Fas was a cell surface protein (Rouvier et al, 1993). However, the isolation and characterisation of Fas ligand was proving to be more difficult than anticipated. We postulated that if the Fas-Fc protein bound a "cell surface ligand" it could then be located on FACS analysis using a FITC-conjugated anti-mouse antibody. Using this strategy we tested various cell lines and peripheral blood mononuclear cells with the purified Fas-Fc protein. Cells were incubated with the Fas-Fc protein as a "primary antibody" for FACS analysis and secondary anti-mouse FITC conjugated antibody was used to localise the Fas-Fc binding. A dose dependant increase in Fas-Fc binding was seen on activated T cell blasts with the best staining on FACS analysis seen at 50ug/ml **(figure 3.2.3.1a).**

To ensure that the Fas-Fc staining was not via nonspecific Fc receptor binding a number of control measures were introduced. All samples for analysis included negative control samples stained with equivalent concentrations of isotype matched mouse IgG antibody. Fc receptor expression was tested on the various cell types and none was detectable on Jurkat T cell lines nor on activated T cell blasts **(figure 3.2.3.1a),** whilst large amounts of FcR staining was detectable on neutrophils and monocytes. In addition all FACS analysis was conducted after saturating amounts of human IgG were used to bind any Fc receptors that may have been present in low amount.

Therefore these results demonstrated that the Fas-Fc protein was binding activated T cells via its Fas component and not by Fc binding to Fc receptors.Thus the Fas-Fc protein was functioning in effect as a "Fas ligand probe" as predicted and could consequently be used for staining a variety of cell types.

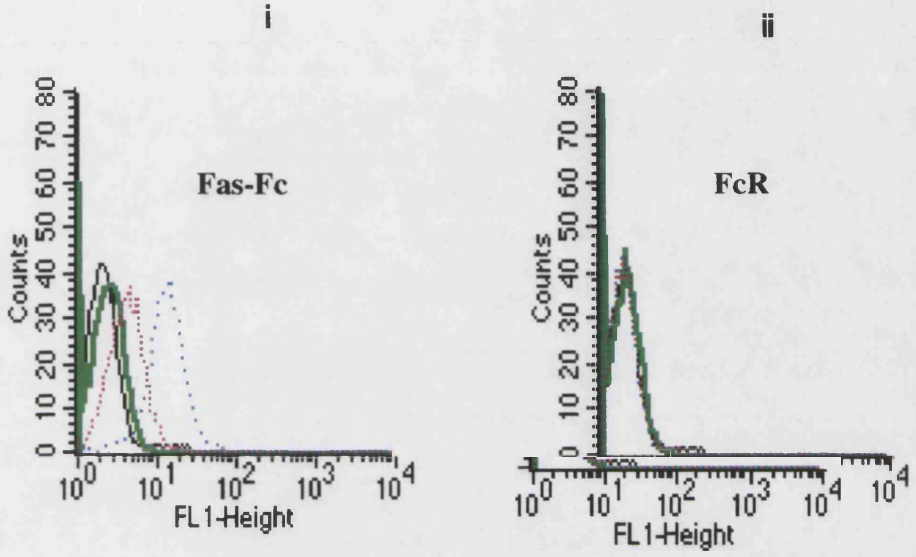


Figure 3.2.3.1a : T cell blasts - Fas-Fc binds specifically to activated T cells but not via Fc binding to the FcR.

The histograms show FACS analysis of T cell blasts four days after activation with PMA / ionomycin.

i) Fas-Fc binding to activated T cell blasts was seen in a dose-dependant manner (green histogram = 1ug/ml, pink dotted histogram = 10 ug/ml, blue dotted histogram = 50 ug/ml). Negative control staining of cells (black histograms) was carried out with 10ug/ml mouse IgG. FITC-labelled anti-mouse IgG was used for secondary detection.

ii) There was no evidence of FcR expression on T cell blasts (green histogram ii) using anti-human FcR antibodies, therefore also supporting the results that Fas-Fc binding was not via the FcR but rather by specific Fas binding to a putative ligand.

3.2.3.1 b) Fas-Fc staining of various cell types for Fas ligand (FasL)_

In order to search for expression of FasL the Fas-Fc protein was used as a primary antibody on a variety of cell types. Fas-Fc staining and FACS analysis showed the presence of a "putative Fas ligand" on the surface of Jurkat T cells and activated T cell blasts, but no detectable surface FasL was found on neutrophils, monocytes, B cells, transformed B cell lines, nor on the T cell line H9 (**figure 3.2.3.1b**). The absence of nonspecific cell binding and also the fact that no universal T cell staining was found (no FasL on H9 cells or resting T cells) gave us confidence that we were seeing a putative FasL identified by the Fas-Fc fusion protein.

These results therefore suggested specificity of staining with the Fas-Fc and a restricted expression of FasL on T cell blasts and Jurkat T cells.

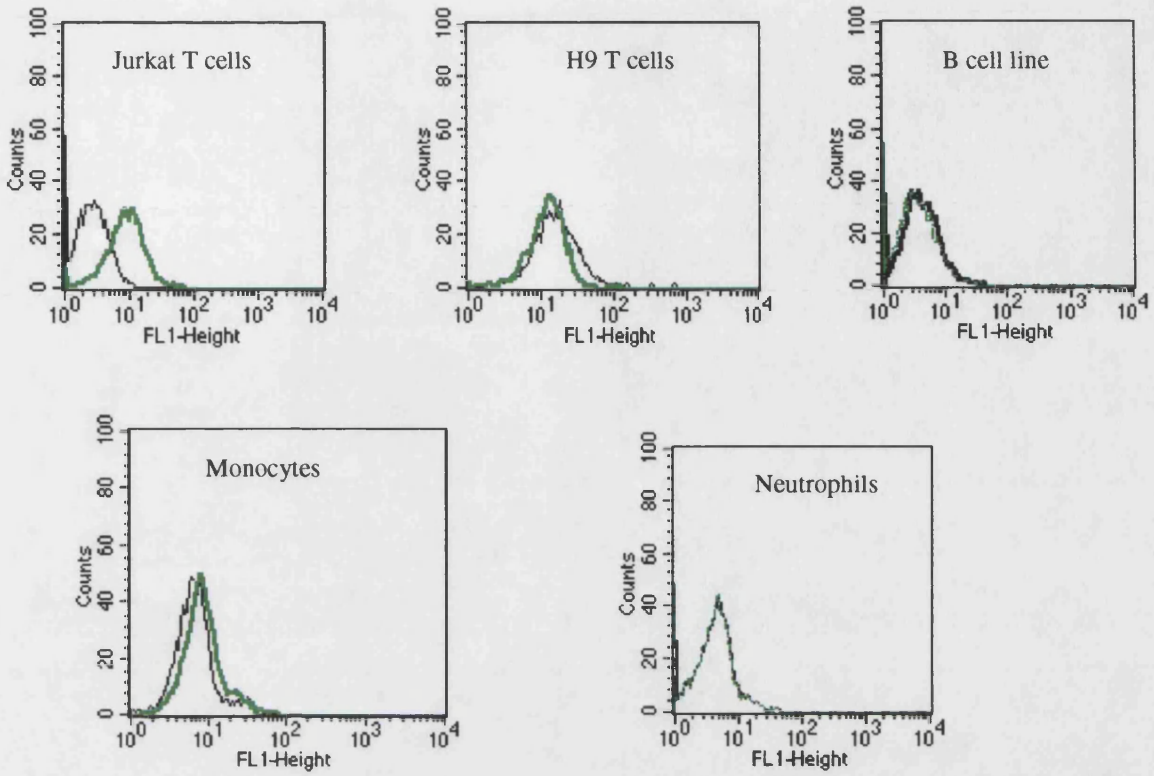


Figure 3.2.3.1 b: Fas-Fc binding on a variety of cell types.

FACS analysis was carried out following staining of cells with 10ug/ml Fas-Fc protein and a secondary FITC-conjugated anti-mouse IgG. All negative control cells were incubated with 10ug/ml mouse IgG (black histograms). Fas-Fc binding to a "putative" FasL was seen on Jurkat cells but not on the H9 T cells (green histograms). Also there was no evidence of FasL expression on an activated B cell line, monocytes, or neutrophils using the Fas-Fc protein. Results are representative of five independent experiments.

3.2.3.1 c) Fas Ligand expression on activated T cells

To investigate if the T cell staining with Fas-Fc was evident on resting T cells PBMCs were purified and T cells were isolated as described (methods chapter 2.1.3.2). Fas-Fc staining was carried out and FACS analysis showed no "FasL" expression on the surface of resting T cells but only on activated T cells from peripheral blood of normal volunteers (**figure 3.2.3.1c**). It was therefore evident that resting T cells did not express surface FasL as detected by the Fas-Fc protein.

Kinetics of expression of FasL

To assess the kinetics of expression of FasL following activation of T cells Fas-Fc staining was carried out at daily time points on purified T cells.T cells were activated by mitogenic stimulation with PMA and ionomycin and kept in culture with IL-2 for up to seven days. **Figure 3.2.3.1c** shows the FACS analysis where ligand expression was seen by day one after activation and continued to increase up to day three before returning to baseline by day five. Thus the Fas-Fc protein has demonstrated specificity in binding to a putative FasL only on T cells and has also revealed a pattern of expression of FasL following activation of T cells. The FasL expression on the T cell surface following mitogenic activation appeared to be transient, returning completely to baseline after five days. However, FasL expression was seen on resting Jurkat T cells and this may reflect other factors operative in these transformed cells. These findings have been subsequently confirmed by others using monoclonal antibodies developed after the cloning and identification of Fas ligand and will be discussed at the end of this chapter.

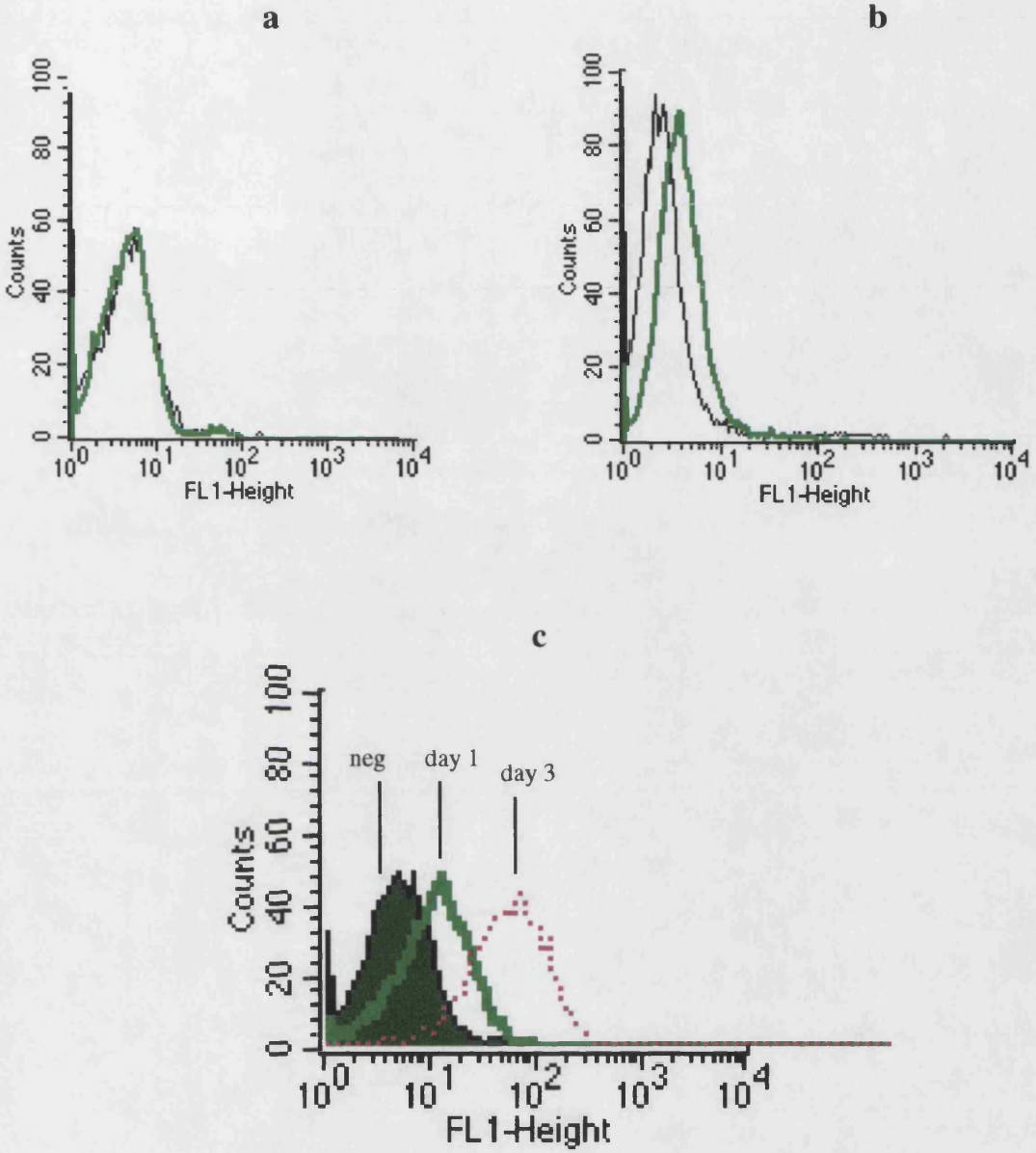


Figure 3.2.3.1 c : FasL expression on activated T cells as detected with Fas-Fc.

FACS analysis of T cells with Fas-Fc showed no resting ligand expression (a). FasL expression increased following activation of T cells with PMA / ionomycin (b). There was a time dependant increase in FasL expression upto day 3 following T cell activation (c) (negative = shaded histogram, green unshaded histogram = day 1, pink dotted histogram = day 3). Results reflect more than five independent experiments.

3.2.3.1 d) Variability of staining with Fas-Fc

The above experiments were reproducible on at least five occasions but showed a considerable decrease in sensitivity when using a subsequently-prepared batch of Fas-Fc (**figure 3.2.3.1d**). There was no evidence for any alteration in the immunogenicity of subsequent batches (compared on western blotting) nor any evidence of change in the mRNA expression of Fas-Fc in transfected cell lines. It has been suggested that expression in COS cells is associated with the development of mutations over time (Calos et al, 1983) but we did not pursue sequence analysis of the various transfectants from the batches shown in figure 3.2.3.1d.

The loss of sensitivity did limit subsequent use of the Fas-Fc protein for FACS analysis of Fas-L expression, however the protein continued to show functional activity in the inhibition of Fas-mediated apoptosis (see later). Other groups using a similar approach with Fas-Fc have experienced similar problems with low level binding to Fas-L. One possible explanation may be the nature of Fas-ligand and its variability in expression on the cell surface. It became evident following the cloning of the ligand and development of antibodies that other factors influence surface expression of FasL, amongst these are included the metalloproteinase inhibitors which were shown to influence TNF cleavage from the cell surface (Kayagaki et al, 1995). At the time we were not able to test for the possible influence of metalloproteinase inhibitors on the highly sensitive Fas-Fc batch.

To summarise, the results presented in this section have therefore shown the successful generation of a recombinant Fas-Fc cDNA and the validation of the construct in a number of experiments. The recombinant Fas-Fc protein was produced in cell culture supernatants and demonstrated immunoreactivity both on dot blots and western blotting. In addition, Fas-Fc displayed the ability to block anti-Fas antibody binding to Fas-expressing H9 cells. Specific binding by the Fas-Fc protein helped identify potential FasL expression on the surface of activated T cells and Jurkat T cells. It was therefore time to test if Fas-Fc could inhibit Fas-mediated apoptosis by acting as a receptor decoy.

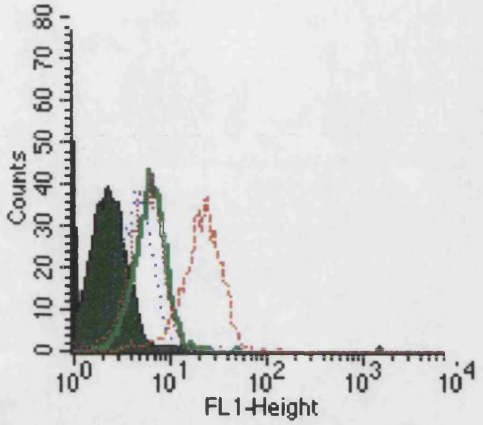


Figure 3.2.3.1 d: Variability of Fas-Fc binding.

FACS analysis of Jurkat cells is shown with various batches of Fas-Fc. The level of binding seen with the original batch (orange histogram) could not be reproduced with any subsequent batches (light green unshaded, blue dotted and pink dotted histograms). The latter batches showed consistent but reduced levels of binding to Jurkat cells when compared to the negative control cells (dark green shaded histogram).

3.2.3.2) Inhibition of Fas-mediated apoptosis by Fas-Fc in vitro

3.2.3.2 a) JAM assay of apoptosis in Jurkat cells expressing Fas

Given the immunoreactivity of the Fas-Fc protein we investigated whether the Fas-Fc could be used as a receptor decoy to block Fas-mediated apoptosis in vitro. In order to test this it was necessary to establish a number of apoptosis assays in the laboratory to measure Fas-mediated apoptosis. Jurkat cells normally express large amounts of Fas on their surface and die by apoptosis on engagement of Fas by its natural ligand or monoclonal anti-Fas antibodies. Jurkat cells were thus largely chosen for the functional apoptosis assays but we also tested similar effects in activated T cells blasts and also in the previously generated CHO-Fas transfectants. A JAM assay (chapter 2.5.1) is shown in which apoptosis of Jurkat cells was measured following Fas engagement. The results in **figure 3.2.3.2(a)** show how monoclonal CH11 antibody resulted in apoptosis (reflected as a decrease in cell survival). Control Jurkat cells without antibody stimulation had a 100% survival and Fas-Fc protected from CH11-mediated killing of the Jurkat cells to maintain cell survival up to 95% in the presence of CH11. In addition Fas-Fc was tested in a Jurkat apoptosis assay using soluble ligand instead of CH11. The soluble ligand was generated as part of this project and will be described later (chapter 4). Fas-Fc was seen to inhibit sFasL-induced apoptosis of Jurkat cells in a manner comparable to an inhibitory anti-Fas antibody (ZB4) which is also shown in **figure 3.2.3.2(b)**.

The results confirmed previous findings of Jurkat cell apoptosis following Fas engagement but in addition they demonstrate the ability of the Fas-Fc protein to inhibit Fas-induced killing with both monoclonal CH11 and sFasL. Therefore our hypothesised use of Fas-Fc as a receptor decoy in preventing Fas-mediated apoptosis was confirmed.

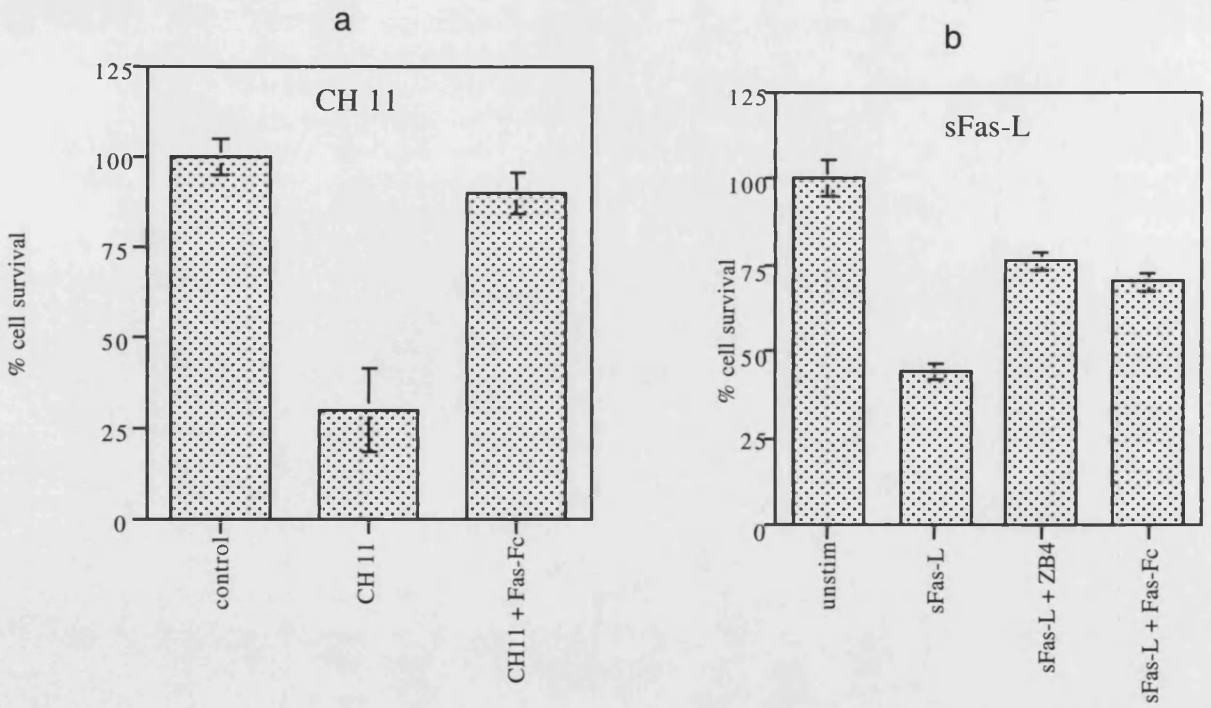


Figure 3.2.3.2 a: Fas-Fc inhibition of Fas mediated apoptosis.

This figure shows a JAM assay in Jurkat T cells with Fas-induced apoptosis by monoclonal anti-Fas antibody CH 11 in (a) and soluble Fas-Ligand (sFasL) in (b). In both cases Fas-Fc pre-incubation improved the % cell survival, and in the case of sFasL (b) the inhibition of apoptosis by Fas-Fc was seen to be comparable to a Fas-blocking antibody ZB4. In all cases the % cell survival was calculated from the change in ^{3}H-Thymidine incorporation compared to unstimulated Jurkat cells. The results represent four independent experiments.

3.2.3.2 b) Fas-Fc inhibition of apoptosis in bioassays

To test whether Fas-Fc was active in a more physiological system two bioassays were established. These were designed to test if Fas-Fc could inhibit cell-mediated killing via FasL.

Superantigen (Staphylococcal enterotoxin B, SEB) induced cell death is inhibited by Fas-Fc.

Fas-Fc inhibition was tested in an SEB blast bioassay. To achieve this superantigen (SEB) stimulation of T cells was carried out (section 2.1.3) to produce activated T cell blasts. Prior to re-exposure of the eight day old blasts to SEB for 6 hours, ^{3}H-thymidine labelling was conducted on the blasts as for a standard JAM assay (section 2.5.1). The percentage death by apoptosis measured on re-exposure to SEB was then calculated as in a JAM assay. This process of cell death following restimulation by superantigen has been described as "activation-induced cell death" (Ettinger et al, 1995) and was thought to be related to upregulated expression of Fas and FasL with consequent ligation of Fas and death by apoptosis.

Figure 3.2.3.2b (i) shows in the histogram (a) the percent cell survival in the control ^{3}H-thymidine-labelled day 8 SEB blasts left overnight in culture medium alone without SEB. With reexposure to SEB (10ug/ml) however, the blasts underwent ±50 - 60% apoptosis (histogram b) which was prevented by preincubation of the T cell blasts with Fas-Fc prior to rechallenge with SEB (histogram c).

This bioassay thus demonstrated that Fas-Fc inhibited "activation-induced cell death" induced by the superantigen SEB.

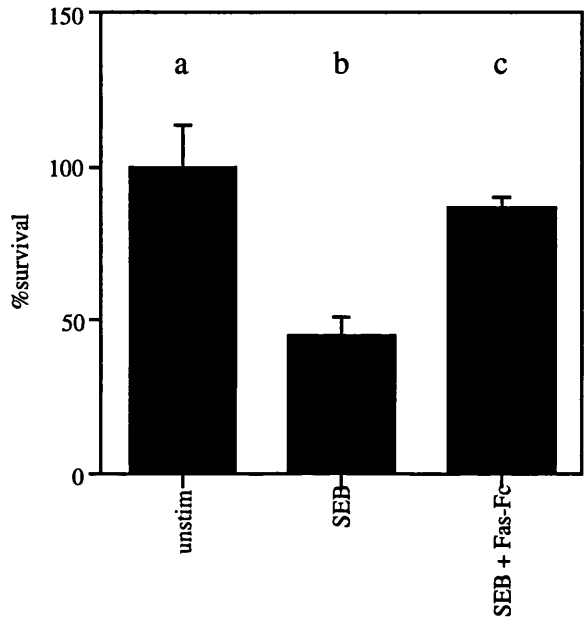


Figure 3.2.3.2 b (i): Fas-Fc inhibiting Fas-mediated "activation-induced cell death".

One in vitro bioassay in which Fas-Fc inhibition was seen is represented here. In histogram **b**, SEB rechallenge of T cell blasts previously stimulated with SEB resulted in apoptosis (activation induced cell death). Histogram **a** shows the T cell blasts which did not receive restimulation with SEB. Fas-Fc pre-incubation before SEB rechallenge protected from apoptosis (**c**) by blocking the FasL-Fas interaction. All histograms represent the mean and standard error of the mean from triplicate samples. The result above is representative of three independent experiments.

Jurkat cell bioassay to assess Fas-Fc inhibition of Fas-FasL interactions.

Fas-Fc inhibition was tested in another bioassay using Jurkat cells as Fas-positive targets which could be killed by activated T cells expressing an upregulated Fas ligand. Apoptotic death of Fas-positive Jurkat cells was measured by the JAM assay (section 2.5.1) and FasL upregulation on the effector cells was achieved by SEB restimulation of T cell blasts (Boshell et al, 1996). In such a bioassay the FasL expressed on the activated T cell blasts engages Fas receptors on the Jurkat T cells in order to induce apoptosis via a cell-cell interaction of the ligand-receptor pair.

To test the activity of Fas-Fc, it was used in this bioassay to block Fas-mediated apoptosis of the Jurkat cells. **Figure 3.2.3.2b(ii)** shows the Jurkat bioassay. Histograms b and a show the baseline survival of Fas-positive Jurkat cells with and without Fas-Fc, respectively. In the presence of unstimulated (day 8 after initial SEB stimulation) T cell blasts there was some altered survival of Fas-positive Jurkat cells as seen by a reduction in cpm in histograms c and d (compared to a and b) but no significant protection was offered by Fas-Fc preincubation (compare d and c). However, when the T cell blasts were restimulated with SEB for six hours prior to incubation with Jurkat cells there was significant apoptotic death of the Fas-positive Jurkat cells (compare histogram e to a and c), indicating an upregulation of FasL on the T cell blasts induced by SEB. In the latter situation Fas-Fc protected from SEB blast-induced Jurkat cell apoptosis (compare histogram f to e).

The results of this second bioassay thus also demonstrated the ability of Fas-Fc to act as a receptor decoy to prevent Fas-mediated apoptosis. In addition this assay also confirmed by an independant method our previous findings that FasL was upregulated on activation of T cells.

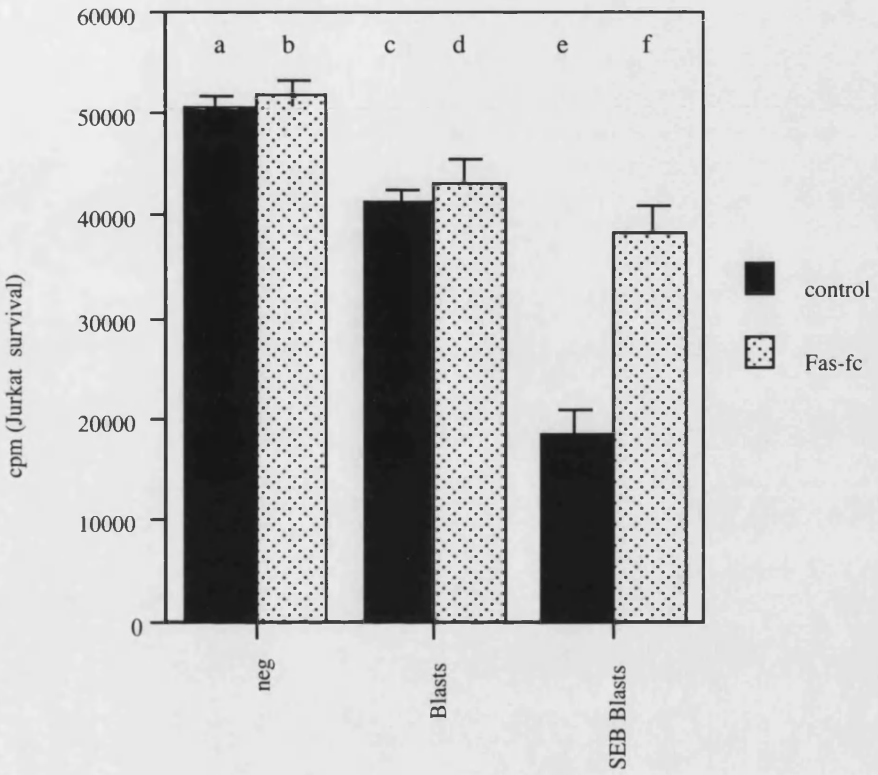


Figure 3.2.3.2 b (ii): Fas-Fc inhibiting Fas mediated cell-cell killing.

A second in vitro bioassay in which Fas-Fc inhibition was seen is represented here. Jurkat T cells were used as Fas-positive target cells to measure cell-cell mediated death induced by FasL on activated T cell blasts. Unstimulated day-8 T cell blasts only had a minor death inducing effect on Jurkat cells (c compared to a). Fas-Fc in the latter situation did not have a noticeable effect (d compared to c). However, SEB stimulated day-8 T cell blasts did induce significant apoptotic death of Jurkat cells (e compared to a) which was inhibited by pre-incubation of Jurkat cells with Fas-Fc 10ug/ml (f compared to e).
The results represent the mean / standard error of triplicate samples from one of three independent experiments.

The ability of Fas-Fc to block Fas-related apoptosis thus gave us a reagent that was useful in defining the role of Fas in functional assays in future experiments. It was also intended to use the purified Fas-Fc protein as an immunogen in a rabbit to attempt to produce a polyclonal anti-Fas antibody for use in the laboratory, but limitations on time and resources precluded this from being achieved. Other groups have since used a similar approach to produce various monoclonal and polyclonal anti-Fas antibodies (Alderson et al, 1994). With the availability of a variety of anti-Fas antibodies as a result it seemed unnecessary to spend any more time pursuing this.

3.2.3.3) Immunoprecipitation of a putative Fas ligand from Jurkat cells

Since the Fas-Fc protein appeared to identify a putative FasL on the surface of activated T cells and Jurkat cells it was decided to test if it could be used to immunoprecipitate the "putative ligand" from Jurkat cell lysates. **Figure 3.2.3.3** shows a Coomassie stained gel from such immunoprecipitates which demonstrates a specific 37-40 Kd immunoprecipitated protein.The immunoprecipitated protein was only evident in Jurkat lysates (lane 2) and not on Cos cell lysates nor on H9 T cell lysates (lanes 4 and 3 respectively). The latter would be consistent with our previous FACS analysis which failed to reveal FasL expression on H9 T cells. Isotype matched mouse IgG coupled to sepharose beads also failed to immunoprecipitate any protein (lane 1).

This experiment therefore clearly demonstrated a specific band of protein (a putative FasL) immunoprecipitated by Fas-Fc coupled to sepharose beads.The precipitated protein was remarkably similar in size to the mouse and rat Fas-L described by Suda and Nagata (1994) a few months later. Subsequent descriptions in the literature following the cloning of human Fas-L have confirmed that Jurkat cells express Fas-ligand (Martinez-Lorenzo et al, 1996).With the subsequent development of anti-FasL antibodies we tested if FasL

could be detected on Jurkat cells. As seen in **figure 3.2.3.3** FasL was found on the surface

of Jurkat cells with the Nok1 antibody but much more ligand was seen with intracellular staining. Therefore the results showing FasL expression with Fas-Fc have been confirmed with the antibody staining. In addition the finding of increased levels of intracellular FasL despite low levels on the surface may explain why the Fas-Fc was able to immunoprecipitate the FasL in Coomassie-stainable quantities.

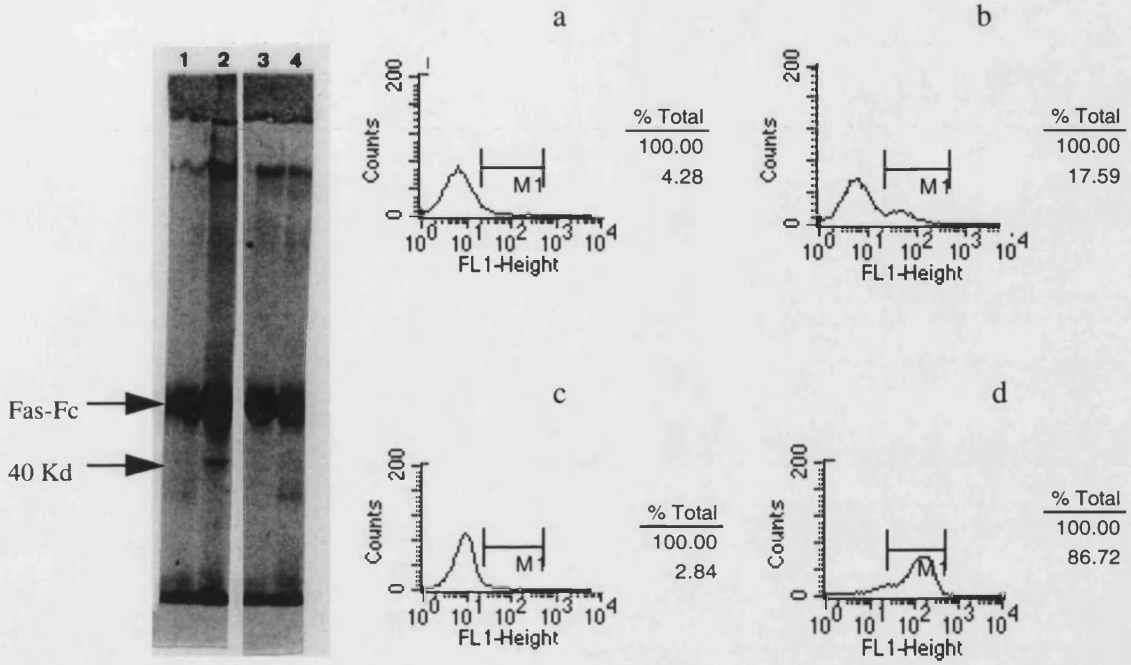


Figure 3.2.3.3 : Immunoprecipitation of "Fas ligand".

The Fas-Fc protein was used to immunoprecipitate "FasL" from Jurkat cells. Immunoprecipitates were electrophoresed on SDS-PAGE gels and stained with Coomassie Blue. Lane 2 shows the presence of a ±40 Kd band which co-immunoprecipitated with Fas-Fc but not with control mouse IgG (lane 1). H9 T cells (lane 3) and COS cells (lane 4) did not reveal any co-precipitate with Fas-Fc. All lanes show the 50 Kd Fas-Fc protein which was used as the immunoprecipitating antibody at 10ug/ml (coupled to proteinG-sepharose).
FACS analysis was conducted on Jurkat T cells when anti-FasL antibodies became available. The FACS histograms show surface staining (a and b = negative and positive staining respectively) and intracellular staining (c and d = control negative and positive respectively) with Nok1 antibody.

3.2.3.4) Jurkat cells kill each other in culture

The finding that Jurkat T cells expressed both Fas and FasL led us to speculate at the time that activated T cells may have the ability to kill each other in order to regulate cell number and maintain lymphocyte homeostasis. It was noted that when Jurkat cells were grown to high density in culture there was a high rate of cell death. We needed to determine if this was due to Fas-mediated apoptosis.

To test this we established an assay based on a ^{51}Chromium cytotoxicity method (section 2.1.6). Jurkat cells expressed FasL as shown from the evidence above, and PMA/ ionomycin was seen to induce FasL expression on activated T cell blasts, so we postulated that if we stimulated Jurkat cells with PMA / ionomycin they may upregulate FasL and thereby kill Fas-positive Jurkat cells within the same culture. In order to obtain cell - cell contact the assay was established in U-bottomed 96 well culture plates where stimulated ^{51}Cr-labelled Jurkat cells were left overnight under various conditions (**figure 3.2.3.4**).

The results of this experiment showed that a Fas-mediated mechanism was operative in the Jurkat cell death that was seen under these culture conditions. Jurkat cells stimulated with PMA / ionomycin had a high level of spontaneous death as measured by chromium release (histogram a), which was very nearly as high as the total induced chromium release with detergent (histogram b). Inhibition of cell death was evident when the Jurkat cells were incubated with both blocking Fas antibodies (M3 and M33) and our Fas-Fc protein (histograms c, d and e, respectively). Control mouse antibody did not inhibit Jurkat cell death when compared to Fas-Fc (histogram f). These results therefore support the concept of expression of FasL on Jurkat cells and may explain the phenomenon of cell death in culture especially when Jurkat or T cell blasts are grown to very high density. In the latter situation close cell contact may allow "fratricide" through FasL-Fas interactions.

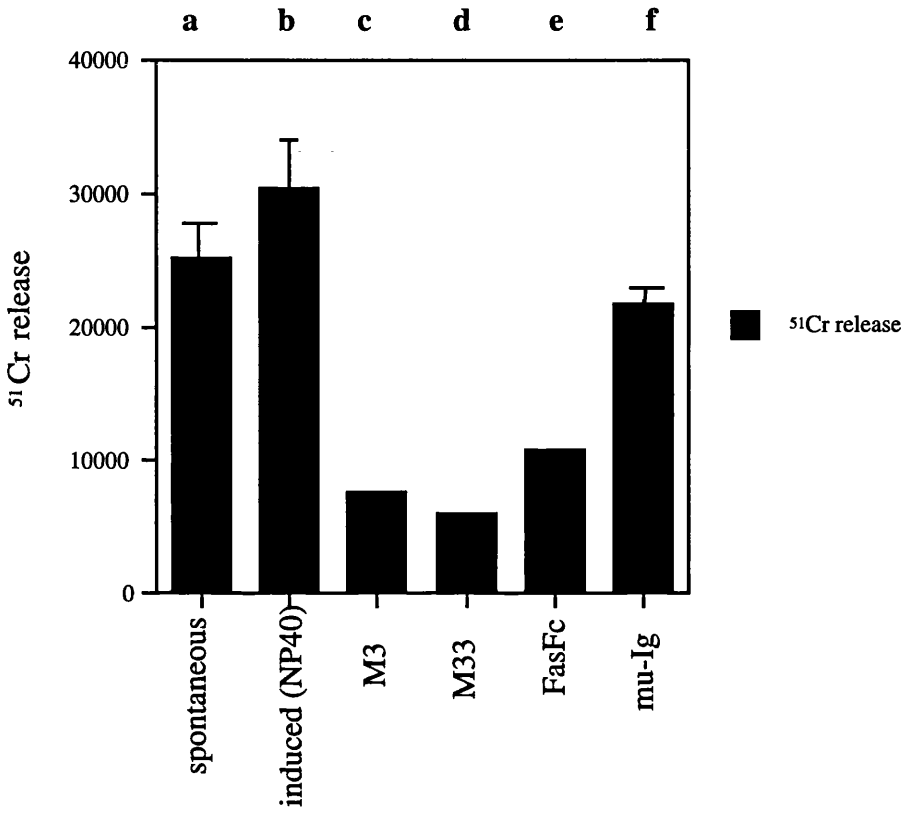


Figure 3.2.3.4: Jurkat cells kill each other via a Fas-mediated mechanism.

Jurkat cells were labelled with ^{51} Cr and stimulated with PMA / ionomycin before incubation in 96 well plates overnight. Cell death was measured in a standard chromium release assay. Cell death was seen in Jurkat cells left in close proximity in a well overnight (a), which was inhibitable by pre-incubation with blocking Fas antibodies (c and d) or Fas-Fc (e), but not with isotyp-matched mouse IgG (f).
The results represent means and SEM of triplicates from one of three independent experiments.

DISCUSSION.

A major aim of the initial work was the generation of a number of reagents for the study of Fas biology in humans. Accordingly the human Fas gene was successfully cloned. The cloning of human Fas cDNA from a cell line found to express large amounts of surface Fas was a good starting point in a project that was to involve a substantial amount of molecular biology. It provided experience with many of the molecular techniques required later for the cloning, characterisation and expression of the recombinant products generated both for Fas and also FasL (chapter 4). In addition it provided a useful handle for PCR conditions and oligonucleotide primer design for the SSCP analysis of the Fas gene (chapter 5).

The cloned Fas gene was functionally expressed under the control of a heterologous promoter in CHO cells. This development of a transfectant model of Fas in a CHO system was achieved through a number of selection processes. We speculated at first that it would be difficult to achieve stable transfectants of Fas given the nature of the molecule as a death protein. Hence it was expected that transfectants would die during the selection process as a result of exposure to anti-Fas antibodies. An explanation as to why this did not occur may be that the antibody incubations were taking place at 4^{o}C rather than the requisite 37^{o}C for Fas-mediated apoptosis. Why the cells did not die on returning to 37^{o}C after the selection process is still unexplained. It may be possible that the dynamic cellular changes on re-warming allowed cleavage or disposal of antibody complexes thus preventing the required four to six hour exposure for apoptosis to occur. It may be argued that the selection processes favoured the propagation of cells resistant to Fas-mediated apoptosis. However, the CHO-Fas transfectants maintained sensitivity to Fas-mediated apoptosis when challenged with anti-Fas antibody under appropriate conditions (37^{o}C for 4 - 6 hours), with a death rate of 30 - 50 %. It has been reassuring to note that other

groups working with murine Fas have also been able to establish stable Fas expressing cell lines without the problem of excessive cell death during the selection process (Itoh et al 1991). These also appear to have maintained sensitivity to Fas-mediated apoptosis in the range of 30 - 50%. A retrospective analysis of these early experiments shows that the cell death was still relatively heterogeneous, which may be explained in the light of current knowledge about the need for recruitment of other death domain proteins like FADD and FLICE (discussed below). Essentially this early finding of cellular survival under conditions where we expected almost certain and total apoptosis should have alerted us that not all Fas exposure leads to cell death. This theme will be explored further in the next chapter.

In any event the stable expression of human Fas in the CHO cell line has provided a useful transfectant model for in vitro analysis of Fas-mediated apoptosis. One reason for this is that negative control cells were easy to come by in the form of either untransfected cells or mock transfected CHO cells, unlike the situation with lymphocyte cell lines where all cells express Fas. Therefore the CHO-Fas transfectants allowed identification of Fas-specific mechanisms of apoptosis and this became particularly useful with the validation of the soluble FasL reagents generated later in this project. Also, the successful expression of Fas on the CHO cells confirmed that the original cDNA for Fas which was cloned from the H9 T cells was the correct fragment of DNA.

The CHO-Fas transfectant model could also serve a useful purpose in dissecting the individual protein interactions identified more recently. For example, it is now known that in order for an apoptotic signal to be transduced following surface Fas engagement, a number of other death domain associating interactions must occur (Cascino et al, 1996). Death domain proteins like FADD and FLICE have been shown to be essential for Fas-mediated apoptosis (Chinnaiyan et al, 1995 and Muzio et al, 1996) but other proteins also interact with these and the exact role of each needs to be defined. What is the influence of FLIP or FAP (Irmler et al, 1997 and Su et al, 1995) on the interactions of the death

domain proteins and can these be influenced in any way by dominant negative mutants? These interacting proteins first need to be identified within the CHO-Fas system which we have established before we can proceed to appropriate step-by-step introduction of the relevant genes to study their influence on the outcome of Fas engagment on the surface. It may then be useful to study the death-domain-associating proteins by targeted mutagenesis and introduction into the Fas transfectant system. One shortcoming of the CHO-Fas transfectant system may be that the CHO cells may not necessarily reflect the exact physiological and protein components involved in lymphocyte apoptosis.

Initial steps were taken towards defining Fas-mediated signalling in the CHO-Fas system. We investigated the role of the MAPK/SAPK pathways and found that the MAPK and JNK pathways of signalling were intact in the CHO system. JNK was stimulated via Fas in the CHO-Fas transfectants, but not in negative CHO controls. This provided an example where negative CHO controls were useful in identifying a Fas-specific effect on JNK signalling in the transfectant system. Similar effects were observed in the CHO-Fas transfectants with regards to sphingomyelinase signalling. It is known that ceramide (which can be generated by sphingomyelinase activity) can also stimulate JNK activity (Verheij et al, 1996).However it remains unclear if this is a critical step in a cascade of enzyme activation leading to apoptosis or if it is another independant pathway for JNK activation. In addition, the actual role of JNK activation in Fas-mediated apoptosis remains in question (Nishina et al, 1997). Whilst JNK activation is seen with Fas surface engagement it appears that apoptosis may occur independant of JNK activity, and that apoptosis is more critically dependant on Caspase activation (Chen et al, 1996). There is also evidence of JNK activation following T cell activation with CD3 and CD28 (Su et al, 1994), which is clearly a proliferative signal rather than a death trigger. Thus it appears that JNK activation does not necessarily equal death, and further research is required to clearly define the role of JNK signalling following Fas engagement. The balance of Fas effects on multiple signalling pathways may determine the ability of a cell to undergo apoptosis under given conditions and the CHO-Fas system should prove to be a useful

model for dissecting out the components of these pathways if we can introduce the various genes and / or inhibitors under controlled transfectant conditions as we have done with the Fas gene.

Another major objective was achieved through the generation of the Fas-Fc protein. An approach with recombinant PCR technology was used to generate a hybrid cDNA comprising human Fas and mouse IgG. The successfully engineered DNA was cloned and validated by restriction mapping and also limited sequence analysis. Subcloning was carried out in order to express the Fas-Fc in a mammalian cell culture system using COS-7 cells. The transfected COS cells were found to express a soluble protein which was characterised for immunoreactivity in a variety of experiments. The soluble Fas-Fc protein which was thus successfully produced in the culture system was put to use in predominantly two types of experiments. Firstly it was used as a primary "antibody" for binding and identifying a putative FasL on the cell surface by FACS analysis, and secondly it was used as a receptor decoy to identify Fas specific mechanisms of apoptosis.

The Fas-Fc protein was used successfully to identify a putative FasL which was found to have restricted surface expression on activated T cells and Jurkat T cell lines. We could not detect any expression of FasL on H9 T cells suggesting that the Fas-Fc was not binding non-specifically to a T cell marker. In addition the absence of binding to other cell types including B cell lines and PBMC's confirmed that the Fas-Fc identified a specific T cell ligand for Fas. Rigorous efforts were made to ensure that we were not seeing non-specific binding of Fas-Fc via its Fc component to Fc-receptors (FcR) on the cells. In any case all FACS staining was carried out in the presence of saturating amounts of human IgG even though we did not detect any human FcR expression on the Jurkat and T cell blasts which expressed the FasL. We were also able to explore the expression kinetics of FasL using the Fas-Fc and showed that resting T cells did not express FasL but upregulation of FasL expression occurred by day one after stimulation with PMA /

ionomycin. The upregulation continued up to day three and returned to baseline by day five after the initial stimulation. This finding of a T cell restricted expression of FasL and its regulation by mitogenic stimulation was very interesting at the time especially because there were no data on FasL except for speculation from the gld mouse model (which is discussed later in chapter 4).

Our findings also turned out to be consistent with data on murine FasL expression described by Suda and Nagata (1994). The latter group first cloned the FasL gene which was the rat ligand for Fas using a very similar Fas-Fc construct. However the fundamental difference was that our construct comprised human Fas and therefore identified a human ligand on Jurkat cells and activated T cell blasts. Suda et al also found a T cell restricted expression of the rat FasL (Suda et al, 1993). Another important difference in the approach of Suda et al was in the use of a cytotoxic cell line that was heavily sorted (sixteen times) before they could see good murine Fas-Fc binding. The sorted cytotoxic cell line was used to clone the Fas ligand which turned out to be the rat ligand derived from the rat hybridoma originally used to create the cell line.

FasL expression was thought to be restricted to activated T cells and cytotoxic T cells for some time after the publications of Suda and Nagata (1994), thus supporting our findings with the Fas-Fc protein. However, more recently with the development of anti-FasL antibodies FasL expression has been found on a variety of cell types (to be discussed further in the next chapter). Amongst the more intriguing findings have been the demonstration of FasL expression on non-lymphoid immune privileged sites (Griffith et al, 1995 and Bellgrau et al, 1995, and discussed in chapter 4) such as the eye and testis. Therefore our identification of a human ligand for Fas on activated T cells and Jurkat cells using the Fas-Fc construct proved to be correct at the outset, and only more refined detection systems have been able to identify other cell types with FasL expression. These latter methods of detection of FasL expression still depend very heavily on mRNA expression and intracellular expression of ligand. Unfortunately, our efforts to progress

rapidly in this field were somewhat hampered by less potent batches of Fas-Fc as discussed.

As a result of the identification of a FasL on Jurkat and T cell blasts we proceeded to attempt to immunoprecipitate the ligand using Fas-Fc as an immunoprecipitating antibody. Consequently a protein was successfully immunoprecipitated specifically from Jurkat cell lysates with a molecular weight of approximately 40 Kd. Before we could proceed further with protein sequencing and an attempt at cloning the ligand, Suda et al (1993) published results on the rat and mouse ligand for Fas. It was interesting that the 40Kd protein immunoprecipitated by Fas-Fc from Jurkat cells had a striking resemblance to the mouse and rat ligand described by Suda et al (1993). We thus were able to use the Fas-Fc protein not only to identify a putative surface ligand for human Fas on Jurkat and activated T cells but also to immunoprecipitate the ligand specifically from Jurkat T cells.

The problem that prevented rapid progress was the difficulty in consistent identification of surface FasL with new batches of Fas-Fc and this proved to be an obstacle at the time. Also, considering that our best staining with Fas-Fc was seen at concentrations of 50ug/ml this suggested that the affinity of Fas-Fc and FasL was not very high. However we needed to pursue rigorously the possibility of non-specific binding as described above, and at the end of that analysis it appeared that the Fas-Fc was specifically detecting a putative FasL on activated T cells and Jurkat cells. One explanation for some of our difficulties was provided by recent evidence that the FasL protein is unstable by nature and requires metalloproteinase inhibitors to keep it on the cell surface. Most researchers working with FasL have found difficulty in identification of FasL on the surface of cells because of the cleavage by metalloproteinases (Kayagaki et al, 1995). At the time of preparation of the first "super-batch" of Fas-Fc we had no idea nor any means of testing for contamination of the Fas-Fc sample with metalloproteinase inhibitors. Our attempts to define the batch problem was limited to an analysis of transfectant mRNA expression and protein immunoreactivity, both of which proved to be normal. There is also the

possibility that gene mutations within the cloned expression plasmid may have accounted for an altered level of binding without affecting the immunoreactivity of the Fas-Fc protein. Other authors have previously described a phenomenon of gene mutations in long term COS cell expression systems (Calos et al, 1983). Despite the reduced intensity of surface staining of FasL we were able to use the protein to immunoprecipitate a putative FasL from lysates of Jurkat cells. This may be explicable by our later results of FasL staining of Jurkat using monoclonal antibodies to FasL which became available after the cloning of FasL. Using the monoclonal antibodies we could confirm that abundant FasL expression was seen on intracellular staining (figure 3.2.3.3) as compared to the cell surface of Jurkat cells probably because FasL requires metalloproteinase inhibition for FasL to remain on the cell surface. In addition, another explanation may be provided by recent evidence suggesting that transfer of intracellular FasL to the cell surface varies according to the nature and intensity of the stimulus and occurs in a calcium dependent manner (Anel et al, 1994; Vignaux et al, 1995). It was reassuring to see that we were not alone in having problems with consistent identification of surface FasL, but in any case the T cell expression data and immunoprecipitation of FasL by Fas-Fc all proved to be confirmed by later work (Suda et al, 1994 and Lynch et al, 1994).

The second major use of the Fas-Fc protein was as a receptor decoy to inhibit Fas-mediated apoptosis. Our hypothesis that the soluble Fas-Fc protein would act as an inhibitor by binding anti-Fas antibodies thus preventing Fas receptor engagement proved to be correct in a number of assays. After testing the ability to block anti-Fas staining of cells in the FACS immunoreactivity assay the Fas-Fc was tested in apoptosis assays and found to block Fas-mediated apoptosis. We were able to exploit the latter use to show the role of Fas-mediated apoptosis in the SEB system and also in other bioassays presented in the results above. Subsequently, we have shown that apoptosis of SEB-activated T cells occurred on rechallenge with SEB in a Fas-specific manner (Boshell et al, 1996). This "activation-induced cell death" which occurs following various stimuli including CD3, superantigen, and phorbol esters has been ascribed largely to Fas-mediated mechanisms

with some minor role for TNF-mediated pathways in some cases (Tucek-Szabo et al 1996, Sytwu et al 1996). Of particular note is our findings of a Fas-based "fratricide" system in Jurkat cell culture. This idea was supported by later research showing FasL expression on Jurkat and the release of soluble FasL from Jurkat cells under stimulated conditions (Dhein et al 1995). The Fas-mediated death of Jurkat cells and T cells in culture may have some relevance in vivo particularly in lymphoid tissue such as the spleen and lymph nodes where lymphocytes are in close proximity to each other. Here activated cells may undergo death under appropriate conditions via a Fas-mediated fratricide or suicide pathway (Brunner et al 1995 and Ju et al 1995). Thus the Fas-Fc protein has fulfilled its potential in defining biological systems in which Fas-mediated apoptosis is operative.

The potential use of Fas-Fc as an immunogen for generation of our own supply of anti-Fas antibodies was also considered. However with the rapid rise in interest in Fas related research a number of commercial biomedical companies have used a similar approach to prepare a large variety of anti-Fas antibodies (Alderson et al 1994). It therefore seemed unnecessary to pursue antibody production given our limited time and resources. It was interesting to see a similar Fas-Fc protein used in very recent research relating to directed mutagenesis based on the crystal structure of Fas to show two critical ligand binding sites (Starling et al 1997), suggesting that the Fas-Fc protein could be used for epitope binding analysis of newly developed anti-Fas antibodies.

In summary this chapter has demonstrated the generation of a number of reagents which have contributed to our understanding of human Fas biology and the identification of a human FasL. Much of this work has been confirmed in mouse models of Fas but new questions arise including the concept of resistance to Fas-mediated apoptosis. The next chapter will attempt to address some aspects of this in addition to the generation of reagents for the study of FasL.

CHAPTER 4

Fas Ligand (FasL):

Generation and validation of a soluble human FasL

INTRODUCTION

After the cloning of Fas and identification of its critical role in disease pathogenesis in the *lpr* mouse, a worldwide search ensued for the counter-receptor to Fas. The elusive nature of the ligand earned it the title of an "immunological holy grail". We made attempts to isolate the gene for human FasL from Jurkat cells following our finding that Fas-Fc identified and immunoprecipitated a putative "FasL". However, during this time the rat and mouse ligand were cloned and sequence data became available (Suda et al, 1994; Takahashi et al, 1994; Lynch et al, 1994). We therefore took an approach using oligonucleotide primers designed from conserved regions of the sequences for mouse and rat FasL, with the premise that the sequence conservation may apply across species to humans. This approach proved unsuccessful and with the cloning of the human FasL by other groups it soon became apparent that the sequence chosen because of its conservation between mouse and rat was not conserved in the human ligand (Takahashi et al, 1994). We eventually obtained the human FasL cDNA from Dr D. Lynch of the Immunex corporation, which allowed us to embark on a project to generate a recombinant FasL protein not very dissimilar in design to the Fas-Fc protein. The aim was to generate reagents to enable further study of functional aspects of Fas engagement, using the natural ligand as compared to monoclonal antibodies to Fas. In our experience with other molecules like CD28, it was known that different effects could be observed when natural ligand was used rather than monoclonal antibodies (Nunes et al, 1994).

RESULTS

4.1) FasL-FLAG: cDNA construct and protein production

In an attempt to generate a soluble ligand a molecular approach was used to generate a cDNA comprising the extracellular region of the FasL without the transmembrane and cytoplasmic domains. We reasoned that the protein produced by such a gene expressed in vitro would be shed into the cell culture medium because of the lack of a transmembrane domain to anchor it onto the cell membrane. Since no antibodies were available against FasL at the time we incorporated a nucleotide sequence encoding an eight amino acid FLAG peptide, to enable identification by anti-FLAG antibodies (Kodak).

4.1.1) Generation of Fas ligand-FLAG cDNA (sFasL) by PCR

In order to generate the recombinant soluble epitope-tagged FasL (sFasL), oligonucleotide primers were designed from the C-terminal extracellular encoding region of the FasL sequence (genbank accession no: UO3470) as shown in **figure 4.1.1.** From the cloned FasL seqence it was predicted by Suda and Nagata (1994) that the FasL protein was a type II transmembrane protein with the extracellular region encoded by the C terminal domain and the cytoplasmic portion by the N terminal domain. The sequence encoding 8 amino acids of the FLAG peptide (Kodak) was incorporated into one of the FasL primers as shown in the figure. A PCR reaction was used to generate the recombinant Fas-ligand-FLAG (sFasL) cDNA. **Figure 4.1.2** outlines the molecular steps involved in the production of the sFasL and shows the gel fragments generated by PCR. The agarose gel in figure 4.1.2 shows the ± 600bp FasL-FLAG cDNA compared to the full length FasL cDNA of ± 1000 bp. Therefore this PCR reaction produced the predicted truncated cDNA fragment comprising the extracellular region of the human FasL. In order to check the cDNA it was cloned into a plasmid vector (pCR3) using a TA cloning method, and then restriction mapped. Thus the experiment designed to generate and clone a DNA construct comprising the extracellular domain of human FasL with an epitope tag was successfully completed as indicated by the restriction mapping information. It remained to test whether a soluble protein would be produced by expression of the sFasL cDNA in vitro.

```
5'
gggtcccgtc cttgacacct cagcctctac aggactgaga agaagtaaaa        50
ccgtttgctg gggctggcct gactcaccag ctgccATGCA GCAGCCCTTC       100
AATTACCCAT ATCCCCAGAT CTACTGGGTG GACAGCAGTG CCAGCTCTCC       150
CTGGGCCCCT CCAGGCACAG TTCTTCCCTG TCCAACCTCT GTGCCCAGAA       200
GGCCTGGTCA AAGGAGGCCA CCACCACCAC CGCCACCGCC ACCACTACCA       250
CCTCCGCCGC CGCCGCCACC ACTGCCTCCA CTACCGCTGC CACCCCTGAA       300
GAAGAGAGGG AACCACAGCA CAGGCCTGTG TCTCCTTGTG ATGTTTTTCA       350
TGGTTCTGGT TGCCTTGGTA GGATTGGGCC TGGGGATGTT TCAGCTCTTC       400
CACCTACAGA AGGAGCTGGC AGAACTCCGA GAGTCTACCA GCCAGATGCA       450
CACAGCATCA TCTTTGGAGA AGCAAATAGG CCACCCCAGT CCACCCCCTG       500
AAAAAAAGGA GCTGAGGAAA GTGGCCCATT TAACAGGCAA GTCCAACTCA       550
AGGTCCATGC CTCTGGAATG GGAAGACACC TATGGAATTG TCCTGCTTTC       600
TGGAGTGAAG TATAAGAAGG GTGGCCTTGT GATCAATGAA ACTGGGCTGT       650
ACTTTGTATA TTCCAAAGTA TACTTCCGGG GTCAATCTTG CAACAACCTG       700
CCCCTGAGCC ACAAGGTCTA CATGAGGAAC TCTAAGTATC CCCAGGATCT       750
GGTGATGATG GAGGGGAAGA TGATGAGCTA CTGCACTACT GGGCAGATGT       800
GGGCCCGCAG CAGCTACCTG GGGGCAGTGT TCAATCTTAC CAGTGCTGAT       850
CATTTATATG TCAACGTATC TGAGCTCTCT CTGGTCAATT TTGAGGAATC       900
TCAGACGTTT TTCGGCTTAT ATAAGCTCta agagaagcac tttgggattc       950
tttccattat gattctttgt tacaggcacc gagaatgttg tattcagtga      1000
gggtcttctt acatgcattt gaggtcaagt aagaagacat gaaccaagtg      1050
gaccttgaga ccacagggtt caaaatgtct gtagctcctc aactcaccta      1100
atgtttatga gccagacaaa tggaggaata tgacggaaga acatagaact      1150
ctgggctgcc atgtgaagag ggagaagcat gaaaaagcag ctaccaggtg      1200
ttctacactc atcttagtgc ctgagagtat ttaggcagat tgaaaaggac      1250
acctttttaac tcacctctca aggtgggcct tgctacctca agggggactg     1300
tctttcagat acatggttgt gacctgagga tttaagggat ggaaaaggaa      1350
gactagaggc ttgcataata agctaaagag gctgaaagag gccaatgccc      1400
cactggcagc atcttcactt ctaaatgcat atcctgagcc atcggtgaaa      1450
ctaacagata agcaagagag atgttttggg gactcatttc attcctaaca      1500
cagcatgtgt atttccagtg caattgtagg ggtgtgtgtg tgtgtgtgtg      1550
tgtgtgtgtg tgtatgacta aagagagaat gtagatattg tgaagtacat      1600
attaggaaaa tatgggttgc atttggtcaa gattttgaat gcttcctgac      1650
aatcaactct aatagtgctt aaaaatcatt gattgtcagc tactaatgat      1700
gttttcctat aatataataa atatttatgt agatgtgcat ttttgtgaaa      1750
tgaaaacatg taataaaaag tatatgttag gatacaaata                 1790
                                                    3'
```

Figure 4.1.1: Human Fas ligand (FasL) Gene Sequence

The sequence for human FasL is shown (Genbank accession no: UO3470). The transmembrane domain is indicated as a boxed area and the untranslated regions shown in lower case. The 842 bp coding region is shown in upper case, and the sites for binding of the oligonucleotide primers used to amplify the soluble FasL (sFasL) sequence are underlined. A sequence for the Flag peptide was incorporated into primer A as shown below:

5' primer (A):
cccatggactacaaggacgacgatgacaagCAGCTCTTCCACCTACAGAAGGAGCTGG
(Flag sequence in lower case, FasL sequence in upper case)

3' primer (B): GGAAAGAATCCCAAAGTGCTTC

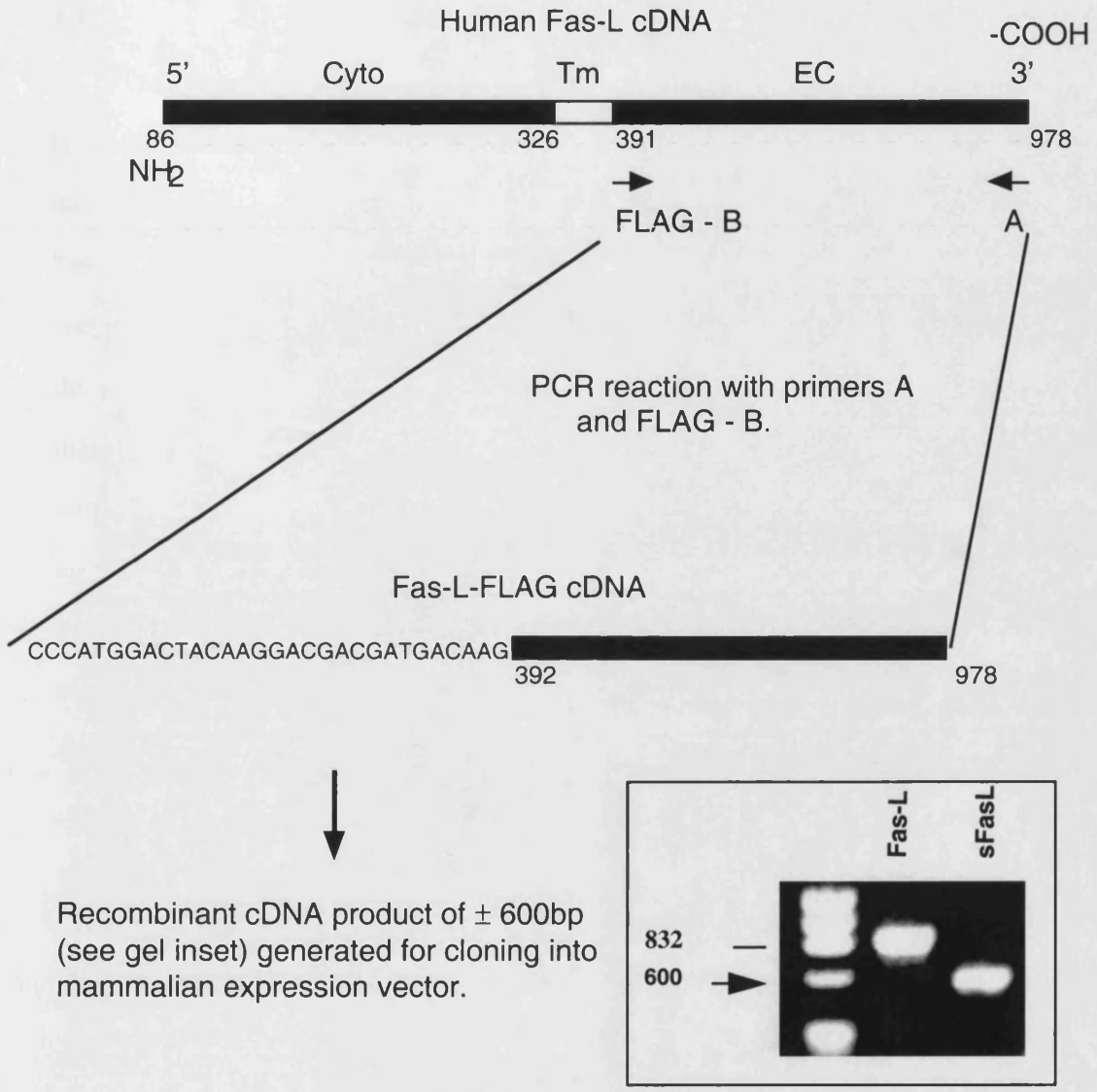


Figure 4.1.2 : Strategy for the generation of Soluble FasL.

The line drawings outline the general strategy employed to generate a cDNA for sFasL by PCR. Essentially, the -COOH terminal extracellular domain encoding region of the gene was amplified together with a sequence for the FLAG peptide as shown. The agarose gel shows the sFasL-FLAG PCR product (± 616 bp in lane 3) in relation to the cDNA for the coding region of FasL (± 842 bp in lane 2). Molecular weight markers are shown (ϕX-174 *Hae* III digest in lane 1).
Tm = transmembrane region, Cyt = cytoplasmic region , EC = extracellular region.

4.1.2) Expression of sFasL in culture

In order to achieve protein expression in cultured cells, the cloned sFasL cDNA was introduced into COS-7 cells using electroporation. Transfected cells were selected in Neomycin and cell culture supernatants were tested for apoptosis-inducing activity (see section 4.2). The results in **figure 4.1.3** shows that COS-7 cells mock transfected with the plasmid vector not containing the sFasL sequence produced no apoptotic activity in their culture supernatants, whilst a dose dependant increase in apoptotic activity was seen with the sFasL transfectant supernatants. In addition it was possible to test that the apoptosis was induced in a Fas-specific manner by using the previously generated Fas-Fc. As shown in chapter 3 section 3.2.3.2, Fas-Fc inhibited Fas-mediated apoptosis induced by both CH11 and sFasL in a variety of assays. Therefore these results suggest that the sFasL was appropriately expressed in culture supernatants of transfectants as predicted and was biologically active. Fas-Fc inhibition of the sFasL-induced apoptosis supported a Fas-specific mechanism of action.

Despite the tagged FLAG sequence there was technical difficulty with the isolation and demonstration of the sFasL protein on gels as well as immunoblots. This was ascribed to small quantities of protein produced, which may have been insufficient to see on a gel but which retained potent and Fas-specific apoptosis-inducing activity. Also post-translational protein modification through incorporation of the FLAG sequence may have altered the sFasL conformation and thereby epitope recognition by anti-FLAG antibodies.

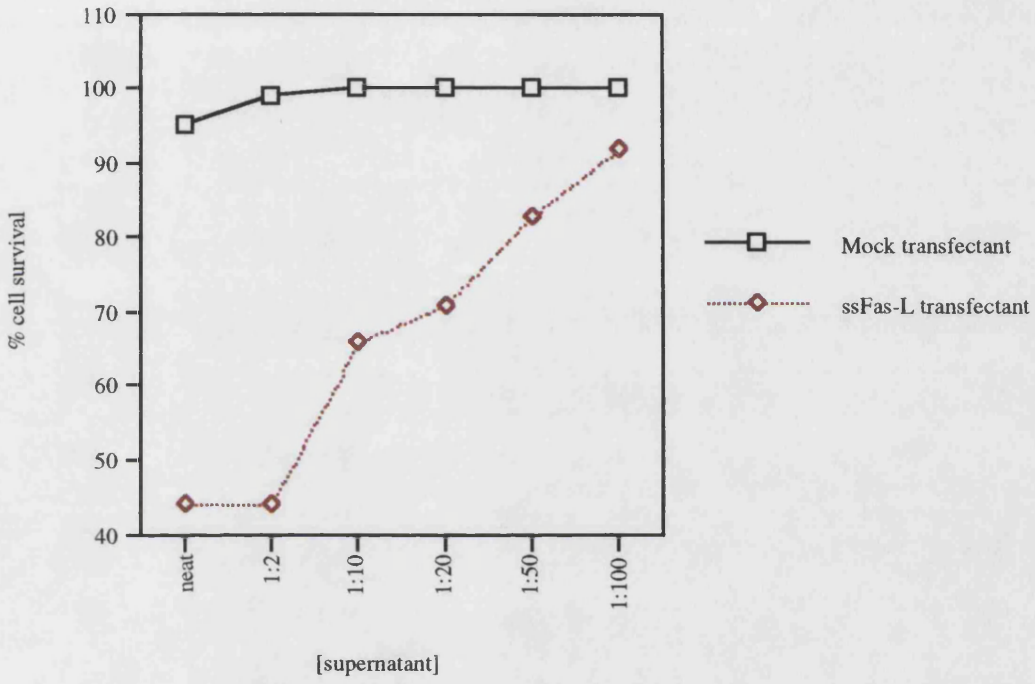


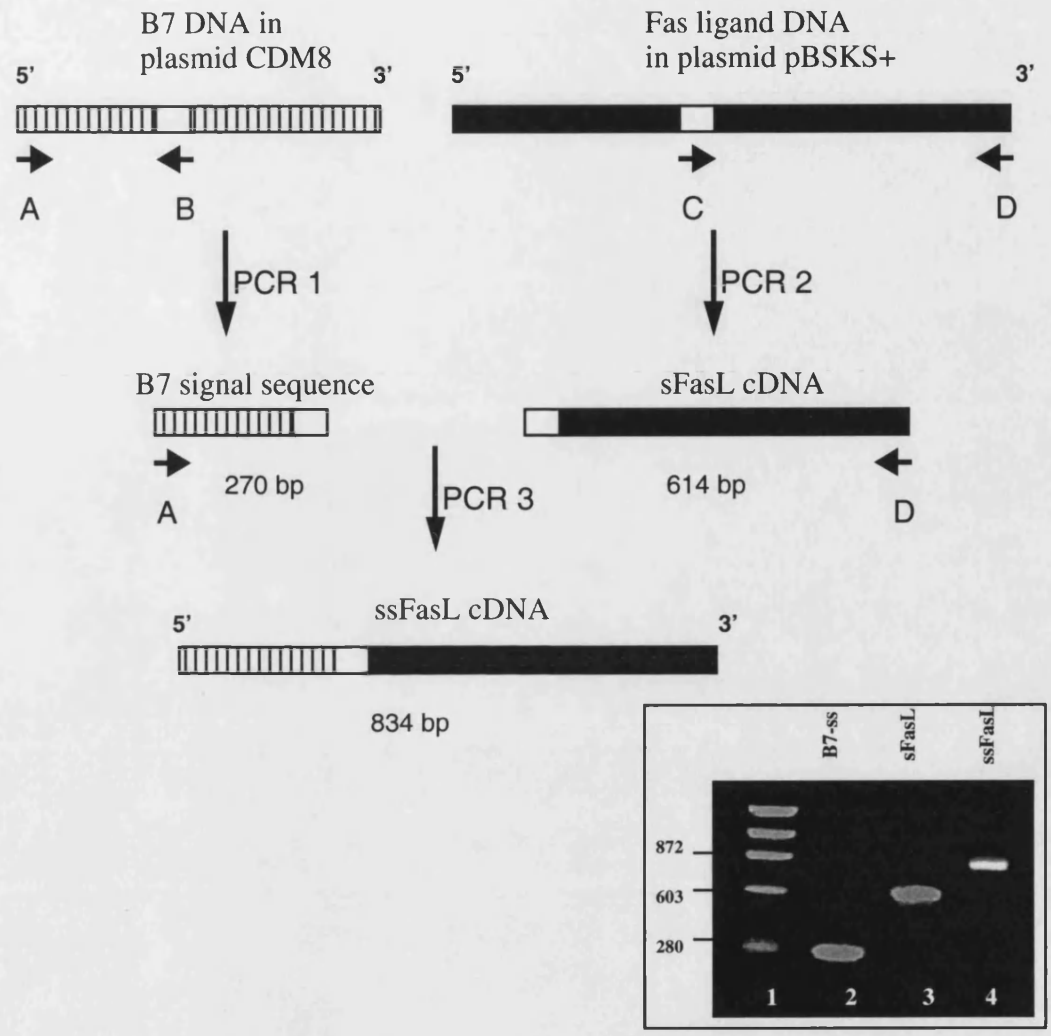
Figure 4.1.3: sFasL supernatants induce apoptosis (dose response).

Apoptosis of Jurkat cells was measured in a JAM assay and expressed as % cell survival versus dilution of cell culture supernatant. Apoptosis of Jurkats occurred in response to increasing concentrations of sFasL supernatants (red curve) but supernatants from mock transfected cultures did not induce Jurkat cell apoptosis (black curve).

4.1.3) Incorporation of a signal sequence onto sFasL by recombinant PCR

Due to poor protein production and the resultant difficulty with isolation of the sFasL, further modification of the sFasL was undertaken using a molecular approach to incorporate a signal sequence onto the sFasL cDNA.

In an attempt to improve protein production, it was decided to incorporate a signal sequence 5' of the FasL encoding region. With the signal sequence for the B7 molecule which was already in use in the laboratory, a strategy was planned as outlined in **figure 4.1.4.** Using specifically designed primers as indicated the B7 (CD80) signal sequence was amplified using PCR, as was the sFasL cDNA using newly designed primers. Unbound oligonucleotides and dNTP's were removed and the two fragments annealed in an overlap PCR reaction using the flanking primers. In the agarose gel of figure 4.1.4 the various PCR products are shown. In this figure it can be seen that the overlap PCR produced the predicted larger ±840 bp fragment comprising the added signal sequence. Hence this PCR based approach to attach a signal sequence of CD80 5' of the sFasL cDNA was successful. Further mapping and expression of the signal sequence FasL (ssFasL) was necessary.



Primer A: CTCCAATCTCTGTGTGTTTTG

Primer B: CTTGTCATCGTCGTCCTTGTAGTC catgggaccagccagcaccaagag

Primer C: ctcttggtgctggctggtcccatg GACTACAAGGACGACGATGACAAG

Primer D: GGAAAGAATCCCAAAGTGCTTC

Figure 4.1.4: Attaching a Signal Sequence to sFasL.

The line drawings outline the recombinant PCR approach used to generate a signal sequence onto the sFasL cDNA. Primers used are shown above with the complementary sequences for the overlapping primers (B and C) represented in lower case (B7 sequence) and upper case (sFasL sequence). The inset shows an agarose gel of the PCR products of PCR 1 (±270bp B7 signal sequence, lane 2), PCR 2 (±600 bp sFasL product, lane 3) and the overlap PCR 3 (± 834 bp signal sequence sFasL, lane 4). Molecular weight markers are shown in lane 1(ϕX-174 *Hae* III digest).

4.1.4) Cloning and restriction mapping of ssFasL

In order to confirm the appropriate splicing of the signal sequence the recombinant ssFasL cDNA was TA-cloned and mapped as described in **figure 4.1.5**. Details are provided because the vector chosen was different from those in previous cloning experiments. In this case the pCR3 vector provided the opportunity for TA cloning directly into a mammalian expression vector. The miniprep screen with *Eco* R1 digests shown in gel A (figure 4.1.5) revealed a number of clones with inserts (lanes 4-10 and 14, 15) which were then selected for restriction mapping. *Apa* 1 cuts in the FasL sequence and the multiple cloning site of pCR3 to reveal a clear difference between sense (±209bp fragment) and anti-sense (± 728 fragment) clones and gel B of figure 4.1.5 shows this clearly (sense refers to the correct 5' to 3' orientation of the cloned insert in relation to the CMV promoter of the plasmid vector). Of the nine clones depicted all were sense clones except three (lanes 6, 7, and 8 gel B, figure 4.1.5).

Similar findings were seen with *Nco* 1 restriction mapping to support the data with the *Apa* 1 digests (see figure 4.1.5, lanes 12-20). Thus the results suggested a high cloning efficiency with this plasmid and revealed a number of sense clones of ssFasL for in vitro expression.

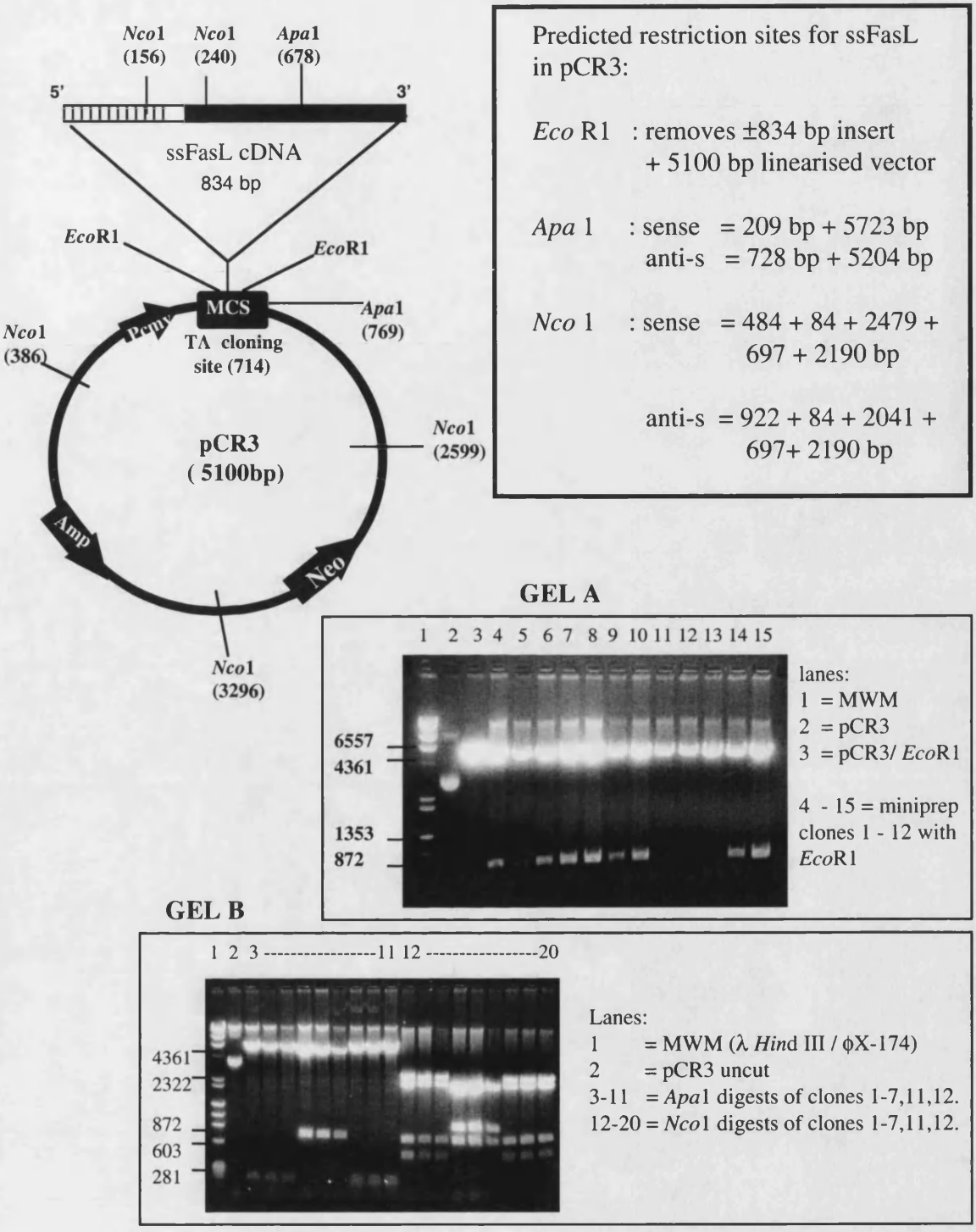


Figure 4.1.5: Cloning of Signal Sequence FasL (ssFasL) into pCR3.

A cloning map and predicted restriction sites are shown for ssFasL in pCR3. Agarose gel A shows randomly selected miniprep clones digested with EcoR1 to reveal positive clones in 9 of the 12 selected (lanes 4-10 and 14, 15 in gel A). Further restriction mapping using *Apa*1 and *Nco*1 revealed that clones 1, 2, 3, 7, 11 and 12 were in the correct orientation for mammalian cell expression whilst clones 4, 5 and 6 (lanes 6, 7, 8 and 15, 16, 17 in gel B)were anti-sense clones.

MWM = molecular weight markers (λ *Hin*d III / ϕX-174), MCS = multiple cloning site, Amp = ampicillin resistance gene, Neo = neomycin resistance gene, Pcmv = cytomegalovirus promoter sequence for in vitro expression , anti-s = antisense clones.

4.1.5) Expression of ssFasL in culture

In order to produce the ssFasL protein in COS-7 cell supernatants one sense clone of ssFasL/pCR3 was chosen for expression in mammalian cell culture. After electroporation and selection under neomycin, COS cell supernatants were harvested and pooled.

To test the efficiency of the ssFasL produced in culture comparitive assays of apoptosis were established for the sFasL (no signal sequence) and the ssFasL (with signal sequence) proteins. **Figure 4.1.6.** shows the results. The addition of a signal sequence appeared to enhance the apoptotic activity of the ssFasL (as compared to sFasL) produced in culture supernatants as seen in both the JAM assay as well as the Annexin assay shown in figure 4.1.6. In the JAM assay (i) the killing potential of the FasL was compared to vector transfected control supernatant (histogram a), and ssFasL consistently gave 20 - 25% more apoptosis (histograms d and e representing independant transfectants) compared to sFasL (histograms b and c).

Similar observations were made in the Annexin assay (ii) where 20 - 25% more apoptosis was seen with ssFasL (FACS histograms in figure 4.1.6). Thus the addition of a signal sequence to the sFasL enhanced its potential to kill Jurkat cells as observed in two independent in vitro assays of apoptosis.

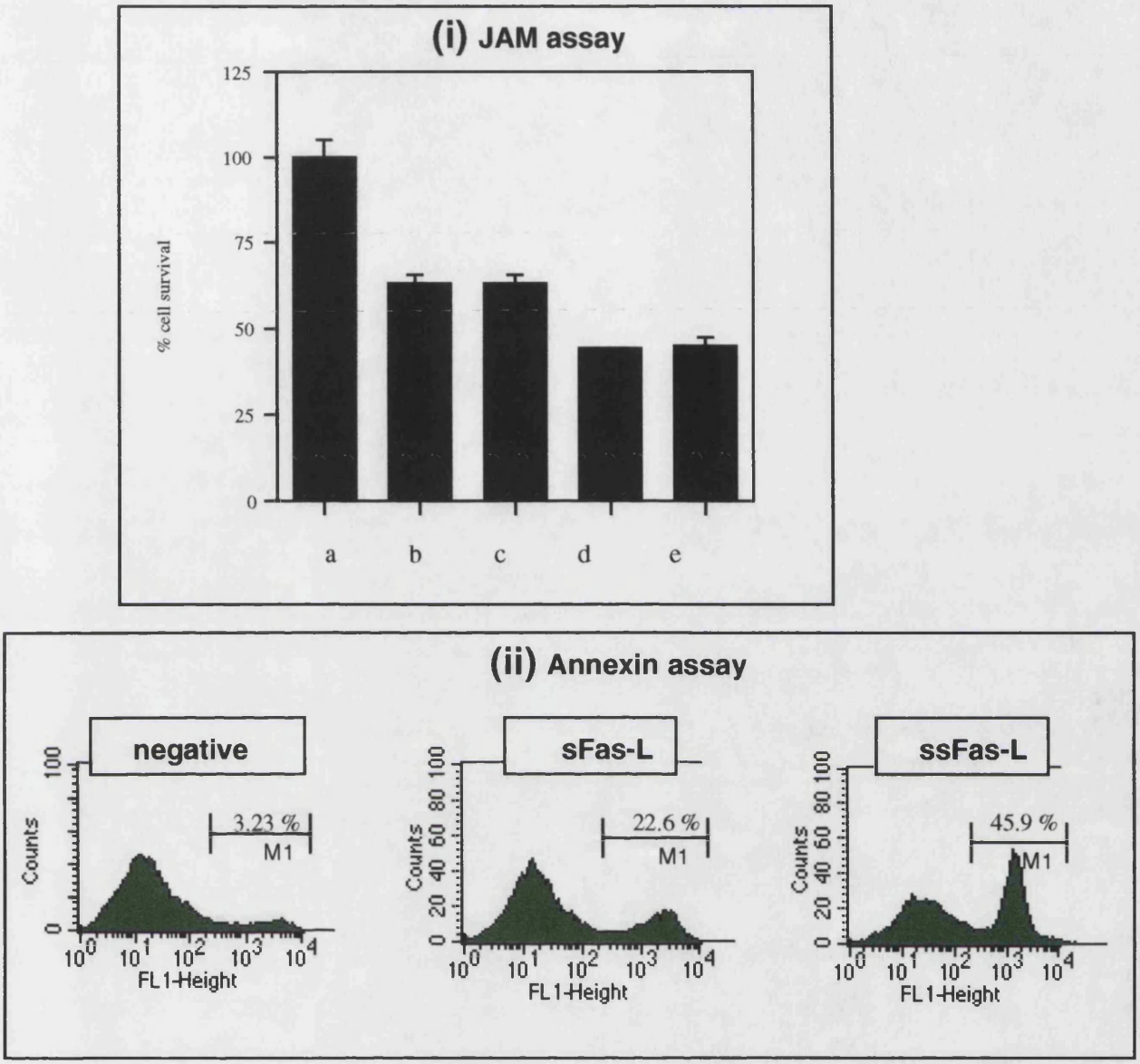


Figure 4.1.6: Enhanced effectiveness of ssFasL compared to sFasL.

A comparison of apoptosis induced by the sFasL versus ssFasL is shown in two assays. In **(i)** a JAM assay on Jurkat cells shows 20 - 25% greater apoptosis induced by two independent batches of ssFasL (d and e) compared to sFasL (b and c). All comparisons were made to mock transfected supernatants at the same concentration (1:10 , histogram a). A similar pattern of enhanced apoptosis (± 25%) was seen with ssFasL in an Annexin assay for apoptosis in Jurkat cells **(ii).** In the latter experiment percent apoptotic cells are indicated above the M1 marker as determined on FACS analysis. All cell stimulations were carried out for 6 hours. Results are representative of three independent experiments.

4.2) Soluble Fas ligand (sFasL) in apoptosis assays

Following the successful generation and production of the soluble ligand as described above it was compared to the monoclonal anti-Fas antibody CH 11 (regarded as the gold standard for Fas-induced apoptosis). In order to assess this it was necessary to establish a number of apoptosis assays.

4.2.1) JAM assay

In order to assess apoptotic changes induced by sFasL as compared to CH11 the JAM assay (method in 2.5.1) was employed on a variety of cell types. **Figure 4.2a** shows the results in the U937 cell line, CHO-Fas transfectants and T cell blasts and Jurkat cells, all of which express Fas on their surface. Comparable killing was induced by the sFasL in all four cell types with a tendency to increased killing activity in T cell blasts. It is unclear if this reflects a true biological difference in response to the "natural ligand" as opposed to the antibody. However the results of a second assay of DNA fragmentation, namely the TUNEL assay (chapter 2.5.3) did not show an enhanced killing with sFasL compared to CH 11 (figure 4.2b). Thus these results suggest that sFasL can induce apoptosis via the Fas receptor in a variety of Fas expressing cell types and that the apoptosis induced by sFasL is largely comparable to CH 11.

4.2.2) Annexin V-FITC assay

In order to confirm that the apoptosis detected in the JAM assay could be substantiated by other methods we used the Annexin-FITC assay (methods 2.5.2). The Annexin-FITC assay depends on detection of externalised phosphatidyl-serine (PS) by Annexin V-FITC and FACS analysis. PS is a phospholipid molecule usually situated on the inner leaflet of the plasma membrane. It is rapidly externalised and exposed on the surface of apoptotic cells. Fas-induced apoptosis has also been shown to be associated with a rapid externalisation of PS (Martin et al, 1995). Our results (**figure 4.2c**) show that Fas-induced apoptosis was detected by 4-6 hours using an Annexin-FITC assay and that both

the CH 11 antibody and the sFas-L induced early annexin changes (histograms d and b respectively). The specificity of Fas-mediated apoptosis was demonstrated in blocking annexin changes by preincubation of the Jurkat T cells with the ZB4 anti-Fas antibody (reduction of apoptotic population from 45.9% to 6.08%).The CHO-Fas transfectants generated earlier were also sensitive to sFas-L induced apoptosis when measured by the Annexin-FITC assay. Thus these results confirmed by an independent measurement that the sFasL was as capable of inducing apoptosis as CH 11.

4.2.3) Cytospin analysis of nuclear morphology

Morphologic changes in apoptotic cells with cytoplasmic shrinkage, nuclear condensation and membrane blebbing are best visualised on electron microscopy or confocal microscopy. We attempted to study nuclear morphologic change by fluorescence microscopy with simple propidium iodide staining of cells stimulated with the sFasL (methods 2.5.4). **Figure 4.2d** shows our results where gross nuclear changes were seen with sFasL on Jurkat T cells. Apoptotic cells showed reduced or irregular speckled staining with propidium iodide (picture b compared to a). It was also evident that the Fas-mediated apoptotic changes could be inhibited by Fas-Fc (picture c). It was of interest that the apoptotic changes were also inhibited by chloroquine (picture d), possibly on the basis of sphingomyelinase inhibition. As described in figure 3.1.8 we found that chloroquine inhibited sphingomyelinase signalling following Fas engagement. Thus the cytospin analysis showed that gross morphologic changes of apoptosis could be detected by six hours after exposure to sFasL and that the changes were inhibited by Fas-Fc blockade of Fas receptors or by inhibition of signalling pathways of apoptosis.

All the above assays essentially measured apoptosis in response to sFasL. However each method reflected a different manifestation of the apoptotic process and thereby confirmed the activity of the sFasL as an apoptosis-inducing protein. In general the sFasL appeared to have comparable activity to the monoclonal anti-Fas antibody CH11. We have thus successfully manipulated the FasL cDNA by recombinant PCR and expressed a functional soluble form of human FasL with pro-apoptotic activity.

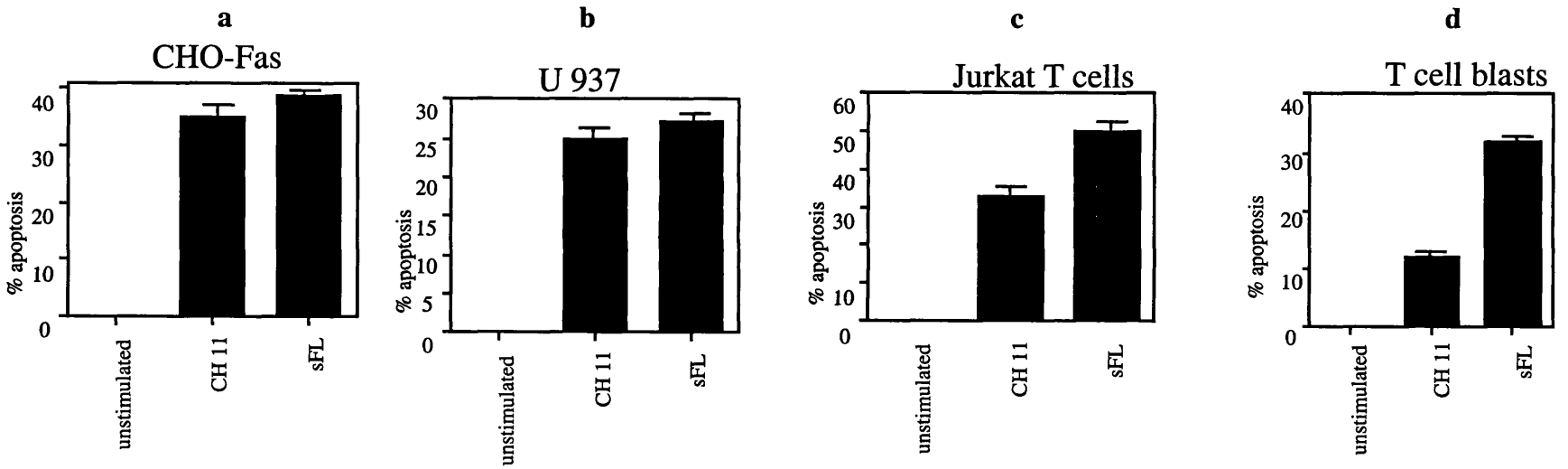


Figure 4.2 a : sFasL induces Fas-mediated apoptosis in a variety of cell types.

JAM assays were carried out on a variety of cell types as represented above. The percent apoptosis was calculated following exposure to monoclonal anti-Fas (CH 11 1ug/ml) or sFasL (1: 10 supernatant). For CHO-Fas transfectants and U937 monocytic cell lines (a and b respectively) the CH 11 and sFasL had comparable effects, whilst it appeared that sFasL was more effective than CH 11 at inducing apoptosis in the Jurkat T cells (c) and activated T cell blasts (d). Data are representative of three independent experiments.

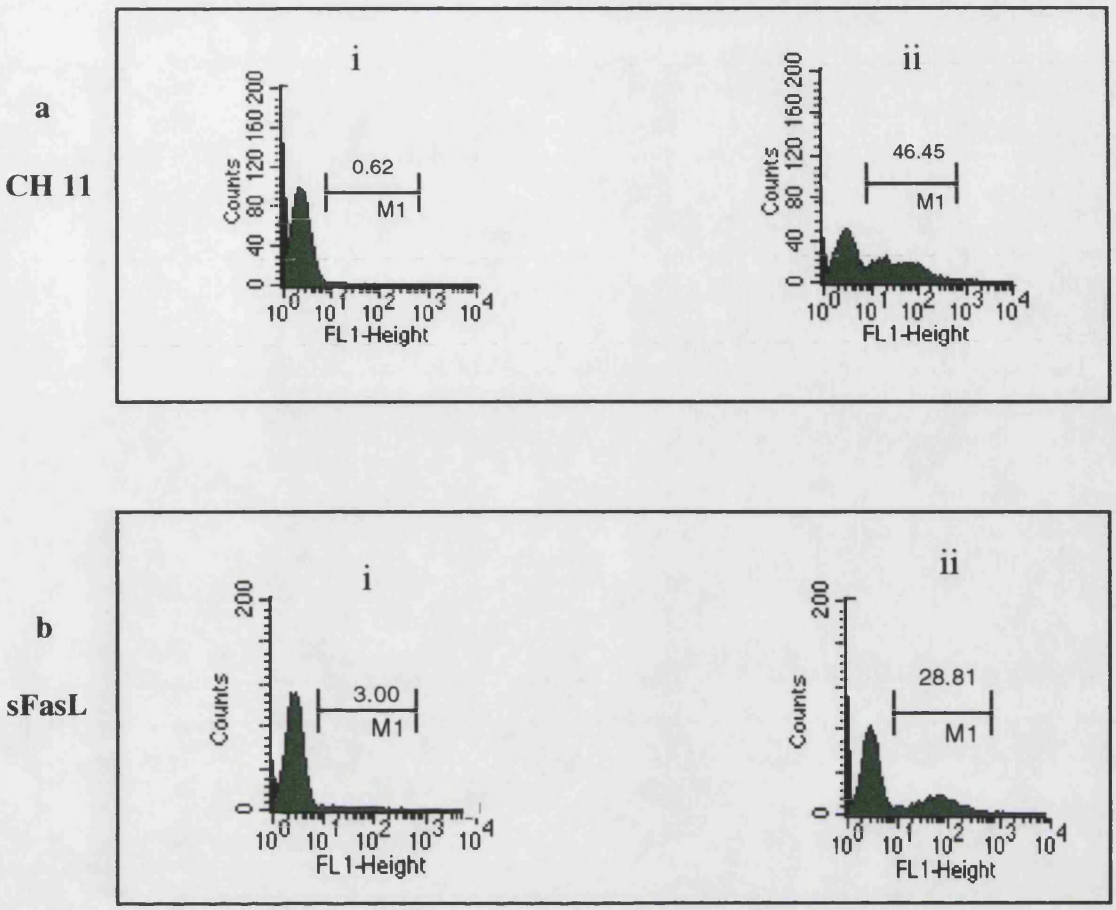


Figure 4 . 2 b: TUNEL assay of apoptosis induced by sFasL.

A TUNEL assay showing FACS histograms is depicted here with apoptosis reflected by a shift to the right. Both anti-Fas antibody (**a** ii) and the sFas-L (**b** ii) induced apoptosis of Jurkat T cells (percent cell death is reflected above the M1 marker as determined by FACS analysis). Negative cells were incubated with isotype-matched mouse antibody (a i) or cell culture supernatant from mock-transfected COS cells (b i). Cells were all stimulated for 6 hours before the TUNEL assay was conducted.

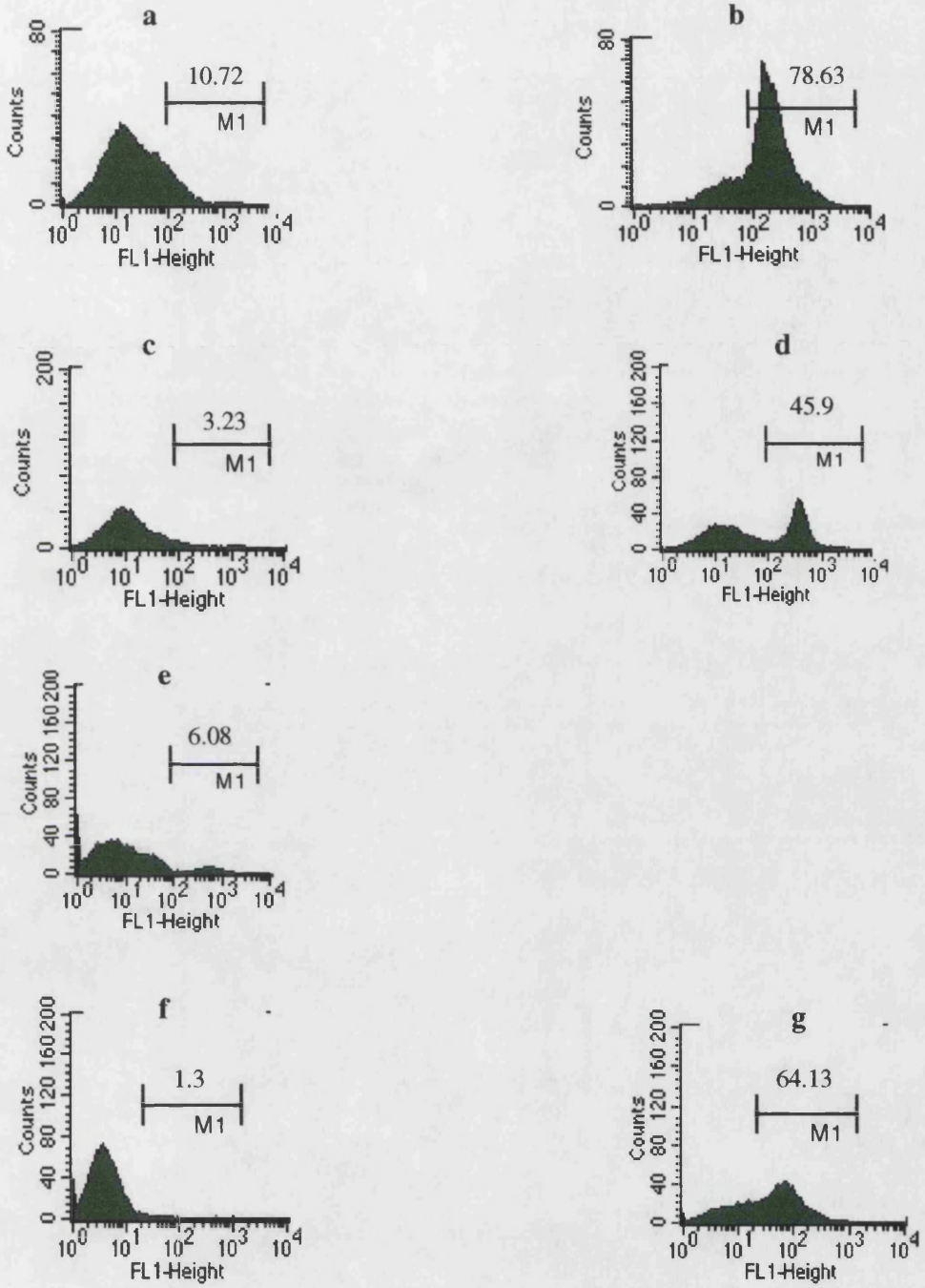


Figure 4 . 2 c : Annexin-FITC assay of apoptosis induced by sFasL.

FACS pictures are shown of an Annexin-FITC assay in Jurkat cells depicting apoptosis as a shift in the histogram to the right. Both CH 11 (b) and sFasL (d) induced apoptotic changes compared to control cells (a and c). The effect of sFasL was inhibited by a Fas blocking antibody ZB4 (e). Apoptosis measured by Annexin was also detectable in the CHO-Fas transfectants on exposure to sFasL (g) as compared to mock supernatant (f). Gates on the apoptotic population (M1)of cells are shown with percentages reflected. The results are representative of three independent experiments.

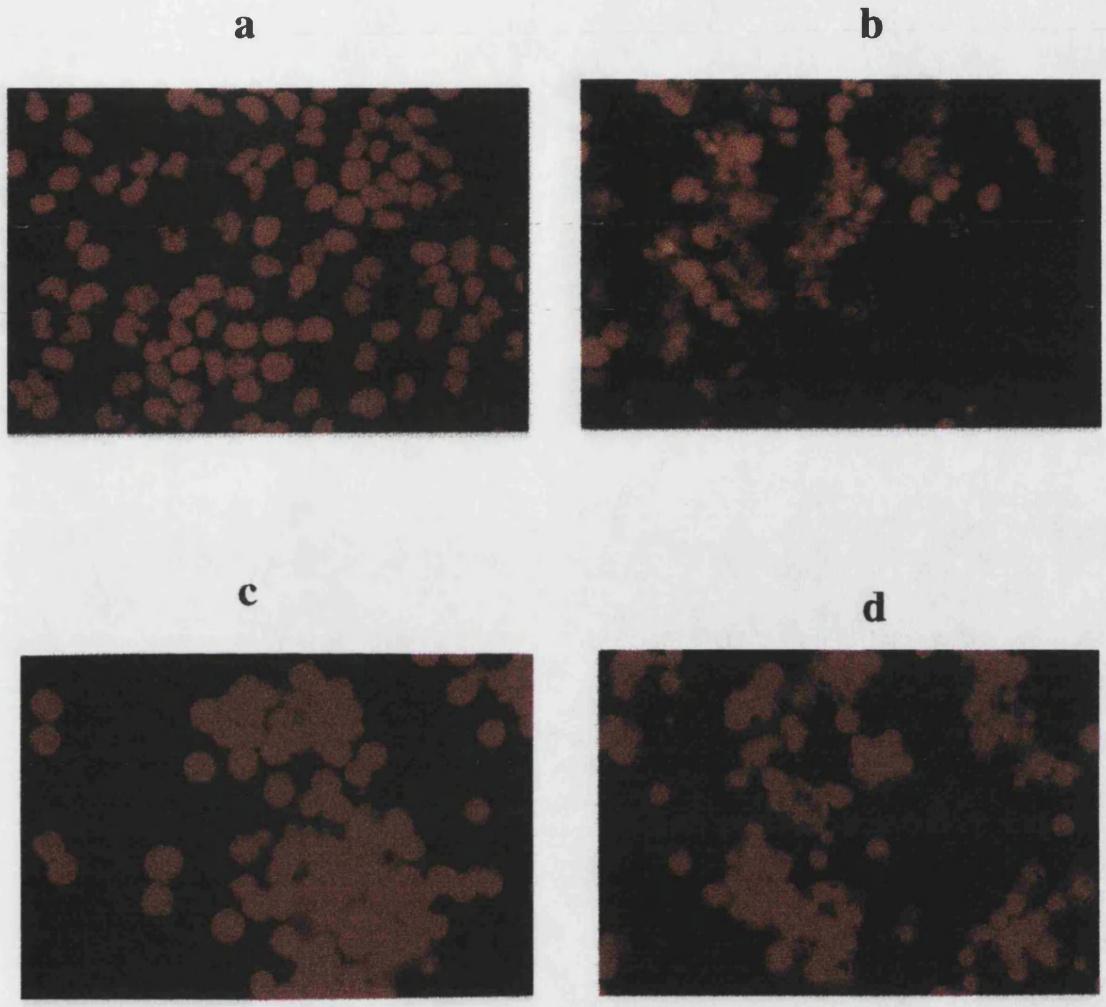


Figure 4.2 d: Cytospin analysis for apoptosis by sFasL.

Cytospin pictures (40X magnification) are shown of Jurkat T cells with propidium iodide (PI) staining of nuclei following various stimuli. Jurkat cells were incubated with control supernatant (a) or sFas-L supernatant (b) for 4-6 hours and centrifuged onto slides for PI staining. Intact nuclei stain homogeneously with PI (a) whilst the apoptotic nuclei in b can be seen with very irregular speckled staining and much reduced PI fluorescence overall. Inhibitors of Fas-mediated apoptosis like Fas-Fc restored normal nuclear staining (c) and an interesting inhibition of Fas mediated apoptotic change was also seen with chloroquine (d).

4.3) Apoptosis in human T cell blasts

4.3.1) Resistance to Fas-mediated apoptosis : Lack of correlation between Fas expression and Fas-mediated apoptosis.

Analysis of the apoptosis assays revealed that not all cells were equally sensitive to Fas-induced killing. A significant proportion of cells (Jurkat and T cells) survived exposure to anti-Fas reagents despite surface Fas expression. It has previously been reported that activation of T cells was required for five to six days before Fas-mediated apoptosis was seen (Itoh et al 1991, and Trauth et al, 1989). In our activated T cell cultures there was clear evidence of variation in sensitivity to Fas-mediated killing **(figure 4.3.1).**

In an attempt to study the resistance to Fas-mediated apoptosis, another student in the laboratory (Lucy Walker) generated a Fas resistant Jurkat T cell line (JLW) by culturing Jurkat cells with low concentrations of anti-Fas antibody over time. These cells were resistant to Fas-induced killing despite expressing wild type levels of Fas. To see if the JLW cells were resistant to the sFasL generated above, a JAM assay was conducted comparing wild type Jurkat cells to the JLW cells. Resistance to Fas-mediated apoptosis was found in the JLW cell line when using either anti-Fas antibody or the recombinant sFasL (**figure 4.3.1**). From the figure it is apparent that despite equivalent expression of surface Fas the JLW cells were not as sensitive to apoptosis as the Jurkat T cells (5% death compared to 30 - 35% after 6 hours stimulation).

Activated T cell blasts also vary in their susceptibility to apoptosis by Fas stimulation and an example is shown in figure 4.3.1 (iii) of a stimulated T cell culture with good surface Fas expression but resistance to Fas-induced apoptosis. The factors influencing resistance to Fas-mediated apoptosis were not clear but a number of protein interactions may be implicated including anti-apoptotic factors like Bcl-2, Bcl-XL, and the more recently identified death-domain-interacting proteins (FADD (Chinnaiyan et al, 1995), FLICE (Los et al, 1995) and FLIP (Irmler et al, 1997) or phosphatases like FAP (Su et al, 1995). It remains for these interactions to be defined in our JLW cell line and also in the resistant

T cell blasts. Defects of signalling pathways could also conceivably influence outcome after Fas engagement.

Thus whilst our data did not identify the mechanisms operative in Fas resistance, they did reveal at an early stage of our investigation that Fas surface expression did not necessarily correspond to apoptotic death following Fas receptor engagement. This concept of resistance to Fas-mediated apoptosis has now become an intense focus of research efforts after years of dogmatic reports that "all Fas-positive cells died by day 6 after activation".

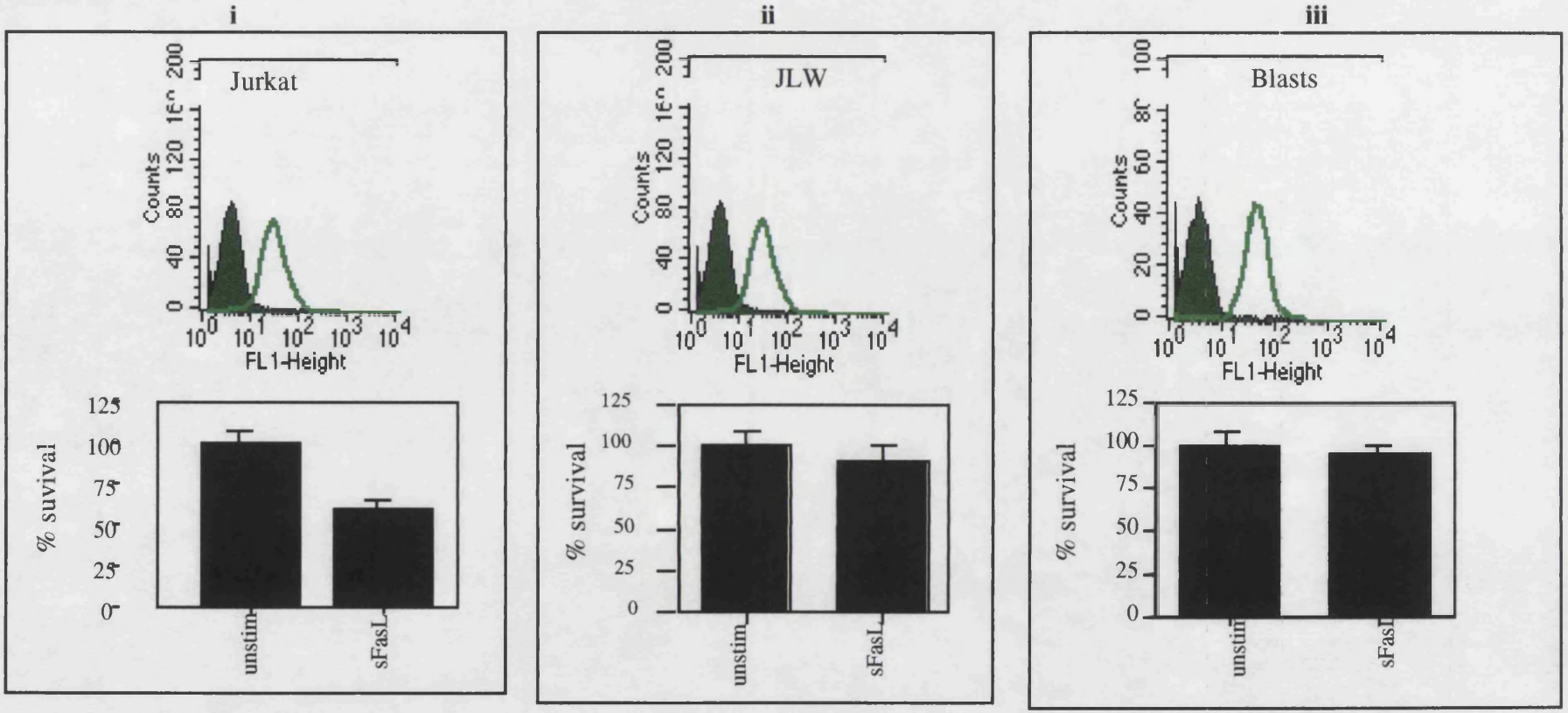


Figure 4.3.1: Fas-induced apoptosis does not correlate with surface Fas expression.

FACS analysis of various cell types are shown for Fas surface expression above. No significant differences in surface expression of Fas could be seen for Jurkat cells, JLW cells, and T cell blasts (unshaded histograms). In vitro apoptosis as measured by a JAM assay is shown below each FACS histogram where it is evident that JLW cells (ii) and T cell blasts (iii) did not die as readily as Jurkat cells (i) after exposure to sFasL for 6 hours despite similar levels of surface Fas expression. The data are representative of more than six experiments.

4.3.2) Resistance to Fas-mediated apoptosis is not associated with defective JNK signalling.

4.3.2.1) Time course for JNK signalling following Fas engagement in Jurkat cells.

Since we had already established that phosphorylation of JNK ocurred as a signalling mechanism after Fas engagement (chapter 3.1.5c) we investigated whether there was any abnormality in JNK signalling to account for the Fas resistance of the JLW cells ; i.e., is the defect in these cells above or below the level of JNK signalling?

In order to do this an in vitro JNK assay was established using known stimuli of JNK activity. However, a time course experiment was necessary to find the optimum duration of stimulation necessary following Fas engagement. **Figure 4.3.2** shows a time course experiment for both sFasL and CH 11 and indicates that the JNK activation following Fas receptor stimulation was rapid with activity seen by 5 minutes and a decay of activity apparent by 60 minutes.

Thus it was decided that JNK signalling would be assessed after fifteen minutes of Fas receptor stimulation in order to optimally detect changes within this signalling pathway.

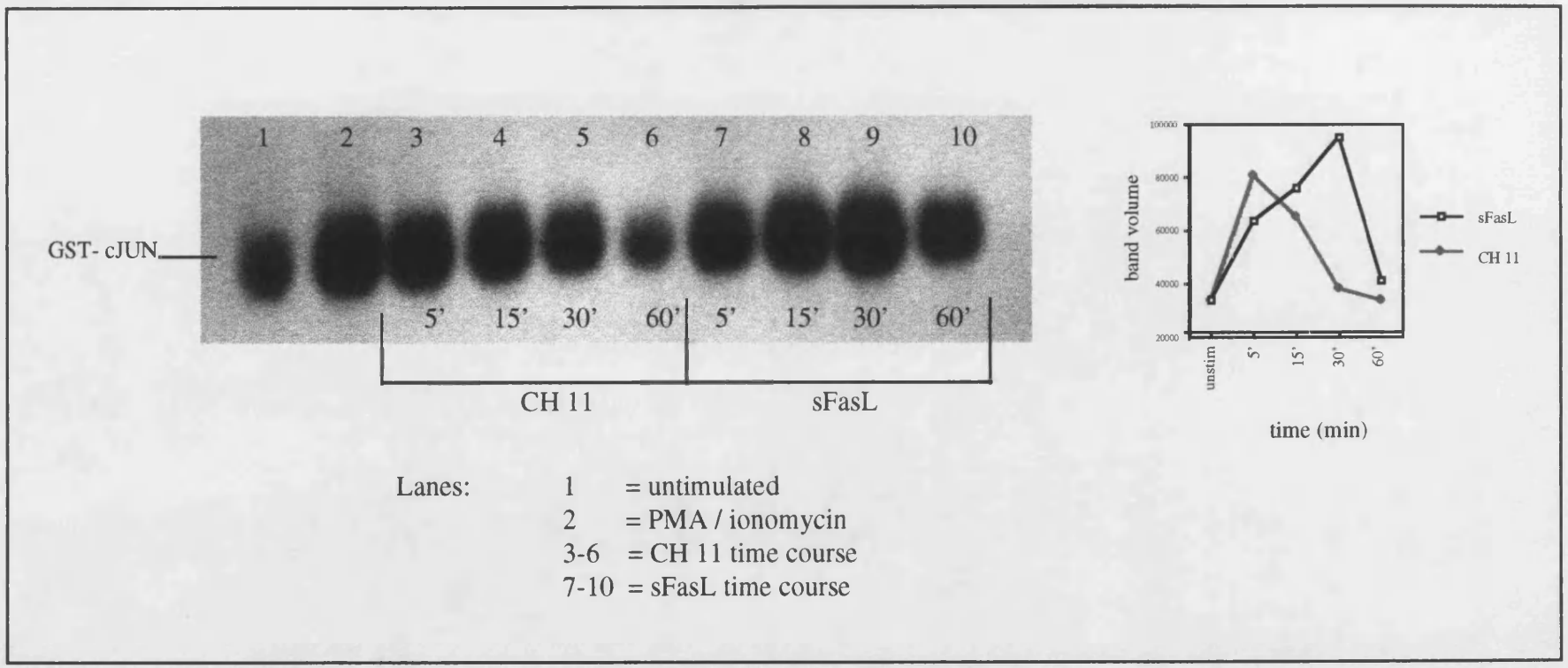


Figure 4.3.2: In vitro Jun kinase (JNK) assay - time course of activation after Fas stimulation

An in vitro JNK assay is shown using Jurkat cell lysates following stimulation by PMA / ionomycin, CH 11, and sFasL. Fas stimulations by CH 11 (0.5ug/ml) and sFasL (1:10) were carried out for the times indicated below the gel lanes (in minutes). Both antibody and sFasL stimulated JNK activity as measured by in vitro GST-cJun phosphorylation with upregulation evident by 5 minutes (lanes 3 and 7 compared to lane 1) and persisting for upto 60 minutes (lanes 6 and 10. The plotted curves represent the kinetics of GST-cJun phosphorylation for CH 11 and sFasL when the gel bands were quantitated using Phoretix 1D gel analysis software.
abbreviations: unstim = unstimulated, min = minutes.

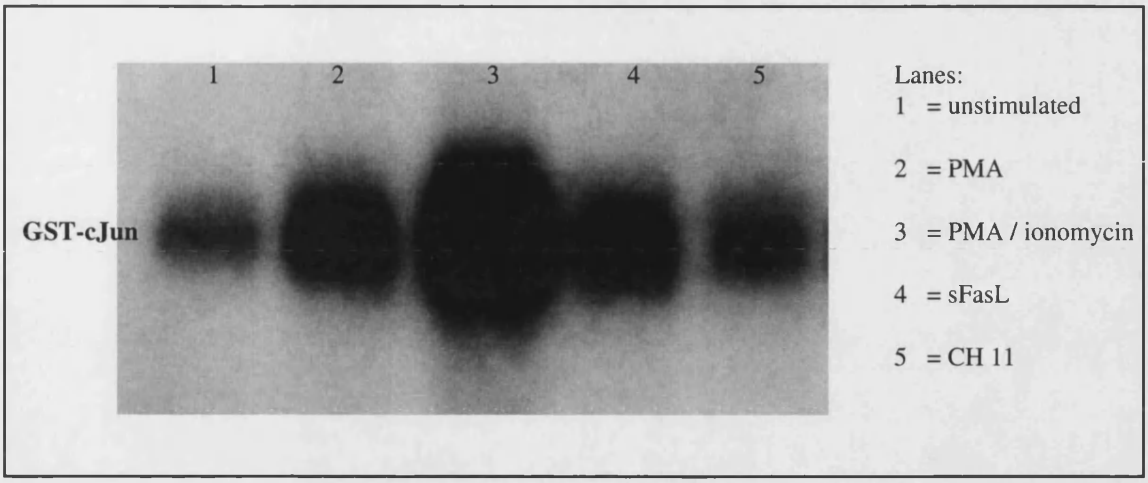
4.3.2.2) Comparison of JNK signalling in Jurkat and JLW cell lines.

In order to assess the JNK signalling pathway in the Fas-resistant JLW cells an in vitro JNK assay was applied using the conditions established above. Fas-mediated activation of JLW cells was compared to Jurkat cells and lysates were analysed for in vitro JNK activity fifteen minutes after stimulation.

Figure 4.3.3 shows the results of an in vitro kinase assay measuring JNK activity by phosphorylation of a GST-cJun substrate. As compared to unstimulated cells both PMA alone and PMA/ionomycin induced marked activation of JNK. Both CH11 and sFasL also induced JNK activity but to a lesser extent than the phorbol ester stimulation. Whilst the resistant JLW cells maintained a Fas-related upregulation of JNK activity (compare lanes 4 and 5 to lane 1 for JLW cells) similar to the sensitive Jurkat cells, there appeared to be a generally lower overall signalling response to Fas stimulation within the JLW cells (compare lanes 4 and 5 for JLW cells to equivalent lanes for Jurkat cells). Similarly, T cell blasts that showed resistance to Fas killing still maintained the ability to activate JNK on Fas engagement (data not shown).

These results therefore suggested that the JNK signalling pathway, whilst activated by Fas stimulation may be somewhat altered in resistant cells with a lower level of activity compared to Fas sensitive cells. However, no obvious major defect of JNK signalling was evident to account for the resistance to Fas-mediated apoptosis, and therefore other signalling pathways must be implicated.

JURKAT CELLS



JLW CELLS

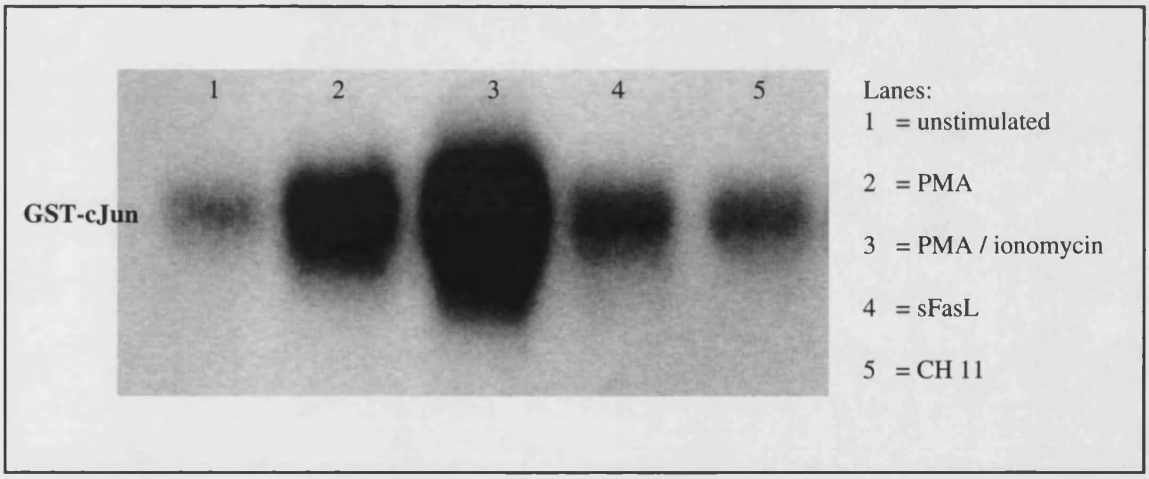


Figure 4.3.3: Comparison of JNK signalling in Jurkat and JLW cells.

An in vitro kinase assay is shown for both Jurkat and JLW cells with various stimuli. Comparisons were usually made with control unstimulated cells as shown in lanes 1 of each gel. PMA / ionomycin was used as a positive control (lanes 3) and was consistently greater in its stimulation of JNK activity than PMA alone (lanes 2). Both CH 11 (lanes 5) and sFasL (lanes 4) stimulated JNK activity in JLW (Fas-resistant cells) as well as Jurkat cells (Fas-sensitive), but the phosphorylation of GST-cJun was somewhat reduced in the Fas-resistant JLW cells following Fas engagement. Results are representative of three experiments.

DISCUSSION

Having previously identified a human FasL on T cells using our Fas-Fc protein, we were also successful in generating a soluble FasL protein as a useful reagent for further study of Fas-FasL interactions. The sFasL protein was generated by manipulation of the human FasL cDNA which required a recombinant PCR approach, followed by cloning and expression in cell culture. The soluble ligand was produced in vitro in a COS-7 cell culture system and was shown to be specific and highly effective in mediating Fas receptor stimulation and apoptosis. The sFasL was comparable to the gold standard monoclonal anti-Fas antibody (CH11) in a range of in vitro apoptosis assays. It is unclear from our data if the sFasL was more effective than the CH11 antibody because of a natural ligand conformation. The enhanced apoptosis seen in Jurkat cells and T cell blasts with sFasL was not always consistent but this may also be due to other factors. Firstly, it was difficult to quantitate the sFasL which was used as a supernatant from transfectant cell cultures, and therefore a dose-for-dose comparison could not be conducted in order to compare its effectiveness to the purified CH11 antibody. However despite this the sFasL was at least as effective as 1ug/ml purified CH11 antibody in mediating Fas-specific apoptosis. It is interesting that differences in activity of the natural ligand as compared to anti-Fas antibody have been described (Suda et al, 1996; Zipp et al, 1997). A second explanation for the inconsistency in the killing potency may be in the heterogeneous nature of cell sensitivity to Fas-mediated apoptosis, and will be discussed below.

FasL belongs to the TNF family of proteins, of which TNF has been identified to require a trimeric structure for effective TNF-R stimulation (Kriegler et al, 1988). Consequently it has been hypothesised that the Fas receptor would also trimerise or oligomerise with FasL engagement. The requirement for cross linking of a variety of IgG anti-Fas antibodies supported this idea (Dhein et al, 1992), whilst it seemed that the CH 11 antibody did not require prior cross linking because of its IgM structure. It was interesting to see that our sFasL did not require any prior manipulation in order to induce apoptosis. This would

suggest that the protein was being appropriately translated in a timeric or multimeric form in the COS cell transfectants. An alternative model would suggest that the sFasL protein was produced in monomeric form and inducing timerisation of the Fas receptor following engagement by recruitment of other death domain binding proteins. Unfortunately, although we could detect specific and highly effective apoptotic activity in the transfectant supernatants we were not able to isolate and purify the sFasL from the supernatants in order to define its structure further. Although the epitope tag attached to the sFasL was detectable with anti-FLAG antibodies there was difficulty with consistent immuno-detection of sFasL. In light of the pressures of time and resources further isolation and purification of sFasL was not pursued. However, given its potent and specific activity the sFasL supernatants were used for a variety of assays in the laboratory. More recently the NMR structure of Fas has been identified as being trimeric (Huang et al, 1996).

The role of sFasL in Fas-mediated apoptosis has been investigated by others more recently. Soluble FasL has been found to be produced naturally by Jurkat cells (Dhein et al, 1995) following metalloproteinase cleavage and may play a role in regulating cell survival under appropriate activation conditions. It has been suggested that activation of T cells may upregulate not only surface FasL for cell-cell killing via Fas but may also result in production of soluble FasL which may then influence cell death through an autocrine or paracrine mechanism (Brunner et al, 1995). Our findings with a recombinant sFasL would certainly support the idea that cells can die by apoptosis when presented with a soluble FasL as opposed to a cell surface expressed FasL. Further research with molecules like the sFasL may eventually yield new therapeutic approaches particularly in situations where Fas-induced apoptosis needs to be enhanced, as for example in lymphoid malignancies.

It was noted that some cells did not undergo apoptosis on Fas engagement despite high levels of Fas expression on FACS, and not all activated T cell blasts died after 6 days of activation as suggested in the literature (Owen-Schaub et al, 1992). More recently other papers, including our own have also suggested that not all activated cells with Fas

expression are susceptible to apoptosis (McLeod et al, 1998; Ogasawara et al, 1995). One important signalling pathway that was thought to be critical for apoptosis was the JNK pathway (Chen et al, 1996).An attempt was made to define if JNK was implicated in the phenomenon of resistance to Fas-mediated apoptosis in our cultures. Although we observed upregulation of JNK activity even in the Fas resistant cells, there appeared to be less overall activity of JNK within these cells compared to Fas-sensitive Jurkat cells. However the JNK activity may be influenced by other enzymes induced on engagement of the Fas receptor, for example sphingomyelinase, and may not necessarily correlate with susceptibility to apoptosis. The role of JNK in apoptosis has come into question more recently and it may be that JNK plays different roles under different apoptosis-inducing conditions. Whilst JNK activation is critical for apoptosis induced by UV irradiation or osmotic stress (Chen et al, 1996), the evidence suggests that with Fas stimulation JNK activation occurs independently of any susceptibility to apoptotic death (Nishina et al, 1997). The exact role of JNK activation after Fas engagement remains uncertain. It could be that JNK is not critical for Fas-mediated apoptosis and that it may mediate other Fas effects such as transcription factor induction and cell proliferation as is the case with its family member, the TNF-R (Ponton et al, 1996; Hsu et al, 1995). Another possibility is that JNK activation occurs earlier than the critical apoptosis signalling events more recently identified such as activation of Caspases (Chen et al, 1996), and that the latter may play a part in the resistance to Fas-mediated death in the JLW cell line. Other death domain associated factors like FADD (Chinnaiyan et al, 1995), FLICE (Los et al, 1995) and FLIP (Irmler et al, 1997) or phosphatases like FAP (Su et al, 1995) may also play a role in modulating the Fas death pathway within these resistant cells. For example, it is known that inhibitory proteins like FLIP bind to the death-effector-domains of FADD and thereby inhibit their association with FLICE which is an essential requirement for the formation of the death-inducing signalling complex (DISC) (Irmler et al, 1997; Thome et al, 1997). Further characterisation of resistance to Fas-mediated apoptosis and the role of inhibitory molecules such as FLIP needs to be conducted in our JLW cell system.

This chapter has therefore shown the generation of a sFasL reagent which has been validated and used in a variety of assays in the laboratory. During this analysis we observed the phenomenon of resistance to Fas-mediated apoptosis and this was explored further. Whilst the preliminary signalling experiments suggested no overwhelming role for JNK in Fas-mediated apoptosis, there remains nevertheless a large number of questions about the possible interaction of this pathway with other recently identified death signalling pathways. We have a good system with the JLW Fas resistant cell lines and CHO-Fas transfectants to explore this further in the future.

CHAPTER 5

FAS in Human SLE

INTRODUCTION

There are a number of mouse models of lupus, one of which is the MRL-*lpr* /*lpr* mouse which spontaneously develops features very similar to human SLE. The homozygous*lpr* mouse develops accelerated autoimmune features with arthritis, glomerulonephritis, skin changes and autoantibody production including amongst others anti-nuclear antibodies and antibodies to dsDNA (Cohen and Eisenberg, 1991). Following the identification that the *lpr* autoimmune phenotype was due to a defect of the Fas gene (Watanabe-Fukunaga et al, 1992) the importance of the gene was demonstrated by the dramatic reduction of autoimmune features in these mice with transgenic correction of the Fas gene within the T cells (Wu et al, 1994). The *lpr* mouse has a retrotransposon insertion into intron 2 of the Fas gene resulting in altered transcription and production of a non-functional Fas protein resulting in an inability to induce apoptotic cell death, particularly manifested in defective Fas-mediated peripheral deletion of activated lymphocytes and defective Fas-mediated cytotoxicity (Adachi et al, 1993). An allelic variant, *lpr* ^{cg} was discovered which has a single base mutation within the cytoplasmic death domain of the Fas gene and manifests with a very similar autoimmune syndrome (Watanabe-Fukunaga et al, 1992), highlighting the critical function of the cytoplasmic death domain of Fas. Thus, given the critical nature of this single gene in murine lupus, it was decided to explore the role of the Fas gene in human SLE. To study the role of Fas in SLE, we used an approach of looking for Fas gene polymorphism by PCR-SSCP analysis. Functional apoptosis assays were also assessed in the context of Fas engagement by both ligand (generated earlier in this project) and monoclonal anti-Fas antibody. An analysis of the clinical parameters in relation to apoptosis was carried out to see if any correlations were evident.

RESULTS

5.1) SSCP ANALYSIS OF THE FAS GENE

With the cloning of Fas as described in chapter 3 we had a human Fas cDNA probe which could be used for restriction fragment length polymorphism (RFLP) analysis. However RFLPs can only be detected when DNA polymorphisms are present in the recognition sequences for the corresponding restriction endonucleases. On the other hand, single stranded conformational polymorphisms (SSCP) have been shown to be an efficient and useful method for detection of point mutations at various positions in a fragment of DNA (Orita et al, 1989). SSCP analysis depends on the separation of single stranded DNA's of the same nucleotide length by polyacrylamide gel electrophoresis. Altered conformations of single stranded DNA as a result of even a single point mutation within the DNA gives rise to a different migration pattern on polyacrylamide gel electrophoresis. Therefore an approach using SSCP was employed to search for defects within the Fas coding region in human SLE.

5.1.1) Optimisation of SSCP gels for the detection of polymorphism.

In order to optimise conditions to be sensitive enough for the detection of minimal gene variation a set of Fas primers were designed to generate a mutant Fas cDNA differing from normal by only two nucleotide bases. The polyacrylamide gel in **figure 5.1.1** demonstrates that with optimum gel running conditions at 4°C, the mutant Fas PCR product was readily differentiated from the normal. However standard agarose gel electrophoresis was not sensitive enough to detect these minute changes in DNA sequence as seen in **figure 5.1.1 (b)**. Thus the technique of SSCP analysis was established within the laboratory with sufficient sensitivity to detect point mutations in the Fas cDNA. We could therefore proceed with analysis of the Fas gene in human SLE.

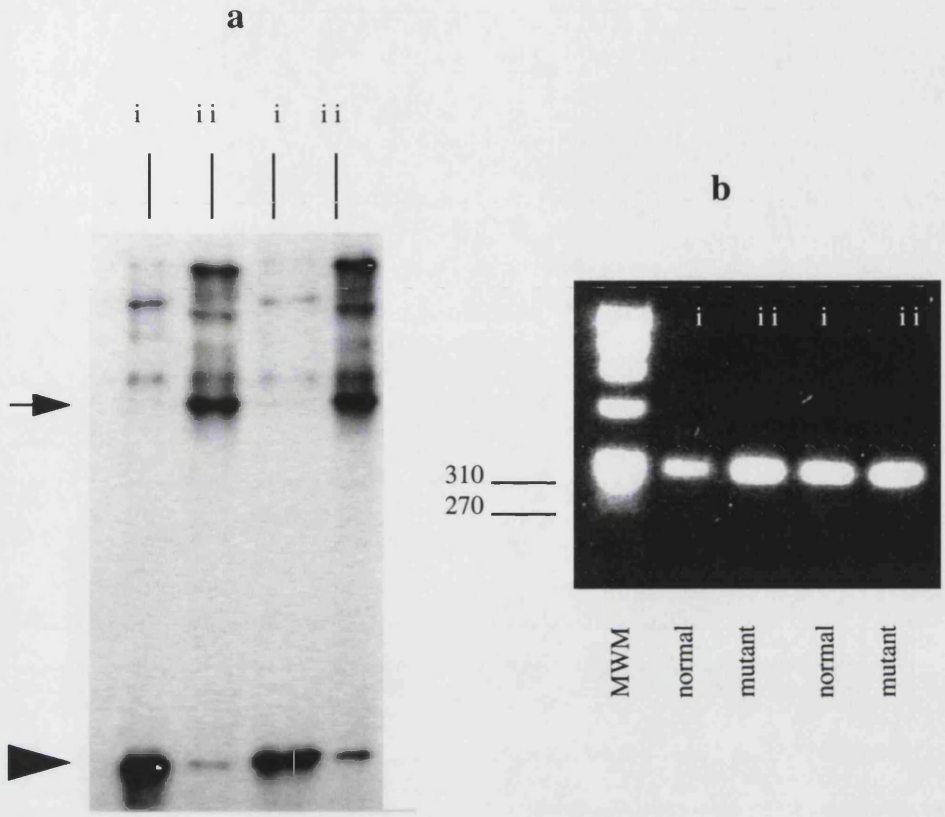


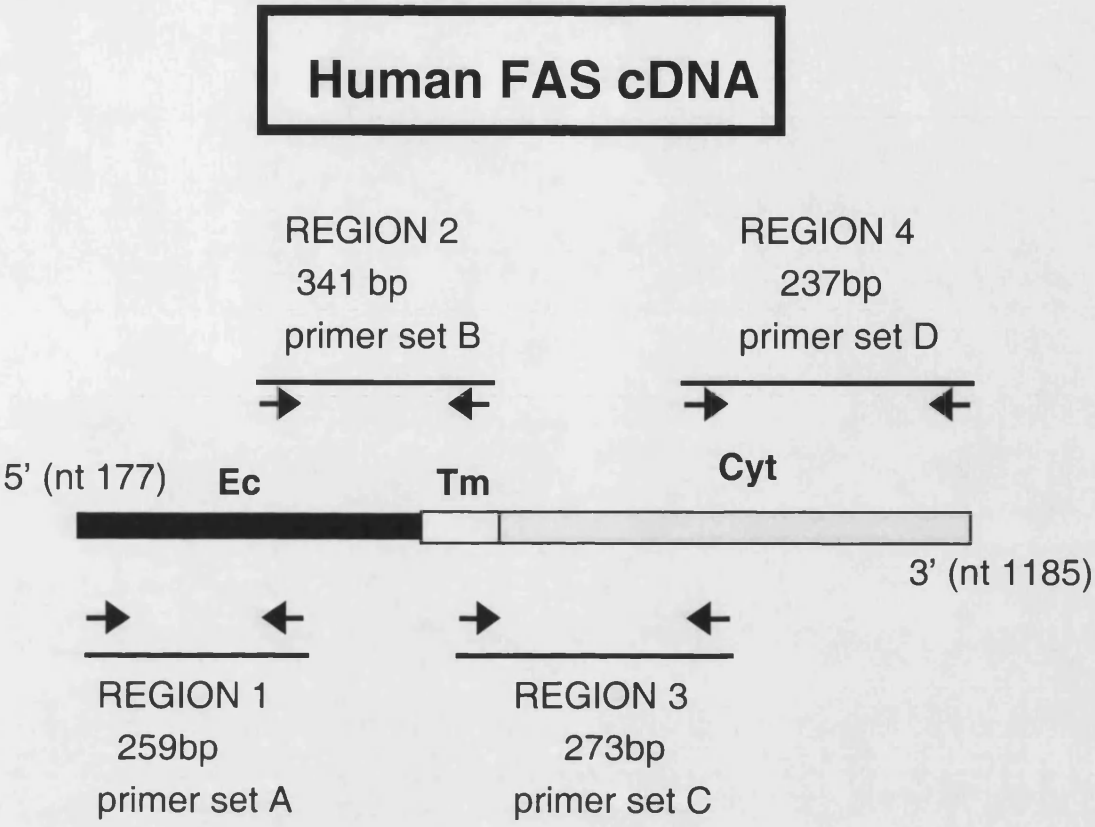
Figure 5.1.1: Optimisation of SSCP gels.

Optimisation of SSCP gels was achieved by using a mutant Fas product generated by PCR. A mutant primer (CATTCTCATTCAAGATCTCATC) was designed to produce a Fas PCR product which differed from the normal by two bases (AA replacing the normal TT at the position underlined). Identical PCR products of 270 base pairs were seen on 2% agarose gel electrophoresis **(b)** but non-denaturing polyacrylamide gel electrophoresis **(a)** showed a clear difference in mobility of the mutant Fas PCR products (lanes marked ii in both gels). Lanes marked (i) show the normal Fas 270 bp product which has a predominant lower band (large arrowhead) whilst the mutant has a retarded migration pattern (smaller arrow). Other conformational patterns are often evident with SSCP and can be seen in the upper bands as minor differences in DNA migration.

5.1.2) Analysis of Human Fas cDNA by SSCP analysis

In order to analyse the Fas cDNA, blood samples from patients and normal controls were obtained and processed by Ficoll gradient separation. Cells were stimulated with PMA / ionomycin to upregulate Fas expression and total RNA was isolated for cDNA generation by reverse transcription. Radiolabelled PCR was carried out for the SSCP polymorphism analysis under controlled conditions (chapter 2.4). Patients with SLE were chosen at random from a Connective Tissue Diseases clinic at the Royal National Hospital for Rheumatic Diseases in Bath, and blood from normal volunteers was taken from amongst staff at the hospital and the Bath Institute for Rheumatic Diseases. A total of 60 samples were analysed comprising 30 SLE patients and 30 normal healthy donors.

In order to study the Fas gene by SSCP polymorphism the cDNA was divided into four overlapping fragments as defined by the oligonucleotide primers shown in **Figure 5.1.2.** It was necessary to study the ±1000bp cDNA in four fragments because the sensitivity of the SSCP technique is reduced at larger fragment sizes (optimum DNA analysis on sizes up to ±350 bases).The line diagram in Figure 5.1.2 depicts the four regions amplified by PCR with region 1 corresponding to the extracellular domain of the Fas cDNA, region 2 comprising extracellular and transmembrane encoding domains and the cytoplasmic domain of Fas cDNA was represented within regions 3 and 4. The exact nucleotide and amino acid details of the four arbitrarily defined regions are provided in the legend to figure 5.1.2.



primer set A:	5' CGGAGGATTGCTCAACAACCATGCTG	(nt 177-202)
	3' TTCATCCCCATTGACTGTGCAGTCCC	(nt 433-408)
primer set B:	5' GGGACTGCACAGTCAATGGGGATGAA	(nt 408-434)
	3' TTGGCAAAAGAAGAAGACAAAGCCAC	(nt 744-720)
primer set C:	5' CTTTGTCTTCTTCTTTTGCCAATTC	(nt 725-747)
	3' CATTGTCATTCTTGATCTCATC	(nt 994-975)
primer set D:	5' GATGAGATCAAGAATGACAATG	(nt 975-995)
	3' CACTCTAGACCAAGCTTTGGATTTC	(nt 1210-1185)

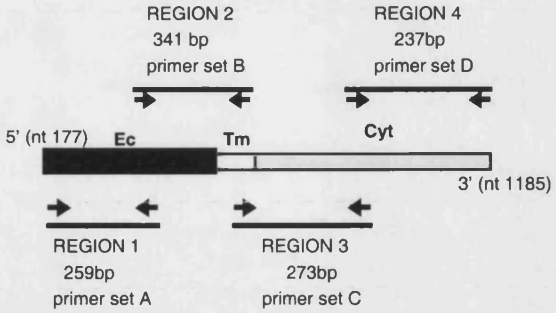
Figure 5.1.2 : Regions of the Human Fas cDNA defined for SSCP analysis.

A line diagram depicts the cDNA for human Fas. Primer sets were designed for each of the four regions which were defined arbitrarily on the basis of size of the DNA fragment (250 - 350 bp being the optimum for SSCP analysis). Region 1 corresponds to the extracellular (Ec) 77 amino acids of Fas, whilst region 2 includes amino acids 70 - 182 (AA 70 - 165 of Ec region and the 17 AA's of the transmembrane region (Tm). The cytoplasmic (Cyt) amino acids of Fas were divided between regions 3 and 4 (amino acids 175-264 and 258-336 respectively) with the "death domain" represented within region 3. The *lpr*^{cg} mutation of the mouse localises to region 3 (amino acid transition from Ile-225 to Asn-225 corresponding with a Val-238 to Asn-238 transition in humans).

5.1.3) SSCP polymorphism in Human SLE.

The results are shown in representative polyacrylamide gels demonstrating SSCP analysis of the FAS cDNA in **Figures 5.1.3a, b and c.** There was no evidence of polymorphism within the regions of the extracellular and transmembrane domains of the Fas cDNA **(figure 5.1.3a)**. The proximal part of the cytoplasmic domain (defined as region 3, **figure 5.1.3b)** which represents amino acids 175-264 revealed two predominant alleles (marked A and B) but there was no difference in the number or zygosity of alleles A and B when patients and normal samples were compared. As shown in figure 5.1.3b the predominant pattern was that of homozygosity for allele B, but there were no differences between normals and SLE patients. Equal numbers of patients and controls were also represented in alleles AA and AB.

Only one patient with SLE was found with a polymorphic band **(figure 5.1.3c)** in region 4 which comprised the Fas cDNA encoding the last 78 amino acids of the Fas protein (cytoplasmic region). Independent SSCP analysis confirmed the polymorphic band as shown in **figure 5.1.3d** and sequence analysis revealed a point mutation with an A to G transition at nucleotide position 1098 (translating to an Asp-300 replacing Asn-300). There were no unusual characteristics in the SLE phenotype of this patient. The results therefore show that except for the one patient with SLE no other genetic defects were evident in the Fas coding region in the sixty samples analysed.



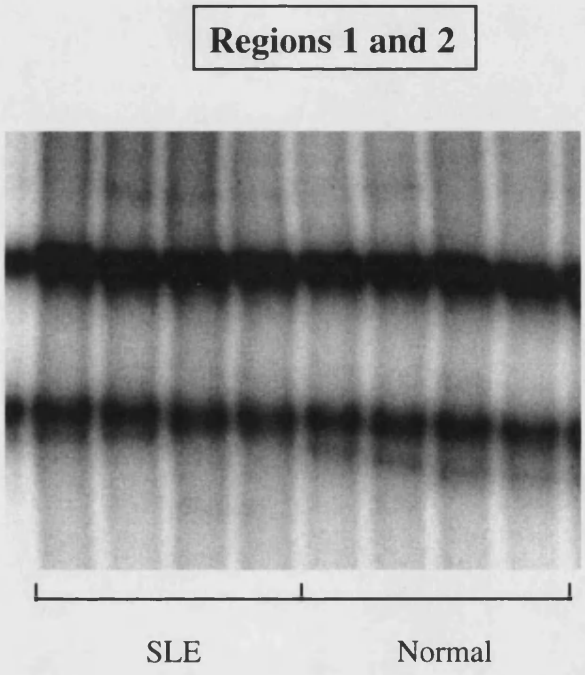
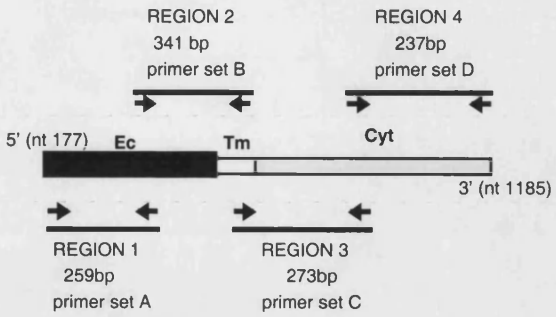
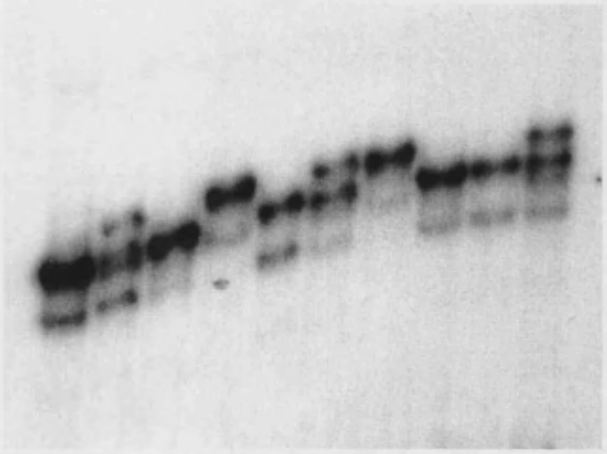


Figure 5.1.3 a: SSCP gel for regions 1 and 2.

Regions 1 and 2 as defined in the line drawing shown above and representing both the Ec (extracellular) domain and the Tm (transmembrane) domain of Fas cDNA showed no polymorphism by SSCP analysis. The SSCP gel shown is a representative autoradiograph of the 60 samples analysed, half of which were normal controls and half unselected SLE samples. Band patterns were identical for regions 1 and 2.



Region 3



Allele	Norm	SLE
AA	7	6
AB	6	6
BB	17	18

Figure 5.1.3 b: SSCP gel for region 3 of the Fas cDNA.

A representative SSCP gel for region 3 (as defined in the line drawing above) is shown. This region represents the cytoplasmic domain of Fas (amino acids 175-264) which contains the "death domain". Two predominant alleles were seen in this region (labelled A and B). Analysis by groups showed no differences between normal controls and SLE patients for either the number or zygosity of alleles. The table shows allele frequencies for normal (Norm) and SLE samples.

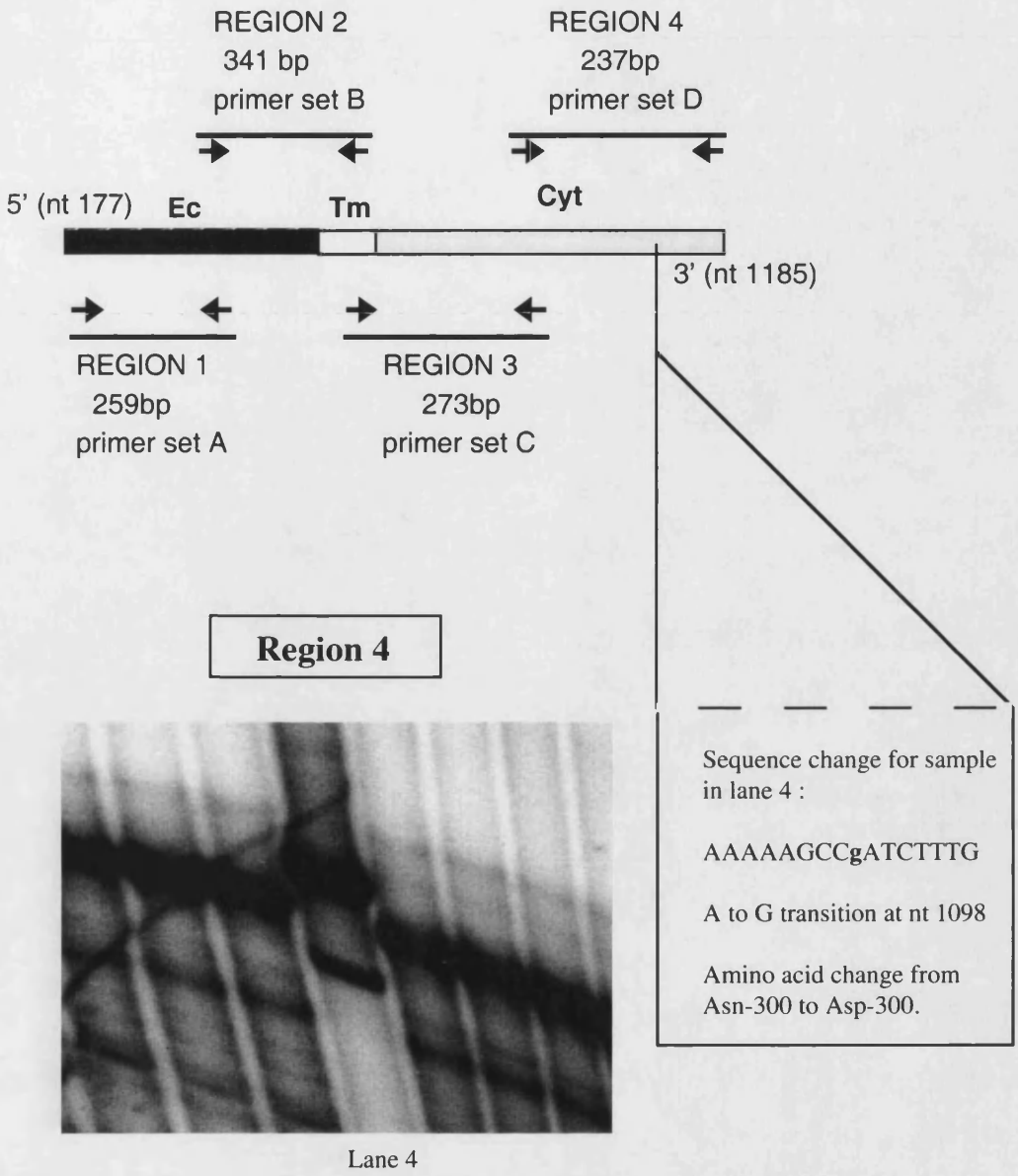


Figure 5.1.3 c: SSCP gel for region 4 of the Fas cDNA.

SSCP analysis for region 4 (as defined in the line drawing above) revealed one patient with SLE (lane 4) who had a polymorphic band as shown. This was independantly confirmed on two subsequent SSCP gels (one shown in figure 5.1.3d), and sequencing information revealed a point mutation at nucleotide 1098 (A to G transition resulting in amino acid change from Asn-300 to Asp-300). Further functional characterisation of this patient's T cells was carried out in the JAM assay and is shown in figure 5.1.3d.

In order to assess if the cytoplasmic mutation resulted in impaired apoptosis as occurs with the point mutation of cytoplasmic Fas in the *lpr*^{cg} mouse, functional analysis was carried out on activated T cells of the patient with the SSCP mutation. This was done using a JAM assay. Fas-mediated apoptosis was tested using both CH 11 and our sFasL on day six after activation of the T cells with PMA / ionomycin. FACS analysis for Fas expression was also carried out and did not reveal any defect in surface Fas expression on this patient's T cells.

Results of the in vitro apoptosis (as measured by a JAM assay) did not show any impaired apoptosis as expected from the *lpr*^{cg} model (figure 5.1.3d). In fact the Fas-mediated apoptosis was increased compared to normals. Therefore our in vitro analysis showed that the cytoplasmic mutation in our patient with SLE was associated with increased rather than decreased apoptosis. As a result, the question that arose was whether the enhanced apoptosis was due to the mutation occurring in the terminal "regulatory" domain of the Fas molecule. Deletion studies have shown that the terminal 15 amino acids of Fas have a downregulatory function (Itoh et al, 1993). With this in mind we undertook to study further patients and controls with the in vitro apoptosis assay to see if the above mutation at nucleotide position 1098 was in fact specifically associated with increased apoptosis in this patient.

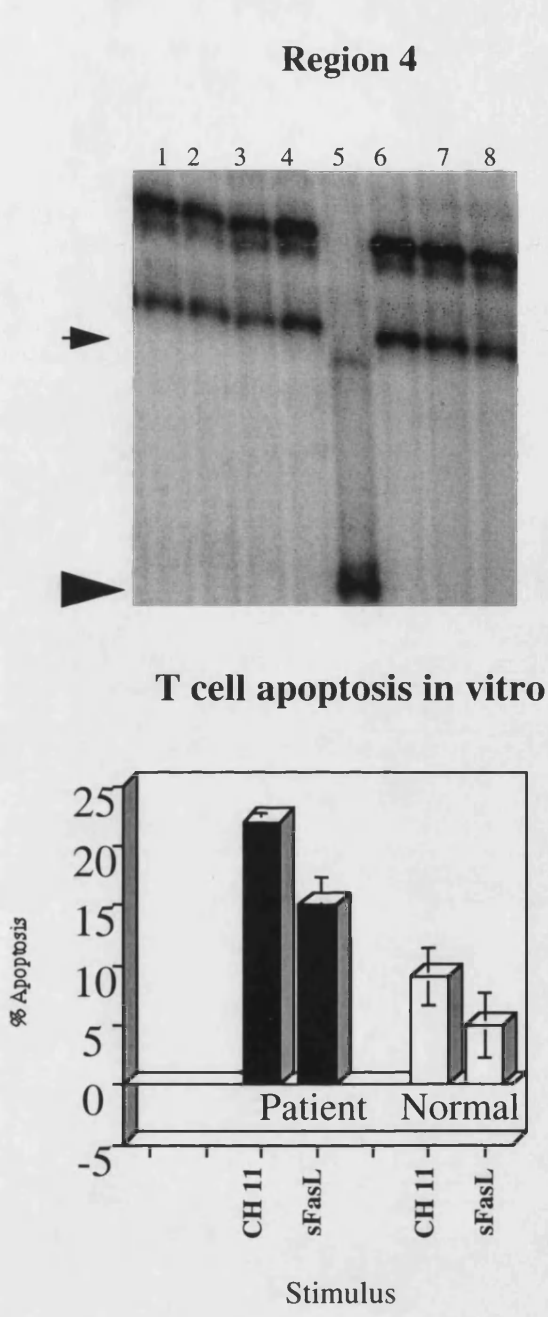


Figure 5.1.3 d: Functional characterisation of polymorphism from region 4.

An independant confirmation of the SSCP polymorphism is shown in the gel above (lane 5 corresponds to the same patient as in lane 4 figure 5.1.3c). An altered band migration can be seen (large and small arrows) compared to the surrounding normal and SLE samples. A point mutation was found in the cytoplasmic region of the patient cDNA (shown in figure 5.1.3c) and the T cells from this patient were further tested for in vitro apoptosis using the JAM assay. The results shown above suggest enhanced in vitro Fas-mediated apoptosis of the patient's T cells compared to normal.

5.2) APOPTOSIS IN SLE

Despite the absence of detectable genetic defects in the Fas cDNA of the SLE patients as a whole, it could still be possible that Fas function may be altered, and in light of the findings of increased apoptosis in the one patient with the cytoplasmic mutation it was essential to study the phenomenon in additional SLE patients. In the MRL-*lpr* mouse model the Fas gene abnormality is associated with impaired apoptosis, so that the autoimmunity is thought to be related to prolonged survival and function of activated lymphocytes. Thus an apparent discrepancy in our findings of increased apoptosis in this SLE patient needed to be addressed. We attempted to address the issue by studying the T cells from 20 samples (10 normal and 10 SLE). Therefore T cells from patients with SLE and normal controls were isolated and expanded as described (chapter 2.1.3) using PMA and ionomycin to induce T cell activation.

5.2.1) Upregulation of Fas surface expression on T cells

Published data have suggested that Fas-mediated apoptosis of T cells only occurred 5-6 days after activation (Owen-Schaub et al, 1992). In order to therefore optimise Fas expression for the apoptosis assays we first established a time course of upregulation of Fas surface expression using PMA/ionomycin stimulation. The results (**Figure 5.2.1**) confirmed maximal upregulation of Fas expression on T cells by six days after the mitogenic stimulus in our culture conditions. Therefore all samples obtained from patients and normal controls were handled under the same conditions so that apoptosis analysis could be conducted when Fas expression was at a maximum.

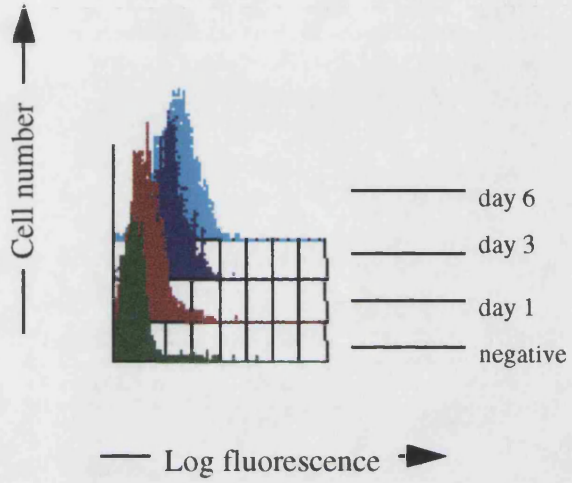


Figure 5.2.1: Upregulation of Fas expression on activated T cells.

Following PMA/ionomycin stimulation Fas surface expression was upregulated until day 6-7 as shown in this FACS analysis for Fas surface expression. Negative controls were stained with isotype-matched mouse IgM antibody and secondary detection was with anti-mouse IgM-FITC.

5.2.2 a) FACS analysis of T cells for Fas and FasL expression in SLE

In order to assess surface expression of Fas and FasL, T cells from SLE and normal controls were obtained as described (chapter 2.1.3). FACS analysis was conducted on day six activated T cells using CH 11 and Fas-Fc staining for Fas and FasL, respectively.

The results of the log scale mean fluorescence intensity (MFI) have been presented on a box plot in **figure 5.2.2**. In figure 5.2.2 (a) it can be seen that the Fas surface expression on activated-day-six T cells was significantly higher in SLE compared to normal controls ($p = 0.049$, Mann Whitney U test) but there was no significant difference in FasL expression ($p = 0.85$, Mann Whitney U test).

Therefore under the controlled culture conditions established there was increased surface expression of Fas on activated T cells from SLE compared to normal T cells.

5.2.2 b) In vitro Fas-mediated apoptosis of activated T cells in SLE

To assess in vitro apoptosis in the twenty samples, DNA fragmentation analysis by JAM assay was used. T cells from the cultures were removed on day six for FACS analysis and labelled with ^{3}H-thymidine for the JAM assay.

The results obtained from each sample have been computed as a box plot in **figure 5.2.2b.** Apoptosis induced by both CH 11 and sFasL showed a tendency to be increased in SLE compared to normal T cells. However, the differences did not reach statistical significance (p values 0.10 and 0.12 respectively for CH11 and sFasL). This may be explained by a relatively small number of samples, and the differences may become more significant if larger sample sizes are analysed. Based on an anticipitated 10 -

15% difference in the rate of apoptosis with a standard deviation of between 5 and 8, a sample size of 20 was enough to detect a difference at 5% level of significance. The power of such a study was calculated at 80% from standard nomograms. However it remains difficult to define what level of difference of apoptosis within such an in vitro assay may be biologically and medically relevant, despite statistical significance.

Therefore we can only conclude from these data that SLE T cells have a higher level of expression of Fas compared to normals and there is a tendency to enhanced apoptosis on Fas engagement.

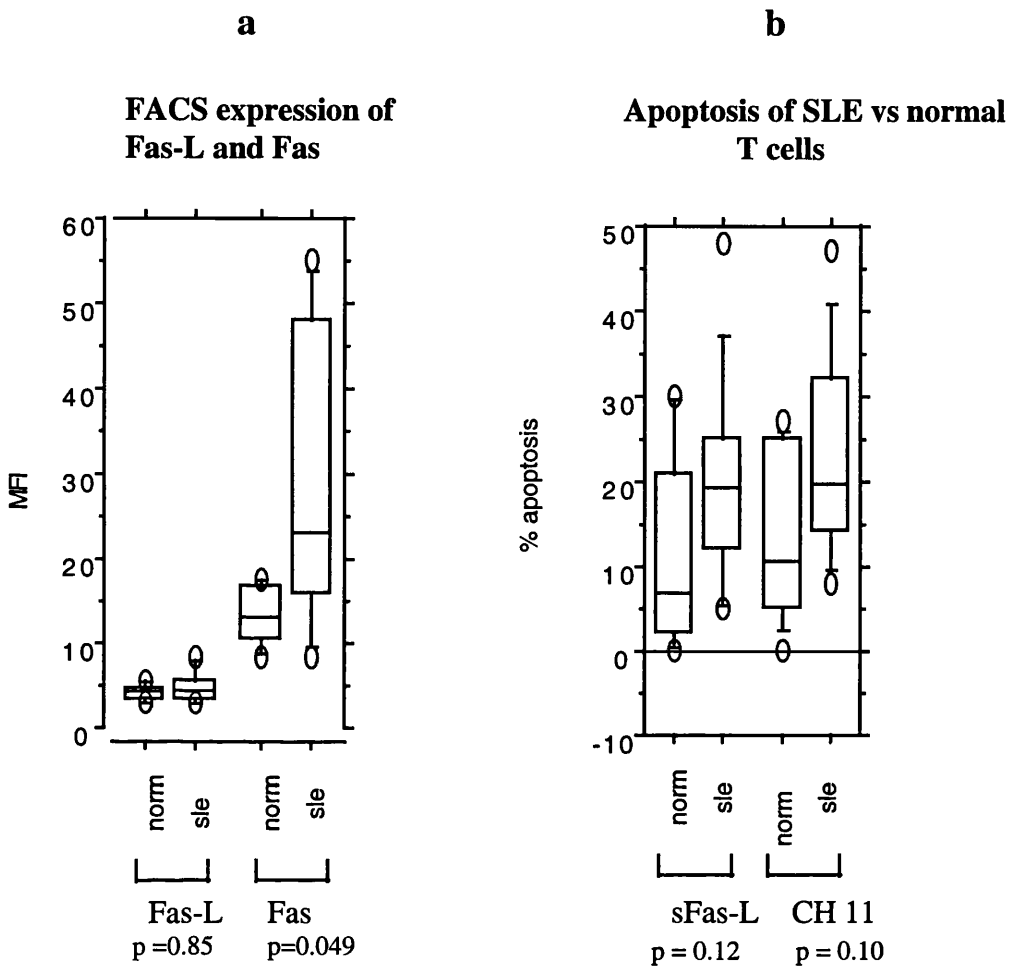


Figure 5.2.2 : Analysis of Fas expression and apoptosis in SLE.

FACS analysis revealed significantly increased surface expression of Fas (a) (p value 0.049) in the SLE patient group compared to normals, but there was no difference detectable in FasL expression using the Fas-Fc protein for staining (the results are shown as a box plot of the mean fluorescence intensity derived from FACS analysis of 10 samples in each group). Although there was a trend to increased in vitro apoptosis in SLE mediated by Fas (b) in the case of both sFasL and CH 11 there was no significant difference statistically (p values shown , using Mann-Whitney U test).

5.2.3) Correlation of apoptosis and clinical features of SLE:

The degree of in vitro apoptosis is not associated with clinical disease parameters in our group of SLE patients.

In order to assess if the increased apoptosis was associated with any clinical manifestations of SLE, statistical correlation analysis was conducted. The SLICC index is a validated score of total tissue damage in SLE (Gladman et al, 1992) and it was decided to assess if apoptosis as measured in the in vitro assay correlated with the amount of tissue damage seen in SLE.

Figure 5.2.3 (a) shows the statistical correlation plot for apoptosis versus the SLICC index and there was no statistically significant correlation ($p = 0.495$, Spearman correlation). Similarly there was no statistically significant correlation between the degree of apoptosis in SLE and peripheral lymphocyte counts (shown in figure 5.2.3 b) or levels of double stranded DNA antibodies (dsDNA). The sample size was too small to analyse with any confidence the relationship between percent apoptosis and autoantibody status (for example u1RNP positivity).

Therefore our data suggested a trend towards an inverse correlation between the degree of in vitro apoptosis and lymphocyte counts and to a lesser extent between apoptosis and organ damage (SLICC index). However once again the results were not statistically significant and further analysis may be warranted on a larger sample size.

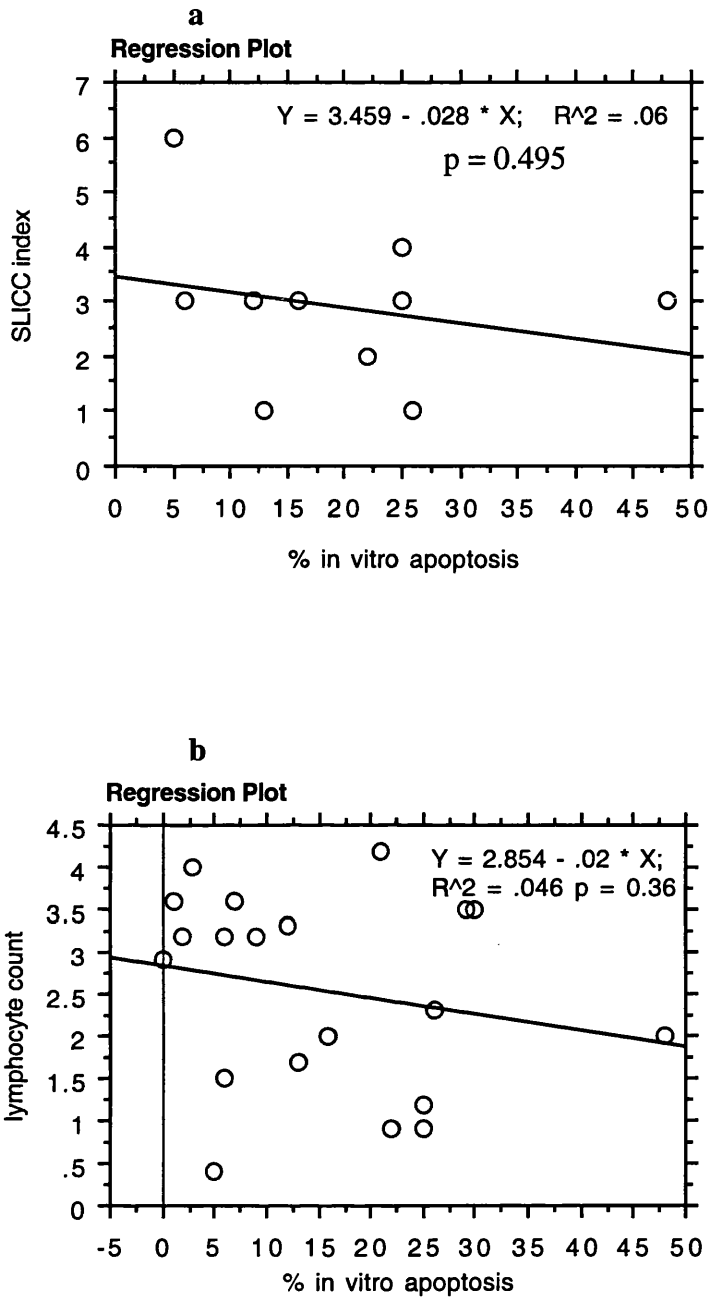


Figure 5.2.3 : Correlation analysis of Apoptosis versus SLE disease parameters.

Statistical analysis of the 20 samples (10 normal and 10 SLE) for correlation between the percent in vitro apoptosis and SLE disease parameters was conducted using Spearman correlation and regression plots as shown above. No significant relationship was found between organ damage (as defined by the SLICC index) and apoptosis in vitro (a). Similarly although there was a trend towards an inverse relationship between the degree of apoptosis and the lymphocyte count (b), no statistically significant association could be found.

DISCUSSION

It was very clear from the MRL-*lpr* mouse model of SLE that the Fas gene played a critical role in the development of the autoimmune manifestations within this mouse (Watanabe-Fukunaga et al, 1992). The critical nature of the Fas gene defect was highlighted by the fact that the autoimmune features were abrogated by a transgenic correction of the Fas gene defect within these mice as already mentioned (Wu et al, 1994). Interestingly the *lpr* ^{cg} mouse also developed the same autoimmune features with only a single point mutation within the cytoplasmic region of the Fas gene (Watanabe-Fukunaga et al, 1992). Consequently, we undertook to study the role of Fas gene defects in human SLE. In order to achieve this we established the technique of SSCP analysis. SSCP analysis for DNA polymorphism was established for the first time in our laboratory using primers designed to generate a PCR mutant of Fas. A comparison of the mutant and normal Fas products which differed by only two bases allowed us to establish optimal gel electrophoresis conditions for the detection of mutations within the Fas gene. Consequently, PCR-SSCP analysis was conducted on sixty samples in total (thirty with SLE and thirty normal controls). The results showed no significant polymorphism within the Fas cDNA. Despite interesting allelic patterns evident in region 3 (figure 5.1.3b) there was no segregation of any particular allelic pattern with disease phenotype. However, one SLE patient revealed a band shift in the cytoplasmic region of the Fas cDNA. This SSCP band shift was confirmed on two further occasions before sequence analysis was conducted. Sequence analysis on the latter patient showed a point mutation within the cytoplasmic death domain but unlike the *lpr* ^{cg} mouse there was no impairment of Fas-mediated apoptosis. In fact this patient had evidence of enhanced in vitro apoptosis when measured by a JAM assay of DNA fragmentation, and its significance in relation to the site of the mutation has been discussed with the relevant results (section 5.1.3). However, as a result of the finding of increased death of lymphocytes by apoptosis in this patient with SLE, it was decided to explore if this occurred in other patients with SLE. Thus the apoptosis assays and reagents generated and described in the previous two chapters were exploited to study functional aspects of Fas biology in the context of human

SLE. Contrary to the findings of decreased Fas expression and apoptosis in the MRL-*lpr* mouse, we found increased Fas expression on the surface of activated T cells from SLE patients and a tendency to enhanced in vitro Fas-mediated apoptosis. However, we could not find any statistically significant correlations with disease manifestations and the increased Fas-mediated apoptosis.

Other research groups studying the Fas gene in SLE have found no significant polymorphism related to disease, thus confirming our findings of a lack of Fas gene polymorphism in SLE (Fiucci et al, 1994). The latter study identified point mutations in three patients which proved to be insignificant because they caused no amino acid change in the protein. Therefore our approach of SSCP analysis whilst novel in its application to the analysis of the Fas gene in human disease, has not revealed any major genetic defects in human SLE. It remains to be seen if genetic defects can be identified for other Fas-associated molecules like FADD, FLICE, FLIP etc.

Much controversy surrounds the possibility that alternative splice variants producing a soluble Fas molecule may be elevated in SLE. The presence of elevated soluble Fas molecules could theoretically inhibit Fas-mediated apoptosis and thereby produce an *lpr*-like phenotype in humans (Cheng et al, 1994). However, the detection of soluble Fas has not been consistently reproducible in different laboratories and elevations have been found in diseases other than SLE (Hasunuma et al, 1997; Knipping et al, 1995; Goel et al, 1995). In addition the method of identification of levels of soluble Fas is questionable. Cheng et al (1994) first described an ELISA for measurement of soluble Fas but this was based on a variant of Fas found in one patient which was subsequently cloned and expressed. It would seem inappropriate to base a general population analysis of soluble Fas levels on an assay derived from a recombinant mutant Fas variant. There was no evidence of splice variants on our SSCP analysis where we would have expected that splice variants producing soluble Fas would be detectable as a change in size of the cDNA. Now that the genomic structure of the human Fas gene is available (Cheng et al, 1995) it may be possible to consider an approach of SSCP analysis of genomic DNA

from our SLE population. This may yield information about defects in the intronic sequences of Fas which would not have been detectable with our analysis of cDNA.

In addition to the genetic analysis we pursued functional analysis of apoptosis in an attempt to study if Fas was playing a role in the pathogenesis of SLE. We found increased in vitro apoptosis in some patients with SLE, but the overall analysis of the group (10 patients compared to 10 normals) did not show significantly enhanced apoptosis. The trend may however prove to be statistically significant with a larger sample size. Enhanced apoptosis in SLE has also been reported by Emlen et al (1994) in patients with SLE and subsequently by other authors (Ohsako et al, 1994; Amasaki et al, 1995). However, most conclusions have been extrapolated from the enhanced Fas expression seen on FACS. Our study identifies a direct Fas-mediated in vitro assay for measuring apoptosis using a natural ligand for Fas and could now be applied to the study of more patients with SLE and other autoimmune diseases. The identification of enhanced in vitro apoptosis in human SLE appears to be contradictory to the findings of impaired apoptosis in the MRL *lpr /lpr* mouse. However in vitro studies in the *lpr* mouse have also shown increased apoptosis (Van Houten and Budd 1992). Whether these in vitro findings of increased apoptosis represent an artefact or not is uncertain. The enhanced apoptosis may simply reflect the activated state of lymphocytes from the SLE patients on removal from the body and this will need further analysis in the context of other diseases. Better in vivo analysis of apoptosis will also be needed to define the relevance of these in vitro findings.However, a suggested hypothesis that may explain the increased apoptosis and autoimmunity is provided by data from Casciola-Rosen et al (1994). They reported specific autoantigen presentation within apoptotic blebs after ultraviolet exposure of skin keratinocytes. In addition the u1RNP protein and other autoantigens like PARP, Topo-1, Ro and La antigens (Casiano et al, 1996) have been found in apoptotic particles following Fas stimulation. SLE skin is particularly sensitive to ultraviolet irradiation and it is conceivable that the apoptotic blebs containing cleaved autoantigenic products may flood the macrophage phagocytic system and allow antigen processing with a resultant autoimmune response including the autoantibody production reflected in these disorders.

In view of our findings of increased apoptosis and the supportive data from the literature it was decided to analyse our patient group for any relationship between the level of apoptosis and the clinical manifestations of SLE. No significant correlation was detectable between the level of in vitro apoptosis and the clinical and serological features of SLE. The SLICC index is a validated damage index in SLE (Gladman et al, 1992) which reflects systemic tissue damage. Given the exaggerated apoptosis in vitro in the SLE samples it appeared reasonable to look for a correlation between the degree of apoptosis and tissue damage, but no statistically significant correlation could be found. Similarly there appeared to be no significant correlation between the degree of apoptosis and the lymphocyte count which is characteristically depressed in SLE. However, the trend of the correlation with lymphocytes suggested an inverse relationship and other workers have also found an inverse relationship between lymphocyte counts and percentage apoptosis (Amasaki et al, 1995). The difference in results may be explained by a difference in methods of apoptosis detection. Statistical significance of any relevant correlations may only become apparent with a greater sample size. Also subset analysis of larger groups of patients with SLE may allow us to identify if any associations exist between autoantibody production (such as u1RNP) and the enhanced in vitro apoptosis.

It must be noted that the *lpr* gene only causes autoimmune features on certain backgrounds in mice (Izui et al 1984). For example the *lpr* gene defect only results in significant autoimmunity in MRL mice but not in many other strains of mice. This would be in keeping with human SLE which is probably a multi-genetic disorder with a strong influence of environmental factors. From our analysis so far the Fas gene appears not to have any major defects in human SLE, but interesting functional defects may be present as outlined above. We have not conducted any analyis on the ligand for Fas, but one paper suggests that from a sample of 75 SLE patients only one had a defective FasL gene with resulting impaired apoptosis (Wu et al, 1996). It is possible that the Fas / FasL genes may play a role in only a subset of patients with SLE having specific features such as lymphadenopathy or glomerulonephritis. Given the heterogenous nature of SLE it may

be necessary to identify particular sub-groups by clinical parameters or autoantibody profiles for further analysis of functional Fas-mediated apoptosis defects. This will be necessary in order to complete the analysis of the role of Fas as a candidate gene in the pathogenesis of SLE.

Increased Fas-related apoptosis has been associated with the development of diseases like Hashimoto's thyroiditis (Giordano et al, 1997), Fulminant hepatitis (Kondo et al, 1997), Insulin dependent diabetes (Chervonsky et al, 1997) and graft versus host disease(Baker et al, 1997). Apart from the features of autoimmunity in some of these conditions, they appear to be unified in their pathogenesis by the process of Fas-mediated destruction of tissue. Despite the disappointing lack of evidence so far of a direct role for Fas gene defects in human SLE, a very interesting group of patients have been identified with Fas gene defects and disease resembling the *lpr* mice in that they develop marked lymphoproliferative disease. It appears to affect mainly children and has been reported as various diseases including Autoimmune Lymphoproliferative Syndrome (AILPS) and the Canale Smith Syndrome (Fisher et al, 1995; Rieux-Laucat et al, 1995; Drappa et al, 1996). These probably represent the same disorder which is characterised by non-malignant lymphoproliferation with varying grades of autoimmune manifestations, associated with a variety of Fas gene defects and impaired apoptosis. Most of the defects are associated with dominant Fas gene defects but curiously a number of family members with heterozygous Fas gene defects had some in vitro abnormalities without the associated clinical features of disease (Rieux-Laucat et al, 1995). Whether or not the association with lymphoma development in some of the family members is real needs to be defined. There has been an association between SLE and malignancy (Banks et al, 1979) and it would be interesting to look for Fas-related defects in these subgroups of SLE patients. This may have implications in the future identification and management of patients at high risk for the development of malignancy either de novo or secondary to the immunosuppressive drugs like azathioprine which are used in the treatment of SLE.

Is there a unifying hypothesis to explain Fas-associated increased apoptosis in the pathogenesis of the SLE, whilst simultaneously explaining a possible role of impaired apoptosis in the subgroup of patients with malignancy? Given the number of associated death domain proteins that determine Fas function it is possible that a link could be found. One theory suggests that retroviruses may play a role in the pathogenesis of SLE (Izui et al, 1981). Whilst retroviral insertions into the Fas gene of the *lpr* mouse have not been seen thus far in human SLE, it is conceivable that as yet unidentified viral proteins may interact with the Fas signalling mechanisms to increase apoptosis. It is known that the terminal 15 amino acids of Fas plays a regulatory role in reducing apoptosis (Itoh et al in 1993) and viral proteins could theoretically interfere with this death domain function to "release the brake" as it were. Lymphotrophic viruses may explain the lymphocyte selective effect in SLE. On the other hand, it is possible that other as yet unidentified viral proteins resembling CrmA or Baculovirus p35 may block Fas signalling via the Caspase pathway leading to impaired apoptosis and the development of malignancy. Very recent identification of a viral like protein (FLIP) (Irmler et al, 1997) which inhibits apoptosis and another protein (Daxx) (Yang et al, 1997b) which enhances Fas associated apoptosis, have shown that the hypothesis is reasonable and other viral interacting proteins may well be identified in the future. It is possible that our understanding of the relationship between "genes and the environment" may take on an entirely new meaning in relation to Fas and SLE pathogenesis.

CHAPTER 6

FINAL DISCUSSION and CONCLUSIONS

In order to place this work in context it must be emphasized that when the project commenced essentially no reagents were available for the study of human Fas except for the CH 11 and anti-Apo-1 antibodies. Therefore in order to embark on a study relating to human Fas one of the primary objectives was the generation of appropriate reagents. This was undertaken using molecular and cellular techniques, firstly to modify and express a human Fas-Fc protein, and secondly to generate a soluble Fas ligand (sFasL) protein, both of which have been demonstrated to have specific effects in chapter 3 and 4 respectively. In addition, both reagents have proven useful in extending our understanding and contribution to current knowledge of Fas-FasL biology in human T cells (Boshell et al, 1996; McLeod et al, 1998; Walker et al, 1998). These reagents, together with the expertise acquired in doing the DNA manipulation to generate them, also allowed us to extend our analysis to the role of the Fas gene in human SLE (chapter 5).

As described in chapter 3, the Fas-Fc protein was successfully generated by expression of a cloned Fas-Fc cDNA in a COS cell culture system. After validation and testing of the Fas-Fc, it was used as a blocking reagent in a variety of Fas apoptosis assays and, excitingly, particularly in the context of the time at which this work was carried out, Fas-Fc was useful in identifying a putative Fas ligand on the surface of activated T cells and Jurkat T cells. Specific immunoprecipitation of a 40Kd putative ligand was also carried out at that time when no other information was available on FasL. However, as it turned out, we encountered some problems with batch variation which took some months to explain, and other research workers using a very similar approach with a murine Fas-Fc protein were able to identify and clone the first ligand for Fas, albeit from a rat hybridoma (Suda et al, 1995). Subsequent data from them confirmed our findings that FasL was expressed specifically on activated T cells, but with further study it became evident that mRNA expression of FasL could be detected in a variety of other lymphoid and non-lymphoid cells. More recently, expression of FasL has also been found on immune privileged tissue such as

the testis and cornea (Bellgrau et al, 1995; Griffith et al, 1995) and also on certain maligant tumours (section 1.3.7). Thus it was established over the recent years that FasL-Fas interactions were important in a variety of situations including lymphocyte cell death following activation (Nagata and Golstein, 1995), cytotoxicity mechanisms (Carter et al, 1995; Podack et al, 1995), NK cell function (Arase et al, 1994), neutrophil cell death (Liles et al, 1996) , eosinophil function (Tsuyuki et al, 1995), monocyte function (Shinohara et al, 1995), as well as some incompletely defined role in the function of hepatocytes (Galle et al, 1995), muscle cells (Seino et al, 1996), a variety of brain cells (Dowling et al, 1996; Dsouza et al, 1996), synoviocytes (Kawakami et al, 1996; Matsumoto et al, 1996), keratinocytes (Sayama et al, 1994), and thyroid cells (Giordano et al, 1997). The implications for therapeutic manipulation have therefore become far wider than originally anticipated, where Fas-FasL interactions were thought to be solely of importance in the maintenance of immune homeostasis.

As regards FasL surface expression, it also became clear that FasL itself was largely unstable on the cell surface and required metalloproteinase inhibitors to allow retention on the cell surface for detection by FACS analysis (Kayagaki et al, 1995). Besides offering some explanation for our findings of heterogenous expression of FasL on T cells when using our Fas-Fc construct as a "primary antibody", this finding that FasL could be secreted as well as maintained as a cell surface protein offered a variety of possibilities in terms of FasL function. It became clear that FasL could act to mediate apoptosis via the Fas receptor not only by cell-cell contact but also by acting as a soluble mediator, similar to its prototype family member, TNF (Dhein et al 1995). Thus in situations where activated lymphocytes need to be eliminated, cell-cell contact such as occurs in the tight compartments of lymphoid tissue, will allow FasL-induced killing of sensitive Fas-positive targets cells. Similar cell-cell contact is probably responsible for the Fas-mediated apoptosis operative in cytotoxic and NK cell function, and in the maintenance of immune privilege (Brunner et al, 1995 and Ju et al,

1995). In other situations however, where cell-cell contact may not be as evident, soluble FasL secreted into the cellular environment may play a role. It is unclear if this latter mechanism is the major one responsible for target cell destruction in situations like fulminant hepatitis, inflammatory muscle disease, cardiac disease and so on, where not as much cell-cell contact may be seen when compared to lymph nodes. As an extension of this hypothesis, one needs to consider if the small number of activated lymphocytes within these tissues may be responsible for soluble FasL release and innocent bystander killing of the Fas positive cells within the environment. Thus our findings that a human sFasL generated in vitro could effectively mediate apoptosis is consistent with recent descriptions of naturally produced soluble FasL (Dhein et al 1995). Our sFasL may therefore prove even more useful as a reagent for analysis of Fas-related cell-cell killing versus non-cognate cellular mediated apoptosis in a variety of diseases. With further isolation and purification of the sFasL it may even have some role in future for appropriate manipulation of the Fas system to enhance apoptosis where defective (e.g. lymphoid malignancies and drug resistance).

In order to manipulate the Fas system for therapeutic benefit, a better understanding is required of the signalling mechanisms which are engaged following Fas receptor engagement. We have shown that Fas engagement with both antibody and sFasL was associated with signalling via the sphingoymelinase and JNK pathways. However, both these signalling pathways are also activated by T cell proliferative signals, in particular CD28 (Su et al, 1994). It is unclear if these signalling pathways mediate multiple effects or whether the activation simply reflects bystander activation secondary to activation of other enzymes. Alternatively, activation of sphingomyelinase and JNK may reflect cellular activation of a cascade of reactions common to a number of stimuli, both proliferative and apoptotic and thereby serve to integrate subsequent signalling and cellular outcome. More work is required to understand the complex signalling events and their relevance to Fas-mediated apoptosis in particular.

It appears that the Caspase cascade may be more specific to apoptosis signalling, but this also does not have specificity for Fas-based signalling (Martin and Green, 1995). Other protein interactions have been defined that suggest a hierarchy of protein-protein interactions with the Fas death domain (Cascino et al, 1996; Chinnaiyan et al, 1995 and Muzio et al, 1996). Thus it appears that following Fas engagement, a death-inducing-signalling complex (DISC) is assembled on the Fas cytoplasmic death domain. This comprises proteins like FADD (Chinnaiyan et al, 1995; Muzio et al, 1996) and FLICE (Enari et al, 1996; Muzio et al, 1996) which may then induce the Caspase cascade and mitochondrial changes with committment to apoptosis. Negative regulation of this signalling pathway appears to be achieved through other death domain interacting proteins like FLIP (Irmler et al, 1997) and Daxx (Yang et al, 1997b). A number of other signalling mechanisms have now been described following Fas engagement, including FAP and other phosphatases, but the exact significance within the death signalling cascade is not clearly defined as yet. Our transfectant system and reagents generated in this project could prove useful in dissecting some of these signalling pathways in order to define their significance. In particular, given that single genes regulating each step in the cascade of signalling can be introduced in a step by step manner into the CHO-Fas system, we may also be able to define the hierarchy of events following Fas receptor stimulation.

One consequence of the multiple interactions required for Fas-induced apoptosis is that not all Fas stimulation results in death by apoptosis. It has become evident that the appropriate engagement of death domain interactions is required in order to commit a cell down the death pathway (Chinnaiyan et al, 1995; Muzio et al, 1996). These discoveries shed tremendous light on our findings of resistance to Fas-induced apoptosis which were recorded at a very early stage of our investigations. In retrospect the original publications relating to Fas/Apo-1 also recorded a sensitivity to Fas stimulation of around 30-35% but the excitement of the discovery of Fas as a "death

molecule" was probably the reason for this observation being overlooked (Owen-Schaub et al, 1992; Klas et al, 1993). However, subsequent publications by the same authors have now acknowledged the concept of resistance to Fas-induced apoptosis, and there is a flurry of activity underway to attempt to explain it. We have described a Fas resistant cell line generated from Jurkat T cells (JLW, chapter 4), which displayed Fas surface expression whilst demonstrating resistance to Fas-induced apoptosis. Our findings of intact but slightly reduced JNK signalling needs to be explored further, but an analysis of the role of the other death-domain-interacting proteins is clearly indicated. Resistance to Fas-mediated apoptosis may explain some of the diseases associated with Fas / CD95, including rheumatoid arthritis (Nishioka et al, 1998), Sjogren's syndrome (Elkon, 1997), and drug resistant lymphoid malignancies. In these diseases Fas expression has been noted in lesional tissue but there appears to be an obvious survival, if not proliferation of the diseased tissue as is the case in the rheumatoid synovium, the lymphoid aggregates in sjogrens exocrine glands, and the persistance of Fas-positive lymphoma cells. More research work is clearly indicated before we can make any attempts to manipulate the Fas system in these diseases.

In order to test the reagents that were generated a number of apoptosis assays have been established and validated. Using one of these apoptosis assays (JAM assay for DNA fragmentation) we have been able to apply one of our generated reagents (sFasL) to study a prototypic autoimmune disease, SLE. Whilst no significant Fas genetic defects have been found in our SLE patients there are some interesting questions that arise from the evidence of increased in vitro apoptosis. Other approaches can also still be used to complete the analysis of the role of Fas and FasL in human SLE. It should be remembered that SLE is much more complex in its clinical phenotype than the MRL *lpr /lpr* mouse. Whilst the Fas gene defect in the mouse appears critical to the development of disease, this is clearly not the case from our studies thus far in human SLE . In order to define if the Fas-FasL system plays any role in the pathogenesis of human SLE, it will be necessary to study specific subgroups of patients with features

of lymphadenopathy, malignancy and autoantibody defined subgroups. The analysis must include not only genetics of Fas and FasL but also functional studies of apoptosis in these groups of patients especially in light of our results that functional changes can occur despite normal genetic sequences.

Future work - a personal perspective.

Research into Fas and FasL biology is progressing at a rapid rate but much remains to be learnt about the mechanisms of resistance and sensitivity to Fas. With the identification of many new proteins that interact with the Fas signalling pathway, there will be many years of work ahead to try to identify key signalling interactions that determine cellular sensitivity to Fas engagement. Only with further understanding of these mechanisms could the Fas-FasL system be fully exploited for possible therapeutic intervention in relevant lymphoproliferative disorders and autoimmunity.

It remains to be seen if subsets of SLE with clinical features more closely resembling the *lpr* phenotype can be identified to have structural Fas gene defects. Clearly genetic studies need to be completed for Fas-L in SLE and other autoimmune diseases. Given the data on selective autoantigen presentation within apoptotic blebs it may be important to subset patients with SLE and other autoimmune diseases like Scleroderma by their autoantibody profiles to see if there is any link between Fas-mediated apoptosis and disease.

The question of enhanced apoptosis rather than impaired apoptosis in autoimmune pathogenesis needs to be addressed. This may be approached also through an analysis

of patients by autoantibody subsets but further supportive evidence will be needed for this hypothesis in vivo possibly using animal models.

The relevance of apoptosis in vivo in human disease is a difficult issue, but with rapid advancement in cellular technology, it is not unreasonable to aim in the future to label patient cells in order to track their outcome in vivo with appropriately designed "tools of measurement". This may be very useful for example to define if the lymphopaenia of SLE is due to enhanced destruction by apoptosis.

It is the case so often in medical science that the discovery of a new molecule usually comes with the expectation that answers and cures will be found. In true fashion also, the discovery of Fas has brought with it many more questions that need to be answered before the knowledge can be fully applied for the benefit of patients.

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Journal of Immunology *159*, 2108-2115.

APPENDIX 1

A list of suppliers and contact telephone numbers follows in alphabetical order.

Amersham International plc (Little Chalfon, Bucks, UK)	01494 544000
Appligene (Britley, Chester-le-Street , UK)	0191 4920022
BDH (Merck Ltd)	0800 223344
Becton Dickinson UK Ltd (Cowley , Oxford, UK)	01865 748844
Boehringer Mannheim (Lewes, East Sussex, UK)	01273 480444
BIO 101 Inc (La Jolla , California , USA) via Stratagene UK	
BioRad Laboratories Ltd	01442 232 552
CP Pharmaceuticals Ltd (Wrexham,UK) via Fisons UK.	01509 231166
Dynal (UK) Ltd (Croft Business Park, Bromborough,UK)	0151 3461234
Fahrenheit Lab. Supplies (Kingswood, Bristol, UK)	0117 9701667
Fisons Ltd (Loughborough, Leics, UK)	01509 231166
Genetic Research Instruments Ltd (Dunmow,Essex,UK)	
ICN Biochemicals Ltd (UK)	01844213366
Kodak (IBI limited)	0800 581700
Life Technologies (Paisley , Scotland, UK)	0141 8146100

LKB (see Wallac Ltd)	01509 231166
NBL Gene Sciences Ltd (Cramlington, Northumberland,UK)	01670 732992
New England Biolabs (Hitchin, Hertfordshire,UK)	01462 420616
Novagen (via AMS Biotechnology UK Ltd)	01993 706 500
Nycomed UK Ltd (Sheldon, Birmingham, UK)	0121 7422444
Pharmacia Biotech Ltd (St Albans, Herts,UK)	01727 814000
Phoretix International	0191 230 2121
Promega Ltd (Enterprise Road, Southampton,UK)	01703 760225
Sigma Aldrich Ltd (Poole, Dorset,UK)	01202 733114
Skatron Ltd (Newmarket, Suffolk, UK)	01638 660600
Stratagene	01296 714 071
Unipath Ltd (Basingstoke, Hants,UK)	
Wallac Ltd (Turka, Finland) - distributed via Fisons , UK.	01509 231166
Whatman Scientific (UK)	01622 692022