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Bi-directional Tumour-Host Interaction in a Murine Model

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In memory of my father-in-law, John Slattery, who lost his battle with cancer, after only 4 weeks, on 27th May 2000 and in honour of my mother, Eileen O'Brien, who after 6 years seems to be winning her fight with cancer. What we know, or think we know, is only the beginning.

'Cancer is unimaginably complex. Nevertheless, one must steadfastly, systematically, and unpretentiously hew through the vast thicket of the unknown toward a distant envisaged goal, knowing full well that one may never reach it but only approximate it. Goals have a tantalizing way of receding as one proceeds. Yet they give guidance. One should strive for answers to unsolved questions and not trim questions to answers already available.'

(Weiss 1971)

Abstract

Tumours evoke immunologic responses yet they continue to grow and develop. The majority of people who develop tumours are immunocompetant, evoke immune responses and yet metastases occur in lymphnodes, potentially the most hostile immune environment. This is the paradox that we are attempting to unravel. It is hoped that this work would eventually lead to improved immunotherapeutic strategies.

The primary objective of the work was to study the mechanisms by which tumours of differing origins and antigenic spectra circumvent the anti-tumour immune response to grow and develop. Responses were characterised for two histologically distinct murine tumours grown in immunocompetant, syngeneic mice. Immunological response profiles were defined in terms of lymphocyte proliferation, cytotoxic function and lymphocyte subsets in naive non-tumour bearing mice and mice with increasing tumour burden. The effect of this anti-tumour immune response on the developing tumour was examined in a model of mixed tumour antigenicity established in our laboratory. The influence of tumour derived immunosuppressive factors on the immune response was examined. These factors were partially characterised and compared to determine if the differing response profiles could be attributed to differing immunosuppressive factors. The possible effect of tumour antigens shed into and processed by the gut associated lymphoid tissue with consequent down regulation of the systemic immune response (Oral Tolerance) to a developing tumour was examined.

Abbreviations

ACK ammonium chloride buffer
ADCC antibody-dependant cellular cytotoxicity
APC antigen presenting cell
bFGF basic fibroblast growth factor
cdk cyclin dependent kinase
CIN cervical intraepithelial neoplasia
CML chronic myelogenous leukaemia
ConA Concavalin A
CPL compartment for peptide loading
CPM counts per minute
CTA cancer testis antigen
CTL cytotoxic T-lymphocyte
DAG diacylglerol
DISC death inducing signal complex
DMBA Dimethylbenzanthrene
DMEM Dulbecco's modified Eagle's medium
DTH delayed type hypersensitivity
DTT Dithiothreitol
EBV Epstein-Barr virus
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
EGF epidermal growth factor
ELISA enzyme-linked immunosorbent assay
FACS fluorescence-activated cell sorter
FADD Fas-associated death domain
FCS foetal calf serum
FLICE Fas-associated death domain like IL-1ß-converting enzyme
FLIP FLICE-inhibitory protein
GM-CSF granulocyte macrophage colony-stimulating factor
H&E haematoxylin and eosin
H+N head and neck
HB-EGF heparin-binding epidermal growth factor-like growth factor
HLA human leucocyte antigens
HPV human papilloma virus
HRF homologous restriction factor
IAP Immunosuppressive Acidic Protein
ICAM intercellular adhesion molecule
ICE Interleukin 1β converting enzyme
IELs intraepithelial lymphocytes
Ii invariant chain polypeptide
IL- interleukin-
IFN interferon
IP_3 inositol 1,4,5-triphosphate
IRF immunoregulatory factors
ISF immunosuppressive factor
KIR killer-cell inhibitory receptor
LAK lymphokine activated killer
LFA leucocyte function associated antigen
LMP low molecular weight protein
MAGE melanoma antigen E
MCAF monocyte chemotactic and activating factor
MFI mean fluorescence indices
MHC major histocompatibility complex
NCAM neural cell adhesion molecule
NF-AT nuclear factor of activated T-cells
NK natural killer
NO nitric oxide
PBL peripheral blood lymphocyte
PBS phosphate-buffered saline
PDGF platelet-derived growth factor
PI propidium iodide
PI-PLC-γl phosphatylinositol phospholipase C-γl
PKC protein kinase C
PMA phorbol myristate acetate
SCC squamous cell carcinoma
SFDMEM serum free DMEM
SRBCs sheep red blood cells
SV40 simian virus 40
TAM tumour associated macrophage
TAP transporter associated with antigen processing
TCA trichloroacetic acid
TCR T-cell receptor
TDCF tumour derived chemotactic factor
TGF transforming growth factor
TIL tumour infiltrating lymphocyte
TNF tumour necrosis factor
TPA 12-O-tetradecanoylphorbol 13-acetate
TRAIL TNF-related apoptosis-inducing ligand
TSTA tumour specific transplantation antigen
TUNEL terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
VCAM vascular cell adhesion molecules
VLA very late activation molecules

Presentations

"Active Down Regulation of Specific Anti-Tumour Immune Responses During Tumour Progression"

Barrett, J, O'Brien, MG, Collins, JK and O'Sullivan, GC
Irish Society of Gastroenterology, June 1994.

"Mechanism of Immune Escape in Immunologically Heterogeneous Tumours"

O'Brien, MG, Daly, S, Barrett, J, Collins, JK, Shanahan, F and O'Sullivan, GC
The American College of Surgeons, Surgical Forum, San Francisco, October 1996.

"Tumour Antigen Specific Oral Immune Tolerance"

O'Brien, MG, Collins, JK, Shanahan, F and O'Sullivan, GC.
Irish Society of Gastroenterology, November 1996.
(Winner of The President's Prize)

The Sylvester O'Halloran Surgical Scientific Meeting, March 1997.

Poster presentation at The American Gastroenterology Association, Washington, May 1997.

"British Association of Paediatric Surgeons, XLV Annual International Congress, Bristol, July 1998

Publications

Mechanism of Immune Escape in Immunologically Heterogeneous Tumors.
O'Brien, MG, Daly, S, Barrett, J, Collins, JK, Shanahan, F and O'Sullivan, GC.
Surgical Forum (1996); XLVII: 472-475.

Innate Resistance to Listeria Monocytogenes in Tumour Bearing Mice.
Gahan, GC, Barrett, JR, O'Brien, MG, O'Sullivan, GC, Shanahan, F and Collins, JK.
Journal of Leucocyte Biology (1997); 62(6): 726-732.

Adenocarcinoma of the oesophago-gastric junction. What is the role of suppression of the T-lymphocyte function?
O'Sullivan, GC, O'Brien,MG, O'Connell, J, Collins, JK and Shanahan, F.
In: The Esophago-Gastric Junction. (1998) Eds. R. Giuli, Galminche, J-P, Jamieson, GG and Scarpignato, C. John Libbey Eurotext, Paris.

Oral Immune Tolerance to Tumor Specific Antigens May Confer Growth Advantage to Esophageal and Gastric Cancers
O'Brien, MG, Collins, GC, Collins, JK, Shanahan, F and O'Sullivan, GC.
Diseases of the Esophagus (in press).

Chapter 1

Literature Review

1.1 Introduction

Over the past fifty years the age adjusted annual incidence rates for cancer have progressively increased (Polednak 1994). This increase is partly artefactual due to better reporting and recording of malignant disease as a diagnosis on death certificates. Improved detection appears to account for most of the increase in breast and prostate cancer (Garfinkel 1994; Lenhard 1996). Cigarette smoking has been a major contributor to the rise in lung cancer among women. Acquired immunodeficency syndrome has led to increases in non-Hodgkin's lymphoma and Kaposi's sarcoma and increased sunlight exposure has affected the trends in melanoma (Devesa, Blot et al. 1995). Primary prevention, mainly involving behavioural changes such as exposure to known carcinogens, is the key to reducing incidence rates. Scientific and epidemiological research into the aetiology of common cancers whose causes are poorly understood (e.g. breast and prostate cancer) and more rare cancers with a recent increased incidence (e.g. testicular cancer and Hodgkin's disease) will enable further focused preventative measures.

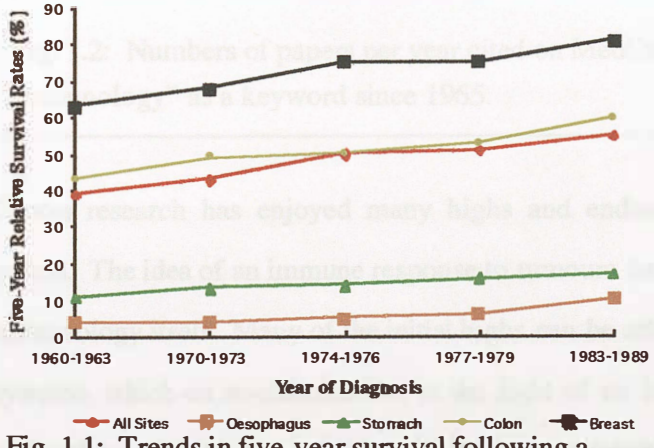


Fig. 1.1: Trends in five-year survival following a diagnosis of malignant disease in Caucasians in United States of America (Boring, Squires, et al 1994).

Mortality rates for cancer on the other hand have remained stable or declined slightly depending on the site of origin of the tumour (Devesa, Blot et al. 1995). Surgical technique

and post-operative care have reached their limit in terms of their potential to cure cancer. Henceforth improvements in these fields will relate to morbidity rather than mortality. Similarly the toxicity, largely attributable to their lack of specificity, of the currently available adjuvant therapies (i.e. chemotherapy and radiation therapy) prohibits more prolonged or aggressive treatment. Immunotherapy, theoretically, offers the potential for antigen specific eradication of localised and disseminated cancer cells with minimal if any systemic side effects. It is for these reasons that the prospect of immunotherapy is so exciting. Tumour immunology has therefore justifiably received considerable and increasing attention over the past number of years (Fig. 1.2).

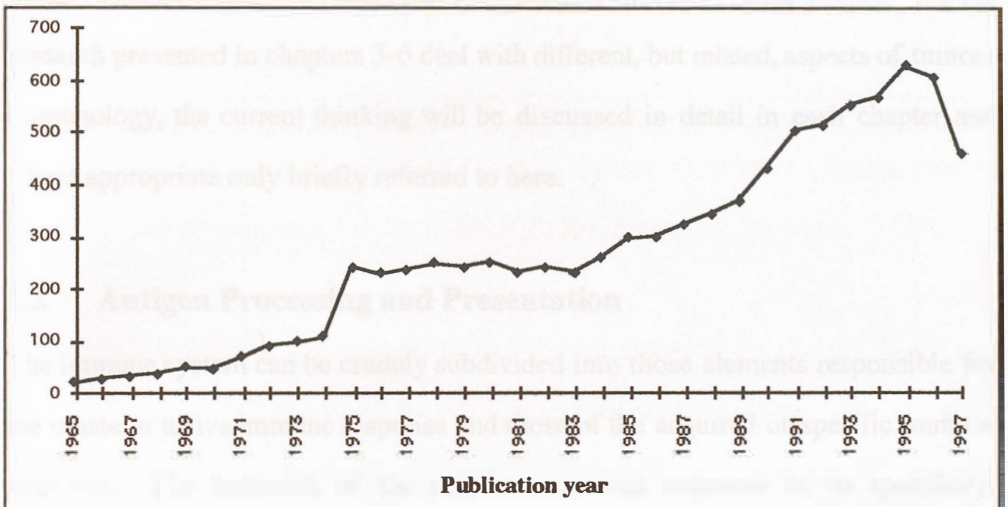


Fig. 1.2: Numbers of papers per year cited on Medline database with "tumour immunology" as a keyword since 1965.

Cancer research has enjoyed many highs and endured some lows during this period. The idea of an immune response to tumours has existed almost as long as immunology itself. Many of the initial highs can be attributed to the use of model systems, which on re-examination, in the light of an increasing understanding of molecular immunology, were seen to be inappropriate. In the past two decades there has been marked progress in our basic knowledge of immunology and

genetics which, coupled with better research techniques and tools, has led to renewed optimism and hope in the field of tumour immunology.

I intend in this introductory chapter to give the reader a broad overview of the field of tumour immunology both to provide a context in which the following work takes place and to illustrate the complex and ever evolving state of our knowledge. The review will be presented in progressive fashion starting with our current understanding of antigen processing, presentation and T cell activation. The evidence for, and nature of, tumour antigens will be followed by that for an immune response to tumour antigens. This will lead to a discussion of the mechanisms by which tumours avoid detection and/or elimination by the immune system. As the research presented in chapters 3-6 deal with different, but related, aspects of tumour immunology, the current thinking will be discussed in detail in each chapter and where appropriate only briefly referred to here.

1.2 Antigen Processing and Presentation

The immune system can be crudely subdivided into those elements responsible for the innate or native immune response and those of the acquired or specific immune response. The hallmark of the acquired immune response is its specificity, provided by T- and B-lymphocytes, which respectively constitute the cellular and humoral immune responses. There are fundamental differences in the way that the cellular and humoral arms of the immune system recognise antigens (Table 1.1). Both cellular and humoral immune responses to tumour antigens have been demonstrated *in vivo*. However the potential for antibody-mediated tumour cell death, either by antibody-dependent-cell-mediated cytotoxicity (ADCC) or by complement activation has largely been shown only *in vitro*. T-cells have been shown to play a critical role in the anti-tumour immune response *in vivo*. Rosenberg and others have shown that both circulating and tumour infiltrating T-

Table 1.1 Differences between specific cellular and humoral immunity.

	Cellular	Humoral
Responding cell	T-Lymphocyte	B-Lymphocyte
Recognising molecule	T-cell Receptor	Immunoglobulin
'Self' molecules required?	Yes	No
Co-Stimuli required	Yes	No
Nature of antigen	Protein/Glycoprotein	Protein / Nucleic acid / Polysaccharide
State of antigen	'Processed'	Native or denatured
Position of antigen	Cell surface	Cell surface or free
Immunity passively transferred by	Lymphocytes	Serum

cells can be shown to lyse autologous tumour cells *in vitro* and to reduce tumour burden *in vivo* (Rosenberg, Spiess et al. 1986; Rosenberg, Packard et al. 1988).

Using molecular cloning techniques tumour associated antigens (TAAs) have been identified that can be recognised by autologous lymphocytes (Boon, Cerottini et al. 1994). These two findings, together, provide strong evidence of immune recognition and activation by autologous tumours. How this recognition occurs and why the activation fails to eliminate the tumour are fundamental questions that must be answered before we can attempt to develop successful immunotherapeutic strategies.

As indicated in Table 1.1, above, the basis of antigen recognition by the cellular immune response is the interaction between T-cell receptor on the T-cell and processed antigen presented by antigen presenting cells in association with 'self' molecules and with additional co-stimuli. These 'self' molecules are the products of the Major Histocompatibility Complex (MHC), a region of highly polymorphic

genes that are closely linked together on chromosomes 6 and designated HLA in humans and on chromosome 17 in mice where they are designated H-2. The initial discovery of murine MHC was made by Snell *et al.*, who were analysing the rejection of transplanted tissues. Using differing inbred strains of mice they observed that skin grafts from one animal to itself (autografts) or grafts between animals of the same inbred strain (syngeneic grafts) were not usually rejected. Grafts between animals of different inbred strains or between outbred animals (allografts) were almost always rejected (Snell 1958). The genes responsible for the distinction between native and foreign tissue were designated histocompatibility genes. Utilising congenic strains of mice they went on to demonstrate that although several different genes could contribute to rejection that a single genetic region was responsible for most rejection phenomena. This region was linked to a gene encoding a polymorphic blood group antigen called Antigen II and hence the region was called histocompatibility-2 or H-2. Obviously this approach could not be used to identify the MHC genes in humans. In humans the approach was a serological one based on observations by Dausset and others of patients who rejected renal transplants and blood transfusions.

It was discovered that in the presence of complement the recipient's serum (alloantisera), which contained alloantibodies, would lyse lymphocytes (which expressed alloantigens) from the donor and also from some but not all third party donors. Because the rejection antigens were expressed on human leucocytes they were designated human leucocyte antigens or HLA. Researchers then went on to map these genes and ended up with two subclasses of MHC molecules. Class I are derived from HLA-A, -B, -C in the human and H-2K, D, L in the mouse. Class II molecules derive from HLA-DP, -DQ, -DR in humans and I-A and I-E in mice. As well as their differing origins there are a number of other important differences between class I and class II molecules which are summarised in Table 1.2. MHC molecules are receptors for peptide molecules serving to present them to the T cell

receptor. The assembly and physical relationships between antigen and MHC molecules have been worked out in great detail and are summarised in Table 1.2 and Fig. 1.3 (Bjorkman, Saper et al. 1987; Bjorkman, Saper et al. 1987; Brown, Jardetzky et al. 1993). The construction and expression of MHC/antigen comlex on the cell surface involves a large and diverse array of enzymes, transporter proteins (e.g. TAP-1 and –2), stabilization proteins (e.g. Calnexin, invariant chain polypeptide) and many more (Restifo and Wang 1996).

Table 1.2 Differences between MHC class I and class II molecules

	MHC CLASS I	MHC CLASS II
Location	HLA-A, -B, -C in humans H-2 K, D, L in mice	HLA-DP, -DQ, -DR in humans H-2 I-A, I-E in mice
Length of peptide	shorter, 8 -10 residues	arbitrary length, 12 - 20+
Nature of antigen binding cleft	Closed at both ends H^+ at both ends	Open ended
Number of binding site pockets	3	5
Size	45kDa α chain, B 2 microglobulin	34kDa α chain, 28 kDa B chain
Recognising cell	CD -8^+	CD - 4^+
Source of antigen	Intracellular	Extracellular

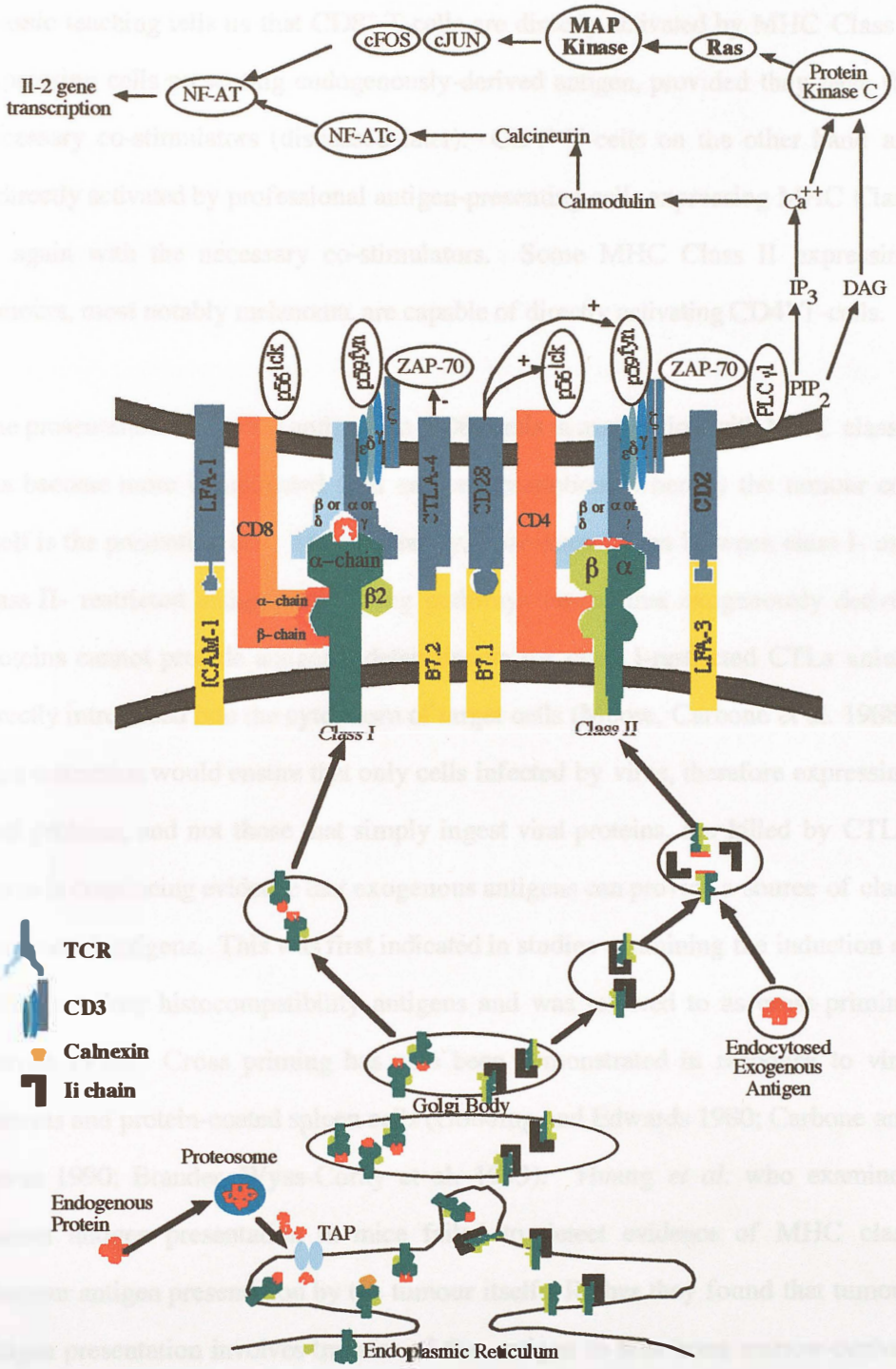


Fig. 1.3: A simplistic diagrammatic representation of the principle mechanisms involved in antigen presentation, recognition and subsequent signalling events leading to T-cell activation.

Classic teaching tells us that $CD8^+$ T-cells are directly activated by MHC Class I expressing cells presenting endogenously-derived antigen, provided they have the necessary co-stimulators (discussed later). $CD4^+$ T-cells on the other hand are indirectly activated by professional antigen-presenting cells expressing MHC Class II, again with the necessary co-stimulators. Some MHC Class II expressing tumours, most notably melanoma, are capable of directly activating $CD4^+$ T-cells.

The presentation of tumour antigens to $CD8^+$ cells in association with MHC class I has become more complicated than earlier assumptions whereby the tumour cell itself is the presenting cell. The, apparently, clear demarcation between class I- and class II- restricted antigen processing pathways means that exogenously derived proteins cannot provide antigenic determinants for class I-restricted CTLs unless directly introduced into the cytoplasm of target cells (Moore, Carbone et al. 1988). This restriction would ensure that only cells infected by virus, therefore expressing viral proteins, and not those that simply ingest viral proteins, are killed by CTLs. There is convincing evidence that exogenous antigens can provide a source of class I-restricted antigens. This was first indicated in studies examining the induction of CTLs to minor histocompatibility antigens and was referred to as cross-priming (Bevan 1976). Cross priming has also been demonstrated in response to viral proteins and protein-coated spleen cells (Gooding and Edwards 1980; Carbone and Bevan 1990; Brander, Wyss-Coray et al. 1993). Huang *et al.* who examined tumour antigen presentation in mice failed to detect evidence of MHC class I/tumour antigen presentation by the tumour itself. Rather they found that tumour antigen presentation involves transfer of that antigen to host bone marrow-derived cells before presentation to $CD8^+$ T cells (Huang, Golumbek et al. 1994). The induction of CTLs to ovalbumin-laden spleen cells has not only been shown to occur by cross-priming on host bone marrow-derived APCs but also to require $CD4^+$ T-cell help (Bennett, Carbone et al. 1997). Furthermore they demonstrated

that both the $CD4^+$ and the $CD8^+$ T-cells needed to recognise antigen on the same APC. No clearly defined mechanisms for this have yet been defined, however a subset of splenic APCs, phenotypically macrophages, can present exogenous antigens on MHC class I to $CD8^+$ T-cells *in vitro* (Rock, Rothstein et al. 1993). Selective depletion of macrophages has been shown to effectively eliminate influenza-specific CTL priming (Debrick, Campbell et al. 1991). Heat shock protein gp96, which binds MHC class I restricted antigenic peptides, is known to be involved in the intracellular transfer of antigens to MHC Class I, may also be involved in the transfer of class I-restricted antigens to other cells *in vivo*. Recently Kurts *et al.* have demonstrated constitutive cross-presentation of exogenous self-antigens which occurs in the absence of any obvious T cell help and leads to deletion of autoreactive $CD8^+$ T-cells (Kurts, Kosaka et al. 1997). Taken together these findings suggest that cognate $CD4^+$ T-cell help, or the absence thereof, is a pivotal step in directing $CD8^+$ T-cells either towards activation or towards tolerance.

1.3 Antigen Recognition and T-cell Activation

1.3.1 T-cell Receptor

The T-cell receptor is a heterodimer of 2 polypeptide chains, a 40-60 kDa acidic glycoprotein alpha-chain and a 40-50 kDa basic glycoprotein beta-chain. A member of the Ig superfamily, TCR shares at least 15% sequence homology with molecules as functionally diverse as IgG, CD2, ICAM-1, MHC Class I + II and IL-2R. The alpha and beta chains of the TCR have a marked resemblance to Immunoglobulin (Ig) with variable and constant regions and serve a similar function, to facilitate diversity of receptor expression. The constant region anchors the variable region to the cell surface and extends through the cell membrane into the cytoplasm, where it is non-covalently associated with four or five other proteins to form the TCR complex. Three of these proteins are called CD3 proteins and include a 25-28 kDa glycosylated γ-chain, a 20 kDa glycosylated δ-chain and a 20

kDa non-glycosylated ε-chain. In addition 90% of TCR complexes contain a homodimer of 16 kDa non-glycosylated ζ-chains and the remaining 10% contain a heterodimer of a ζ-chain with a 22 kDa non-glycosylated -η-chain. All the components of the TCR complex must be present for functional TCR to be expressed at the cell surface. All of the elements are synthesised in excess of that needed for cell surface expression except the ζ -chains. Because these are all produced in excess the rate-limiting step is the production and assembly of the ζ -chains into the TCR complex.

There has recently been described a second type of TCR designated γδTCR after the two chains that make up the TCR heterodimer in place of the αβ chains of the more common TCR. These γ and δ chains are not the same as those of the CD3 complex. γδTCR account for approximately 5% of all lymphocytes and their precise function is still being investigated but it is known that there is a predominance of γδ T-cells in epithelial tissues, in the gut associated lymphoid tissue in particular where they account for almost 10% of the intraepithelial lymphocytes in humans but up to 50% in mice. It is thought that they may initiate immune responses to common micro-organisms at mucosal sites prior to the recruitment of more specific αβ T-cells.

1.3.2 Accessory Molecules on the T-cell Surface

In addition to the TCR, T-cells also express other molecules on their cell surface that are involved in the response to antigen presentation. These surface molecules serve to bind other molecules on the surface of other cells such as antigen presenting cells. They serve to strengthen the bond between antigen presenting cell and T-cell to allow more time for the T-cell to be stimulated and for any effect of the stimulated T-cell to occur. Many accessory molecules may also transduce signals thereby amplifying the TCR stimulus.

CD4 and CD8 are good examples of cell surface glycoproteins expressed on differing subsets of T-cells. They serve as accessory molecules intricately associated with TCR function and therefore are often referred to as co-receptors. CD4 is a 55 kDa transmembrane glycoprotein present on the surface of approximately 65% of αβ-positive T-cells. It serves as a cell adhesion molecule with a specific affinity for MHC class II molecules. CD4 molecules may serve as signal transducers independently or as amplifiers of the effect of ligand binding to the TCR. Bifunctional antibodies directed against CD3 and CD4, simultaneously, are more potent activators of T-cell responses than either alone. CD8, present on 35% of αβ-positive T-cells, is a more variable molecule depending on the stage of maturation of the T-cell in question. In adult humans it is composed of either a heterodimer of a 32 kDa CD8 α–chain and a 34 kDa CD8 β-chain or as a homodimer of CD8 α–chains. The functions of CD8 positive T-cells are similar to those for $CD4^+$ cells except that they have a specific affinity for class I MHC molecules.

There are many other accessory molecules some of whose functions are known some not. Leucocyte free antigen-1 (LFA-1) is a member of the β_2 subunit of the lymphocyte family of integrins. It is present on 90% of mature lymphocytes and thymocytes and it is responsible for strengthening the adhesion between T-cells and the APCs. The avidity of LFA-1 for its ligands is enhanced shortly after TCR stimulation through an unknown mechanism. Intercellular adhesion molecules 1, 2 and 3 (ICAM-1, -2, -3) are three of the ligands for LFA-1. ICAM-1 is expressed on a wide variety of cells and as well as strengthening the adhesion between two cells it has been shown to independently enhance TCR mediated activation of T-cells. Very late activation (VLA) molecules are also members of the integrin family but from a different subunit, β_1. One of the groups of ligands for VLAs are the vascular cell adhesion molecules (VCAM) and the interaction between the two may regulate the traffic of lymphocytes out of the blood stream. They also bind to

extracellular matrix proteins such as fibronectin and laminin possibly to facilitate retention in the tissues.

CD28 is a homodimer of two 44 kDa polypeptide chains. A homologous molecule CTLA -4 is expressed on activated T-cells. The known ligand for both is a 60 kDa molecule expressed on dendritic cells and on stimulated monocytes/macrophages and B-cells called B7. The binding of B7 to CD28 provides a necessary co-stimulus for the activation of T-cells. A second ligand for these receptors has been identified on APCs and designated B7-2. There are many other accessory molecules that have varying functions such as, intercellular adhesion (CD2), signal transduction and activation (CD45, CD5, Ly6, Thy-1) and the retention of T-cells in the extravascular space (CD44).

1.3.3 T-cell Activation

The result of antigen binding to the TCR complex in the appropriate setting with the necessary co-stimulators is a series of events called T-cell activation. Activation of T-cells results in proliferation, clonal expansion, development of antigen specific memory T-cells and initiation of effector functions. Binding of antigen to the TCR, triggers, via antigen recognition motifs on CD3 and ζ-chains, a rise in intracellular Ca++ and activation of a number of phosphorylation and kinase enzyme systems (Altin, Pagler et al. 1994; Trowbridge and Thomas 1994).

The binding of antigen/MHC complexes to the TCR alone is not sufficient to induce T-cell activation but rather may induce anergy. For T-cell activation to occur there is a requirement for additional co-stimulatory signals, the best defined of which is the B7/CD28 interaction alluded to earlier. There are other co-stimulators that may act through different receptors and at different stages in T-cell activation.

Within minutes of TCR activation the T-cells begin transcribing genes. The genes that are transcribed early can be subdivided into cellular proto-oncogenes/transcription factor genes whose products are involved in cell growth and differentiation and cytokine genes and cytokine receptor genes, many of which act in an autocrine fashion on the T-cells which produce them. The cytokine most studied in this regard is IL-2 along with it's α-receptor, both of which are used as activation markers in T-cell research.

1.4 Tumour Antigens

When the study of tumour immunology began at the turn of the century early investigators found striking evidence of rejection of tumour transplants. Unfortunately they were using outbred strains of mice and found that resistance to tumour grafts could also be induced with grafts of normal tissue and hence what they were seeing were the responses to allografts. Further developments awaited the establishment of inbred or syngeneic strains of mice by repeated father/daughter breeding. The first demonstration of tumour antigens capable of inducing specific rejection of transplantable tumours was seen in mice with methylcholanthrene induced tumours (Prehn and Main 1957; Klein, Sjogren et al. 1960). Following inoculation with syngenic tumour cells the developing tumours were excised and the mice proved resistant to subsequent inoculations with the same tumour cell line. Similar results were seen with tumours induced by ultraviolet radiation (Kripke and Fisher 1976) and in virally induced tumours. In the virally induced tumour group it was also possible to immunise against tumour development by prior immunisation with virus (Sjogren, Hellstrom et al. 1961). Hewitt, however, reported that when using spontaneous murine tumours he was unable to demonstrate any evidence of antitumour immunity (Hewitt 1982). This has since been shown not to be the case. From what has already been discussed

we know how the immune system recognises antigens, now we must examine the nature of tumour antigens that potentially may be recognised and responded to.

1.4.1 T-cell Defined Tumour Antigens

Four different experimental approaches have been used to identify proteins presented in conjunction with MHC class I to tumour specific cytotoxic T-cells (CTLs). The first approach was to fractionate cell extracts or membrane preparations and to test individual fractions for their ability to confer protective immunity or to stimulate T-cells *in vitro*. This approach was largely unsuccessful. It was used with some success, by Ullrich *et al.* in 1989, to isolate tumour specific transplantation antigens from the methylcholanthrene induced sarcoma Meth A.

A more sophisticated method with a similar origin is based on the biochemical purification, using high performance liquid chromatography, of peptides, which were eluted from MHC class I molecules of tumour cells. The peptide fractions were then incubated with mutant cells capable of expressing MHC class I molecules and examined for lysis by CTL clones. The positive fractions are then further purified and sequenced using mass spectrometry (Cox, Skipper et al. 1994).

A genetic approach to tumour antigen detection relies upon construction of DNA libraries from tumour cells and subsequent transfection of recombinant DNA into cells that express MHC molecules. The transfection cosmid usually also contains a plasmid which confers resistance to the antibiotic Geneticin (or some such molecule) which allows positive selection of successfully transfected cells based on geneticin resistance. CTLs are then used to identify the transfected cells that are expressing the antigen to which they respond. Fragments of the transfected gene are then transfected to define the region that encodes the antigenic peptide. Antigen P815A on mouse mastocytoma cells was identified in this way (Uyttenhove,

Godfraind et al. 1997). They established antigens P815A- and P815B- loss variants of the mastocytoma P815 cell line. These antigen loss variants were transfected with a cosmid library of the DNA of the parent P815 cell line. The transfected cells were tested for their ability to stimulate proliferation of an anti-P815A CTL clone thus identifying cells expressing P815A. The cosmid was retrieved and the gene coding for the antigen isolated and designated P1A. This gene was sequenced and was found to be homologous with the sequence from normal cells of the syngenic mouse. Furthermore transfection of the gene isolated from normal tissue also induced the expression of P815A antigen. However gene P1A has little or no expression in normal cells and hence antigenicity results from its activation and consequent expression in tumour cells. Similarly the Melanoma Antigen E (MAGE) gene family, discussed below, was isolated in this way.

A fourth method, the reverse of the previous two approaches, starts with proteins that are known to be overexpressed or mutated in tumour cells. From these proteins putative antigenic peptides are generated with consensus anchor motifs for a frequent HLA and loaded onto antigen presenting cells. These are then used, *in vitro*, to stimulate lymphocytes from patients and normal controls to obtain CTLs that specifically recognise the peptide/HLA combination. To demonstrate that the peptide is indeed a true tumour antigen these CTLs must recognise the tumour cells with the gene encoding the peptide and expressing the relevant HLA class (Jung and Schluesener 1991; Celis, Tsai et al. 1994). Dahl *et al.* used similar methods to obtain CTLs specific for peptide fragments of mdm2, a tumour associated protein. They retrieved a series of CTL clones with varying avidity and found that low avidity CTLs would lyse peptide-pulsed cells but not cells expressing mdm2 endogenously, these were lysed by the high avidity CTLs only (Dahl, Beverley et al. 1996).

1.4.2 Serologically Defined Tumour Antigens

Serological identification of tumour antigens has become the latest tool with which to further demonstrate and define the immune response to developing tumours. It was Lloyd Old's group, in the 1970's, who had the most success with attempts to define tumour antigens serologically using autologous serum and tumour cell lines from cancer patients. Though they were able to identify and define only a few antigens they clearly demonstrated that sera from some cancer patients, but not from normal healthy volunteers, specifically recognised antigens on autologous tumour, but not on normal, cells providing compelling evidence for the existence of tumour specific antigens and *in vivo* immune recognition of these. Further advances were hampered by the cumbersome nature of this approach and awaited the establishment of a new approach. Serological identification of antigens by recombinant expression cloning (SEREX) was established by Sahin and colleagues in Germany in 1995 (Sahin, Tureci et al. 1997). Using this approach a cDNA library is constructed from fresh tumour specimens, packaged into lambda phage vectors and expressed recombinantly in *E. coli*. The recombinant proteins that are generated are then transferred onto nitrocellulose membranes and identified as antigens by their reactivity with high titre IgG antibodies in the patients' serum which are detected using enzyme conjugated secondary antibodies specific for the Fc portion of human IgG. Positive clones are subcloned and ultimately the nucleotide sequence of the inserted DNA is determined. Using this technique this group alone have identified more than 100 different antigens which can broadly be divided into 3 groups (a) previously known tumour antigens e.g. MAGE; (b) transcripts identical to or highly homologous with known genes not previously known to elicit immune responses in humans e.g. restin; (c) antigens from previously unknown genes e.g. HOM-RCC-3.1.3 from renal cell carcinoma.

These serologically defined tumour antigens have different patterns of antigen expression. 'Shared' tumour antigens are expressed in a variety of neoplasms and

not in normal tissue except the testis e.g. MAGE; Differentiation antigens are expressed in tumours and in normal tissue in a lineage specific manner; Antigens encoded by mutated genes e.g. p53; Overexpressed genes e.g. HOM-RCC 3.1.3; Splice variants e.g. restin; Cancer related autoantigens and cancer independent autoantigens; Finally virus-encoded antigens e.g. HERV-K10 in renal cell cancer.

Some known tumour antigens share common features. MAGE, BAGE and GAGE all originally identified by CTLs are expressed to varying degrees on different cancers as well as being expressed in the testis and therefore have been designated cancer/testis antigens or CTAs. Several more CTAs have been identified using SEREX methodology. The coincident expression of CTAs in malignancy and the testis may be due to hypomethylation, which is constitutive in the testis and also occurs in tumours. As testicular germ cells do not express MHC class I molecules gene expression should not result in antigen presentation to CTLs. Furthermore CTA expression in the testis does not preclude their use as targets for immunotherapy because (a) the testis is a site of immunological privilege and difficult for T-cells to access and (b) possesses potent immunosuppressive mechanisms of it's own. Mice immunised with the antigen encoded by the P1A gene produced strong P1A specific CTL responses that did not cause testicular inflammation or attenuation of fertility (Uyttenhove, Godfraind et al. 1997).

Utilising three different strategies serologically defined antigens are being examined for T-cell reactivity. For the 'peptide approach' sequences of serologically identified antigens are screened for peptides containing binding motifs for MHC class I molecules and predicted peptides are tested for binding, loaded onto APCs expressing MHC class I and used to expand specific $CD8^+$ T-cells. In the 'whole protein approach' professional APCs are transfected with cDNA encoding the serologically defined antigen and used to prime T-cells. T-cells generated by either method are then tested for specific recognition of antigen-

positive tumour cell lines. The SEREX approach by utilising fresh tissue restricts the analysis to genes that are expressed *in vivo* and not the entire genome. Furthermore by utilising the patients' serum SEREX identifies antigens, which have been expressed and detected in vivo. Some antigens may however undergo conformational change in the processing of the samples and avoid detection.

Tumour antigens were formerly subdivided into tumour-specific antigens and tumour-associated antigens. However the number of tumour antigens that are recognised by T-cells grows daily and therefore it is better to classify them into subgroups based on their pattern of expression. This facilitates comment on their suitability or otherwise for use in potential immunotherapeutic strategies.

1.4.3 Nature of Tumour Antigens

1.4.3.1 Tumour-Specific Shared Antigens

This group consists of those tumour antigens that are encoded by genes normally silent in most normal tissues but activated in a number of tumours of varying histological types. Examples of antigens in this group are P1A gene encoded antigens P815A and P815B in mice. In humans the best-known tumour specific shared antigens are those encoded by the MAGE genes. The MAGE-1 gene was identified using cosmid libraries as described above. It belongs to a family of 12 closely related genes all located on chromosome X. Using PCR technology a wide range of tumours has been examined for expression of MAGE antigens. The percentages of expression are shown in Table 1.3 (Coulie 1996). MAGE -1, -2 and -3 were not detected in normal tissue except the testis. MAGE-1 encodes for the antigen MZ2-E that is presented by HLA-A1 molecules. Approximately 26% of Caucasians express HLA-A1 and about 49% of melanoma patients bear tumours expressing MAGE-1, therefore, about 13% of melanoma patients are

expected to express antigen MZ2-E on their tumour. This antigen therefore has a potential use as a tumour vaccine.

Table 1.3. MAGE gene expression as assessed by PCR amplification on tumour specimens.

Percentage of tumours positive for MAGE-1, -2, -3 or -4					
	1,2,3 or 4	**1**	**2**	**3**	**4**
Melanomas (148)	75	36	63	65	23
HNSCC (44)	68	25	29	48	50
Lung Carcinomas (55)	53	34	33	31	36
Sarcomas (18)	50	11	22	22	28
Mammary Carcinomas (146)	26	18	8	11	5
Colorectal Carcinomas (32)	25	0	9	16	16
Prostatic Carcinomas (20)	20	15	15	15	0
Renal Carcinomas (49)	0	0	0	0	0
Bladder Carcinomas (59)	42	19	29	34	31
Leukaemias/Lymphomas (27)	0	0	0	0	0
Brain (9)	0	0		0	

(numbers in parentheses represent the sample number per group)

By stimulating draining lymph node cells of patients with pancreatic carcinoma with a pancreatic tumour cell line Finn *et al.* generated CTLs capable of lysing not only pancreatic tumour cell lines but also most breast cell lines in a non-MHC restricted manner. They found that these cells could be stimulated with purified mucin. Similar results were obtained with nodes from breast cancer patients (Jerome, Domenech et al. 1993). Normally breast and pancreatic mucins, encoded by the gene MUC-1, are multiple tandem repeats of a 20 amino acid motif that are fully glycosylated. In cancer patients the mucins are underglycosylated and therefore recognised by CTLs. Mucin underglycosylation also occurs in the breast

of lactating women but only at the extracellular apical surface, which is not accessible to T-cells. Because underglycosylated mucins appear to be very specific for tumour cells and are not HLA restricted they may be suitable for vaccination trials.

1.4.3.2 Differentiation Antigens

A large number of cytotoxic T-lymphocytes isolated from melanoma patients were found to recognise not only melanomas but also normal melanocytes. This group of antigens has been designated differentiation antigens. A good example is tyrosinase. This enzyme transforms tyrosine into dihydroxyphenylalanine (DOPA) an intermediate product in the synthesis of melanin. It was discovered, using the cosmid library transfection approach described above, in the pursuit of antigen Ab which had been isolated by immunoselection, using CTLs, from a group of melanoma antigens from patient SK29(AV) (Brichard, van Pel et al. 1993). Other differentiation antigens that have been identified include carcinoembryonic antigen and prostate-specific antigen. As these antigens are also expressed to varying degrees on some normal tissues such as the choroid layer of the eye and normal bowel the potential autoimmune side effects of active immunotherapy against these antigens needs to be carefully evaluated.

1.4.3.3 Antigens Resulting from Mutations

Table 1.4 Incidence of p53 and ras mutations in common cancers

	p53	ras
Colon	70%	50%
Breast	30-50%	<5%
Lung	50%	
SCC Lung	100%	
Pancreas		90%
AML		25%

This category consists of peptides generated by point mutations in the genes that encode the normal cell proteins. It is becoming increasingly apparent that a significant proportion of antigenic point mutations, as well as generating tumour antigens, may also be involved in oncogenesis. The majority

of murine tumour antigens identified to-date are the result of point mutations. This may well be the result of the use of chemical carcinogens. However they have also been found in spontaneous murine and human tumours. The melanoma antigen encoded by a mutated cyclin-dependant kinase (cdk) 4 gene has lost the ability to bind its inhibitory protein p16. This affects cell cycle regulation favouring uncontrolled cell growth and tumour development. Oncogenesis may also result from mutations resulting in persistent activation such as results from the mutation in ß-catenin. The mutated protein is more stable as are the complexes it forms with transcription factor Lef-1, which results in persistent transactivation, stimulating cell proliferation (Rubinfeld, Robbins et al. 1997). Oncogenesis may be the result not only of increased cell proliferation but also of a reduction in programmed cell death, apoptosis. Recently a mutation in the CASP-8 gene appears to impair the ability of its product, a protease caspase-8, also known as FLICE (Fas-associated death domain like IL-1ß-converting enzyme) or MACH alpha 1 (mediator of receptor induced toxicity associated CED-3 homologue) to induce apoptosis as compared with the wild-type caspase-8 (Mandruzzato, Brasseur et al. 1997). As with most human tumour antigens this subclass was initially identified on melanoma cells but mutant FLICE was detected on a squamous carcinoma. Because these proteins are the result of point mutations they are frequently unique and therefore despite superb tumour specificity are unsuitable for use as tumour vaccine targets. Sporadic mutations in p53 and the ras protooncogenes are amongst the most common genetic alterations observed in human cancers (Bos, Fearon et al. 1987; Almoguera, Shibata et al. 1988; Hollstein, Sidransky et al. 1991) (Table 1.4). Germline mutations in p53 have been described in 'hereditary cancers' such as Li-Fraumeni syndrome, patients with multiple primary neoplasms (Malkin, Li et al. 1990; Malkin, Jolly et al. 1992). Ras oncogenes are activated by point mutations that occur almost exclusively in codons 12, 13 and 61 which are easily defined and therefore were selected as the first oncogenes to be used as potential immunotherapeutic targets (Barbacid 1987). CTLs against these most

frequently mutated oncogenes have only been generated through artificial stimulation with synthetic peptide fragments. Other potentially more useful tumour antigens are the chimeric proteins that arise as a result of chromosomal translocations. More than 95% of patients with chronic myeloid leukaemia carry a chromosomal translocation of the c-abl protooncogene on chromosome 9 to the breakpoint cluster region (bcr) of chromosome 22 producing the Philadelphia chromosome (Shtivelman, Lifshitz et al. 1985; Hermans, Heisterkamp et al. 1987). $CD4^+$ T cells have been raised in vitro against the protein, bcr-abl, that results from this translocation. Because identical translocations occur frequently in certain types of leukaemias these represent another source of strictly tumour-specific antigens that might be exploited in immunotherapy.

1.4.3.4 Overexpressed Tumour Antigens

Some antigens are not the result of mutations themselves but are expressed in normal and tumour tissue. However CTLs against these antigens will only lyse the tumour cells and not the normal tissue because in normal tissue the antigen is expressed at levels that are below the threshold required for CTL recognition (Viola and Lanzavecchia 1996; Lethe, van der Bruggen et al. 1997). Ikeda *et al.* characterised a CTL clone directed against a metastatic melanoma cell line that had lost all but one of its HLA class I molecules. The CTL clone recognised a peptide, presented in conjunction with HLA-A24, derived from a previously unknown gene, PRAME, which is expressed at high levels in many tumours and at low levels in certain normal tissues (testis, ovary, endometrium and adrenals). Interestingly the CTL clone did not lyse the initial melanoma cell line despite the presence of PRAME mRNA in this cell line. This is because the CTL clone expresses, p58.2, a natural killer inhibitory receptor which has an affinity for certain HLA-C molecules including HLA-Cw7 which has been lost in the CTL sensitive cell line (Ikeda, Lethe et al. 1997). Hence this receptor represents a specificity booster because normal cells expressing the same antigen should not be lysed. T-cells which

express p58.2 may play an important role in attacking tumours that have escaped the specificity of T-cells through loss of or decreased expression of MHC class I molecules.

Her-2/neu is another antigen expressed in some normal tissues and overexpressed in 30% of breast tumours and ovarian carcinomas (Hesketh 1997). A peptide derived from Her-2/neu was found to be the target of lymphocytes infiltrating some HLA-A2 ovarian carcinomas (Fisk, Bleevins et al. 1995). It remains to be seen whether immunisation against these types of antigens will lead to autoimmune damage in normal tissues or whether they will escape through sub-threshold levels of antigen expression.

1.4.3.5 Viral Antigens

Antigens derived from oncogenic viruses constitute another category of potentially useful tumour antigens. The first evidence of rejection antigens induced by oncogenic viruses was based on studies of polyoma virus induced tumours (Habel 1961; Sjogren, Hellstrom et al. 1961). These studies demonstrated that in contrast to tumour-specific transplantation antigens (TSTAs), induced by chemical carcinogens, rejection antigens in polyoma virus induced tumours have identical or cross-reactive specificities even if produced in different species (Hellstrom and Sjogren 1966). A number of virally induced murine tumours have been studied including Friend leukaemia virus, SV 40 and Gross virus. In humans, adenovirus, Epstein-Barr virus, papillomavirus, human T-cell leukaemia virus-1 and Hepatitis B virus have all been studied. Perhaps the best studied is the papilloma virus and in particular the E7 oncoprotein of human papilloma virus (HPV) 16 which is present in most cervical carcinomas. Tumour specific CTLs have been elicited through *in vivo* immunisation with E7 peptides presented in association with HLA-A0201 molecules (Feltkamp, Smits et al. 1993).

1.5 Effector Mechanisms In Anti-Tumour Immunity

There are a number of different observations that infer the existence of an anti-tumour immune response *in vivo*. These include the spontaneous regression of tumours, the development of tumours in immunosuppressed patients and their subsequent resolution upon cessation of immunosuppressive therapy and the correlation, in certain tumours, between an immune infiltrate and a better prognosis (Svennevig, Lunde et al. 1984). The tumour antigens discussed above are largely of academic interest unless there is *in vivo* recognition and an immunologically based attempt at eradication. This response involves a diverse range of immunological effector mechanisms from both the innate and adaptive immune systems and both the humoral and cellular immune systems. Three criteria must be met for the successful elimination/containment of a developing tumour by immunological mechanisms. i) The tumour must possess and appropriately express tumour antigens to be sufficiently antigenic to be immunogenic (dealt with above); ii) The immune system must be sufficiently intact and functioning to recognise and respond to the antigenic stimulus (to be dealt with here); iii) The immune response must proceed unhindered and unimpeded (dealt with later). Lymphocytes are pivotal in the response to developing tumours as they provide the receptors, which recognise potential tumour antigens and also determine the specificity of the immune response by distinguishing self from non-self. Particular attention is paid to T-lymphocytes, as they are capable not only of directly lysing tumour cells but also of orchestrating the other elements of the immune response.

1.5.1 T-Lymphocyte Development

In order to comprehend the nature of the cellular immune response to developing tumours it is necessary to have some understanding of T-cell development. The immune system is capable of responding to between 10^{10} and 10^{15} different antigens. Clearly there is insufficient space for each individual receptor to be encoded for directly in the genome. For years we have known that this antigenic

diversity arises from rearrangement of variable (V), diversity (D) and joining (J) gene segments to result in productive genes that encode either disulfide-linked heavy and light chains of the immunoglobulin receptor on B cells or the dilsufide-linked α and β TCR proteins. Straightforward combinatorial gene rearrangement alone is capable of generating 8.1×10^6 different T-cell receptors and 2.4×10^7 immunoglobulins in the mouse. Further diversity is ensured through (i) variable boundary recombinations of V, D & J regions to produce junctional sequences; (ii) nucleotide insertion in the D & J regions; (iii) interchain amplification i.e. random association between α and β chains; (iv) somatic hypermutation, which probably only applies to B cells, doesn't increase diversity but rather occurs during the generation of memory cells and is thought to fine tune the response towards increased affinity.

Because of the essentially stochastic nature of antigen-receptor formation, there will not only be receptors for foreign antigens but also receptors that will react with self antigens and also non-functioning or useless receptors. These useless and potentially harmful lymphocytes must be removed from the immune system and this is brought about through positive and negative selection exerted during lymphocyte development.

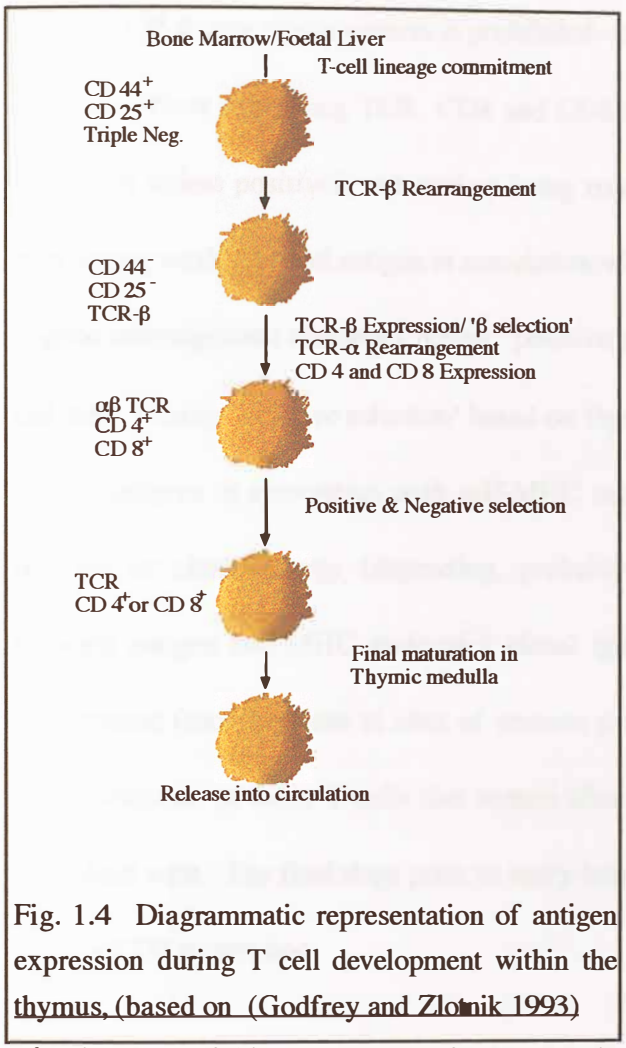


Fig. 1.4 Diagrammatic representation of antigen expression during T cell development within the thymus, (based on (Godfrey and Zlotnik 1993)

The main site of T-cell maturation is the thymus, though humans with a congenital absence of the thymus, as in DiGeorge syndrome, and athymic nude mice do possess some functional, mature T-cells (Fig. 1.4). Precursor cells originate in either the foetal liver or bone marrow and migrate to the thymus, possibly related to their expression of CD 44 -a potential homing receptor, where under an unknown stimulus they quickly become committed to develop into lymphocytes and express CD 25 or Interleukin 2 receptor α chain. They are no longer at this stage multipotential cells as they have lost myeloid and erythroid precursor activity. Within the thymus these immature precursor cells are located in the cortex and as they mature migrate into the medulla from whence they are released into the circulation. During this process approximately 95% of cortical precursor cells will die.

Once committed to T-cell development these pre-T-cells must progress thru' a series of selection stages (see Fig. 1.4). 'β-selection' permits only those cells that stably express TCR β chain (in association with gp33) to survive, but

further TCR-β gene rearrangement is prohibited – a phenomenon known as allelic exclusion. Now expressing TCR, CD4 and CD8 these thymocytes will undergo apoptosis unless positively selected as being reactive to thymic epithelial cells expressimg weakly bound antigen in association with self-MHC molecules. TCR α-gene rearrangement continues during 'positive selection' until selected or the cell dies. Finally 'negative selection' based on thymic epithelial cell presentation of self-antigens in association with self-MHC molecules results in either clonal deletion or clonal anergy (depending, probably, on the strength of binding between antigen and MHC molecule); clonal ignorance for those antigens not encountered (such as those at sites of immune privilige i.e. testis and eye); and clonal selection of those T-cells that remain after all the self-reactive cells have been dealt with. The final stage prior to entry into the circulation is loss of either CD4 or CD8 expression.

Lymphocyte development takes approximately three weeks and is not a random free for all but is in fact organised chaos (which probably really only reflects the current state of our ignorance) as evidenced by the emergence in waves of T-cells that are restricted by the TCRs they express. This temporal order of emergence is related to the expression of TCR genes and arises in a colinear fashion with their physical order on the TCR chromosome. Despite the massive progress in the past two decades there are large gaps in our current understanding, with many intriguing questions to be answered. For instance how does TCR cross linking at three different stages in lymphocyte life cycle elicit three different responses? Cross linking during positive selection permits survival, during negative selection encourages cell death and in mature T cells induces activation and proliferation.

1.5.2 MHC-Restricted Effector Mechanisms

1.5.2.1 Cytotoxic T-lymphocytes

The first report of cytotoxic activity in lymphoid cells, by Govaerts in 1960, demonstrated that thoracic duct lymphocytes from dogs which had previously rejected a renal allograft were able to specifically kill donor kidney epithelial cells *in vitro* (Govaerts 1960). So began the study of cytotoxic T-lymphocytes or CTLs. The majority of CTLs are $CD8^+$ but approximately 5% are $CD4^+$. CTLs are not fully differentiated when they leave the thymus and the appearance of fully functional CTLs requires antigen recognition and cytokine stimulation. These cytokines IL-2, IFN-γ and possibly also IL-4, IL-6, IL-7 and IL-12 may arise from the $CD8^+$ CTLs themselves in an autocrine manner thus giving rise to 'helper-independent' CTLs. The more common source of these cytokines is from $CD4^+$ Th1 cells that respond to tumour antigens that have been processed and presented by MHC class II expressing antigen presenting cells such as dendritic cells or macrophages. As referred to earlier T-cell activation also requires additional cell surface signals (see Fig. 1.3). Once activated successfully the CTL will divide to produce many daughter clones with identical specificity to produce a pool of effector cells capable of mediating target cell lysis. Some of the progeny will be dedicated to become memory cells to respond to future contacts with antigen more rapidly.

1.5.2.2 CTL mediated target cell lysis

Over the last twenty years research has revealed that cytotoxic lymphocytes induce target cell death by at least three distinct mechanisms - secretion of cytotoxic cytokines and either calcium dependant or calcium independent contact mediated cytotoxicity. Examples of such cytotoxic cytokines are TNF-α or IFN-γ, which, as well as being directly cytotoxic in themselves, upregulate Fas expression on malignant cells rendering them more susceptible to contact dependant cell death. Recent studies suggest that different cytotoxic mechanisms may operate in

specific types of CTL under different circumstances. The two major mechanisms responsible for contact dependant CTL induced target cell death exert their effects either through the exocytic secretion of lytic granules which contain a variety of proteins and enzymes (Fig. 1.5) or through ligand mediated cell surface induction of apoptosis.

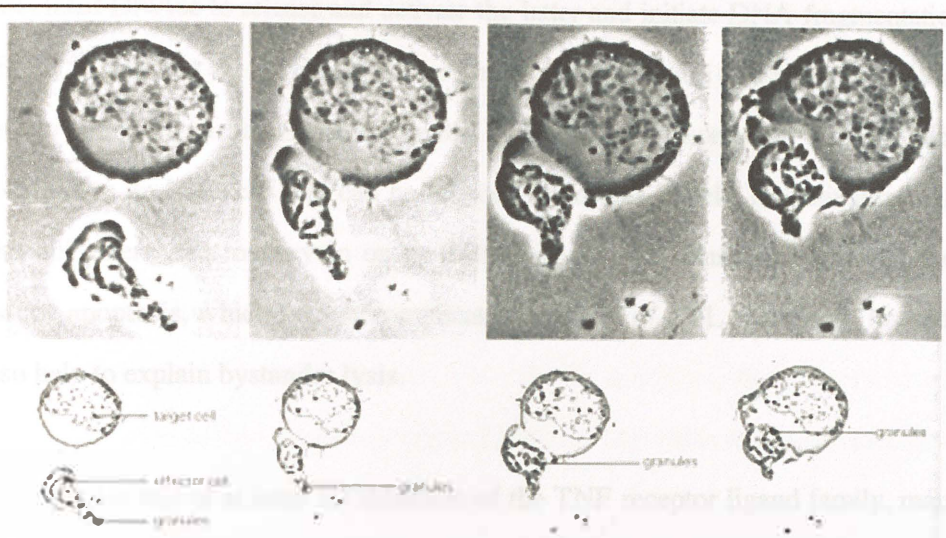


Fig. 1.5: Electronmicrographic pictures of T cell granule exocytosis in response to target cell recognition (Yannelli, Sullivan et al. 1986)

TCR target cell recognition is associated with a rise in intracellular calcium and cytoskeletal rearrangements along which lytic granules move to the point of contact (Griffiths 1995). These granules release Perforin, a 70kD protein which polymerises in the cell membrane of the target cell forming pores of variable size (5-20nm) some of which are large enough to permit entry of Granzymes, serine proteases that induce chromatin condensation and DNA fragmentation i.e. apoptosis (Heusel, Wesselschmidt et al. 1994; Berke 1995). The cytotoxic T-cells themselves are resistant to the effects of the released Perforin and Granzyme (Zalman, Wood et al. 1987; Jiang, Persechini et al. 1989; Jiang, Ojcius et al. 1990).

CTLs may also induce tumour cell apoptosis by cross linking target cell Fas receptors. Fas, a 48kDa transmembrane glycoprotein, and its ligand, FasL, a 40

kDa type II transmembrane protein, are structurally related to the TNF and NGF receptor families. Engagement of the Fas receptor results in aggregation of its intracellular death domains and recruitment of Fas-associated death domain (FADD) and procaspase 8 to form the death inducing signal complex (DISC). This triggers a cascade of caspases, which eventually cleave an inhibitor of caspase-associated DNAse to release and activate the latter and initiate DNA fragmentation (Enari, Sakahira et al. 1998). Whether Granzyme B induces apoptosis directly or by activating the caspase enzymes is still unclear. FasL expression appears to be constitutive on NK cells and can be induced rapidly on T-cells by activation with phorbol esters and ionomycin or by TCR engagement. Purified FasL can also induce apoptosis, which further complicates the issue of CTL specificity, but may also help to explain bystander lysis.

Fas ligand is one of at least 10 members of the TNF receptor ligand family, many of which including TNF are capable of inducing programmed cell death or apoptosis. A recently described protein TNF-related apoptosis-inducing ligand or TRAIL has been shown to induce apoptosis in melanoma cells that are resistant to FasL or other TNF family members (Thomas and Hersey 1998). At least three receptors have been described for TRAIL. R_1 or death receptor 4 is found in most tissues including activated T-cells. R_2, again widely distributed, unlike R_1 induces apoptosis by mechanisms involving interaction with FADD. The third receptor, R_3, lacks a cytoplasmic domain and therefore is incapable of transducing an apoptotic signal. It has a postulated decoy role in protecting normal tissues, on which it is expressed, from TRAIL induced apoptosis and leaving tumour cells, on which it has not been detected, susceptible. TRAIL has been shown to mediate $CD4^+$ CTL killing of melanoma cells and may also be involved in MHC I restricted $CD8^+$ T-cell killing of melanoma cells. In the absence of TCR engagement TRAIL is not expressed and therefore TRAIL induced apoptosis is target cell specific.

The Fas death signal is not only relevant to the target cell. Mice that carry spontaneous autosomal recessive mutations in chromosome 19 (lymphoproliferative or lpr) or chromosome 1 (generalised lymphoproliferative disease or gld) develop lymphadenopathy and splenomegaly. Lpr/lpr mice have defective expression of Fas and are resistant to apoptotic signals delivered by either Fas antibodies or perforin-free CTLs. Activated CTLs from gld/gld mice have defective FasL expression and do not lyse Fas^+ target cells (Takahashi, Tanaka et al. 1994). Both defects result in an abnormal accumulation of lymphocytes suggesting that Fas/FasL may be involved in normal lymphocyte death. Positive and negative selection processes in the developing thymus are not affected but peripheral clonal deletion and elimination of activated T-cells are affected (Smyth 1995). Within 24 hours of TCR stimulation T-cells upregulate Fas expression and become sensitive to Fas-mediated apoptosis several days later and this mechanism serves to down-regulate the immune response.

This apparent distinction between the granule exocytosis mechanism of CTL induced target cell death and the FasL/Fas receptor induction of apoptosis has become blurred. Bossi and Griffiths, 1999, have recently demonstrated that newly synthesised FasL is stored in the lytic granules of activated T-cells and NK cells. In the majority, but not all, of these granules FasL has been shown to co-localise with Granzyme B and Perforin. Its release upon TCR engagement may be mediated via a tyrosine kinase signalling pathway as the cytoplasmic tail of FasL interacts with distinct domains of Fyn tyrosine kinase (Hahne, Lowin et al. 1995). Its release is associated with degranulation of the lytic granules in response to a calcium flux triggered by target cell recognition. As shown above the granule exocytosis and Fas pathways of CTL mediated cytotoxicity are distinguishable by their differences in calcium dependence. This distinction is still valid, as Perforin activity is calcium dependent at every step, degranulation, binding to the target cell

membrane and polymerisation. In contrast only FasL release requires calcium, binding and activation are calcium independent (Bossi and Griffiths 1999).

Approximately 90% of $CD8^+$ CTL killing is attributable to Perforin/Granzyme induced cell death whereas less than 10% of $CD4^+$ CTL killing is by this mechanism. $CD4^+$ CTL killing can be ascribed to Fas induced cell death but the majority, 60%, is through unknown mechanisms possibly soluble cytotoxic cytokines (Shresta, Pham et al. 1998).

1.5.2.3 Tumour-infiltrating lymphocytes

There is a great deal of *in vivo* evidence of T-cell reactivity against tumour antigens from both observations on the clinical course of malignant disease and from experimental manipulations. The best demonstration of specific T-cell reactivity comes from the extraction of MHC-restricted $CD8^+$ T-cells from tumour specimens, which have been shown *in vitro* and *in vivo* to exhibit specific tumouricidal activity. These cells referred to as tumour infiltrating lymphocytes or TILs have been shown to preferentially localise to tumour sites(Griffith, Read et al. 1989). They have also been shown to be 50 to 100 times more effective at killing tumour cells than lymphokine activated killer (LAK) cells. These will be dealt with in detail in the section on tumour immunotherapy.

1.5.2.4 $CD4^+$ T-cells in the Antitumour Response

Most of the focus in tumour immunology to date has been on $CD8^+$ T-cells for two reasons, firstly, most tumours express MHC class I antigens but not MHC II and secondly, $CD8^+$ CTLs are able to induce tumour cell killing directly following recognition of tumour antigens. Recent evidence suggests that $CD4^+$ T-cells are an equally critical component of the antitumour immune response. Initially their role was largely thought to be to facilitate the development of CTLs by secreting cytokines, in particular Interleukin-2, in response to antigen stimulation, hence the

name helper T-cells. Evidence is accumulating that, not unexpectedly, their role is much more complex and includes the following functions:

- •Activation of $CD8^+$ T cells through cytokine release
- •Activation of antigen presenting cells to present antigens to $CD8^+$ CTLs
- •Activation of Macrophages to produce reactive oxygen metabolites such as superoxide and nitric oxide
- •Activation of Eosinophils
- •$CD4^+$ T cell tolerance permitting tumour growth through a failure of immune containment.
- •Direct cytotoxic activity

The dependence on 'help' mediated by $CD4^+$ T-cells for the priming of antigen-specific $CD8^+$ CTLs has been well documented (Dranoff, Jaffee et al. 1993; Qin, Richter et al. 1998). A widely held theory is that help for CTLs is mediated by cytokines (e.g. IL-2) produced by $CD4^+$ 'helper' cells activated in close proximity to $CD8^+$ cells at the surface of the antigen presenting cell - so called three cell interaction (Keene and Forman 1982). It leaves a lot to chance to assume that the helper and cytotoxic T-cells would encounter the antigen presenting cell at the same time. As we already know $CD8^+$ cells which encounter antigen in the absence of co-stimulation will become tolerant and not activated. An alternative theory is that this help comes via activation of the antigen presenting cell - the sequential two hit theory (Ridge, Di Rosa et al. 1998). This theory proposes that CD40 ligand expressed on the surface of activated $CD4^+$ T-cells interacts with CD40 on the antigen presenting cell surface enabling the latter to co-stimulate $CD8^+$ CTLs. CD40 ligand (also known as gp39 or CD154) is a 33kDa type II membrane glycoprotein member of the TNF gene family that is preferentially expressed on activated $CD4^+$ T-cells and mast-cells. Its receptor CD40 is, a 40kDa glycoprotein member of the TNF-receptor family, found on B-cells, dendritic cells, activated

macrophages and follicular dendritic cells. There is a good deal of evidence to support this theory. Anti-CD40 antibodies could replace $CD4^+$ help in the generation of CTLs (Bennett, Carbone et al. 1998; Ridge, Di Rosa et al. 1998). Blocking of the CD40 ligand inhibited the development of activated CTLs (Schoenberger, Toes et al. 1998). Ligation of CD40 on the surface of APCs such as dendritic cells, macrophages and B-cells greatly increases their antigen presentation and co-stimulatory capacity. The CD40 ligand gene maps to human chromosome Xq24 (Banchereau, Bazan et al. 1994). It's expression is defective in the X-linked hyper IgM syndrome in which patients have high levels of IgM but severely reduced levels of other immunoglobulins because their B-cells are unable to switch to the production of other classes of Ig (Grewal and Flavell 1996). The inability to induce experimental allergic encephalomyelitis in CD40 $^{-/-}$ mice can be overcome by providing them with B7-1^+ APCs by adoptive transfer. These findings suggest that CD40 ligand is required for the induction of co-stimulatory activity on APCs, which in turn is required for activation, and priming of T-cells (Grewal and Flavell 1996). Levels of B7-1 (CD80) and B7-2 (CD86) on resting APCs are generally low becoming upregulated following APC activation. Monoclonal antibodies against CD40 ligand and CD40 inhibit activated T-cells from inducing CD80 and CD86 on B-cells (Ranheim and Kipps 1993). Ligation of CD40 on dendritic cells by CD40 ligand triggers the production of extremely high levels of IL-12 and is also a potent stimulus for upregulating expression of ICAM-1, CD80 and CD86 (Cella, Scheidegger et al. 1996). These effects result in increased capacity of the dendritic cells to trigger proliferative responses and IFN-γ production by T-cells. To summarise the above findings recognition of the MHC-peptide complex by the TCR leads to the induction of CD40 ligand on T-cells enabling simultaneous engagement of the MHC-peptide with the TCR and CD40 by CD40 ligand leading to upregulation of B7-1 and B7-2 on the APCs. These co-stimulators in turn amplify the response of the T-cells.

If the only contribution of $CD4^+$ T-cells to antitumour immunity were the provision of help for the generation of $CD8^+$ CTLs then depletion of $CD4^+$ T-cells from vaccinated animals immediately prior to tumour challenge should not lead to a reduction in antitumour immunity. However significant reductions in antitumour immunity are observed. Also adoptive transfer of tumour-specific $CD4^+$ T-cells confers antitumour immunity. Other groups have shown that eradication of $CD8^+$ CTLs does not necessarily prevent tumour rejection. The presence of activated macrophages and eosinophils at the site of tumour challenge in vaccinated animals is dependant on the presence of $CD4^+$ T-cells and independent of $CD8^+$ CTLs (Pardoll and Topalian 1998). This interaction between $CD4^+$ T-cells and macrophages is dependent on cell-cell contact and CD40-CD40L interaction (Wagner, Stout et al. 1994; Stout, Suttles et al. 1996). There is direct evidence that different subclasses of $CD4^+$ T-cells are responsible for different effects. T-helper 1 cells activate macrophages to produce nitric oxide and superoxides whereas T-helper 2 cells recruit and activate eosinophils.

Recent evidence has demonstrated that $CD4^+$ T-cells specific for tumour antigens can become actively tolerised to undergo clonal division or become anergic. Following vaccination with irradiated weakly immunogenic TS/A cells (a spontaneous mammary adenocarcinoma) normal mice developed a tumour upon subsequent challenge whereas μMT mice, who are deficient in mature B-cells, rejected the challenge (Qin, Richter et al. 1998). Reconstitution of these μMT mice with either crude spleen cell suspensions or with purified syngenic B-cells led to tumour development, implying that B-cells or their products were involved. Inoculation of naïve mice with serum from naive or immunised animals prior to challenge did not affect tumour development. Hence it was not antibodies that inhibited tumour development. $CD4^+$ T-cells from naive or immunised mice did not proliferate *in vitro* in response to stimulation with TS/A cells whereas $CD4^+$ T-cells from μMT mice did. $CD4^+$ T-cells were required at the time of immunisation

but not at the time of challenge. The mechanism by which B-cells appear to inhibit $CD4^+$ T-cells is not yet known. They proposed that B-cells compete with other APCs for tumour-derived antigens and that presentation by B-cells of these antigens to $CD4^+$ T-cells diverts antitumour immunity toward a 'non-productive' Th2-type humoral immune response and away from a 'productive' Th1-type cellular response.

There is increasing evidence that $CD4^+$ T-cells may themselves posess potent specific cytotoxic activity against autologous tumour cells both *in vivo* and *in vitro*. Rosenberg *et al.* in their preliminary report on the effect of adoptive transfer of tumour-infiltrating lymphocytes (TILs) and Interleukin-2 therapy in the treatment of patients with metastatic malignant melanoma reported objective tumour regression in 40 - 60% of patients (Rosenberg, Packard et al. 1988). These TILs were derived from tumour deposits in various locations from subcutaneous, lymph node, soft tissue, bone, liver and lung. The relative numbers of $CD4^+$ and $CD8^+$ cells varied. Of the 20 patients reported in the study 9 patients had greater than 80% $CD4^+$ TILs of whom 7 had an objective partial regression in tumour volume. More recently Konomi *et al.* examined the *in vitro* cytotoxic activity of fractionated T-cells against autologous tumour cells in a series of 8 paired (i.e. autologous tumour and lymphocyte) human samples of varying histological origin (Konomi, Sekine et al. 1995). They report that when using fractionated T-cells, with greater than 90% purity in respect of CD4 or CD8 expression and using both a modified ^{51}Cr-release assay and a colorimetric cytotoxicity assay, $CD4^+$ T-cells have cytotoxic activity as strong as that of $CD8^+$ T-cells towards autologous tumour cells. They demonstrated that $CD4^+$ T-cell cytotoxic activity against autologous tumour cells was detectable at low levels in 6 out of the 8 cases using the standard 4 hour ^{51}Cr-release assay. However by modifying the ^{51}Cr-release assay such that spontaneous release was kept to a minimum they were able to prolong the study to 20 hours and at that time period cytotoxic activity was detectable in all cases.

Furthermore the cytotoxic activity was increased 67-fold (range: 5 - 197) over the 4 hour study. These levels of cytotoxic activity were confirmed in a 24 hour colorimetric assay. The mechanism of this cytotoxic effect remains to be elucidated.

1.5.3 MHC-Unrestricted Effector Mechanisms

1.5.3.1 Natural Killer Cells

The term 'immunological surveillance' was first used by Burnet in a lecture given in June 1963 and was the result of work that had taken place over more than a decade (Burnet 1963). Burnet's original concept was that "in large long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character" (Burnet 1970). This immunological effector function was originally assumed to be the domain of antigen-specific T-cells. However the theory fell into disrepute when it was noted that athymic nude mice, which lack functioning T-cells, did not have a greater incidence of spontaneous tumours than normal mice. Beige mice, which lack functioning natural killer (NK) cells, on the other hand do have increased incidences of spontaneous tumours. It appears therefore that Burnet's concept of immunological surveillance may be valid and that the frontline effector cells may be NK cells.

Natural killer cells, so named because of their ability to spontaneously, without the need for prior sensitisation, eliminate tumour cells, but not normal tissue cells, in *in vitro* cytotoxicity assays are morphologically large granular lymphocytes and therefore phenotypically and functionally distinct from T and B cells. Gorelik and

Herberman showed that removal of NK cells from mice with surgically resected B16 melanoma resulted in uncontrolled metastatic disease and death (Gorelik and Herberman 1986). Conversely, Barlozzari et al. showed that the adoptive transfer of purified large granular lymphocytes could restore resistance to metastases in immunosuppressed rats (Barlozzari, Reynolds et al. 1983). NK cells constitute up to 20% of peripheral blood lymphocytes (Baxevanis and Papamichail 1994), but are resident in varying proportions in other tissues, liver associated lymphocytes - 50% (Hata, Van Thiel et al. 1991), lymphocytes in the spleen - 10%, lymph node - 2-7%, lung - 30% (Whiteside and Herberman 1990). They have also been detected in skin, intestine and placenta (Taglibue, Befus et al. 1982; Linnemeyer and Hamilton 1990). Fairly convincing evidence exists to support that absent or low NK activity in humans or experimental animals is associated with a high frequency of viral disease and cancer (Pross and Lotzova 1993; Whiteside and Herberman 1994).

NK cells do not undergo Ig or TCR gene rearrangements nor do they express CD3. Many attempts have been made to fractionate NK cells into subsets based on either cytokine elaboration or membrane expression of CD56, an isoform of neural cell adhesion molecule (NCAM) also designated ANK-1, or expression of CD16, also known as FcγRIII, a low affinity receptor for the Fc portion of Ig. Based on their response to IL-2 Whiteside and Herberman have defined two functionally and phenotypically distinct subsets of NK cells, designated activated, adherent NK cells (A-NK) and activated, non-adherent NK cells (NA-NK) (Whiteside and Herberman 1995). They demonstrated that only the A-NK cells are able to efficiently enter solid tumour tissues, eliminate established tumour metastases or prolong survival when adoptively transferred together with IL-2 (Yasumura, Lin et al. 1994). Furthermore they showed that pre-A-NK cells, which express ANK-1, upon stimulation with IL-2 readily adhere to vascular endothelium and extravasate through into tissues and therefore are ideally equipped to perform

antitumour surveillance. This is supported by evidence that rhodamine labelled A-NK cells selectively accumulate in B16 lung metastases within 24 hours of adoptive transfer (Basse, Golgfarb et al. 1994).

NK cells appear to be able to distinguish between abnormal and normal cells by two different mechanisms. Firstly, the NKR-P1 receptor recognises oligosaccharide molecules on the surface of target cells and may trigger target cell killing. Secondly, a group of NK cell receptors, designated 'killer-cell inhibitory receptors' (KIRs), have been proposed to negatively regulate NK cell activity upon specific recognition of class I molecules in association with self proteins (Garrido, Ruiz-Cabello et al. 1997). Lysis will only occur when the target cell lacks a particular class I molecule, the 'missing self' hypothesis. Some of these KIRs belong to the immunoglobulin superfamily. Human NK cells display different KIRs that interact with specific HLA class I molecules. For example p58.1 and p58.2 are glycoproteins with two Ig-like domains involved in the recognition of HLA-Cw2, 4, 5 and 6 or HLA-Cw1, 3, 7 and 8 respectively. Similarly receptors for HLA-A and -B alleles have been identified. A second type of KIR has been identified bearing a C-type lectin domain, designated CD94. This in association with a 43kDa glycoprotein, kp43, forms an inhibitory heterodimer involved in the recognition of HLA-C, -A and Bw6 alleles. The diversity of expression of KIRs has yet to be defined but all NK cells will express at least one functional KIR thereby preventing autoimmune reactivity. The situation is made more complex by the fact that both the CD94 and p58 KIRs have homologous receptors involved in NK cell activation. Lobo and Patel have recently reported that MHC class II antigen expression may also protect tumour cells against lysis mediated by NK and LAK cells (Lobo and Patel 1994). Obviously whether the NK cell is ultimately activated or not will depend on the interplay between all these factors.

In normal tissues pre-A-NK cells do not readily mature into A-NK cells. However in abnormal tissues local availability of IL-2, IFN-γ, IL-12 and other cytokines as well as extracellular matrix proteins such as fibronectin initiate signal transduction and maturation of pre-A-NK into A-NK cells. Once functionally activated, A-NK cells eliminate cells that do not express appropriate class I molecules or do not express them at a sufficiently high level. Target cell killing by NK cells involves similar mechanisms as killing by CTLs, namely granule exocytosis and induction of target cell DNA fragmentation.

Recently the demarcation between T-cells and NK cells has become blurred with the discovery of cells that co-express NKRP-1 and TCR and have been designated 'natural T-cells' (Bix and Locksley 1995). We know that they have a potent effector function as evidenced by their ability to produce high levels of cytokines within 1 hour of activation. The integration of signals generated at the TCR with those from NKRP-1 may underlie novel functions of these cells that remain to be elucidated.

1.5.3.2 Lymphokine Activated Killer Cells

The first report of the lysis of fresh solid tumour cells by autologous human lymphocytes cultured in IL-2, then known as T-cell growth factor, was made by Rosenberg's group in 1981 and followed on similar work in animals over the preceding years (Yron, Wood Jr. et al. 1980; Lotze, Grimm et al. 1981). They termed the effector cells "lymphokine activated killer" cells or LAK cells and proceeded to characterise them. They reported that activation of peripheral blood lymphocytes, from cancer patients or normal individuals, in lectin free Interleukin-2 containing supernatant for a brief period of 2-3 days resulted in LAK cytotoxicity toward NK-resistant fresh tumour cells (Grimm, Mazumder et al. 1982). They went on to demonstrate LAK cell cytotoxicity against autologous and allogenic tumour cells of varying histological types. Using monoclonal antibodies and

complement they determined that LAK cells were generated from precursor cells that were neither T-cells nor NK cells (Grimm, Ramsey et al. 1983). They were able to maintain LAK cells in culture for more than 2 months expanding them to greater than 10^{20}-fold without loss of lytic activity thus confirming their potential as candidates for immunotherapy (Eberlein, Rosenstein et al. 1982; Rayner, Grimm et al. 1985). Their subsequent use in immunotherapy will be dealt with later.

1.5.3.3 γδ **T-cells**

A relatively new subset of T-cells, designated γδ T-cells has been described and as such our understanding of their nature and function is in its infancy. γδ T-cells express an alternative form of TCR. The γδ TCR is a heterodimer of γ and δ subunits which contain constant and variable regions. The receptor exists in disulphide-linked and non-disulphide-linked forms, co-expressed in association with CD3. The ligands for γδ TCR are still being identified. There are grounds for believing that γδ T-cells recognise simple ligands rather than 'compound' ligands (e.g. MHC-peptide), although there may be exceptions, and may bind to surfaces or epitopes in the same way that antibodies do. A range of ligands for γδ TCR have been identified including heat shock proteins and both naturally and synthetically created phosphorylated non-peptidic ligands(Born, Hall et al. 1990; DeNagel and Pierce 1993; Fu, Cranfill et al. 1993; Weintraub, Jackson et al. 1994; De Libero 1997). Some γδ TCR recognise ligands in the form of small processed protein fragments bound to antigen-presenting molecules (Born, Hall et al. 1990).

γδ gene rearrangement ontogenically precedes the αβ gene rearrangement and they are found in all species suggesting that they must provide some survival benefit. They constitute 5 – 15% of peripheral blood T-cells of mice and humans but up to 50% in some other species (Davis and Chien 1995). In humans γδ T-cells constitute 5% of peripheral T cells and 10% of intra-epithelial lymphocytes. In mice 5% of peripheral T-cells and 50% of thymocytes are γδ TCR^{+}. Thirty to

seventy percent of murine intra-epithelial lymphocytes (IELs) are γδ TCR^+ and this rises to 95% in germ free mice. This suggests that exposure to gastrointestinal bacteria alters the homeostasis in intra-epithelial lymphocytes.

The function of γδ T-cells is poorly understood. Because of their preferential localisation to epithelial surfaces and their more primitive, less specific, antigen triggering mechanism a sentinel function of protection at mucosal surfaces seems likely. They have been implicated in the host defence against infectious agents such as *Listeria monocytogenes* and *mycobacteria* (Rakhmilevich 1994; Chandler, Frater et al. 1995). They have also been implicated in the pathogenesis of acute graft versus host disease and autoimmune diseases such as rheumatoid arthritis (Ellison, MacDonald et al. 1995). As well as producing cytokines that aid in the direct elimination of certain pathogens γδ T-cells may also contribute to the cytokine milieu that influences the differentiation of antigen-specific $CD4^+$ T-cells into either Th1 or Th2 cells (Ferrick, Schrenzel et al. 1995).

The relevance of γδ T-cells to anti-tumour immunity is currently being investigated. Given the selective localisation of $γδ^+$ T-cells to mucosal sites and the association between lymphocyte infiltration and prognosis in colorectal cancer, gastrointestinal malignancies would seem a logical research subject. Watanabe *et al.* used flow cytometric analysis of TILs obtained from 13 patients with colorectal cancer and simultaneously analysed PBLs and normal IELs in the same patient (Watanabe, Hizuta et al. 1995). They found that the frequency of γδ TCR expression in TILs was significantly lower than in IELs. However the tumour generated a larger quantity of TILs than normal tissue generated IELs such that the number of γδ T-cells per unit weight was the same in tumour and normal tissue. They also found that approximately half of TILs and IELs that were γδ TCR^+ were also $CD8^+$ whereas the majority of γδTCR^+ PBLs were $CD8^-$ and $CD4^-$. They conclude that γδTCR^+ TILs and IELs are the same subset of $γδ^+$ T cells and different from

γδTCR$^+$ PBLs. Given that γδ T-cells are present in human IELs at relatively low levels and not significantly upregulated in TILs it remains difficult to define what role if any they play against cancer cells *in vivo*.

As mentioned above heat shock proteins are ligands for γδ T-cells (Fu, Cranfill et al. 1993). Yu-quan Wei *et al.* have demonstrated that heating fresh tumour cell isolates at 42°C for 30 – 60 minutes lead to induction of HSP70 with surface expression of same (Wei, Zhao et al. 1996). They reported an increase in killing of freshly isolated tumour cells by autologous peripheral blood lymphocytes, as measured by Cr^{51} release assay and confirmed by trypan blue dye exclusion, following heat stressing in 42% of cases. This autologous killing was enhanced in 80% of cases by the combination of heat stressing with OK432 treatment prior to surgery. Furthermore they report an expansion of γδ T-cell subpopulation in PBLs following co-culture with heat stressed tumour cells. They attributed the increase in autologous tumour killing to an expansion of γδ T-cells which recognised HSP70 expression on the heat stressed cells, a conclusion which is substantiated by inhibition of this cytotoxicity with anti-HSP70 monoclonal antibody treatment of the target cells or anti-Vδ2 mAb treatment of the effector cell population. The relevance of this paper lies in the fact that HSPs are induced by stress other than heat and if they can be induced *in vivo* would recruit another limb of the immune system in the fight against cancer.

1.5.4 Non-lymphoid Cytotoxic Effector Cells

1.5.4.1 Tumour-associated Macrophages

It was Virchow, in 1863, who first noted the frequent occurrence of a lymphoreticular infiltrate in human neoplasms. He proposed that this reflected the origin of cancer at the sites of previous or chronic inflammation. Numerous attempts, with dramatically differing results, have been made to correlate the degree of inflammatory infiltrate with prognosis (Svennevig, Lunde et al. 1984;

Håkansson, Adell et al. 1997). Where an improved prognosis has been associated with an inflammatory infiltrate there have also been confounding factors. The prognosis for melanoma is better for superficial lesions i.e. Clark's level II, which also tend to have the highest lymphocytic infiltrate possibly because activated blood vessels of the superficial dermis express high levels of the adhesion molecule ELAM-1 which may recruit lymphocytes. The improved survival of patients with colorectal cancer associated with eosinophil infiltration, which may accumulate because of their presence in the lamina propria of the intestine, disappears when adjusted for Duke's stage (Mantovani, Bottazzi et al. 1992). The proportions of the various cell subsets that constitute the infiltrate change with tumour growth/development (Håkansson, Adell et al. 1997). There is great variability in the chemotactic ability of clones derived from the same primary tumour to attract monocytes.

There are a number of soluble factors that may be involved in the recruitment and activation of monocytes. The most studied is monocyte chemotactic protein 1 (MCP-1), a 76 amino acid polypeptide that is chemotactic for monocytes and not for neutrophils or lymphocytes. Levels of tumour associated macrophages (TAM) have been shown in a murine sarcoma model to correlate with the expression of MCP-1 mRNA (Walter, Bottazzi et al. 1991). Other chemotactic agents include macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), platelet-derived growth factor (PDGF) or vascular permeability factor (VPF) (Soiffer, Lynch et al. 1998).

When examining the functions of TAMs we find that they can have conflicting effects on tumour growth and tumour cell death depending on the activation state of the macrophages and the intrinsic properties of the tumour cell. This is what Mantovani refers to as macrophage balance (Mantovani, Bottazzi et al. 1992). Macrophages have been shown *in vitro* and *in vivo* to have potent tumour cytotoxic

abilities. The mechanisms of cell killing include the release of tumour necrosis factor (TNF), interleukin-1 (IL-1), free radicals, proteases and nitric oxide. These cytokines in turn recruit secondary immune cells to amplify the non-specific tumouricidal effects. On the other hand they are known to produce growth factors such as PDGF, epidermal growth factor (EGF) and TGF-ß and have been shown to promote tumour cell growth *in vitro* and *in vivo*. They have also been shown to possess direct angiogenic activity and to produce proteases thereby facilitating tumour growth and metastasis. Tumour associated macrophages have been shown to inhibit tumour specific T-cells through the elaboration of suppressive cytokines such as IL-10 and TGF-β and other suppressive factors such as prostaglandin or by inducing alterations in the signal transducing apparatus of T-cells (dealt with below) (Fujii, Igarashi et al. 1987). It is obvious, given the diversity of macrophage functions, why a clear relationship between macrophage infiltration and prognosis is not forthcoming. Tumour derived molecules have been shown to deactivate macrophages that are in close proximity to the tumour while activating distal macrophages. The picture is becoming more complicated as our understanding increases, for instance, tumours have been shown to control macrophage production of effector molecules, for instance diverting L-arginine away from nitric oxide production to favour the synthesis of ornithine, a precursor for polyamine tumour growth factors required for cell replication; (This has been comprehensively reviewed in Elgert, 1998). The use of TAM in immunotherapy may prove to be a double-edged sword and should be used with care.

1.5.4.2 Eosinophils

Eosinophilia has been described in association with Hodgkin's Disease and other lymphomas and less frequently with cancers of the colon, stomach, ovary, uterus, cervix, breast and thyroid (Samoszuk 1997).

Activated CD4+ T-cells have been shown to produce C5a, IgE and Interleukins 4 and 5, potent eosinophils chemoattractants (Vadas and Clark-Lewis 1985; Stefanini, Claustro et al. 1991; Pericle, Giovarelli et al. 1994; Hung, Hayashi et al. 1998). Il-2 administration has been shown to induce a marked eosinophilia (Ishimitsu and Torisu 1993; Rivoltini, Viggiano et al. 1993). Once recruited to the tumour site eosinophils become activated, by some as yet unknown mechanism(s), possibly involving monocyte-derived IL-2 and/or IFN-γ (Vadas, Nicola et al. 1984; Valerius, Repp et al. 1990; Rivoltini, Viggiano et al. 1993).

Eosinophils are also capable of antibody-independent cytotoxicity. Mouse eosinophils have been shown to bind to allogenic MCA-38 colon adenocarcinoma cells in the absence of antibody and without utilising integrin molecules. Binding was also fibronectin independent but was prevented by pre-treatment of eosinophils with inhibitors of protein tyrosine kinases (Taylor, Lee et al. 1998). Eosinophils have also been shown to adhere to target cells via major basic protein which is itself cytotoxic for tumour cells (Kubo, Loegering et al. 1999). As well as being directly cytotoxic themselves eosinophils have been shown, by releasing eosinophil peroxidase, to augment the tumour cell cytotoxicity of mast cells and macrophages (Henderson, Chi et al. 1981; Nathan and Klebanoff 1982).

The relevance of eosinophils within tumours has been much debated with most papers finding no prognostic relationship (Samoszuk 1997). However some authors have reported that tumour-associated tissue eosinophilia is associated with an improved prognosis whereas tumour-associated blood eosinophilia is associated with tumour dissemination and a poor prognosis (Lowe, Jorizzo et al. 1981). The picture becomes even more complicated by the finding that tumour-infiltrating eosinophils express TGF-β_1, a known immunosuppressant, and TGF-α, an angiogenic factor thereby possibly facilitating tumour growth (Wong, Elovic et al. 1991; Ghiabi, Gallagher et al. 1992). The relationship between tumours and

eosinophils is unfolding and becoming increasingly more complex with the evidence suggesting that the eventual effect of eosinophils will depend on the tumour microenvironment.

1.5.5 Humoral Immune Responses

It is generally accepted that an effective antitumour response depends largely on cell mediated immunity. However antitumour antibodies may, by inducing antibody-dependant-cell-mediated cytotoxicity (ADCC), recruit non-specific effector cells such as NK cells to attack and eliminate tumour cells, which have been coated (opsonised) with antibody. There have been reports of the successful utilisation of ADCC in the elimination of tumours in animal models (Bright, Shearer et al. 1994).

Perhaps the most important role for antibodies in cancer therapy will be the use of monoclonal antibodies to target tumour antigens either for imaging of metastases or for the delivery of cytotoxic agents to tumour deposits.

Table 1.5: Possible tumour escape mechanisms.

- Poor immunogenicity
- Immunoselection of weakly antigenic tumours
- Tumour-induced Immunosuppression
- Induction of suppressor cells
- Immune blockade
- Tumour microenvironment
- Tolerance of tumour antigens
- Alterations in TCR

1.6 Mechanisms of Immune Evasion

Having examined the armamentarium that is available to the immune system to target tumour cells it is surprising that in immunocompetant individuals tumours not only develop and grow but metastasise to lymph nodes potentially the

most hostile immune environment. This failure of immune containment, or tumour escape, may be the result of several possible mechanisms (Table 1.5). For an antigenic tumour to grow and metastasise in an immunocompetant host it must either avoid detection or in the event of being detected avoid elimination.

1.6.1 Poor Immunogenicity

Immunogenicity is a function of both the antigenicity of the developing tumour and the competence of the host immune response. It is ideally dependant upon all the tumour cells possessing a novel antigen, to which the immune system has not previously been tolerised, which is not shared with normal tissues and expressing that antigen on the cell surface appropriately complexed with MHC molecules for presentation to a fully functional immune system together with the appropriate co-stimulators.

1.6.1.1 Alterations in MHC expression

Ever since 1975 when Zinkernagel and Doherty demonstrated that CTL efficacy was restricted by surface molecules encoded by genes of the class I region of the major histocompatibility complex (MHC) researchers have postulated that they may be involved in the immune response to tumours. This lead to the discovery that MHC class I molecules presented intracellular proteins, derived from mutated genes in case of a malignant-cell, on the cell surface. MHC class I gene expression is controlled by a variety of trans-acting factors which bind to regulatory elements on the cis-MHC genes, (reviewed in David-Watine, 1991). Though expressed constitutively on the majority of somatic cells the level of expression is variable being highest on cells of lymphoid origin and very low or absent on hepatocytes, skeletal muscle and lymph vessel endothelium but can be upregulated at sites of inflammation by cytokines such as IFN-γ and TNF-α.

Now there was the possibility that T-lymphocytes would be able to recognise cells expressing abnormal proteins on their surface. Animal models, largely virally induced tumours, were the first to provide direct evidence for a role of class I molecules in the rejection of syngeneic tumour cells. Class I-restricted CTLs have also been documented in some human tumours, most notably melanoma, but not yet for the majority of tumours. The loss of an MHC antigen was first described in a mouse lymphoma in 1976 and in human tumours in 1977. An increasing proportion of tumours are being found with alterations in MHC expression (Table 1.6).

Anti-tumour activity of $CD8^+$ CTLs has been demonstrated in a wide variety of animal tumours including lymphoma, sarcoma, glioma, mastocytoma and melanoma. In humans class I-restricted CTLs have been described in ascites from ovarian cancer, renal cell carcinoma, squamous cell cancers of the head and neck and lung and also in perhaps the most extensively studied human cancer, melanoma. In the majority of these, despite the demonstration of tumour antigen-

Table 1.6: Frequency (%) of HLA class I altered phenotypes in invasive tumours.

	HLA-loss					
Tumour type	HLA-A+B+C	HLA-A	HLA-B	HLA-A+B	HLA-allelic	Total
Breast	52	4	8	9	15	88
Cervix	18	3	19	2	21	63
Colon	21	8	5	5	26	65
Larynx	9	19	16	9	26	79
Melanoma	16	3	5	2	25	51
Pancreas	12	19	8	ND	ND	39
Prostate	34	ND	ND	ND	51	85

ND = not determined. (Adapted from Garrido et al. 1997)

specific CTLs, the tumours were not rejected. Some viruses such as the Epstein-Barr virus (EBV) and human papilloma virus (HPV) are able to maintain infection of cells whilst expressing a minimum number of viral genes. In the EBV-associated Burkitt's lymphoma the only viral gene expressed in the cell is the EBNA-1 antigen. Recent evidence has shown an internal glycine-alanine repeat region of this antigen which effectively inhibits it's processing for presentation by class I, thereby allowing EBV to maintain infection in the absence of cell surface antigen expression. Other viruses such as Adenovirus type 12 through viral oncogene E1A have been shown to exert a strong down-regulatory influence on HLA expression, whereas Cytomegalovirus achieves the same effect by inhibition of the transport of HLA molecules through the Golgi apparatus to the cell surface. Thus loss/reduction of MHC expression was postulated to facilitate immune evasion. This was further corroborated by enhanced immunogenicity of poorly immunogenic tumours following transfection with allogenic MHC class I. Furthermore whilst high levels of class I expression were associated with enhanced immunogenicity, low levels of the same class I molecules failed to induce CTLs but these cells remained sensitive to CTL lysis.

Between 39 and 88% of tumours derived from HLA^+ epithelia are HLA deficient (See Table 1.6). This deficiency can take different forms but can crudely be divided into four phenotypes based on monoclonal antibody staining of cryostat sections (Garrido, Ruiz-Cabello et al. 1997). Increasingly flow cytometric analysis of cell surface molecules is being used (Coventry, Neoh et al. 1994; Diederichsen, Ostenhom et al. 1998).

Phenotype I: total HLA loss could result from structural defects in the MHC genes but is more commonly associated with defects in $ß_2$-microglobulin ($ß_2m$) or TAP synthesis, i.e. molecules pivotal to the construction and assembly of class I/antigen complexes. Complete loss of expression of functional $ß_2m$ was first

observed in the Burkitt lymphoma cell line, Daudi, in which one copy of the $ß_2$m gene was lost and a point mutation in the initiating codon of the other $ß_2$m gene resulted in a failure to translate the mRNA. Studies of the $ß_2$m gene in colorectal tumour cells and colorectal tumour cell lines identified a short sequence of four CT nucleotide repeats in exon 1 of the gene which appear to act as a 'hot spot' for mutations in the gene. Colorectal tumours with mutations in the $ß_2$m gene or other types of HLA expression also exhibited the mutator phenotype, which was described in association with defects in DNA mismatch repair. Hence in colorectal cancer, at least, HLA loss may be associated with deficient DNA mismatch repair systems (Garrido, Cabrera et al.). Lack of expression of class I molecules has also been linked to a lack of expression of TAP gene products in a variety of tumours including non-small cell lung cancer, cervical carcinoma, colorectal cancer and renal cell carcinoma. In the case of non-small cell lung cancer and renal cell carcinoma class I expression was restored by treatment with IFN-γ indicating a regulatory rather than a structural defect in TAP gene expression (Seliger, Höhne et al. 1996). Furthermore transfection of TAP genes into a TAP-deficient cell line restored class I expression and induced resistance to NK cell lysis.

Phenotype II: HLA haplotype loss in only a few tumour cell lines and may be related to chromosomal non-dysjunction or mitotic recombination.

Phenotype III: HLA locus loss has been described in several tumours (see Table 1.6). The mechanisms are not yet known but may be transcriptional. In melanomas increased c-*myc* transcription has been correlated with selective HLA-B downregulation, whereas n-*myc* downregulates all HLA class I genes. This type of transcriptionally mediated downregulation is relevant to immunotherapy as it can often be overcome by cytokine treatment.

Phenotype IV: HLA allelic loss possibly resulting from point mutations, partial deletions or as a consequence of chromosomal breakage, though, the basis for allelic loss has not been identified in individual cases. One of the most striking examples of selective allelic loss is the high frequency of loss of expression of HLA-A11 on Burkitt lymphoma cell lines derived from HLA-A11 individuals. This is strongly suggestive of immunoselection based on a presumed CTL response to EBV-encoded antigens presented in association with HLA-A11 molecules.

That tumour cells have a relatively high frequency of HLA loss/downregulation is well documented. The debate centers much more on whether this correlation is merely incidental or biologically significant, conferring some sort of selective growth advantage. Given the genetic instability of malignant cells the simplest explanation would be that the observed changes in MHC expression are merely coincidental. Demonstrating that HLA and associated genes do not have a higher rate of mutation than other genes, which are not essential for tumour growth, would support this hypothesis. Some indirect evidence exists to support this argument. Firstly, the frequency of HLA loss has not been shown to be significantly higher in tumours with demonstrable tumour-specific CTL response as compared to tumours without. Secondly, HLA loss has been demonstrated in tumours with little or no inflammatory infiltrate. In both of these cases however the immune response may have exerted it's influence earlier in tumour development, selected the resistant phenotype, and abated. Thirdly, a tumour suppressor gene, WAF-1 has recently been identified on chromosome 6, the same chromosome as the MHC, raising the possibility that interference with WAF-1 gene expression may reflect genetic instability in this region.

At present the argument in favour of immunoselection is stronger. MHC antigen loss variants have been generated, under selective pressure of CTL, both *in vitro*

and *in vivo*. HLA allelic changes were detected in two metastatic melanoma lesions obtained five years apart in the same patient. Levels of MHC class I mRNA were strongly reduced in a renal cell carcinoma cell line when compared to normal renal tissue and were reduced further in metastatic renal cell carcinoma cell line derived from the same patient (Seliger, Höhne et al. 1996). Whereas 30 to 63% of cervical carcinomas show some alteration in class I expression there is no evidence of changes in MHC expression in cervical intraepithelial neoplasia (CIN) (Connor and Stern 1990). There is evidence of an increased incidence of class I down regulation in cervical carcinoma lymph node metastases compared with the primary cancers. In a study of over 700 tumours of several types and corresponding normal tissue, little or no HLA loss was observed in premalignant or carcinoma in situ tissue. Taken together these studies would tend to imply that HLA loss is a late event in the evolution of a cancer that may be associated with a poorer prognosis.

The emergence of HLA loss variants would facilitate avoidance of elimination by $CD8^+$ CTLs but should render them susceptible to NK cell lysis and consequent eradication. Why doesn't this happen? Tumours with total loss of class I molecules should be universally sensitive to NK cell lysis but this doesn't appear to be the case. It may be that these cells have not lost all HLA class I expression but appear to because either the monoclonal antibodies employed have low affinity or the cells have been assumed to lack class I expression because they lack functioning TAP. However, in this latter case, some class I expression is possible if endogenous proteins can make their own way to the endoplasmic reticulum, at least in theory. There may be other surface antigens, not yet identified, involved in the inhibition of NK cell cytotoxicity of allogenic cells. Finally all of the other tumour associated immunosuppressive mechanisms, to be discussed below, may prevent elimination of an NK sensitive cell.

Given that HLA loss would be advantageous to tumour growth and metastasis by allowing escape from CTL lysis, then sufficient loss to avoid detection by CTL but without inducing susceptibility to NK cell lysis would be selected. Therefore patients who are homozygous for a HLA subtype recognised by a particular KIR would have to lose both alleles to become NK sensitive. Conversely it would be expected that tumours that have evaded a CTL response would be more likely to be homozygous for HLA alleles that are ligands for KIRs. This is supported by data obtained from cervical cancer patients, where homozygotes for HLA-Bw4 and -Bw6 or two groups of HLA-C allele homozygotes recognised by p58 inhibitory molecules show an increased frequency compared with local controls.

It is possible to determine the HLA-expression phenotype of any tumour and in the future it should be possible to determine the rate of HLA allele loss in different cancers. This will enable the design of immunotherapeutic strategies based on either cytokine therapy to overcome transcriptional downregulation of class I molecules or transfection with class I genes to restore CTL susceptibility. However intratumoural heterogeneity with regard to HLA phenotype will present a difficult obstacle to overcome in the development of any single immunotherapeutic approach.

1.6.1.2 Lack of Co-Stimulatory Molecules

T-cell activation in response to antigen recognition involves at least three different cell surface interactions and probably more. Firstly, cell to cell contact involving adhesion molecules such as ICAM-1 and LFA-1 or LFA-3 and CD2. Secondly, TCR recognition of the MHC/antigen complex and subsequent intracellular signalling. Thirdly, the delivery of the co-stimulatory signal commonly provided by the antigen presenting cell, referred to earlier.

It is almost 30 years since Bretscher and Cohn first proposed that the generation of an antigen-specific immune response required at least two distinct signals from the antigen presenting cell (Bretscher and Cohn 1970). We now know that the first signal occurs via the TCR-CD3 complex and is responsible for the specificity of the response, while antigen-presenting cells provide the second signal, referred to as co-stimulation. Many molecules can enhance a suboptimal proliferative response: nutrients, hormones, trophic factors, interleukins, and cell adhesion molecules. Some of these act on the T-cell to improve its survival in culture, others act on the antigen-presenting cell to enhance its overall effectiveness. Adhesion molecules facilitate the interaction between the two cells and enhance signal transduction by increasing occupancy of the TCR.

Additionally there is a unique set of molecules, in the absence of which, TCR engagement results in clonal anergy (Gimmi, Freeman et al. 1993). This co-stimulatory signal is transmitted through CD28. It was first identified as a differentiation antigen expressed by $CD4^+$ and some $CD8^+$ cells. CD28 is a 44kD homodimeric glycoprotein member of the immunoglobulin supergene family. It is highly conserved between species (Linsley and Ledbetter 1993). CD28 is expressed on resting lymphocytes, 95% of $CD4^+$ and 50% of $CD8^+$, and is upregulated following activation (Dalgleish 1996). Signal transduction following ligand binding by CD28 occurs through either calcium dependant or calcium independent pathways (Linsley and Ledbetter 1993). The calcium dependant pathway through mechanisms similar to CD3 signal transduction i.e. protein tyrosine kinase activation of phospholipase c-γ1 and may represent a point of interaction with CD28 (Schwartz 1992).

T-cell activation upregulates CD28 expression, which in turn upregulates IL-2 and IL-2 receptor. CD28 ligation upregulates IL-2rα, IL-2rβ and IL-2rγ, increased

transcription of IL-2 mRNA and increased expression of cytokine genes (including IFN-γ, GM-CSF and TNF-α), allowing cells to pass from G_0 to G_1 phase of the cell cycle (Greenfield, Nguyen et al. 1998). TCR engagement in the absence of CD28 signalling results in a state of long-term specific unresponsiveness termed anergy. This anergic state can be generated by blocking the interaction of CD28 with its ligand either with anti-CD28 Fab fragments, antibodies to B7 ligands or CTLA-4Ig during antigen presentation.

Cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4 or CD152) was originally discovered, by screening mouse CTL derived cDNA libraries, in a search for lymphocyte surface molecules having a role in T-cell cytotoxicity (Brunet, Denizot et al. 1987). They are located in close proximity on chromosome 1 in the mouse and on chromosome 2 in humans (Harper, Balzano et al. 1991). CTLA-4 is not detected on resting cells but is induced during T-cell activation and is equally expressed on human $CD4^+$ and $CD8^+$ T-cells but is higher in murine $CD8^+$ than $CD4^+$ T-cells. On activated T-cells CTLA-4 expression is approximately 2-3% that of CD28 (Linsley 1995). However it has been shown to have ~ 20-fold higher affinity for their common ligand B7 (Krummel and Allison 1995). CTLA-4 is therefore an activation-induced, high avidity, low abundance receptor for B7 molecules. Ligand binding by CTLA-4 induces, through a series of enzymatic steps, a protein tyrosine phosphatase Syp. CTLA-4 bound Syp congregates in the area of cell-cell contact in close proximity to the TCR where the enzymatic activity of Syp dephosphorylates $p52^{SHC}$ associated with CD3ζ thereby damping down TCR signals (See Fig. 1.3) (Waterhouse, Marengère et al. 1996). It has been shown that the ordered phosphorylation of CD3ζ of TCR establishes the threshold for T-cell activation and by interfering with this CTLA-4 may antagonise TCR signal transduction (Lee, Chuang et al. 1998). This inhibitory effect on T-cell activation only occurs when CTLA-4 molecules are ligated in close proximity to signalling TCR complexes (Fraser, Rincón et al. 1999). Thus the activation state of

the T-cell is determined by the competing effects of TCR induced activation and CTLA-4 interference with the TCR signal transduction.

As well as being involved in determining the activation status of T-cells upon antigen recognition CTLA-4 stimulation is involved in terminating the immune response. Peripheral lymphoid homeostasis must be maintained for the health of the organism as uncontrolled lymphocyte activation would lead to autoimmune disease and grossly enlarged peripheral lymphoid tissues such as occurs in *lpr/lpr* or *gld/gld* mice which lack, respectively, Fas molecules and Fas ligand expression. T-cell stimulation via CD3/CD28 not only induces the aforementioned activation signals and cytokines but also initiates the mechanisms for it's own demise by upregulating CTLA-4 mRNA and Fas expression rendering themselves susceptible to activation induced cell death or propriocidal regulation. In $CD4^+$ cells Fas-mediated apoptosis is the main mechanism for TCR/CD3-induced cell death. In resting cells Rel family proteins, such as c-Rel and NF-κB, are sequestered in the cytoplasm by association with IκB (an endogenous inhibitor of NF-κB) proteins. Following CD3/CD28 signal transduction proteolytic degradation of IκB proteins occurs releasing active NF-κB to translocate to the nucleus and activate transcription of responsive genes (Pioli, Gatta et al. 1999). CTLA-4 stimulation prevents IκB degradation, thereby increasing the cytoplasmic levels of IκB and reducing the nuclear Rel protein content with obvious consequences for cytokine gene transcription (Fraser, Rincón et al. 1999). CTLA-4 binding has other effects, which may contribute to its antagonistic effect in CD28 stimulation. CTLA-4 binding has been shown to induce TGF-ß production by $CD4^+$ T-cells, a potent inhibitor of T cell responses, potentially countering the stimulatory effect of CD28 induced IL-2 (Chen, Jin et al. 1998).

Memory T-cells have a lower threshold for activation through the TCR and are thought to be less dependent on costimulation than naive T-cells. However CD4

engagement away from TCR results in non-activation of memory T-cells and involves CTLA-4 ligation. Unlike naive T-cells CTLA-4 is expressed at low levels on memory T-cells and is continually recycled from cytoplasmic pools. This CTLA-4-mediated inhibition of memory T-cell activation following low intensity TCR engagements serves to raise the threshold for activation and is felt to extend the lifespan of memory T-cells by avoiding inappropriate activation (Metz, Farber et al. 1998).

The first CD28 ligand, or counter-receptor as it is sometimes known, BB-1 now known as B7.1 (CD80), was identified by Yokochi as a B lymphoblast antigen recognised by a monoclonal antibody BB1 (Yokochi, Holly et al. 1981). Freeman *et al.* in 1993 uncovered B7.2, an alternative ligand for CTLA-4 in B7-deficient mice (Freeman, Borriello et al. 1993). B7.2 shares only 26% homology with B7.1 and that it was constitutively expressed on resting cells (Freeman, Gribben et al. 1993). Both belong to the Ig superfamily composed of an extracellular region with 2 Ig-like domains. B7.1 has a short cytoplasmic domain, which may associate with the intracellular actin based cytoskeleton. B7.2 has a longer cytoplasmic domain, which contains potential phosphorylation sites for protein kinase C suggesting that it may have a signalling function. B7.2 is upregulated within 6 hours of activation of B-cells and macrophages and maximal levels are achieved between 18 and 24 hours. It is constitutively expressed on monocytes, dendritic cells and T-cells. B7.1 expression occurs 24 to 48 hours following activation reaching maximal levels after a further 48 to 72 hours (Greenfield, 1998; Lenschow, Walunas et al. 1996). Expression of B7.1 and B7.2 is controlled by cell-cell contact and by cytokines. Antigen binding to B-cell surface Ig induces B7.2 expression as does CD40 receptor on the B-cells interacting either with T-cell expressed CD40L or anti CD40 antibodies. An additional level of control of B7.1 and B7.2 expression results from the effect of cytokines. IL-4 is a potent inducer of B7.1 and to a lesser extent B7.2. IFNγ increases expression of B7.2 and B7.1 on monocytes but

down regulates B7.1 expression on peritoneal macrophages. IL-10 blocks expression of both on peritoneal macrophages and downregulates B7.2 but not B7.1 on human dendritic cells. Thus the effects of individual cytokines depends on the cytokine in question, the target cell type and its state of activation.

As if the situation wasn't complex enough it appears that B7.1 or B7.2 may influence or direct Th1 versus Th2 development respectively. There are many studies that yield apparently conflicting results and are reviewed in (Sperling and Bluestone 1996). In short they speculate that under physiological conditions of low antigen density inhibition of CD28 signalling leads to no response due to clonal deletion by apoptosis. Co-stimulation via CD28 may be more important for the development of Th2 responses whereas Th1 responses seem to have less of a requirement, for instance CD28-deficient mice have normal or enhanced Th1 responses and impaired Th2 cytokine (IL-4, IL-5 and IL-10) production (Greenfield, Nguyen et al. 1998). Naive T-cells could be activated in the absence of CD28 costimulation by increasing the strength of signal through the TCR, however, B7.2 mediated costimulation was essential for the development of a Th2 cytokine response suggesting that B7.2 may be a preferred costimulator for Th2 cells (Manickasingham, Anderton et al. 1998). Finally high levels of costimulation together with high TCR occupancy may induce "high zone tolerance" or "clonal exhaustion" (Fig. 1.6).

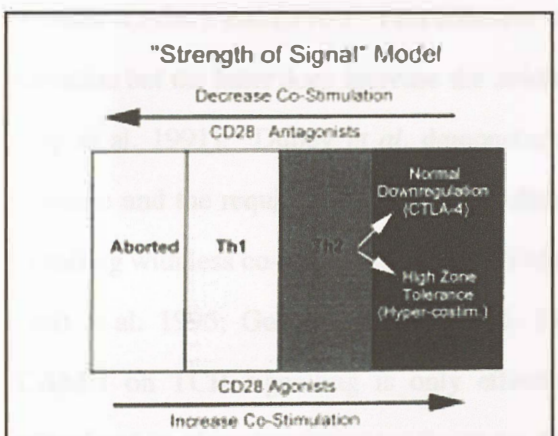


Fig 1.6: Effect of strength of co-stimulatory signal on the T-cell outcome

1.6.1.3 Additional Co-stimulators

The two-signal model of lymphocyte activation is an oversimplification. The co-stimulatory effect of adhesion molecules was attributed simply to their ability to prolong cell-cell contact and thereby TCR occupancy. At least three additional cell surface molecules on antigen presenting cells, all members of the Ig supergene family, have been shown to costimulate T-cell activation. These are intercellular adhesion molecule-1 (ICAM-1 or CD54) which interacts with leucocyte function associated antigen-1 (LFA-1 or CD18) on the T-cell, leucocyte function associated antigen-3 (LFA-3 or CD58) which interacts with LFA-2(CD2) on the T-cell and vascular cell adhesion molecule-1 (VCAM-1 or CD106) which interacts with very late activation antigen-4 (VLA-4 or CD49). Each of these has been shown individually to co-stimulate T-cell activation.

ICAM-1 is the most studied of the three and has been shown to mediate adhesion between ICAM-1 and LFA-1. This adhesive effect occurs in the absence of T cell activation but the latter does increase the avidity of LFA-1 for ICAM-1 (Kuhlman, Moy et al. 1991). Dubey *et al.* demonstrated that the magnitude of the T cell response and the requirement for co-stimulation depends on the intensity of TCR signalling with less co-stimulation required when the TCR signal is strong (Dubey, Croft et al. 1995; Goldstein, Chen et al. 1998). The co-stimulatory effect of ICAM-1 on TCR signalling is only effective when the ligands are presented colocalised in close proximity on the same surface (Kuhlman, Moy et al. 1991). ICAM-1 binding to LFA-1 alone does not elicit any detectable intracellular events however when associated with TCR signal transduction, induces prolonged inositol phospholipid hydrolysis and a sustained increase in intracellular levels of free Ca^{++} (Van Seventer, Bonvivi et al. 1992). A number of reports using plate bound anti-CD3 with or without plate bound ICAM-1 have demonstrated higher levels of cytokine (IL-2, IL-4, IFN-γ and TNF-α) production by $CD4^+$ T-cells and $CD8^+$ T-cells as well as enhanced T-cell proliferation (Damle, Klussman et al. 1992; Fan,

Brian et al. 1993; Chen, Goldstein et al. 1999). Furthermore these studies found differences in the pattern of cytokines produced in response to ICAM-1 co-stimulation between naïve and activated T cells and between different T-cell subsets. Damle *et al.* found naïve $CD4^+$ $CD45RA^+$ T-cells proliferated more in response to ICAM-1 or VCAM-1 than to B7 or LFA-3 co-stimulation whereas antigen preactivated T cells were most efficiently costimulated by B7. This raises the possibility that ICAM-1 and VCAM-1 are responsible for the initiation of proliferation while B7 and LFA-3 may facilitate sustained proliferation of antigen primed T-cells.

The current state of our understanding suggests that features of antigen/TCR interaction (density in particular), cytokine availability and the relative density of several distinct costimulatory molecule/receptor interactions, which may synergise with each other, are all integrated by the T-cell during its activation for cytokine secretion and proliferation.

1.6.1.4 Immunoselection of Weakly Antigenic Tumours

It is now generally accepted that some, if not all, tumours express surface antigens that under the correct conditions a competent immune system is capable of recognising and responding to. Unlike single cell cultures in the laboratory developing tumours are heterogeneous in terms of antigen expression. The strength of the response to developing tumours *in vivo* ranges from weak and ineffectual to total elimination depending again on a variety of factors including the immunogenicity of the antigen. This antigen-specific T-cell response causes a selective outgrowth of antigen-negative tumour variants. By the time a cancer is clinically detectable the most immunogenic elements/cells have been eliminated leaving those cells, which due to their weak immunogenicity have a growth advantage. This concept will be discussed more comprehensively in chapter 5.

1.6.2 Tumour-Induced Immunosuppression

1.6.2.1 Fas (CD95)

Programmed cell death or apoptosis is an organised, active, form of cell suicide that occurs during embryogenesis, metamorphosis, endocrine induced tissue atrophy and normal tissue turnover. Furthermore, as mentioned earlier, apoptosis is involved in CTL mediated cytotoxicity and peripheral deletion of activated mature T-cells at the end of an immune response. There is a wide variety of intrinsic and extrinsic signals that induce apoptosis including oxidative stress, intracellular proteolysis, corticosteroids, ultraviolet irradiation, tumour necrosis factor, lymphotoxin, Apo3 ligand, Apo2 ligand and most importantly in the current context Fas ligand or CD95L (reviewed in (Ashkenazi and Dixit 1998). Briefly Fas ligand binding to Fas receptor induces trimerization of the Fas receptors with aggregation of their death effector domains that then recruit Fas-associating protein with death domain (FADD or MORT1). FADD then binds to and activates caspase-8 previously known as FLICE (FADD-like ICE (Interleukin 1β Converting Enzyme)). Caspase-8 binds to FADD via two death effector domains inducing self-activation of the protease domain and further downstream activation of the Caspase cascade (Nagata 1997).

FasL was originally thought to be expressed only on cells of lymphoid/myeloid origin such as T-cells, B-cells, NK-cells and macrophages. Subsequently stromal cells of the eye and Sertoli cells of the testis have been shown to costitutively express FasL (Suda, Takahashi et al. 1993; Bellgrau, Gold et al. 1995; Griffith, Brunner et al. 1995). These are two areas of the body, which along with the brain, ovary, placenta and pregnant uterus have been described as 'immunologically privileged' as they appear to be exempt from immunological responses. Immune privilege is best demonstrated in the eye by the success of human corneal allografts, where the hundreds of thousands of transplants performed world-wide

every year enjoy one-year survival rates approaching 90% without tissue matching or immunosuppressive therapy (Griffith and Ferguson 1997). For many years this privilege was attributed to a lack of lymphocytic drainage and to the presence of a blood-ocular barrier. It is now known that immunological privilege in the eye is much more complex involving many mechanisms including FasL expression. This is corroborated by almost complete rejection of $FasL^-$ corneal allografts in a murine model as compared with >50% survival of $FasL^+$ grafts (Griffith and Ferguson 1997). Furthermore corneas grafted heterotopically to the skin are promptly rejected. More recently Lau *et al.* prolonged islet cell graft survival and restored normoglycaemia in diabetic animals by co-transplanting with $FasL^+$ myoblasts (Lau, Yu et al. 1996).

The ability of tissue expressing FasL to kill inflammatory cells that come into contact with it encouraged examination of FasL expression on tumour cells. It is not surprising therefore that leukemias and lymphomas were amongst the first malignancies to be shown to express FasL (Walker, Saas et al. 1998). An ever increasing number of non-lymphoid tissues and tumours are being characterised as expressing FasL. This is not a universal phenomenon with both inter- and intra-tumoural heterogeneity of expression. Hahne *et al.* while screening sera for soluble FasL observed sFasL in 18 of 35 patients with melanoma and proceeded to investigate if the sFasL had originated from melanoma cells rather than from activated T cells (Hahne, Rimoldi et al. 1996). They discovered substantial quantities of FasL in lysates of melanoma cells lines established from patients with melanoma. Immunohistochemical examination of sections of metastatic melanoma lesions revealed tumours to express FasL but not Fas. FasL was not detected in normal melanocytes in the surrounding skin indicating that FasL upregulation probably occurs during tumourigenesis. In three out of seven hepatocellular carcinomas (HCCs) examined FasL mRNA was detected (Strand, Hofmann et al. 1996). The presence of FasL protein was confirmed in two $mRNA^+$ and not found

in one mRNA$^-$ HCCs examined by western blot analysis. FasL expression has also been detected in colon cancer cells (O'Connell, O'Sullivan et al. 1996) and two out of seven primary colonic adenocarcinomas (Shiraki, Tsuji et al. 1997). Fas ligand mRNA and protein has also been detected in vivo in all 8 oesophageal carcinomas (6 squamous and 2 adenocarcinoma) examined (Bennett, O'Connell et al. 1998). They found both FasL positive and negative areas within individual tumours. Shiraki *et al.* detected FasL mRNA and protein in all four hepatic metastases of colonic adenocarcinoma that they examined.

The next question to be answered is whether or not FasL expression is of any clinical relevance, does it confer immune privilege on the tumours? There is a good deal of *in vitro* evidence of killing of Fas$^+$ target lymphocytes by FasL expressing tumour cells including hepatoblastoma cells and colonic adenocarcinoma cells (O'Connell, O'Sullivan et al. 1996; Strand, Hofmann et al. 1996; Shiraki, Tsuji et al. 1997). There is also evidence that TILs have enhanced expression of Fas compared to matched and control peripheral blood samples and therefore increased susceptibility to FasL mediated apoptosis (Cardi, Heaney et al. 1998). *In vivo* evidence has largely been indirect with for example rapid growth of FasL expressing melanoma cells in normal mice compared with delayed tumourigenesis in Fas deficient *lpr* mice (Hahne, Rimoldi et al. 1996). The first direct, quantitative evidence of FasL induced TIL apoptosis is from Bennett *et al.* Using immunohistochemical and in-situ hybridisation techniques they detected FasL positive and negative areas in oesophageal carcinoma specimens (Bennett, O'Connell et al. 1998). Furthermore, using anti-CD45 antibodies, they reported a fourfold depletion of TILs in Fas$^+$ as compared with Fas$^-$ areas within the same tumour. Using dual staining of CD45 (common leucocyte antigen) and DNA strand breaks (TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) techniques they demonstrated a twofold increase in apoptotic cell

death of TILs in Fas^+ versus Fas^- areas. They have called this the Fas counterattack.

The consequences of tumour expression of FasL are not restricted to the immediate tumour milieu. Membrane bound FasL undergoes metalloproteinase-mediated proteolytic cleavage to generate soluble Fas ligand, which in mice is not capable of inducing apoptosis, but in humans does, though not as efficiently as membrane bound FasL (Nagata 1997). Soluble FasL has been detected in the sera of 18 of 35 patients with malignant melanoma and also in sera of patients with lymphoma (Hahne, Rimoldi et al. 1996; Tanaka, Suda et al. 1996). Whether soluble tumour-derived FasL contributes to the systemic consequences of malignant disease such as the systemic immunosuppression seen in end stage disease remains to be seen. It is noteworthy that peripheral blood lymphocytes from renal cell carcinoma patients have lower levels of Fas expression then PBLs from normal healthy controls (Cardi, Heaney et al. 1998).

Why are these tumour cells not killed by the FasL that they themselves express? It has previously been shown that normal colonic epithelial cells and hepatocytes express Fas. The physiological significance of colonic epithelial cells expressing both Fas and FasL is not known but it has been suggested that as Fas is expressed on the basolateral surface and is only present on the luminal surface in abnormal epithelial cells binding at that stage of soluble FasL shed by epithelial cells eliminates potentially harmful cells (Krammer, Galle et al. 1998). In malignant disease resistance to apoptosis may be fundamental to tumour progression (Djerbi, Screpanti et al. 1999). The resistance to Fas induced apoptosis may result from down-regulation or depletion of Fas expression as in the case of hepatocellular carcinoma where 9 out of 22 specimens were shown to lack any expression of Fas and the remaining 13 had reduced expression (Strand, Hofmann et al. 1996). The interference with Fas-induced apoptosis may occur at the level of signal

transduction either through mutations in the various enzymes in the cascade or through the expression of inhibitors of apoptosis. Irmler *et al.* identified a protein which they have called FLIP (FLICE-inhibitory protein) which is predominantly expressed in muscle and lymphoid tissue. It is present in high levels in melanoma cell lines and malignant melanoma tumours but not in normal melanocytes. It exerts its effect by interfering with the FADD-FLICE interaction (Irmler, Thome et al. 1997). Evidence has been presented of failure of FLICE to associate with DISC, again impairing apoptotic signal transduction (Peter, Kischkel et al. 1997). FLICE expression has been shown to be capable of mediating immune escape by tumour cells in vivo, how this is possible is not clear as they will only block Fas mediated apoptosis leaving perforin-mediated cytotoxicity intact (Medema, de Jong et al. 1999). Upregulation of anti-apoptotic genes such as bcl-x_L has also been implicated.

A note of caution though, at a recent workshop on "Mechanisms of Immune Evasion by Tumours" controversial results were presented concerning false-positive results for FasL expression when using certain commercial reagents. Protein and mRNA data were presented showing that melanoma cells, previously reported to express FasL, do not actually express this molecule on their membranes (Finke, Ferrone et al. 1999).

1.6.2.2 Soluble Immunosuppressive Factors.

It is now well established that animals and humans with advanced malignant disease have a definable impairment in immune function. To what extent this is a consequence of or a cause of the progressive tumour burden remains to be elucidated. Soluble factors capable of non-specifically suppressing *in vitro* and *in vivo* assays of immune function (e.g. DTH responses, lymphocyte proliferation and cytotoxicity, skin graft rejection and lymphokine production) have been found in the culture supernatant of tumour cells of a wide variety of histological subtypes,

as well as in the sera and effusions of animals and humans with malignant disease (See Table 4.1, Chapter 4). Some of these immunosuppressive factors (ISFs) have been purified and identified, such as TGF-β, but the majority have only been partially purified and classified. Cytokines, proteins, amino acids, nucleic acid analogues and other substances have all been shown to possess immunosuppressive activity. These ISFs will be discussed in detail in Chapter 4.

1.6.2.3 Suppressor Cells

Since the 1960's a large body of circumstantial evidence has been accumulated to suggest that as well as helper and cytotoxic functions T-cells can also suppress the activity of B- and T-cells. Some of the strongest evidence for the existence of suppressor T cells comes from Robert North's group in New York (North 1985). In their studies they found that infusion of splenocytes (largely T-cells) from immune donors failed to have any effect on a tumour growing in immunocompetant recipients. However, infusion of the same cells into mice, which have been rendered T-cell deficient, by thymectomy, lethal γ-irradiation and protected with bone marrow 6 weeks earlier, caused complete regression of similar sized tumours in these mice. Furthermore transfer of splenocytes from immunocompetant, tumour bearing mice 3 hours after the above infusion prevented this tumour regression, whereas splenocytes from naïve, non-tumour bearing mice did not. The suppressor activity of these splenocytes was completely abolished by prior incubation with anti-T-cell (anti-Thy 1.2) antibodies and complement. These studies suggest the tumour induced development of T-cells capable of suppressing immune eradication of a developing tumour. Further analysis revealed that the suppressor cells were of the Ly $1^{+}2^{-}$ phenotype, or $CD5^{+}$, $CD8^{-}$ T-cells. Susceptibility to passive immunotherapy, following irradiation, relapses after 2 to 4 weeks unless the animals were thymectomised or treated with anti CD4 monoclonal antibodies. Regeneration of this barrier to immunotherapy is also dependent on the presence of tumour (Awwad and North 1990). They postulated that the presence

of $CD4^+$ tumour-induced suppressor cells was responsible for some of the documented failures to cause tumour regression by adoptive immunotherapy. These suppressor cells are sensitive to chemotherapeutic agents such as cyclophosphamide, vincristine and bleomycin (Xu 1988; Awwad and North 1989; Awwad and North 1990). Analysis of TCR Vβ gene expression in rat lung cancer by Gelber *et al.* revealed tumour infiltration with tumour containing T-cells expressing TCR Vβ-5 or –6 and tumour-growth enhancing T-cells expressing TCR Vβ-11 (Gelber 1992). Similar work by Wang *et al.* demonstrated protection of mice from developing pulmonary melanoma metastases by antibody depletion of TCR $V\alpha\text{-}3^+$ T-cells (Wang and Taniguchi 1995). Whether tumour infiltrating lymphocytes exert overall a positive or a negative effect on tumour growth depends on a balance between these opposing cell populations which in turn depends on unknown factors.

As with many biological systems it will depend on the activation status of individual cells and therefore in part on the pattern of cytokines/chemokines that they release. Th-2 cells capable of secreting the immunosuppressive cytokines IL-4 and IL-10 have been identified in melanoma infiltrating lymphocytes (Kharkevitch 1994). Natural killer cell derived TGF-β has been shown to suppress the generation of CTL in the murine melanoma model B16 (Tamada 1997). Prostaglandin E2 secreted by non-small cell lung cancer has been shown to induce IL-10 production by normal peripheral blood mononuclear cells (Huang 1996).

As discussed above tumour-specific T-cells, which encounter tumour cells in the absence of costimulators are rendered anergic and there is now *in vitro* and *in vivo* evidence that these anergic T-cells may themselves be immunosuppressive (Diaz-Gallo 1992; Lombardi 1994).

Other tumour-infiltrating cells, notably macrophages as mentioned above, may exert an immunosuppressive effect (Mantovani, Bottazzi et al. 1992). Recently, Seung *et al.*, induced rejection of a progressively growing tumour by preventing granulocyte infiltration with antigranulocyte monoclonal antibodies (Seung 1995). The $CD8^+$ cells responsible for rejection were also present, but ineffective, in untreated animals. Young *et al.* in a study of 37 patients with head and neck squamous cell cancer found four times as much GM-CSF in the tumour specimens of the 20 patients who developed recurrent disease within 2 years compared with the tumours of the 17 who remained disease free (Young 1997). They also found 2.5 times as many natural suppressive cells with the $CD34^+$ precursor phenotype in the tumour specimens of patients who subsequently developed recurrent disease.

1.6.3 Immune Blockade

1.6.3.1 Soluble Intercellular Adhesion Molecules

From what we have already discussed we know of the importance of intercellular adhesion molecules in the interaction between CTLs and target cells. Melanoma cells express large amounts of CD58 (LFA-3) and the patients' serum has been shown to contain soluble CD58 that inhibited melanoma cell lysis which was to a great extent dependant on CD58 – CD2 interactions (Altomonte 1993). Becker *et al.* described secretion of soluble CD54 (ICAM 1) from melanoma cell lines at levels able to block conjugate formation between melanoma-specific TIL clones and tumour cells (Becker 1993).

1.6.3.2 Shed Tumour Antigens

Tumours are known to shed tumour antigens and this can cause a smoke screen for the immune response. This subject will be dealt with below under tumour microenvironment and also in Chapter 4, which discusses tumour elaborated immunosuppressive factors.

1.6.4 Tumour Microenvironment

The tumour microenvironment has been shown to possess a number of features that influence the generation and effectiveness of tumour-reactive lymphocytes. Tumour location has been shown to influence local cytokine production, which in turn affects the systemic immunological and physiological consequences of the tumour-bearing state. Experimental liver tumours produced similar systemic, TNF and IL-6, cytokine responses to experimental subcutaneous tumours that were ten times larger by weight (Fong, Moldawere et al. 1992). This was not due to a mechanical or destructive effect on the liver but rather was attributed to the proximity of the tumour to hepatic Kupffer cells, which subsequently produced the myriad of cytokines.

The physiology of tumours differs from that of normal tissues in a number of important aspects, which are largely due to differences between the two vasculatures. Solid tumour growth requires continuous new vessel growth or angiogenesis. This is due in part to tumour cell induction of vascular endothelial growth factor promoter activity (Fukumura, Xavier et al. 1998). This neovascularisation results in a disordered vasculature composed of distended leaky capillaries with sluggish blood flow (Brown and Giaccia 1998). This disordered vasculature is associated with, possibly caused by, areas of hypoxia which in turn contribute to genetic instability of the tumour cells, and also with tumour resistance to radiotherapy and some anti-cancer drugs. These hypoxic areas induce anaerobic metabolism with consequent production of lactic acid resulting in an acidic microenvironment within solid tumours. This low pH is associated with cell death or decreased colony forming ability and also with a selective pressure for tumour cells that are able to survive in this milieu. This combination of hypoxia and acidic pH is toxic to mammalian cells which would include responding lymphocytes if

they reach there (Tannock and Rotin 1989). The acidic microenvironment has also been shown to impair the generation of non-MHC-restricted killer cells and in general to impair the induction of anti-tumoural immune responses within solid tumours (Muller, Fischer et al. 2000).

Tumour cells elaborate a number of factors, which have been shown either to enhance their tumour/metastasis forming ability, by protecting against apoptosis, or to inhibit the antitumour immune response. Small cell lung cancer has been shown to have an extensive stroma of extracellular matrix proteins including Fibronectin, Laminin and Tenascin. Adhesion of these cells to extracellular matrix proteins enhances tumourigenicity and confers resistance to some chemotherapeutic agents as a result of ß1 integrin-stimulated tyrosine kinase activation, which can prevent entry into programmed cell death or apoptosis (Sethi, Rintoul et al. 1999). Gangliosides are complex glycosphingolipids found primarily on the external membrane of cells, which have been shown to be shed by tumour cells and to be potent inhibitors of the cellular immune response in vitro (Ladisch, Gillard et al. 1983; Gonwa, Westrick et al. 1984). Using a number of clones of a murine lymphoma cell line Ladisch *et al.* found that differences in tumourigenicity correlated directly with differences in ganglioside content and ganglioside shedding. Purified gangliosides enhanced the tumourigenicity of the most weakly tumourigenic clone. Co-culture of highly tumourigenic/ganglioside shedding cells with poorly tumourigenic/low ganglioside shedding cells augmented the tumourigenicity of the latter cell line. Gangliosides have been detected in significant quantities in the plasma of a particularly aggressive human tumour, neuroblastoma.

Tumours have also been shown to elaborate soluble tumour antigen. Thomson described three stages of immune response during the development of a syngeneic methylcholanthrene-induced sarcoma in hooded rats. During the first stage there

are relatively few tumour cells, which shed minimal quantities of weakly tumour specific antigens and hence stimulates relatively few reactive cells. The middle stage is characterised by a developing tumour but a strong rejection response, which prevents the development of an identical tumour at a remote site. He hypothesised that the primary tumour is not rejected because of a high local concentration of shed tumour antigen capable of binding to tumour specific immunoglobulins and to cell-surface receptors reducing or inhibiting their ability to react with membrane bound antigen. The third stage is characterised by the appearance of soluble tumour antigen in the circulation and a waning of the anti-tumour immune response (Thomson 1975). He concludes that soluble tumour antigen present in the local microenvironment of the tumour in the early stages of tumour growth interferes with the ability of immune cells to cause tumour rejection. As the tumour progressively grows sufficient soluble antigen is produced and released systemically to suppress the effector arm of the host's anti-tumour immune response at distant sites, facilitating the development of metastases.

Tumour cells have also been shown to express Fas ligand which induces apoptosis in infiltrating lymphocytes, discussed earlier and to secrete immunosuppressive factors such as TGF-ß discussed later.

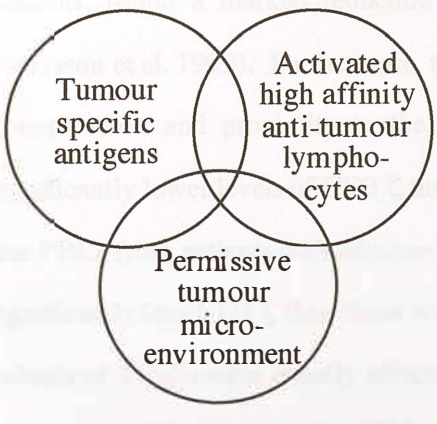


Fig. 1.7: Interplay of factors in the tumour micro-environment

The tumour microenvironment is a critical parameter that independently of other factors can suppress lymphocyte extravasation and/or function inside tumours (Ganss and Hanahan 1998). The factors necessary for the development of an effective anti-tumour immune response can be diagrammatically represented in Fig.1.7 from Ganss.

1.6.5 Alterations in Signal-Transducing Molecules

Recent work has demonstrated abnormalities in the signal-transducing mechanisms of T and NK cells in tumour-bearing mice and in humans with colorectal, ovarian, hepatic, gastric, pancreatic and cervical carcinomas and in melanomas. This impairment was first described in a slowly growing murine model of colon carcinoma (Mizoguchi, O'Shea et al. 1992). They reported a blunted Ca^{++} flux in response to anti-CD3 antibodies, altered protein tyrosine phosphorylation resulting in decreased $p56^{lck}$ and $p59^{fyn}$ expression despite normal or elevated lck or fyn mRNA, implying post-transcriptional impairment. When they examined the structure of the TCR they found normal amounts of α, β and ε subunits reduced amounts of γ but CD3ζ was absent. They linked these changes to the observed reduction in lytic function of $CD8^+$ T-cells and decreased expression of mRNA for TNF-α and granzyme B. Interestingly $CD4^+$ T-cells had preserved proliferative responses and lymphokine production. They reported similar findings in a murine renal cell adenocarcinoma, Renca, and absent expression of ζ chains in peripheral blood T cells of 7 out of 12 human cancer patients. More interestingly a correlation between these signal transducing abnormalities and the stage of disease in cancer patients has been uncovered. Matsuda *et al.*, in a study of PBLs, TILs and IELs from patients with colorectal carcinoma and age and sex matched controls, found a marked reduction in CD3 ζ chain levels in TILs (Matsuda, Petersson et al. 1995). Furthermore, they found a direct relationship between CD3 ζ expression and proximity to the tumour. IELs from near the tumour had significantly lower levels of CD3 ζ than IELs from more distant sites. They found that PBLs from patients with advanced colorectal cancer, Dukes stages C or D, had significantly less CD3 ζ than those with more localised Dukes A or B disease. All subsets of T-cells were equally affected. A marked reduction in the percentage of NK cells in TILs compared to PBLs and a significantly reduced CD16 ζ in NK cells close to the tumour relative to distant IELs or PBLs as also found. Finally, they reported that 1-3 days of *in vitro* culture in the presence of IL-2, PHA, anti-

CD2 or anti-CD3 induced increasing levels of CD3 ζ that were fully restored by day 7. Kono *et al.* demonstrated a marked decrease in CD3 ζ chain expression in PBLs from patients with cervical carcinoma when compared with PBLs from normal healthy controls; the levels of CD3 ζ in PBLs from patients with cervical intraepithelial neoplasia (CIN) were intermediate between the two (Kono 1996). This raises the possibility that alterations in signal-transducing molecules may occur early in the development and progression of some cancers and may even be useful as prognostic indicators. This is substantiated by Zea *et al.* who demonstrated that melanoma patients with low TCR ζ levels had significantly shorter overall survival than patients with normal TCR ζ levels (Zea 1995). TCR ζ-deficient patients also tended to have faster growing tumours.

Decreased TCR ζ expression in other disease pathologies is relevant to attempts to ascertain the causative agent/agents behind this phenomenon. T-cells in peripheral blood and synovial fluid of patients with rheumatoid arthritis are known to have poor mitogenic responses and are associated with decreased zeta chain expression (Maurice 1997). Lymphocytes from leprosy patients have alterations in nuclear transcription factors and in CD3 ζ chain expression. This together with the knowledge that all subsets of T-cells and NK-cells are equally affected implies that the stimulus is not tumour antigen dependant and may be related to chronic inflammation. A link came with the discovery of suppressor macrophages (Mantovani, Bottazzi et al. 1992). Aoe *et al.* in a murine colon carcinoma model reported the selective loss of CD3 ζ chain in the splenocytes of tumour-bearing mice (Aoe, Okamoto et al. 1995). They also found that tumour growth was associated with the accumulation of macrophages in the spleen that correlated with the loss of ζ chain expression. Coculture of these macrophages from tumour-bearing mice with T-cells induced the loss of ζ chain expression in the T-cells. More importantly naïve macrophages non-specifically activated with zymosan A and LPS were able to induce the same abnormality in T-cells. This finding was

confirmed by Kono et al. who went on to investigate the interactions between the two cell populations (Kono, Salazar-Onfray et al. 1996). They found that macrophages isolated from metastatic lymph nodes of patients with malignant melanoma or peripheral blood monocytes activated with LPS or PMA down-regulate levels of CD3 ζ in autologous peripheral blood T-cells. They also found that coculture with activated monocytes impaired Ca^{++} flux in stimulated T-cells and impaired cytotoxic function. Catalase a scavenger of H_2O_2, almost totally abrogated the inhibitory effect of activated monocytes on melanoma specific CTLs. Kono *et al.* were able to induce loss of ζ chain expression in T-cells by pretreatment with non-toxic doses of H_2O_2. In patients with stage III or IV oral carcinoma, the absence or low expression of ζ chain in TILs independently predicts a significantly shorter 5 year survival than that of patients with a normal ζ expression. In patients with ovarian carcinoma the presence of normal ζ expression prior to the onset of Il-2 therapy is predictive of a clinical response to Il-2 (Whiteside 1999). Both of these facts point to the biological significance of determining ζ chain status prior to the onset of biological therapies.

There is some controversy over whether or not the decreased expression of ζ chains can be reversed. Matsuda *et al.* implied that *in vitro* culture of CD3 ζ deficient cells for up to 7 days in IL-2 resulted in complete recovery (Matsuda, Petersson et al. 1995). This may have been due to selective outgrowth of ζ expressing cells. Salvadori *et al.* used retroviral vectors to produce an IL-2 secreting murine fibrosarcoma (Salvadori, Gansbacher et al. 1994). They found that IL-2 secretion by tumours was able to prevent the loss of ζ chain expression preventing immunosuppression facilitating the generation of antitumour responses. Farace *et al.* reported that the decrease in ζ chain expression they found in patients with advanced cancer was not reversed by *in vivo* IL-2 treatment (Farace 1994). Reversibility of CD3 ζ defects has been described in humans in vivo in a lymphoma patient treated with standard chemotherapy (Massaia 1994). Again this

may reflect selective outgrowth of T-cells with normal TCR. This question can only be answered with studies of T-cells at the single cell level. Recently, Deakin *et al.*, have reported that using flow cytometry, western blot analysis and immunohistochemistry they failed to find evidence of CD3 ζ chain downregulation in patients with colorectal carcinoma, therefore TCR receptor alterations like many other aspects can only be a piece of the overall jigsaw (Deakin, Singh et al. 1999). Other signal transduction defects are being identified affecting transferrin receptor, Jak 3 and Stat5a and Stat5b (Kudoh 1994; Kolenko 1997; Pericle 1997). Obviously this aspect of tumour immunology has to be further investigated as it currently presents an obstacle to successful immunotherapy and its reversal may be a novel immunotherapeutic strategy.

1.7 Immune Stimulation

As I alluded to in the introduction the way forward in the treatment of established malignant disease may well lie with immunotherapeutic approaches. These approaches would be, and have been, based on the information presented above and will be briefly discussed below. A note of caution though as not all of the effects of immune recognition and response to a tumour are detrimental to its growth. A number of researchers have shown evidence of paracrine stimulation of tumour growth and metastasis associated with a tumour-infiltrating T-cell response. This concept was first proposed by Richmond T. Prehn in the 1970s and reviewed by him in 1994 (Prehn 1994). He examined published studies where either the immune response or tumour immunogenicity were titrated and concluded that probably all tumours including spontaneously occurring ones are immunogenic to varying degrees. He proposed that weakly immunogenic tumours induced stimulation of tumour growth, the extent of which was related to the degree of tumour immunogenicity. Eventually a threshold is reached in tumour immunogenicity where the effect of the immune response is inhibition of tumour

growth rather than stimulation. His theory is diagrammatically represented in Fig. 1.8. He makes a coherent argument and is even able to explain the findings of people such as Hewitt who claimed to show evidence that tumours were not immunogenic. Hewitt attempted to immunise mice with irradiated spontaneous tumour cells prior to challenging with live tumour cells. The tumours grew faster in all seven immunised animals when compared to non-immunised controls, a result that Hewitt interpreted as artefact (Hewitt, Blake et al. 1976). Prehn goes on to suggest that immunotherapy will eventually succeed but may need greater boosts than previously suspected or indeed very poorly immunogenic tumours may be better treated with immunosuppression, perhaps an added benefit of chemotherapy.

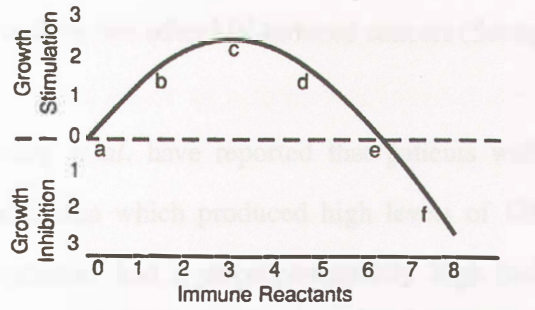


Fig. 1. Relationship between tumor stimulation or inhibition and the concentration and/or quality of the immune reactants.

Fig 1.8 Prehn's theory of the relationship between the quantity of immune reactants and tumour inhibition or stimulation

The scientific evidence to support Prehn's theory was not long in coming. Pekarek *et al.* in January 1995 investigated the growth dynamics of a UV-induced murine tumour, 4102-RE, which grew in nude mice but was rejected when transplanted into syngenic euthymic mice and an antigen related variant 4102-PRO which grew in euthymic mice (Pekarek, Starr et al. 1995). They found that the 4102-PRO line grew faster than the parent line *in vivo* in athymic mice and that *in vitro* growth of 4102-PRO but not 4102-RE was stimulated by soluble factors released from peritoneal exudate cells cultured with tumour cells. Furthermore the 4102-PRO cell line and not 4102-RE was stimulated *in vitro* by human growth factors TGF-ß, PDGF, BFGF and EGF. Conditioned media from 4102-PRO cell line had a significantly greater chemoattractant effect on peritoneal exudate cells *in vitro* than media conditioned with the parent line 4102-RE. Finally pretreatment of nude mice

with anti-granulocyte antibodies reduced the growth rate of 4102-PRO to a growth rate similar to that of 4102-RE in athymic nude mice. They conclude that the 4102-PRO cell line attracts monocytes *in vivo* which in turn produce soluble growth factors that in turn stimulate its growth. They went on to show that treatment of euthymic mice with antigranulocyte antibody enabled rejection of the otherwise progressively growing 4102-PRO without interfering with the $CD8^+$ T-cell-mediated immune response (Seung 1995). They found similar evidence that tumour variants had evolved to attract monocytes, which in turn stimulated tumour growth in two other UV-induced cancers (Seung, Seung et al. 1995).

Young *et al.* have reported that patients with Head and Neck Squamous Cell Carcinoma which produced high levels of GMCSF or had a high $CD34^+$ cell population had a disproportionately high incidence of recurrence or metastatic disease (94% and 100% respectively) due to intratumoural suppression of T-cells by $CD34^+$ cells but also found tumour stimulating effects due to soluble mediators produced by $CD34^+$ cells (Young, Wright et al. 1997). It is not just monocytes that can enhance tumour growth. Peoples *et al.* have reported that tumour infiltrating lymphocytes, from ovarian and breast cancers, can be induced *in vitro* to produce heparin-binding epidermal growth factor-like growth factor (HB-EGF) and basic fibroblast growth factor (bFGF) (Peoples, Blotnick et al. 1995). Their production was enhanced by serum depletion and exposure to a progressively acidotic environment – conditions that mimic the tumour microenvironment. Immunohistochemical studies confirmed the *in vivo* production by TILs of HB-EGF and bFGF, which was shown *in vitro* to stimulate tumour cells.

The relevance and significance of this apparent immune induced stimulation of tumour growth remains to be established. Tumours that have been genetically engineered to express GM-CSF for use as cancer vaccines have conferred resistance to tumour challenge (Dranoff, Jaffee et al. 1993). The conditions under

which GM-CSF can inhibit or facilitate tumour growth remain undefined but as with a lot of biological systems it may relate to a cumulative or quantitative effect.

1.9 Concluding Remarks

Clearly our understanding of the immune system is still relatively naïve. Given this assumption it is amazing what successes have been achieved with therapeutic interventions. An improved understanding of the immune pathophysiology of T-cells in cancer patients is fundamental to their intelligent manipulation and use in therapeutic approaches, if we are to improve upon these results. This is however a two-edged sword as with increased knowledge the picture is becoming more and more complex. Tumour immunology initially started with work in animal models, which fell into disrepute as assumptions were made and incorrect conclusions drawn. Subsequently attempts to control all the experimental variables together with a social/moral drive to avoid animal experimentation led to the increased reliance on *in vitro* models. These certainly have advanced our knowledge significantly, but their limitations are becoming increasingly clear. We need to use this knowledge to establish and develop accurate and relevant animal models.

With this aspiration in mind the overall objective of my work was to study the mechanisms by which tumours of differing origins and antigenic spectra circumvent the anti-tumour immune response to grow and develop. The tumour cell lines to be used are JBS, an antigen-loss variant of a virally (SV-40) transformed murine fibrosarcoma cell line and its large-T expressing parent cell line SV3T3. The other cell line is a chemically induced and potentiated squamous carcinoma, CarB. All three cell lines produce tumours in the immunocompromised athymic nude mouse, which lack functioning T-cells. Growth of JBS and CarB tumours in immunocompetant mice is classically MHC-restricted to Balb/c mice (SV3T3 is too antigenic to grow in immunocompetant mice).

To define the effect of a progressively growing tumour on the host immune system, JBS and CarB tumours will be established in immunocompetant Balb/c mice. Control, non-tumour-bearing or naïve mice and mice with progressive tumour burdens will be sacrificed and lymphocyte (splenocyte) function assessed. Immune response will be defined by examining lymphocyte numbers and phenotype i.e. T-cell (CD3), B-cell (CD40), helper (CD4) or cytotoxic (CD8) T-cell and activation status (CD25, Interleukin-2 receptor) in the spleens harvested from tumour-bearing mice. The function of these lymphocytes will be investigated both in terms of their proliferative ability, inherently and in response to exogenous stimulation with cytokine (IL-2) or ConcavalinA and their cytotoxic activity against the host tumour and a control cell line.

The potential influence of tumour-derived immunosuppressive factors on that immune response will be investigated. These factors will be partially characterised and compared to determine if differences in the immune response to developing tumours correlate with the potential elaboration of differing ISFs.

To investigate whether the elaboration *in vivo* of tumour-derived ISFs is sufficient to protect a more antigenic and therefore immunogenic tumour from elimination by the host response, mixed tumours of either JBS or CarB together with an equal number of strongly antigenic SV3T3 cells will be used to establish tumours that will be sacrificed at progressive tumour burdens and histologically examined for the presence of viable SV3T3 (large-T antigen expressing) cells. Should the ISF fail to protect the more strongly antigenic cells then we will be able to determine if the attraction of immune cells and their consequent elimination will result in bystander elimination of the more weakly antigenic cells.

As discussed earlier tumours have also been shown to induce cellular mediators of immunosuppression. Oral tolerance is a phenomenon whereby antigens, which if

given by any other route would induce an immune response, when given orally induce a specific systemic immune hyporesponsiveness. We ppostulated that the Gut-Associated Lymphoid Tissue (GALT) may process shed tumour antigens and consequently down-regulate the systemic immune response to tumours of the GI tract. Using the same cell lines as before, JBS and CarB, we investigated to what extent, if any, feeding mice tumour fragments could alter the immune response to developing tumours. Specifically the effect of feeding on tumour growth rates and lymphocyte proliferative and cytotoxic ability were investigated. In accordance with other workers in the field of oral tolerance the tolerising effect of feeding on Delayed-Type Hypersensitivity (DTH) responses was investigated.

It is intended that having defined a murine model of weakly antigenic tumours and explored possible mechanisms of tumour-host interaction that the work will be taken forward by the next researcher to look for *in vivo* evidence of ISF activity and to begin to investigate mechanisms of utilising this information either for diagnostic/tumour monitoring purposes or by counteracting it for therapeutic purposes.

Leonardo daVinci (1452 – 1519) wrote "The supreme misfortune is when theory outstrips performance". This has certainly been the case until now, however, our understanding is catching up with our expectations.

Chapter 2

General Materials and Methods

2.1 Cell Lines

2.1.2 SV3T3

For strongly antigenic cells, an SV40 transformed BALB/c 3T3 cell line was used. SV40 is a highly oncogenic virus, which induces malignant transformation and expression of large-T antigen, a potent viral coded tumour specific transplantation antigen (TSTA). This cell line, designated SV3T3, is tumourigenic only in immunocompromised mice, e.g. MF1 athymic nude mice and not in the immunocompetant syngeneic BALB/c animal. The original cells were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland, and maintained in our laboratory.

2.1.3 JBS

This is a large-T antigen negative variant of the SV3T3 cell line that was established in our laboratory by Dr. John Barrett. The cell line was developed from a tumour that occurred in one of a panel of immunocompetant BALB/c mice inoculated with tumourigenic doses of SV3T3. The JBS cell line is classically MHC restricted to the immunocompetant BALB/c mouse in which it is both antigenic and tumourigenic. The minimum tumourigenic dose was 1 X 10^5 cells per mouse with 1 X 10^6 cells consistently producing tumours in mice. The standard tumourigenic dose used was 2 X 10^6 cells/mouse.

2.1.4 CarB

The CarB cell line is a Dimethylbenzanthrene (DMBA, $C_{20}H_{16}$, Class II carcinogen) induced, 12-O-tetradecanoylphorbol 13-acetate (TPA) potentiated squamous carcinoma, which is MHC restricted to and tumourigenic in BALB/c

mice (Buchmann, Ruggeri et al. 1991). This line was a generous gift of Dr. A. Balmain, Beatson Research Institute, Glasgow. The minimum tumourigenic dose is 1 X 10^6 cells per mouse while 2 X 10^6 cells produced tumours in 100% of mice. The standard dose for tumour induction was 2 X 10^6 cells/mouse.

2.1.5 HL-60

This human promyelocytic cell line was derived from the peripheral blood lymphocytes of a patient with acute promyelocytic leukaemia (Collins, Gallo et al. 1977; Gallagher, Collins et al. 1979). It is known to have a large deletion in the p53 gene and therefore to lack 53 expression. This line was used to standardise the suppressive activity between different production batches of supernatant and to examine the effects of various treatments of the supernatant on its ISF activity. These results were always confirmed using activated and non-activated splenocytes.

2.1.6 NSO

This is a murine myeloma cell line that was used to determine the specificity and potency of the ISFs.

2.1.7 L929

This murine fibroblast cell line was grown to confluence in sterile petrie dishes and after loading with neutral red dye was used to determine if the ISFs were cytotoxic.

2.1.8 Others

From time to time it was necessary to utilise some of the other cell lines available in the laboratory such as K-562 - an erythroblastoid cell line established from a pleural effusion of a patient with CML in terminal blastic crisis that is positive for the Philadelphia chromosome (Lozzio and Lozzio 1975). They are highly sensitive to NK cell killing (Lozzio and Lozzio 1979). This line was again used to examine the specificity of the ISF activity in the supernatants. NRK – normal rat kidney cells and U-937- a monocytoid cell line derived from the pleural effusion of a patient with diffuse histiocytic lymphoma (Sundström and Nilsson 1976). Again this cell line was used to examine the specificity of the suppressive supernatants. Daudi, derived from a 16 yr old male Negro with Burkitt's Lymphoma was used in some cytotoxicity studies as it is particularly sensitive to lymphokine activated killer cells.

2.2 Tissue Culture

2.2.1 Subculture of Adherent Cell Lines

Cells were grown in 72 or 162cm^2 tissue culture flasks (Costar) and maintained at 37°C in a humidified atmosphere with 5% CO_2 in air. All cell lines were maintained by twice weekly passage in Dulbecco's Modified Eagles Medium (DMEM, Gibco Ltd.) supplemented with Glutamine and 10% Foetal Calf Serum (Gibco Ltd.). Adherent cell lines (i.e. JBS, CarB, L929) were grown to 80-90% confluence. In a class II sterile hood the spent medium was removed and disposed off safely in a 1% Vircon (Antec International) solution. The cell monolayer was washed twice in pre-warmed, sterile PBS to remove all traces of serum, which would inhibit the action of trypsin. Then 3-5mls of 0.25% trypsin

(Gibco Ltd.) was added to the flask and left for 1 minute. The trypsin was removed and disposed of safely and the flasks returned to the incubator. The flasks were gently agitated at regular intervals to dislodge the adherent cells, usually 5 – 20 mins. Strongly adherent cells were either re-bathed in 0.25% trypsin, 0.25% trypsin with 1mM EDTA or removed using a sterile cell scraper (Costar). Once the cells were dislodged 1-3mls of foetal calf serum was added to the flask to neutralise any further tryptic activity. The cells were then removed from the flasks, placed in 10ml centrifuge tubes and topped up with DMEM. The cell suspension was gently centrifuged out of this medium and resuspended in DMEM for counting, reseeding flasks or culture slides etc. All cell lines were replaced with fresh stocks from liquid nitrogen storage four times a year.

Non-adherent cell lines were subcultured in a similar fashion without the need for tryptic removal.

2.2.2 Cryopreservation of Cell Lines

At regular intervals of 3 to 4 months cell stocks were replenished. Cell suspensions were generated, as described above, and centrifuged out of suspension into a pellet of cells that was resuspended in freeze medium. Freeze medium consisted of 91% FCS, the solutes of which enabled slow freezing and the proteins of which were cryoprotectant and 9% Dimethyl Sulfoxide (DMSO, Sigma) a known cryoprotectant which prevents intracellular crystal formation. The cells in freeze medium were placed in 1ml aliquots in freeze vials and placed in freeze pods in –80°C freezer for 24 hours. This provided a freeze rate of 1°/min. The freeze vials were then transferred for long term storage to liquid

nitrogen where they continued to freeze down at a rate of 2°/min to their final temperature of –196°C.

For recovery from storage the freeze vials were rapidly thawed to 37°C and the 1ml aliquots diluted in DMEM to be washed out of the DMSO-containing solution. The cells were washed three times in DMEM before being resuspended in normal tissue culture medium.

2.2.3 Mycoplasma Testing

The lines were checked at regular intervals for mycoplasma contamination by fluorescence microscopy and supernatant culture and periodically by ELISA. Fluorescence microscopy was carried out on cells that had been grown on sterile cover slips in antibiotic free medium for 48-72 hours. The slides were washed once in PBS, stained for 15 mins in DAPI solution and analysed with an immunoflourescence microscope (Russell, Newman et al. 1975; Uphoff, Gignac et al. 1992). Mycoplasma contamination would show up as a thin blue fluorescent rim around the unstained cytoplasm. Supernatant from cultured cell lines, obtained by centrifugation at 150g for 15 mins, was incubated overnight with [^{3}H] thymidine and then assayed for the presence of tricloroacetic acid precipitatable material (Hudson and Hay 1991). At no stage was evidence of Mycoplasma contamination detected.

2.3 Animals

2.3.1 Balb/c mice

Eight to 10 week old Balb/c mice (Harlan Olac Laboratories, Oxfordshire, U.K.) of either sex were obtained from the Biological Services Unit, University

College Cork, Ireland. Throughout the experiment the animals were housed and cared for at that unit. The animals were maintained on a standard diet of lab chow and water ad libitum in accordance with the guidelines of the Department of Health., Licence Number B100-2800.

2.3.2 Athymic Nude Mice

For growth curve determinations, 6-8 week old MF1 Nu/Nu athymic nude mice obtained from the same source and maintained under the same conditions, were also used.

2.3.3 Others

There were occasions, which will be detailed in the text, when it was necessary to avail of some other animals that were available such as lpr mice, c57 Black mice and Wistar rats.

2.4 Tumour Growth and Harvest

2.4.1 Tumour Induction

Cell suspensions were generated from tissue culture monolayers as described above and viable cell numbers were ascertained by Trypan Blue Dye Exclusion (see below). These cells were than resuspended at a concentration of 1 X 10^7 cells/ml in a 50/50 dilution of DMEM in PBS. Each animal received a subcutaneous inoculation, in the nape of the neck, of 200μls, equivalent to 2 X 10^6 tumour cells/mouse. This volume of inoculum and location yielded the most consistent tumours in terms of lack of puncture site extravasation of cell suspension following injection and tumour shape. Tumour bearing mice were

sacrificed at various stages of tumour burden for experimental reasons or sooner if skin ulceration occurred.

2.4.2 Tumour Volume Growth Curves

Following tumour establishment, tumours were allowed to grow and develop and were monitored by alternate day measurements in two dimensions using a Verniers Calipers. Tumour Volume was calculated according to the formula Volume = $\pi/6(l+s^2)$ where l = longest dimension and s = shortest. Calculating the volume of a three dimensional mass based on two measurements is prone to error but this formula gave the smallest margin of error (Fiennes 1988). From these volumes tumour growth curves were constructed. Given the potential inaccuracies in estimating tumour volume for those experiments where it is necessary to euthanase the animal, i.e. for spleen harvest, the tumours were also excised and weighed in order to more accurately assess the effect of tumour burden, in terms of tumour weight, on the lymphocyte function being assessed.

2.4.3 Sacrifice and Tumour Harvest

Non tumour-bearing control mice and mice with tumours of a desired burden were sacrificed by anaesthetising with ether (Merck) followed by cervical dislocation. The mice were sterilised by placing in alternating baths of 70% ethanol and 1 % Vircon and transferring to a laminar flow hood (Danlaf). The spleens and tumours were then aseptically removed and weighed.

2.4.4 Spleen Cell Suspensions

The spleens were placed on a stainless steel mesh screen in a sterile petri dish (Greiner) with 10 ml of medium (Brooks and Fernandez-Botran 1994) and gently pressed through the mesh into the medium using a syringe plunger as a pestle. The membranous sac was discarded, a pipette used to dissociate clumps and the resultant cell suspension pelleted by centrifugation at 300g (Hermle Z 380) for 10 min. The resultant pellet of cells was resuspended in NH_4Cl at 37°C for 5 min to remove erythrocytes from the suspension. After 5 min the cells were washed out of this solution and resuspended in PBS. Clumps of cells that could not be dissociated were removed by sedimentation. This washing step was repeated 3 times. Once a single cell suspension had been obtained the splenocytes were placed in a tissue culture flask with an appropriate volume of complete medium with 2mmol Glutamine and placed in a humidified incubator at 37°C with 5% CO_2 in air. This facilitates the removal of phagocytic cells by adherence to the tissue culture plastic. Two hours later the non-adherent cells were removed to a fresh flask of medium.

2.4.5 Enumeration of Cell Number

Cell viability was assessed by Trypan Blue dye exclusion (Hudson and Hay 1989). The cell pellet is resuspended in a known volume of medium, usually 5-10 mls. An aliquot of this suspension is diluted, usually 1:10, with 0.2% trypan blue (Gibco Ltd.). Viable cells exclude the trypan blue and thus nonviable cells appear blue. After incubating for 5 mins the viable cells were counted using a haemocytometer. The dilution of cell suspension in trypan blue was routinely adjusted such that at least 100 cells were counted on the haemocytometer

(Improved Neubauer, BDH) (Brooks and Fernandez-Botran 1994). Once the number of cells in the aliquot was known the total number of viable cells in the cell suspension could be calculated.

2.5 Functional Assays

2.5.1 Proliferation Assays

Proliferation assays were used to quantify the endogenous proliferative ability of splenocytes from tumour bearing mice and as a general measure of immunocompetance. The splenocytes from naïve and tumour-bearing mice were resuspended in DMEM at a concentration of 3 X 10^7/ml. Twenty microliters of this suspension was added to wells of 96 well U or round bottom tissue microtitre culture plates with low evaporation lids (Nunc). The final well volume was 220μls; therefore the final cell concentration in the wells was 3 X 10^6 splenocytes/ml. To the **control endogenously activated** wells 200μls of complete medium was added. All experimental wells were set up in replicates of a minimum of three but often six and sometimes more. To the **control activated** wells 100μls of SFDMEM and 100μls of ConA (Concavalin A, Sigma) working solution (see Appendix II) or IL-2 (Interleukin-2, Sigma Product No. I-4517) working solution (see Appendix II) was added. To the **immunosuppressive** wells 100μls of the conditioned medium and 100μls of ConA working solution were added. The plates were incubated at 37°C in 5% CO_2 in air for 72 hours at which time 20μls of [^{3}H]Thymidine (NET-027, DuPont) working solution (equivalent to 1μCi per well) was added to each well. The plates were incubated for a further 18 hours to permit incorporation of the [^{3}H]Thymidine. At the end of this period the cells were manually harvested onto glass-fibre filter paper

(Schleicher & Schuell) in a Minifold Microsample Filtration Manifold (Biorad). Large intracellular macromolecules were precipitated and unincorporated [^{3}H]Thymidine removed by washing through the filter paper twice with ice-cold 7.5% TCA (see Appendix II). The filter paper was allowed to air dry before being cut into individual pieces corresponding to individual wells and placed in scintillation vials with 10 ml of scintillation fluid (EcoLite, ICN Biomedicals). Each vial was sealed, labelled and left overnight prior to being analysed in a Beckman Scintillation counter. All results were analysed and replicates were averaged.

2.5.2 ISF Assays

ISF assays were based on the proliferation assays and were used to determine if supernatants or conditioned media possessed suppressive activity, the effect, if any, of various treatments on the suppressive activity of conditioned media and to standardise the suppressive activity between different production batches of conditioned media. For the majority of these experiments haemopoietic cell lines were used, to reduce the need for splenocytes, but all relevant results were confirmed using activated murine splenocytes. The indicator cells used were either HL-60s or occasionally NSOs. These cells were resuspended at a concentration of 2 X 10^6 cells/ml and again 20μls was added to each well. As these cells are transformed there was no need for exogenous activation with ConA or IL-2. Thus the wells were set up with control wells and with wells containing serial dilutions of conditioned media and supernatant from various sources such that the final concentration of cells was 2 X 10^5/ml. Because these cells have a rapid doubling time they only needed to be incubated with ISF for 24

hours prior to the addition of [^{3}H]Thymidine. After this step the samples were handled in the same manner as described above.

2.5.3 Cytotoxicity assay

Cytolytic activity was quantitated using a Chromium release assay based on that described by Martz (Martz 1993). Target cells which had been growing in log phase as a monolayer were harvested in the usual manner by washing, twice with sterile PBS to remove dead cells and complete medium, once with trypsin to remove dying but still attached cells, which were discarded, and then finally bathed in fresh trypsin for two minutes. This solution was removed and discarded and the cells left to detach over a period of 15 to 30 minutes. Once detached the cells were suspended in Complete Medium to neutralise any further tryptic activity. The cells were pelleted from this solution, washed once more and finally resuspended at a conc. of 5 X 10^5 per ml in DMEM to which was added 100μCi of sterile $Na_2{}^{51}CrO_4$ (NEZ-030 (S), DuPont). This solution was placed in a 37°C, humidified incubator with 5% CO_2 in air for 1 hour, and gently agitated every 10 mins. Following incubation the cells were washed 3 times in sterile PBS to remove extracellular ^{51}Cr and resuspended to a concentration of 5 X 10^4/ml in complete medium. One hundred microliters of this suspension was added to individual wells of a 96-well round-bottomed plate (Costar) to which the effector cells had been added to each well at varying effector target ratios from 100:1 to 1:1 in an equal volume of 100μls of complete medium. All ratios were repeated in triplicate sets of wells. To determine the spontaneous release of ^{51}Cr (designated 'c') the effector cells were omitted from a triplicate set of wells and the final volume of the wells corrected accordingly. Once set up the plate

was centrifuged at 300g and incubated for 4 hours in a humidified atmosphere of 5% CO_2 in air at 37°C. To determine the maximum release of ^{51}Cr (designated 't') 100μl aliquots of labelled cells were placed in capped centrifuge tubes with an equivalent volume of distilled de-ionised water. These vials were then subjected to two freeze/thaw cycles. After 4 hours both the plate and vials were centrifuged at 500g and 100μl aliquots of each sample's supernatant (designated 'e') carefully removed into pre-labelled centrifuge, screw cap vials (Sarstedt). All samples plus the residue of the maximum release vials (designated 'r') were then placed in a gamma counter (CompuGamma, Model 1282-001, LKB Wallace) and read for 120secs per sample. Only assays where the spontaneous release of ^{51}Cr was less than 30% of the total uptake, were considered interpretable.

i.e. $c < (30(t + r)/100$

The cytolytic effect of incubating effector cells with ^{51}Cr labelled target cells was calculated according to the formula

Percentage corrected release (cytotoxicity) = $100(e - c)/(t - c)$

2.6 ISF Production

2.6.1 Conditioned Media

Mice bearing non-ulcerating tumours of 1-2cm^3 were sacrificed as described above and in a sterile laminar flow hood the tumours were aseptically excised from their subcutaneous location. The tumours were placed in SFDMEM. The tumours were dissected into small fragments of 1-2mm^3 in size and approximately 0.75 gm of minced tumour was placed in a 25cm^2 tissue culture flask with 7.5 ml of SFDMEM, sufficient to completely cover the tumour

fragments. This was incubated for 24 hours at 37°C in 5% CO_2 in air after which the initial media, which contained large amounts of probable extracellular matrix protein, was discarded, the fragments were washed to clarity in PBS and resuspended in fresh SFDMEM. After a further 48 hours the medium was removed and centrifuged at 500 g to remove any debris and passed aseptically through a 0.2μm filter. At this stage the conditioned medium was used in experiments. Having established that freeze/thawing neither generated nor abrogated ISF activity, in terms of units of activity per ml, in the conditioned media, unused or excess media was aliquoted and stored at –20°C until required.

2.6.2 Cell Culture Supernatant

Cell lines were seeded into 172cm^2 tissue culture flasks and allowed to attach and proliferate. Once the cell monolayer was 50 – 60% confluent the serum-containing medium was removed and the monolayer washed three times with PBS to remove any residual sera before replenishing the flask with 20 ml of SFDMEM. The flasks were incubated for 24 hours at 37°C in 5% CO_2 after which the medium was removed, centrifuged and filtered through a 0.2μm filter as above.

2.6.3 Ultrafiltration

Initial experiments into the nature and function of any suppressive activity in conditioned media were undertaken with crude freshly prepared supernatant. It will become clear from Chapter 4, Tumour Derived Soluble Immunosuppressive Factors, that all of the immunosuppressive activity was in solutes < 0.5kD in size. Once this finding was confirmed, all conditioned media were ultrafiltered

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to remove solutes > 1kD. Fifty mls of freshly generated conditioned medium is placed in a clean ultrafiltration cell (Stirred Ultrafiltration Cell, 8050, Amicon) that has been assembled correctly beforehand. The assembly process involves correct insertion of the filter membrane (Diaflo Ultrafilters, Amicon) into the apparatus. The device can be used either to filter molecules smaller than the nominal cut-off or to concentrate larger molecules. Because the conditioned medium contains a large and diverse number of molecules it was necessary to filter the medium in stages, firstly through 100kDa, then 10kDa, 1kDa and finally 0.5kDa cut-off membranes. The device when assembled and containing the solution to be filtered is sealed, connected to CO_2 cylinder and pressurised. Prior to pressurising the filtration cell is placed on top of a magnetic stirring table (Stuart Scientific) to initiate spinning of the integral stirring bar. This keeps the fluid circulating past the membrane and delays clogging of the membrane. Once the solution is being sufficiently stirred to generate a vortex approximately one third the depth of the fluid. The cell is then pressurised to a maximum of 45*psi* . The membrane will take higher pressures up to 75*psi* but with increasing pressure the membranes seem to clog faster. Once the unit is pressurised the ultrafiltrate needs to be collected from the exit tubing. Once the solution has been filtered the apparatus should be depressurised slowly. If interested in the larger molecules, i.e. above the membranes nominal cut-off the stirring bar should be left spinning for a few mins in the unpressurised container to resuspend the material. To improve recovery of material below the nominal cut-off size for the membrane (membranes permit passage of 95% of smaller material through) the retentate can be resuspended, diluted and filtered again. Once the conditioned media has been ultrafiltered to the desired size it is passed

through a 0.2μm filter to sterilise it prior to use or storage. The recovery rates for ISF activity after passage of conditioned media through the various membranes will be expanded on in Chapter 4.

Chapter 3

Immunological Response Profiles

(Part of this chapter was published in the Journal of Leucocyte Biology, 62: 726-732; 1997)

3.1 Introduction

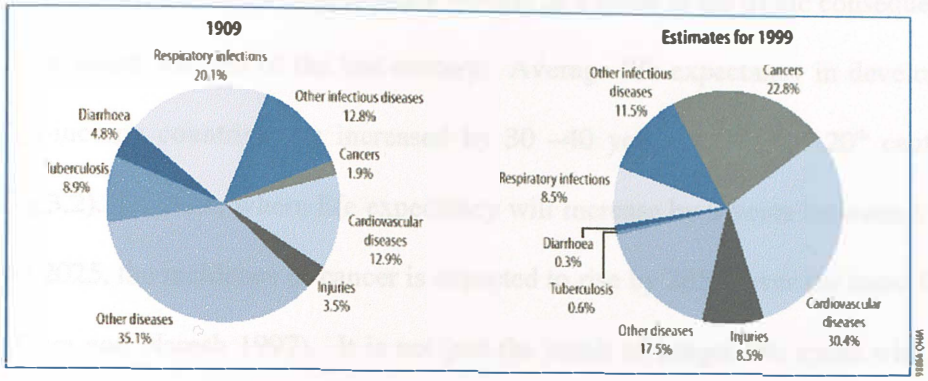


Fig. 3.1: Changes in cause of death in Chile over the 20th century (Jameson, Creese et al. 1999)

The state of world health is in transition. At the beginning of the last century (20th) infectious diseases were the major cause of human suffering and death. Now at the start of a new millennium epidemics in non-communicable diseases (cardiovascular, diabetes and cancer) have taken over (Jameson, Creese et al.

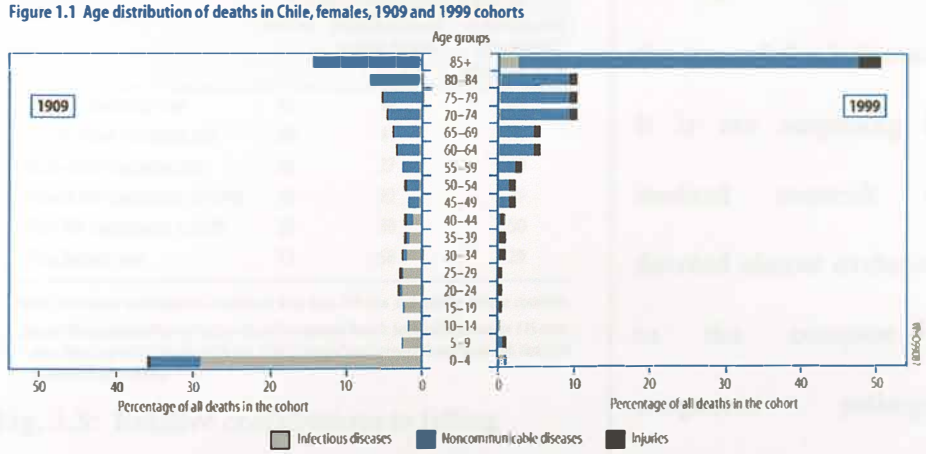


Fig. 3.2: Demonstration of the shift in age at death, including causes in Chile over the course of the last century (Jameson, Creese et al. 1999).

1999). Fig. 3.1 graphically demonstrates the change in reported causes of death in Chile that occurred over the course of the last century. This shift from communicable to non-communicable disease as a cause is the ironic consequence of the health success of the last century. Average life expectancy in developed high-income countries has increased by 30 –40 years during the 20th century (Fig.3.2). In India, where life expectancy will increase by 9 years between 1995 and 2025, the incidence of cancer is expected to rise by 285% over the same time (Mittra and Naresh 1997). It is not just the result of longer life spans with the obviously increased risk of malignancy that has caused this shift. It results also from improved control of infectious diseases, note the world-wide eradication of smallpox, and better education of civilians. A great deal of this success can be attributed to the generation and application of increased knowledge regarding disease processes (Fig. 3.3).

Table 1.2 Sources of mortality reduction, 1960–1990

Reduction	Percentage contribution of gains in		
	Income	Educational level of adult females	Generation and utilization of new knowledge
Under-5 mortality rate	17	38	45
Female adult mortality rate	20	41	39
Male adult mortality rate	25	27	49
Female life expectancy at birth	19	32	49
Male life expectancy at birth	20	30	50
Total fertility rate	12	58	29

Note: The results are based on analysis of data from 115 low and middle income countries.

Source: **Wang J et al.** *Measuring country performance on health. Selected indicators for 115 countries.* Washington DC. The World Bank, 1999 (Human Development Network, Health, Nutrition and Population Series).

Fig. 3.3: Relative contributions to falling mortality (Jameson, Creese et al. 1999).

Given that infection was the major cause of death at the turn of the last century it is not surprising that medical research was devoted almost exclusively to the conquest of exogenous pathogens. Understandably, all the research at that time was interpreted in the light of the body's attempt to fend off invading organisms. In this research climate emerged the concept of self/non-

self discrimination. This led Lewis Thomas to propose a concept, which was expanded on by Jodhua Lederberg, who at the time was working for McFarlane Burnet who coined the term and whose name is synonymous with 'Immune surveillance' (Burnet 1963). The original theory was adapted by Bretscher & Cohn to a 'two signal' model to explain the induction of tolerance in mature T cells (Bretscher and Cohn 1970). Their model was modified by Lafferty & Cunningham to allow both the primary antigen recognition signal and the secondary co-stimulatory signal to be delivered by the same APC (Lafferty and Cunningham 1975). For the past 25 years this model has been the cornerstone of tumour immunology thinking and the basis for interpretation of results and the construction of immunotherapeutic approaches. When athymic mice, who lack functioning T cells, did not exhibit the predicted increase in the incidence of spontaneous tumours the immune surveillance theory fell into disrepute (Sanford, Kohn et al. 1973). Into this void came Ephraim Fuchs and Polly Matzinger with an alternative hypothesis of how the immune system is organised. They call their theory the 'danger model', which proposes that the immune system has evolved to distinguish dangerous stimuli from safe (Fuchs and Matzinger 1996). According to their model developing tumours do not present a danger signal and therefore do not elicit an immune response. Any tumour cells that did encounter the immune system would present signal one only (antigen) in the absence of co-stimulation and therefore not generate an immune response.

Whichever model you choose to subscribe to, there is ample evidence of immune response to tumour antigens. Evidence that the immune system could cause the

regression of established cancers dates back as far as 1774 when de Lisle, a French physician, reported the disappearance of a breast cancer following the injection of pus, and subsequent evolving infection, into the patients leg (Old 1996). William B Coley, a surgeon at Memorial Hospital, New York, from 1892 – 1936, having noted the tumouricidal effects of erysipelas formulated a preparation consisting of the soluble toxins from erysipelas and *Serratia marcescens* (Coley 1894). His success, though overshadowed by the development of chemotherapy, was the basis for the use of BCG, which has been shown in randomised trials to be effective in the treatment of superficial bladder cancer. In 1980 Nauts reviewed the literature and found 449 reported cases of spontaneous tumour regression. Erysipelas from *Streptococcus pyogenes* was the most commonly associated feature.

The development of tumours in immunosuppressed patients and their subsequent resolution upon cessation of immunosuppressive therapy lends further support to a role for the immune response in the control of malignant disease. The development of cancer following organ transplantation has been reported as affecting 4-5% of transplant recipients (Sheil 1998). However with lengthening recipient survival it has been predicted that by 33 years post cadaveric renal transplant all recipients will have skin cancer and 30 to 40% will have or will have died of non-skin cancer (Sheil, Disney et al. 1997). The aetiology of cancer in immunosuppressed patients is probably multi-factorial and related to the induction of immunosuppression and oncogenic effects of the immunosuppressive agents themselves. Immunosuppression has been shown to increase susceptibility to viral infections and many of the cancers that occur in

immunosuppressed individuals have a viral origin (Ioachim 1990). Furthermore more aggressive immunosuppression is associated with earlier detection of cancers (Hiesse, Kriaa et al. 1995). Azathioprine, a potent immunosuppressant in use for over 35 years, has been shown to cause chromosomal breaks and nuclear abnormalities in humans and animals (Sheil 1998). It is not just patients with therapeutic immunosuppression that are at increased risk of developing malignant disease, patients with secondary immunodeficiency from other causes such as AIDS, or autoimmune diseases and patients with primary immune deficiencies as in SCID, DiGeorge etc. are also at an increased risk. The risk of developing a malignant tumour in persons with primary immune deficiencies is 4%, approximately 10,000 times higher than normal people (Ioachim 1990). Finally the reporting of spontaneous tumour regressions in immunosuppressed patients following correction of their immune deficiency and the correlation, in certain tumours, between an immune infiltrate and a better prognosis encouraged the research which has been presented in chapter 1 (Svennevig, Lunde et al. 1984).

3.1.2 Aims

There is a good deal of evidence in humans that tumours of differing histological types are associated with differing immunological responses. In our laboratory workers have published evidence of a field of regional immunosuppression surrounding oesophageal squamous carcinoma that contrasts dramatically with the lymphocyte activation seen in the immediate environs of colorectal adenocarcinoma (O'Mahony, O'Sullivan et al. 1993). What we cannot answer is whether these observations truly reflect different responses to histologically

distinct tumours or whether they can be attributed to variations in aetiology or anatomical location. Or could they represent analysis at different points in the timescale from tumour induction to detection? To attempt to analyse changes in the pattern of immune responses with progressive tumour burden in humans would not be feasible in the vast majority of patients because of the urgency of treatment. It would only be ethically possible in those patients who elected not to undergo any intervention at all, most likely to be end-stage disease patients. Analysis f events early in the course of of the disease would be equally improbable given the potential for surgical cure. Inter-patient variability in terms of age, pre-morbid health, immune status, risk factors for the development of malignant disease, aetiological variability and differences in clinical status and tumour burden at the time of diagnosis render their collective use liable to error.

Similar work with animal models has shown a wide variation in the nature and strength of the immune response to developing tumours. Researchers who have attempted to look at the response to progressive tumour burden have either compared non-tumour-bearing mice with tumour-bearing mice at either a time-dependent point or with mice with a tumour burden greater than a given dimension (Adler, Takiguchi et al. 1971; Walker, Yurochko et al. 1992). Of those who have attempted to look at the progressive response most have used tumour dimensions, with their large inherent error, as their measure of tumour burden (Ghosh, Komschlies et al. 1995). This research has been undertaken using a wide variety of tumour models in a wide range of animal models in a large number of labs worldwide. Each has contributed a piece of the puzzle but

it is difficult to know if differences between some reports are a genuine difference or reflect artefact due to methodological variation.

The aims of the work presented in this chapter are therefore to use the most accurate assessment of tumour burden i.e. tumour weight to define the immune response to increasing tumour burden. The lymphocyte response will be defined in terms of crude non-adherent splenocyte numbers and lymphocyte subsets, specifically T-cell (CD3), B-cell (CD40), T-helper cell (CD4), cytotoxic T-cell (CD8) together with Interleukin-2 receptor (CD25) expression as a marker of their activation status. Lymphocyte function with progressive tumour burden will be assessed by endogenous, non-mitogen, stimulated, mitogen- (ConA) and cytokine- (IL-2) stimulated proliferation and cytotoxic function. This will enable the direct comparison between the immune responses to two aetiologically and histologically distinct tumours and will define the tumour model to be used in subsequent chapters.

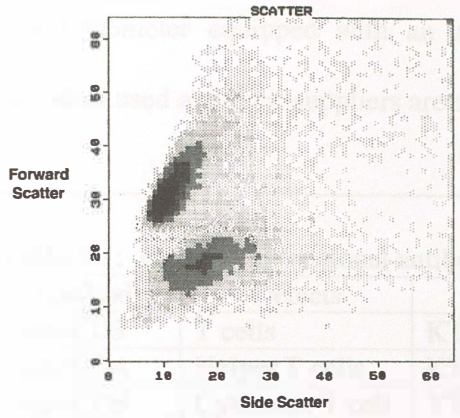
3.2 Materials and Methods

The cell lines, animals, proliferation and cytotoxicity assays were performed as described in Chapter 2, General Materials and Methods (Sections 2.1, 2.3, 2.5.1 & 2.5.3).

3.2.1 Flow Cytometric Analysis

A flow cytometer is a complex piece of equipment designed to analyse cells in suspension. The cells are made to flow in single file in a stream that is perpendicularly intersected by a light stream, usually from an argon laser. The

deflection in the light stream that is caused by the light hitting a cell is detected by a photodiode in the direct path of the light beam. This deflection is referred to as forward scatter, the intensity of which is proportional to cell volume (see left).



The light beam is scattered in all directions, when it strikes a cell, so a series of light detectors, called photomultiplier tubes, as they amplify the weak signal and convert light into an electrical signal, are set up at right angles to the light beam. If the beam does not hit a cell it is not scattered and therefore nothing is detected. The first photomultiplier tube or PMT1 detects light that has been scattered at 90° to the original beam and is called side scatter. The intensity of side scatter also reflects cell volume to an extent but also cell granularity and cell surface topography. The combination of forward and side scatter can be used to distinguish monocytes, granulocytes, lymphocytes or platelets in a sample of whole blood based on morphology. To increase the analytical capabilities of the cytometer further the cells can be labelled with fluorochrome conjugated monoclonal antibodies in a manner analogous to immunofluorescent histochemistry. Fluorochromes are molecules that when excited by light, of a set wavelength, emit light at a different wavelength. In our analysis we used a dual labelled staining technique. The two fluorochromes that we used were Fluorescein Isothiocyanate (FITC) and R-Phycoerythrin (PE). FITC absorbs light at 460 – 510nm and emits at 510 – 560nm, with a peak at 525nm (green). PE absorbs

light at 480 – 565nm and emits at 578, with a peak at 575nm (orange). The advantage of this combination of fluorochromes is that they are both excited by light from an argon laser and emit light in ranges that are distinct enough to be resolved. . For this work the samples were analysed using a Coulter Epics Elite Flow Cytometer equipped with an argon laser that emits at 488nm. The antibodies used and their suppliers are detailed in Table 3.1

Table 3.1: Panel of monoclonal antibodies used, including their supplier.

Antibody	Detects	Clone	Code No.*	Supplier
anti-CD3	T cells	KT3	MCA500F	Serotec
anti-CD4	Helper T cells	KT6	MCA612PE	Serotec
anti-CD8	Cytotoxic T cell	YTS 106-18	MCA1108PE	Serotec
Anti-CD8	Cytotoxic T cell	KT15	MCA609PE	Serotec
Anti-CD40	B cells		MCA1143PE	Serotec
FITC Neg Control	Dinitrophenyl Hapten	LO-DNP-16	MCA1212F	Serotec
PE Neg Control	Dinitrophenyl Hapten	LO-DNP-16	MCA1212PE	Serotec
Biotin-anti-CD25	IL-2 receptor, α chain		K5121204	Pharmingen
Avidin-FITC	Biotin		H511353	Pharmingen
Biotin-anti-IgM	Non-specific control		R613355	Pharmingen

* The suffix F denotes FITC conjugated antibodies and PE denotes PE conjugated antibodies.

When the murine splenocytes were analysed on the flow cytometer as well as a lymphocyte population a second population of cells was found. The origin and nature of these cells was not known, though morphologically they were smaller and less granular than the lymphocyte population. A review of the literature did not reveal any similar published profiles, though it must be said that most papers did not publish forward and side scatter dot plots and in those that did the gates

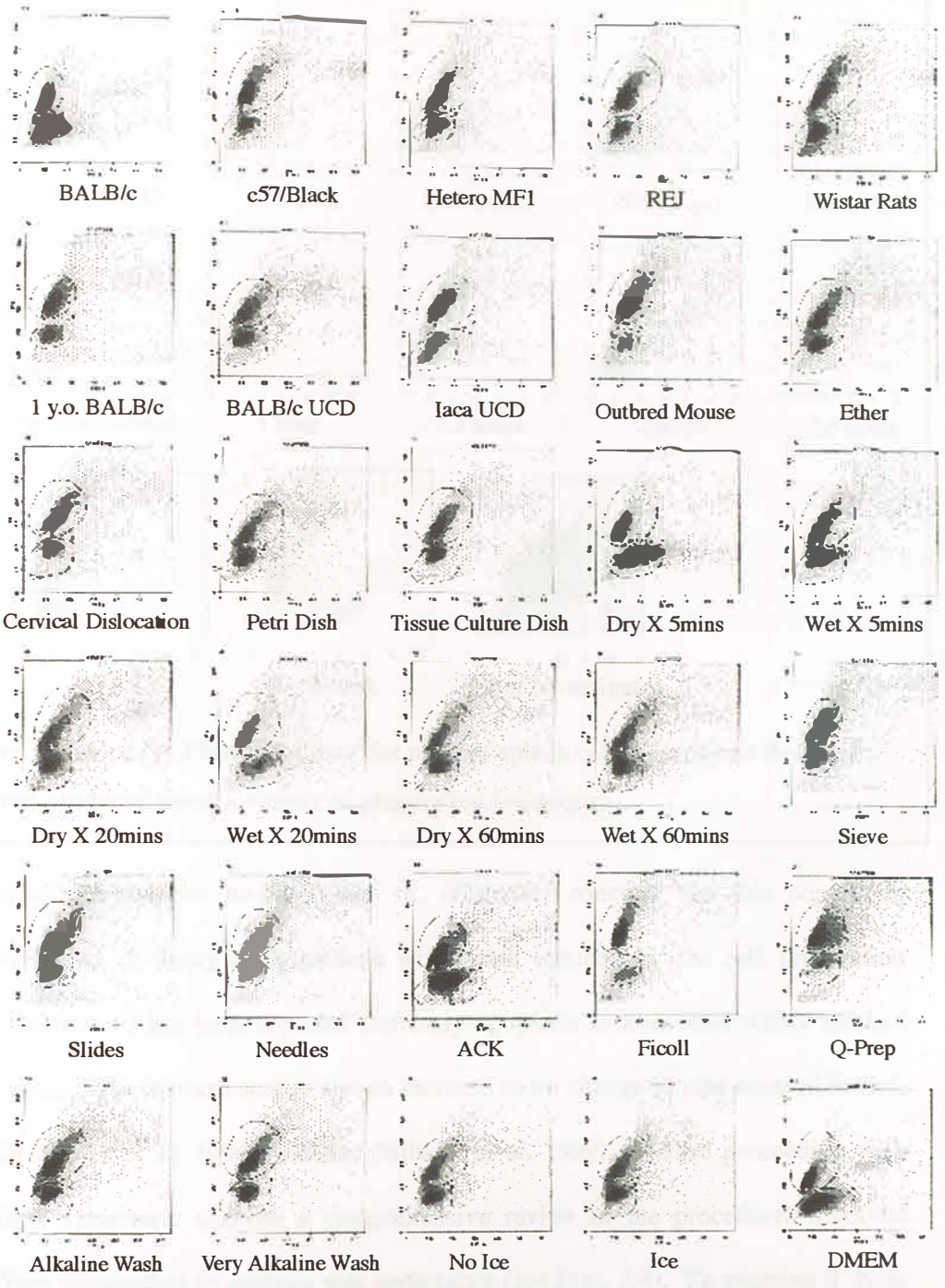


Fig. 3.4: Flow cytometer dot plots of spleen cell suspensions that have been produced using a variety of preparation techniques.

were set very high (Kuroi, Sato et al. 1990; Phyu, Sornes et al. 1999). We did not know whether this population represented immature lymphocytes from the

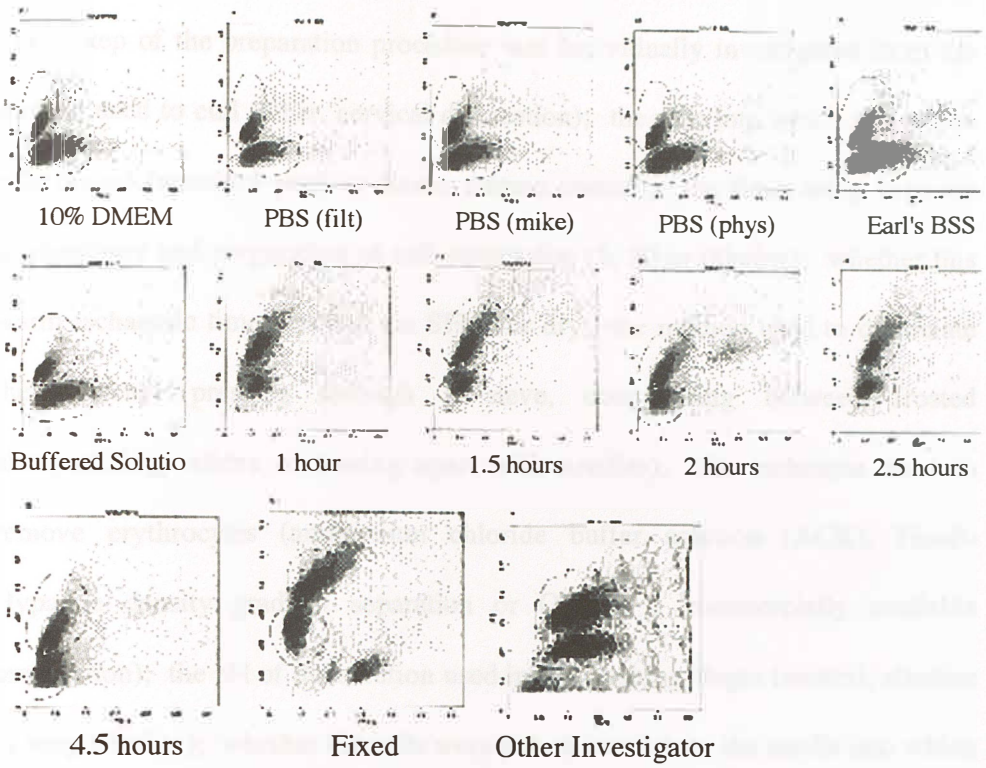


Fig. 3.4 (Cont.): Flow cytometer dot plots of spleen cell suspensions that have been produced using a variety of preparation techniques.

lymphoid follicles in the spleen or, of greater concern, that this population consisted of dying cells perhaps with death induced by the cell preparation methods. It has been reported that early apoptosis is associated with a marked reduction in forward scatter and an increase or no change in side scatter (Ferlini, Di Cesare et al. 1996; Scherer, Stillwell et al. 1999). Before proceeding with flow cytometric analysis a comprehensive review of the procedures involved from preparation to analysis was undertaken (see Figs. 3.4). To examine if these cells were specific to Balb/c mice other species (c57/Black, REJ, Hetero MF1) were also analysed, as were the splenocytes of an inbred rat strain, Wistar. mice from another animal facility (Balb/c and Iaca, from University College Dublin) as well as an outbred strain were examined to determine if the abnormal

population was due to a subclinical infection in our facility or due to inbreeding. Every step of the preparation procedure was individually investigated from the method used to cull (ether, cervical dislocation); the dish into which the spleen was placed (standard petri or tissue culture coated); the time delay between splenectomy and preparation of cell suspension (5, 20 or 60mins); whether this warm ischaemic time was wet (in SFM) or dry; the method used to dissociate the spleens (pressing through a sieve, compressing between frosted histopathology slides or teasing apart with needles); the technique used to remove erythrocytes (ammonium chloride buffer solution (ACK), Ficoll-Hypaque density gradient separation or Q-prep, a commercially available preparation); the pH of the solution used in the washing stages (neutral, alkaline or very alkaline); whether the cells were put on ice or not; the media into which the cells were resuspended for antibody labelling (DMEM, 10% DMEM, PBS from three different sources, Earl's balanced salt solution, buffered media); the delay between sample preparation and analysis (1 – 4.5 hours); and whether or not the cells were fixed prior to analysis and finally using a different investigator to prepare the sample. These factors were varied individually and in various combinations and permutations. Despite exhaustive analysis this population was a constant feature.

Next the cells were analysed for viability both visually by Trypan Blue Dye exclusion and cytometrically with Propidium Iodide staining. From the cytometer the suspicious population of cells constituted from 25 to 40% of the total population but total cell viability by dye exclusion was consistently 95% or more. Propidium Iodide (PI) is a fluorochrome that is intercalated into DNA or

RNA and fluoresces at >620nm when excited by light at 488nm. It is used for cell cycle analysis in fixed cells but can be used to assess cell viability of unfixed cells as the cell membrane of dying cells becomes leaky permitting entry of PI into the cell where it becomes intercalated and fluoresces when excited. From a stock solution of 1mg PI /ml of PBS 20μls was added to 10^6 cells in 1ml of medium and incubated for 5 mins before analysis on the flow cytometer.

It can be seen that treatment of the splenocyte cell suspensions with ACK while not significantly affecting the numbers of cells in the two populations (data not shown) did significantly increase the permeability of the cell membrane to PI particularly in the suspicious population (Table 3.2). Therefore all further cytometric analysis of splenocytes was undertaken without the use of ACK in the splenocyte preparation.

Table 3.2: Percentages of cells taking up PI in the two groups under investigation comparing the ACK treated and non-treated groups.

	ACK Treated	Non-ACK Treated	t-test
Lymphocyte	13.4 +/- 7.2	3.4 +/- 0.6	p= 0.035
Suspicious	88.3 +/- 10.6	16.4 +/- 7.6	p= 0.000008

The apparent disagreement between the >95% viability of splenocyte preparations, as determined by Trypan Blue dye exclusion, and 88% membrane permeability of cells in the suspicious population, as determined by Propidium Iodide incorporation, requires explanation. Clearly these cells cannot be necrotic as they lack any morphological features of necrosis on light microscopy. Perhaps they are in early stages of apoptosis or programmed cell death.

Apoptosis is an organised form of cell death common to many physiological processes from embryology to lymphocyte turnover. The possibility that these cells were in an early stage of apoptosis that permitted trans-membrane passage of PI but not Trypan Blue was addressed. One of the earliest events in programmed cell death is the externalisation, or "flipping", of phosphatidylserine (PS), a membrane-bound phospholipid normally restricted to the inner leaflet of the lipid bilayer (Blankenberg, Katsikis et al. 1998). It is believed that exposure of PS enables recognition of apoptotic cells and their subsequent removal by phagocytes (Stuart, Damoiseaux et al. 1998). Annexin V is a Ca^{++} dependent, endogenous human protein with a molecular weight of 36kD and a high affinity for membrane bound PS (Vermes, Haanen et al. 1995). It has been shown, by Vermes and others, to be possible, to detect early apoptosis before loss of membrane integrity. Apoptosis has been detected in thymocytes within 4 hours of exposure to dexamethasone a known inducer of apoptosis, at a stage when PI uptake was not altered (Donner, Becker et al. 1999). Thus we postulated that if the suspicious population of cells were representative of very early apoptosis, even before morphological changes in cell size and shape then PS should be detectable on their cell surfaces by Annexin V staining.

We used dual labelling with membrane (Annexin V) and nuclear (PI) stains to ascertain what, if any, stage of apoptosis these cells were in. However no significant difference in PS expression was detected between the two populations of cells regardless of whether ammonium chloride or density gradient centrifugation was used to separate the erythrocytes (Fig. 3.5). As one might anticipate there were slightly more Annexin V positive cells in the suspicious

population of splenocytes treated with ammonium chloride, this difference was not significant.

The Annexin V staining has not resolved the initial conflict between PI and Trypan Blue. Of note some authors have reported that PS exposure is not always the earliest indicator of apoptosis. When camptothecin is used to induce apoptosis in HL-60s nuclear fragmentation the hallmark of apoptosis is

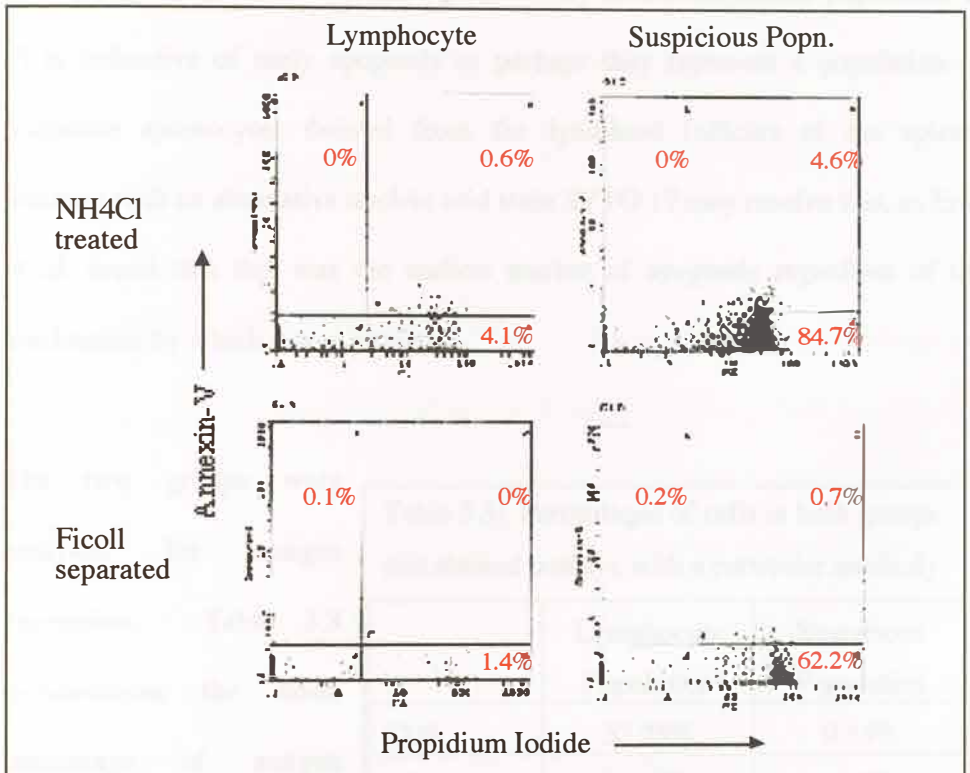


Fig. 3.5: To compare the viability of cells in the morphologically lymphocytic group with those in the 'suspicious' population erythrocytes were removed either by lysis with NH_4Cl or separated by density centrifugation with Ficoll Hypaque. The remaining spleen cells were dual labelled with Annexin V and Propidium Iodide. The lymphocyte population has 95 – 98.5% viability whereas the suspicious population has 10 – 33% viability. Percentages of cells in each group are highlighted in red.

detectable within 3 hours but Annexin V binding could not be detected until after 4 hours (King, Radicchi-Mastroianni et al. 2000). This difference may be related to the mechanism by which apoptosis is induced. Eray *et al.* using HF-1 cells, a follicular lymphoma cell line, found that if apoptosis was induced via the B-cell receptor an increased uptake of PI preceded labelling with Annexin V (Eray, Matto et al. 2001). When apoptosis was induced using a calcium ionophore, A23187, cells became positive for Annexin V earlier than for PI. We are left wondering whether the membrane permeability of the suspicious population to PI is indicative of early apoptosis or perhaps they represent a population of immature splenocytes derived from the lymphoid follicles of the spleen. Staining with an alternative nucleic acid stain SYTO 17 may resolve this, as Eray *et al.* found that this was the earliest marker of apoptosis regardless of the mechanism by which it was produced.

The two groups were analysed for antigen expression. Table 3.3 demonstrates the mean percentage of antigen expressing cells in each of the two groups of interest. It can be seen that very few of the antigens of interest to us are expressed on the morphologically suspicious group of cells. What binding is present could be non-specific and related to those cells that are dying

Table 3.3: Percentages of cells in both groups that stained positive with a particular antibody

	Lymphocyte Population	Suspicious Population
CD3	57.25%	9.15%
CD4	33.65%	4.0%
CD8	16.95%	1.05%
IL-2	13.62%	4.92%
CD4/IL-2	4.15%	1.85%
CD8/IL-2	0.75%	0.85%

based on PI uptake. The reduced expression of cell surface markers of mature lymphocytes would support the hypothesis that this population was derived from the lymphoid follicles of the spleen.

The splenocytes were stained with a panel of antibodies to CD3, CD4, CD8, CD40 and CD25 (see Table 3.1). The anti- CD3, CD4, CD8 and CD40 monoclonal antibodies were directly conjugated to the relevant fluorochrome. Detection of CD25 required a two-step approach firstly with Biotinylated anti-CD25 monoclonal antibody followed by an Avidin conjugated FITC. All staining procedures required staining with a non-specific isotype control antibody to enable exclusion of artefact. We used dual staining with one FITC and one PE conjugated antibody. Individual samples were set up for analysis as shown (Table 3.4). A single cell suspension having been made the cells are pelleted and resuspended in PBS to a final concentration of 1 X 10^7 per ml. Aliquots of 100μls of this cell suspension (1 X10^6 cells) were transferred to eppendorf tubes. To these tubes 10μls of the directly conjugated antibodies were added (samples A, B, C, D, G, I) and incubated in the dark on ice. After 30 minutes the cells were washed to remove unbound antibody and resuspended in 200μls of ISOTON II, an azide free balanced electrolyte solution. The cells were then stored in the dark, on ice, until

Table 3.4: For each spleen cell suspension, samples were labelled and stained as follows

A: FITC/RPE negative controls
B: CD3-FITC/CD4-RPE
C: CD3-FITC/CD8-RPE
D: CD40-RPE
E: RPE/Avidin-Biotin negative controls
F: CD25-FITC
G: CD4-RPE
H: CD4-RPE/CD25-FITC
I: CD8-RPE
J: CD8-RPE/CD25-FITC

all samples were ready to be analysed. For analysis of IL-2 receptor (CD25) expression the cells were initially incubated for 30 mins as before with 2μls of the biotynilated primary antibody or the isotype control antibody. After washing the cells were resuspended in PBS and 2μls of the avidin-FITC, secondary reagent together where appropriate, with 10μls of the directly conjugated anti-CD4 or CD8 antibody were added. Thirty minutes later the cells were again washed and resuspended in ISOTON II prior to analysis on the cytometer. The samples were analysed using a Coulter Epics Elite Flow Cytometer equipped with an argon laser that emits at 488nm. The isotype control samples were used to set the gates for analysis of the test samples. In each sample a minimum of 15,000 events or cells were analysed. Data will be graphically presented as isometric dot plot or contour displays but the majority of the results will be presented as percentages of events analysed. As IL-2 receptor (CD25) status is a quantitative measure, i.e. the number of IL-2 receptor positive cells may or may not change but the number of IL-2 receptors per cell may, these samples were also analysed for mean fluorescence indices using the WIN MDI analysis software.

3.2.2 Measurement of Interferon-γ (IFN-γ)

Spleen cell suspensions were prepared as described in Chapter 2 from naïve and tumour-bearing mice. Splenocytes (6 X 10^5) were co-incubated with 5ng/ml phorbol myristate acetate (PMA, Sigma) and 500 ng/ml ionomycin (Sigma) in complete medium at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 24 hours in culture the medium was harvested, centrifuged to remove any cellular debris and assayed using an enzyme-linked immunosorbent assay

(ELISA) kit specific for murine IFN-γ (BioSource International). The assay was carried out according to the manufacturer's instructions. Samples representing splenocyte cultures from three separate mice with similar tumour burdens were pooled, diluted and analysed as a single sample. This was done in duplicate for each tumour stage such that 6 mice were assayed per group. IFN-γ was also assayed in the sera of tumour bearing mice. Anaesthetised mice were culled by cardiac puncture exsanguination. The blood was allowed to clot and the clots to retract overnight at 4°C. The resultant serum samples were again pooled from three separate mice, diluted and analysed for murine IFN-γ by ELISA in accordance with the manufacturer's (BioSource International) instructions.

3.3 Results

3.3.1 Tumour Growth Rates

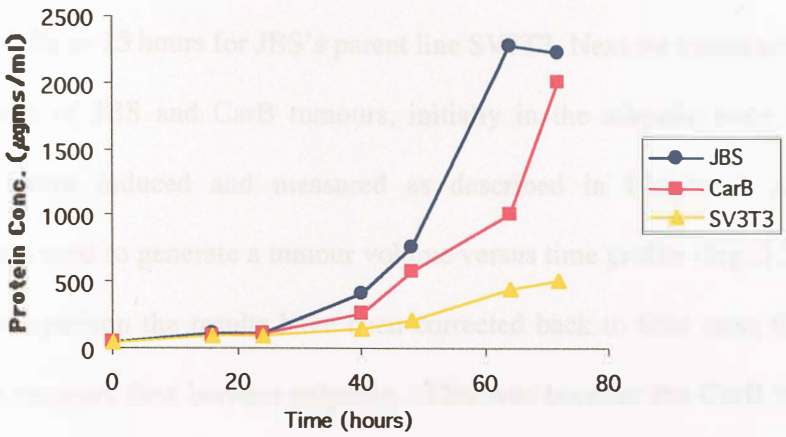


Fig. 3.6: *In vitro* growth curves for individual cell lines grown as monolayers and determined by protein concentration

Having set flasks up in the method described above and using the Bradford method for protein determination it was possible to construct graphs of protein concentration, crudely indicative of cell number against time (Fig. 3.6). This

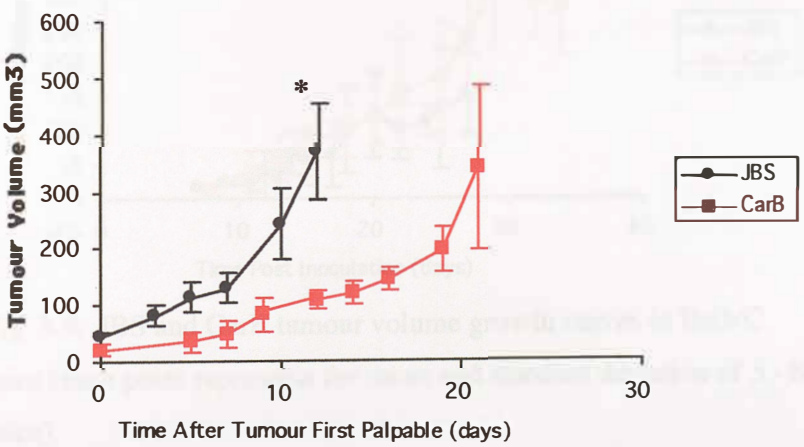


Fig. 3.7: Volume growth curves for JBS and CarB tumours grown in athymic nude mice (each point represents the mean and standard deviation of a panel of 7 mice). JBS tumours grow faster than CarB with $p<0.01$ on day 12* of tumour growth.

graph was then used to estimate the doubling time of individual cell lines, during the log phase of cell growth i.e. when growing fastest. It can be seen that *in vitro* JBS cells grow the fastest with a doubling time of 8 hours, compared to 10 hours for CarB cells or 15 hours for JBS's parent line SV3T3. Next we examined the *in vivo* growth of JBS and CarB tumours, initially in the athymic nude mouse. Tumours were induced and measured as described in Chapter 2 and the information used to generate a tumour volume versus time profile (Fig. 3.7). For ease of comparison the results have been corrected back to time zero, the time when the tumours first became palpable. This was because the CarB tumours were slower in becoming established. This graph confirms the *in vitro* finding in that it demonstrates JBS tumours growing faster *in vivo* than CarB tumours.

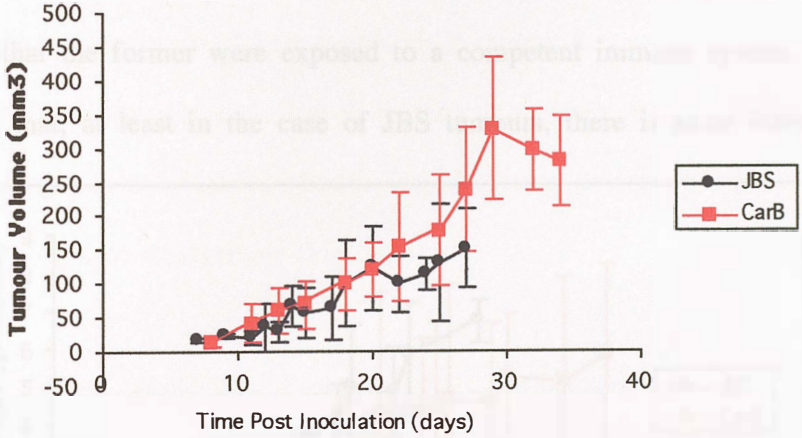


Fig. 3.8: JBS and CarB tumour volume growth curves in Balb/C mice (each point represents the mean and standard deviation of 5 - 8 mice).

Whether this growth advantage would still manifest itself in immunocompetant mice was next investigated. Tumours were generated in immunocompetant Balb/C mice, measured and volume growth curves constructed as before (Fig.

3.8). The clear growth advantage of JBS over CarB seen *in vitro* and *in vivo* in nude mice has disappeared. These results were consistently found in three repeat experiments. Now there is no statistically significant difference between the growth rates of the two differing tumours in immunocompetant animals. The possibility that this loss of difference in growth rates was due to artefact related to the use of a formula to calculate tumour volume, which is known to have a large margin of error was considered. Therefore it was decided to construct a growth curve related to tumour weight at excision (Fig. 3.9). This graph is based on the wet weights of over 100 freshly excised tumours of each histological type. Tumours that were significantly delayed in development, of either type, were excluded from analysis. Again no clear growth advantage of one cell type over another appears. The differences between the tumours, which developed, in Balb/C mice and those which developed in nude mice or cell line growth rates *in vitro* is that the former were exposed to a competent immune system. This suggests that, at least in the case of JBS tumours, there is some interaction

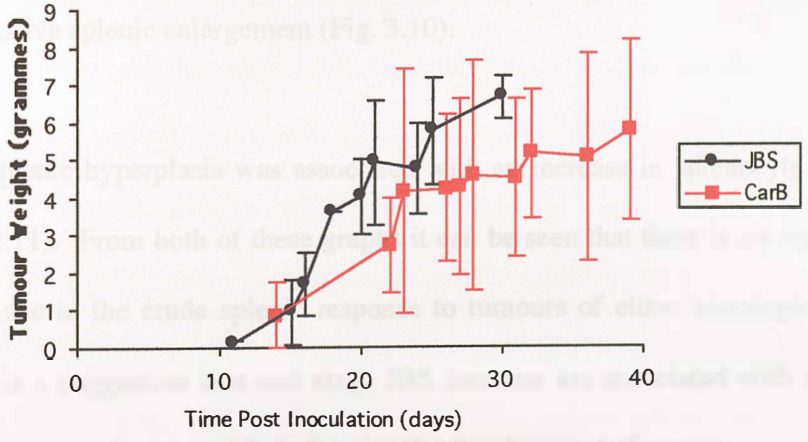


Fig. 3.9: JBS and CarB tumour growth curves in Balb/C mice based on weight of excised tumours. (there were more than 100 mice for each tumour type).

between the developing tumour and the host immune system and encouraged further research.

3.3.2 Spleen Response to Tumour Growth

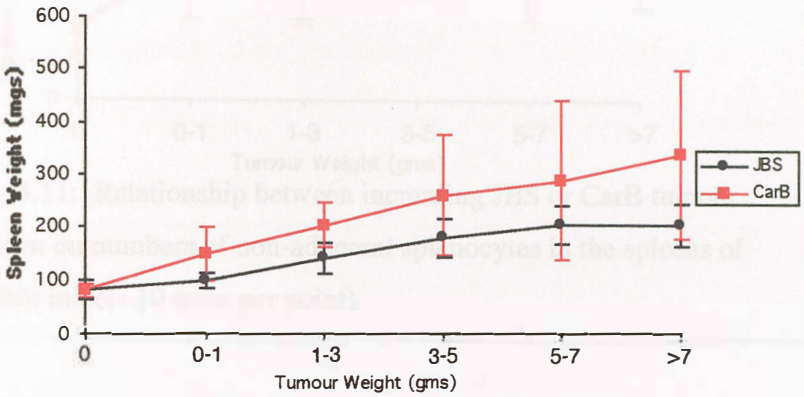


Fig. 3.10: Relationship between spleen weight and excised tumour weight in Balb/C mice with increasing JBS or CarB tumour burden (there were more than 100 mice for each tumour type)

The spleen is a major site of haematopoiesis in mice and as such has been shown to enlarge in response to a number of developing tumours (Rayner 1994). Mice with increasing tumour burden of either histological type were found to undergo progressive splenic enlargement (Fig. 3.10).

This splenic hyperplasia was associated with an increase in splenocyte number (Fig. 3.11). From both of these graphs it can be seen that there is no significant difference in the crude splenic response to tumours of either histological type. There is a suggestion that end stage JBS tumours are associated with a fall in splenocyte numbers possibly indicating the development of anergy.

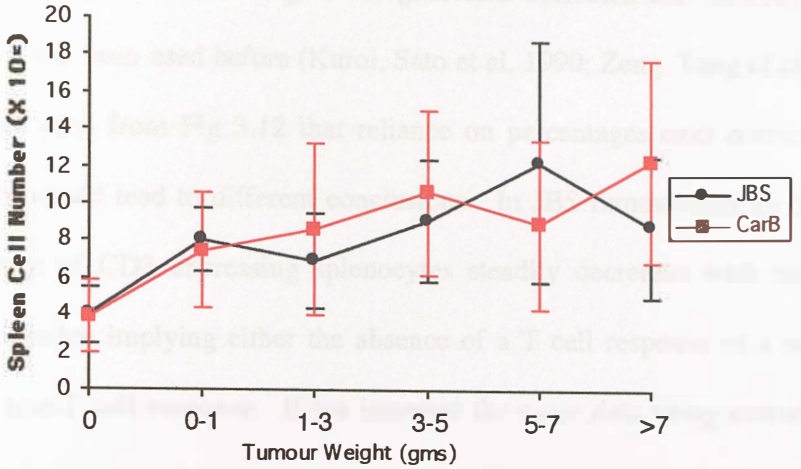


Fig. 3.11: Relationship between increasing JBS or CarB tumour burden on numbers of non-adherent splenocytes in the spleens of Balb/c mice(>10 mice per point).

3.3.3 Lymphocyte Subsets

Before analysing splenocyte function we looked at lymphocyte subsets in the spleens of these tumour-bearing mice. Spleen cell suspensions were examined for expression of T-cell markers, CD3, CD4 and CD8, a B cell marker, CD40, and Interleukin –2 receptor expression, which is upregulated in activated cells. The flow cytometer generates data as a series of dot plots from which percentages of stained cells can be extracted. To show all of the dot plots would be confusing and not necessarily helpful therefore typical examples of cytometer plots are included in some of the data figures for completeness. As I have already stated the cytometric analysis generates results in terms of percentages of events (i.e. cells) analysed. We were concerned that because of the recognised splenic hyperplasia and increase in splenocyte cell numbers associated with increasing tumour burden, (see Fig. 3.11), analysis of percentages may mask or exaggerate changes in lymphocyte subsets. Therefore we adopted an approach, which used these cytometer-generated percentages and together with the mean

cell numbers given above (Fig. 3.11) generated corrected cell numbers. This approach has been used before (Kuroi, Sato et al. 1990; Zeng, Tang et al. 1995). It can be seen from Fig 3.12 that reliance on percentages over corrected cell numbers would lead to different conclusions. In JBS tumour-bearing mice the percentage of CD3 expressing splenocytes steadily decreases with increasing tumour burden implying either the absence of a T cell response or a relatively greater non-T cell response. If we interpret the same data using corrected cell

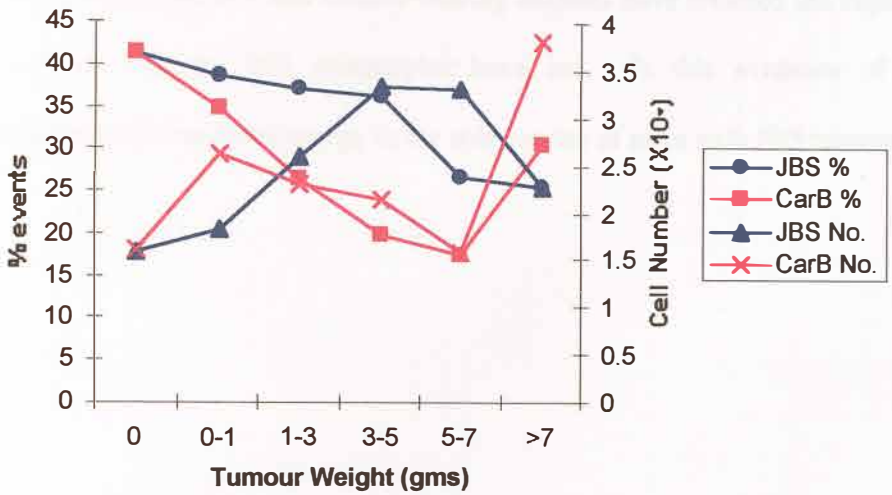


Fig. 3.12: Relationship between surface expression of T cell marker, CD3, on splenocytes from JBS or CarB tumour-bearing mice with progressive tumour burden comparing the differences when presented as percentages of analysed events (JBS%, CarB%) and as corrected cell numbers (JBS No., CarB No.)

numbers then we see an gradual increase in T cell numbers up to a maximum at a tumour burden of 5-7gms with a decline above this. Similar differences are seen in the early stages of CarB tumour-bearing mice. The results used to generate Figs. 3.12 to 3.21 are summarised in Table 3.5 for easy reference .

From Fig. 3.12, we can conclude that mice who develop JBS tumours have a progressive T cell hyperplasia which peaks in mice with tumours of 3-7 gms and then falls with further increases in tumour load. CarB tumour bearing mice on the other hand do not exhibit any significant increase in T cell numbers until the animals have an extensive tumour size, > 7 gms. This contrasts significantly with the response to JBS tumours. This may be related to impairment of skin integrity seen over very large CarB tumours and any ensuing immune response to secondary, subclinical, pathogen invasion. If this is the case, it is interesting that the splenocytes of CarB tumour-bearing animals have retained the capacity to respond whereas, JBS splenocytes have not. Is this evidence of the development of functional anergy in the splenocytes of mice with JBS tumours?

Table 3.5: Results of cytometric assessment of splenocyte expression of designated cell markers in tumour bearing mice (mean +/- standard deviation)

JBS

	Tumour Weight	Cell No.	$CD40^+$	$CD3^+$	$CD4^+$	$CD3^+/CD4^+$	$CD8^+$	$CD3^+/CD8^+$	$Il\text{-}2R^+$	$CD4^+/Il\text{-}2R^+$	$CD8^+/Il\text{-}2R^+$	$Il\text{-}2R^+$ MFI	$CD4^+/Il\text{-}2R^+$ MFI	$CD8^+/Il\text{-}2R^+$ MFI
Naïve		3.99 +/-1.54	1.27 +/-0.65	1.59 +/-0.59	1.18 +/-0.35	1.21 +/-0.22	0.48 +/-0.19	0.56 +/-0.13	0.65 +/-0.49	0.26 +/-0.25	0.06 +/-0.04	*	*	*
0-1gm	0.48 +/-0.31	8.07 +/-1.68	1.95** +/-0.04	1.84 +/-0.48	1.34* +/-0.26	1.29 +/-0.34	0.29 +/-0.35	0.29 +/-0.42	0.72* +/-0.23	0.91* +/-0.32	0.11 +/10.11	33.4	53.3	52.4
1-3gm	2.10 +/-0.51	6.95 +/-2.59	3.20** +/-0.71	2.60* +/-0.71	1.94* +/-0.37	1.90 +/-0.45	0.86** +/-0.44	0.85 +/-0.47	1.85* +/-1.04	0.56* +/-0.21	0.16** +/-0.09	39.8	75.5	94.6
3-5gm	3.37 +/-0.31	9.13 +/-3.26	4.16* +/-0.01	3.33* +/-0.95	2.53* +/-0.64	2.25 +/-0.52	0.69 +/-0.33	0.72 +/-0.33	1.54* +/-1.16	0.83 +/-0.78	0.19* +/-0.08	24.6	55.7	133.5
5-7gm	5.99 +/-0.50	12.4 +/-6.47	4.71** +/-0.79	3.31** +/-0.50	2.41** +/-0.22	2.19 +/-0.24	0.99** +/-0.27	0.83 +/-0.32	2.12 +/-1.29	0.56* +/-0.04	0.23 +/- 0.1	44.0	62.1	158.1
>7gm	8.50 +/-1.84	8.86 +/-3.87	*	2.27** +/-0.18	2.14* +/-0.41	*	1.15* +/-0.19	*	0.57** +/-0.07	0.37 +/- 0.05	0.31* +/-0.03	*	*	*

CarB

	Tumour Weight	Cell No.	$CD40^+$	$CD3^+$	$CD4^+$	$CD3^+/CD4^+$	$CD8^+$	$CD3^+/CD8^+$	$Il\text{-}2R^+$	$CD4^+/Il\text{-}2R^+$	$CD8^+/Il\text{-}2R^+$	$Il\text{-}2R^+$ MFI	$CD4^+/Il\text{-}2R^+$ MFI	$CD8^+/Il\text{-}2R^+$ MFI
Naïve		3.92 +/-1.95	1.25 +/-0.64	1.62 +/-0.59	1.19 +/-0.35	1.18 +/-0.22	0.50 +/-0.20	0.55 +/-0.13	0.59 +/-0.48	0.28 +/-0.24	0.06 +/-0.04	*	*	*
0-1gm	0.46 +/-0.19	7.49 +/-3.06	3.54** +/-0.33	2.62* +/-0.79	1.90** +/-0.45	1.66 +/-0.52	0.70 +/- 0.40	0.52 +/-0.40	1.89** +/-0.40	0.46 +/-0.29	0.02* +/-0.03	35.9	64.2	90.6
1-3gm	1.60 +/-0.52	8.68 +/-4.61	3.95** +/-0.59	2.31* +/-0.55	1.77* +/-0.36	1.51 +/-0.42	0.30* +/-0.20	0.26 +/-0.18	2.39* +/-0.99	0.35 +/-0.11	0.02* +/-0.04	40.0	47.5	107.2
3-5gm	3.35 +/-0.33	10.7 +/-4.47	4.19* +/-1.29	2.15 +/-0.75	1.68* +/-0.22	1.37 +/-0.38	0.30 +/-0.20	0.17 +/-0.20	2.47* +/-0.33	0.39 +/-0.13	0.05* +/-0.05	34.1	66.9	159.3
5-7gm	5.37 +/-0.40	8.99 +/-4.55	3.63** +/-0.25	1.59* +/-0.30	1.30* +/-0.10	1.10 +/-0.11	0.20** +/-0.10	0.18 +/-0.09	1.64	0.42	0.02	36.2	115.5	289.9
>7gm	8.40 +/-1.27	12.5 +/-5.46	5.70* +/-0.21	3.81** +/-0.24	2.68* +/-0.26	2.48 +/-0.04	1.20 +/-1.20	1.25	3.54** +/-1.26	0.58* +/-0.13	0.06	35.6	74.5	74.2

Red'*' - p value < 0.05 when compared to naive mice

* no results available

Green'*' - p value < 0.05 when compared to JBS or CarB of similar weight

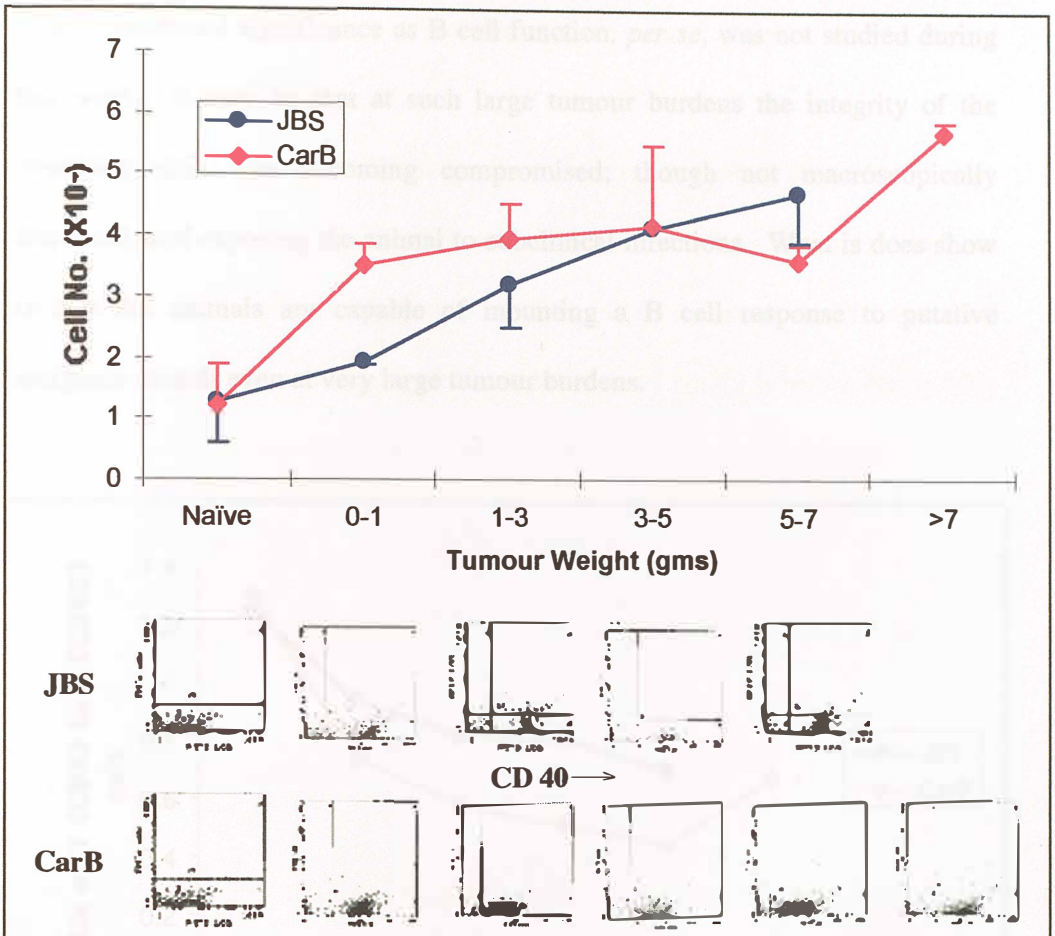


Fig. 3.13: Trends in surface expression of B-cell marker, CD40, on splenocytes from JBS or CarB tumour-bearing mice with progressive tumour burden. Typical flow cytometer dot plots of CD40 expression for each tumour burden group are included.

The development and progression of both tumours is associated with a B cell hyperplasia in the spleens of tumour-bearing animals (Fig. 3.13). The gradual and steady increase in B cell numbers in JBS tumour-bearing mice differs from the more rapid and sustained B cell response in animals with CarB tumours. In CarB tumour-bearing mice the B cell hyperplasia appears to be waning with larger tumour burdens apart from the sudden rise in those mice with tumours

greater than 7 gms in weight. The reason for this is unclear and I am not aware of any functional significance as B cell function, *per se*, was not studied during this work. It may be that at such large tumour burdens the integrity of the overlying skin was becoming compromised; though not macroscopically disrupted, and exposing the animal to subclinical infections. What is does show is that the animals are capable of mounting a B cell response to putative antigenic stimuli even at very large tumour burdens.

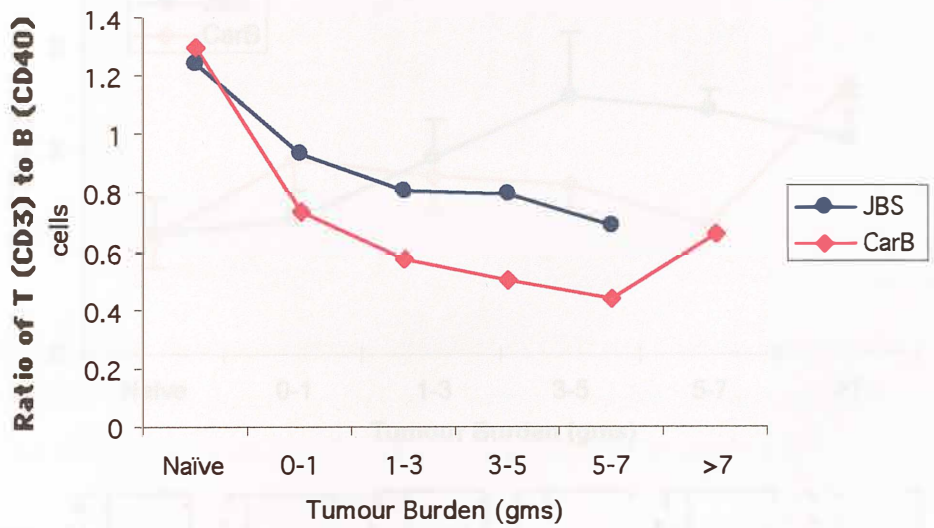


Fig. 3.14: Changes in ratio of T cells to B cells in JBS or CarB tumour bearing mice with progressive tumour burden.

Fig 3.14 demonstrates the change in splenocyte subsets from predominantly a T cell population in naïve mice to a preponderance of B cells in tumour-bearing mice. Both tumour types are associated with a fall in the ratio of T cells to B cells. This shift is more obvious in CarB animals, which is not surprising given their marked B cell response and equivocal T cell response. Mice with very

large (>7 gm) tumours display return towards the normal ratio, due to a relatively greater increase in T than B cells at that stage of tumour burden.

To further investigate the T cell response cell surface expression of CD4 (helper T cells) and CD8 (cytotoxic T cells) was examined, both in single colour analysis (see Table 3.5) and in dual antibody staining combined with anti-CD3 monoclonal antibodies. The trends in expression of singly labelled and doubly

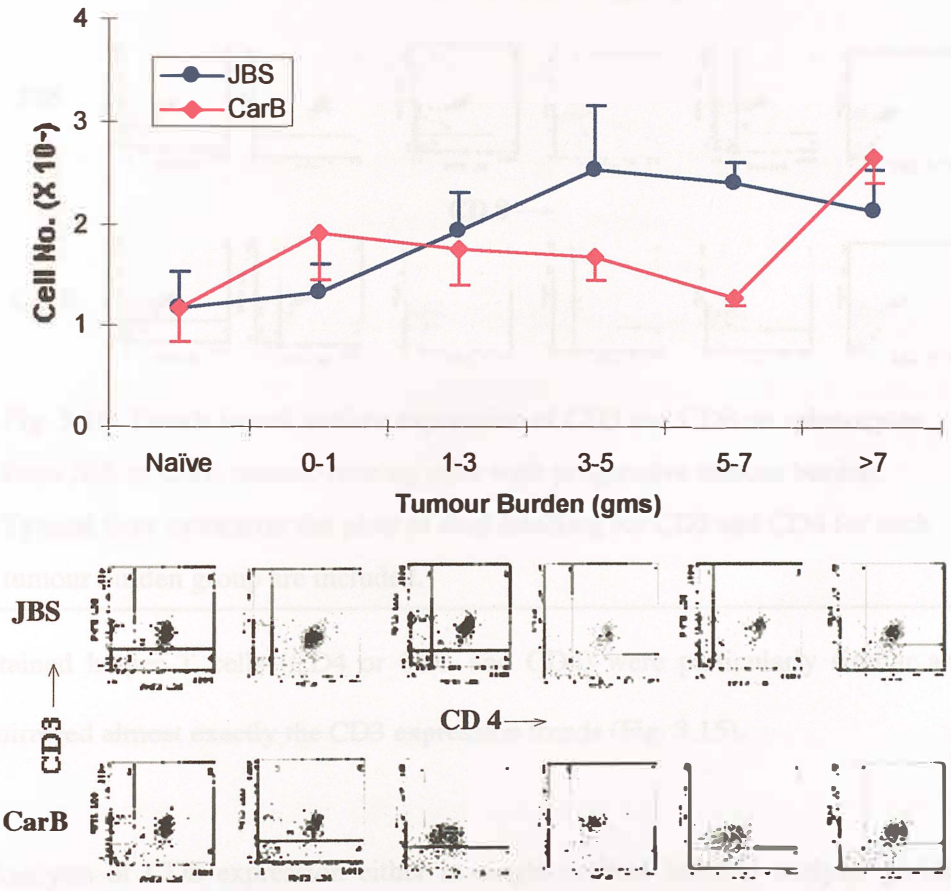


Fig. 3.15: : Trends in cell surface expression of CD3 and CD4 on splenocytes from JBS or CarB tumour-bearing mice with progressive tumour burden. Typical flow cytometer dot plots of dual labelling for CD3 and CD4 for each tumour burden group are included.

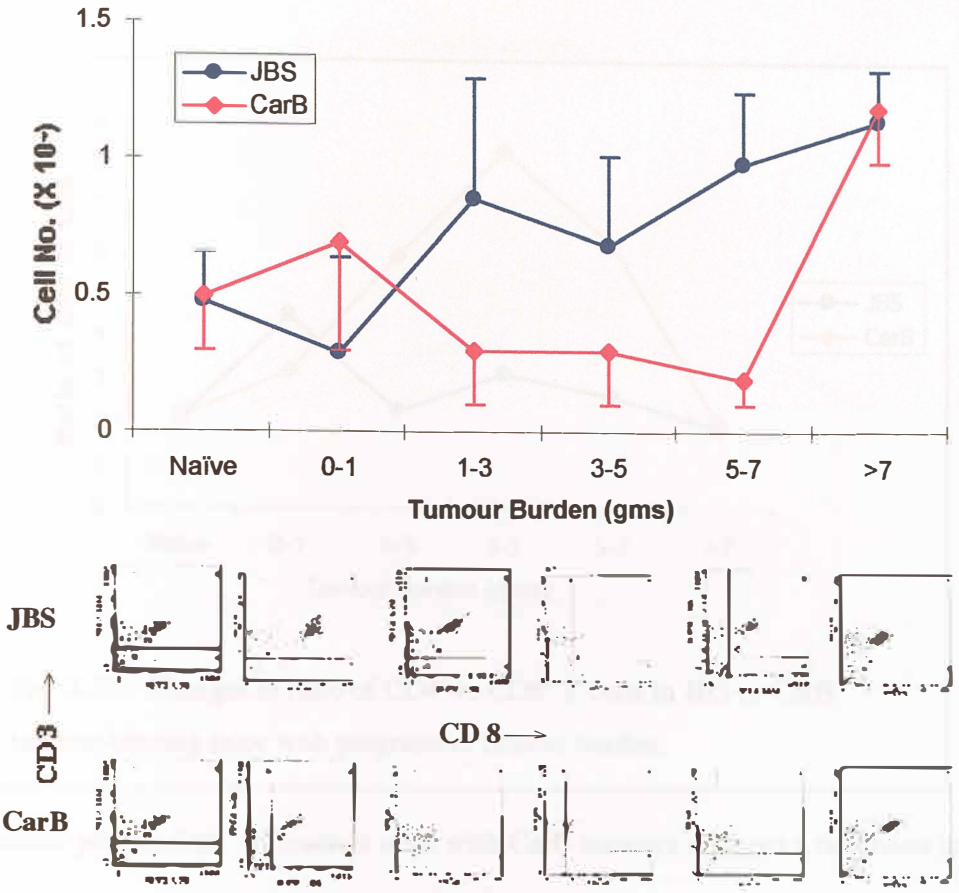


Fig. 3.16: Trends in cell surface expression of CD3 and CD8 on splenocytes from JBS or CarB tumour-bearing mice with progressive tumour burden. Typical flow cytometer dot plots of dual labelling for CD3 and CD4 for each tumour burden group are included.

stained helper T cells (CD4 or CD3 and CD4) were particularly similar and mirrored almost exactly the CD3 expression trends (Fig. 3.15).

Analysis of CD8 expression either in single or dual labelled analysis yielded interesting contrasts with the CD3 and CD4 trends (Fig. 3.16). The $CD8^+$ population of cells in JBS tumour-bearing mice do not show the same tendency to a reduction in cell numbers in mice with the greatest tumour load as seen with $CD3^+$ and $CD4^+$ cells. Rather there is a steady increase in $CD8^+$ cells throughout

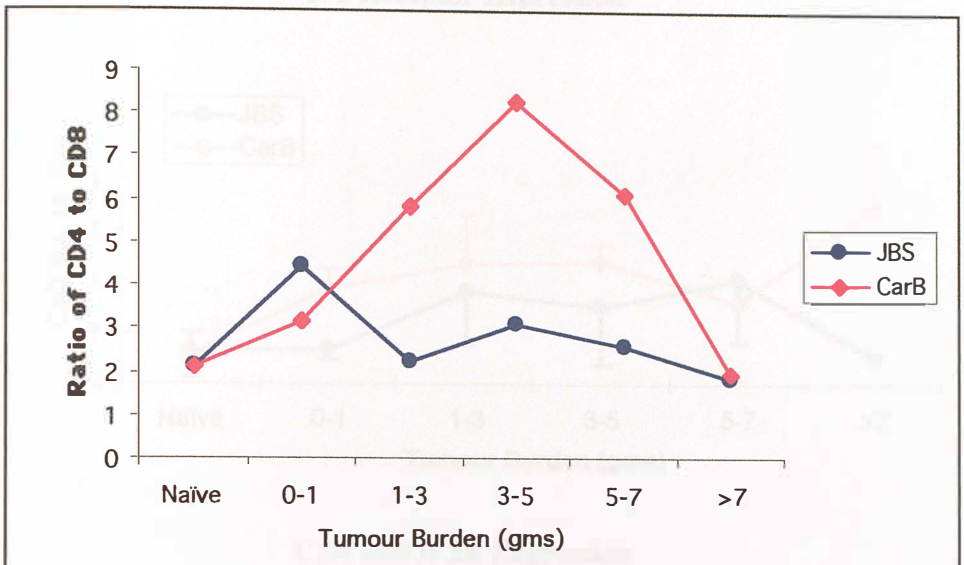
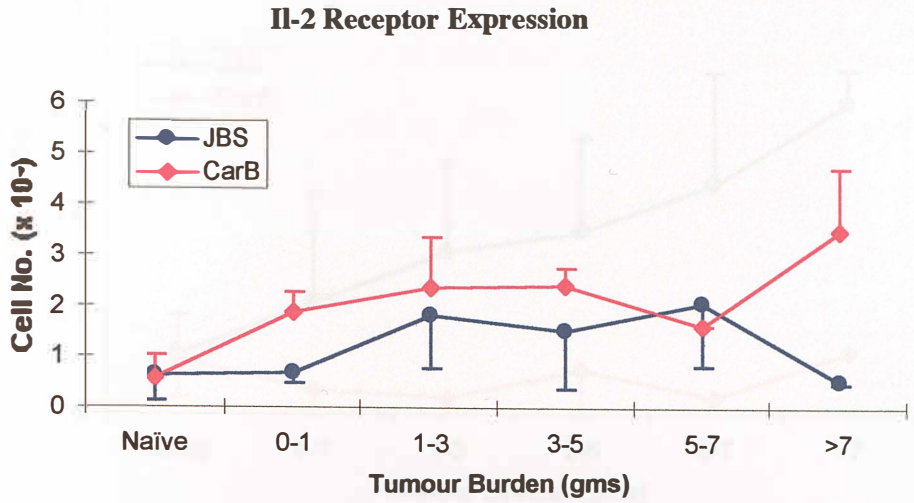


Fig. 3.17: Changes in ratio of $CD4^+$ to $CD8^+$ T-cells in JBS or CarB tumour-bearing mice with progressive tumour burden.

tumour progression. In contrast mice with CarB tumours undergo a reduction in $CD8^+$ cells, except at the largest tumour volume, a similar trend to that seen for CD3 and CD4 cells in CarB tumour bearing mice.

Fig. 3.17 shows the ratio of $CD4^+$ T cells to $CD8^+$ T cells for both tumour types. JBS tumour- bearing mice seem to show an increase in $CD4^+$ cells that is probably an artefact related to the apparent fall in $CD8^+$ cells in mice with tumour burdens of 0-1 gms. Otherwise there is no real change in T cell subsets of mice with progressive JBS tumour burdens. CarB Tumour bearing mice on the other hand undergo a relative increase in $CD4^+$ splenocytes, largely the consequence of a fall in $CD8^+$ cells.



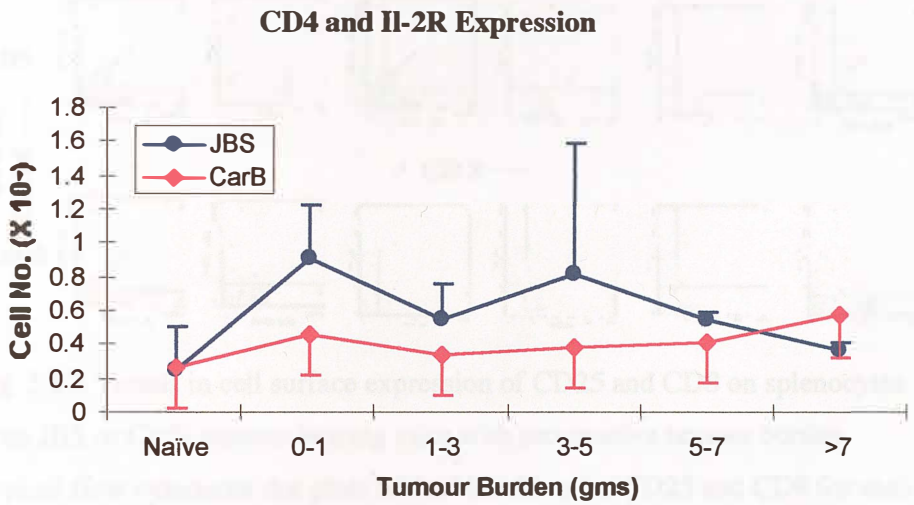


Fig. 3.18: Trends in cell surface expression of CD25 (Il-2R) alone or dual-labelled CD25 and CD4 on splenocytes from JBS or CarB tumour-bearing mice with progressive tumour burden.

Understandably we were also interested in the activation status of these cells, therefore we investigated the cell surface expression of CD25 or the Interleukin (Il-2) Receptor using dual staining with either anti-CD4 or anti-CD8 antibodies (Fig. 3.18). Both tumour types are associated with an increase in Il-2 receptor expressing cells with increasing tumour burden, though CarB more so than JBS. Interestingly at the largest tumour volumes CarB tumour-bearing mice have a

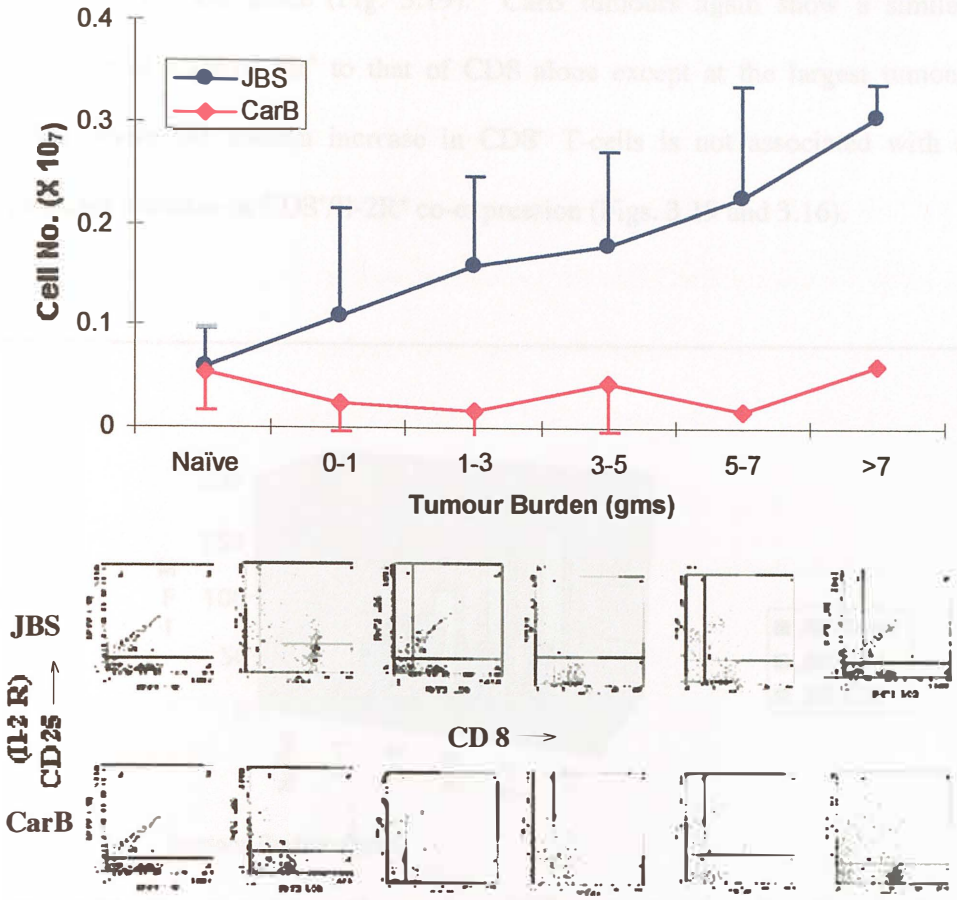


Fig. 3.19: Trends in cell surface expression of CD25 and CD8 on splenocytes from JBS or CarB tumour-bearing mice with progressive tumour burden. Typical flow cytometer dot plots of dual labelling for CD25 and CD8 for each tumour burden group are included.

marked increase in IL-2R expressing splenocytes whereas JBS tumour-bearing mice exhibit a fall.

Trends in the co-expression of CD4 and Il-2R in JBS tumour bearing mice mirror exactly those of singly labelled $CD4^+$ cells (Fig. 3.18). In CarB tumour-bearing mice on the other hand there is minimal change in the co-expression of CD4 and IL-2R even at the largest tumour burdens. JBS tumours are associated with a progressive increase in $CD8^+/Il\text{-}2R^+$ splenocytes again similar to single

expression of CD8 alone (Fig. 3.19). CarB tumours again show a similar expression of $CD8^+/Il\text{-}2R^+$ to that of CD8 alone except at the largest tumour burden where the sudden increase in $CD8^+$ T-cells is not associated with a significant increase in $CD8^+/Il\text{-}2R^+$ co-expression (Figs. 3.19 and 3.16).

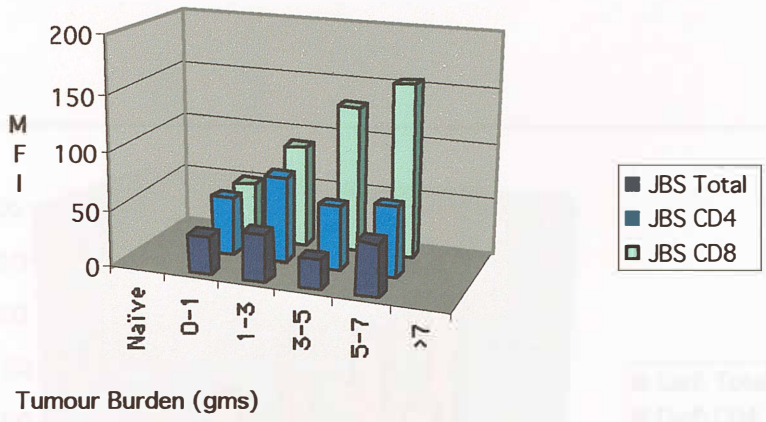


Fig. 3.20: Trends in Mean Fluorescence Indices (indicative of an increase in number of receptors on the cell surface, rather than number of cells expressing the receptor) of Interleukin-2 receptor expression on surfaces of all splenocytes (JBS Total) or splenocytes co-labelled with either CD4 (JBS CD4) or CD8 (JBS CD8) from JBS tumour-bearing mice.

Interleukin-2 receptor upregulation is a marker for cellular activation and therefore simply looking at the number of cells expressing Il-2R could mask upregulation of those cells already expressing low levels of Il-2R. Mean Fluorescence Indices (MFIs) offer an opportunity to overcome this obstacle by calculating the fluorescence intensity, which is related to the number of fluorochromes bound to the cell surface itself related to the number of receptors expressed, of each cell analysed and then calculating the means for each population of cells examined. MFIs were calculated using WIN MDI software.

Figs 3.20 and 3.21 show the Interleukin-2 receptor MFIs for splenocytes from both histological tumour types. From the information available there appears to be no significant change in the MFIs for total Il-2 receptor expression or Il-2R co-expression with CD4 for either tumour type. The MFIs for Il-2R in $CD8^+$ cells rises with increasing tumour burden in JBS and CarB tumours. Again in animals with >7gm CarB tumours the mean fluorescence indices fall off, this is despite the increase in absolute number of $CD8^+$ T cells.

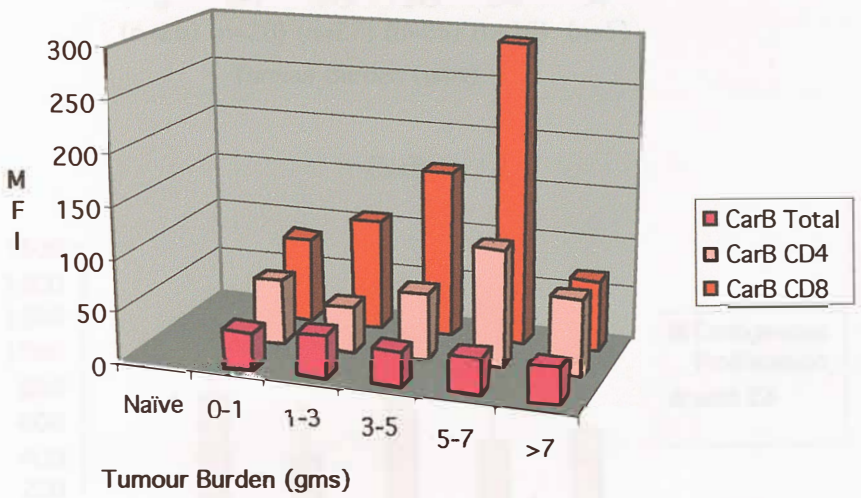


Fig. 3.21: Trends in Mean Fluorescence Indices (indicative of an increase in number of receptors on the cell surface, rather than number of cells expressing the receptor) of Interleukin-2 receptor expression on surfaces of all splenocytes (CarB Total) or splenocytes co-labelled with either CD4 (CarB CD4) or CD8 (CarB CD8) from CarB tumour-bearing mice.

3.3.4 Functional Studies

3.3.5 Lymphocyte Proliferation Assays

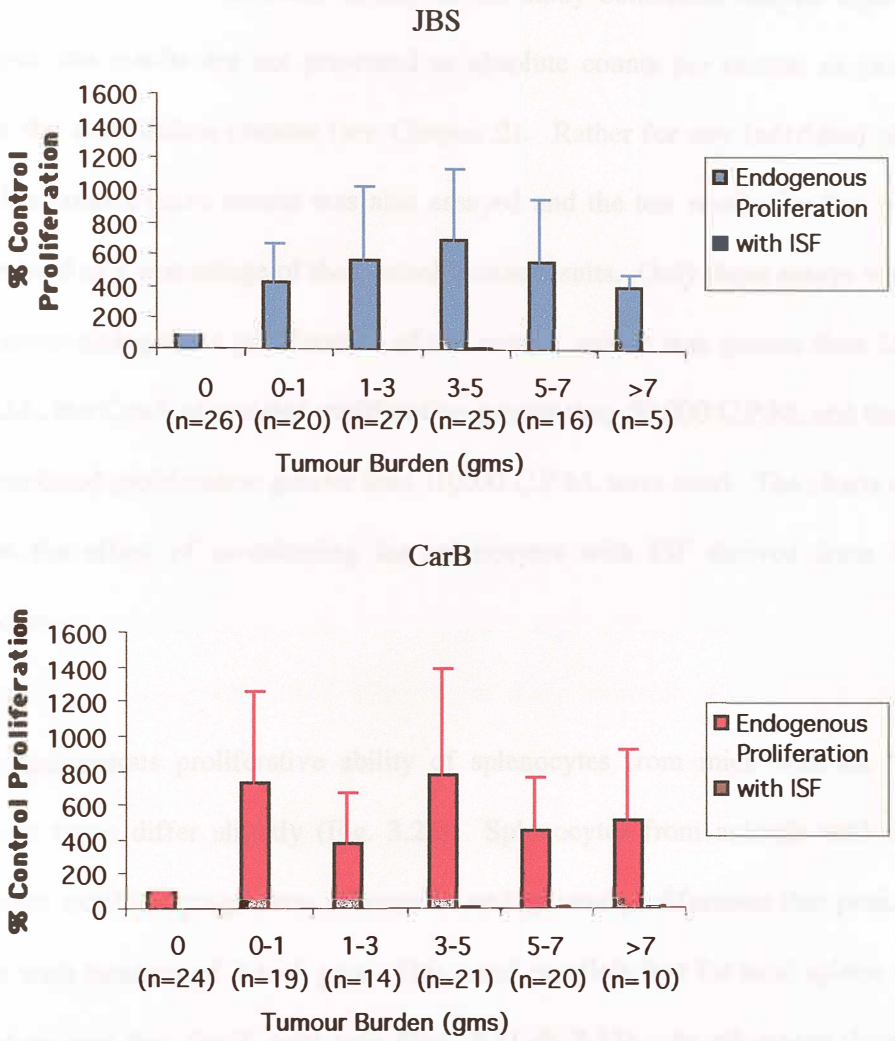


Fig. 3.22: Trends in endogenous proliferation of splenocytes, expressed as a percentage of control naïve mouse splenocytes, from JBS or CarB tumour-bearing mice with increasing tumour burden. The effect of co-culture with ISF (inhibition of proliferation) is included for all tumour burden groups but in some, is too small to register on this scale. The numbers in brackets represents the number of mice per group.

Figs 3.22 to 3.24 demonstrate the endogenous (or non-exogenously activated) proliferative ability of splenocytes from mice bearing either JBS or CarB tumours. Because the assays were carried out on many different days, with the potential for minor variations in any of the study conditions despite rigorous control, the results are not presented as absolute counts per minute as gained from the scintillation counter (see Chapter 2). Rather for any individual plate work a control/naïve mouse was also assayed and the test results for that plate expressed as a percentage of the control mouse results. Only those assays where the mean endogenous proliferation of the control mouse was greater than 1500 C.P.M., the ConA stimulated proliferation greater than 50,000 C.P.M. and the Il-2 stimulated proliferation greater than 10,000 C.P.M. were used. The charts also show the effect of co-culturing the splenocytes with ISF derived from JBS tumours.

The endogenous proliferative ability of splenocytes from mice with the two tumour types differ slightly (Fig. 3.22). Splenocytes from animals with JBS tumours exhibit a progressive increase in endogenous proliferation that peaks in mice with tumours of 3 to 5 gms. This trend parallels that for total spleen cell numbers and that for T cells (see Figs. 3.11 & 3.12). At all stages there is negligible proliferation in splenocytes which are co-cultured with ISF, though there is a suspicion of some very slight recovery of proliferative function, up to a maximum of 16%, in the presence of ISF with increasing tumour burden. This fits with data on tumour-derived immunosuppressive factors, which suggest that with prolonged exposure cells become slightly less sensitive to the suppressive effect (see Chapter 4). Splenocytes from animals with CarB tumours appear to

have a bi-phasic endogenous proliferative response with peaks in animals with 0-1 and 3-5 gm tumours. This pattern does not correspond with the changes in cell populations described earlier. The early and more rapid rise in endogenous proliferation seen in mice with CarB tumours weighing between 0-1 gms is more marked than in animals with JBS tumours of a similar weight and does correspond with the initial trends in cell numbers.

ConcavalinA (ConA) is a potent non-specific stimulator of T cells and is widely used to determine the proliferative potential of lymphocytes. Here we see that splenocytes from JBS tumour bearing mice, as with endogenous proliferation, have a progressive increase in mitogen induced proliferation which peaks at 3-5 gm tumour burden and then declines (Fig. 3.22). In marked contrast splenocytes from CarB tumour-bearing mice have an impaired ability to respond to ConA (Fig. 3.22). This functional impairment also contrasts with the bi-phasic endogenous proliferation response profile, which suggests that splenocytes from CarB tumour-bearing animals are already maximally, endogenously stimulated and thus incapable of responding to the stimulatory effects of ConA. Splenocyte proliferation in response to ConA, regardless of tumour type, is profoundly impaired by co-culturing with ISF. Again there appears to be some minor recovery of proliferative ability, up to a maximum of 30-35%, in response to ConA stimulation in the splenocytes from mice with larger tumours.

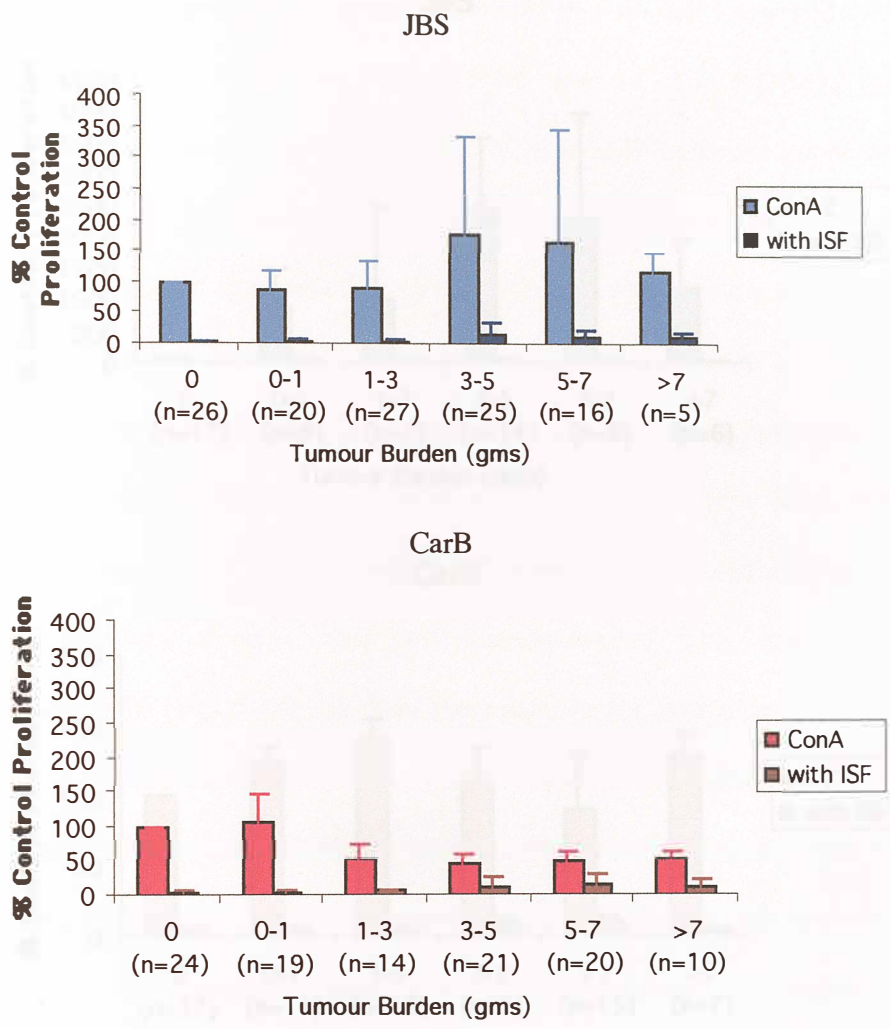


Fig. 3. 23: : Trends in mitogen (ConA) stimulated proliferation of splenocytes, expressed as a percentage of stimulated naïve mouse splenocytes, from JBS or CarB tumour-bearing mice with increasing tumour burden. The effect of co-culture with ISF (inhibition of proliferation) is included for all tumour burden groups. The numbers in brackets represents the number of mice per group.

The effects of exogenous Il-2 stimulation on the proliferative ability of these splenocytes is shown in Fig. 3. 24. What it does show is that again in JBS

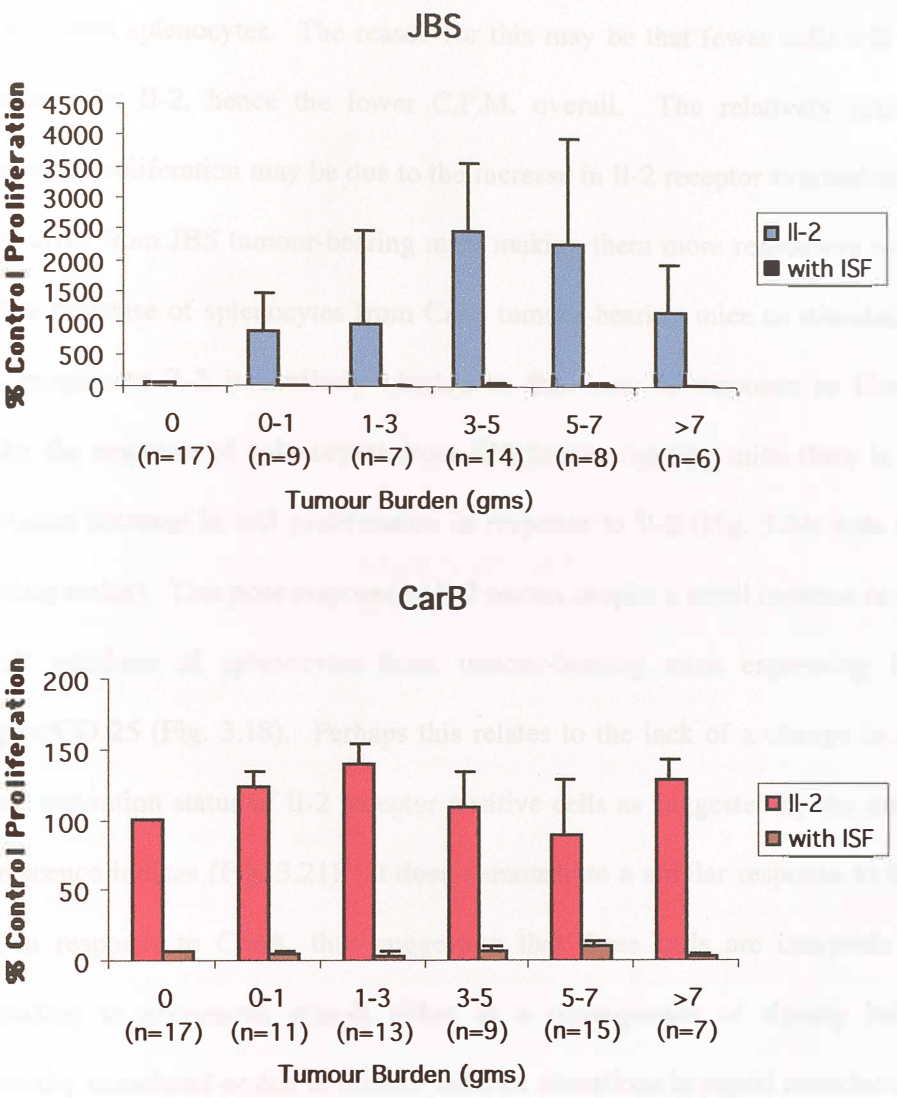


Fig. 3.24: Trends in cytokine (Il-2) stimulated proliferation of splenocytes, expressed as a percentage of stimulated naïve mouse splenocytes, from JBS or CarB tumour-bearing mice with increasing tumour burden. The effect of co-culture with ISF (inhibition of proliferation) is included for all tumour burden groups. The numbers in brackets represents the number of mice per group.

tumour-bearing mice there is a gradual increase in proliferative response up to mice with 3-5 gm tumours. Furthermore the relative increase in proliferation seen in response to IL-2 is significantly greater than that seen in response to

ConA, though the absolute counts per minute (C.P.M.) are four fold higher in ConA treated splenocytes. The reason for this may be that fewer cells will be stimulated by Il-2, hence the lower C.P.M. overall. The relatively greater increase in proliferation may be due to the increase in Il-2 receptor expression of splenocytes from JBS tumour-bearing mice making them more responsive to Il-2. The response of splenocytes from CarB tumour-bearing mice to stimulation with exogenous Il-2 is similarly blunted to that seen in response to ConA. Unlike the response of splenocytes from JBS tumour-bearing mice there is no significant increase in cell proliferation in response to Il-2 (Fig. 3.24: note the differing scales). This poor response to Il-2 occurs despite a small increase in the overall numbers of splenocytes from tumour-bearing mice expressing Il-2 receptor/CD 25 (Fig. 3.18). Perhaps this relates to the lack of a change in the overall activation status of Il-2 receptor positive cells as suggested by the mean fluorescence indices (Fig. 3.21). It does demonstrate a similar response to that seen in response to ConA, thus suggesting that these cells are incapable of responding to exogenous stimuli either as a consequence of already being maximally stimulated or due to tumour induced alterations in signal transduction mechanisms.

3.3.6 Interferon –γ

Interferon-γ is a potent cytokine secreted by both CD4 and CD8 positive lymphocytes. In conjunction with another researcher in our unit looking at susceptibility of tumour-bearing mice to infection with Listeria monocytogenes (C. Gahan) we analysed the production of IFN-γ in JBS tumour-bearing mice. IFN-γ did not reach detectable levels in the sera of tumour-bearing mice at any

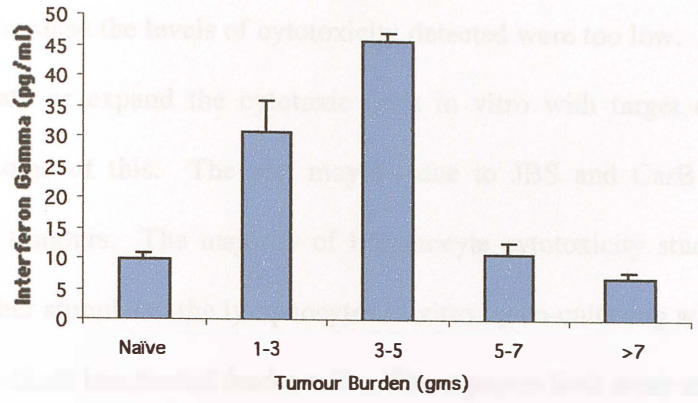


Fig. 3.25: Detection of IFN-γ in the supernatant culture medium of PMA/Ionomycin activated splenocytes taken from mice bearing JBS tumours of differing size.

stage of tumour progression (results not shown). However, a clear and familiar trend in IFN-γ production was detectable in the culture supernatants of PMA/Ionomycin-stimulated splenocytes from JBS tumour-bearing mice (Fig. 3.25). There is a marked increase in the production of IFN-γ with increasing tumour-burden up to a maximum of 5gms and thereafter a decline in its production. This 'bell-shaped' response curve mirrors that for cell numbers and proliferative responses to tumour progression in JBS tumour-bearing mice.

3.3.7 Cell-mediated Cytotoxicity

Cell mediated cytotoxicity was determined using the standard ^{51}Cr release assay described in Chapter 2. It was not possible to generate sufficient effector cells to analyse a range of effector-target ratios from individual mice. To overcome this splenocytes from 3 to 5 mice within the same tumour weight categories were pooled. Therefore each point on the chart represents the result from a group of mice rather than a single mouse. The ideal with ^{51}Cr release cytotoxicity studies is to use the series of effector:target ratios to calculate the number of units of

lytic activity per sample. In the analysis that was performed this was not possible because the levels of cytotoxicity detected were too low. As we did not re-stimulate or expand the cytotoxic cells in vitro with target cells this will explain some of this. The rest may be due to JBS and CarB being poorly antigenic tumours. The majority of lymphocyte cytotoxicity studies published have further stimulated the lymphocytes in vitro by co-culturing with target cells with or without inactivated feeder cells. These papers look more at the inducible

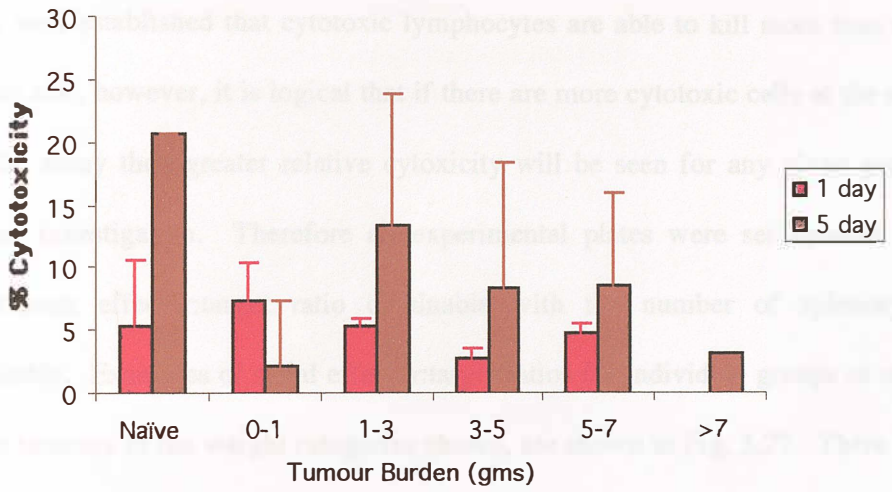


Fig. 3. 26: ^{51}Cr Cytotoxicity of splenocytes from CarB tumour-bearing mice, at increasing tumour burdens, against CarB cells, at an effector:target ratio of 25:1, assayed within 24 hours of harvest and after 5 days.

cytotoxic potential of lymphocytes from tumour-bearing hosts with a view to immunotherapy. For this work we were interested in whether or not the tumour target cells were specifically recognised by host lymphocytes and what, if any, was the inherent level of cytotoxicity. Previous work in our laboratory, using a different cytotoxicity assay, had shown the absence of cytotoxic function in splenocytes from JBS tumour-bearing mice when assayed within 24 hours of harvest (Barrett 1990). However if allowed to recover for 5 days in vitro,

without additional antigen stimulation, then cytotoxic function could be detected. A similar impairment of cytotoxic function was seen in this study, which diminished if the harvested splenocytes were allowed to recover in vitro for 5 days (Fig. 3.26). Whilst one could postulate that this enhanced cytotoxicity is attributable to removal from the tumour-bearing host and thus any suppressive factors that may be in circulation, this cannot be the case as an increased cytotoxicity is also seen in splenocytes from naïve, non-tumour-bearing mice.

It is well established that cytotoxic lymphocytes are able to kill more than one target cell, however, it is logical that if there are more cytotoxic cells at the start of the assay then greater relative cytoxicity will be seen for any given period under investigation. Therefore all experimental plates were set up with the maximum effector:target ratio obtainable with the number of splenocytes available. Examples of serial effector:target ratios for individual groups of mice with tumours in the weight categories shown, are shown in Fig. 3.27. There is a minimum of 5 mice per group. As expected increasing effector:target ratio is associated with enhanced cytotoxicity. This could be taken to imply either a greater number of tumour-antigen specific effector cells or simply the addition of greater numbers of non-specific cytotoxic cells such as NK cells or LAK cells.

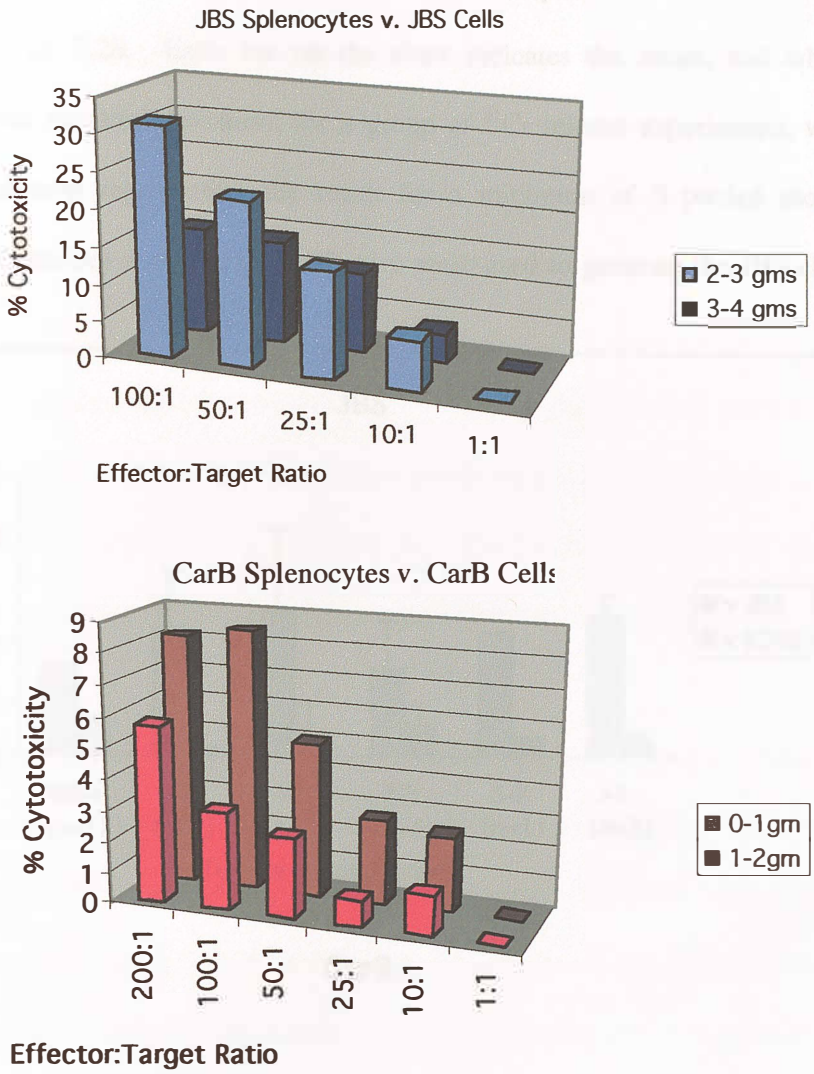


Fig. 3.27: Examples of ^{51}Cr Cytotoxicity, using serial effector:target ratios, of splenocytes from JBS or CarB tumour-bearing mice, with the tumour burdens indicated in the legends against the immunising tumour cell line.

To address this question the cytotoxicity of splenocytes from tumour-bearing mice was assessed not only against the host cell line but also against a control cell line K562. K562 is an erythroblastoid cell line derived from the pleural effusion of a patient with chronic myelogenous leukaemia. It is sensitive to

killing by both NK and LAK cells (Hudson and Hay 1989). The results are shown in Fig. 3.28. Each bar on the chart indicates the mean, and where available the standard deviation, for a group of ^{51}Cr release experiments, with each experiment representing the result for a minimum of 5 pooled mouse spleens. Therefore a minimum of 80 mice were used to generate the JBS chart

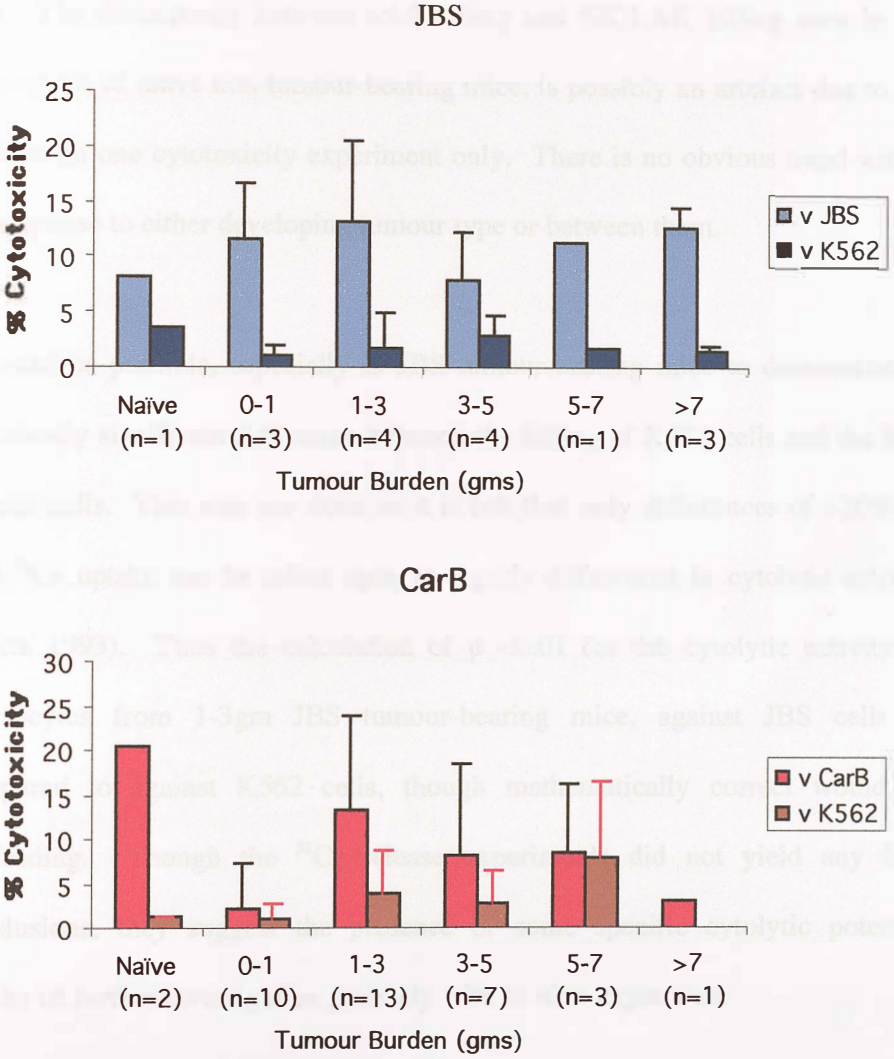


Fig. 3.28: ^{51}Cr Cytotoxicity of splenocytes from JBS or CarB tumour-bearing mice, 5 days after harvest, against the tumour cell line and a control cell line (K562). The results are those for an effector:target ratio of 25:1. Numbers of experiments are included in parentheses.

and 180 the CarB chart. The levels of cytotoxicity seen are low, perhaps due to the decision not to expand the cytotoxic population *in vitro* prior to the assay but also to the low effector:target ratio of 25:1. The charts do demonstrate the existence of some degree of cytotoxic potential against the sensitising tumour cell line. They also suggest that though some of this cytotoxicity may be due NK and LAK cells, their presence does not explain all of the tumouricidal activity seen. The discrepancy between total killing and NK/LAK killing seen in the splenocytes of naïve non-tumour-bearing mice, is possibly an artefact due to the reliance on one cytotoxicity experiment only. There is no obvious trend within the response to either developing tumour type or between them.

It would be possible, especially in JBS tumour-bearing mice to demonstrate a statistically significant difference between the killing of K562 cells and the host tumour cells. This was not done as it is felt that only differences of >20% of total ^{51}Cr uptake can be relied upon to signify differences in cytolytic activity (Martz 1993). Thus the calculation of $p < 0.01$ for the cytolytic activity of splenocytes from 1-3gm JBS tumour-bearing mice, against JBS cells as compared to against K562 cells, though mathematically correct would be misleading. Though the ^{51}Cr release experiments did not yield any firm conclusions, they suggest the presence of some specific cytolytic potential worthy of further investigation probably with *in vitro* expansion.

3.4 Discussion

In this chapter evidence has been presented of bi-directional tumour host interaction in the form of increasing splenomegaly and spleen cell numbers with progressive tumour burden. There are also tumour-induced alterations in the subsets of lymphocytes in these developing spleens. The increasing tumour burden is associated with changes in the proliferative ability of splenocytes and with a suggestion of acquisition of target cell specific cytolytic activity. Perhaps the most significant finding is the differing response profiles to the two histologically distinct tumours, JBS and CarB.

The data presented in this chapter add to the large and growing body of evidence of immune reactivity against tumours. The evidence for the existence of tumour antigens that could be identified by the immune system and the immune effector cells capable of responding to them has been dealt with extensively in chapter 1. In the introduction to this chapter, I alluded to the circumstantial clinical evidence of effective immune responses to malignant disease, which I will expand on here. There is good evidence that immune suppression increases the incidence of come cancers (Table 3.6). As these cancers are not typically encountered in immuno-competant people, many researchers

Table 3.6: Incidence and increase in cancers in immunosuppresed patients (Penn, 1988).

	Increase	Proportion of Cancer (%)
Kaposi sarcoma	X 400	5.7
Lymphoma	X 90	13
Vulva/perineum	X 100	3
Skin	X 29	38
Cervix	X 14	16
Liver	X 30	10
Breast	?	10
Prostate	?	2

have been sceptical of their significance. However the relatively lower incidence in immunocompetant people could imply that effective immune surveillance has been operational in these people. Penn also reported that more aggressive pharmaceutical immunosuppression was associated with a greater likelihood and more rapid onset of cancer (Table 3.7). Most patients on immunosuppressive drugs will have been encouraged to stop smoking thus reducing their risk of lung cancer. Renal transplant recipients, the single largest group receiving immunosuppressant therapy will have had periods of strict protein restriction while on dialysis awaiting transplantation. As saturated animal fats are a known risk factor for the development of breast and colon cancer, this dietary restriction may explain the lack of an increased risk of these more common cancers. Experimental data has shown that an immune response may promote growth of breast carcinoma and that immune suppression with a resultant decrease in $CD4^+$ and $CD4^+/CD8^+$ ratio inhibits tumour promotion in mice (Stewart, Tsai et al. 1995). Furthermore, Stewart showed that, the incidence of breast cancer is lower in chronically immunosuppressed women than that expected for the general female population. The incidence of breast cancer in women receiving immunosuppressant therapy following cardiac and renal transplant is statistically significantly less than that expected for the normal female population (33.2 per 10,100 over 1-11 year follow-up versus 44 per 10,000, p=0.009). For all other major cancers the incidence was higher in

Table 3.7: Development of lymphoma after transplantation (Penn, 1988).

Immunosuppressant	Months to onset	Proportion of patients with cancer
Azathioprine	48	11
+ cyclosporin	15	23
+ OKT3	7	64

immunosuppressed women. Cervical carcinoma or cervical intraepithelial neoplasia (CIN) has been reported to occur in 16 – 21% of immunosuppressed patients. Given its prevalence in the normal population, its association with the human papillomavirus and the potential for cure if detected early enough it has been suggested as a control for the quality of follow-up and screening of patients post transplant (Jonas, Rayes et al. 1997).

There is also a suggestion that tumours arising in immunosuppressed patients are at a more advanced stage than similar tumours in immunocompetant people although not necessarily less differentiated (Penn 1988). Tumours arising in immunosuppressed individuals have less frequent loss of HLA class I and II antigens. Testicular carcinoma presents at a more advanced stage, both pathologically and clinically, in HIV infected individuals than in normal people but the cure rate is not affected.

The documentation of cases of 'spontaneous regression' of malignant disease gives further circumstantial evidence of an effective immune response against malignant disease. Spontaneous regression has been reported for most types of tumours; however, lymphoma, melanoma and renal carcinoma predominate (Table 3.8). Though anecdotally

Table 3.8: Reported cases of spontaneous regression of cancer from 1900-1987

	No. of Cases
Leukaemia/lymphoma	121
Melanoma	69
Renal cell cancer	68
Neuroblastoma	41
GI cancer	34
Retinoblastoma	33
Lung & bronchus	25
Breast	22
Testis	16
Others	75
Total	504

unexplained cases of spontaneous regression appear rare the more closely it is examined the more frequent it seems. Possinger, in 1988, reported spontaneous regression occurring in fewer than 1 in 500 patients with renal carcinoma. When Oliver, in 1989, looked at this is greater detail with monthly x-rays in patients with kidney cancer not receiving treatment they reported regression in more than 1 in 20 patients. While these cases represent a minute proportion of the total number of malignancies detected annually and lack solid objective scientific proof of effective immune orchestrated elimination of the tumours they do support the possibility of anti-tumour immune function.

Further support comes from the research into tumour infiltrating lymphocytes and the prognostic significance of tumour infiltrates discussed in detail in Chapter 1. Those conclusions are corroborated by investigations into the phenomenon of "concomitant immunity". Concomitant immunity is the paradoxical ability of a host with a progressively growing tumour to reject, in an antigen specific manner, an inoculation of the same tumour cells at a site distant to the existing tumour. Originally described by Paul Ehrlich in 1906, it has been demonstrated in a wide variety of animal models and in at least one human study (Ehrlich 1906). With 'informed consent', Southem *et al.* injected, patients with advanced disseminated cancer, subcutaneously $< 10^7$ of the patient's own tumour cells and found that some patients with widely disseminated cancers were able to reject such autotransplants (Southem 1966). Some patients followed for up to 18 months did not develop tumours at the inoculation site. He also injected autologous cancer cells admixed with autologous lymphocytes and found that in patients in whom the development of a tumour at the inoculation site was

inhibited by the addition of lymphocytes, as compared with a control in the same patient, 9/20 (45%) had widely disseminated disease. This compares favourably with 18/22 (82%) of patients whose lymphocytes did not prevent the development of tumours having widely disseminated disease. Thus patients with widely disseminated disease were less likely to be able to inhibit tumour development at the inoculation site. Whether this apparent effect of autologous lymphocytes is evidence of immune containment or a consequence of less advanced disease was not addressed. The continued growth of the primary tumour despite the display of concomitant immunity has lead to the phenomenon being largely ignored, however, failure to generate concomitant immunity has been associated with shorter survival times due to more rapid dissemination and growth of tumour metastases (Milas, Hunter et al. 1974). This supports the concept of immune containment.

Crude suggestive evidence of immune containment has also been found in the data presented here. Note the difference in tumour growth rates, based on volume, seen when tumours are grown in immunocompetant Balb/C mice compared to those grown in athymic nude mice (Figs. 3.7 versus 3.8). The marked difference between the growth rates, for the two different tumours, seen in nude mice has disappeared in the immunocompetant mice. If the growth curves are corrected back to begin when the tumours are first palpable it can be seen that this is due almost solely to a change in the growth kinetics of JBS tumours in Balb/C mice (Fig. 3.29).

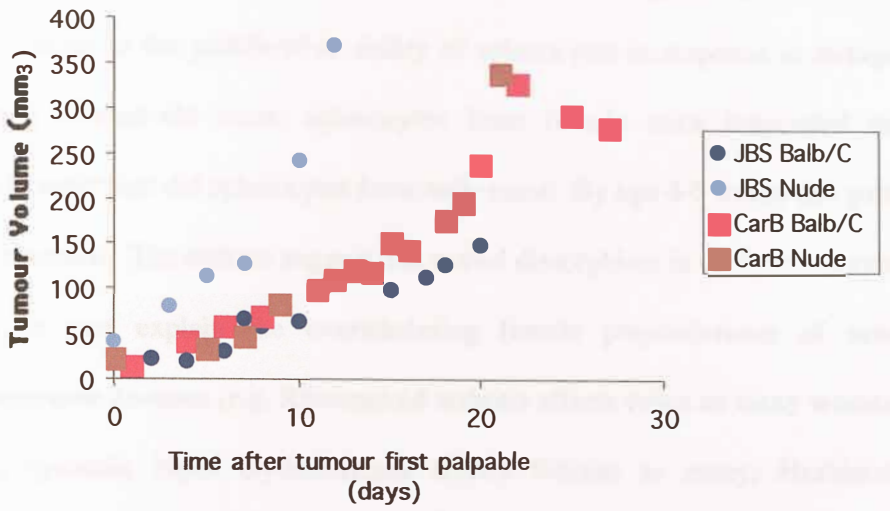


Fig. 3.29: Volume growth curves for JBS and CarB tumours grown in Balb/C and athymic nude mice corrected back to day when tumours were first palpable. (Error bars have been excluded to avoid clutter, but can be seen in Figs. 3.7 & 3.8)

That this growth confinement is immune-mediated is suggested by the changes seen in spleen weight and non-adherent spleen cell numbers with increasing tumour burden (Figs. 3.10 & 3.11). Despite the lack of any evidence of immune containment of CarB tumours there is a suggestion, though not statistically significant, of greater tumour-induced splenomegaly in CarB tumour-bearing mice. This increase in spleen weight is associated with an increase in spleen cell numbers. The changes in spleen weight and cell number are not attributable to normal physiological changes associated with age. Mice have been shown to undergo significant increases in splenic T-cells as evidenced by flow cytometric analysis of splenocytes from mice aged 3 – 9 weeks with markers for CD3, CD4 and CD8 (Rosen, Tran et al. 1999). They also reported a decrease in spleen mass between the ages of 6 and 8 weeks. All of these changes were related to puberty,

which in mice occurs before 8 – 9 weeks. Interestingly they found sexual dimorphism in the proliferative ability of splenocytes in response to mitogens. Among 3-week-old mice, splenocytes from female mice responded more significantly that did splenocytes from male mice. By age 4-6 weeks this pattern was reversed. The authors suggest that sexual dimorphism in the immune system may in part explain the overwhelming female preponderance of several autoimmune diseases (e.g. Rheumatoid arthritis affects twice as many women as men; systemic lupus erythematosus affects 9-times as many; Hashimoto's thyroiditis affects 40-times as many women as men). It is not likely that this sexual difference would have the same significance in tumour immunology, or, at least in the setting of the work presented herein, sufficient confounding potential, to warrant restricting my research to a single gender. Hence this work was carried out using post-pubertal mice of either gender. In the relatively longer term (given a mouse's life expectancy), over two years there is evidence of an age-related decline in immune function and proliferative ability (Kariv, Ferguson et al. 1992; Hsiung, Wang et al. 1994). However, for the period of time that the mice were involved in the study (8–16 weeks of age) the spleen weight, spleen cell numbers and proliferative ability were all constant (Hsiung, Wang et al. 1994; Watanabe, Iwatani et al. 1995).

While Table 3.5 gives the absolute numbers, Figs 3.11-3.13 & 3.15-3.16 show the trends in non-adherent cell number, T-cell, B-cell, helper T-cell and cytotoxic T-cell with increasing tumour burden for both tumour types. In JBS tumour-bearing mice there is a general trend towards increased cell numbers in all groups with increasing tumour burden with a decline in $CD3^+$ and $CD4^+$ cell numbers in

mice with the largest tumour burden. CarB tumour-bearing mice while demonstrating a similar progressive trend in overall non-adherent splenocyte cell and B-cell numbers does not have the same increase in $CD3^+$, -4^+ or -8^+ cell numbers until they reach the largest tumour burden category, a time when JBS numbers are beginning to decline. This difference is reflected in Fig. 3.14, which shows that both tumours are associated with a reversal of T:B cell ratio that is more pronounced in CarB tumour-bearing mice. Fig. 3.17 shows the remarkable stability of the CD4:CD8 ratio in JBS tumour-bearing mice in stark contrast to the almost 4-fold increase seen in CarB mice (due more to a greater loss of $CD8^+$ cells than $CD4^+$ cells).

In Chapter 1 the essential contribution of $CD4^+$ T-cells to an effective immune response to malignant disease was discussed in some detail. $CD4^+$ T-cells have been shown to be necessary during the priming and effector stages of the anti-tumour immune response (Hung, Hayashi et al. 1998). Some researchers would suggest that $CD4^+$ T-cells are even more vital than $CD8^+$ T-cells as optimal induction of the latter requires the former (Baxevanis, Voutsas et al. 2000). This is supported by resistance to the development of tumours in a significant number of CD8 knockout mice but not in CD4 knockout mice (Toes, Ossendorp et al. 1999). This may be due to the induction of cytotoxic macrophages or eosinophils or direct cytotoxicity of $CD4^+$ T-cells, as discussed in Chapter 1.

Changes in lymphocyte subsets have been reported in patients with malignant disease, have been shown to correlate with stage of disease and proposed as a marker of response to therapy. Kuroi *et al.* looked at lymphocyte subsets in

patients with Gastric cancer and found that increasing tumour stage was associated with a decrease in $CD3^+$ and $CD4^+$ T-cells and a decrease in CD4:CD8 ratio (Kuroi, Sato et al. 1990). $CD8^+$ T-cell numbers remained relatively constant, so most of the reduction in $CD3^+$ T-cell number was due to a fall in $CD4^+$ T-cell. The greatest reduction in the latter population was in cells co-expressing CD4 and CD45RA – a marker for naïve T-cells (Male, Champion et al. 1992). They concluded that the reduction in $CD4^+/CD45RA^+$ cells in peripheral blood was due to a relative accumulation in the spleen where they were exerting a suppressive effect. An alternative theory is that they have become activated, trafficked to the tumour site and undergone activation induced cell death. This finding of a reduction in $CD4^+$ T-cells and in CD4:CD8 ratio in patients with increasing stage of gastric cancer has been confirmed by other groups (Lee, Chang et al. 1994). These researchers found a correlation between the reduction in CD4:CD8 ratio and tumour size, invasion and lymph node metastasis but not with histological type. A reduction in $CD4^+$ T-cell number and CD4:CD8 ratio has also been described in patients with invasive transitional cell carcinoma (TCC) of the bladder (Kaver, Pecht et al. 1992). This reduction was not seen in patients with superficial TCC of the bladder. They reported that male patients with invasive TCC were more likely than female patients to have a reduction in $CD4^+$ T-cell numbers, thus possibly explaining the more favourable prognosis in the latter. Not all tumours have a reduction in $CD4^+$ T-cell numbers as demonstrated by Kaver *et al.* who reported a reduction in $CD8^+$ T-cell numbers and a consequent rise in CD4:CD8 ratio. Still others have reported a decrease in $CD4^+$ T-cells and a simultaneous increase in $CD8^+$ T-cells leading to a marked reduction in CD4:CD8 ratio (Zeng, Tang et al. 1995). This group

investigated lymphocyte subsets in patients with surgically verified unresectable hepatocellular carcinoma (HCC) who received radiotherapy and ensuing resection. They found that those patients who became suitable for subsequent resection had less of a reduction in CD4:CD8 ratio before and a greater recovery of CD4:CD8 ratio following radiotherapy. Similarly patients whose HCC had a fibrous capsule displayed less of a reduction in CD4:CD8 ratio and were more likely to undergo later resection. They propose that determination of lymphocyte subset numbers and in particular the CD4:CD8 ratio may be useful as a prognostic tool.

There is a dilemma associated with investigating lymphocyte subsets in humans with malignant disease, the work of Southem, discussed above, probably would not receive ethical approval today. In humans it is possible to determine lymphocyte subset numbers in patients at the time of presentation and following therapy, but ascertaining the natural history is not as easy. In particular, establishing whether the alteration in lymphocyte populations is a cause or a consequence of the developing tumour. This dilemma is best answered using carefully chosen animal models. Certainly

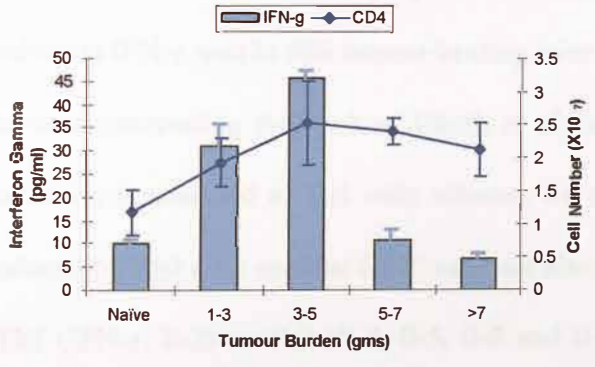


Fig. 3.30: Trends in $CD4^+$ cell number and Interferon-γ production by PMA/Ionomycin stimulated non-adherent splenocytes in JBS tumour-bearing mice with increasing tumour burdens

similar tumour induced changes in T-cell populations, reduction in $CD4^+$ and an increase in $CD8^+$ T-cells, have been seen in murine models (Walker, Yurochko et al. 1992). This group compared splenocytes from naïve mice and mice 21 days post tumour induction, a time at which they say coincided with maximal tumour-induced immune suppression, but did not show the effect of progressive tumour growth. Others have demonstrated temporal changes in lymphocyte populations following tumour induction. Ghosh *et al.* have demonstrated changes in cytokine production pattern with progressive tumour growth of either renal cell carcinoma or MCA-38 colon carcinoma (Ghosh, Komschlies et al. 1995). They reported that anti-CD3 antibody stimulated splenocytes from tumour-bearing mice produced less IFN-γ and Il-2 but more Il-4 than splenocytes from naïve mice. Furthermore using nuclear extracts from splenocyte preparations and from isolated $CD4^+$ splenocytes they found a transient increase in DNA-binding pattern corresponding to the IFN-γ core promoter region followed by a gradual disappearance as the tumour progressed. This corresponds well with the production IFN-γ seen in JBS tumour-bearing mice (Fig. 3.25). The only caution I have in interpreting the work of Ghosh *et al.* is their assumption that IFN-γ production is confined to Th1 cells whereas we now know that IFN-γ is also produced by Th0 cells and that $CD8^+$ cells are also being shown to exhibit either a Th1 (IFN-γ, Il-2) or Th2 (Il-4, Il-5, Il-6 and Il-10) type pattern of cytokine production (Kemeny, Noble et al. 1994; Openshaw, Murphy et al. 1995). A similar trend in Il-2 production, to that seen for IFN-γ production, was demonstrated in mice bearing a syngeneic tumour - CSA1M (Zou, Shimizu et al. 1992). Endogenous spleen cell production of Il-2 initially increased following tumour induction but thereafter gradually decreased with progressive tumour

growth. This reduction in IL-2 production could be recreated by the addition of tumour cell culture supernatant or ascitic fluid and restored by the addition of anti-TGF-β antibody. In the work presented here we have looked at the production of IFN-γ and at CD4 expression and found a good correlation in JBS tumour-bearing mice (Fig. 3.30). This suggests that the change in $CD4^+$ T-cell numbers is associated with an initial shift towards and then away from a Th1 pattern of cytokine secretion.

The relevance of the marked B cell (CD40) hyperplasia has not been examined in great detail as it was beyond the scope of this work. Both tumour types induce a significant B-cell response (Figs 3.13 & 3.14). Tumour immunity is largely mediated by $CD8^+$ CTLs whose activation requires $CD4^+$ T-cell help. Because most tumours do not express MHC class II, $CD4^+$ T-cells require priming by MHC class II expressing antigen-presenting cells (APC). These APCs can be dendritic cells, macrophages or B-cells. Tumour cell antigens because they are complex and occur in small amounts are suitable for B-cell uptake and presentation. The importance of B-cells to the anti-tumour immune response was demonstrated by Qin *et al.* who, using a B-cell deficient hybrid of Balb/C, reported resistance to tumour development in the hybrid mice but not in the B-cell competent mice (Qin, Richter et al. 1998). By reconstituting the hybrid mice with spleen cells or B-cells they became vulnerable to tumour development. Injection of the hybrid mice with serum from naïve or immunised mice did not facilitate tumour development. These experiments suggest that B-cells themselves and not their product – antibodies – are necessary if the hybrid mice are to become susceptible to tumour development. They also found that $CD4^+$ T-

cells were necessary for the immunisation phase but not for the challenge phase. They postulate that the effect of B-cells in promoting tumour development may be through competition with other APCs for antigen to present. Experienced T-cells respond to antigen presented by B-cells, whereas virgin T-cells are rendered tolerant (Fuchs and Matzinger 1992). An alternative possibility may be that B-cells may drive the $CD4^+$ T-cell response towards a Th2 response, which promotes the development of a humoral rather than a cellular T-cell response (Paul and Seder 1994; Sotomayor, Borello et al. 1996).

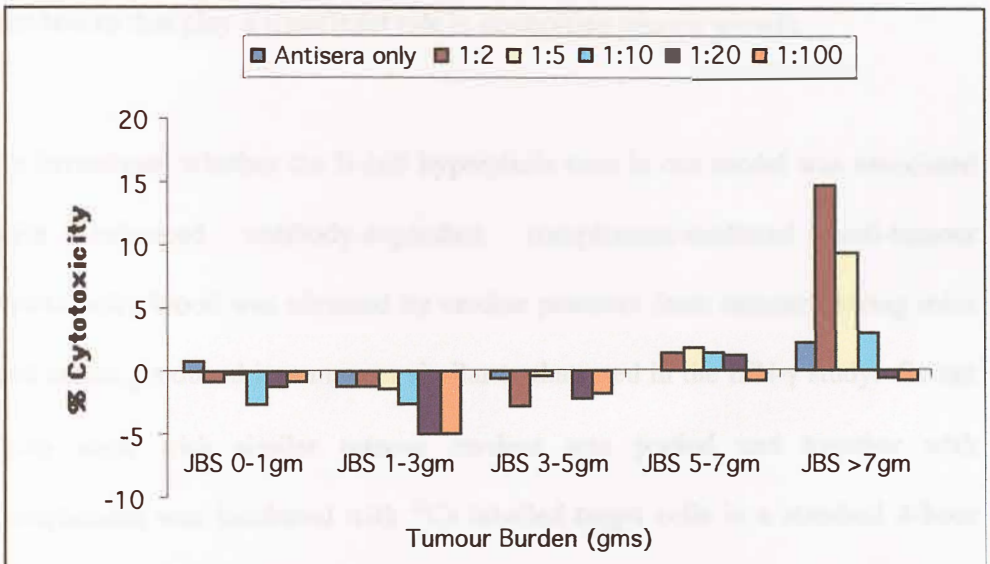
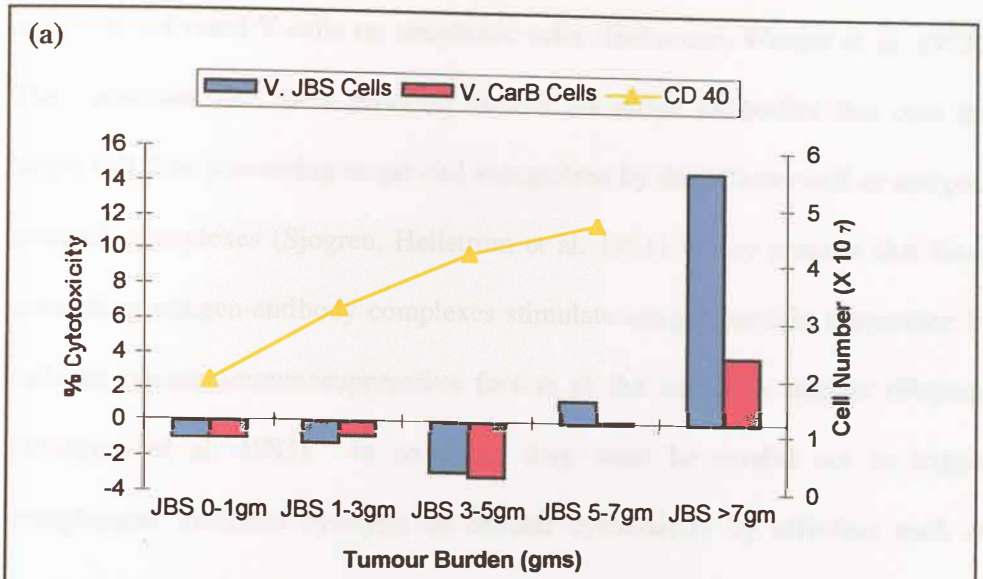


Fig. 3.31: Complement mediated cytoxicity of serum (using serial dilutions shown) from JBS Tumour-bearing mice against JBS target cells in a ^{51}Cr release assay

Apart from their interaction with the activity of T-cells, B-cells have an end function of their own, the production of antibodies. A detailed discussion of the humoral arm of the immune system is clearly beyond the scope of this treatise, but does require some elaboration. The existence of antibodies capable of recognising tumour antigens has been discussed in Chapter 1, a fact utilised in

one of the most successful approaches to the identification of tumour antigens. It is known that these antibodies may help or hinder the growth of a tumour. Antibody responses may be important in generating antibody-dependant cellular cytotoxicity (ADCC) or antibody-dependant complement mediated tumour cell lysis (Vile, Chong et al. 1996). The varieties of tumours in which antibodies have been found have been discussed already. Another group in whom tumour cell specific antibodies have been identified are patients treated with cancer vaccines that have been shown to have some clinical efficacy such as in melanoma (Mittleman, Chen et al. 1994). There is however a paucity of antibodies that play a significant role in controlling tumour growth.

To investigate whether the B-cell hyperplasia seen in our model was associated with enhanced antibody-dependant complement-mediated anti-tumour cytotoxicity blood was obtained by cardiac puncture from tumour-bearing mice and serum produced in a manner similar to that used in the IFN-γ study. Serum from mice with similar tumour burdens was pooled and together with complement was incubated with ^{51}Cr labelled target cells in a standard 4-hour release assay described before (Fig. 3.31). Cytotoxicity is only seen in the serum of mice with greater than a 7 gm JBS tumour burden. The negative cytotoxicity is a result of subtraction of the cytotoxic effect of complement alone in the absence of serum. The specificity of this cytotoxicity, where serum was available for testing, is shown in Fig. 3.32. Killing of K562 cells was not evident in sera from either JBS or CarB tumour-bearing mice (data not shown).



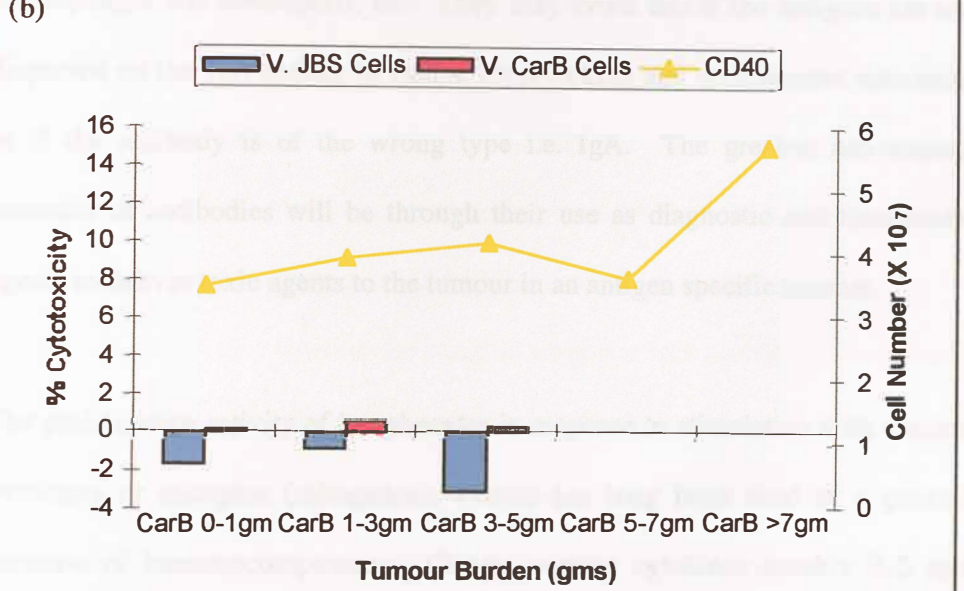


Fig. 3.32: Antibody-dependant complement-mediated cytotoxicity of 1:2 dilution of serum from JBS (a) and CarB (b) tumour-bearing mice against ^{51}Cr labelled target and control cells, also showing trends in CD40 (B-cell) numbers with progressive tumour burden

Antibodies have also been implicated in aiding tumour growth by facilitating immune evasion. The Hellstroms and their co-workers have placed great emphasis on the ability of 'blocking factors' in the host serum, which prevent the

action of activated T-cells on neoplastic cells (Hellstrom, Warner et al. 1973). They postulate that these blocking factors are either antibodies that coat the target cell thus preventing target cell recognition by the effector cell or antigen-antibody complexes (Sjogren, Hellstrom et al. 1971). They propose that these circulating antigen-antibody complexes stimulate antigen-specific suppressor T-cells to release immunosuppressive factors at the site of a tumour (Nepom, Hellstrom et al. 1983). In so doing they must be careful not to trigger complement mediated cytolysis or cellular cytotoxicity by effectors such as macrophages and neutrophils, etc. They may avoid this if the antigens are too dispersed on the cell surface to enable cross-linking and complement activation or if the antibody is of the wrong type i.e. IgA. The greatest anti-tumour potential of antibodies will be through their use as diagnostic and therapeutic agents to deliver toxic agents to the tumour in an antigen specific manner.

The proliferative activity of lymphocytes in response to stimulation with various cytokines or mitogens (mitogenesis assays) has long been used as a general measure of immunocompetance. Clearly various cytokines notably Il-2 and TGF-β have been used. Polyclonal activators, though less specific are cheaper, are widely used. The most commonly used, and the ones used in this work, being Concavalin A (ConA) and Phytohemagglutinin (PHA), which are all largely T-cell activators; Lipopolysaccharide (LPS) and Pokeweed mitogen (PWM) act largely on B-cells and Phorbol esters (PMA) with Ionomycin which act on both T and B-cells. This specificity is blurred as T-cell activators will induce the T-cells to secrete cytokines that in turn activate B-cells and vice versa.

As with much of the other work described so far, people who have looked at proliferative activity in animals have tended to compare tumour-bearing with non tumour-bearing and in humans to compare TILs with PBLs. Adler *et al.* for instance, found enhanced endogenous proliferation (as determined by [^{3}H] Thymidine incorporation) in the splenocytes of sarcoma-bearing mice when compared with controls (Adler, Takiguchi et al. 1971). There was variability in the extent of proliferation depending on the particular subtype of sarcoma the animal was afflicted with. All tumours were associated with a profound blunting of their splenocyte proliferative response to stimulation with PHA as compared with controls. This response appears to be similar to that observed in CarB tumour-bearing mice but in marked distinction to JBS tumour-bearing mice (Figs. 3.22 & 3.23). However, as the mice in Adler's work had "greater than 1cm diameter" tumour burden when sacrificed we do not know the homogeneity of tumour burden and whether the trend in response was upward or downward. Similar depression of lymphocyte proliferation has been documented in humans with malignant disease. Miescher *et al.* examined the proliferative ability of TILs and PBLs from humans with cancers of varying histologies – melanoma, breast, colon, oesophageal, lung, glioma and skin (Miescher, Whiteside et al. 1986). They found that in all cases (bar 1 of 22) the endogenous proliferative activity of TILs and PBLs was reduced compared with controls and in all but 2 there was little or no response of TILs to PHA, ConA or PMA when compared with autologous controls (PBLs). Interestingly the histology of the tumour did not influence the proliferative response. This conflicts with my data, which clearly shows a different pattern of response for the two distinct tumours (Fig. 3.23). It may be that the tumours here are either very early (unlikely) or very late

tumours, times at which the difference in responses are less marked (again unlikely). This does show the merit of, and need for, establishing the natural history of lymphocyte responsiveness with progressive tumour growth.

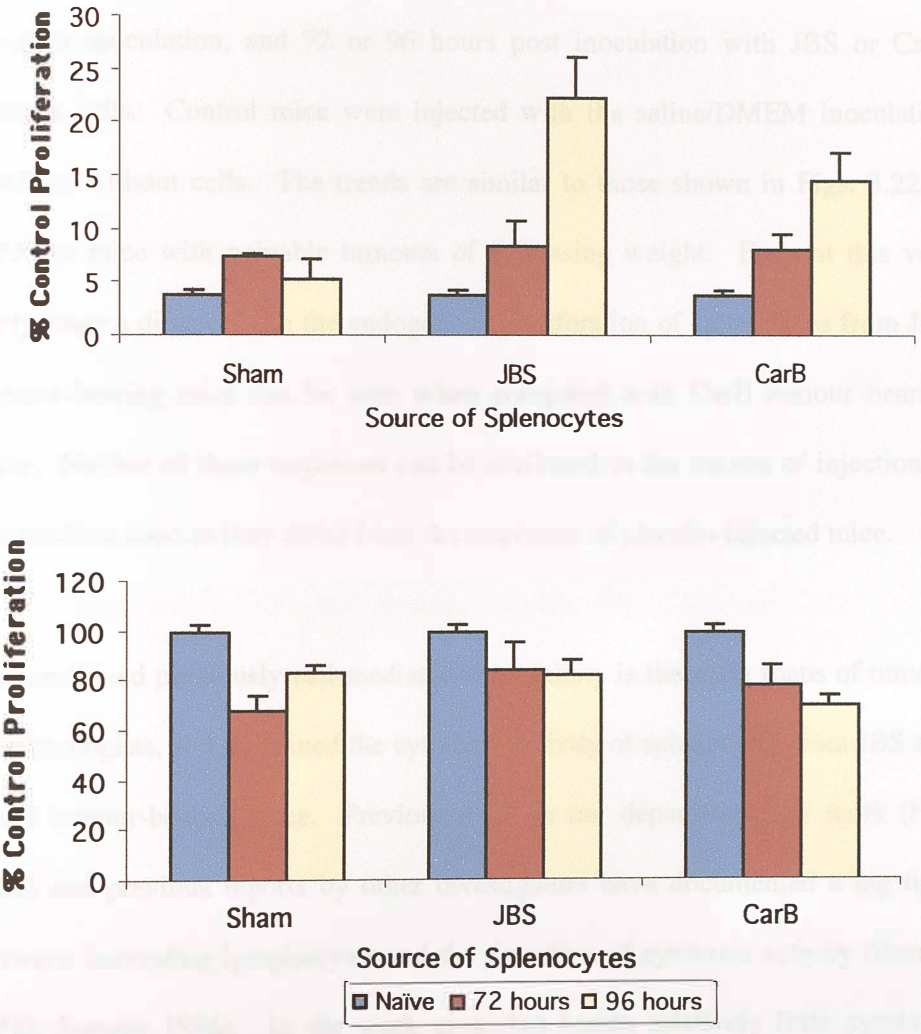


Fig. 3.33: Endogenous (top) and ConA stimulated (bottom) proliferation (expressed as a percentage of activated naïve splenocytes) of splenocytes from naïve Balb/C mice and mice 72 or 96 hours post injection with medium only (Sham), JBS cells or CarB cells at tumourigenic doses.

An essential aspect of this natural history is knowledge of what happens to lymphocyte function prior to a tumour becoming palpable or clinically manifest. Some preliminary work in this area was undertaken and is shown in Fig. 3.33. These experiments examined the proliferative ability, both endogenous and potential when activated by ConA, of splenocytes from three groups of mice, prior to inoculation, and 72 or 96 hours post inoculation with JBS or CarB tumour cells. Control mice were injected with the saline/DMEM inoculation medium without cells. The trends are similar to those shown in Figs. 3.22 & 3.33 for mice with palpable tumours of increasing weight. Even at this very early stage a difference in the endogenous proliferation of splenocytes from JBS tumour-bearing mice can be seen when compared with CarB tumour bearing mice. Neither of these responses can be attributed to the trauma of injection or the medium used as they differ from the responses of placebo-injected mice.

As mentioned previously cell-mediated cytotoxicity is the main focus of tumour immunologists, so I examined the cytotoxic activity of splenocytes from JBS and CarB tumour-bearing mice. Previous work in our department, my work (Fig. 3.26) and previous reports by other investigators have documented a lag time between harvesting lymphocytes and the detection of cytotoxic activity (Barrett 1990; Somers 1996). In the work presented herein relatively little cytotoxic activity was detected (max. was 30% for splenocytes from 2-3gm JBS tumour-bearing mice against JBS cells; Fig. 3.27). Most researchers in this field utilise a period of in vitro "expansion" which often involves re-stimulation with tumour cells and supplementation with Il-2 (Fujiwara, Hamaoka et al. 1977; Mukherji,

Wilhelm et al. 1986; Miescher, Stoeck et al. 1988). This is because most researchers are interested in the cytolytic potential of these cells from an immunotherapeutic point of view, whereas I wished to establish the endogenous level of cytotoxicity and it's trend in response to increasing tumour burden. Previous workers have endeavoured to ascertain the effect of tumour burden on cytotoxic activity (Loeffler, Smyth et al. 1992). They reported an increase in cytotoxic activity in splenocytes (after *in vitro* expansion) from early tumour-bearing mice (tumours for < 21 days) as compared to naïve mice with a subsequent fall in cytotoxicity, to below that of naïve mice, in late tumour-bearing mice (tumours for >30 days).

There was no previous data for comparison with my results for Il-2 receptor expression and mean fluorescence indices so as such this data stands on its own (Figs. 3.18 – 3.20). The upward trend in absolute cell numbers and mean fluorescence indices for cells doubly staining with CD8 and CD25 is not reflected in any increased cytotoxicity *in vitro* (Fig. 3.28). This may be due to immunosuppressive factors elaborated by the developing tumour (discussed in detail in chapter 4).

3.5 Conclusion

The work presented in this chapter was undertaken to evaluate the immune response, in terms of lymphocyte phenotype and reactivity, to two, histologically distinct, developing tumours. Though not exhaustive, and largely confined to T-cell activities, the results presented herein document the changes that occur in splenocyte morphology, endogenous and stimulated proliferative ability and

endogenous cytotoxicity in response to the development of two histologically and aetiologically distinct tumours. This work establishes the background against which work in the ensuing sections takes place, by providing a context in which to search for tumour-derived immunosuppressive factors and defining the nature of the immune response to overcome by oral tolerance. This is, to the author's knowledge, the first detailed analysis of the immune response to progressive tumour burden, using accurate measurement of that tumour burden, in tumour-bearing animals and is also the first direct comparison of the immune responses to two distinct tumours in the same animal model. As such the documentation of these contrasting response profiles to the two tumours represents a worthy contribution to our understanding of the natural history of malignant disease albeit in a mouse model.

Chapter 4

Tumour Derived Soluble Immunosuppressive Factors

4.1 Introduction

The development of a malignant growth is the consequence of either enhanced/uncontrolled cell division and/or reduced cell death. In chapter 3, evidence was presented of changes in immune parameters of splenocyte number, lymphocyte subsets, lymphocyte proliferation and cytotoxic functions concurrent with increasing tumour burden. In chapter 6 evidence of immune recognition of JBS and CarB cells, in the form of a marked DTH response, following prior immunisation will be presented. Given that JBS and CarB express antigens that the immune system is capable of recignising and that in the early stages of tumour growth the host lymphocytes have enhanced proliferative and cytotoxic functions how this neoplastic growth can survive and grow progressively in the face of this specific and potentially tumouricidal immune response is the central paradox of tumour immunology. Some mechanisms by which developing tumours may evade detection or elimination such as altered immunogenecity, immune blockade or a suppressive microenvironment have already been discussed in the introductory chapter. In Chapter 1, I alluded to the existence of soluble immunosuppressive factors elaborated by, or induced by, tumours; these will be discussed further here.

A large and diverse number of substances, derived from human or animal tumours, capable of inhibiting lymphocyte function have been reported in the literature over the past few decades. The majority of these factors have only been partially characterised at best, but in increasing detail, particularly with the advent of newer technology. Some of those that have been completely identified are detailed in Tables 4.1 & 4.2. These tables demonstrate that ISFs have been

found in many locations (surface, culture supernatant, serum and tissue mRNA) and isolated from a variety of histological tumour types. Furthermore these substances have been shown to correlate with immune suppression in vivo and with in vitro immunosuppressive effects (see text).

4.1.1 Tumour Derived ISFs – Surface Origin

4.1.1.1 CD95L

Soluble Fas ligand (sFasL) has been detected in the sera of 18 of 35 patients with malignant melanoma and also in patients with lymphoma or leukaemia (Hahne, Rimoldi et al. 1996; Tanaka, Suda et al. 1996). Tanaka also reported finding sFasL in the effusion of a patient with lymphoma who had pleural invasion. Soluble FasL is a 27kD glycoprotein, generated by a matrix-metalloproteinase-like protease cleavage from the 40kD membrane bound FasL (Muschen, Moers et al. 2000). Soluble FasL has been shown to induce apoptosis in Fas positive human cells but not murine cells (Nagata 1997). The induction of apoptosis by soluble FasL is not as efficient as that induced by membrane bound FasL. In patients with breast cancer Muschen *et al.* reported that FasL expression by the tumour correlated well with peripheral blood depletion of $CD4^+$ and $CD8^+$ lymphocytes (Muschen, Moers et al. 2000). They also demonstrated that supernatants from cultured breast cancer cells induced apoptosis in Jurkat T-cells via the Fas pathway.

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Table 4.1: Examples of tumour derived immunosuppressive factors.

Tumour Derived ISF	Tissue of Origin	Location	Findings	Reference
Surface				
CD95L	Breast	Surface/ Soluble	Decreased $CD4^+$ and $CD8^+$ T-cells correlate with CD95L. Induction of Apoptosis on Jurkat T cells	Muschen M. *et al.*, Immunology. 99(1): 69-77, 2000
	Multiple Myeloma	Surface	Killing of T-ALL cells	Villunger A. *et al.*, Blood. 90(1):12-20, 1997
	Astrocytoma	Surface	Killing of T cells	Saas P. *et al.*, J. Clin. Invest. 99(6):1173-8, 1997
Gangliosides	Erythro-leukaemia	Soluble	97% reduction in the generation of CTLs 72% reduction in thymidine uptake by T-cells	McKallip R. *et al.*, J Immunol. 163(7):3718-26, 1999
	YAC-1 Lymphoma	Soluble	Inhibition of Il-2 stimulated T-cells by blocking IL-2 receptors	Lu P. *et al.*, Cell Immunol. 173(1):22-32, 1996
	Brain	Soluble	43% reduction of NK activity in non-adherent PBMC	Grayson G. *et al.*, Cell Immunol. 139(1):18-29, 1992
	Murine Lymphoma	Soluble	Inhibition of mitogen stimulated [^{3}H]Thymidine, [^{14}C]-leucine and [^{3}H]-lysine by T cells	Krishnaraj R. *et al.*, Eur J Ca & Clin Oncol. 18(1):89-98, 1982
Mucins	Colon Cancer	Surface	Inhibition of cytokine production	Kim JA. *et al.*, Ca Immunol, Immunoth. 48(9):525-32, 1999
	Adeno-carcinoma	Soluble	Suppression of T-cell proliferative responses	Agrawal B. *et al.*, Nature Med. 4(1):43-9, 1998
Tumour Antigens	Murine Lymphoma	Soluble	Inoculation with soluble antigen lead to accelerated tumour growth and delayed rejection	Srinivasa Rao V. *et al.*, Cancer Res. 37(9):3385-9, 1977

Table 4.1 (Cont.): Examples of tumour derived immunosuppressive factors.

Cytokine/ Chemokines				
TGF β	Prostate	Tissue	Overproduction of TGF β1 was associated with a shorter median survival	Wikstrom P. *et al.*, Prostate. 37(1):19-29, 1998
	Small Cell Lung Ca	Soluble	Tumour produced TGF β1 inhibited IL-2 stimulated T-cell proliferation	Fischer JR. *et al.*,Eur J of Cancer. 30A(14):2125-9, 1994
	Glioblastoma	mRNA	Glioblastomas, known to be associated with impaired immunity have significantly elevated levels of TGF β1 mRNA	Bodmer S. *et al.*, J. Immunol. 143(10):3222-9, 1989
PGE 2	Varied	Soluble	PGE2 was immunosuppressive and Indomethacin was associated with inhibition of immunosuppression *in vitro* and faster tumour growth *in vivo*	Plescia OJ. *et al.*, Proc. Natl. Acad. S. 72(5):1848-51, 197
IL-10	Gastric	mRNA	62% of Gastric Ca specimens expressed IL-10 mRNA compared with 17% normal stomach	Morisaki T. *et al.*, J Surg Oncol. 63(4):234-9, 1996
	Melanoma	mRNA/ Soluble	Elevated IL-10 mRNA expression in 62% of melanoma cell lines	Chen Q. *et al.*, Int J of Cancer. 56(5):755-60, 1994
αFP	Hepatoma	Soluble	Inhibition of ConA reactivity and mixed lymphocyte reactivity of splenocytes	Wang XW. *et al.*, Eur J of Phar. 351(1):105-11, 1998
βHCG	Cervical	Tissue	Tissue expression of βHCG	Naughton MA. *et al.*, Cancer Res. 35(7):1887-90, 1975

Table 4.1 (Cont.): Examples of tumour derived immunosuppressive factors.

Other				
Nitric Oxide	Prostate	Tissue	All 16 Prostatic Ca specimens expressed inducible Nitric Oxide Synthase (iNOS) but 0/10 cases of Benign Prostatic Hypertrophy	Klotz T. *et al.*, Cancer. 82(10):1897-903, 1998
	Colon	Tissue/ Soluble	Significant elevation of tissue iNOS compared to control tissue; Inhibition of PHA stimulated lymphocyte proliferation by supernatant	Kojima M. *et al.*, J of Surg Oncol. 70(4):222-9, 1999
	Bladder	Tissue	Bladder carcinoma has a high iNOS content compared to normal tissue	Klotz T. *et al.*, Urology. 54(3):416-9, 1999
Lipocortin-1	Gastric Ca.	Soluble	Significant inhibition of lymphocyte proliferation	Koseki H. *et al.*, Surgery Today. 27(1):30-9, 1997
Adenosine	Adeno-carcinoma	Soluble	Extracellular fluid contains immunosuppressive levels of Adenosine	Blay J. *et al.*, Cancer Res. 57(13):2602-5, 1997
Alanine	Murine Lymphoma	Soluble	Alanine & lactate inhibited *in vivo* priming for secondary cytotoxic responses	Droge W. *et al.*, Cancer Detection & Prevention. 11(3-6):151-6, 1988
Peptide	Cancer Patients	Serum	Inhibition of *in vitro* lymphocyte activation	Nimberg RB. *et al.* Cancer Res. 35(6):1489-94, 1975
	Hepatoma	Ascites	Inhibition of lymphocyte activation	Namba M. *et al.*, Gann. 68(6):751-6, 1977
	Brain	Serum	Inhibition of lymphocyte activation	Kikuchi K. *et al.*, Acta Neurochir.. 103(1-2):52-61, 1990

Table 4.2: Examples of tumour-induced, host-derived immunosuppressive factors

Host Derived ISF	Cell of Origin	Location	Findings	Reference
Prostaglandin E2	Macrophages	Soluble	Suppression of mixed lymphocyte reactions	Malick AP. *et al.*, J Leuk Biol. 42(6):673-81, 1987
	Macrophages	Soluble	Suppression of mixed lymphocyte reactions	Garner RE. *et al.*, Cell Immunol. 108(2):255-68, 1987
IL-12(p40)2	Spleen Cells	Soluble	Antagonism of IL-12 stimulation of NK activity and IFNγ production	Schmidt C. *et al.*, Int J of Cancer. 77(3):460-6, 1998
Immuno-globulin	Lymphocytes	Serum	Inhibition of lymphocyte activity	Levy JG. *et al.*, Immunology. 32(4):475-81, 1977
IL-10	Monocytes	Soluble	Culture of monocytes with colon cancer cells induced production of IL-10	Kucharzik T. *et al.*, Clin & Exp. Immunol. 110(2):296-302, 1997
H_2O_2	Macrophages	Soluble	Inhibition of T-cells through down-regulation of CD3ζ chain expression	Kono K. *et al.*, J. Immunol. 26(6):1308-13, 1996
Lipocortin 1	Macrophage	Soluble/ MRNA	Inhibition of lymphocyte activation	Sakata T. *et al.*, J Immunol. 145(1):387-96, 1990
Nitric Oxide	Macrophages	Soluble	Inhibition of tumour lymphocyte infiltration	Lejeune P. *et al.*, J Immunol. 152(10):5077-83, 1994

4.1.1.2 Extracellular Matrix Proteins

The extracellular matrix (ECM) has recently being recognised as having biological purposes other than providing a stromal milieu for cell survival. Increasingly the components of the ECM are being identified and immunological functions attributed to them. Matrix metalloproteinases have been shown to facilitate tumour invasion and metastasis and as seen above to liberate soluble FasL which in turn can induce apoptosis in CD95 expressing immune cells. ECM proteins have been shown to protect small cell lung cancer against apoptosis, probably via β1-Integrin stimulated tyrosine kinase activity (Sethi, Rintoul et al. 1999). Other integrin members of the ECM, Fibronectin and Laminin, have been shown to enhance T-cell activation via co-stimulatory pathways. Tenascin, is a large glycoprotein present in the ECM, which has been shown to inhibit the proliferation of T-cells in response to a number of stimuli (IL-2, phorbol ester and anti-CD3 antibody with Fibronectin) possibly by interacting with VLA-4 or –5 integrin receptors (Hemesath, Marton et al. 1994). Some solid tumours have been shown to surround themselves, and their metastases, with an extensive ECM stroma (Sethi, Rintoul et al. 1999). It may be that the biological role of Tenascin is to counter that of Fibronectin and Laminin and that large amounts in the ECM of solid tumours inhibits a functional T-cell response.

4.1.1.3 Gangliosides

Gangliosides are complex glycosphingolipids found primarily in the external surface of cells but also shed into the ECM of tumours where they have been shown to be potent inhibitors of the cellular immune response. Tumour derived

gangliosides, at 5-20 μM concentrations, have been shown to inhibit *in vitro* CTL generation by 80-100% and to reduce the response to secondary challenges *in vivo* by 42-74% (McKallip, Li et al. 1999). Co-injection of 1pmol of purified tumour-derived ganglioside with a poorly tumourigenic cell line increased the tumour incidence at 2 weeks from 8 to 83%; p=0.001 (Ladisch, Kitada et al. 1987). Serum levels of gangliosides have been found to correlate with tumour burden in a murine model (Krishnaraj, Lengle et al. 1982). Gangliosides isolated from clinically more aggressive/advanced Neuroblastoma tumours (stages 3&4 versus stages 1&2) were found to be more immunosuppressive than those from less advanced tumours (Floutsis, Ulsh et al. 1989). The validity of this latter finding was not pursued in that they did not attempt to see if the gangliosides at different stages were structurally different. There have been conflicting reports as to whether structurally more complex gangliosides are more immunosuppressive or not (Sharom, Chiu et al. 1990; Grayson and Ladisch 1992). The number of sialic acid residues on the ganglioside has been shown to correlate with immunosuppressive activity particularly if the sialic acid is at the terminal end. This correlation was confirmed by abrogation of immunosuppressive function by total desialylation (Ladisch, Becker et al. 1992). The result of ganglioside inhibition is cytostasis not cytotoxicity as treatment with gangliosides results in a reduced uptake of [^{3}H]Thymidine, [^{3}H]Leucine, but secretion of IL-2 is maintained, no increase in uptake of Trypan Blue and recoverability of proliferative potential if the cells are washed out of the ganglioside-containing medium. The putative mechanism of action is via competition with IL-2 receptor for IL-2 as increased exogenous IL-2 can

overcome the suppression and gangliosides reduce the surface binding of [^{125}I] labelled IL-2 (Chu and Sharom 1993).

4.1.1.4 Mucins

Mucins are yet another group of surface glycoproteins, that are also secreted, those in normal tissues are fully glycosylated. In patients with adenocarcinoma (breast, colon and pancreas) underglycosylated mucins have been found on the cell surface and in sera or ascites. This underglycosylation exposes tandem repeats of a 20 amino acid motif, which can be recognised by the immune system and is potentially a subject for immunisation (see Chapter 1; tumour specific shared antigens). High levels of mucins have been correlated with poor prognosis, and have been shown to inhibit lymphocyte proliferation *in vitro* (Agrawal, Krantz et al. 1998). Mucins (MUC1 and MUC2) from colonic adenocarcinoma have been shown to alter Th1 activity, in that they inhibited IL-2 mRNA expression and secretion of IL-2 by $CD4^+$ T-cells but did not affect IL-10 or TGF-β secretion (Kim, Dayton et al. 1999).

4.1.1.5 Soluble Tumour Antigens

That tumours possess antigens capable of being recognised and responded to has been dealt with at length in Chapter 1. We know that cell membranes are not static structures but rather are continually shedding and regenerating surface molecules. For cell or antibody mediated cytotoxicity to occur the target cell must stably express the target antigen for a period of time. It would seem logical therefore that if the tumour cell could shed the antigen quickly enough then it would evade death. What remains to be proven is that tumour cells do shed

antigen and that this incurs a degree of protection from the host immune response. There is surprisingly little published regarding shed tumour antigens. Most of the publications date back to the 1970's. For example Srinvasa and Bonavida described the detection of a 50-60kD fraction in the serum of tumour bearing rats, which inhibited the cytotoxicity of anti-tumour antibodies, and they conclude that the fraction must represent soluble antigen that is cold competing. Furthermore they correlated the peak serological levels of this fraction with the period of fastest tumour growth (Srinivasa Rao and Bonavida 1977). It may be that researchers moved away from the search for tumour-specific antigens as the immunological surveillance theory fell into disrepute or with improving technologies that the tumour derived antigens became better defined e.g. mucins etc. Again much of the work is this area is historical with little or no publications in the last 15-20 years. The same could be said of blocking antibodies and circulating antibody antigen complexes both of which in the past have been proposed as tumour associated immunosuppressive entities. For a historical synopsis I refer you to Currie, 1980.

4.1.1.6 Intercellular Adhesion Molecules

Intercellular adhesion, essential for the correct functioning of the cellular immune system, can be disrupted by an excess of any of the intercellular adhesion molecules in solution. This is exactly the case with Intercellular Adhesion Molecule-1 (ICAM-1 or CD54). Purified ICAM-1 and melanoma cell culture supernatants have both been shown to inhibit conjugate formation between T-cell clones and autologous melanoma cells *in vitro* (Becker 1993). Furthermore soluble ICAM-1 was found in the sera of patients with melanoma

and was found to inhibit melanoma cell lysis by NK cells in a dose dependant manner *in vitro* (Altomonte 1993). If ICAM-1, by interfering with effector/target cell interaction can be shown to be immunosuppressive *in vitro* then it must only be a matter of time before other adhesion molecules are found to have the same potential.

4.1.2 ISFs – Secreted

The growth of some tumours may depend on their ability to induce local or systemic immunosuppression through the secretion of cytokines. However, the picture is, as always, more complex than this with cytokines such as TGF-β being a prime example.

4.1.2.1 Transforming Growth Factor β

TGF-β is a 25kD homodimeric protein held together by disulfide bonds. It is extremely well conserved with remarkable species homology between human, murine, bovine, and porcine TGF-β, differing in only 1 of the 112 amino acids. It is formed from monomers derived by cleavage of a 391 amino acid precursor and it is only active in its dimeric form. It was originally described because of it's ability to stimulate proliferation of normal rat kidney (NRK) fibroblasts in soft agar in the presence of epidermal growth factor (EGF), possibly by upregulating EGF receptors in NRK cells (Kehrl, Wakefield et al. 1986). Since the original description of TGF-β there are now 3 related forms designated TGF-$\beta_{1\text{-}3}$ and three types of receptor referred to as type I (RI), type II (RII) and type III (RIII) (Brattain, Markowitz et al. 1996). RI and RII are serine/threonine kinases, both of which are necessary for TGF-β signal transduction, RIII does not appear to have much of an intracellular signalling motif (Wrana, L et al. 1994).

TGF-β is possibly the most functionally diverse growth factor, currently known, a detailed discussion of which is beyond the scope and brief of this dissertation. It has been implicated in the promotion or inhibition of proliferation, differentiation and chemotaxis in a cell type-specific manner from embryogenesis to adult life (reviewed in (Sporn and Roberts 1988; Roberts, Flanders et al. 1990). In the field of tumour immunology TGF-β has been shown to act in both an autocrine and a paracrine fashion to induce immunesuppression. It has been shown to inhibit T- and B- cell proliferation (Kehrl, Wakefield et al. 1986), inhibit the growth and function of NK and LAK cells (Cerwenka, Bevec et al. 1994), inhibit macrophage oxygen metabolism and down-regulate MHC expression (Sulitzeanu 1993). It has been isolated from the serum, culture medium, ascitic fluid and mRNA of a variety of tumours (see Table 4.1). The levels of TGF-β in the plasma of tumour-bearing mice were 300% higher than non-tumour-bearing animals (Gohongi, Fukumura et al. 1999). The paradox of how a tumour that is sensitive to the suppressive effects of TGF-β can continue to grow and develop is partially explained by the discovery of mutations in TGF-β RII expression. Progression of non-malignant colonic polyp cell lines to malignancy is associated with loss of sensitivity to TGF-β, loss of RII expression has been documented in colonic tumours, gastric tumours and lymphoma (Brattain, Markowitz et al. 1996). The action of TGF-β is best summarised by Michael Sporn "If something is off, TGF-β will switch it on. If something is on, TGF-β will switch if off" (Balkwill 1993).

4.1.2.2 Prostaglandins

Prostaglandins have been observed to play a role in tumour-induced immune suppression. Their suppressive effects have been attenuated by treatment with cyclo-oxygenase inhibitors such as Indomethacin (Mertens, Bramwell et al. 1992). The role of Prostaglandin E_2 (PGE_2) in particular in experimental lung cancer has been extensively studied (Young and Knies 1984; Chiabrando, Broggini et al. 1985). The PGE_2 has been seen to be released by both the lung tumour cells and tumour-associated macrophages. The release by the host's immune cells of PGE_2 may be related to other factors released by the tumour cell. Release by murine mammary adenocarcinoma of a GM-CSF like factor has been shown to induce PGE2 release from murine spleen, bone marrow and peritoneal cells (Fu, Watson et al. 1991). Huang *et al.* have proposed a possible mechanism of action for PGE_2 (Huang, Sharma et al. 1996). They found a 10-100 fold increase in PBL IL-10 production, following exposure to cell supernatants of non-small cell lung cancers (NSCLC), which was blocked by up to 60% following pre-treatment with indomethacin. They speculate, with good evidence, that the tumour induced IL-10 may serve to both inhibit lymphocyte function and decrease the capacity for APC antigen presentation and cytokine release thus shifting the immune response away from cell-mediated toward a Th2 response.

4.1.2.3 Interleukin-10

IL-10 was initially identified as a Th2 cell product that inhibited cytokine secretion by Th1 cells. It is a 17-21kD protein, which is again highly conserved, human IL-10 shares 80% homology with murine IL-10 (Taga and Tosato 1992). Like many other cytokines it has multiple biological effects (reviewed in(de

Waal Malefijt, Yssel et al. 1992). IL-10 secreted by tumours into their microenvironment may have tumour protective effects by down-regulating MHC class I molecule expression on tumour cells, and thus protecting them from cytotoxic T-cells (Matsuda, Salazar et al. 1994). Alternatively IL-10 may also have a tumour protective effect by, as mentioned above pushing the cellular response towards a Th2 type response (Chouaib, Asselin-Paturel et al. 1997). It has been proposed that IL-10 favours the development of a Th2 response over Th1 by inhibiting the production of IL-12 (a potent and independent inducer of Th1 response) by accessory cells such as monocytes and macrophages (D'Andrea, Aste-Amezaga et al. 1993). The IL-10 may come from the tumour itself or as with so many immunosuppressive cytokines from the hosts own responding cells. TGF-β has been shown to stimulate host macrophages to produce suppressive quantities of IL-10 (Maeda, Kuwahara et al. 1995).

There have been other secreted ISFs, which have been fully identified and are mentioned in Tables 4.1 and 4.2. There are even more partially/incompletely defined ISFs that have been described some of which may prove to be those already mentioned and some undoubtedly novel. The relevant ones will be referred to in the discussion that follows the results of the experiments presented herein. It will be clear by now that though it is right to pursue the identification of all suppressive factors elaborated by tumours that it is unlikely that a single omnipresent ISF will be found, neutralisation of which would be of therapeutic benefit to patients. Rather it is increasingly apparent that tumour-induced immune-suppression is the consequence of a complicated, often interrelated network of suppressive factors. This complexity is best exemplified in a paper

by Alleva *et al.* who reported that tumour-derived TGF-β, IL-10 and PGE2, all proven suppressors in their own right, promote distal macrophage suppressor activity by increasing macrophage production of nitric oxide and TNF-α (Alleva, Burger et al. 1994). Complete negation of all elements may not be possible but elimination of some may tip the balance back in favour of the host.

4.1.2.4 Aims

The key question at the centre of this chapter is whether the different immunological response profiles described in chapter 3 can be attributed to the elaboration by JBS and CarB tumours of different immunosuppressive factors. In order to answer that question we must first establish whether these tumours produce any immunosuppressive factors. The use of tumour explant cultured supernatants for the production and investigation of tumour derived ISFs is a widely recognised and published technique that will be employed here. Having established the validity of using [^{3}H]Thymidine incorporation as a measure of ISF activity. A variety of tissue sources will be used to determine/ascertain the tumour tissue specificity of ISF production and a range of haematogenous and non-haematogenous cell lines will be used to ascertain if the ISF is tissue-specific in its effect.

Having established the existence of ISF activity in crude culture supernatants the next step will be to fractionate the supernatant using High Performance Liquid Chromatography or Ultrafiltration (described in chapter 2) to obtain more pure samples for further analysis. These fractions will be subjected to a variety of physical and chemical stresses to determine the biophysical properties of the

putative ISF(s). It is hoped that the combination of the above approaches will guide towards the next step in isolation of the ISFs. The possibility that any suppressive activity we find may be due to previously described ISFs will be investigated by a variety of approaches including size exclusion, antibody neutralisation, biophysical treatments and amino acid analysis.

At all stages the results for treatment of JBS and CarB derived ISFs will be compared to look for potential differences/similarities.

4.2 Materials and Methods

The cell lines, animals, thymidine incorporation assays were performed as described in Chapter 2, General Materials and Methods. The generation of tumour explant supernatant and cell culture monolayer supernatant has been dealt with in Chapter 2. The same approach was used to generate supernatants for various murine tissues. As the ISF used throughout was the <1kD ultrafiltered fraction the technique of ultrafiltration is also described in Chapter 2.

4.2.1 Protein Estimation

Protein concentration of supernatants was determined using a Bio-Rad Protein Assay, which is based on Bradford's dye-binding procedure (Bradford 1976). It is a colorimetric assay based on the colour change of Coomasie brilliant blue G-250 dye, which binds primarily to basic (especially arginine) amino acid residues. A stock solution of 1mg/ml of Bovine Serum Albumin (Sigma) in SFM is made and used to generate serial dilutions of 50, 25,20, 15,10, 5, 2.5, 2, 1

and 0 μgms/ml to construct a standard curve. Each time a protein estimation assay is carried out a new set of standards and standard curve are created. Serial ten-fold dilutions up to 1:10,000 in SFM of the samples whose protein concentration is being calculated, are made. All dilutions, standard and test, are produced in centrifuge vials (Eppendorf) to which the Bio-Rad reagent (Biorad, Cat. No. 500-0006) is added and centrifuged prior to aliquoting into quadruplicate wells of a 96 well flat-bottom microtiter plate for subsequent analysis on a plate reader. The Bio-Rad reagent is diluted 1:5 with SFM and filtered through a 0.2μm filter, to remove any precipitate, prior to addition to the assay. All samples, standard and test, are added to quadruplicate repeat wells of the microtiter plate remembering to put SFM and reagent alone into the first row of the plate such that the plate reader can be 'blanked' prior to sample analysis. The absorbance at 600nm was measured in an ELISA plate reader. All samples must be analysed within 2-20 minutes of addition of reagent, as after this time the protein/reagent complexes precipitate out of solution and alters the light absorbency of the sample. The standard samples are used to generate a standard curve to which a best-fit regression line is applied and the formula for the slope of that line is used to determine the protein concentration of the test samples.

4.2.2 Flow cytometry of cell cycle analysis

Fresh splenocyte suspensions from naïve, non-tumour bearing mice were generated as described in Chapter 2, co-cultured in medium containing ISF for the required duration and then prepared for cell cycle analysis as described in Chapter 3.

4.2.3 MTT-based Cytotoxicity Assay

3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT, for short) is a soluble tetrazolium salt that is converted by viable cells into an insoluble formazan precipitate. The purple-coloured formazan crystals may be dissolved in a variety of organic solvents and the optical density of the resulting solution measured at 600nm on an ELISA plate reader. It was originally described by Tim Mosmann and can be used to measure cytotoxicity, proliferation or activation without the need to resort to potentially harmful radioisotopes or involving washing steps which can result in sample variation (Mosmann 1983). We used a modified version of Mosmann's assay where DMSO was used to solubilise the formazan crystals prior to colorimetric analysis (Twentyman and Luscombe 1987).

4.2.4 Acid activation

The medium was acidified by the addition of HCl until a final pH of 3.2 was attained. The acidified medium was incubated for 1 hour at 4°C, then reneutralised by the addition of an appropriate volume on NaOH.

4.2.5 High performance liquid chromatography (HPLC) fractionation

Prior to undertaking HPLC fractionation the explant supernatant had to be concentrated as the column could only take a 2ml load volume but produced 2ml per collection point causing a dilutional effect. Fifty mililitres of supernatant were concentrated by lyophilization, in an Edwards Freeze Dryer, and reconstituted with 2mls of sterile water. Samples were fractionated by gel permeation HPLC using a Superdex 75 Hi-Load 26/60 Prep Column (Pharmacia

LKB). Two mls of the concentrated sample were applied to the column and eluted at 1ml/min in an isocratic system with PBS as the mobile phase. Using a P100 HPLC System(Spectra Physics) and a detector with an ultraviolet light source at 280nm, peaks were recorded on an integrator. Two ml fractions were collected from the eluate of the column using an automatic fraction collector (Pharmacia). The HPLC column was kindly set up and run by Maurice O'Donoghue but I carried out sample preparation and collection. Following HPLC individual samples were sterilised by filtration through 0.2μm filters and stored at 4°C until analysed.

4.2.6 Biophysical Properties

4.2.6.1 Heat Treatment

Screw cap, sealed, freeze vials (Eppendorf) containing supernatant or SFM were placed in Styrofoam inserts, protruding from the base, to enable them to float. These were then placed in a water bath that had been heated to 56°C and stabilised at that temperature for at least 1 hour before starting. The vials were kept at 56°C for 30 mins and having been allowed to cool, slowly, back down to room temperature were used in standard assays of ISF activity as before. Similarly prepared vials were placed in a beaker of water at 90°C and maintained at that temperature over a Bunsen burner for 5 mins.

4.2.6.2 Enzymatic Digestion

Trypsin is a proteolytic enzyme that cleaves proteins at Arginine and Lysine amino acid residues. A stock solution of 500μgms/ml of Crude Pancreatic Trypsin (Sigma) in SFM is made. This is added to centrifuge vials (Eppendorf)

containing ISF to produce a final concentration of 50μgms of Trypsin/ml. This is then incubated at 37°C for 2 hours. The reaction is terminated by the addition of Soybean Trypsin Inhibitor (Sigma) to a final concentration of 100μgms of inhibitor/ml of solution.

Proteinase K: A stock solution of Proteinase K (Sigma) in SFM is made and added to vials of ISF to yield a final concentration of 20μgms/ml. This is incubated at 37°C for 1 hour and the reaction terminated by heat inactivating the enzyme to 90°C for 5 mins.

Thymidine Phosphorylase: From a stock solution of 1mg/ml of Thymidine Phosphorylase in PBS varying volumes were added to ISF to yield differing final concentrations. This is incubated at 37°C for 1 hour and the reaction terminated by heat inactivating the enzyme at 90°C for 5 mins.

Dithiothreitol (DTT) reduces disulfide bonds and as such denatures proteins. From a stock solution of 0.65M DTT (Sigma) in SFM 100μls is added to 900μls of ISF to yield a final concentration 0.065M DTT. This solution is left at room temperature for 1 hour and terminated by dialysing against PBS at 4°C for 24 hours.

Trichloroacetic acid (TCA) is traditionally used to precipitate proteins. Equal volumes of 10% TCA (Sigma) and ISF were added to a centrifuge tube and allowed to stand at 4°C for 1 hour. Following centrifugation at 10,000g for 10mins, the supernatant was removed into glass centrifuge tubes. The TCA was

extracted by washing three times with 10 mls of ether. After the third washing step the lower layer was removed and dialysed against PBS at 4°C for 24 hours.

4.3 Results

4.3.1 Suppressive Effect

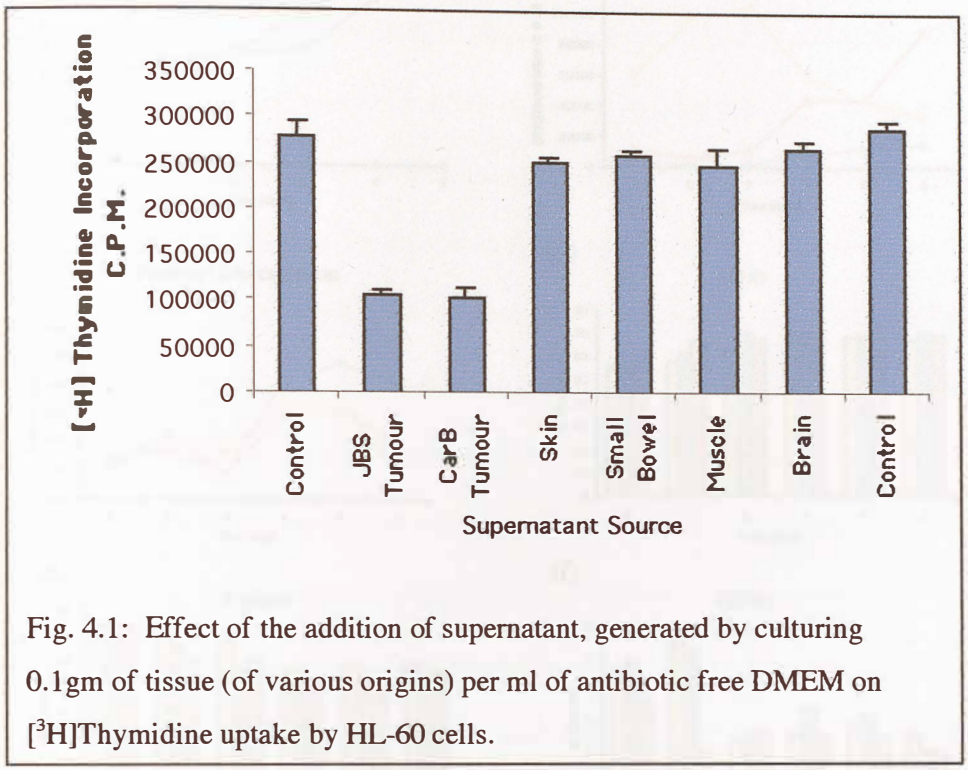


Fig. 4.1: Effect of the addition of supernatant, generated by culturing 0.1gm of tissue (of various origins) per ml of antibiotic free DMEM on [^{3}H]Thymidine uptake by HL-60 cells.

The effect of tumour explant supernatants on [^{3}H] Thymidine uptake by HL-60s is demonstrated in Fig. 4.1. Ten microliters, or a 1:22 dilution, of explant supernatant was enough to reduce thymidine incorporation by 66% for JBS ($p=0.001$) and CarB tumours ($p=0.006$). This reduction or suppressive effect could not be achieved with supernatants generated from normal murine skin, small bowel, muscle or brain ($p>0.126$). [^{3}H]Thymidine incorporation is a well-established and widely used assay for immunosuppression, however, it was possible that we were not seeing a suppressive effect but rather a toxic effect or competition from unlabelled thymidine in the ISF. The suppressive effect therefore had to be validated.

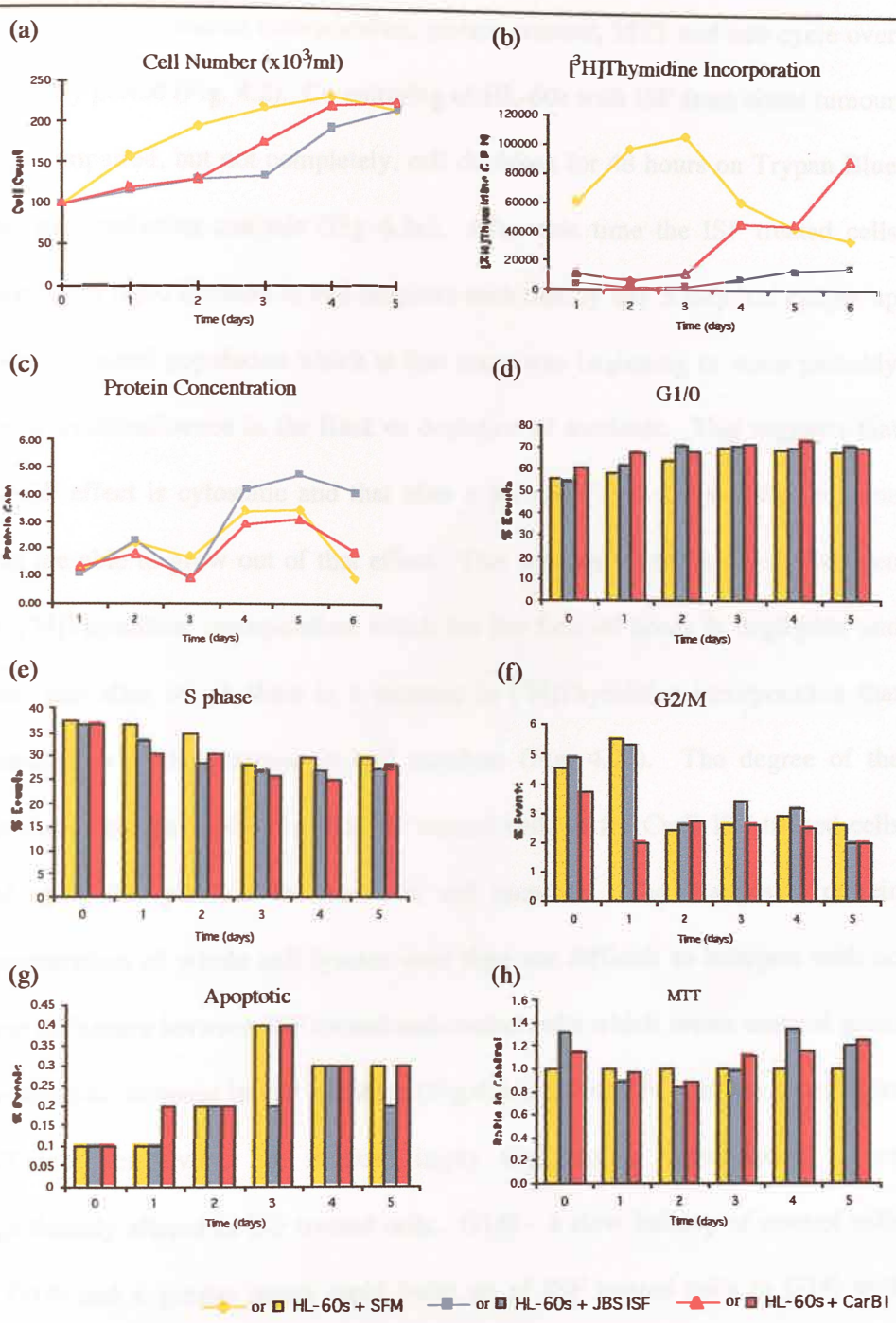


Fig. 4.2: Effect of culturing Hl-60s over a period of 5 days in medium containing SFM or JBS or CarB derived ISF on (a) viable cell number, (b)[^{3}H]Thymidine incorporation, (c) protein concentration of whole cell lysates, (d-g) cell cycle phase and (h) MTT assay.

A series of experiments were set up, which monitored the effect of ISF on cell

number, [^{3}H]Thymidine incorporation, protein content, MTT and cell cycle over a five day period (Fig. 4.2). Co culturing of HL-60s with ISF from either tumour source impaired, but not completely, cell doubling for 48 hours on Trypan Blue vital dye exclusion analysis (Fig 4.2a). After this time the ISF treated cells underrwent rapid increase in cell numbers such that by day 5 they had caught up to the untreated population which at that stage was beginning to wane probably due to overconfluence in the flask or depletion of nutrients. This suggests that the ISF effect is cytostatic and that after a period of cell turnover the immune cells are able to grow out of that effect. This response is the same as that seen for [^{3}H]Thymidine incorporation which for the first 48 hours is negligible and static and after which there is a increase in [^{3}H]Thymidine incorporation that coincides with the increase in cell numbers (Fig. 4.2b). The degree of the increase is not as marked in JBS ISF treated cells as for CarB ISF treated cells and again this parallels the trends in cell numbers. The changes in protein concentration of whole cell lysates over time are difficult to interpret with no clear difference between ISF treated and control cells which seems unusual given the absolute increase in cell numbers (Fig.4.2c). It may be that the assay lacks sufficient sensitivity, but it does imply that protein manufacture is not significantly altered in ISF treated cells. G1/0 – a slow buildup of control cells in G1/0 and a greater /more rapid build up of ISF treated cells in G1/0 with reciprocal decreases in cells in S phase (Fig. 4.2 d&e). In the control population there is an initial increase in cells in G2/M with a subsequent decline. This pattern is the same in JBS ISF treated cells but the decline in G2/M cells is immediate in CarB ISF treated cells (Fig. 4.2f). At all stages there was only a very small population of apoptotic cells which began to peak after 48-72 hours

(Fig. 4.2g). The addition of ISF to HL-60s had an initial decrease, but not abrogation, in the MTT assay suggesting a cytostatic rather than cytotoxic effect, from which the cells recover after 72 hours (Fig. 4.2h). In summary coculturing HL-60s with ISF from either tumour induced a cytostatic effect from which with time they appeared to recover some functions.

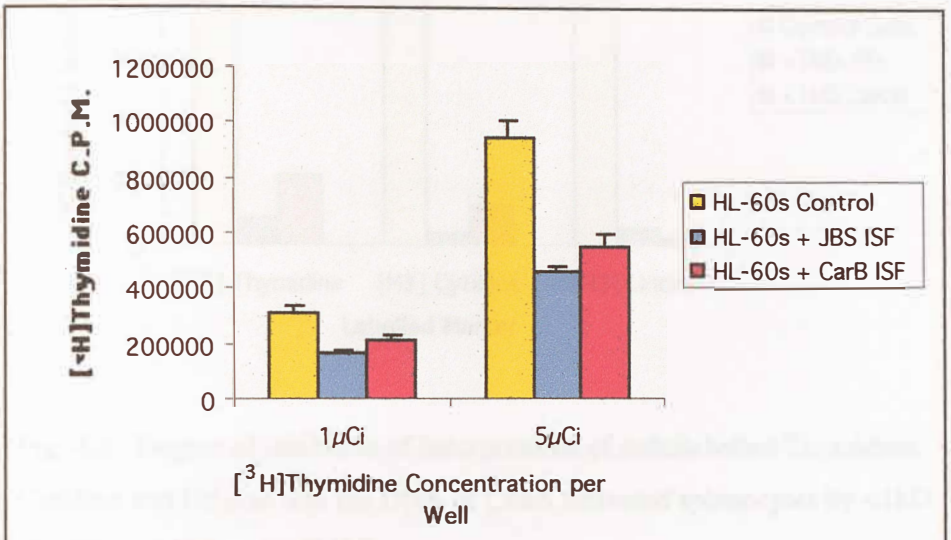


Fig. 4.3: The suppressive effect of ISFs on HL-60 incorporation of [^{3}H]Thymidine at two different concentrations.

Explant supernatant therefore does exert a genuine cytostatic effect on immune cells but as the assay that was primarily used was the [^{3}H]Thymidine incorporation to have full confidence in the validity of that assay and to ensure that the 'ISF effect' wasn't simply cold competition for the labelled thymidine the effect of increasing the amount of labelled thymidine was investigated (Fig. 4.3). Increasing the concentration of [^{3}H]Thymidine per well, while increasing the overall counts per minute, did not diminish the suppressive effect of either

ISF which remained statistically significant with a p value of less than 0.008 for 1μCi [^{3}H]Thymidine per well and p value less than 0.002 for 5μCi per well.

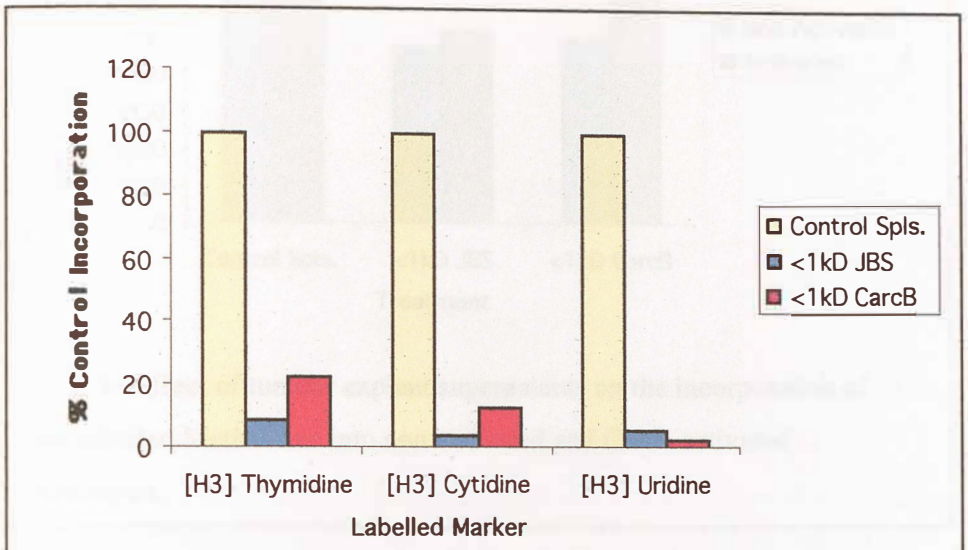


Fig. 4.4: Degree of inhibition of incorporation of radiolabelled Thymidine, Cytidine and Uridine into the DNA of ConA activated splenocytes by <1kD ultrafiltered JBS or CarB ISF.

Further evidence that the suppressive effect is real and not an artefact of the Thymidine incorporation assay used to measure suppression is gained from Figs. 4.4, 4.5. Explant supernatants were equally effective at inhibiting the incorporation of other radio-labelled nucleic acids, [^{3}H] Cytidine and [^{3}H]Uridine into active splenocytes. It also inhibits the incorporation of labelled Methionine into both activated and non-activated splenocytes.

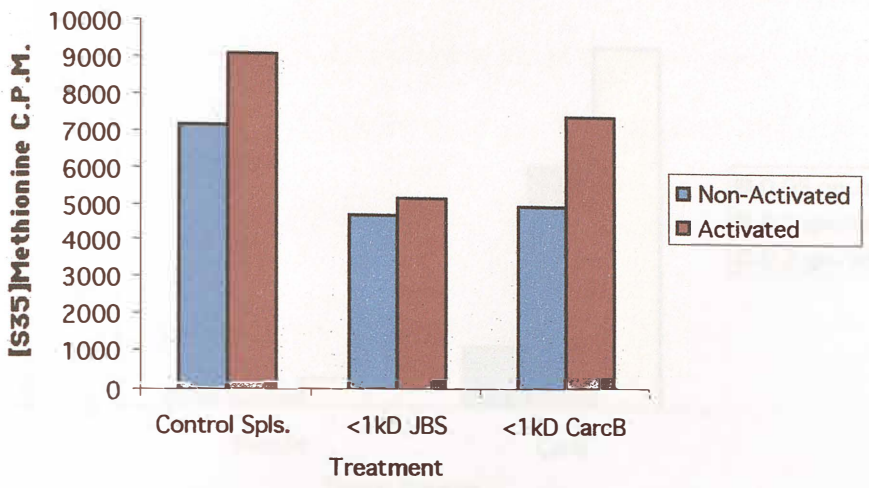


Fig. 4.5: Effect of tumour explant supernatants on the incorporation of radiolabelled Methionine into non-activated and ConA activated splenocytes.

To determine if the amount of tissue per ml used in the generation of explant supernatant influenced the ISF effect tumour tissue and control tissue were cultured at varying concentrations with medium and the resultant supernatant assayed to determine the number of units of ISF activity per well (Fig. 4.6). A unit of ISF activity is defined as that which causes a 50% reduction in [^{3}H]Thymidine uptake. Muscle tissue supernatants generate very little ISF activity even when large amounts are used. The ISF activity that is generated is probably related to metabolic by-products such as lactate and alanine, which will be dealt with later. Tumour explant supernatants on the other hand generate significant amounts of ISF activity that increases with larger concentrations of tissue. JBS tumour supernatant demonstrated the same dose/response curve (data not shown). To ensure consistency in production, all explant supernatants were

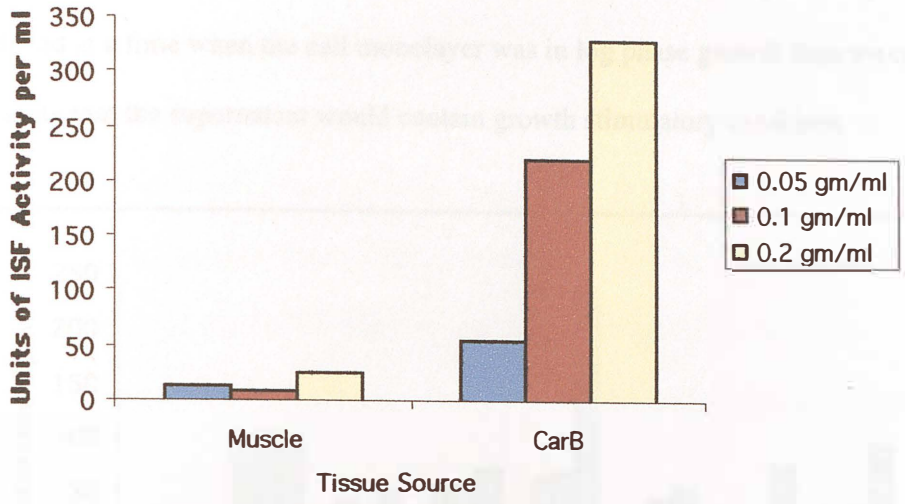


Fig. 4.6: Relationship between the amount of tumour tissue per ml used in the generation of explant supernatant and the resultant number of units of suppressive activity per ml. One unit of activity is that volume of supernatant required to inhibit [^{3}H]Thymidine incorporation into HL-60s by 50%.

generated using 0.1gm of fresh tumour tissue per ml thus maximising ISF generation while minimising metabolic by-products.

The next question to be answered was whether the growth of the tumour in an immunocompetant mouse was necessary for ISF generation or could tumours grown in athymic nude mice or even cell culture monolayers be used to generate ISF activity. Fig. 4.7 displays the results from three separate series of assays for ISF activity in supernatants generated from tumours grown in immunocompetant Balb/c mice, Athymic Nude mice or cell culture monolayer. The test cells were ConA activated murine splenocytes. The immunosuppressive effect of tumour explant supernatants was a consistent finding regardless of the animal in which the tumour was grown. Supernatants generated from cell culture monolayers on the other hand had variable effects from suppression to no effect to stimulation.

Though this wasn't investigated further it may be that if the supernatant was produced at a time when the cell monolayer was in log phase growth then we can postulate that the supernatant would contain growth stimulatory cytokines.

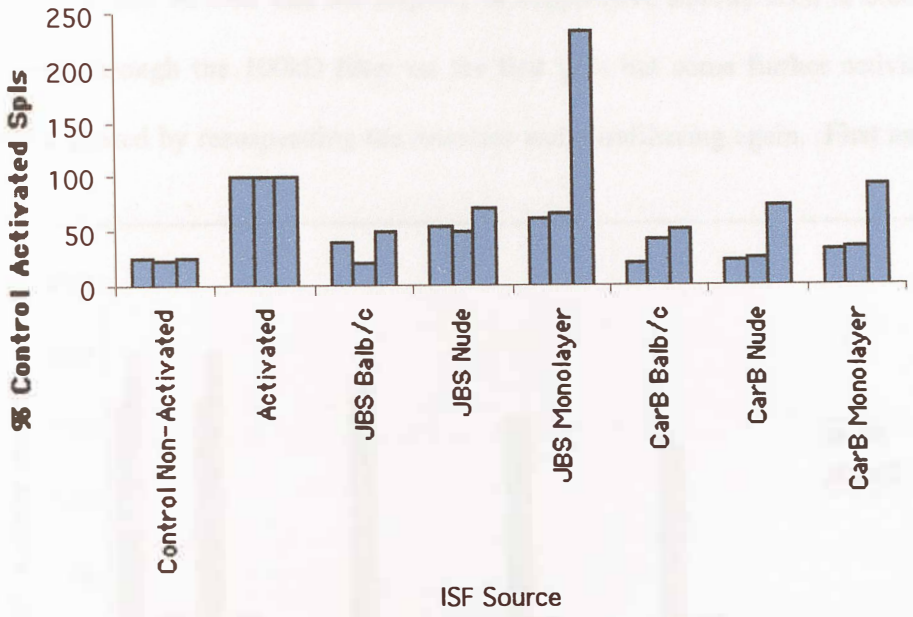


Fig. 4.7: Three different series of experiments detailing the effect of JBS or CarB supernatants from different sources (tumours grown in Balb/c or athymic nude mice or cell culture monolayers) on [^{3}H]Thymidine incorporation by activated splenocytes from naïve mice.

4.3.2 Fractionation

Explant supernatants, by virtue of the production technique, would be expected to contain a mixture of cytokines and metabolic by-products. Ultrafiltration was therefore used to identify the approximate weight or weights of the ISF activity (Fig.4.8). It can be seen that the majority of suppressive activity seen in crude ISF came through the 100kD filter on the first pass but some further activity could be passed by resuspending the retentate and ultrafiltering again. First and

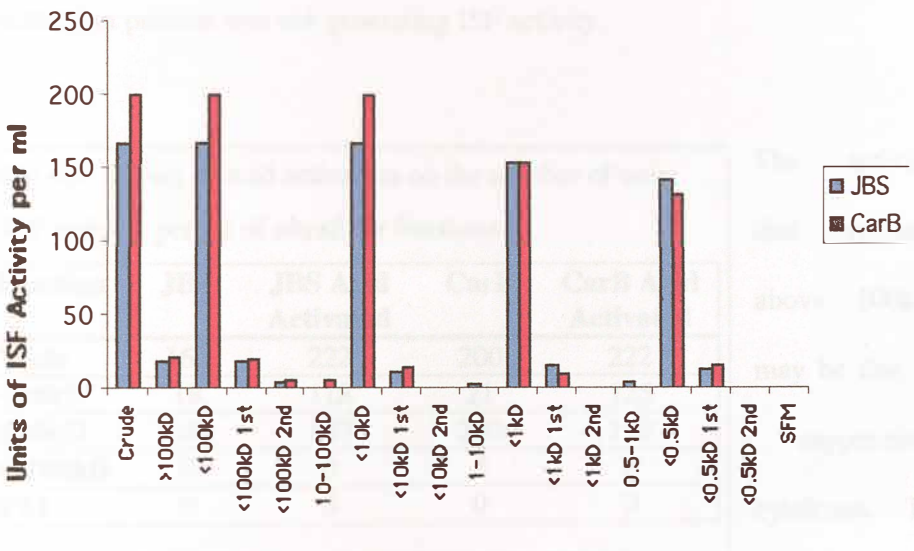


Fig. 4.8: Units of ISF activity per ml of crude explant supernatant and ultrafilter fractions. >100kD = that retained above the 100kD cutoff membrane after the resuspension/washing steps; <100kD the supernatant that passed through the 100kD cutoff membrane; <100kD 1st the supernatant that passed through the 100kD membrane after the first resuspension in PBS; <100kD 2nd the supernatant that passed through the 100kD membrane after the second resuspension in PBS; 10-100kD the supernatant that had passed thru the 100kD cutoff membrane at first pass but was retained by the 10kD cutoff membrane after two resuspension steps. Subsequent groups are similarly labelled

second refer to the first and second resuspension and filtration stages. What remained after the second washing stage was deemed to be too large to pass through the membrane. The majority of ISF activity passed sequentially through 10kD, 1D and 0.5kD membranes with minimal loss of activity. With the exception of some activity, which was retained above 100kD, the majority of activity could be attributed to molecules smaller than 500D in size. Serum free medium treated in the same way and ultrafiltered down through the various membranes did not have any ISF activity, (data not shown), proving that the ultrafiltration process was not generating ISF activity.

Table 4.3: Effect of acid activation on the number of units of ISF activity per ml of ultrafilter fractions

Fraction	JBS	JBS Acid Activated	CarB	CarB Acid Activated
Crude	167	222	200	222
>100kD	18	118	21	125
<100kD	167	167	200	118
10-100kD	0	0	5	6
SFM	0	0	0	0

The activity that remained above 100kD may be due to suppressive cytokines. To ascertain if some of this was due to TGF-β the fractions were acid activated and re-analysed for ISF activity. It has been shown that TGF-β can be secreted in a biologically inert form that is unmasked by acid treatment of the culture medium (Kehrl, Wakefield et al. 1986). Acid activation increased the suppressive effect of crude supernatant by 10 to 33% (Table 4.3). All of this increase was in the >100kD fraction, the suppressive activity of which was increased 6-6.5 fold.

This finding would suggest that crude ISF contains acid activatable cytokines. Therefore all further experiments on ISF activity were carried out on <1kD fractionated ISF.

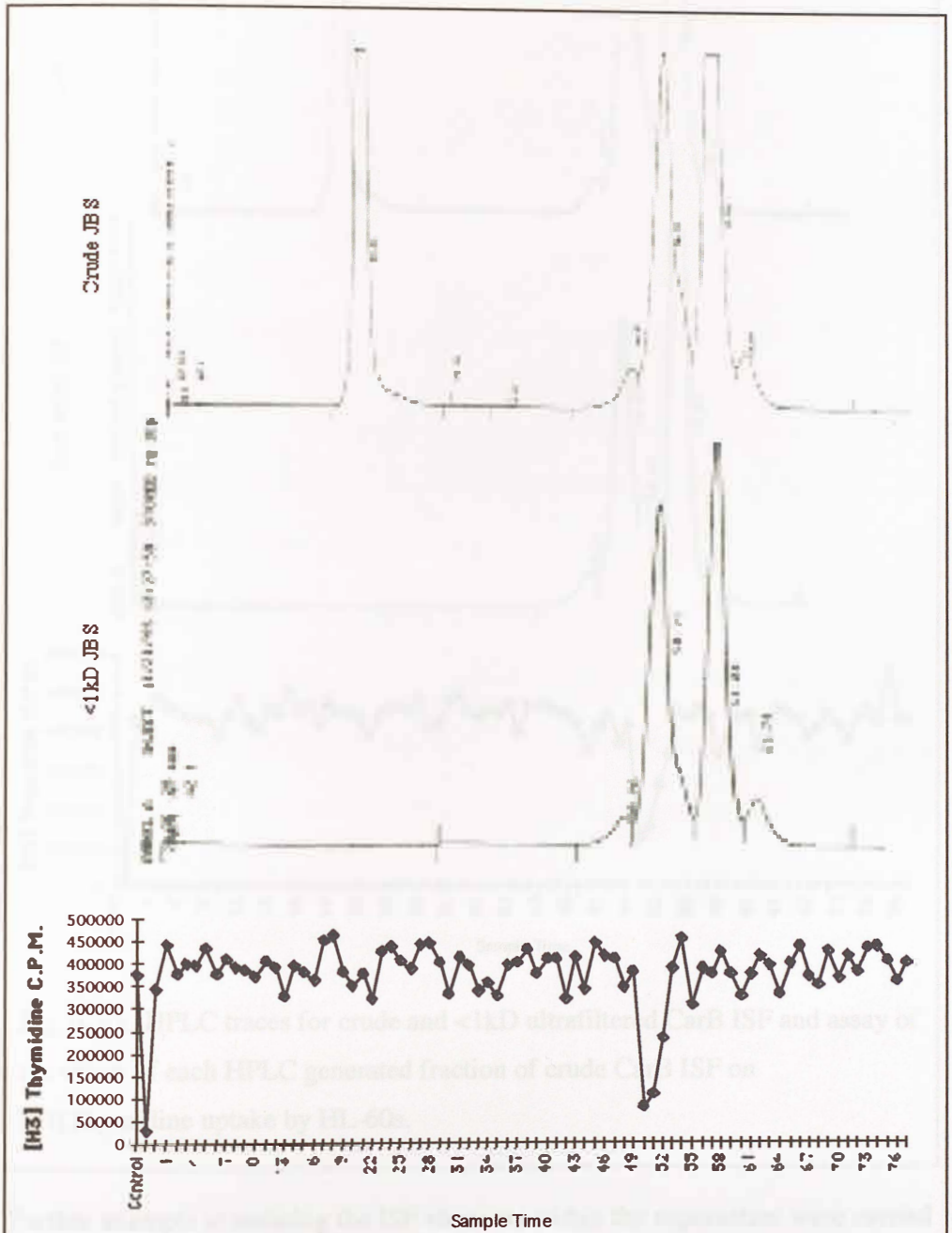


Fig. 4.9: HPLC traces for crude and <1kD ultrafiltered JBS ISF and assay of the effect of each HPLC generated fraction of crude JBS ISF on [^{3}H]Thymidine uptake by HL-60s.

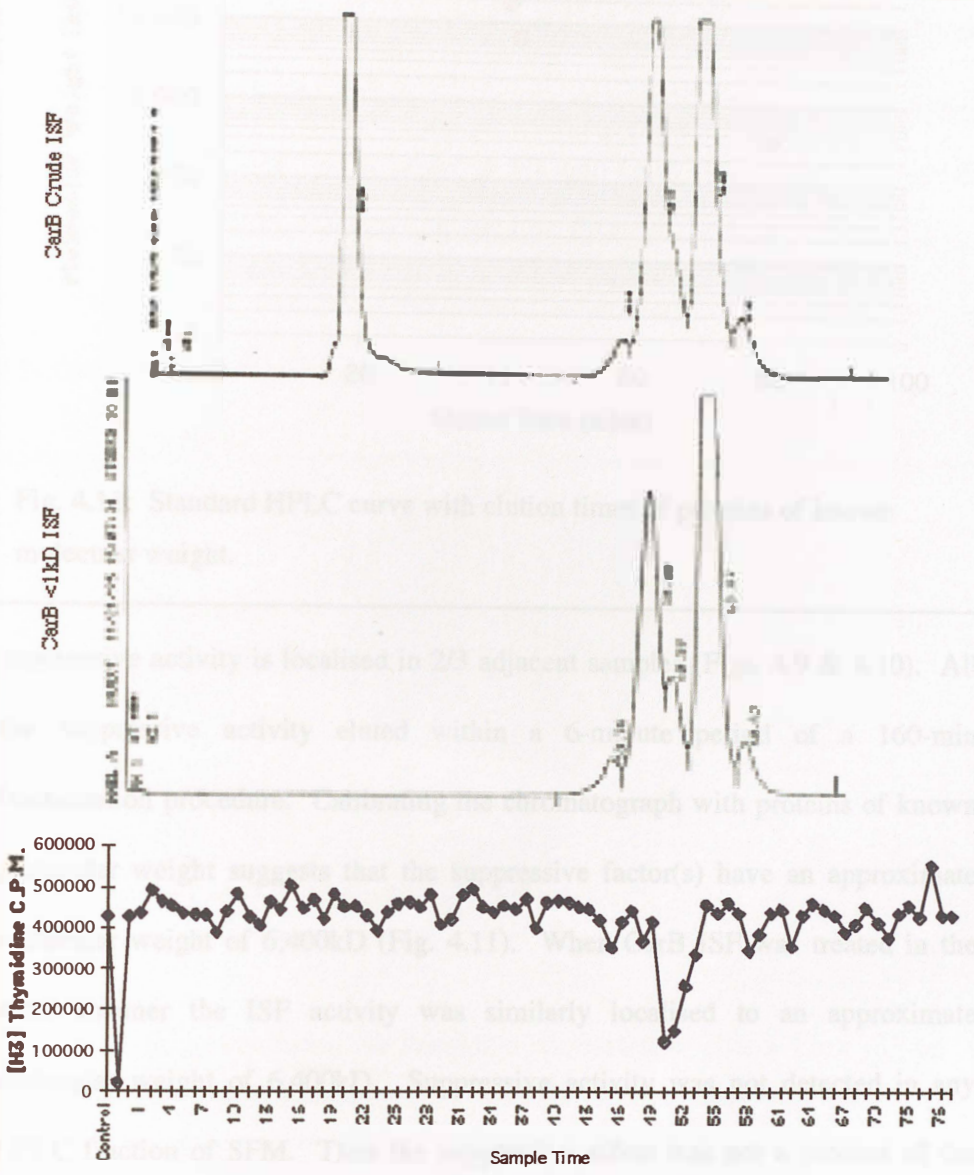


Fig. 4.10: HPLC traces for crude and <1kD ultrafiltered CarB ISF and assay of the effect of each HPLC generated fraction of crude CarB ISF on [^{3}H]Thymidine uptake by HL-60s.

Further attempts at isolating the ISF elements within the supernatant were carried out using high performance liquid chromatography (HPLC) fractionation. The chromatograph generates elution fractions every 2 minutes. When each of these timed fractions are assayed for inhibition of [^{3}H]Thymidine incorporation the

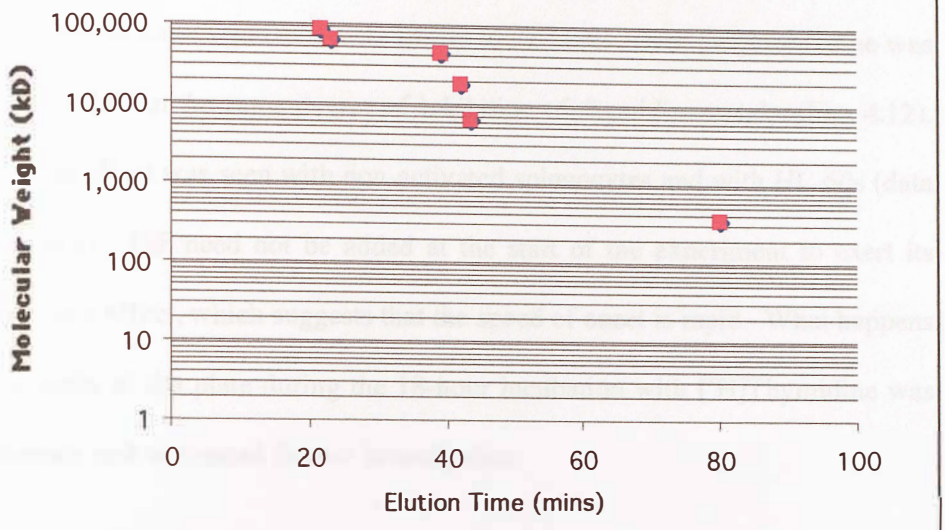


Fig. 4.11: Standard HPLC curve with elution times of proteins of known molecular weight.

suppressive activity is localised in 2/3 adjacent samples (Figs. 4.9 & 4.10). All the suppressive activity eluted within a 6-minute period of a 160-min fractionation procedure. Calibrating the chromatograph with proteins of known molecular weight suggests that the suppressive factor(s) have an approximate molecular weight of 6,400kD (Fig. 4.11). When CarB ISF was treated in the same manner the ISF activity was similarly localised to an approximate molecular weight of 6,400kD. Suppressive activity was not detected in any HPLC fraction of SFM. Thus the suppressive effect was not a product of the HPLC fractionation process.

4.3.3 Time Course

To determine the speed of onset of the ISF effect an ISF assay of non-activated and activated splenocytes was set up as described in Chapter 2. This time however the ISF was added to the plate at different time intervals but the experiment was otherwise conducted as described. Addition of ISF to the

splenocytes at any stage from plate set-up to 72 hours when [^{3}H]Thymidine was added resulted in the same degree of inhibition of thymidine uptake (Fig. 4.12). The same effect was seen with non-activated splenocytes and with HL-60s (data not shown). ISF need not be added at the start of the experiment to exert its suppressive effect, which suggests that the speed of onset is rapid. What happens in the wells of the plate during the 18-hour incubation with [^{3}H]Thymidine was not known and warranted further investigation.

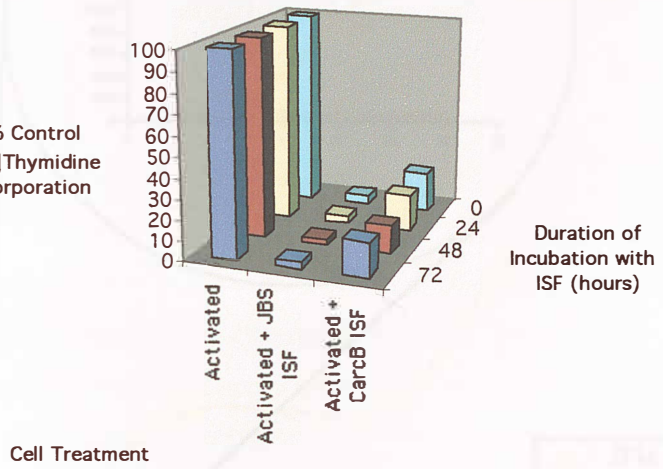


Fig. 4.12: Effect of altering the duration of incubation of JBS or CarB ISF on the uptake of [^{3}H]Thymidine by ConA activated splenocytes

To more accurately define the speed of onset of the ISF effect splenocytes from naïve non-tumour-bearing mice were activated with ConA in the usual manner for 72 hours. These activated splenocytes were then incubated in the presence of ISF and [^{3}H]Thymidine in sterile Eppendorf tubes. At progressive intervals the incubations were terminated by placing the vials on ice, which stopped all cellular activity and limited any further active thymidine incorporation. At the end of the 5-hour period all of the vials were processed in the usual manner and

thymidine counts analysed. It can be seen from Fig 4.13 that ISF inhibition of thymidine incorporation begins almost immediately with divergence obvious after 10 mins (see insert Fig.4.13). This difference becomes more pronounced as the experiment progresses.

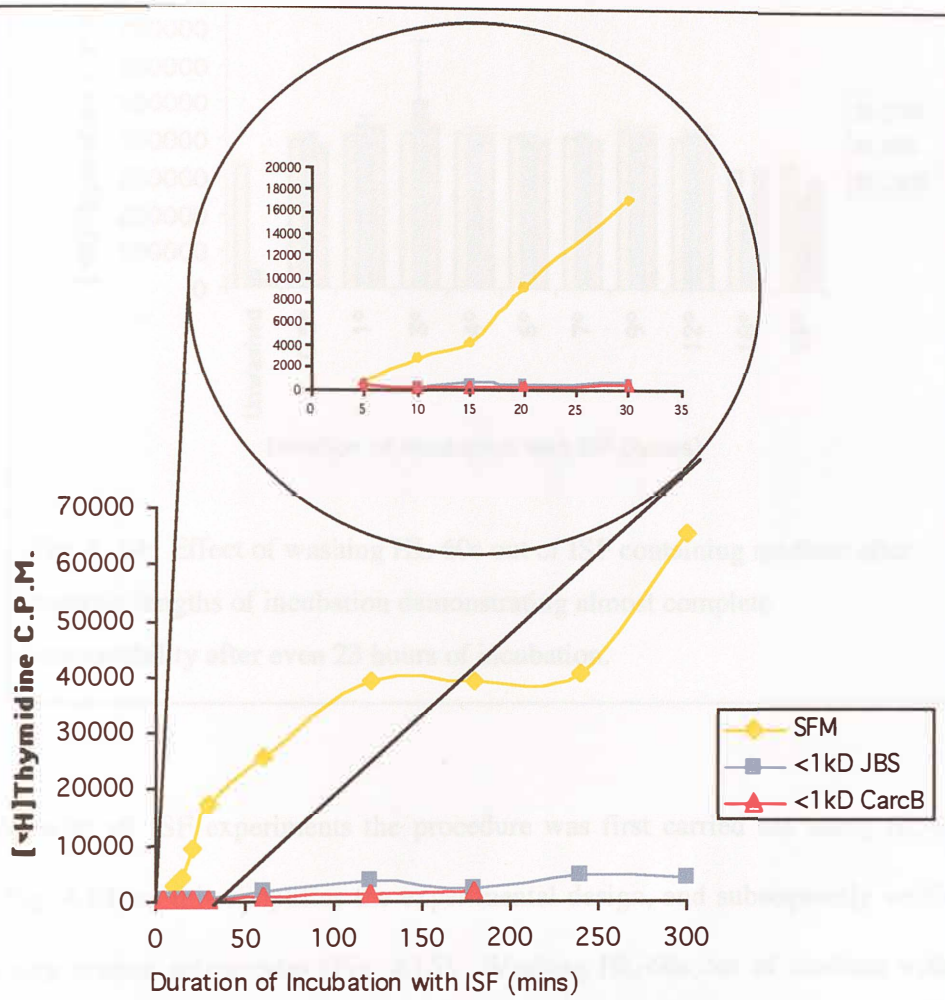


Fig. 4.13: Effect of reducing the duration of incubation of ConA activated splenocytes prior to precipitation and analysis demonstrating the speed of onset of ISF effect.

4.3.4 Reversibility/Recoverability

The flow cytometric data seen in Fig. 4.2 suggested that if given sufficient time the immune cells could recover or grow out of the suppressive effect of ISF.

Having established that ISF can exert its suppressive effect within 10 mins we next wished to see what effect removing exposed cells from the suppressive milieu had *in vitro*.

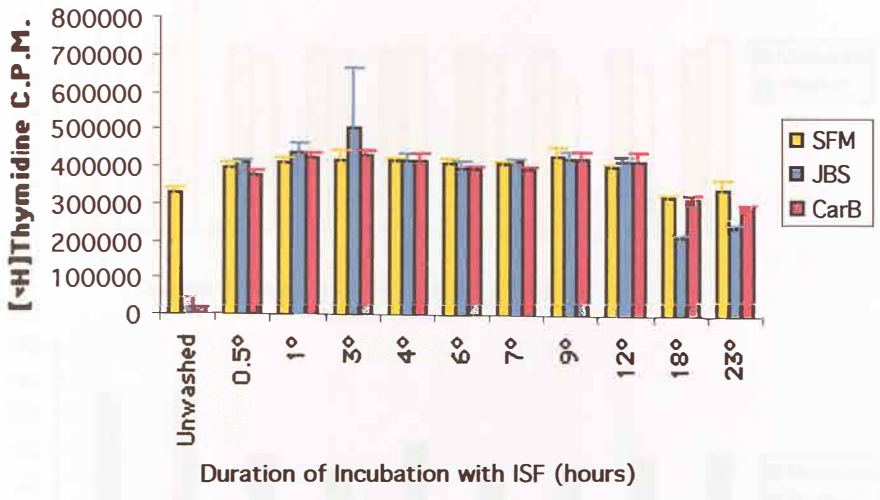


Fig. 4. 14: Effect of washing HL-60s out of ISF containing medium after variable lengths of incubation demonstrating almost complete recoverability after even 23 hours of incubation.

As with all ISF experiments the procedure was first carried out using HL-60s (Fig. 4.14) as this simplifies the experimental design, and subsequently verified using murine splenocytes (Fig. 4.15). Washing HL-60s out of medium with 2 units of ISF activity after varying incubation times of 0.5 to 23 hours, before the addition of [^{3}H]Thymidine resulted in complete recovery of thymidine incorporation (Fig.4.14). In fact thymidine incorporation in some of the samples was enhanced, in the control group also, possibly due to the replenishment of nutrients in the culture medium. Almost identical results were found when the HL-60s were substituted with splenocytes that had been pre-activated *in vitro* with ConA (Fig. 4.15). The thymidine counts have been adjusted and expressed

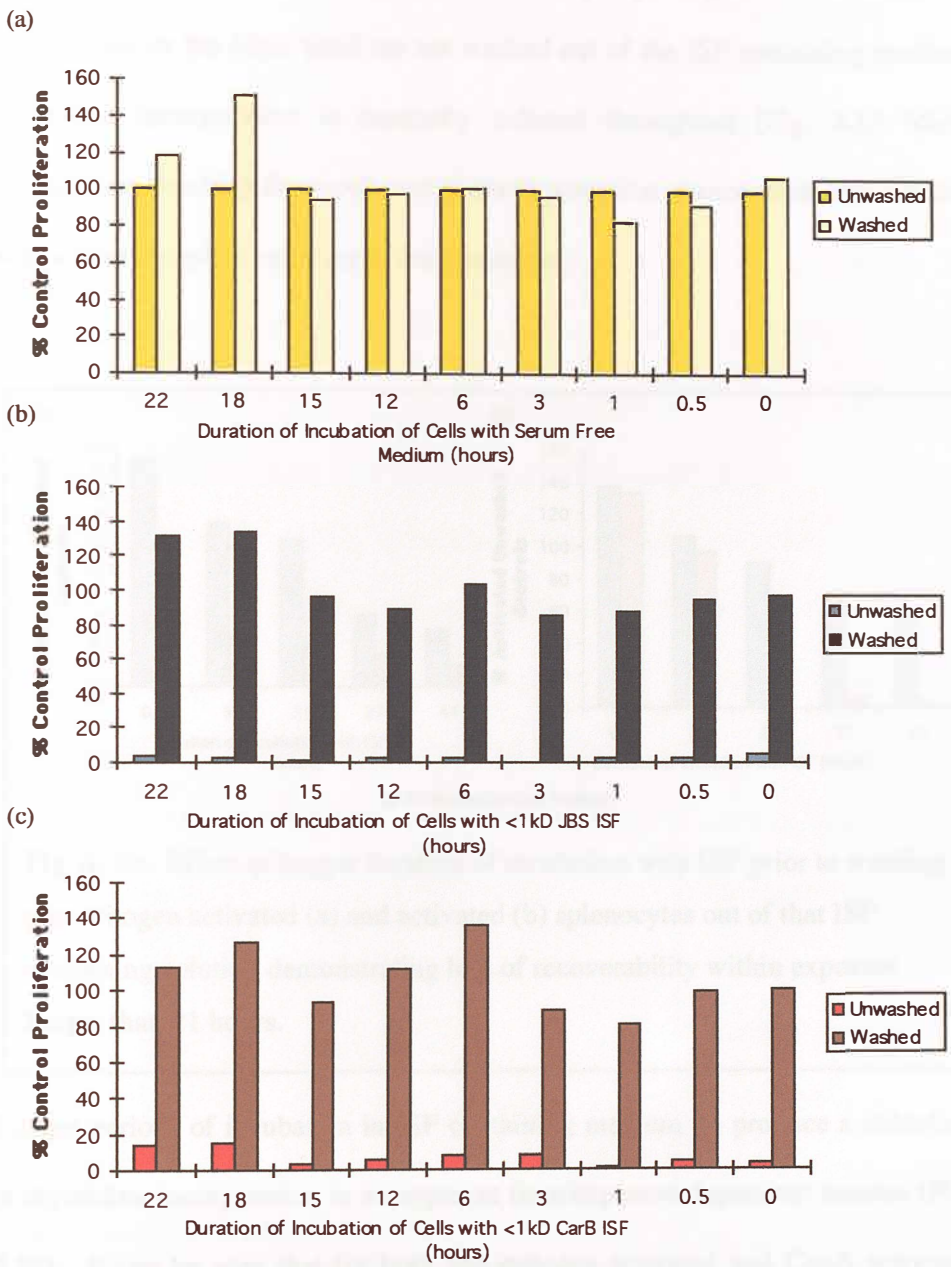


Fig. 4.15: Effect of washing ConA activated splenocytes out of medium containing (a) SFM, (b) <1kD JBS ISF and (C) <1kD CarB ISF after varying durations demonstrating reversibility of ISF effect up to 22 hours.

as a percentage of the count for thymidine incorporation by activated unwashed splenocytes for each time point. This was done to facilitate graphic expression of the data as otherwise the scale of the axis would be too wide. It can be seen

that washing is not associated with significant changes in thymidine incorporation in the serum free medium treated groups (Fig. 4.15a). If activated splenocytes on the other hand are not washed out of the ISF containing medium thymidine incorporation is markedly reduced throughout (Fig. 4.15 b&c). Furthermore washing these cells out of the suppressive environment is associated with a near complete recovery of incorporation.

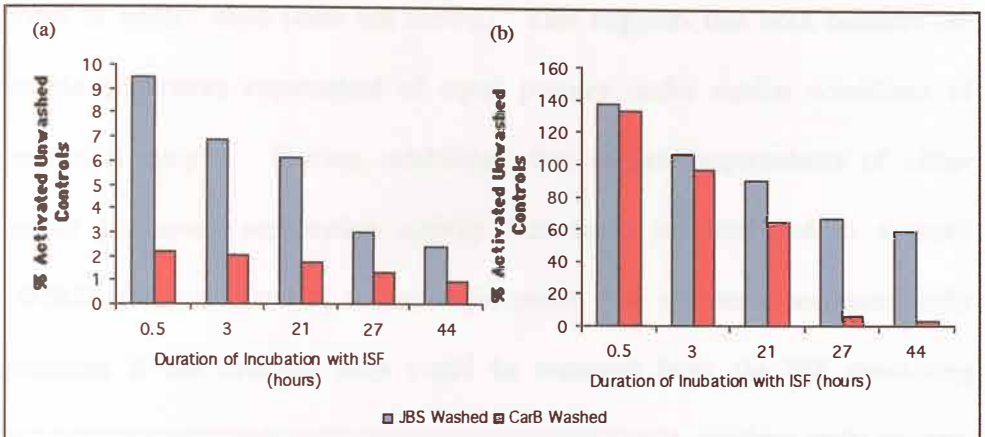


Fig. 4. 16: Effect of longer duration of incubation with ISF prior to washing non-mitogen activated (a) and activated (b) splenocytes out of that ISF containing solution demonstrating loss of recoverability within exposure longer than 21 hours.

Longer periods of incubation in ISF containing medium do produce a reduction in thymidine incorporation in an apparent time/exposure dependent manner (Fig. 4.16). It can be seen that for both non-mitogen activated and ConA activated splenocytes that exposure to ISF for periods in excess of 21 hours induces a time related reduction in thymidine incorporation. Furthermore it appears that the supernatant derived from CarB tumour explant supernatants is more potent (less reversible) than that derived from JBS despite both sets of experiments being set up with 2 units of ISF activity (determined as described above) per well or 1 unit

per 100μls. This anomaly was examined by carrying out ISF assays looking for extent of suppression with differing dilutions of supernatant of known protein concentration (Fig. 4.17). It can be seen that if the supernatants from JBS or CarB tumours are diluted to a known protein concentration and then assayed for ISF activity there is no significant difference between the two tumour sources. The same dose response trend was seen if the tumours used to generate the supernatant had been grown in nude mice rather than if the tumours had been grown in Balb/c mice (data not shown). This suggests that both tumours are capable generating supernatant of equal potency under similar conditions of production/analysis. Having established that explant supernatants of either tumour possessed suppressive activity that could be attributed to a small (<0.5kD) fraction that had a very rapid onset, was cytostatic and potentially reversible if the immune cells could be removed from the ISF containing medium early enough, we next wished to examine the biophysical properties of the ISF fraction to see if we could abrogate the suppressive effect.

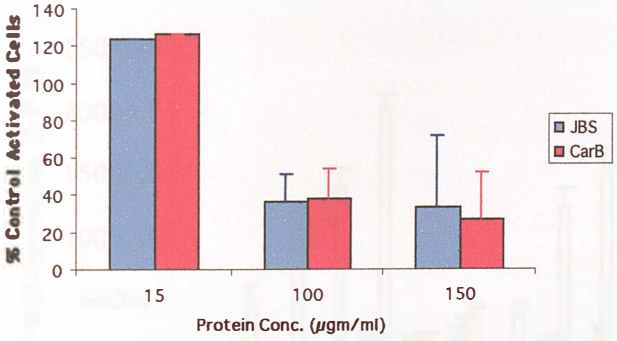


Fig. 4.17: Suppressive effect of JBS or CarB ISF of known protein concentration.

4.3.5 Specificity

Having established that ISF production was specific to tumour tissue we investigated the effect of tumour explant supernatant on thymidine incorporation

in a wide variety of haematogenous and non-haematogenous cell lines. It can be seen from Fig. 4.18 the ISF effect is not restricted to cells of haematological origin (Jurkatt, HL-60, Daudi, K562 and NSO) only. Notably the cells that produce the ISF are also markedly sensitive to the suppressive effect of the supernatant themselves. In accord with data from non-exogenously activated and

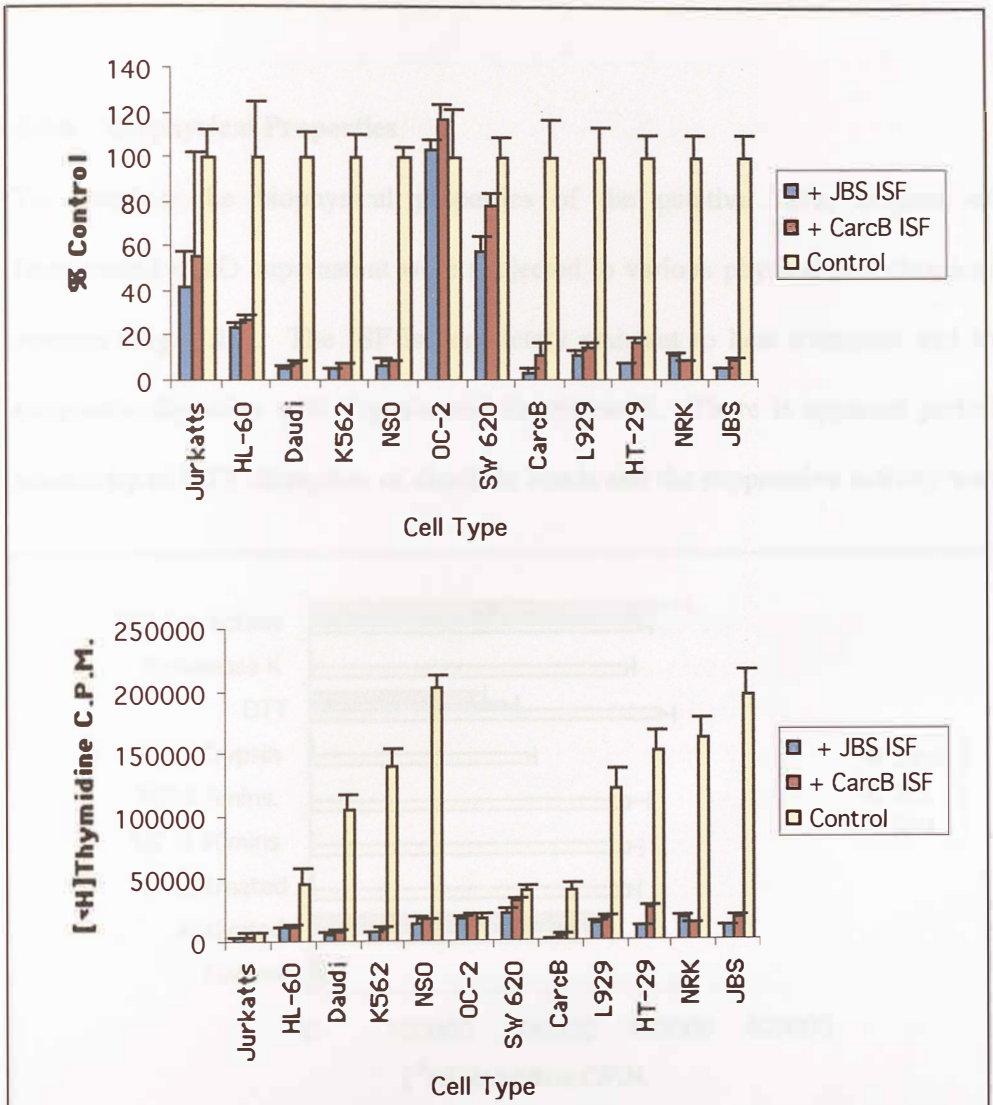


Fig.4.18: Specificity of action of <1kD tumour explant supernatant on a variety of cultured cell lines expressed as a percentage of control [^{3}H]Thymidine incorporation (upper panel) and as absolute counts (lower panel).

activated splenocytes, elevated uptake of [^{3}H]Thymidine by untreated cells is associated with increased sensitivity to the suppressive effect of culture supernatants. The cell lines that were least sensitive to ISF (OC-2 & SW-620) have very low total thymidine incorporation as controls. The exception to this is the CarB cell line, which despite low control thymidine uptake is very sensitive to the ISF effect.

4.3.6 Biophysical Properties

To ascertain the biophysical properties of the putative ISFs, aliquots of fractionated <1kD supernatant were subjected to various physical and chemical stresses (Fig. 4.19). The ISF is completely resistant to heat treatment and to enzymatic digestion with Trypsin and Proteinase K. There is apparent partial sensitivity to DTT disruption of disulfide bonds and the suppressive activity was

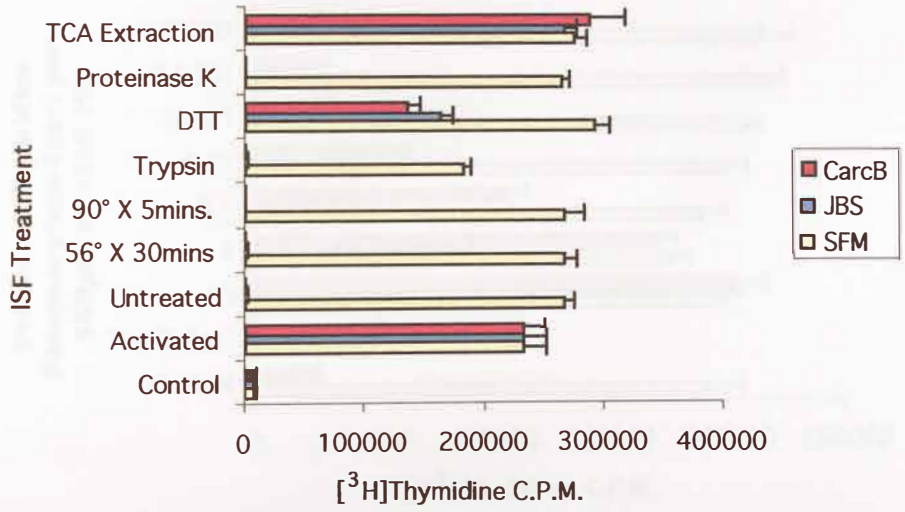


Fig. 4.19: Effect of differing treatments on the suppressive activity of tumour explant supernatant or serum free medium (SFM) on [^{3}H]Thymidine incorporation by HL-60 cells.

completely removed by the TCA precipitation process. However both of these processes were terminated by dialysing the solution against phosphate buffered saline and given the results of ultrafiltration these apparent sensitivities must be viewed with scepticism.

The only enzymatic treatment that appeared to inhibit the suppressive effect of supernatant was treatment with Thymidine Phosphorylase. Thymidine Phosphorylase abrogated completely in a dose dependent manner the inhibitory effect of, supra-normal quantities (normally 10-15μls per well), of supernatant on [^{3}H]Thymidine incorporation by HL-60s (Fig. 4.20). This effect cannot be attributed to persistent enzyme activity destroying unlabelled Thymidine and thus increasing labelled Thymidine incorporation, as the overall levels of Thymidine incorporation never exceed those of the control groups. This dose

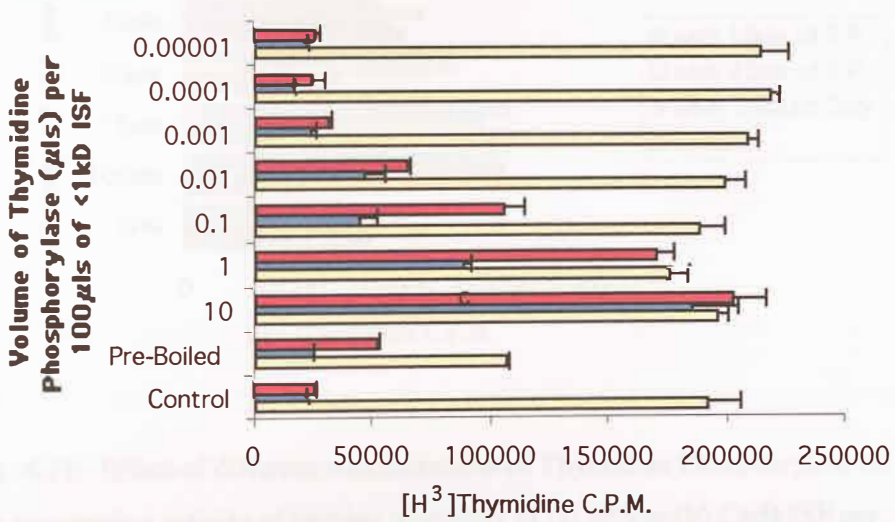
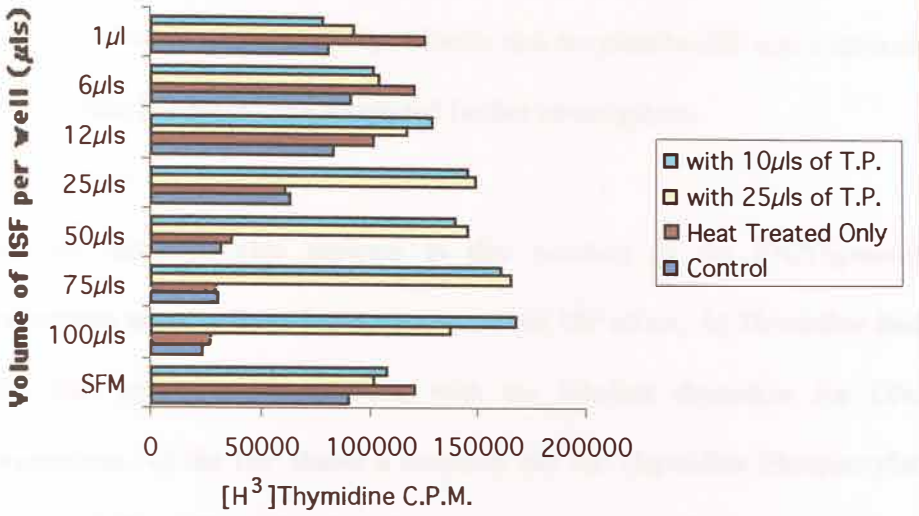
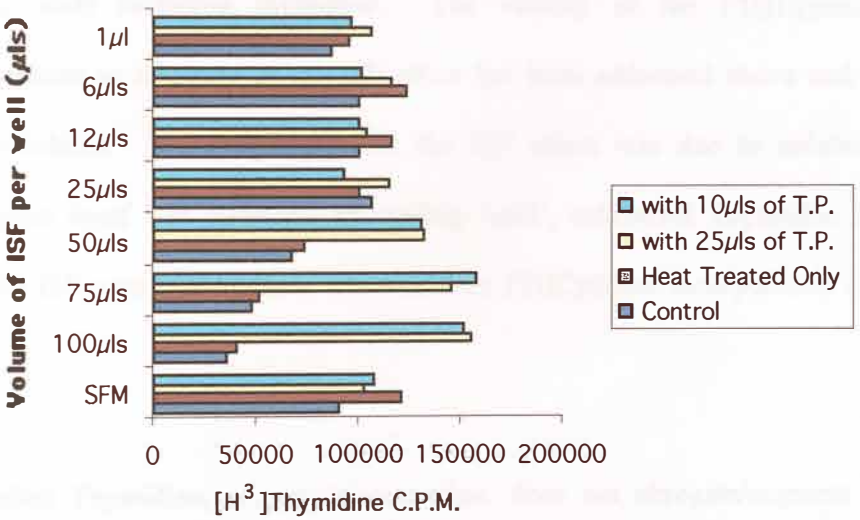


Fig. 4.20: Effect of reducing the quantity of Thymidine Phosphorylase per well on the suppressive activity of fractionated JBS or CarB supernatant or SFM on [^{3}H]Thymidine incorporation by HL-60 cells.

dependant effect was also found in reverse where the amount of ISF per well was titrated against fixed amounts of Thymidine Phosphorylase enzyme (Fig. 4.21).



(a)



(b)

Fig. 4.21: Effect of different concentrations of Thymidine Phosphorylase on the suppressive activity of varying quantities of (a) JBS or (b) CarB ISF per well.

The effect of Thymidine Phosphorylase could be overcome by increasing the amount of ISF per well. The impact of Thymidine Phosphorylase on the suppressive effect was immediate being detectable within minutes of co-incubation (data not shown). The possibility that the putative ISF was a substrate for Thymidine Phosphorylase warranted further investigation.

There are three possible answers to this problem a) the [^{3}H]Thymidine incorporation assay is flawed as a measure of the ISF effect; b) Thymidine itself is the ISF and is cold-competing with the labelled thymidine for DNA incorporation; c) the ISF shares a common site for Thymidine Phosphorylase activity with thymidine either by coincidence or by virtue of being composed of nucleic acids including thymidine. The validity of the [^{3}H]Thymidine incorporation as an assay of the ISF effect has been addressed above and has been validated. The possibility that the ISF effect was due to unlabelled Thymidine itself was addressed by adding 'cold', unlabelled Thymidine to a standard ISF assay to examine it's effect on [^{3}H]Cytidine incorporation (Fig. 4.22).

Unlabelled Thymidine, at any concentration, does not abrogate/augment the suppressive effect of supernatants. Neither does unlabelled Thymidine reduce the incorporation of [^{3}H] Cytidine into HL-60s at any concentration. Both of these observations would mitigate against Thymidine itself being the suppressive factor. This leaves the possibilities that either Thymidine forms part of the suppressive entity or shares a common point of action for Thymidine Phosphorylase with the ISF.

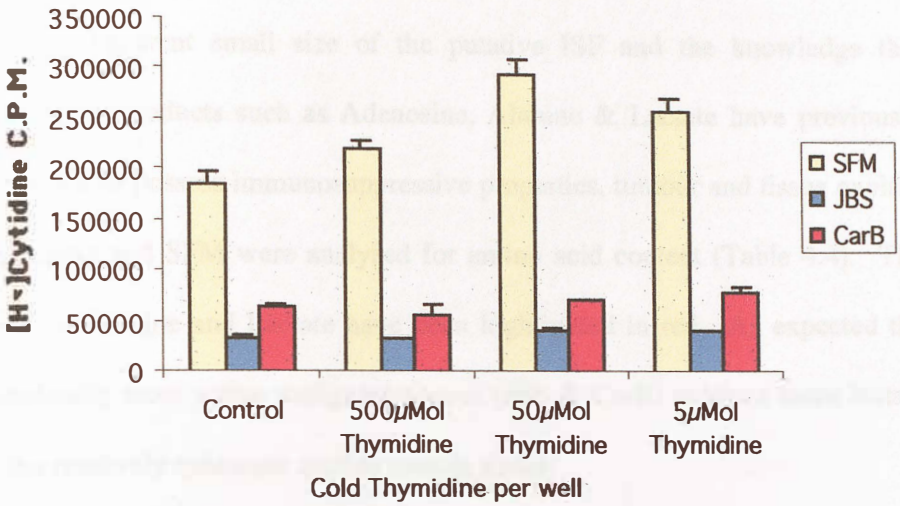


Fig. 4.22: Attempt at cold competition of unlabelled Thymidine with ISF on the suppressive effect of tumour explant supernatant on [^{3}H]Cytidine incorporation.

4.3.7 Amino Acid Analysis

Given the apparent small size of the putative ISF and the knowledge that metabolic by-products such as Adenosine, Alanine & Lactate have previously been shown to possess immunosuppressive properties, tumour and tissue explant supernatants and SFM were analysed for amino acid content (Table 4.4). The results for Alanine and Lactate have been highlighted in red. As expected the metabolically more active malignant tissues (JBS & CarB) produce more lactate than the relatively quiescent murine muscle tissue.

Table 4. 4: Amino acid analysis of serum free medium and tumour explant supernatants

	SFM	CarB <1kD	JBS <1kD	Control Balb <1kD
Alanine	0	169.462	218.548	199.78
Lactate(mM)	0	14.1	15.6	2.78
Glutamic Acid	37.451	204.882	350.094	693.912
Serine	558.758	346.764	297.227	95.532
Threonine	106.234	154.275	167.986	137.49
Glycine	30.526	100.786	122.672	75.996
Cysteine	31.876	29.637	21.452	20.258
Valine	98.409	150.164	165.548	146.738
Methionine	30.039	36.035	46.971	46.215
Isoleucine	107.474	136.429	147.126	140.808
Leucine	111.316	190.269	209.406	185.068
Tyrosine	77.124	107.518	119.196	96.99
Phenylalanine	76.712	103.471	118.333	117.561
Histidine	35.397	44.349	46.465	30.041
Lysine	175.33	374.75	410.929	298.525
Arginine	28.029	-9.545	-4.018	
Cysteic Acid	4.844	3.823	8.586	3.773
Aspartic Acid	2.158	20.143	105.059	12.955
Methionine Sulphone		96.97		50.734
Proline		70.93	98.64	41.15
Tryptophan				

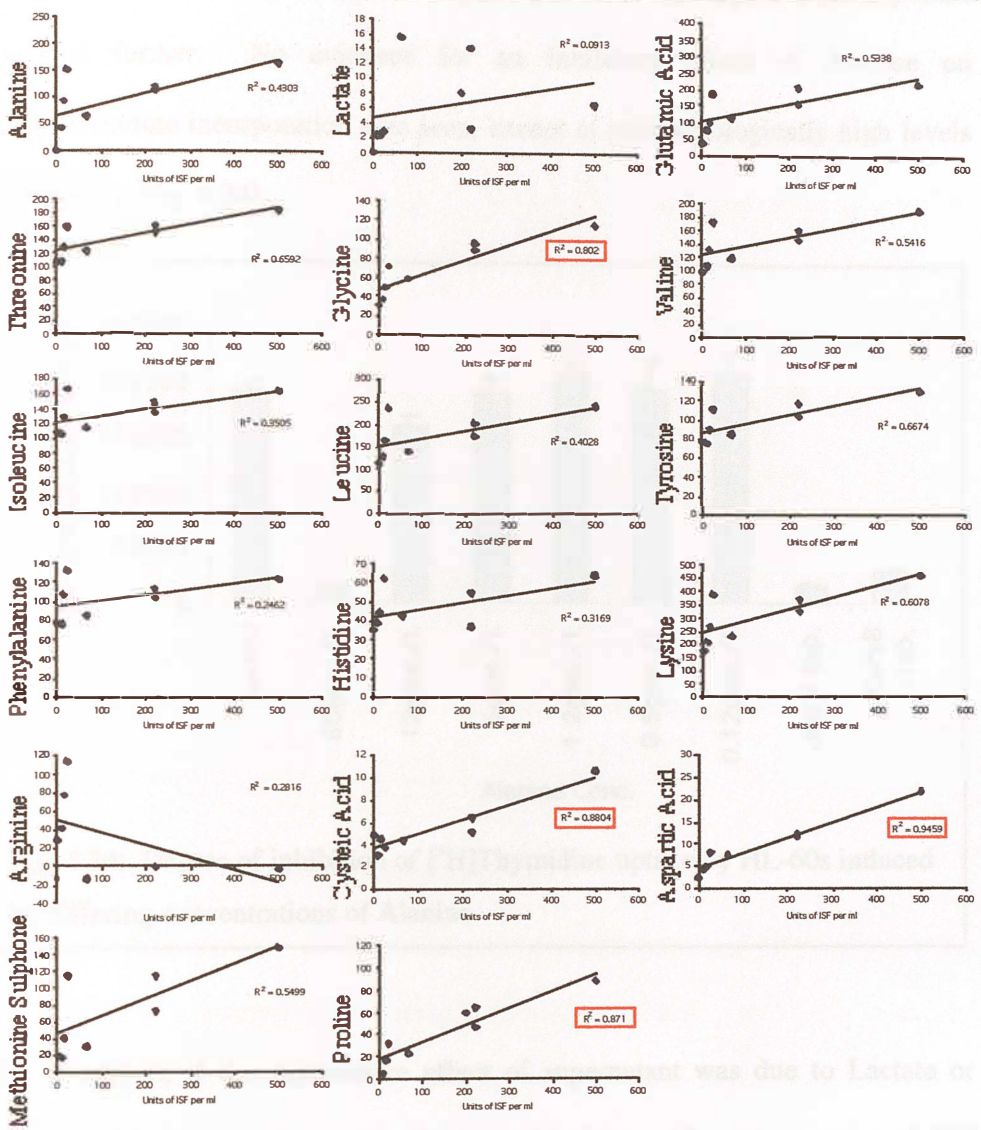


Fig. 4.23: Relationship between individual amino acid concentrations and suppressive activity of explant supernatants from differing tumours and control tissues (the amino acids with the best correlation with suppressive activity are circled in red)

The concentration of each amino acid in turn was graphed to the degree of suppressive activity of seven different supernatant solutions to see if any correlation could be found (Fig. 4.23). The greatest correlation was seen with Aspartic acid, Cysteic acid, Proline and Glycine. Alanine and Lactate levels

showed little correlation with levels of suppressive activity, however as these were the only products to be previously linked to immune suppression they were pursued further. No evidence for an inhibitory effect of Alanine on [^{3}H]Thymidine incorporation was seen, except at pharmacologically high levels of 60gms/l (Fig. 4.24).

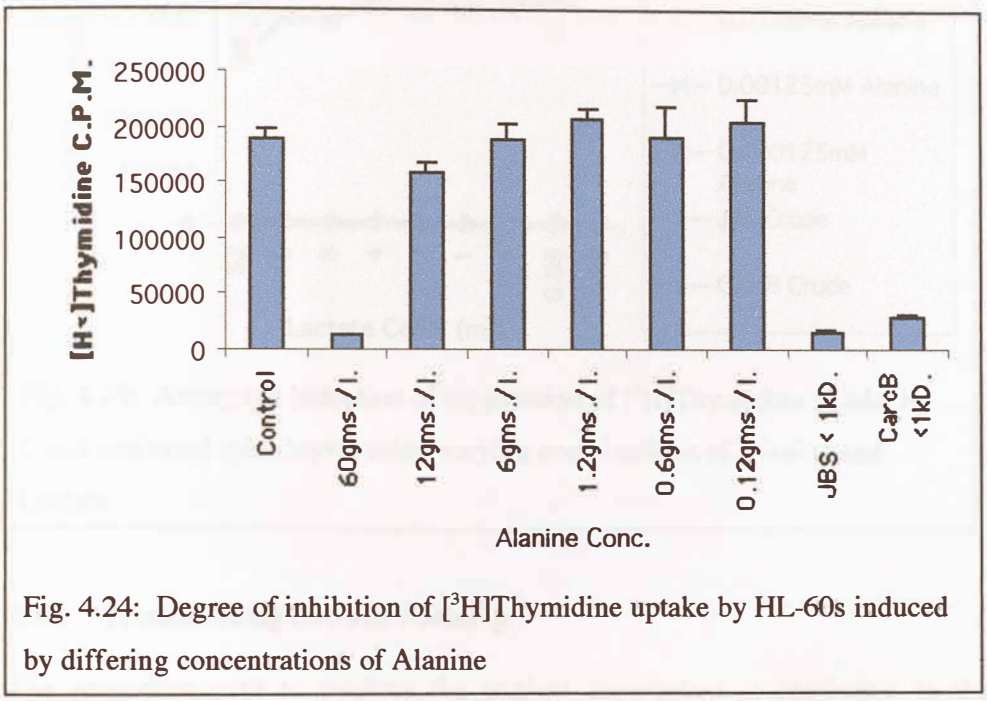


Fig. 4.24: Degree of inhibition of [^{3}H]Thymidine uptake by HL-60s induced by differing concentrations of Alanine

To investigate if the suppressive effect of supernatant was due to Lactate or Alanine alone or to a synergism between Alanine and Lactate a series of ISF assays were set up with varying concentrations of Alanine, Lactate and ISF (Fig. 4.25). Whereas the tumour explant supernatants were consistently inhibitory no inhibition of [^{3}H]Thymidine uptake was seen with Alanine or Lactate alone or in combination. Certainly in this model no evidence that the suppressive effect of tumour explant supernatants is due to Alanine or Lactate could be found. Perhaps given the crude correlations seen in Fig. 4.23 Aspartic acid, Cysteic

acid, Proline and Glycine might be amino acids more worthy of pursuit, particularly Proline in view of it's absence in SFM.

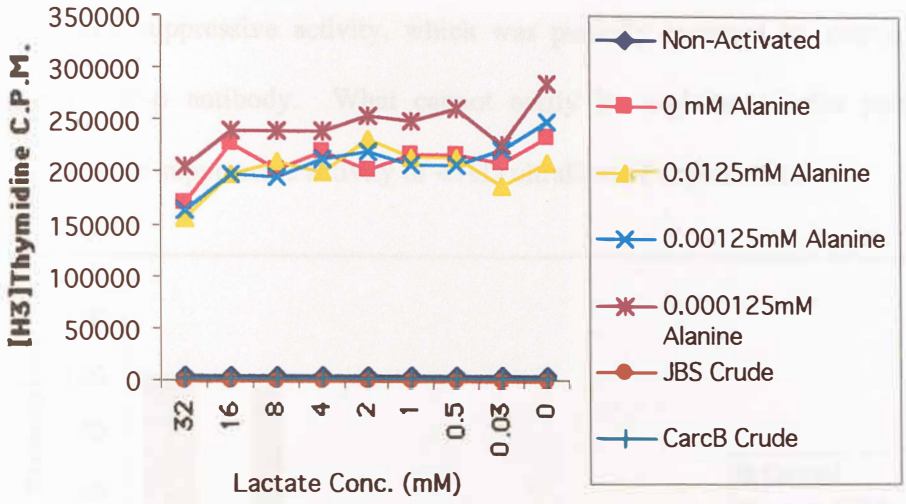


Fig. 4.25: Attempted induction of suppression of [^{3}H]Thymidine uptake by ConA activated splenocytes using varying combinations of Alanine and Lactate

4.3.8 Transforming Growth Factor-β

The procedure used to produce the explant supernatant is conducive to the generation of a number of differing suppressive factors, predictably some of which may be cytokines. I have already demonstrated that acid activation, a process known to activate TGF-β (Kehrl, 1986), of the >100kD retentate leads to a marked increase in suppressive activity (Table 4.3). The presence of TGF-β in the supernatant is further supported by treatment of fractionated and unfractionated supernatant with anti-TGF-β antibody (Fig. 4.26). Recombinant TGF-β produced marked suppression that was prevented by treatment with anti-TGF-β antibodies. OC-2 is a human oesophageal carcinoma cell line established in our unit, which produces large quantities of TGF-β (Irwin, 1996). Supernatant

produced from OC-2 tumours grown in nude mice exhibited marked suppressive activity, some of which was abrogated by prior treatment with anti-TGF-β antibody. Similarly supernatants produced from JBS and CarB tumours demonstrated suppressive activity, which was partially reversed by treatment with anti-TGF-β antibody. What cannot easily be explained is the partial reduction in the suppressive activity of <1kD ultrafiltered supernatants.

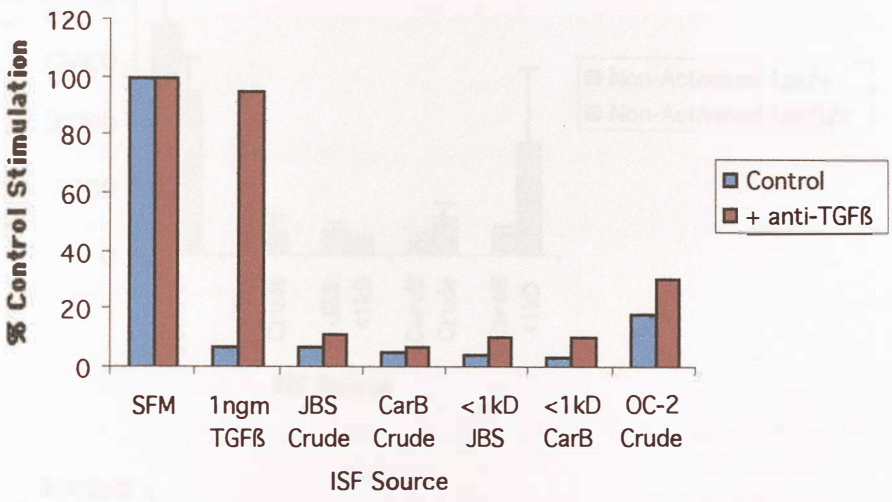


Fig. 4.26: Effect of anti-TGF-β antibody on the suppressive effect of various tumour explant supernatants on [^{3}H]Thymidine incorporation by ConA activated splenocytes.

4.3.9 Lpr Mice

Mice that carry spontaneous autosomal recessive mutations in chromosome 19 (lymphoproliferative or lpr) develop lymphadenopathy and splenomegaly. Homozygous lpr/lpr mice have defective expression of Fas and are resistant to apoptotic signals delivered by either Fas antibodies or perforin-free CTLs. This mouse model was used to investigate if the ISF effect of fractionated explant supernatants worked via the Fas receptor. It can be seen from Fig. 4.27, that

splenocytes from mice that are either heterozygous (Lpr/+) or homozygous (Lpr/Lpr) for the lpr mutation and therefore respectively expressing or not expressing Fas receptor remain sensitive to the suppressive effect whether activated or not. Thus the <1kD suppressive factor(s) do not act via the CD95 pathway.

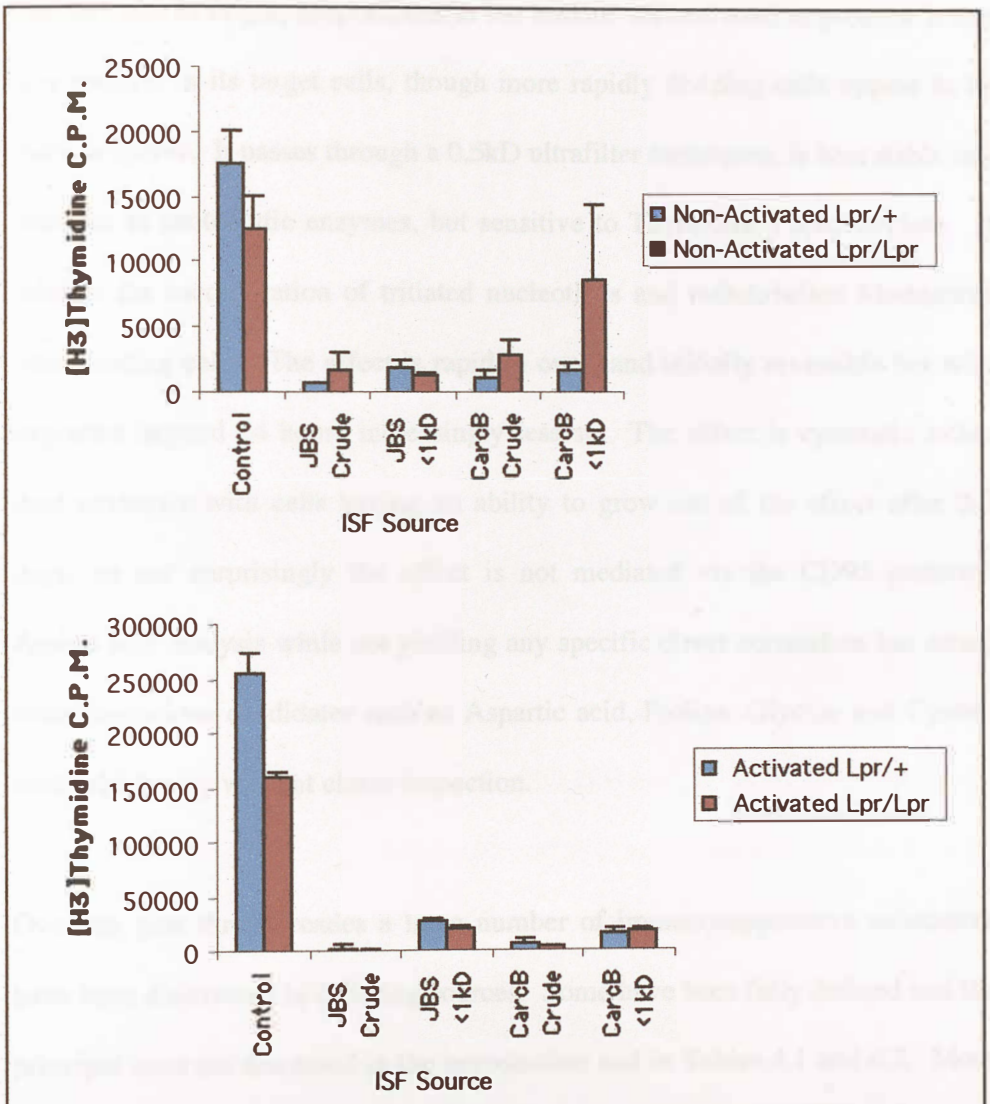


Fig. 4.27: Effect of tumour explant supernatant on [^{3}H]Thymidine incorporation into non-activated and activated splenocytes from Lpr mice.

4.4 Discussion

In this chapter evidence has been presented for the existence of tumour derived immunosuppressive factors. One of which is almost certainly TGF-β - retained by 100kD ultrafiltration membrane, acid activated and neutralised by anti-TGF-β antibody. The other factor has been partially defined as being exclusive to tumour cells in origin, proportional to the tumour volume used to produce it and non-specific in its target cells, though more rapidly dividing cells appear to be more sensitive. It passes through a 0.5kD ultrafilter membrane, is heat stable and resistant to proteolytic enzymes, but sensitive to Thymidine Phosphorylase. It inhibits the incorporation of tritiated nucleotides and radiolabelled Methionine into dividing cells. The effect is rapid in onset and initially reversible but with exposure beyond 24 hours increasingly less so. The effect is cytostatic rather than cytotoxic with cells having an ability to grow out of the effect after 2-3 days, so not surprisingly the effect is not mediated via the CD95 pathway. Amino acid analysis while not yielding any specific direct correlation has raised some suspicious candidates such as Aspartic acid, Proline, Glycine and Cysteic acid, which may warrant closer inspection.

Over the past three decades a large number of immunosuppressive substances have been discovered in differing sources. Some have been fully defined and the principal ones are discussed in the introduction and in Tables 4.1 and 4.2. More have been generated from tumour extracts and culture supernatants or found in sera and ascites of tumours of differing histological origin. Because they have only been partially, biochemically and biologically, defined they have been given a designation by the scientist involved based on their origin or effect. This has

lead to the use of a wide variety of synonyms to describe the suppressive entity in solution; not unlike the chaos that existed before the introduction of the CD system. Examples include Immunosuppressive Factor (ISF, my preferred choice), Lysis Resistance-Inducing Factor (LRIF), HT-29 Factor, Immunosuppressive Substance (IS), Immunosuppressive Acidic Protein (IAP), Immunoregulatory Factors (IRF), Tumour-Derived Immunosuppressive Factors (TDS). Such cumbersome and personalised nomenclature serves to confuse and may hide similar substances under different titles, however until they are fully defined we are encumbered with them.

Other workers in our unit, who have been studying immunosuppression in patients with predominantly gastrointestinal malignancies have reported on the existence of an immune suppressive factor (ISF) derived from oesophageal squamous cell carcinoma (SCC) (O'Mahony, O'Sullivan et al. 1993). O'Mahony described the partial purification of a factor, with an apparent molecular weight of 25kD, from medium conditioned with minced tumour fragments. She reported that incubation of Jurkat cells with ISF containing medium induced a Ca^{++} influx into the cells. Incubation of ISF with mitogen activated PBLs induced a cell-cycle arrest at the G_1/S interphase together with a marked inhibition of [^{3}H]Thymidine incorporation. These effects were not reversible and prolonged exposure to ISF (>24 hours) produced apoptosis in 20% of PBLs and >90% of Jurkat cells. This suppressive activity could not be detected in medium conditioned with normal oesophageal tissue. Treatment of PBLs with ISF while inhibiting proliferation and [^{3}H]Thymidine incorporation did allow the expression of activation markers such as IL-2 and Transferrin receptors. The

suppressive effect was restricted to cells of lymphoid/myeloid origin (Collins and O'Sullivan 1994). Evidence for *in vivo* activity of the putative ISF came by comparing proliferative and cytotoxic responses of lymphocytes draining oesophageal SCC with distal non-draining 'reference' nodes and PBLs from the same patient (O'Sullivan, Corbett et al. 1996). They found relatively depressed responses in cells from tumour draining nodes implying a zonal or regional field of immunosuppression around oesophageal SCC. No such effect was seen in patients with oesophageal adenocarcinoma of whom all but 2 of 26 had normal or enhanced lymphocyte responsiveness in tumour draining nodes.

This ISF differs from that generated in my experiments by virtue of size, lack of reversibility and specificity of effect. However in O'Mahony's work she was using tumour explant cultured supernatant that had been dialysed against PBS across a 5kD membrane. Similar treatment of JBS or CarB cultured supernatant would have resulted in the loss of the ISF that I have described (as happened in DTT and TCA treatment). Contemporary workers in our unit have demonstrated throught the use of *in vitro* 3-D gel culture assays and the use of monoclonal antibodies that what O'Mahony was describing were probably the effects of TGF-β [Irwin, 1996 #715]. I have also shown evidence that suggests the presence of small amounts of TGF-β in the crude culture supernatant of JBS and CarB tumour explants (i.e. suppressive activity that is acid-activatable and can be partially blocked by anti-TGF-β monoclonal antibodies.

The identification of human oesophageal squamous carcinoma (OC-2) derived ISF is currently the subject of two full-time PhD theses in our unit (S. Nolan and

F. Kenny). F. Kenny concentrating on the existence of a smaller ISF in OC-2 conditioned medium has identified activity with identical properties to that described by myself in JBS and CarB supernatants. That is he has evidence of ISF activity that is <1kD in size, rapid in onset, non-specific in effect and exquisitely sensitive to treatment with Thymidine Phosphorylase (F. Kenny, personal communication). S. Nolan on the other hand has been approaching the isolation and identification of OC-2 ISF from a biochemical standpoint. Utilising techniques such as Gas Chromatography Mass Spectroscopy, Liquid Chromatography Mass Spectroscopy and Thin Layer Chromatography she has found evidence suggestive of two peptides each less than 1kD in size which are both immunosuppressive and appear to have a synergistic effect (S. Nolan personal communication)

Ebert *et al.* describe a soluble factor produced by HT-29 (colon cancer cell line) cells that blocks mitogen-induced T cell proliferation and IL-2 production (Ebert, Roberts et al. 1987). Like the factor described herein Ebert's HT-29 Factor is non-toxic, has a very rapid onset of action and is reversible. The factor was not found in media conditioned by normal colonic epithelial cells or fibroblasts. JBS and CarB ISF are not HT-29 factor however as the latter has an apparent molecular weight of 56kD, is heat labile and sensitive to endopeptidases.

Suppressive activity attributable to oesophageal SCC has also been found in aqueous extracts of oesophageal tumours (Mohagheghpour, Parhami et al. 1979). They found that supernatant from homogenised oesophageal SCC (TEx) inhibited [^{3}H]Thymidine and [^{3}H]Leucine incorporation into PBLs. The effect

was reversible by washing. The activity was ascribed to a heat-labile protein with an approximate molecular weight of 70-80kD. The absence of very small molecular weight inhibitory factors may be due to the dialysis step used in supernatant generation.

Immunosuppressive substances have been found in the sera of patients with malignant disease. Yamanaka and colleagues identified the presence of Immunosuppressive Substance (IS) and Immunosuppressive Acidic Protein (IAP) in the sera of patients with Head and Neck carcinomas in differing anatomical locations. Forty to fifty percent of patients with H+N cancer had significantly higher serum levels of IS and IAP than control patients (Yamanaka, Harabuchi et al. 1988). They also found that serum IS levels decreased after treatment of H+N cancers and rose again with recurrent disease. They point out however that IS and IAP may not be tumour-derived but rather products of the host in response to tumour and thus could not be generated in our system of ISF production.

Immunosuppressive Acidic Protein (IAP), though certainly not the suppressive element in JBS and CarB ISF, warrants further mention here, for completeness. IAP is a type of α-1-acid-glycoprotein with a molecular weight of 50kD, initially isolated from ascites of cancer patients (Tamura, Shibata et al. 1981). The serum levels of IAP have been shown to have a significant positive correlation with stage and prognosis in patients with Renal Cell Ca. (Iizumi, Yazaki et al. 1985; Ueda, Yasumasu et al. 1993; Fujikawa, Matsui et al. 2000); testicular cancer (Miki, Saiki et al. 1987): ovarian cancer (Koebl, Tatra et al. 1988); and brain

tumours (Kikuchi, Gotoh et al. 1990). It is also elevated in inflammatory processes such as Multiple Sclerosis, Guillain-Barre (Tsukamoto, Seki et al. 1986) and inflammatory bowel disease, with no correlation between levels of IAP and suppressive effect of sera in patients with Crohn's disease (Watanabe, Tsuru et al. 1984). Given the association between IAP and inflammatory processes, it is not surprising to discover that IAP is produced by macrophages in tumour-bearing hosts (Shibata, Tamura et al. 1983). It has been localised to the cell surface and cytoplasm of macrophages in inflammatory and neoplastic conditions (Aozasa, Ueda et al. 1987). IAP has been shown to selectively suppress NK cell activity (Aso, Tamura et al. 1992), mitogen-induced lymphocyte activation (Kikuchi, Gotoh et al. 1990) and lymphocyte surface CD4 antigen expression (Yamaguchi, Miyahara et al. 1995).

There are precedents for tumour-induced but host derived suppressive factors, for example Lysis Resistance-Inducing Factor (LRIF) which R. Saxena found protected YAC lymphoma cells from NK-cell lysis by inducing up-regulation of MHC class I antigen expression (Saxena, Saxena et al. 1988). LRIF is a heat labile protein with an approximate molecular weight of 12.6kD.

The suppressive factor described in the references cited above is reversible, heat stable and protease resistant. Roth *et al.* found immunoregulatory factors (IRF) with similar properties in supernatants of extracts from melanoma, lung carcinoma and sarcoma tumours. These IRFs inhibited [^{3}H]Thymidine and [^{3}H]Leucine incorporation into mitogen-activated PBLs (Roth, Grimm et al. 1982). They found that IRFs from these histologically different tumours possess

similar biochemical and immunological characteristics. The suppressive effect was non-specific, non-toxic and does not interfere with [^{3}H]Thymidine transport. Based on lectin affinity they determined that the IRFs are glycoproteins but did not estimate a molecular size. The similarities with JBS and CarB ISFs are remarkable but only the melanoma-derived IRF is both heat-stable and reversible. The IRFs are however partially sensitive to trypsin which JBS and CarB-derived ISFs are not.

Immunosuppressive factors have been identified in medium conditioned by Colo 205 cells by Brotherick *et al.* The immunosuppressive properties were abrogated by heat treatment and tryptic enzyme digestion. Further differences with JBS and CarB ISF are a build-up of cells in S-phase with a concomitant fall in G_1/G_0 cell numbers (Brotherick, Shenton et al. 1993). Gel filtration yielded two immunosuppressive fractions with approximate molecular weights of 60 and 20kD, too large to be similar to JBS and CarB ISF. Siwek *et al.* isolated suppressive factors from medium conditioned by a breast cancer cell line but not by a non-malignant breast cell line (Siwek, Lacroix et al. 1997). They describe at least three non-prostanoid factors of low molecular weight (apparent M_w of 0.7, 1.5 and 4kD), the closest in size to JBS and CarB ISF. Siwek's conditioned medium inhibited by 75% DNA synthesis in an osteoblast cell line. The effect was reversible and not due to cytotoxicity. The suppressive effect was specific for osteoblasts, having no effect on [^{3}H]Thymidine uptake by fibroblasts or fibrosarcoma cells. The factors are heat-stable, probable peptides.

The JBS and CarB ISF is too small to be any of the known cytokines described in the introduction. Though there are similarities between the ISF we describe and previously reported, partially defined ISFs. There are sufficient differences to say that none of these represent JBS or CarB ISF. The small size and apparent stability of the latter encouraged the pursuit of possible metabolic/degradation by-products as the source of the suppressive activity.

Nitric oxide (NO) is a cytotoxic and anti-proliferative metabolite of L-arginine, which has been shown in vitro to inhibit lymphocyte proliferation and NK-cell cytotoxicity (Albina, Abate et al. 1991). Though not produced by tumours directly, it has been shown *in vitro* that growing tumours induce macrophage production of NO via the release of unknown soluble mediators (Lejeune, Lagadec et al. 1994). The induction by tumour cells of a non-specific cytotoxic factor such as NO would appear to be suicidal however Lejeune *et al.* found that tumour cell proliferation was only partially inhibited whereas PBLs were totally inhibited by GTN (a NO-releasing compound). It appears that T lymphocytes are more sensitive to the effects of NO than tumour cells. More interestingly Alleva and co-workers looked at differential production of NO and TNF-α by macrophages in tumour-bearing animals. They compared the production of these suppressive products between tumour associated/infiltrating macrophages and distal non-tumour-infiltrating peritoneal macrophages (Alleva, Burger et al. 1994). They found that peritoneal macrophages from tumour-bearing hosts produced more NO than normal mice. Co-culture of non-tumour infiltrating peritoneal macrophages with tumour cell supernatants induced down-regulation of NO production, which was restored by antibody-mediated absorption of IL-

10, TGF-β and PGE_2 from the supernatant. They conclude that tumour growth promotes distal macrophage suppressor activity by increasing production of NO and concomitantly down-regulating local NO and TNF-α production.

Hegardt *et al.* have recently shown in a murine Glioma model that tumour growth is associated with impaired NK and T-cell responses due to adherent spleen cell production of NO which could be counteracted by the NO-synthase inhibitor N-nitro-1-arginine methyl ester(Hegardt, Widegren et al. 2000).

Though not specifically looked for, it does not appear likely that the suppressive effect of JBS and CarB ISF is due to NO because 1) this is a macrophage product and adherent cells are removed during the preparation of splenocytes for assay; 2) the speed of onset of ISF effect mitigates against it; 3) JBS and CarB supernatant inhibit cultured cell line proliferation where no macrophages could be involved; 4) even if there were tumour associated macrophages in the tumour fragments (not specifically looked for) used to generate supernatant, Alleva's work would suggest that these are the least likely to produce NO.

There have been numerous papers and indeed journals devoted to the impact of nutritional status on patient immune function in disease states (cancer, sepsis, burns, post-surgery) and under exercise stress. Glutamine is a good example of an amino acid to have reached such importance. Most intensive care unit patients will have glutamine added to the parenteral nutrition at the first available opportunity, not without evidence. The provision of glutamine to patients following bone marrow transplantation results in lower levels of infection and

shorter hospital stay than control patients on glutamine-free parenteral nutrition (Calder and Yaqoob 1999). The provision of parenteral glutamine may prevent the occurrence of systemic inflammatory response syndrome (SIRS) after major surgery (O'Flaherty and Bouchier-Hayes 1999). The post-operative state has a well-known association with immunosuppression and also of compromised nutrition with low levels of amino acids, particularly branched chain amino acids (Jain, Rush Jr et al. 1981). Perhaps the two are related. Attempts to correlate plasma free amino acids with the presence of malignant disease have met with some success (Scioscia, Snyderman et al. 1998). Others have gone further and suggested a correlation between amino acid profiles and the anatomical site of origin of the tumour (Kubota, Meguid et al. 1992). Parallels between the immune impairment seen with vigorous exercise and after major surgery have been noted previously (Shepard and Shek 1996). Pederson and Toft in a Medline review of available literature concluded that while moderate exercise across a lifespan seemed to increase resistance to upper respiratory tract infections, repeated strenuous exercise suppressed immune function, and cellular immunity in particular (Pedersen and Toft 2000). Some of this exercise-induced immune suppression could be prevented by prior treatment with indomethacin, suggesting an aetiological role for prostaglandins (Asselin, Benquet et al. 1996). Moreover exercising muscles utilise anaerobic glycolysis and consequently produce large amounts of L-lactate and L-alanine. Both of these products together and in isolation have been reported to have immunosuppressive properties. Because tumour cells are reported to have strong glycolytic activity it was hypothesised that tumours might release large quantities of alanine and lactate. This was confirmed by Droge *et al.* in Germany. They reported that Esb, a weakly

immunogenic T-cell lymphoma line, produced substantial amounts of alanine and lactate in culture supernatants (Droge, Eck et al. 1986). A strongly immunogeneic variant Esb-D produced supernatants with lactate but no detectable alanine. They also report that injection of alanine and lactate in vivo impaired the generation of cytotoxic T cells in mice. They suggested that the difference in immunogenecity between the two cell lines was related to the production and release or not of alanine (Droge, Wehrmaker et al. 1988). Certainly both JBS and CarB tumours produce culture supernatants rich in alanine and lactate (Table 4.4). It is noteworthy that Balb/c control tissue (largely derived from muscle, with it's known glycolytic activity) also produced large amounts of alanine but not as much lactate. The lack of any significant suppressive activity in the <1kD Control Balb/c supernatant would support the hypothesis that it is the combination of alanine and lactate that may have the suppressive potential. Attempts to induce *in vitro* suppression of lymphocyte proliferation with exogenous alanine alone (Fig. 4.23) or in combination with lactate (Fig. 4.24) were unsuccessful with the exception of significant suppression seen with 60 gm/l of alanine alone. This concentration of alanine is vastly in excess of those seen in tumour explant supernatants. Droge would argue that at the level of the cytotoxic cell/target cell interface these high concentrations may be achieved and thus alanine would be significant. However this does not explain the suppression attributable to the supernatants detailed above.

It has been known for a long time that all normal subjects excrete in their urine several modified purines and pyramidines. Most of these originate from transfer

RNA (tRNA) (Borek, Sharma et al. 1983). Of the more than 200 enzymes involved in tRNA synthesis, those that methylate tRNA are quantitatively aberrantly hyperactive and qualitatively different from those of normal tissue in patients with malignant disease. The methylation of nucleosides prevents their insertion into the incorrect location during tRNA synthesis and obligates their excretion. It is well known that cancer patients excrete elevated levels of these modified nucleosides (Waalkes, Dinsmore et al. 1973). Borek demonstrated that the elevated levels of modified nucleosides in cancer patients do not originate from massive tumour cell breakdown but rather from increased enzyme activity as the amount of modified nucleosides found in the urine would require tumours as large as 6kg in patients with breast cancer (Borek, Waalkes et al. 1983). Further confirmation of the malignant origin of modified nucleosides came from Masuda *et al.* who demonstrated their immunohistochemical localisation to oesophageal carcinoma only with minimal staining of normal oesophageal epithelium (Masuda, Nishihira et al. 1993). Originally determined on 24-hour urine collections, Borek and co. were able to confirm the validity and reliability of spot urine analysis for modified nucleosides, provided they were expressed as a ratio to urine creatinine. As well as compensating for the contribution of total body mass and diet, assessment of urinary creatinine allows compensation for artificially low creatinine associated with renal dysfunction which can give rise to falsely elevated levels of modified nucleosides (Gehrke, Kuo et al. 1979; Honda, Itoh et al. 1999).

A number of modified nucleosides have been described psuedouridine, 1-methyladenosine, 1-methylinosine, N^2-methylguanosine and N^2,N^2-

dimethylguanosine (Waalkes, Abeloff et al. 1982). Their presence in urine has been shown to correlate with the presence of malignant diseases such as breast (Xu, Schmid et al. 2000); genito-urinary (Koshida, Harmenberg et al. 1985); small cell lung cancer – where it has been shown to relate to the burden and stage of the disease (Lu, Lai et al. 1994); acute lymphoblastic leukaemia – where the urinary levels reflect disease activity (Heldman, Grever et al. 1983); hepatoma – where combined with α-fetoprotein estimation had >90% correlation with diagnosis (Tu, Xu et al. 1995); non-Hodgkin's lymphoma – where excretion of pseudouridine has been identified as an independent prognostic factor (Rasmuson and Bjork 1995); a positive correlation with stage of disease has also been demonstrated between pseudouridine and adenocarcinoma of the large bowel (Motyl, Traczyk et al. 1993). It would appear that modified nucleosides are common to all malignancies, however in a comparison between patients with brain tumours and controls no significant difference in urinary concentrations of pseudouridine could be found (Manjula, Aroor et al. 1993). The use of urinary modified nucleosides as a marker for the presence of malignant disease should be treated with caution as elevated levels have been found in patients with immunodeficiency and psoriasis (Mills and Mills 1985; Clemmensen, Kieffer et al. 1987).

The pervasive presence of these modified nucleosides in serum, urine and ascitic fluid of patients with malignant disease has been described, and very little work on their effect on the immune system has been reported. Ishida's group have demonstrated that methyladenosine markedly enhanced murine susceptibility and mortality to Listeria infection probably by impairing macrophage function

(Takano, Sami et al. 1986). Adenosine, a nucleoside used in RNA and DNA synthesis and in energy transfer intracellularly, has been shown to accumulate at high concentrations in solid tumours, stimulating tumour growth and inhibiting adhesion of immune cells and function of T-cells, macrophages and NK-cells (Spychala 2000). The effects of modified nucleosides have been so remarkable that they have helped to generate a new group of anti-cancer drugs, the purine analogues (Cheson 1998; Cheson, Sorenson et al. 1998). These new drugs have not been without side effects interestingly they have been shown to induce programmed cell death in lymphocytes including quiescent ones probably by an induced acceleration of DNA strand breaks (Dighiero 1996). Unfortunately they are so effective that second malignancies (lymphoma, prostate, lung, colon, bladder and breast) have already been reported in patients who received nucleoside analogue therapy for leukaemia (Cheson, Vena et al. 1999).

The susceptibility of JBS and CarB derived ISF to partial inhibition by treatment with Thymidine Phosphorylase (Figs. 4.20 & 4.21) and the apparent size of the ISF would suggest that the ISF could potentially be modified nucleosides/nucleotides. However the absence of any reduction in suppressive activity by attempts at cold competition with unlabelled Thymidine (Fig.4.22) would suggest that the ISF is not an analogue of Thymidine. Reconciling these two observations is not easy, perhaps the ISF is an alternative modified pyrimidine (uridine or cytidine) or slightly less likely, given the slight structural differences, a modified purine (adenosine or guanosine). The fly in this ointment is the report by Ishida that the modified nucleotides are heat labile, with

destruction of their suppressive activity by heating to 56°C for 30 mins (Takano, Umenai et al. 1983).

4.5 Conclusions

Evidence has been presented that confirms the production by malignant tissue of soluble factors capable on inhibiting a variety of cellular functions such as cell division, nucleotide incorporation, protein manufacture. The inhibitory effect is rapid in onset, initially cytostatic and reversible but not reversible with prolonged exposure. ISFs derived from both tumours exhibited similar functional properties. Neither could they be distinguished on the basis of size or susceptibility to heat and enzyme degradation. It would appear that they may be the same entity or at least from the same family of molecules.

Other investigators in our unit (Kenny and Nolan) are continuing to attempt to isolate and identify the ISF entity in <1kD OC-2 ISF (which appears to have exactly the same physical and functional properties as I have described here) and will hopefully produce methodologies to further elucidate the true nature of the ISF. The ISF has proven very elusive to-date and it was decided to store JBS and CarB tumour cultured supernatants both crude and <1kD fractions to await the definition of the steps necessary to identify it/them.

Chapter 5

Mechanism Of Immune Escape In Immunologically Heterogeneous Tumours

(Part of this chapter was published in Surgical Forum, XLVII: 472 – 475; 1996)

5.1 Introduction

We have established in chapter 3 changes in a wide variety of cellular immune functions and will show in chapter 6 that the JBS and CarB cells possess antigens capable of immune recognition. In chapter 4 *in vitro* evidence of tumour-derived immunosuppressive factors has been presented. We do not know to what extent, if any, *in vivo* production of this ISF is responsible for protecting the developing tumour from immune elimination. We therefore wished to examine in a mixed tumour model whether the putative elaboration of tumour derived ISFs by a weakly immunogenic cell would protect a strongly antigenic cell from elimination or would the additional stimulation of a strongly antigenic (normally not able to produce a tumour in immunocompetant hosts) cell line induce such a potent immune response that both would be eliminated.

With a few exceptions, such as some hereditary tumours, e.g. neurofibromatosis, some invasive carcinomas of the cervix and some parathyroid adenomas, the majority of cancers, at least at the time of diagnosis, appear to arise from the clonal expansion of a mutated single cell (Fialkow 1979). These conclusions were reached through the innovative study of isoforms of the X-linked enzyme Glucose-6-phosphate dehydrogenase (G6PD). Due to the inactivation of one X-chromosome per cell, women who are heterozygous for G6PD will only express 1 isoenzyme per cell. Examining the tumour cell expression of G6PD in heterozygous women with CML, they found expression of only 1 form in 100% of patients studied (Fialkow 1976). Despite this clonal origin, there is extensive evidence of the existence of differing subpopulations of cells between different tumours (intertumoural heterogeneity) and within any given tumour

(intratumoural heterogeneity). Subpopulations have been reported with different cellular morphology, karyotype, growth rate, cell products, receptor expression and metastatic ability (Heppner 1984). That a single cell can give rise to a diverse population is not a new concept, remember 'Dolly' the sheep derived from the nuclear transfer of an established committed cell (Campbell, McWhir et al. 1996).

The emergence of a heterogeneous tumour cell population is believed to be the consequence of tumour evolution or progression. Tumour progression was defined by Foulds as the 'permanent, irreversible qualitative change in one or more of its characters' (Foulds 1954). He attributes the original concept to Rous, who described the progression to carcinoma of virus-induced rabbit papillomas (Rous and Beard 1935). Ironically Rous decried the role of mutations in the genesis of cancer "A favourite explanation has been that [carcinogens] cause alterations in the genes of cells of the body, somatic mutations as these are termed. But numerous facts, when taken together, decisively exclude this supposition" (Bishop 1991). Tumour progression is the evolutionary consequence of the combined effects of genetic instability and tumour micro-environmental/host factors. Nowell, in 1976, suggested that acquired genetic instability was the driving force behind the development of tumour cell heterogeneity (Nowell 1976). He proposed that for most cells, these genetic alterations result in cell death either 'because of metabolic disadvantage or immunologic destruction, but occasionally one has an additional selective advantage with respect to the original tumour as well normal cells, and this mutant becomes the precursor of a new predominant subpopulation'. This

genetic instability is largely the consequence of impaired DNA-repair mechanisms (Devilee and Cornelisse 1994). The genetic basis of carcinogenesis and phenotypic heterogeneity continues to be unravelled, with large numbers of proto-oncogenes and tumour suppressor genes now described (Bishop 1991). Colorectal tumours are amongst the most extensively studied and understood cancers. They provide a good example of the depth of genetic information that is being discovered. Most, if not all, malignant colorectal cancers arise in pre-existing benign adenomas (which themselves may have an inherited predisposition) and progress through defined stages to invasive metastatic carcinoma. *En route,* mutations in 4 or 5 genes are required for the formation of a malignant tumour. The mutations most commonly involved affect chromosome 5q – mutation or loss of the familial adenomatous polyposis (FAP) gene; chromosome 12p – mutation of *ras* gene, usually K-*ras*; chromosome 18q – loss of DCC gene; chromosome 17p – loss of the tumour suppressor gene p53 (Fearon and Vogelstein 1990). The order in which the mutations occur is not critical it is the overall accumulation that is important and the eventual development of cancer, which is governed by unknown factors but the extent of persistent exposure to carcinogens must remain a critical factor.

Once the tumour becomes established the degree of genetic instability seems to escalate. For example early in the development of leukaemias few chromosomal changes are present. However, towards the more advanced stages of the disease the number of karyotypic changes increases significantly (Shtivelman, Lifshitz et al. 1985). Using murine melanoma cell lines, Ling investigated the rate of emergence of metastatic variants from what begin as non-metastatic populations

(Harris, Chambers et al. 1982). They found that cells capable of forming experimental pulmonary metastases were generated at an effective rate if 1.3 X 10^{-5} per cell per generation for the B16F1 cell line and at a rate of 5 X 10^{-5} per cell per generation by the B16F10 cell line (Hill, Chambers et al. 1984). This suggests that the higher rate of generation of variants in the B16F10 population explains its higher metastatic ability. They introduced the concept of 'dynamic heterogeneity' to emphasise that heterogeneous tumours are not composed entirely of stable subpopulations but have additional cells in a state of genetic flux. This genotypic diversity is associated with increased phenotypic variability. For example the rate of appearance of cell variants with different levels of expression of cell surface glycoproteins was found to be several fold higher in breast carcinoma cell lines than in normal breast tissue (Peterson, Ceriani et al. 1983).

The genetic mutations emerge under the influence of selective environmental pressures such as regional differences in oxygen and nutrient supply, inflammatory infiltrates (these have been discussed in other chapters). DNA instability on the one hand and the selection by environmental factors on the other determine the phenotype of a clinically manifest tumour. Despite the random nature of the genetic mutations that occur, non-random cyclical patterns of antigen expression have been reported in subsequent generations of tumour cell lines. For example in a murine mammary tumour model successive generations were seen to go from a dependence on hormone for growth to a hormone independent state (Kiang, King et al. 1982). Over the course of subsequent generations they observed a cyclical reappearance/disappearance of

progesterone and oestrogen receptors and also in DNA ploidy and cellular thymidine kinase activity. Others have also reported the cyclical expression of antigens but their significance or implications have not been fully established. Paradoxically, even though variability is necessary to evolution, the great heterogeneity within tumours could be interpreted as suggesting that selection is not a major determinant in the population biology of cancer. Poste has suggested that in 'polyclonal tumours some form of 'interaction' is occurring between the constituent clonal subpopulations. Thus, in a polyclonal population containing multiple subpopulations an apparent 'equilibrium' exists' (Poste and Greig 1982). Evidence of just such a relationship can be seen in the interactions between three different Ehrlich carcinoma cell lines (Aabo, Vindelov et al. 1994). They report that 2 of the 3 cell lines demonstrated contact inhibition of the other cell lines when grown as mixed tumours or ascites but not if injected separately into opposite flanks of the animal. Clearly, a complete understanding of the impact of tumour cell heterogeneity on the biology of the tumour as a whole, in particular on predicting the response to various therapies, cannot be attained by reductionist, analysis of the individual component subpopulations.

5.1.2 Aims

Tumours evoke variable immunologic responses and are heterogeneous in terms of antigen spectrum and expression. It has been suggested that tumour cells that are destroyed will attract immune cells that may react with poorly antigenic subpopulatons killing them as a bystander effect (Fleuren, Gorter et al. 1995). Others have suggested that immune reactions to tumours would selectively eliminate only the strongly immunogenic cells and allow continued development

of tumours whose cells are weakly antigenic or non-immunogenic (Topalian, Kasid et al. 1990). Or perhaps at the individual cellular level some cells are capable of protecting themselves through mechanisms described in chapters 1 and 4, such as antigen loss, FasL expression or elaboration of ISF.

Using tumours of mixed antigenicity, initially 50:50 weakly antigenic cells JBS or CarB with strongly antigenic SV3T3 cells, this hypothesis was tested *in vivo*. Mixed tumours will be induced and their growth curves constructed and compared with tumours of a single cell line. Tumours will be harvested at various stages and immunohistochemically stained for the presence of large-T antigen, the potent TSTA on SV3T3 cells. Tumour sections will also be stained with Haematoxylin and Eosin to look for the presence of an immune infiltrate. It is not intended to co-localise any inflammatory any inflammatory infiltrate and expression of large-T antigen.

5.2 Materials and Methods

The cell lines and animals were maintained as described in Chapter 2, General Materials and Methods (Sections 2.1 & 2.3). For strongly antigenic cells, we used SV3T3, an SV40 transformed 3T3 cell line, which expresses T-antigen, a potent viral coded tumour specific transplantation antigen (TSTA). Initiation of transformation by simian virus 40 (SV40) requires the expression of only the early region of the viral genome, which encodes two proteins: the large T antigen (MW $\approx$ 94,000) and small t antigen (MW $\approx$ 17,000). Large T antigen is the protein required for initiation of transformation and it has been strongly suggested that the continued expression of functional large T antigen is necessary

for maintenance of the transformed phenotype (Rigby and Lane 1983). Large T antigen, though located predominantly in the nucleus, has been shown to be present on the cell surface of SV40-transformed cells and to act as a transplantation rejection antigen with induction of a CTL response (Gurney, Harrison et al. 1980; Tevethia, Flyer et al. 1980).

For weakly antigenic cells, we used either the JBS cell line, which is a T-antigen negative variant of the original SV3T3 cell line, or the DMBA induced TPA potentiated squamous carcinoma cell line, CarB.

5.2.1 Tumour induction and monitoring

Equal numbers of SV3T3 and JBS or SV3T3 and CarB cells were admixed and 2 X 10^6 cells inoculated subcutaneously into the right flank of 8-10 week old, immunocompetant, Balb/c and athymic nude mice. Developing tumours were measured on alternate days in two dimensions with a Vernier callipers and tumour volumes calculated using the formula ($\pi/6(l+s^2)$) where l = longest dimension and s = shortest. These tumour volumes were used to construct growth curves.

5.2.2 Tumour Extraction

At four to five day intervals animals were sacrificed by anaesthetising with Ether prior to culling by cervical dislocation. Under strict asepsis their tumours were excised and weighed. Tumour segments were snap frozen in liquid nitrogen and stored at –20°C for later preparation of frozen sections and immunostaining.

Alternatively, tumour segments were fixed in 10% formal-saline and then wax embedded for storage and subsequent sectioning.

5.2.3 Tumour Sectioning

Five-micron (5μm) thick frozen sections were cut using a cryostat at –20°C. The sections were placed onto APES (3 Aminopropyltriethoxysilane) coated slides and allowed to air dry prior to fixation by immersing in acetone at –20°C for 5mins. The sections were stored in dust free containers at –20°C until stained. Following standard overnight formalin fixation, the tumour fragments were paraffin wax embedded and sectioned to 3μm thickness.

5.2.4 Coverslip Culture

JBS, SV3T3 and CarB cells were grown to 90% confluence in tissue culture flasks and harvested in the usual manner. Glass coverslips in petri dishes were sterilised by microwaving in an 800Watt oven, on full power for 15 minutes. The petri dishes and coverslips were then allowed to cool and 8 mls of complete medium containing cells were added. The dishes were incubated at 37°C, in 5% CO_2 in air for 2-3 days. Once confluent cell growth was visible microscopically, the medium was removed and the dishes washed twice with PBS. Then 10mls of 50:50 acetone and methanol, at 4°C, were added to the petri dishes and incubated at room temperature for 10 minutes. The acetone/methanol was removed and the coverslips allowed to air-dry. Next 10 mls of sterile PBS was added to the petri dish and the dishes stored at 4°C until stained.

5.2.5 H & E Staining

Haematoxylin and Eosin staining was undertaken at the Histology Department of the Mercy Hospital, Cork with the kind permission of Dr. Gary Lee. The sections were de-waxed using a combination of heat and xylene and subsequently re-hydrated in 3-minute sequential baths of absolute alcohol, 97% alcohol, 95% alcohol, 70% alcohol and distilled water. Sections were stained with H & E, washed and mounted with "Crystal Mount".

5.2.6 Immunological Antigen Detection

5.2.6.1 Flow cytometry

Cells were grown to 60-70% confluence in tissue culture flasks and harvested into suspension. They were fixed in 2% paraformaldehyde at room temperature for 30 minutes, removed by centrifugation at 100Xg for 10 mins and neutralised in 0.2M Glycine. The cells were permeabilised in 70% methanol at 4°C for one hour, washed by centrifugation and resuspended in 0.4mls PBS. Next 100μls of the fixed permeabilised cells were placed in individual, sterile Eppendorf tubes to which 2μls of undiluted, purified mouse anti-SV40 large T, small t monoclonal antibody (Pharmingen, Cat. No. 14121A) were added. To the negative control 10μls of normal mouse serum (generated in our laboratory and diluted 1 in 500 with PBS) was added. After incubating on ice for 30 mins the cells were washed in PBS and resuspended in 100μls of PBS. Next 100μls of unconjugated biotinylated rabbit anti-mouse (LSAB Kit, Dako) was added to both positive and negative controls, which were again incubated on ice for 30 mins. Following another washing step the cells were resuspended in 100μls of

PBS to which 2mls of avidin-FITC (Pharmingen, Cat. No. 13004D) and again incubated on ice for 30 mins. This final incubation stage takes place in the dark, to avoid sample fluorescence deterioration. The cells were centrifuged out of this solution, resuspended in 200μls of Isoton, and analysed on a Coulter Epics Elite cytometer.

5.2.6.2 Immunostaining of Coverslips/Tumour Sections

Coverslips and frozen sections were incubated in a moist chamber for 30mins with 50μls of a 1 in 25 dilution of anti Large T antigen antibody (Pharmingen, Cat. No. 1412180) in PBS. PBS alone was used as the negative control. The slides were washed with Tris-Buffered Saline (TBS; see Appendix 2 – Solutions). Following washing they were incubated with 50μls of biotinylated rabbit anti-mouse IgG antibody (LSAB kit, Dako) for a 30 mins in a moist chamber at room temperature. Following washing the slides were incubated with 50μls of Streptavidin-Alkaline Phosphatase (LSAB kit, Dako) for a further 30mins at room temperature. To enhance the staining intensity the application of the secondary and tertiary reagents were repeated for further 10 min incubation periods. Following the final incubation the slides were washed and substrate (see Appendix 2- Solutions) was filtered onto the slides through Whatman No. 1 filter paper. The substrate was incubated on the slides and allowed to develop for 15 – 20 mins. The slides were then washed in TBS and counter stained with Delafields Haematoxylin for 15 seconds, washed and subsequently mounted using Dako Glycerogel.

5.3 Results

Table 5.1: Mean tumour volumes of differing tumours at 20/21 days post inoculation.

Cells	n =	Mean	S.D.	p Value
JBS/SV3T3	10	103.25	52.79	
JBS 2 X 10^6	10	346.63	87.88	<0.001
JBS 1 X 10^6	10	158.32	83.1	ns
CarB/SV3T3	10	46.67	81.48	
CarB 2 X 10^6	10	121.75	41.05	<0.02
CarB 1 X 10^6	10	59.12	46.48	ns

Students t-test was used for statistical analysis $p<0.05$ is considered significant.

All animals inoculated with tumour cells developed tumours, which became palpable 1 to 2 weeks post inoculation and continued to grow. Mixed tumours became palpable later and grew more slowly that tumours induced with an equivalent number of either JBS or CarB cells alone (Fig. 5.1). The growth trends of the mixed tumours more closely resemble the pattern of tumours induced with an inoculum of 1 X 10^6 cells of either JBS or CarB alone. By day 20 - 21 post tumour induction an obvious divergence in the growth rates can be seen. This difference achieves statistical significance (Table 5.1).

Flow cytometric analysis of the cellular expression of large T antigen for each of the cell lines is shown in Fig 5.2. Panel a, demonstrates the positive and negative control staining of SV3T3 cells, which are known to express the antigen. Panels b & c, demonstrate the loss of expression of large T antigen by JBS cells and the lack of expression in CarB cells. This was confirmed by immunohistochemical staining of cells and tumour sections (Fig. 5.3). This collection of photographs detail the expression of large T antigen on cells grown on coverslips and in frozen sections of tumours grown in athymic nude mice. Positive red staining

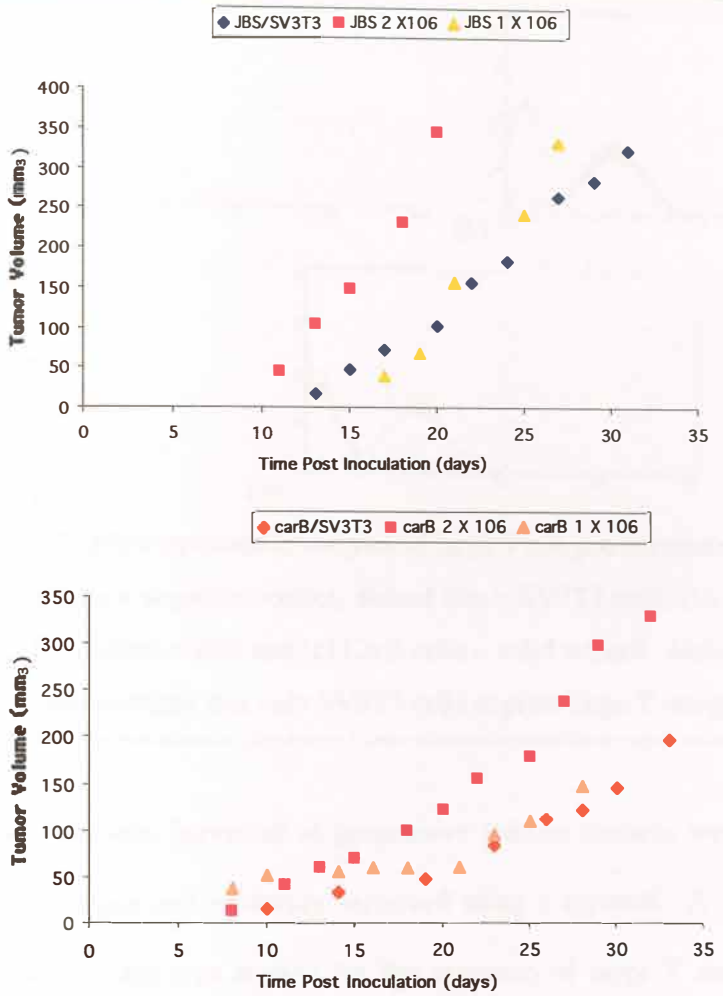


Fig. 5.1: Growth curves for immunologically homogeneous and heterogeneous tumours (JBS/SV3T3 mixtures, Panel A or carB/SV3T3 mixtures, Panel B) in Balb/c mice. Each data point represents the mean of a group of 10 mice. For both JBS and CarB cell lines the growth curve for mixed tumours more closely resembles that of the reduced inoculum dose.

corresponds with antibody detection of large T antigen. As expected, SV3T3 cells and tumour sections both stain strongly positive for large T antigen and neither JBS nor CarB cells or tumours display any evidence of large T or small t antigen expression. Thus the feasibility of staining for the presence of large T antigen in mixed tumours composed of SV3T3 and JBS or CarB cells was established.

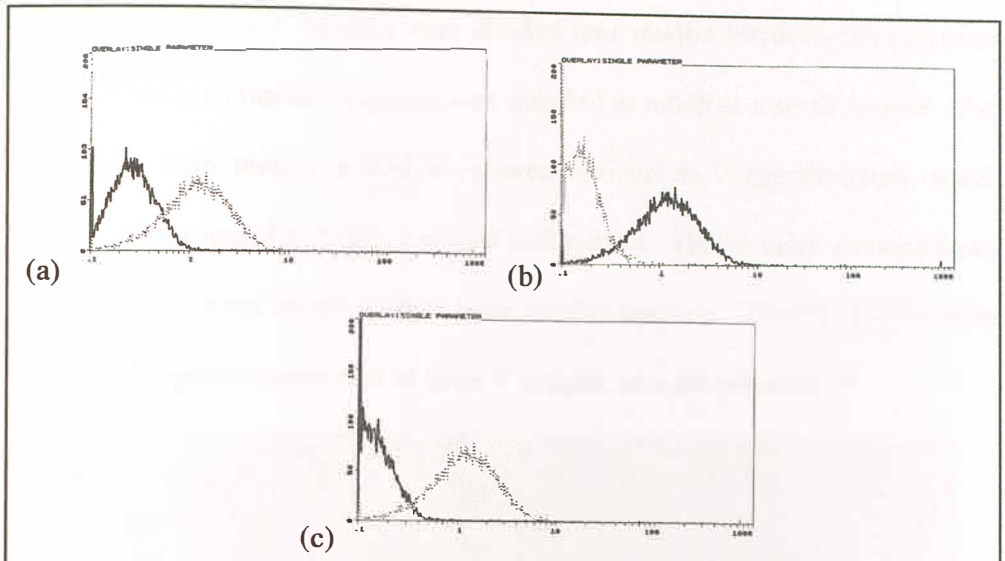


Fig. 5.2: Flow cytometric analysis of large T antigen expression by (a) SV3T3 – solid line = negative control, dashed line = SV3T3 cells, (b) JBS – solid = SV3T3, dashed = JBS and (c) CarB cells – solid = CarB, dashed = SV3T3. This demonstrates that only SV3T3 cells express large T antigen.

Mixed tumours, harvested at progressive tumour burdens were snap frozen in liquid nitrogen and randomly sectioned using a cryostat. A limited sample of tumour sections was stained for the presence of large T antigen. The most positively staining regions from each section were viewed at 400x magnification and photographed. Figs. 5.4 & 5.5 detail photographs of the most strongly, positively staining areas in sections of tumours of increasing size. For both CarB/SV3T3 (Fig. 5.4) and JBS/SV3T3 (Fig. 5.5) mixed tumours there is a progressive decline in the proportion of cells that stain positive for the presence of large T antigen. With very large tumour burdens no evidence of large T antigen expression could be detected. There were a number of possible explanations for the loss of expression of large T antigen. Firstly, technical failure in the performance of APAAP staining, though harvested sequentially tumours were sectioned and stained at random. Secondly, sampling error in the

larger tumours, larger tumours were divided into smaller fractions prior to snap freezing and each tumour fragment was sampled as much as a small tumour. For example a 9gm tumour would have been divided in 9 approximately equal fragments and each fragment sectioned and stained. Hence more sections from larger tumours were examined than from smaller tumours. Thirdly, SV3T3 cells may down regulate expression of large T antigen, as a consequence of

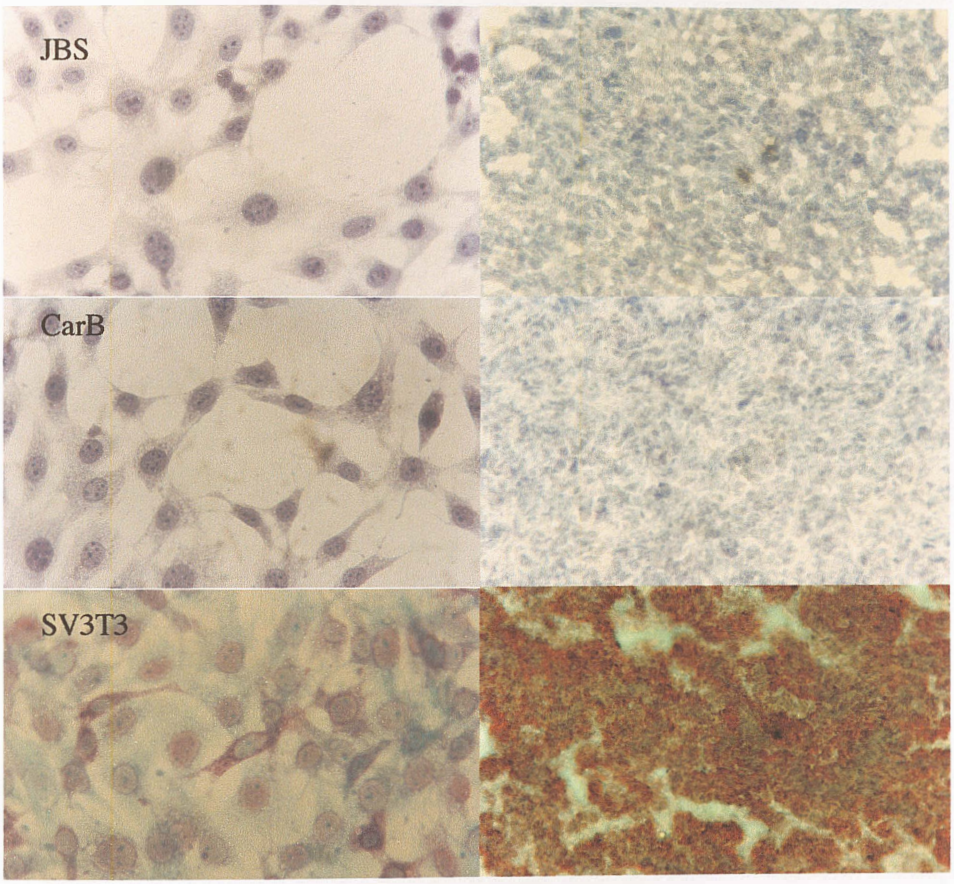


Fig. 5.3: Large T antigen (red) staining of cells grown on coverslips in tissue culture and of tumours excised from athymic nude mice for JBS, CarB and SV3T3 tumours. (Magnified X 400, reduced by about a third).

3-dimensional growth. Tumours grown in athymic nude mice continue to stain positive for large T antigen (Fig. 5.3). The only way to be absolutely certain that this is not the

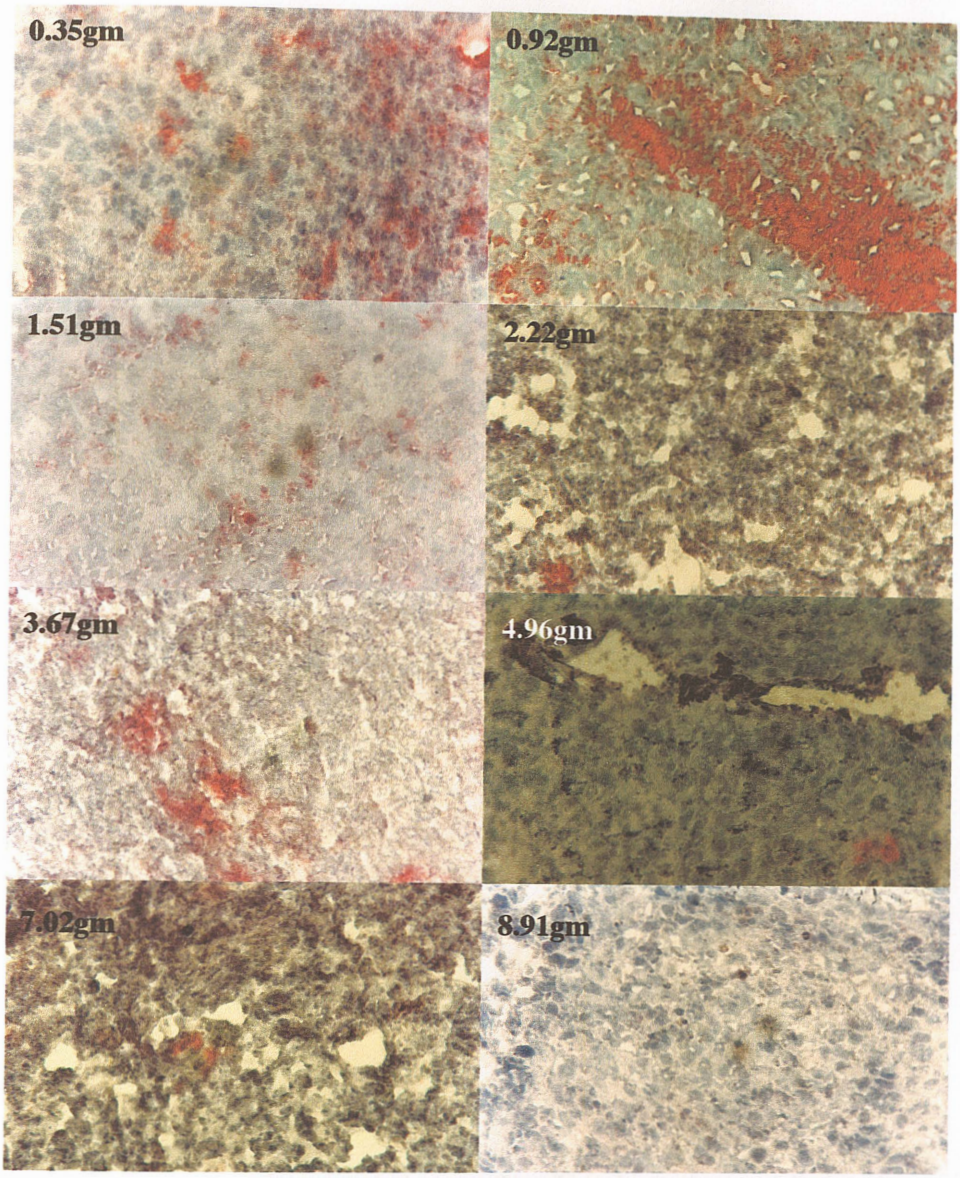


Fig. 5.4: Representative photomicrographs of sections of mixed CarB and SV3T3, tumours grown in Balb/C mice, excised at the tumour burdens stated, stained for the presence of large T antigen (red) and counter stained with Haematoxylin. (X 400, reduced by about a third).

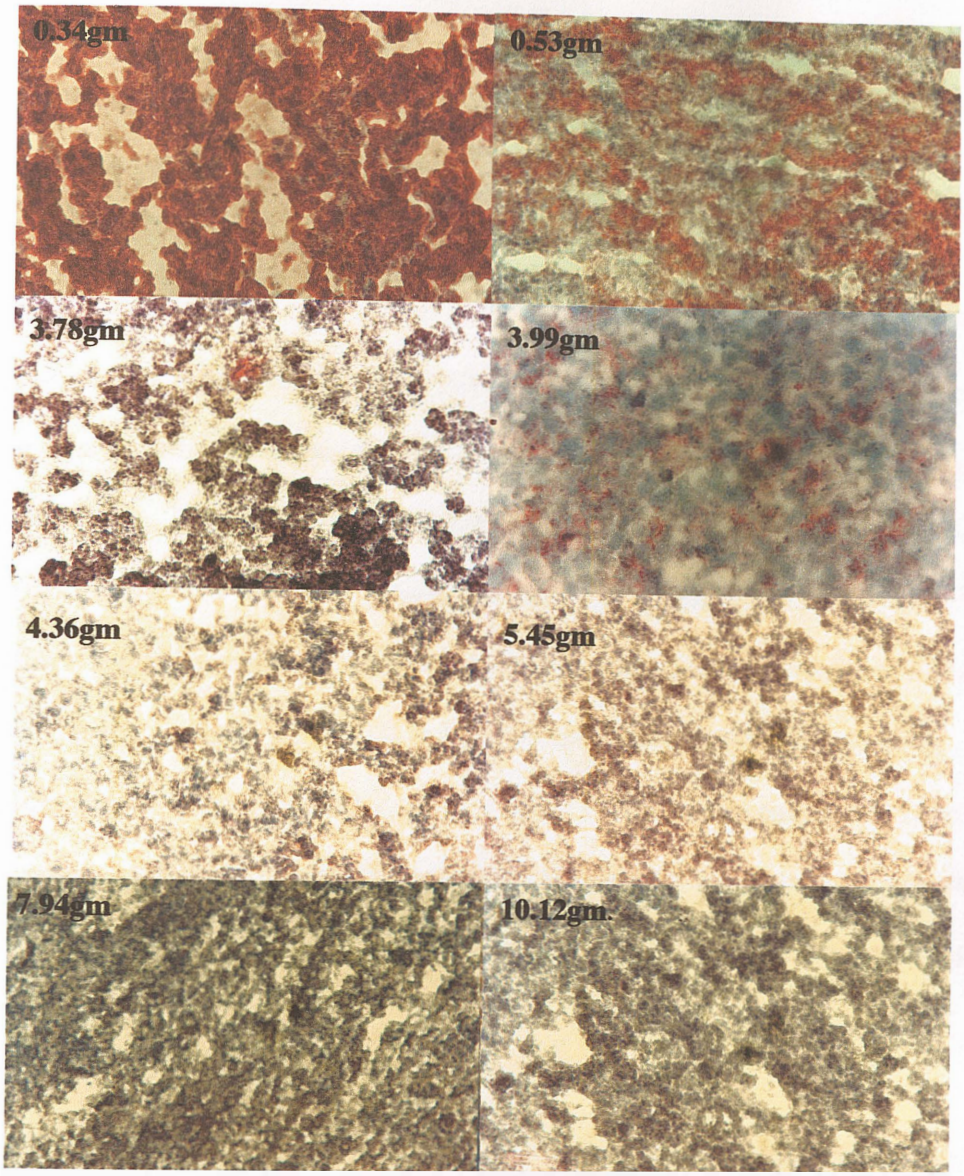


Fig. 5.5: Representative photomicrographs of sections of mixed JBS and SV3T3, tumours grown in Balb/C mice, excised at the tumour burdens stated, stained for the presence of large T antigen (red) and counter stained with Haematoxylin. (X 400, reduced by about a third).

case would be to look for SV40 DNA in mixed tumour preparations from CarB/SV3T3 tumours. It could not be done with JBS/SV3T3 mixed tumour preparations, as JBS cells are a large T antigen negative derivative of SV3T3 cells, with obvious potential overlap in DNA preparations. Fourthly, when

grown as mixed tumours in vivo, SV3T3 cells behave as a subdominant cell line, whose growth is restricted by contact inhibition with JBS or CarB cells, a concept similar to that described by Aabo and alluded to in the introduction to this chapter (Aabo, Vindelov et al. 1994). To address this question mixed JBS/SV3T3 tumours were established in athymic nude mice. To favour SV3T3 cell growth, a ratio of 5 to 1 SV3T3 to JBS cells was used. In those tumours grown in athymic nude mice there was no reduction in large T antigen expression. However mixed JBS/SV3T3 tumours, at a ratio of 5:1, grown in Balb/C mice did exhibit a reduction in large T antigen expression (Fig. 5.6). There is quite a marked difference in large T antigen positive staining of tumours of similar weight grown in immunocompromised athymic nude mice as compared to immunocompetant Balb/C mice. This mitigates against any form of contact inhibition. Finally, the possibility that cellular elements, most probably T-cells, present in the immunocompetant mice selectively eliminate the strongly antigenic SV3T3.

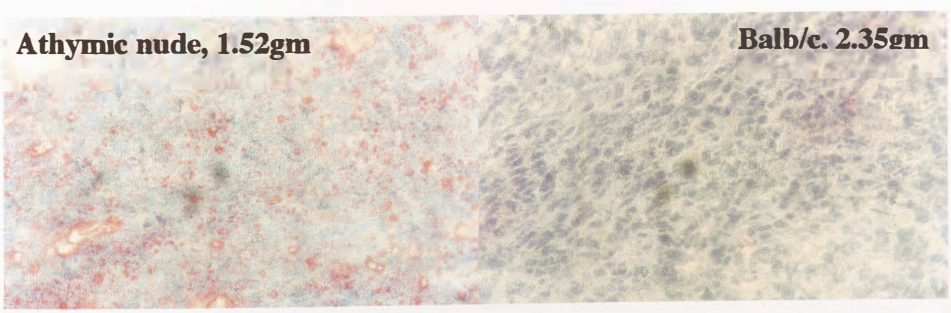


Fig. 5.6: There is a marked difference in large T antigen staining of tumours from athymic nude and Balb/C mice, even when induced with a 5 to 1 ratio of SV3T3 to JBS cells. (X 400, reduced by about a third).

Supportive evidence for this latter hypothesis comes from the inability to grow pure SV3T3 tumours in Balb/C, as they are too strongly antigenic. Evidence of local tumour invasion of adipose tissue, muscle and skin is shown in Fig. 5.7. In Fig. 5.8 evidence of two morphologically distinct tumour cell types is seen, together with areas of necrosis and mixed inflammatory infiltrates.

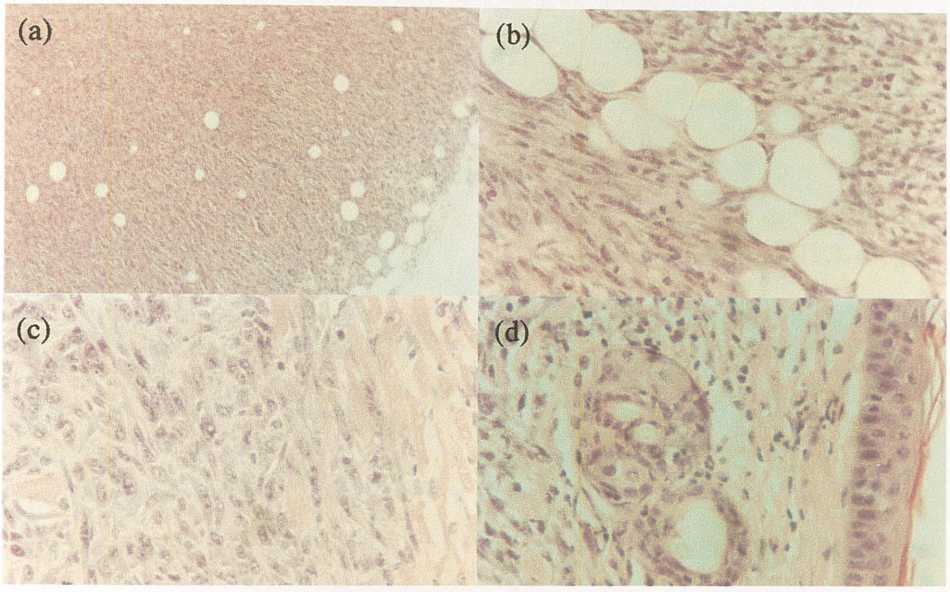


Fig. 5.7: H & E staining of tissue sections from JBS (a & c) and CarB (b & d) tumours, excised from Balb/c mice demonstrating invasion of adipose tissue (a & b), muscle (c) and skin (d). (X 400, reduced by about a third).

Whilst large numbers of lymphocytes are not seen, it must be remembered that following lymphocyte killing of one target cell the lymphocyte can account for up to 10 target cells before undergoing apoptosis.

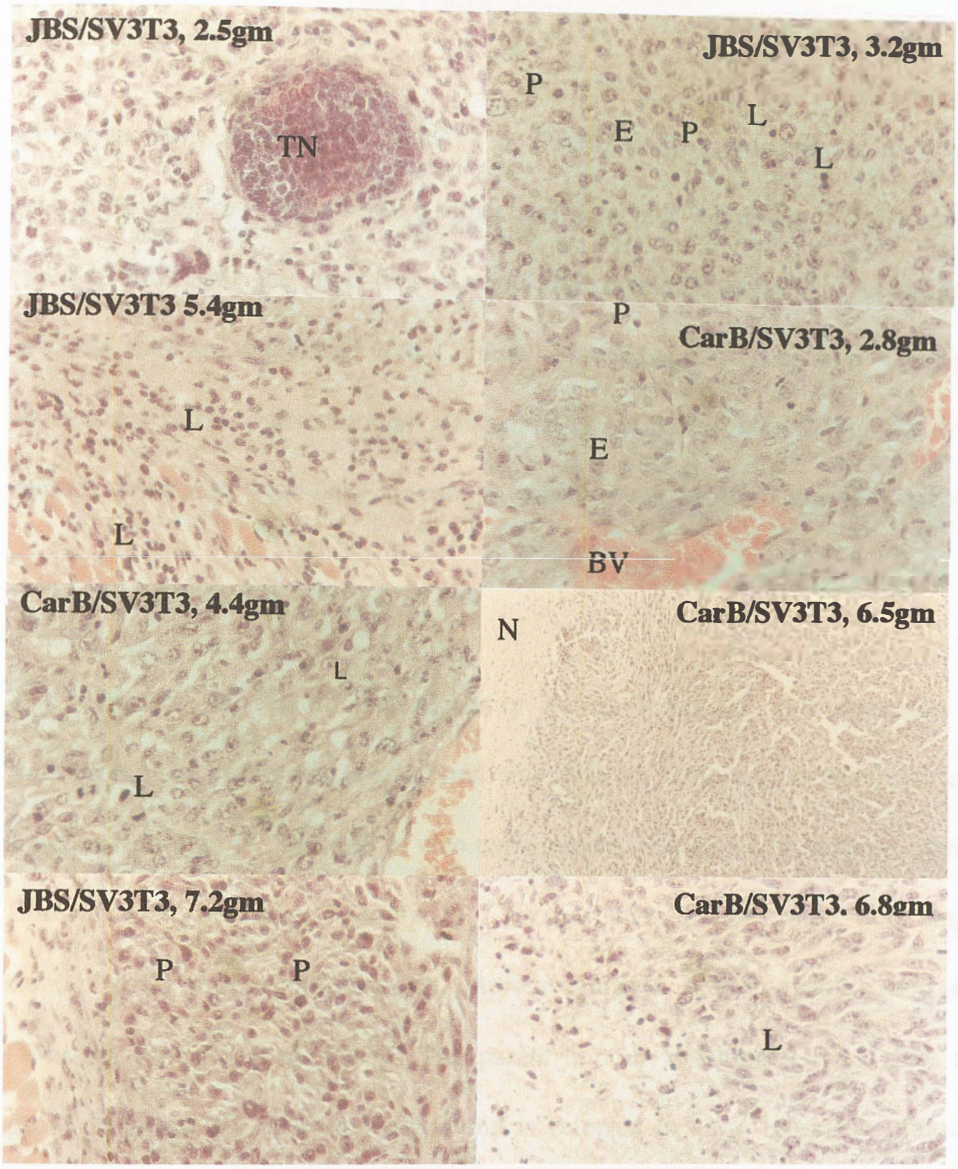


Fig. 5.8: H & E staining of tissue sections of tumours excised from Balb/c mice demonstrating morphological differences between cell types with nodule formation (TN), necrosis (N), inflammatory infiltrates with scattered polymorphs (P), eosinophils (E) and lymphocytes (L). (X 400, reduced by about a third).

5.4 Discussion

Mixed tumours of either JBS/SV3T3 or CarB/SV3T3 cells grow more slowly than pure JBS or CarB tumours alone and at a rate more typical of tumours induced with half the inoculum of pure JBS or CarB tumours. As tumours progress they are associated with a gradual loss of expression of large T antigen expressing cells. Intratumoural inflammatory infiltrates were seen, but were neither correlated with large T antigen positive cell location nor with tumour progression.

The evidence of tumour heterogeneity comes from the full spectrum of immunological resources, i.e. animal and human tumours, spontaneous and induced tumours, *in vitro* and *in vivo* experiments. Using a UV-induced murine fibrosarcoma, Schreiber's group in Chicago have demonstrated that single tumour cells may simultaneously express multiple tumour-specific antigens (Wortzel, Philipps et al. 1983). The parent tumour, designated 1591, does not grow progressively in normal mice and is referred to as a 'regressor tumour'. Antigen loss variants, generated *in vitro* by selection with T-cell clones, grew in immunocompetant mice and were referred to as 'progressor tumours'. They reported a 'hierarchy' of host antigen recognition, such that not all antigens were recognised simultaneously and conclude that tumour progression requires only the loss of the dominant/recognised antigen and not all the recognisable antigens. Evidence of a 'hierarchy' or immunodominance has also been found in an MCA-induced murine sarcoma model, where shared antigens are masked by immunodominant antigens (Dudley and Roopenian 1996). Others have shown that regressor and progressor may co-exist *in vivo* in a regressor tumour (Schmitt

and Daynes 1981). From a different UV-induced murine fibrosarcoma, RD-1024, with a regressor phenotype, therefore grown *in vivo* in immunocompromised animals, multiple (hundreds) sub-clones were isolated in vitro. These were isolated without selection pressure from T-cell clones or antibodies etc. These clones varied enormously in their tumourigenic potential. Though now possessing a progressor phenotype, they still shared rejection antigens with the parent tumour as immunisation with the latter or an alternative regressor sub-clone protected against subsequent challenge with a progressor clone.

One particular aspect of tumour heterogeneity that has been extensively studied is metastatic ability. This is because it is usually as a consequence of metastatic disease and not the primary tumour that patients with cancer succumb. For a tumour to metastasise, it must firstly penetrate the surrounding vascular or lymphatic system and then either grow at the site of penetration or detach and circulate as an individual cell or as small embolic aggregates. In the circulatory system tumour cells must evade host immune responses and survive turbulence. The tumour cells then arrest in the capillaries of distant organs, though some will pass through and reach even more distal capillaries displaying a poorly understood organotropism. Growth at the distant site requires adhesion to or migration through the endothelial cells, continued evasion of the host immune system and the development of a vascular network. B16 is an extensively studied murine melanoma cell line from which Fidler and Kripke derived, *in vitro*, a variety of clones. Using a fluctuation test, originally devised by Luria and Delbruck to distinguish between selection and adaptation in the origin of

bacterial mutants, they investigated the number of pulmonary metastases generated by intravenous injection of the clones compared with the parent line (Fidler and Kripke 1977). Clones were generated in vitro without selection pressure and then injected intravenously. Individual clones exhibited marked variability in numbers of metastases generated. This was not due to the cloning process as when these clones were sub-cloned no difference in metastatic potential was seen. They conclude that cells with variable metastatic potential are present in the parent tumour. This suggests that the generation of a metastasis is not a random event but is linked to properties on the metastatic cell. A concept supported by Fidler's report that less than 0.1% of cells injected intravenously survive to proliferate as metastases (Fidler and Hart 1982). Approaching this from the opposite side, Brodt and Segal used macrophages fed with solubilised membrane extracts of primary and metastatic Lewis lung carcinoma, 3LL and M-3LL, to sensitise normal spleen cells (Brodt and Segal 1982). Splenocytes immunised with extracts of the primary tumour inhibited significantly the development of tumours in the footpad of mice challenged with 3LL. On the other hand mice immunised with extracts from the metastatic cell line failed to prevent 3LL or M-3LL tumour induction. They conclude that emergence of the metastatic variant has coincided with loss of antigenicity.

Tumour heterogeneity has also been demonstrated in spontaneous murine tumours. Murine mammary tumour virus (MuMTV) is involved in mouse mammary tumourigenesis. Using seven spontaneous mouse mammary tumours, one of which was sub-cloned, Heppner's group demonstrated inter- and intra-

tumoural heterogeneity of expression of mammary tumour virus antigen (Hager and Heppner 1982).

Whereas the emergence of antigenic heterogeneity results from genetic instability and local factors described earlier, their significance comes from the acquisition of immune resistance. Mastocytoma P815 is an example of this, though often undergoing near complete rejection in syngeneic hosts, most recover and form progressive tumours (Uyttenhove, Maryanski et al. 1983). This was not due to the development of an immunosuppressed state as the mice still demonstrated concomitant immunity to the parent P815 cell line injected at a remote site. Analysis of the escaping tumour cell populations revealed clones, which were resistant to anti-P815 CTL clones *in vitro*. Using the same tumour model Hines demonstrated in vivo emergence of resistance to CTL killing of P815 cells (Hines 1989). Using adoptive transfer of splenocytes from immune animals he eradicated 102 of 112 P815 tumours in mice. Of the remaining uneradicated 10, 7 were significantly less susceptible *in vitro* to P815-specific CTLs. The probability of failure of adoptive immune therapy was inversely related to the numbers of immune cells transferred, once immunoselection had occurred increasing the number of immune cells had no effect. The reduced CTL sensitivity was not due to alterations in MHC class 1 expression nor to a decreased sensitivity to CTL lytic mechanisms but was concluded to be due to loss of antigen expression. Reduced sensitivity to other mechanisms of host anti-tumour immunity such as decreased sensitivity to NK-cell-mediated lysis and antibody complement cytotoxicity have also been demonstrated (Starkey, Davis et al. 1982). Immunoselection results not only in the emergence of antigen-loss

variants but also in variants with persistent but reduced antigen expression (Topalian, Kasid et al. 1990).

Antigenic heterogeneity has also been documented in human tumours of varying histological origin. Lloyd Old's group in New York demonstrated phenotypic and surface antigen heterogeneity among cell lines derived from different melanoma metastases in the same patient (Albino, Lloyd et al. 1981). Only 1 of 6 clones expressed sufficient antigen to be detectable using autologous serum, the others were defined by morphological differences and reactivity to a panel of monoclonal antibody reagents for antigens on the surface of melanoma cells. This pattern of expression has implications for immunological detection and therapy. With melanoma the entire primary lesion is usually necessary for detailed histological analysis and staging and thus is not usually available for immunological examination, though it has been done and confirmed differences in expression of melanoma-associated antigens between the primary and metastatic lesions (Natali, Cavaliere et al. 1983). With breast cancer however adequate primary and metastatic tissue is obtainable. Analysis of primary tumours and autologous axillary lymph node metastases from 13 patients has shown differences in antigen expression between primary and metastatic lesions (Natali, Giacomini et al. 1983). Antigenic heterogeneity has been demonstrated *in situ* in human osteogenic sarcomas in the primary lesion itself (Byers and Johnston 1977).

Concerns that antigenic heterogeneity is a consequence of *in vitro* or experimental manipulation, though valid, are misplaced. Clonal analysis of

metastatic lesions derived from B16 melanoma cells revealed that the majority (>80%) of experimental metastases produced by intravenous injection are unicellular in origin (Poste, Tzeng et al. 1982). During the first 25 days of their growth analysis of drug resistance reveals that individual metastatic lesions are homogenous but there is interlesional heterogeneity. Progressive growth of the originally clonally homogeneous lesions is accompanied by the emergence of antigenic heterogeneity. By 45 days after initiation of metastatic growth 90% of lesions are heterogeneous. The emergence of the heterogeneous population of tumour cells is as much due to *in vivo* host factors as it is to the inherent genetic instability in the tumours. Using a clonal murine lymphoma split and grown simultaneously *in vitro* and *in vivo* in a syngeneic host, maintenance of clonal homogeneity over a five-month period was observed with *in vitro* growth (Chow and Greenberg 1980). *In vivo* passage on the other hand resulted in the emergence of a heterogeneous population after 3 months. When two sub-lines of P815 that had been maintained by in vivo passage over a 10-year period in two different institutions were compared for antigen expression remarkable stability was demonstrated (Hines 1986). Though differences in susceptibility to in vitro cytotoxicity were seen, reciprocal cross-protection against the growth of either cell line was seen following immunisation with either cell line. Similar stability in antigen expression was seen in two sub-lines of Meth-A fibrosarcoma passaged separately over a decade. This apparent stability supports the concept of an equilibrium between the heterogeneous cell populations described by Poste and discussed in the introduction to this chapter (Poste and Greig 1982). Heppner's group have also presented evidence in support of an intratumoural

homeostatic mechanism, which allows fast- and slow-growing subpopulations to co-exist in the parental tumour (Miller, Miller et al. 1980).

The emergence of phenotypic heterogeneity has been shown to be an *in vivo* phenomenon, requiring the participation of a functional immune system. Whether this is due to the immune system facilitating the emergence of antigen-loss variants by elaborating trophic factors, as Prehn would have us believe, or by selectively eliminating strongly antigenic variants is still unclear (Prehn 1994). Virus-induced tumours give the most convincing evidence for tumour immunogenicity, rejectability and surveillance (Klein and Klein 1977). However, even in papers reporting on *in vivo* immune selection of viral antigen loss variants from a Moloney Murine Leukemia Virus tumour, the authors were unable to prove that this was the result of immune elimination of virus-antigen expressing cells rather than the selective outgrowth of antigen loss/downregulation variants (Jiang and Flyer 1990).

It was in an attempt to answer this question that the work presented herein was carried out. From the immunohistochemical analysis of mixed tumours grown in athymic nude mice it was shown that both cell lines are capable of co-existing in developing tumours. However with progressive tumour growth in immunocompetant mice there is a gradual elimination of the strongly antigenic SV3T3 cells. Interestingly a pure inoculum of SV3T3 cells alone would not result in tumour formation but admixing with JBS or CarB cells affords some degree of protection allowing them to grow initially but ultimately to be eliminated. That they are eliminated rather than down-regulating expression of

large T antigen is supported by the tumour volume growth curves and the nude mouse data. Immune cells (lymphocytes and polymorphs) were found, though not in large quantities, in the mixed tumours, whether they were responding to the tumour or passing through is not known. The data supports the hypothesis that immune cells capable of recognising large T antigen were able to penetrate the tumour and selectively eliminate the strongly antigenic cells leaving weakly antigenic cells to grow and develop.

5.5 Conclusion

The way tumour cell antigens are expressed i.e. the occurrence of phenotypic heterogeneity has major implications for the successful application of various immunotherapeutic strategies. The antigenic heterogeneity observed in most tumours may limit therapeutic efficacy of antibody, T-cell, LAK cell therapy. It had been hoped that tumour cells that are destroyed would attract immune cells that may react with antigen-negative cells – a bystander effect. The above data indicates that the immune system actively selects for a less antigenic cell by eliminating only the more potently immunogenic cells from within heterogeneous and vascularised solid tumours. The recruitment of a potent immune reaction to a tumour by strongly immunogenic cells while creating a dangerous environment does not non-specifically eliminate the weakly antigenic tumour cells. A tumour mass at the lower limits of radiological detection, circa $1cm^3$, may contain as many as 10^9 cells; eradication of 99.9% of these cells a remarkable therapeutic achievement, still leaves 10^6 cells to proliferate, thus providing a large base for the further generation of biological diversity. The challenge for successful immunotherapy is great, as immunologic elimination of

weakly antigenic cells will require specific targeting. Immunotherapeutic strategies based on artificial augmentation of tumour cell antigens by viral or chemical means must ensure targeting of the weakest antigenic cells within heterogeneous tumours.

Chapter 6

Tumour Antigen Specific Oral Immune Tolerance

(This work has been presented in part at The Irish Society of Gastroenterology, Nov. 1996, The Sylvester O'Halloran Surgical Scientific Meeting, March 1997, The American Gastroenterology Association, May 1997, The British Association of Paediatric Surgeons, July 1998 and published in The Esophagogastric Junction, 1998, Eds. R.Giuli, JP Galminche, GG Jamieson and C Scarpignato) and Diseases of the Esophagus (in press).

6.1 Introduction

Over the past 50 years advances in anaesthetic and surgical technique, post-operative care, adjuvant therapeutic modalities and earlier detection have all contributed to improvements in the survival rates for patients suffering from malignant disease (Boring, Squires et al. 1994). Despite these advances, the prognosis for patients suffering from cancer of the foregut remains consistently and significantly worse than patients with distal gastrointestinal tract cancers (Table 6.1) (Boring, Squires et al. 1994; Levi, La Vecchia et al. 1996; Parker, Tong et al. 1996). If we compare patients with similar stages of disease, at the time of

Table 6.1: Changing trends in the relative five-year survival (%) of patients with gastrointestinal tract malignancy in the United States over the past 4 decades.

	1960-63	1970-73	1974-76	1980-82	1986-90
Oesophagus	**4**	**4**	**5**	**7**	**11**
Stomach	**11**	**13**	**15**	**16**	**19**
Colon	**43**	**49**	**50**	**56**	**62**
Rectum	**38**	**45**	**49**	**53**	**60**

Table 6.2: Comparison of five year survival rates (%) by stage at time of diagnosis for patients with gastrointestinal malignancy diagnosed in United States between 1986-91 (Boring, Squires et al. 1994; Levi, La Vecchia et al. 1996; Parker, Tong et al. 1996).

	All Stages	Localised Disease	Regional Disease	Distant Metastases
Stomach	**20**	**57.5**	**21**	**2**
Colon/Rectum	**61**	**93**	**65**	**8**

diagnosis, patients with cancer of the stomach and oesophagus have a poorer prognosis than patients with cancer of the rectum and colon (Table 6.2).

The mucosal immune system is in the front line of protection against invading pathogens. However it is exposed daily to a huge variety of antigens in the diet to which were it to respond would have a detrimental effect on the mucosa and hosts general well-being. This absence of an immune response has been referred to as oral tolerance whereby an antigen induces a state of systemic specific immune hyporesponsiveness when presented by an oral (or nasal) route but if presented by another route induces immune sensitisation. This has been shown in some circumstances to be due to the induction of suppressor cells. We postulate that the GALT may process shed tumour antigen and consequently down-regulate the systemic immune response to tumour antigens in the GI tract.

As we had established murine models of two aetiologically and histologically distinct tumours and partially defined the immune response to those developing tumours. We wished to investigate to what extent, if any, feeding mice JBS or CarB tumour fragments could alter these immune responses. The effect of prior feeding on tumour growth, cytotoxic and proliferative function of tumour-bearing mouse splenocytes and a Delayed-Type Hypersensitivity (DTH) responses will be determined to test this hypothesis.

6.2 Literature Review

The first published report of oral tolerance is attributed to Dakin who, in 1829, in an article concerning contact sensitivity reactions to poison oak and poison ivy describes how "some good meaning, mystical, marvellous physicians or favoured ladies with knowledge inherent" prescribed eating the leaves of the Rhus plants as both a preventative and a cure (Dakin 1829). His own experience of this treatment

was that it induced "an eruption, swelling, redness and intolerable itching, around the verge of the anus". The first scientist to report his research into this phenomenon was the renowned author H.G. Wells who published a series of articles on anaphylaxis. He demonstrated prevention of systemic immune sensitisation to hen's egg protein in guinea pigs by prior feeding with the same antigen (Wells 1911). Chase, in 1946, demonstrated that contact sensitivity responses to 2:4 dinitrochlorobenzene (DNCB) could be suppressed for prolonged periods in an antigen specific manner (Chase 1946). Sulzberger, in 1930, reported similar prevention of contact sensitisation to neoarsphenamine by prior intravenous injection of the antigen (Sulzberger 1930). Because of their work the phenomenon became eponymously known as the Chase-Sulzberger effect. In the 1970's and '80's a diverse range of antigens were investigated in both animal and human models for evidence of an oral tolerizing effect (Asherson, Zembala et al. 1977; Rubin, Weiner et al. 1981; Zhang, Davidson et al. 1991).

Oral tolerance has been clearly and consistently demonstrated in animal and human models to a wide variety of antigenic stimuli. In addition to a wide variety of proteins, both exogenous and endogenous, these antigens include human immunoglobulins (Vives, Parks et al. 1980), contact sensitising agents (Chase 1946), heterologous red blood cells (Mattingly and Waksman 1978), allogeneic leucocytes (Sayegh, Zhang et al. 1992), inactivated viruses (Rubin, Weiner et al. 1981) and bacteria (Challacombe and Tomasi 1980). More recently research attention has focused on antigens known to be associated with pathological processes such as myelin basic protein (Weiner, Mackin et al. 1993), collagen (Nagler-Anderson, Bober et al. 1986) and allogeneic leucocytes (Hancock, Sayegh et al. 1993). The reproducibility of the phenomenon affirms it's credibility, however, no exact dose for the induction of tolerance exists. This is partly because of the differing feeding regimes and end point assays used by various

investigators. It may also reflect inherent differences in the antigen used in terms of size, charge and immunogenicity.

Depending on the nature and complexity of the antigen, systemic tolerance can be induced within 2 days of a single feed of a protein antigen (Friedman and Weiner 1994) but more complex antigens such as sheep red blood cells (SRBCs) require multiple feeds over a 2 week period (Challacombe and Tomasi 1980). During the first few weeks of feeding to induce tolerance there may be an initial immune sensitisation, which, with prolonged feeding becomes down regulated (David 1979). The duration of tolerance varies for different elements of the immune response. Cell mediated immune responses such as delayed-type hypersensitivity responses are particularly easy to tolerize and are long lasting, being still detectable 17 months after a single feed of ovalbumin (Strobel and Ferguson 1987). Humoral immune responses are generally more difficult to tolerize and of shorter duration, peaking during the first few weeks following a single feed of ovalbumin, beginning to wane after 4-6 weeks and not detectable after 6 months (Strobel and Ferguson 1987). The duration of tolerance after feeding more complex antigens is even more short-lived, lasting 2 months for the humoral response to SRBCs (David 1979).

Although a wide range of antigens may induce tolerance certain types of antigens are more likely to induce active immunity than oral tolerance. These include viable antigens such as bacteria and viruses which when killed or inactivated will induce oral tolerance (Rubin, Weiner et al. 1981). Tolerance is also more readily induced to soluble antigens than to particulate antigens. Ovalbumin when coated onto microspheres induces active immunity rather than oral tolerance (McGhee, Mestecky et al. 1992). It may be that soluble antigens enter diffusely across the epithelium and particulate antigens are taken up and processed by M cells in the Peyer's patches (Pappo and Ermak 1989). It is generally not possible to induce

tolerance to thymus-independent antigens, those antigens that are capable of activating B-cells without T-cell help. In general these are large polymeric molecules with repeating antigenic determinants (e.g. Lipopolysaccharide, polymeric bacterial flagellin). At low concentrations they activate specific B-cell clones whereas at high concentrations some are capable of polyclonal B-cell activation. These factors alone help to understand how the GALT distinguishes between non-harmful ingested protein to induce a tolerogenic response and potentially harmful bacteria, which are viable and present a large amount of thymus independent antigen, to activate an immune response.

The induction of tolerance is also influenced by such factors as age, genetic background, intestinal bacterial flora, route of administration, immunological status of the host and probably most importantly the dose of antigen received. There are two periods in the life cycle of a mouse when oral administration results not in tolerance but in immune sensitisation. Neonatal mice fed ovalbumin displayed increased hypersensitivity responses when challenged as adult mice (Strobel and Ferguson 1984). Similarly neonatal mice exposed to gliaden via maternal breast milk had significantly greater systemic immune responses to gliaden than mice from gluten-free colonies (Troncone and Ferguson 1988). Neonatal rats fed myelin basic protein had an enhanced immune response and exacerbated experimental allergic encephalomyelitis when immunised with the same protein in adjuvant as adults (Miller, Lider et al. 1994). This inability to induce tolerance probably reflects systemic immunological immaturity rather than immature antigen processing by the gut as it can be corrected by passive transfer of mature lymphocytes prior to immunisation but not by transfer of serum from tolerised mature animals (Peng, Turner et al. 1989). Interestingly, but not surprisingly, neonatal mice exposed to ovalbumin or bovine serum albumin indirectly via the mother's breast milk rather than by gavage were tolerised to subsequent immunisations. The dose required for such tolerance was significantly lower

1mg/gm/body weight for gavage fed compared with 1-10μgm/gm/body weight for suckling animals (Strobel 1996). The latter dose in mature animals is associated with priming of the immune response. By day 14 of life the magnitude of the immunological tolerance induced by prior feeding of a protein antigen was equivalent to that of an adult mouse. However, if feeding of antigen coincides with weaning no oral tolerance for antibody responses and markedly reduced suppression of cell-mediated immunity is seen (Hanson 1981; Strobel and Ferguson 1984). At the time of weaning there is a change in the bacterial flora of the gut, an exposure to new dietary antigens and a withdrawal of maternal milk any or all of which may contribute to this transient defect in the induction of tolerance. There is an unsubstantiated suspicion that tolerance may be defective in ageing mice (Mowat 1994).

A number of papers have reported differences in susceptibility to tolerance induction by the oral route in mice of differing genetic backgrounds (Kiyono, McGhee et al. 1982; Stokes, Swarbrick et al. 1983). In particular Balb/B mice were found to be much more difficult to tolerize than the congenic Balb/c strain (Lamont, Mowat et al. 1988). These mice differ only at the MHC suggesting that the genes of the MHC may be involved in the development/control of oral tolerance. Furthermore adoptive transfer of serum 1 hour post feed from either Balb/B or Balb/c mice to naive Balb/c mice produced significant suppression of DTH responses, but not if transferred to Balb/B mice (Mowat, G et al. 1987). There is a well-described association between the HLA-DQw2 locus of the MHC and 80-90% of patients with coeliac disease. Furthermore those patients with a particular polymorphic 4.0 kb fragment detected by using a DQ ß chain cDNA probe and the restriction endonuclease Rsa I have a 40-fold increased relative risk of developing coeliac disease (Howell, Austin et al. 1986). It has been suggested that food sensitive enteropathies such as coeliac disease may result from a breakdown of tolerance to orally administered proteins (Mowat 1987).

The induction of oral tolerance relies upon a normally functioning gut with comensal flora. There are higher levels of food specific antibodies in patients with increased intestinal permeability such as Crohn's disease and Coeliac disease (Walker 1987). There is also a well-documented association between the development of food sensitive enteropathies and intestinal infection particularly in children (Harrison, Kilby et al. 1976). This may have the same pathophysiological basis as the defect in tolerance induction seen at the time of weaning. Both may be due to changes in the gut bacterial flora or to alterations in bacterial products such as Lipopolysaccharide (LPS). Germ free mice have been shown to have an impaired systemic immune response as evidenced by an impaired DTH response to parenteral immunization with thymus independent antigens such as SRBCs (MacDonald and Carter 1979). Humoral immune responses are intact, but weakened, and DTH responses are of normal strength if the animal is immunised via a footpad. Daily oral administration of SRBCs to germ free mice regardless of strain resulted in immune sensitisation (Wannemuehler, Kiyono et al. 1982). Prior oral treatment of the germ free mice with LPS resulted in oral tolerance induction. Prolonged oral administration of SRBC to LPS non-responsive C3H/HeJ mice primes systemic immune responses whereas the same treatment in congenic, LPS responsive C3H/HeN mice results in oral tolerance (Kiyono, McGhee et al. 1982). However, oral tolerance to proteins in LPS non-responsive C3H/HeJ mice is entirely normal which suggests that LPS does not appear to be important in the generation of oral tolerance to thymus-dependant antigens (Mowat, Thomas et al. 1986). Similarly serum from ovalbumin fed germ-free Balb/c mice transferred DTH tolerance to naive mice at equivalent levels to conventionally reared mice (Furrie, Turner et al. 1994). In contrast, serum from SCID mice collected 1 hour after feeding ovalbumin was unable to transfer tolerance of DTH responses to naive Balb/c mice. This failure has been linked with a lower number of intraepithelial lymphocytes with a different morphology in the small intestine of

SCID mice as compared with Balb/c mice, suggesting that a functioning GALT is essential for the development of oral tolerance. By reconstituting SCID mice with Balb/c splenocytes it was possible to induce oral tolerance with T-cells but not B-cells. More specifically reconstitution with $CD4^+$ and not $CD8^+$ T-cells (Yoshida, Hachimura et al. 1998). Similarly the inability to induce oral tolerance in germ-free mice is functionally coupled to the innate absence of T-cells in the Peyer's patches of these mice in comparison with normal mice. The requirement for functioning, organised Peyer's patches to establish oral tolerance was also established in a Balb/c model of Peyer's patch null mice where it was not possible to induce tolerance to protein antigens but was not necessary for haptens which were capable of inducing systemic unresponsiveness via the intestinal epithelial cell barrier (Fujihashi, Dohi et al. 2001). Still others have shown the selective emigration of suppressor T-cells from Peyer's patches to the mesenteric lymph nodes (Jeurissen, Sminia et al. 1984). As with all other aspects of immunology there are some conflicting reports. Weiner's group in Boston have, using a murine model have demonstrated that in the presence of mesenteric lymph nodes that Peyer's patches are not an absolute requirement for the induction of either high- or low-dose oral tolerance (Spahn, Fontana et al. 2001; Spahn, Weiner et al. 2002).

Tolerance can be induced most easily by oral administration but also by rectal instillation, portal vein inoculation (Qian, Hashimoto et al. 1985) and inhalation (McMenamin, Oliver et al. 1991; Mestecky, Husby et al. 1996; Staines, Harper et al. 1997; Wolvers, Van Der Cammen et al. 1997).

Oral tolerance can also be induced, though with greater difficulty, after the host has been systemically sensitised to the antigen. It is the induction of tolerance in this setting that is clinically relevant to any therapeutic application in autoimmune disease, though not transplantation, and therefore forms the basis of much current research in the field. A marked reduction in DTH and antibody responses in

immunised animals by oral feeding has been consistently demonstrated (Lamont, Bruce et al. 1988; Peng, Turner et al. 1989). These studies have shown that, as in naive animals, cellular immunity is more susceptible to oral tolerance than humoral immunity. This ability to abrogate the established immune response has also been demonstrated in experimental animal models of autoimmunity including experimental autoimmune encephalitis (Chen, Kuchroo et al. 1994), collagen-induced arthritis (Nagler-Anderson, Bober et al. 1986), experimental autoimmune uveoretinitis (Nussenblatt, Caspi et al. 1990), myasthenia gravis (Wang, Qiao et al. 1993) and immune complex-mediated glomerulonephritis. Successful tolerance induction has been demonstrated in experimental hypersensitivity to a potent systemic immunogen, keyhole limpet haemocyanin, in humans (Husby, Mestecky et al. 1994). Symptomatic improvement without side effects has been reported with the therapeutic application of oral tolerance in humans with established autoimmune diseases, multiple sclerosis (Weiner, Mackin et al. 1993) and rheumatoid arthritis (Trentham, Dynesius-Trentham et al. 1993). The success of these trials has led to the commencement of Phase III trials of oral tolerance in the treatment of autoimmune disease.

The literature is awash with research papers extending back to the start of this century attempting to unravel the complex sequence of events that culminate in oral tolerance. A number of different mechanisms have been implicated which can be broadly divided into two groups potentially at two ends of a spectrum. This is summarised in this schematic (see Fig. 6.1) adapted from Weiner (Weiner 1997). The critical determinant of the mechanism by which tolerance is induced is the dose of antigen ingested. Tolerance to ingested antigens may be attributed to either an active suppressive mechanism or to the induction of clonal anergy/deletion. Low-dose oral tolerance has been shown to induce antigen specific regulatory cells, which upon subsequent recognition of antigen secrete the suppressive cytokine TGF-ß (Miller, Lider et al. 1992). Many papers have reported transferable

suppression mediated by T-cells from Peyer's patches, mesenteric lymph nodes and spleens (Miller and Hanson 1979; Strobel, Mowat et al. 1983). These suppressor cells have been shown to be $CD8^+$ T-cells, which act by secreting TGF-ß (Lider, Santos et al. 1989; Miller, Lider et al. 1992). High dose oral tolerance results from clonal anergy (Weiner, Friedman et al. 1994). Melamed and Friedman have demonstrated evidence of clonal anergy in mice fed high doses of ovalbumin whose splenocytes failed to proliferate in response to ovalbumin or to secrete IL-2 or upregulate IL-2 receptor (Melamed and Friedman 1993).

High and low dose tolerance can further be differentiated by their sensitivity to Cyclophosphamide treatment. The induction of low dose tolerance can be prevented by Cyclophosphamide treatment whereas high dose remains unaffected (Hanson and D 1982; Mowat, Strobel et al. 1982). Other factors have also been shown to abrogate oral tolerance such as the cationization of proteins, 2'-deoxyguanosine, interferon gamma, oestradiol and experimental graft versus host disease. Factors, which favour the induction of tolerance, include interferon beta and oral lipopolysaccharide.

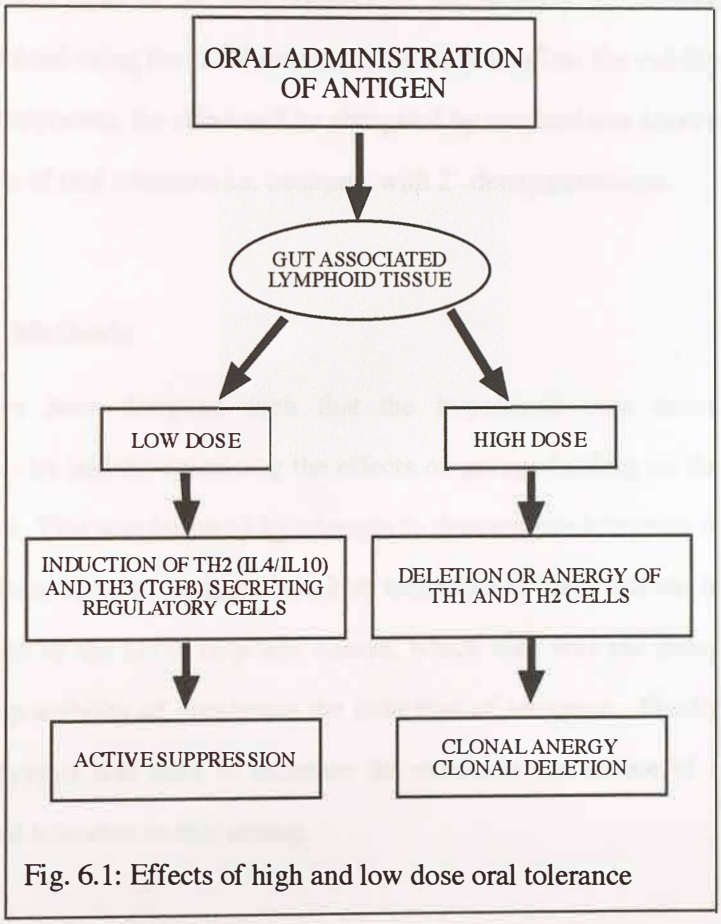


Fig. 6.1: Effects of high and low dose oral tolerance

6.2.1 Aims

Using the two murine tumour models (JBS and CarB) that we have partially defined already the immune response to progressive tumours induced after prior feeding with either tumour in a cross-over controlled manner will be determined in terms of tumour growth curves, cytotoxic potential, endogenous and mitogen-stimulated proliferative ability for comparison with data presented earlier. In addition the effect on antigen induced proliferation and on potential *in vitro* antigen-induced soluble immunosuppression will be investigated using dual chamber transwell culture systems. *In vivo* evidence of suppression of immune responses will be examined using the DTH response. To truly confirm the validity of the effect on DTH responses, the effect will be abrogated by mechanisms known to prevent the induction of oral tolerance i.e. treatment with 2'-deoxyguanosine.

6.3 Materials & Methods

The experiments have been designed such that the hypothesis was tested immediately and *in vivo* by initially examining the effects of gavage feeding on the growth rate of tumours. This was followed by attempts to demonstrate tolerance *in vitro* with the ^{51}Cr release assays. A more sensitive technique to demonstrate *in vivo* tolerance was used in the DTH response assays, which also was the assay used to investigate the possibility of preventing the induction of tolerance. Finally the transwell culture system was used to ascertain the existence and nature of a soluble mediator of oral tolerance in this setting.

The cell lines, animals, thymidine incorporation assays were performed as described in Chapter 2, General Materials and Methods.

6.3.1 Feeding

Following subcutaneous inoculation of 2 X 10^6 tumour cells in Balb/c mice tumours developed and were allowed to attain a size of 1-2cm^3 at which stage the animals were sacrificed and their tumours harvested. The tumours were homogenised in Phosphate Buffered Saline (PBS) using a Moulinex Hand Blender. The homogenate was extensively washed by centrifugation with PBS to remove debris. When the supernatant was clear the homogenate was resuspended in PBS to a final concentration of 0.2gm (wet weight) per ml. This was aliquoted and frozen until use. The PBS vector was also aliquoted and frozen. Using a 18Fr steel ball tipped feeding needle attached to a 1ml syringe, unanaesthetised, animals were fed by gavage 200μls of freshly thawed JBS or CarB tumour homogenate (40mg of tumour) or PBS (Fig. 6.2). Animals were fed daily for 14 days. The amount of feed and the duration of feeding were based on review of the literature, particularly studies which examined tolerance to cellular antigens.

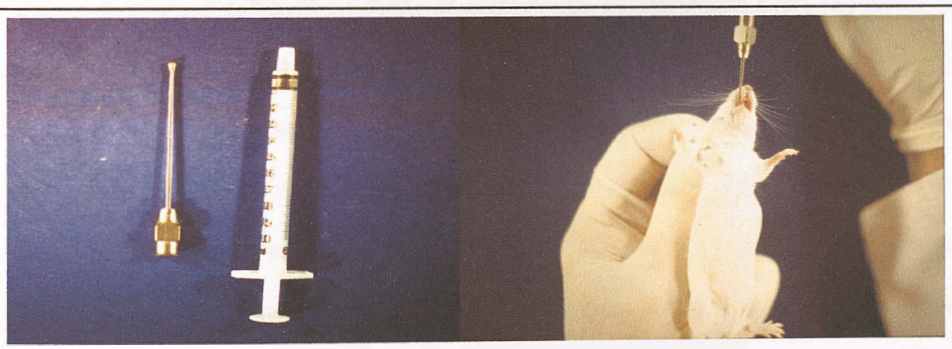


Fig 6.2: Ball-tipped, steel gavage feeding needle and 1ml syringe; in use for gavage feeding of a Balb/c mouse.

6.3.2 Tumour Induction

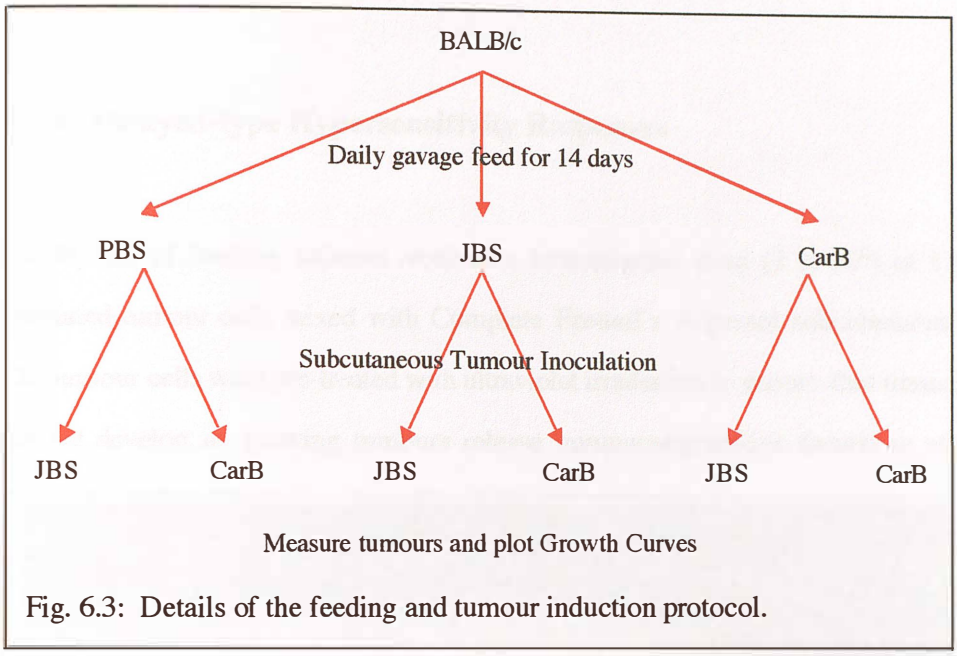


Fig. 6.3: Details of the feeding and tumour induction protocol.

On day 15 animals received a subcutaneous inoculation of 2 X 10^6 tumour cells, in a 50/50 dilution of DMEM in PBS, of either JBS or CarB cell lines (according to the protocol outlined in Fig. 6.3) and were monitored for tumour development daily. When a tumour became palpable alternate day measurements in two dimensions using a Verniers Callipers were performed. Tumour Volume was calculated according to the formula Volume = $\pi/6(l+s^2)$ where l = longest dimension and s = shortest. From these volumes tumour growth curves were constructed.

6.3.3 Spleen Cell Suspensions and Cytotoxicity

On day 25 of tumour growth all animals were sacrificed and their tumours and spleens excised. The mice were divided into groups based on weight within 1gm. The spleens were gently dissociated to generate spleen cell suspensions, according to the protocol described in section 2.4.4. These effector cells were then

resuspended at appropriate concentrations for use in a cytotoxicity assay as described in section 2.5.3.

6.3.4 Delayed-type Hypersensitivity Responses

On day 15, of feeding, animals received a tumourigenic dose (2 X 10^6) of U/V irradiated tumour cells mixed with Complete Freund's Adjuvant subcutaneously. The tumour cells were pre-treated with ultraviolet irradiation to ensure that tumours did not develop, as growing tumours release immunosuppressive factors *in vivo,*



Fig. 6.4: Example of a positive delayed-type hypersensitivity response to JBS cells in the right hind footpad of a Balb/c mouse. Dial gauge callipers, which have been modified to minimise the contact area to facilitate accurate measurement of the footpad in mice. Calipers in use to measure the footpad thickness of a Balb/c mouse.

which would have complicated the experiment. Animals received two further subcutaneous doses of tumour cells in Incomplete Freund's Adjuvant on days 25 and 35. On day 45 animals were anaesthetised and injected, through a 26G needle, with 50,000 tumour cells in 25μls of PBS into the right rear footpad. They received 25μls of PBS into the left rear footpad as a control. Twenty-four hours later the animals were sacrificed and with the observer blinded to the treatments footpad thickness was measured using a modified dial gauge callipers (JED Metrology Ltd, Dublin, Ireland) (Figs.6.4 & 6.5).

All observations were repeated in triplicate by a single observer. The difference between the test and the control foot was recorded. Results are expressed as the mean for each group of five animals $\pm$ SD.

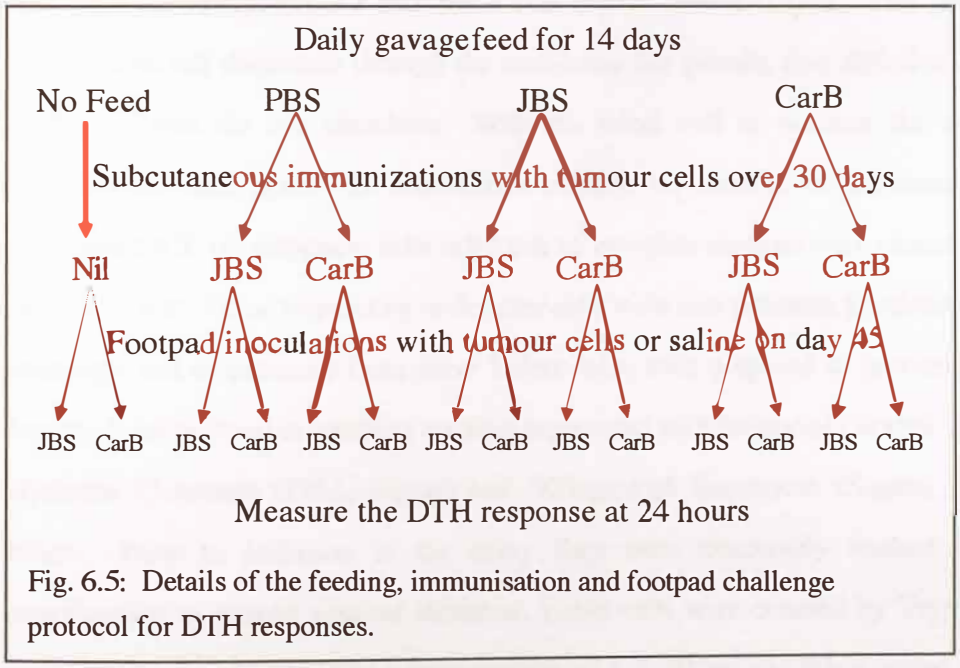


Fig. 6.5: Details of the feeding, immunisation and footpad challenge protocol for DTH responses.

6.3.5 Abrogation

To investigate if the development of tolerance could be prevented, as in previous studies by other units (Mowat 1986), mice were treated with 2'-deoxyguanosine (Sigma) at the same time as feeding. 2'-deoxyguanosine was resuspended in

distilled deionized water to a final concentration of 5 mg/ml. Using a 24G needle each animal was injected with 200μls (1 mg) intraperitoneally at the same time as the gavage feed. Control animals were given an equivalent volume of water intraperitoneally. Intraperitoneal 2'-deoxyguanosine injections were continued for the duration of feeding. Once feeding was stopped animals were immunised and DTH responses determined in the same manner as previously described.

6.3.6 Transwell cultures

Transwell cultures were prepared according to the method of Weiner *et al.* with modifications necessary for our model. The system utilises a dual chamber culture plate (Costar). The bottom chamber is part of the plate into which the upper chamber is placed. The base of the upper chamber or insert well is made from a semi-permeable polycarbonate membrane with a pore size of 0.4μm. This pore size prevents cell diapedesis through the membrane but permits free diffusion of solutes between the two chambers. With the insert well in position the two chambers are 1mm apart. To demonstrate *in vitro* suppression of proliferative responses 2.4 X 10^5 responder cells in 800μls of complete medium were placed in the bottom well. These responding or detector cells were non-adherent, fractionated splenocyte cell suspensions from naive Balb/c mice were prepared as previously described and cultured in complete medium augmented with 5ngms/ml Phorbol 12-Myristate 13-Acetate (PMA, Sigma) and 500ngms/ml Ionomycin (Sigma) for 24hrs. Prior to inclusion in the assay they were extensively washed by centrifugation to remove residual stimulant, viable cells were counted by Trypan blue exclusion, resuspended to a concentration of 3 X 10^6/ml and 80μls added to the bottom well. Spleen cells, 1.2 X 10^6 in 200μls, from control or tolerised mice were added to the upper well. Also to the insert (upper) well 1 X 10^5 JBS or CarB cells, which have been treated with Mitomycin C, were added. Briefly, 1 X 10^7 tumour cells per ml were treated with 50μg/ml for 30 mins at 37°C following which

they were extensively washed to remove residual Mitomycin C (Brooks and Fernandez-Botran 1994). All transwell cultures were set up in triplicate. The transwells were incubated at 37°C in a humidified atmosphere of 5% CO_2. After 54 hrs of culture the cells from the lower chambers were divided equally and reseeded, in the same medium, into 4 wells of a 96-well round-bottomed plate (Nunc). Each well was pulsed with 1μCi of [^{3}H] Thymidine (DuPont). After a further 18hrs each well was harvested onto glass fibre filter paper and counted using liquid scintillation techniques described earlier. Results were expressed as mean $\pm$ standard deviation. Set up in parallel with these transwells were 96 well round bottomed plates with triplicate wells to examine the endogenous proliferative ability of splenocytes from naive, control and tolerised mice as measured by uptake of [^{3}H] thymidine. The response of these splenocytes to stimulation with Mitomycin C treated JBS or CarB cells or to stimulation with PMA and Ionomycin was determined.

6.3.7 Statistics

The results presented here are typical of at least two experiments (in the case of growth curves) but more commonly 3 or more experiments. All animal groups contained a minimum of 5 animals per treatment group. All *in vitro* experiments were with triplicate or quadruplicate replicates. For comparison between groups the Student's t-test or X^2 statistic was used. A p value of less than 0.05 was considered significant.

6.4 Results

The results will show that JBS tumours grew faster in the JBS tumour fed mice than in either the PBS ($p=0.025$) or CarB ($p=0.168$) fed animals. This suggests that the animals have been tolerised systemically to tumour antigens on the JBS cell. Furthermore this tolerance is antigen specific. Splenocytes from the JBS fed, JBS tumour-bearing mice (tolerised) were less cytotoxic for JBS cells than splenocytes from control fed, JBS tumour bearing mice (untolerised). The DTH response in tolerised mice was significantly abrogated ($p<0.01$) compared to controls. The JBS tumour volume growth curves for tolerised and control mice are shown in Fig. 6.6. It can be seen that, in immunocompetant Balb/c mice, JBS tumours grew faster in those fed JBS tumour than those who were fed PBS or CarB tumour as a control. An additional control that is included is a growth curve of JBS tumours in the immunocompromised athymic nude mouse. The growth curve in the JBS tolerised mice is the one which most approximates to the nude mouse, which lacks functioning T-cells.

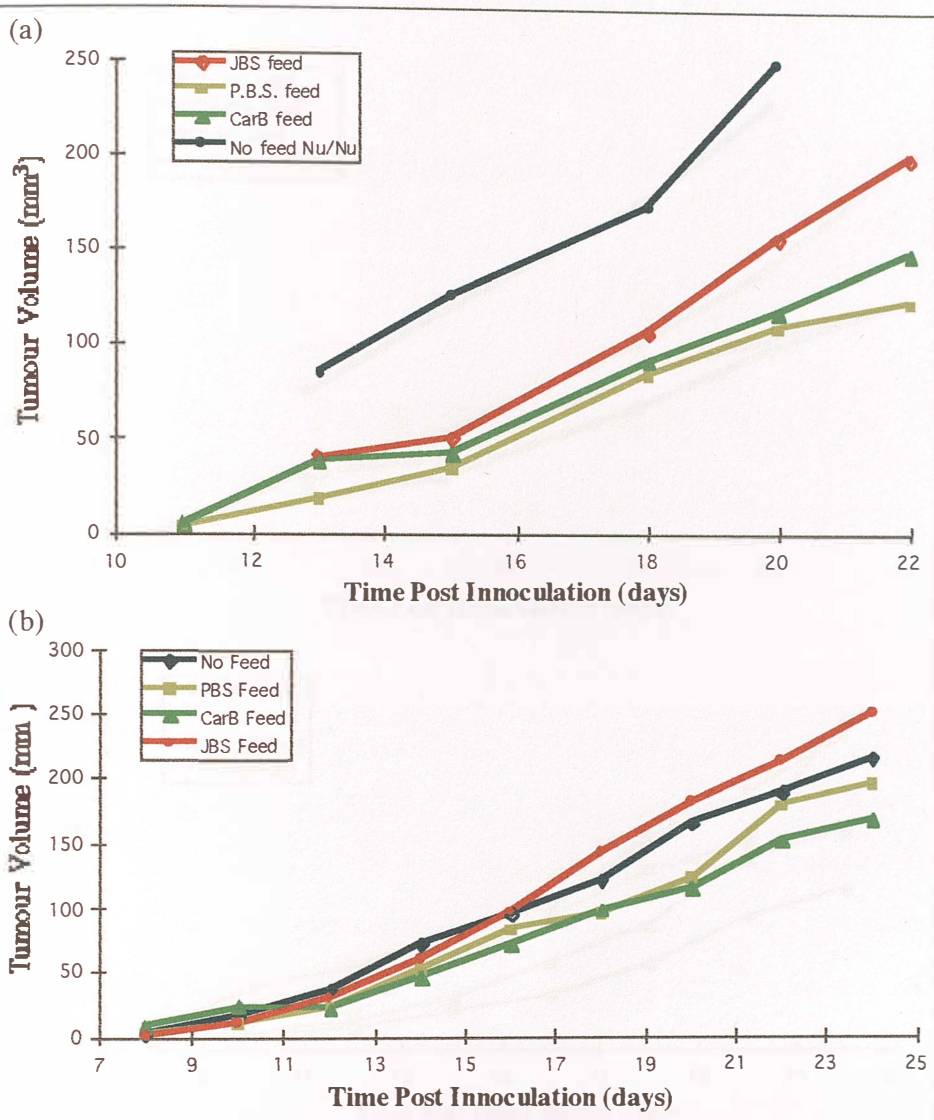


Fig. 6.6: (a) Volume growth curves for JBS tumours that developed in tolerised (JBS fed) and control Balb/c mice (No feed in athymic nude mice, PBS fed and CarB fed). Each point represents the mean of a minimum of 10 mice. (b) A repeat experiment.

The data from a similar series of mice that were inoculated with CarB tumours is shown in Fig. 6.7. There is no effect on CarB tumour growth when mice are fed CarB tumour, JBS tumour or PBS.

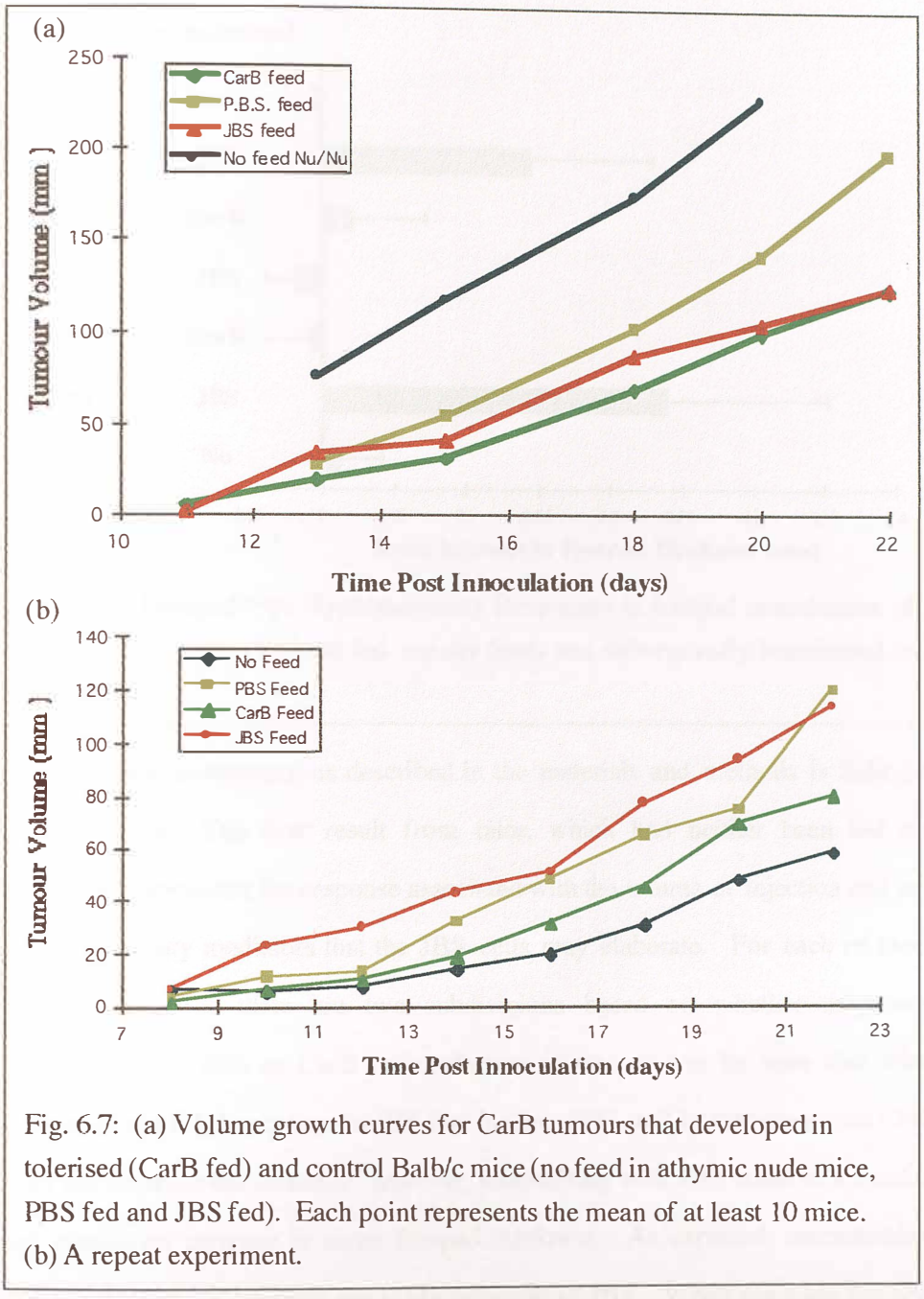


Fig. 6.7: (a) Volume growth curves for CarB tumours that developed in tolerised (CarB fed) and control Balb/c mice (no feed in athymic nude mice, PBS fed and JBS fed). Each point represents the mean of at least 10 mice. (b) A repeat experiment.

The Delayed-Type Hypersensitivity (DTH) Response in tolerised mice was significantly reduced compared to the controls. The data from a series of DTH responses to JBS footpad injections is shown in Fig. 6.8. The X-axis represents the mean (S.D.) difference in footpad thickness between the control (PBS only) and the test (JBS cells in PBS) feet of a panel of 5 mice. Each mouse therefore acts as its own control thereby obviating the need for pre-injection measurements. The various treatment protocols including the nature of the feed and the

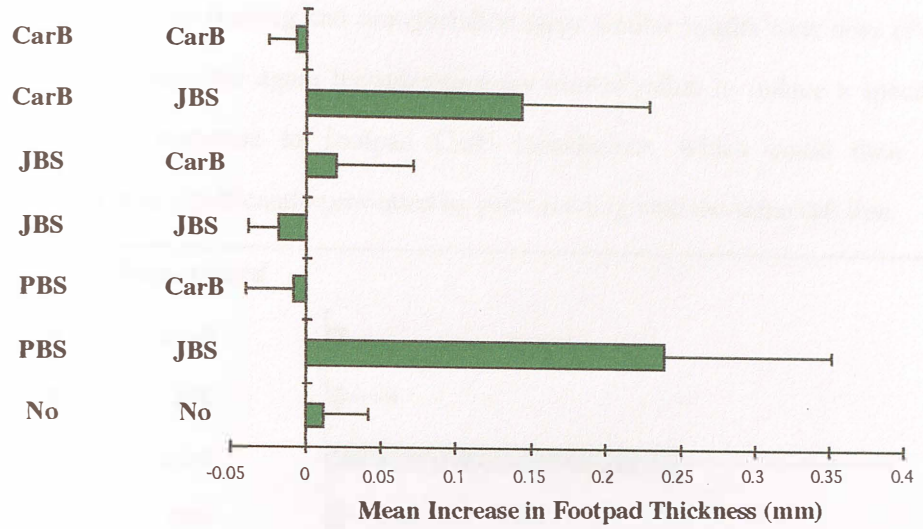


Fig. 6.8: Delayed-type Hypersensitivity Responses to footpad inoculations of JBS cells in mice who been fed various feeds and subsequently immunised to JBS cells.

subcutaneous immunogen as described in the materials and methods is indicated on the Y-axis. The first result from mice, which had neither been fed nor immunised represents the response associated with the trauma of injection and any proinflammatory mediators that the JBS cells may elaborate. For each of the 3 feeding categories there are two subdivisions based on whether they were immunised with JBS or CarB cells subcutaneously. It can be seen that when measuring the DTH response to JBS that feeding PBS and immunizing with CarB does not increase the response, however, immunizing with JBS leads to a marked and significant increase in mean footpad thickness. As expected, immunisation with CarB does not augment the DTH response to JBS. When mice are fed JBS tumour prior to subcutaneous immunization with JBS cells they do not develop a delayed-type hypersensitivity response. This is not seen in mice fed CarB, immunised with JBS and tested with JBS. This series of experiments demonstrates that by subcutaneous immunisation with JBS cells in adjuvant a marked cellular immune response can be induced. Furthermore this response can be negated, in an antigen specific manner, by prior feeding with the immunogen. In

an identical series of experiments where mice were assayed for DTH responses to CarB after various feeding and immunization steps similar results were seen (Fig. 6.9). It was possible again by subcutaneous immunization to induce a specific strong DTH response to footpad CarB inoculations, which could then be specifically and significantly prevented by prior feeding with the same cell line.

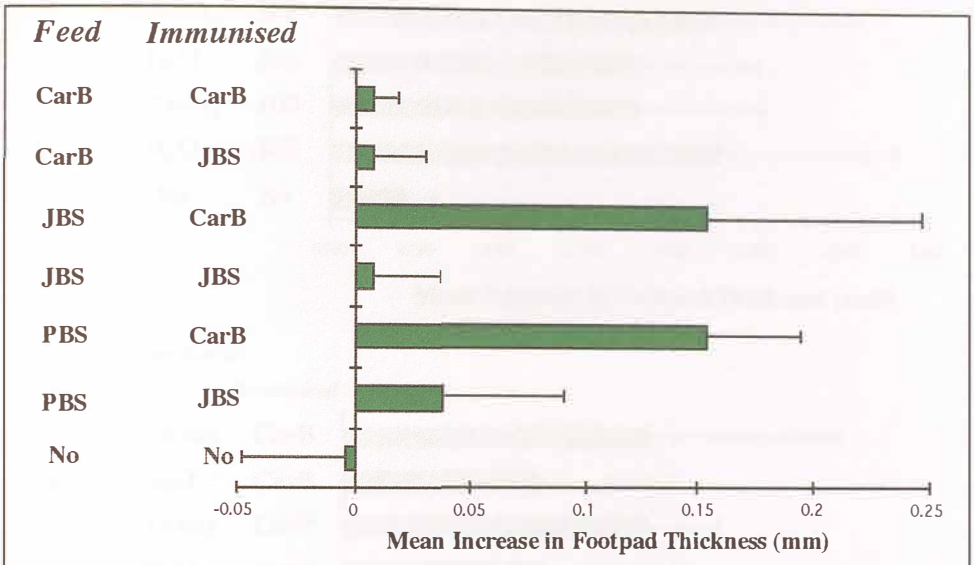


Fig. 6.9: Delayed-type Hypersensitivity Responses to footpad inoculations of CarB cells in mice who been fed various feeds and subsequently immunised to CarB cells.

Furthermore as shown in Fig. 6.10, this prevention of the development of a DTH response could be significantly abrogated by simultaneous intraperitoneal injections of 2'-deoxyguanosine. Fig. 6.10 (a) shows again that it is possible through subcutaneous immunisations to induce a strong DTH response to JBS tumour cells. This response in turn can be completely prevented by prior feeding with JBS tumour. This prevention is itself abrogated by intraperitoneal injections of 2'-deoxyguanosine concurrent with the feeding regime. Intraperitoneal injections of 2'-Deoxyguanosine at the same time as gavage feeding of antigen enabled the development of a normal DTH response. This preventative effect is not seen if the animals are injected intraperitoneally with distilled deionised water, the diluent used to reconstitute the 2'-Deoxyguanosine. Furthermore it cannot be

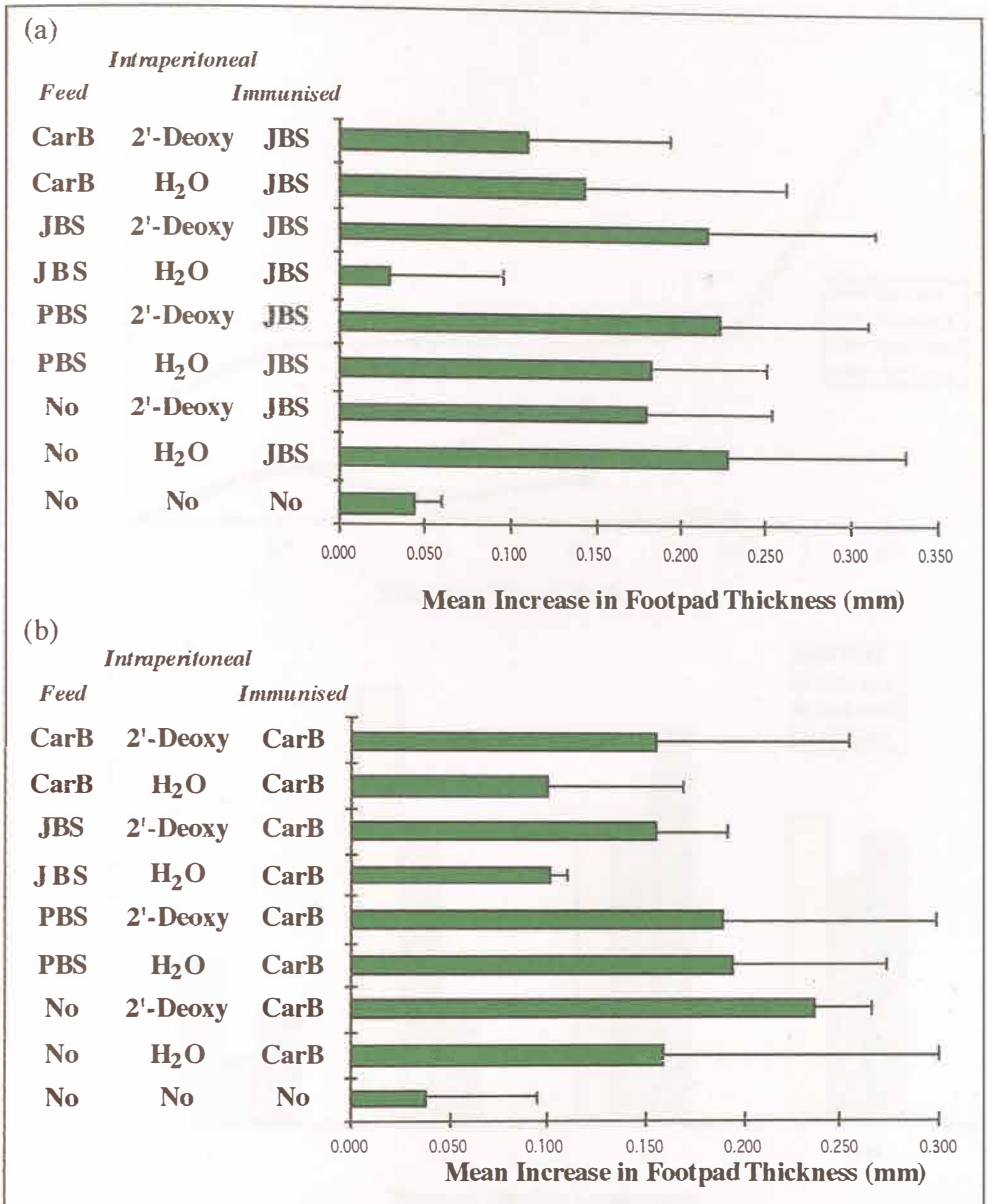


Fig. 6.10: (a) Delayed-type Hypersensitivity Responses to footpad inoculations of JBS tumour cells in mice who have been immunised to JBS cells after various feeding and abrogating treatments. (b) Similar series of DTH responses to CarB following immunisation with CarB

attributed to intraperitoneal injection of 2'-Deoxyguanosine, as it is still possible to induce a potent DTH response in animals that are not fed the tolerogen or are fed PBS. The antigen specificity of the phenomenon is again demonstrated. A similar trend is seen, though not as marked, in those mice tolerised to and immunised with CarB tumour cells (Fig. 6.10 (b)).

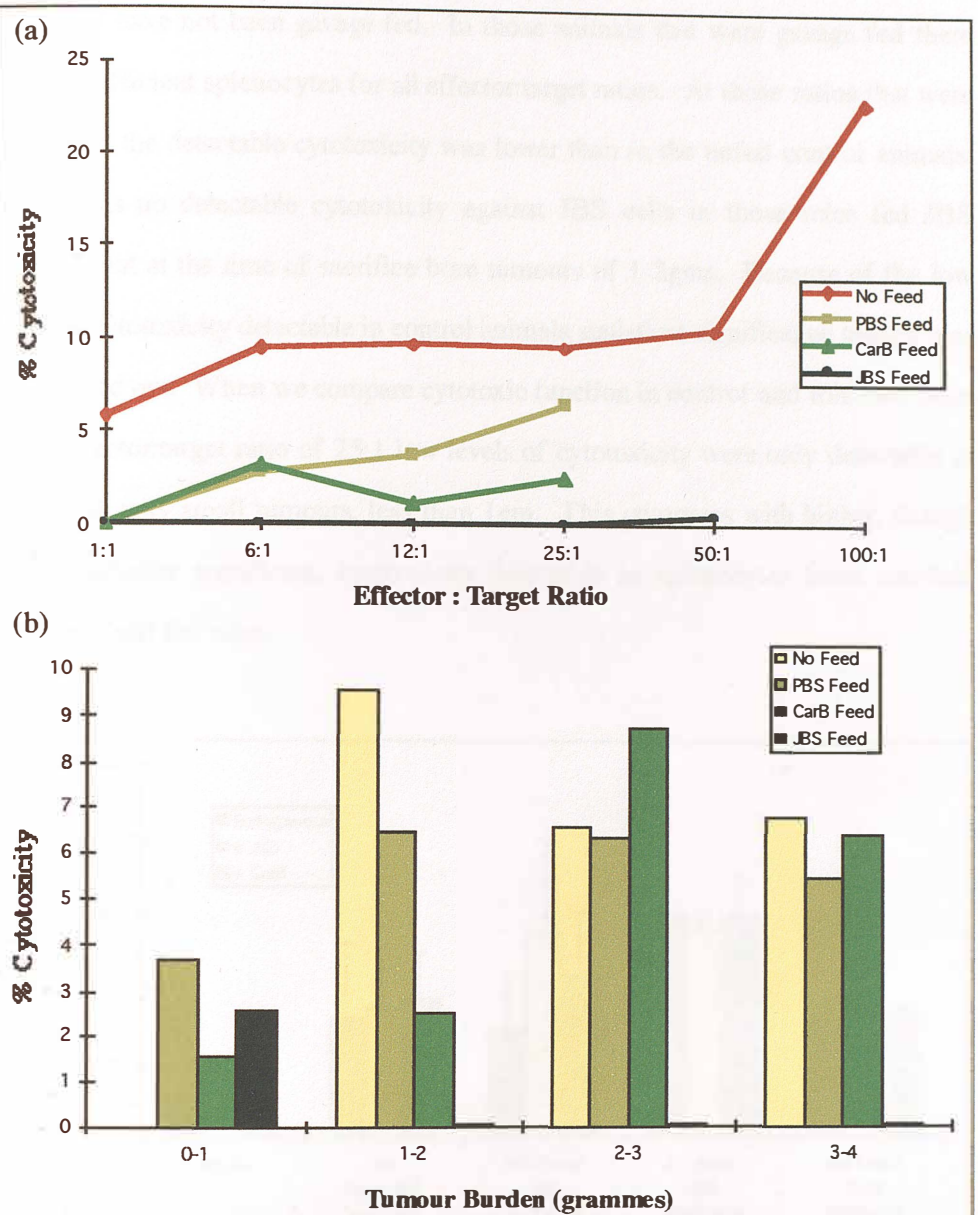


Fig. 6.11: (a) *In vitro* cytotoxicity of splenocytes from tolerised and control Balb/c mice against ^{51}Cr labelled JBS cells at varying effector to target ratios. At the time of sacrifice all mice bore 1-2 gm JBS tumours on their flank. (b) In-vitro cytotoxicity of splenocytes from tolerised and control, JBS tumour bearing, Balb/c mice against ^{51}Cr labelled JBS cells at an effector to target ratio of 25:1.

Attempts to demonstrate *in vitro* cytotoxicity against ^{51}Cr-labelled JBS cells were made (Figs. 6.11 a&b). This figure demonstrates cytotoxicity in splenocyte suspensions of tumour bearing animals, which increases with increasing effector:target ratio. This cytotoxicity is most readily detected in tumour bearing

mice that have not been gavage fed. In those animals that were gavage fed there were insufficient splenocytes for all effector:target ratios. At those ratios that were examined the detectable cytotoxicity was lower than in the unfed control animals. There was no detectable cytotoxicity against JBS cells in those mice fed JBS tumour that at the time of sacrifice bore tumours of 1-2gms. Because of the low level of cytotoxicity detectable in control animals statistical significance testing was not carried out. When we compare cytotoxic function in control and tolerised mice at an effector:target ratio of 25:1 low levels of cytotoxicity were only detectable in mice with very small tumours, less than 1gm. This compares with higher, though not statistically significant, cytotoxicity detectable in splenocytes from non-fed, PBS or CarB fed mice.

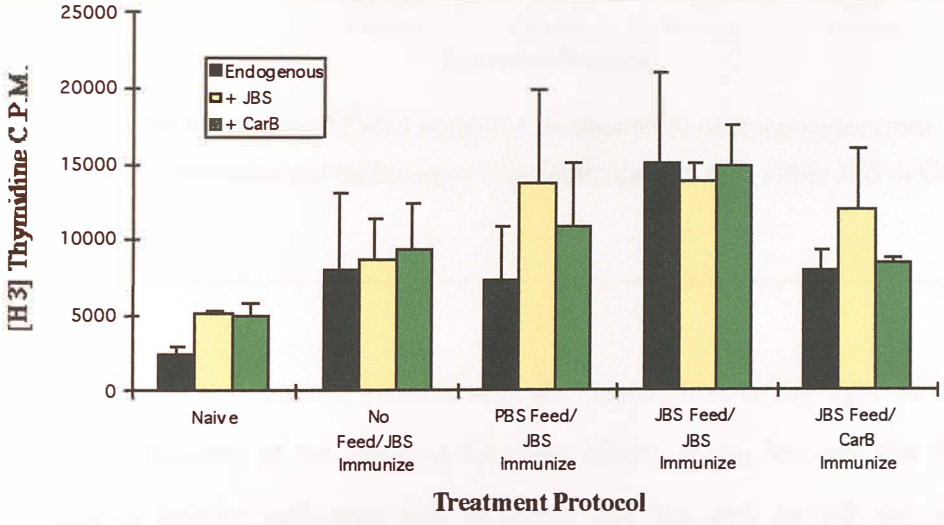


Fig. 6.12: Endogenous and antigen (Mitomycin C treated cells) stimulated [^{3}H] Thymidine incorporation of splenocytes from naive, control and tolerised Balb/c mice after immunisation with either JBS or CarB cells.

To examine if the tolerising effect could be attributed to cellular anergy the endogenous proliferative ability of splenocytes from control and tolerised mice was examined, as was the response of those splenocytes to antigen stimulation and polyclonal PMA and Ionomycin stimulation. When compared to naive animals it can be seen that immunising with either JBS or CarB cells increases the

endogenous proliferation of fractionated splenocytes (Fig. 6.12). As expected, endogenous proliferation was increased in all animals that were immunised. Co-culturing these splenocytes with Mitomycin C treated JBS or CarB cells did not significantly increase the proliferation rate as evidenced by enhanced incorporation of [^{3}H] Thymidine. There was no significant difference in the response of the various treatment groups to stimulation with PMA and Ionomycin (Fig. 6.13).

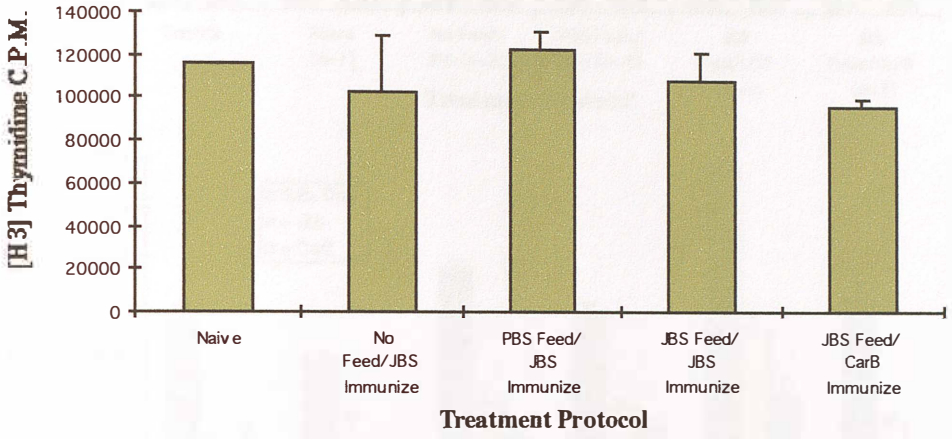


Fig. 6.13: PMA stimulated [^{3}H] Thymidine incorporation of splenocytes from naive, control and tolerised Balb/c mice after immunisation with either JBS or CarB cells.

The results from the transwell cultures were also unable to shed any light on the possible mechanisms of the observed tolerising effect. It can be seen that the responder or detector cells grew well in culture and that their growth was not affected by the presence of Mitomycin C treated JBS or CarB cells in the upper well (Fig. 14,a). The addition of splenocytes from any of the treatment groups (naive, no feed, PBS feed or JBS feed) to the upper well had little if any effect on the proliferative ability of the detector cells in the lower well. However any combination of treatment group splenocytes and Mitomycin C treated tumour cells in the upper well inhibited the proliferation of splenocytes in the lower wells. To ensure that this effect was not attributable to the effect of Mitomycin C treatment of the tumour cells the same series of experiments was carried out using viable, non-

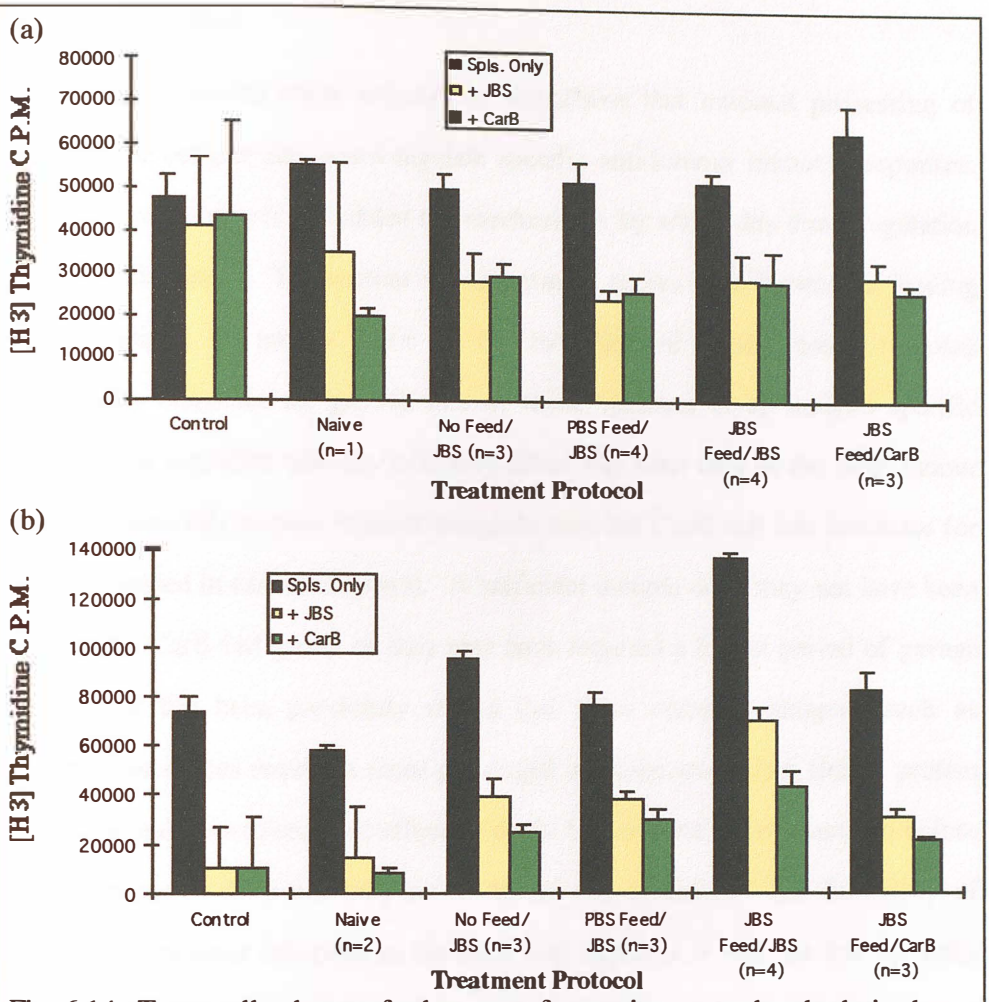


Fig. 6.14: Transwell cultures of splenocytes from naive, control and tolerised mice, showing endogenous and antigen stimulated proliferation. Panel (a) details the response to Mitomycin C treated JBS or CarB cells in the upper well. Panel(b) uses JBS or CarB cells not treated with Mitomycin C.

Mitomycin C treated JBS or CarB cells in the insert wells as appropriate (Fig. 14,b). Unfortunately the addition of viable tumour cells to the insert wells was sufficient to inhibit the proliferative ability of the responder cells. Therefore the inhibition of proliferation in the test wells could not be interpreted as being due to a soluble mediator from the test splenocytes.

6.5 Discussion

The results presented above support the hypothesis that mucosal processing of shed tumour antigen may down regulate specific anti-tumour immune responses. They have not however elucidated the mechanisms by which this down-regulation occurs in this model. The tumour volume growth curves have shown that feeding tumour antigen by gavage prior to the induction of subcutaneous tumours significantly enhances the growth rate of those tumours in an antigen specific manner. We postulate that this tolerising effect was seen only in the JBS tumour group because this tumour is more antigenic than the CarB cell line (evidence for this is presented in earlier chapters). A sufficient antigen dose may not have been given in the CarB fed group or they may have required a longer period of gavage feeding. It has been previously shown that more complex antigens such as allogenic leucocytes require a more prolonged administration than simple protein antigens to induce a tolerogenic effect. It could be said that feeding antigen before tumour induction does not truly reflect the *in vivo* situation. The feasibility of feeding after tumour induction in the flank was explored, it was felt that the daily trauma to the flank associated with immobilisation of the animal for gavage feeding, however minimal could be a complicating factor. Alternative methods of administering the antigen such as tumour cell suspensions in water *ad libitum* or tumour fragments reconstituted with the chow were not pursued because of the impossibility of regulating the dose of antigen each animal received. There are a number of publications based on research into animal models and human autoimmune diseases that demonstrate oral tolerance after the subject has become sensitised to the antigen and there is no reason to suspect that the same could not apply in this model.

To quantify the tolerising effect *in vivo* more precisely, animals were immunised, after feeding, with tumour cells in Freunds adjuvant a powerful immunopotentiator and delayed type hypersensitivity responses were assessed. The DTH response

was chosen because it is a cellular immune response that is easy to measure and has been shown to be susceptible to oral tolerance. Immunising with tumour cells in adjuvant induced a marked swelling that was significantly greater than could be explained by the trauma of injection or attributed to proinflammatory mediators/cytokines released by the tumour cells into the footpad. Furthermore the response was antigen specific as immunisation with one tumour antigen (JBS or CarB) and testing with the alternate antigen elicited a response equivalent in magnitude to non-immunised controls. This DTH response was completely abrogated by prior feeding with the same antigen that was used to immunise and to elicit the response but not if animals were fed the vector-PBS or an alternative tumour antigen. This series of experiments clearly demonstrated the induction of an antigen specific immune response, which was prevented with equal specificity by feeding the offending antigen. This oral tolerance of DTH responses was also seen in animals fed CarB cells perhaps because DTH responses are relatively easy to tolerize. Thus strengthening the hypothesis that the failure to demonstrate an effect on tumour growth was dose related. If, as has been shown in previous work, animals were given daily injections of 2'-deoxyguanosine at the same time as the gavage feed they failed to develop oral tolerance. This abrogation of oral tolerance was directly attributable to the 2'-deoxyguanosine and not to the diluent-H_2O or the trauma of daily intraperitoneal injections. Earlier papers would have suggested that this implied that the mechanism of tolerance in this model was the induction of suppressor T cells (Mowat 1986) as 2'-deoxyguanosine was believed to be specifically toxic for suppressor T cells.

In vitro experiments on the cytotoxic function of splenocytes from tolerised and control mice yielded evidence suggestive of a tolerising effect on cytotoxic function. There are a variety of explanations why this series of experiments failed to yield a statistically significant difference. The cytotoxic function of splenocytes in these tumour-bearing animals is also being suppressed by factors released from

the developing tumour (see previous chapter). The detectable cytotoxicity in the control animals is low for reasons demonstrated and investigated in previous chapters. When the reference cytotoxicity is as low as 25%, at best, the nature and dynamics of the ^{51}Cr release assay make statistical conclusions difficult or invalid (Martz 1993).

By employing a feeding regime arrived at empirically, though based on similar work with allogenic leucocytes, we did not know whether we were inducing a low dose - active tolerance or a high dose - anergic tolerance and we proceeded to investigate this. The endogenous, non-mitogen or antigen activated, proliferative response of splenocytes from naive, control and tolerised animals was examined. We found that compared with naive mice both control and tolerised mice showed enhanced endogenous proliferation. This is probably related to the immunisation with Freund's adjuvant a potent immunopotentiator. There was no evidence of reduced or suppressed proliferative function in tolerised versus control mice. Furthermore, co-culture of splenocytes with tolerogenic or control tumour cells did not significantly alter their proliferation. Neither was a significant difference in the residual proliferative potential of these spleen cell suspensions seen when maximally stimulated with PMA and Ionomycin. These experiments show no evidence of a reduction in splenocyte proliferation either inherently or in response to antigen co-culture to suggest that the mechanism behind the tolerising effect seen *in vivo* is due to active suppression or clonal anergy. This probably reflects the use of crudely fractionated, i.e. non-adherent, and therefore polyclonal spleen cell suspensions. The splenocyte population as a whole could mask any effect of or on a clone or group of clones of splenocytes. Attempts were not made to extract or expand any putative clone of suppressive cells as previous attempts by other investigators in this field have been unsuccessful.

The transwell culture experiments provided a more refined method of examining if the splenocytes from tolerised animals, upon co-culture with the tolerogenic tumour cell line, released a soluble factor capable of mediating antigen non-specific suppression. The addition of Mitomycin C treated tumour cells alone to the insert wells of the dual well chamber did not significantly alter the proliferation of the responder cells in the lower well. We conclude that the treated tumour cells therefore are not secreting any immunosuppressive factors. The addition of splenocytes alone from either naive, control or tolerised mice to the insert wells may have slightly, but not significantly, enhanced the proliferation of the responder cells. Therefore in the absence of exogenous antigen stimulation these splenocytes do not release suppressive molecules. The combination of splenocytes from any source (naive, control or tolerised mice) and Mitomycin C treated tumour cells, either JBS or CarB, in the upper well reduced the incorporation of [^{3}H] Thymidine by the responder cells. There is no significant difference in the degree of suppression between the different groups. The combination of splenocytes and tumour cells in the insert well therefore is capable of non-specifically inhibiting the proliferation of the responder cells. Splenocytes from the control or tolerised groups were no more capable of inducing suppression when co-cultured with tumour cells than were splenocytes from naive mice. We can conclude that co-culture of tumour cells with splenocytes of any source causes the release of a soluble factor capable of suppressing activated splenocytes *in vitro*. Mitomycin C is a potent inhibitor of cell division and exerts its action by irreversibly intercalating into DNA. To determine if the suppression seen was due to the Mitomycin C treatment of tumour cells and perhaps in some way recycling of the Mitomycin C a similar series of transwell culture experiments was carried out using tumour cells from cultures that have not been treated. These experiments demonstrated that the addition of tumour cells to the insert wells with or without splenocytes from any source inhibited significantly the proliferation of the responder cells. These data suggest that tumour cells *in vitro* release soluble factors capable of inhibiting the

proliferation of activated splenocytes. This hypothesis has been dealt with in earlier chapters. Unfortunately the suppression by tumour cells alone renders the remainder of the experiment uninterpretable.

A thorough review of the literature has yielded only one paper examining tolerance to tumour antigens (Hashimoto, Okuno et al. 1984). This paper examined the effect on DTH responses, *in vivo* tumour neutralising assays (Winn assays) and *in vitro* cytotoxicity to tumour antigens after prior sensitisation with tumour antigen via the intragastric route. They were able to demonstrate a reduction in the DTH response by prior feeding of tumour antigen. They also demonstrated reduced *in vitro* cytotoxicity and a markedly reduced *in vivo* tumour neutralising ability of splenocytes from animals that were pre-fed and subsequently immunised by subcutaneous tumour induction, which was excised 7 days later. The work above confirms and expands on these observations in a different animal model. We have presented convincing evidence of *in vivo* tumour antigen specific immune tolerance following antigen feeding, with the DTH response and the tumour volume growth curves. Their *in vitro* results are more convincing than ours probably because prior to use in an assay they expanded the cytotoxic cells *in vitro* by co-culture with irradiated target cells. By excising the developing tumours after 7 days the analysis of their data was not complicated or confounded by tumour derived factors and their effects. The strongest *in vivo* evidence that was presented by Hashimoto *et al.* was the Winn assay or tumour neutralising assay data, which could be interpreted as merely carrying out the *in vitro* cytotoxicity assay subcutaneously rather than in a vial. Similar to ourselves they were unable to determine the mechanism of tolerance induction but did out rule the involvement of suppressor cells. Taken together both papers present strong evidence of tumour antigen specific down-regulation of the anti-tumour immune response both *in vivo* and *in vitro*.

6.6 Conclusions

In conclusion we have clearly and consistently demonstrated *in vivo* evidence that feeding mice tumour antigens prior to immunisation with the same antigens inhibits the development of an immune response in an antigen specific manner. Furthermore feeding mice tumour cells prior to the induction of subcutaneous tumours facilitates a statistically significant enhancement of tumour growth rate. Unfortunately, probably because of the complexity of the antigen we used and the confounding complexity of the influence of tumour-associated immunosuppression we were unable to determine or suggest a mechanism for this oral tolerance.

Chapter 7

Discussion

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Following its inception, the study of tumour immunology was initially carried along on a tide of immunological euphoria only to be decimated by immunological nihilists such as Hewitt (Hewitt,1982). The first demonstration of tumour antigens capable of inducing specific rejection of transplantable tumours was seen in mice with methylcholanthrene-induced tumours (Prehn and Main 1957; Klein, Sjogren et al. 1960). Following inoculation with syngenic tumour cells the developing tumours were excised and the mice proved resistant to subsequent inoculations with the same tumour cell line. Hewitt, however, reported that when using spontaneous murine tumours he was unable to demonstrate any evidence of anti-tumour immunity (Hewitt 1982). This has since been shown not to be the case. Current immunologists are more pragmatic, recognising that neither polarised view is accurate, though the pendulum of evidence has swung back in favour of immunology.

The development of tumours in immunosuppressed patients and their subsequent resolution upon cessation of immunosuppressive therapy supports a role for the immune response in the control of malignant disease. Currently 4-5% of organ transplant recipients will develop cancer, however it has been predicted that by 33 years post cadaveric renal transplant all recipients will have skin cancer and 30 to 40% will have or will have died of non-skin cancer (Sheil, Disney et al. 1997; Sheil 1998). Furthermore more aggressive immunosuppression is associated with earlier detection of cancers (Hiesse, Kriaa et al. 1995). As well as the therapeutically immunosuppressed patients with secondary immunodeficiency from other causes such as AIDS, or autoimmune diseases and patients with primary immune deficiencies as in SCID, DiGeorge etc. are also at

an increased risk. The risk of developing a malignant tumour in persons with primary immune deficiencies is 4%, approximately 10,000 times higher than normal people (Ioachim 1990).

There is a wealth of circumstantial evidence of a successful immune response against tumours in humans. Historically this dates back as far as de Lisle's report in the 18th century of the disappearance of a breast cancer following the injection of pus, and subsequent evolving infection, into the patients leg (Old 1996). This contributed to the development of Coley's toxin, a preparation consisting of the soluble toxins from erysipelas and *Serratia marcescens*, in the 19th century and ultimately to the use of BCG, which has been shown in randomised trials to be effective in the treatment of superficial bladder cancer. (Coley 1894).

It was Mitchison, in 1955, who first reported that the adoptive transfer of lymph node cells from mice that reject tumour allografts would confer accelerated rejection responses in normal recipients on being challenged with the specific tumour allograft (Mitchison 1955). However to mediate tumour regression, at least 10^8 effector cells were required, often needing several donor animals. The lack of suitable/appropriate multiple human donors was overcome with the discovery of Interleukin-2 (Il-2) which has been shown to expand and help to further sensitise immune cells *in vitro* and *in vivo,* so much so that it became routine to administer exogenous Il-2 in conjunction with immune cells (Cheever, Greenberg et al. 1982; Eberlein, Rosenstein et al. 1982). A variety of immune cells have been used.

The increased incidence in the occurrence of spontaneous tumours in Beige mice not seen in athymic mice is attributed to the presence of NK cells, said to be responsible for tumour surveillance, in the latter (Whiteside and Herberman 1995). Following exposure to Il-2 NK cells become LAK cells (Grimm, Mazumder et al. 1982; Gunji, Vujanovic et al. 1989). After some success in animal models and pre-clinical studies Il-2 alone or in combination with LAK was shown to induce response rates of 15 – 20 % in patients with malignant melanoma or renal cell carcinoma (Haas, Hillman et al. 1993; Rosenberg 1999).

Tumour infiltrating lymphocytes (TILs), isolated by enzyme digestion of the tumour and expanded in culture with Il-2 were shown to be 50 – 100 times more potent than LAK cells in mediating tumour regression in animal models (Rosenberg, Spiess et al. 1986; Speiss, Yang et al. 1987). Compared with the non-specific activity of LAK cells 30% of TILs from patients with melanoma have been reported to exhibit specific cytotoxic activity against autologous tumour and not against other allogeneic tumours (Topalian, Soloman et al. 1989). With the addition of Cyclophosphamide to patients receiving TILs and Il-2, Aebersold et al. reported tumour regression in 40% of patients with melanoma (Aebersold, Hyatt et al. 1991). However Bukowski et al. reported no effect in patients with metastatic renal cell carcinoma (Bukowski, Sharfman et al. 1991). So, as before, the biology is more complicated that it first appears.

An alternative source of specific effector cells is the tumour draining lymph nodes (TDLN) based on the probability that the precursor frequency of pre-

effector T-cells that facilitate the development of tumour specific CTLs would be highest in the draining nodes (Yoshizawa, Sakai et al. 1991). Shu *et al.* demonstrated that it was possible to generate tumouricidal TDLNs to a weakly antigenic animal tumour thus overcoming one of the greatest criticisms of the use of animal models (Shu, Chou et al. 1987). Animal and human studies have shown some benefit from TDLN therapy (Hoover Jr., Surdyke et al. 1984; Yoshizawa, Chang et al. 1991; Chang, Yoshizawa et al. 1993; Mitsuma, Yoshizawa et al. 1994). The beneficial effect of LAK, TIL and TDLN therapy has been attributed in large part, though not exclusively, to Il-2 infusions given in association, rather than to the activated cells themselves (Rosenberg, Lotze et al. 1993). A significant drawback of these therapies, apart from the need for tumour tissue, which is not always available, to enable the generation of TILs or TDLNs, is the toxicity associated with Il-2 infusions (Lotze, Chang et al. 1986).

Autolymphocyte therapy is an attempt at overcoming these obstacles by infusing autologous peripheral blood lymphocytes which have been non-specifically expanded *ex vivo* in tissue culture using supernatant from lymphocytes stimulated either in a one-way mixed lymphocyte culture or with anti-CD3 antibodies such as OKT3, with tumour cell extracts also, if available. Autolymphocyte therapy has been shown to prolong median survival time of patients with metastatic renal cell carcinoma 2.5 fold (Osband, Lavin et al. 1990). When the culture supernatant was analysed they found that patients whose supernatant had >500pg Interleukin-1 per ml had a 6-fold survival advantage over those whose IL-1 content was <500pg per ml. The same group reported prolonged survival of patients with metastatic renal cell carcinoma in a

larger clinical series of 355 patients but with limited demonstrable tumour regression, seen only in 4% of patients, with the majority only doing so after several months of therapy (Lavin, Maar et al. 1992). Autolymphocyte therapy has the additional advantage that, in view of its low toxicity, it is carried out on an outpatient basis. Unfortunately, long-term follow-up studies did not confirm significant differences when compared with interferon therapy.

As well as being used to produce culture supernatant for the induction of autolymphocyte therapy anti-CD-3 antibody, OKT3, has been used to stimulate T-cells for direct use as immunotherapeutic agents. This has been designated T-cell receptor activated T cells (TRAC) therapy. TRAC have been shown to have LAK and NK like cytotoxic properties as well as producing IFN, TNF-α and GM-CSF, which may provide anti-tumour effects. TRAC cell therapy has, in animal models, been shown to be 20-fold more effective than LAK cells (Yun, Hargrove et al. 1989). The central principle in TRAC cell therapy is to activate the T cells non-specifically *in vitro* and to expand them *in vivo* with IL-2 (Anderson, Blazar et al. 1989; Loeffler, Platt et al. 1991; Saxton, Longo et al. 1997).

As mentioned earlier optimal activation of T-cells requires co-stimulation via CD28 with, either anti-CD28 antibody, B7.1 or B7.2 molecules. Co-stimulation enhances proliferation, stabilises cytokine mRNA and by inducing Bcl-X_1, confers resistance to apoptosis (Boise, Noel et al. 1995). These anti-CD3, anti-CD28 co-activated T-cells (COACTS) generate a Th1-type cytokine profile, i.e. IL-2 and IFN, and have been shown to help prolong survival in patients with

Non-Hodgkin's Lymphoma possibly by helping to correct T-cell activation defects which had been present prior to therapy thereby reconstituting specific anti-tumour immunity as well as providing non-specific effectors (Harada, Okamoto et al. 1996; Liebowitz 1998).

The ideal would be to transfuse CTLs activated against specific tumour antigens. With our ever-increasing knowledge this day is not far off hastened by the continuing discovery of tumour antigens. The most promising targets would either be oncogenic viral proteins, such as CMV or EBV, or the products of the more common genetic mutations, such as p53, p21, Ras or Her-2/neu (Riddell, Watanabe et al. 1992; Yanuck, Carbone et al. 1993). It is already possible to generate antigen-specific CTL *in vitro* and also to vaccinate against Ras (Gjertsen, Bakka et al. 1996). The day cannot be far away when we will be infusing *in vitro* primed CTL against mutated oncogene products.

Essential to the development of an immune response to cellular antigens are professional antigen presenting cells of which Dendritic cells (DC) are the most effective (Cella, Sallusto et al. 1997). Dendritic cell therapy offers the advantage of minimising toxicity, as there is no need for systemic IL-2, and also obviates the need for *ex vivo* expansion of large numbers of lymphocytes. It has previously been shown that DC respond to antigen encounter by internalising, processing and presenting antigens to T-cells, as well as upregulating expression of MHC molecules, co-stimulatory molecules and cytokines. They can be derived from the peripheral blood or bone marrow and expanded *in vitro* with relative ease (Morse, Zhou et al. 1997). Dendritic cells for use in

immunotherapy can be loaded with antigen from a wide variety of sources, tumour lysates, whole tumour proteins, HLA-A2 restricted peptides and tumour RNA or DNA (Boczkowski, Nair et al. 1996; Celluzzi, Mayordomo et al. 1996; Condon, Watkins et al. 1996; Hsu, Benike et al. 1996; Nair, Snyder et al. 1997). Many researchers have reported an improved prognosis for patients with a wide range of cancers whose tumours are associated with a DC infiltrate (Lotze 1997).

Initial attempts at manipulating the immune system for the elimination of tumours were crude, poorly successful and hampered by intolerable side effects related mainly to the use of pharmacological rather than physiological doses of biologically-active molecules e.g. IL-2 and IFN-γ. The progressive development of increasingly successful immunotherapeutic strategies is a reflection of our increased understanding of tumour immunology specifically tumour antigenicity and immune activation.

The data presented in this thesis add to this already large and growing body of evidence of immune reactivity against tumours. In chapter 6, the ability to induce DTH responses to both JBS and CarB cells provided evidence of the antigenicity and immunogenicty of theses cells (Figs. 6.8 – 6.10). The consistently greater DTH response to JBS when compared to CarB ($p < 0.05$; Students t-test) implies that the latter is perhaps not as strongly antigenic. Crude evidence of immune containment is seen when we examine the growth curves for both tumour types in both immunocompromised athymic mice and immunocompetant Balb/c mice (Fig. 3.29). It can be seen that JBS tumours grow significantly more slowly in the Balb/c mouse than in the athymic mouse.

The difference between the growth curves for CarB tumours is not so marked suggesting again that perhaps it is a less antigenic tumour or at least that it is less immunogenic. That this growth confinement is immune-mediated is suggested by the changes seen in spleen weight and non-adherent spleen cell numbers with increasing tumour burden (Figs. 3.10 & 3.11). Despite the paucity of evidence of containment of CarB tumours there is a suggestion, though not statistically significant, of greater tumour-induced splenomegaly in CarB tumour-bearing mice.

Table 3.5 details the changes in non-adherent cell number, T-cell, B-cell, helper T-cell and cytotoxic T-cell with increasing tumour burden for both tumour types. In JBS tumour-bearing mice there is a general trend towards increased cell numbers in all groups with increasing tumour burden with a decline in $CD3^+$ and $CD4^+$ cell numbers in mice with the largest tumour burden. CarB tumour-bearing mice while demonstrating a similar progressive trend in overall non-adherent splenocyte cell and B-cell numbers does not have the same increase in $CD3^+$, -4^+ or -8^+ cell numbers until they reach the largest tumour burden category, a time when JBS numbers are beginning to decline. This difference is responsible for the more pronounced reversal of T:B cell ratio seen in CarB tumour-bearing mice (Fig. 3.14). Fig. 3.17 shows the remarkable stability of the CD4:CD8 ratio in JBS tumour-bearing mice in stark contrast to the almost 4-fold increase seen in CarB mice (due more to a greater loss of $CD8^+$ cells than $CD4^+$ cells).

$CD4^+$ T-cells have been shown to be necessary during the priming and effector stages of the anti-tumour immune response (Hung, Hayashi et al. 1998). Some researchers would suggest that $CD4^+$ T-cells are even more vital than $CD8^+$ T-cells as optimal induction of the latter requires the former (Baxevanis, Voutsas et al. 2000). This is supported by resistance to the development of tumours in a significant number of CD8 knockout mice but not in CD4 knockout mice (Toes, Ossendorp et al. 1999).

Changes in lymphocyte subsets have been reported in patients with malignant disease, have been shown to correlate with stage of disease and proposed as a marker of response to therapy. Kuroi *et al.* found that increasing tumour stage of Gastric cancer was associated with a decrease in $CD3^+$ and $CD4^+$ T-cells, mainly $CD4^+/CD45RA^+$-naïve T-cells, and a decrease in CD4:CD8 ratio (Kuroi, Sato et al. 1990; Male, Champion et al. 1992). This finding has been confirmed by other groups (Lee, Chang et al. 1994). These researchers found a correlation between the reduction in CD4:CD8 ratio and tumour size, invasion and lymph node metastasis but no relation to histological sub-type. A reduction in $CD4^+$ T-cell number and CD4:CD8 ratio has also been described in patients with invasive transitional cell carcinoma (TCC) of the bladder (Kaver, Pecht et al. 1992). This reduction was not seen in patients with superficial TCC of the bladder. Not all tumours have a reduction in $CD4^+$ T-cell numbers as demonstrated by Kaver *et al.* who reported a reduction in $CD8^+$ T-cell numbers and a consequent rise in CD4:CD8 ratio. Still others have reported a decrease in $CD4^+$ T-cells and a simultaneous increase in $CD8^+$ T-cells leading to a marked reduction in CD4:CD8 ratio (Zeng, Tang et al. 1995). They propose that determination of

lymphocyte subset numbers and in particular the CD4:CD8 ratio may be useful as a prognostic tool.

It is possible in humans to determine lymphocyte subset numbers in patients at the time of presentation and following therapy, but ascertaining the natural history particularly, establishing whether the alteration in lymphocyte populations is a cause or a consequence of the developing tumour is not as easy. This dilemma is best answered using carefully chosen animal models. Certainly similar tumour induced changes in T-cell populations, reduction in $CD4^+$ and an increase in $CD8^+$ T-cells, have been seen in other murine models (Walker, Yurochko et al. 1992). Others have demonstrated temporal changes in lymphocyte populations following tumour induction. (Ghosh, Komschlies et al. 1995). Using nuclear extracts from splenocyte preparations and from isolated $CD4^+$ splenocytes a transient increase in DNA-binding pattern corresponding to the IFN-γ core promoter region followed by a gradual disappearance as the tumour progressed was found. This corresponds well with the production IFN-γ seen in JBS tumour-bearing mice (Fig. 3.25). In the work presented here we have looked at the production of IFN-γ and at CD4 expression and found a good correlation in JBS tumour-bearing mice (Fig. 3.30). This suggests that the change in $CD4^+$ T-cell numbers is associated with an initial shift towards and then away from a Th1 pattern of cytokine secretion.

Both tumour types induce a significant and equivalent B-cell response (Figs 3.13 & 3.14). Tumour immunity though largely mediated by $CD8^+$ CTLs whose activation requires $CD4^+$ T-cell help. Experienced T-cells respond to antigen

presented by B-cells, whereas virgin T-cells are rendered tolerant (Fuchs and Matzinger 1992). It may be that B-cells may drive the $CD4^+$ T-cell response towards a Th2 response, which promotes the development of a humoral rather than a cellular T-cell response (Paul and Seder 1994; Sotomayor, Borello et al. 1996). Or it may be that the B-cell hyperplasia is associated with antibody production which by acting as 'blocking factors' in the host serum, which prevent the action of activated T-cells on neoplastic cells thereby aiding tumour growth (Hellstrom, Warner et al. 1973). Preliminary investigations to find anti-tumour antibodies using ADCC were unsuccessful.

Given its strength, further investigation into the apparent B-cell response to developing tumours is indeed warranted. This could take the form of utilising more sophisticated techniques such as flow cytometry to look at the expression of a wide variety of B-cell markers and to look at their activation status. More refined techniques such as those used in Serex methodology, could be used to look for the presence of tumour antigen recognised and responded to by these B-cells. The clinical significance of the B-cell response could be examined by establishing tumours in B-cell depleted/knockout mice.

The proliferative activity of lymphocytes in response to stimulation with various cytokines or mitogens (mitogenesis assays) has long been used as a general measure of immunocompetance. Two very differing patterns of response to both endogenous and exogenously stimulated proliferation were seen in response to progressive growth of JBS and CarB tumours (Figs. 3.22 – 3.24). The development and progression of JBS tumours is initially associated with an

increase in lymphocyte proliferation, both endogenous and stimulated, but with larger tumour burden begins to wane. CarB tumours on the other hand have a more blunted proliferative response to developing tumours and in particular the inability to enhance proliferation with ConA is highly suggestive of tumour-induced/associated functional anergy. Work by other researchers in our unit compared proliferative responses in lymphocytes from the draining nodes, reference nodes and peripheral blood lymphocytes (PBLs) of patients with oesophageal squamous carcinoma with those from patients with oesophageal adenocarcinoma (O'Sullivan, Corbett et al. 1996). They found that in the latter 24 of 26 patients demonstrated normal or enhanced lymphocyte proliferation in the draining nodes whereas 22 of 23 patients with squamous carcinoma had depressed lymphocyte proliferative and cytotoxic responses in the draining nodes relative to the reference nodes and PBLs.

Adler *et al.*, found similarly enhanced endogenous proliferation (as determined by [^{3}H] Thymidine incorporation) in the splenocytes of sarcoma-bearing mice when compared with controls (Adler, Takiguchi et al. 1971). There was variability in the extent of proliferation depending on the particular subtype of sarcoma the animal was afflicted with. All tumours were associated with a profound blunting of their splenocyte proliferative response to stimulation with PHA as compared with controls. This response appears to be similar to that observed in CarB tumour-bearing mice but in marked distinction to JBS tumour-bearing mice (Figs. 3.22 & 3.23). Similar depression of lymphocyte proliferation has been documented in humans with malignant disease. Miescher *et al.* examined the proliferative ability of TILs and PBLs from humans with cancers

of varying histologies – melanoma, breast, colon, oesophageal, lung, glioma and skin (Miescher, Whiteside et al. 1986). They found that in all cases (bar 1 of 22) the endogenous proliferative activity of TILs and PBLs was reduced compared with controls and in all but 2 there was little or no response of TILs to PHA, ConA or PMA when compared with autologous controls (PBLs). Interestingly the histology of the tumour did not influence the proliferative response. This conflicts with my data, which clearly shows a different pattern of response for the two distinct tumours (Fig. 3.23). Whilst one could argue that it is the proliferative properties of host lymphocytes at the time of diagnosis and initiation of treatment that is clinically important, there is clearly a need to establish the natural history of lymphocyte responsiveness with progressive tumour growth. The immunotherapeutic approach that is adopted will have to be tailored to whether the host lymphocytes are anergic or whether they are capable of responding to exogenous stimuli. Whereas Miescher found no difference in the lymphocyte proliferative ability in 22 different tumours patients I have clearly shown differing responses to two aetiologically and histologically distinct tumours and as such this result merits further investigation as it may partly explain some of the therapeutic failures of non-specific adoptive immunotherapy to date.

In the work presented here relatively little cytotoxic activity was detected (max. was 30% for splenocytes from 2-3gm JBS tumour-bearing mice against JBS cells; Fig. 3.27). Previous workers have endeavoured to ascertain the effect of tumour burden on cytotoxic activity (Loeffler, Smyth et al. 1992). They reported an increase in cytotoxic activity in splenocytes (after *in vitro* expansion) from

early tumour-bearing mice (tumours for < 21 days) as compared to naïve mice with a subsequent fall in cytotoxicity, to below that of naïve mice, in late tumour-bearing mice (tumours for >30 days). A similar trend is seen in the cytotoxicity demonstrated by splenocytes from JBS tumour-bearing mice (Fig. 3.28). Higher levels of cytotoxicity were seen against JBS cells. This again fits with the slightly weaker antigenicity of CarB cells. However the low levels of cytotoxicity seen do not allow for any statistical comparison between differing tumour burdens or between the responses to differing tumours.

Though not exhaustive, and largely confined to T-cell activities, the results presented herein document the changes that occur in splenocyte morphology, endogenous and stimulated proliferative ability and endogenous cytotoxicity in response to the development of two histologically and aetiologically distinct tumours. This work provides context in which to search for tumour-derived immunosuppressive factors and defines the nature of the immune response to be overcome by oral tolerance. This is, to the author's knowledge, the first detailed analysis of the immune response to progressive tumour burden, using accurate measurement of that tumour burden, in tumour-bearing animals and is also the first direct comparison of the immune responses to two, progressively growing, distinct tumours in the same animal model

Perhaps the most significant finding is the differing response profiles to JBS and CarB tumours. It would appear that the JBS cell is the more antigenic of the two weakly antigenic cell lines. It is difficult to believe that the differing trends in

splenocyte subsets and more importantly the apparent anergy seen in proliferative function of splenocytes from CarB tumour bearing mice are related to this weaker antigenicity. Rather it could be the product of tumour-derived factors though both tumours seem to elaborate the same factor *in vitro* (discussed later).

The comprehensiveness analysis of the T-cell response to developing tumours presented above would be enhanced by a variety of further detailed investigative approaches. Further simple cellular techniques such as *in vitro* expansion and activation of splenocytes from tumour-bearing mice in the presence of tumour antigen and antigen presenting cells prior to assessment of their cytotoxic function would yield valuable information about their cytotoxic function. The use of ELISA or cellular biological assays to look at the cytokine content of splenocyte culture supernatant would help to define the nature of the T-cell response. Ideally flow cytometric studies of permeabilised splenocytes from tumour bearing mice would permit surface marker analysis simultaneously with cytoplasmic cytokine markers enabling description of helper and cytotoxic T-cell subsets. Investigation of the B-cell response, touched on here, should yield important information especially regarding the marked B-cell response to CarB tumours. With these more comprehensively defined animal models, the next stage would be to test intervention strategies.

How these neoplastic growths can survive and grow progressively in the face of this specific and potentially tumouricidal immune response is the central paradox of tumour immunology. Tumour-induced or -produced soluble

immunosuppressive factors (ISFs) offer a plausible explanation. Whether differences in the production or nature of any putative ISF would explain the observed differences in response to JBS and CarB tumours was of immense interest. This was proved not to be the case. Both tumours, and less consistently their cell lines, were shown to produce, probably identical, potent ISFs. The production of ISF is limited to the two tumours and proportional to the amount of tissue used in its generation (Figs. 4.1 + 4.6). It is non-specific, having efficacy against all dividing cells with greater potency against more rapidly dividing cells (Fig. 4.18). Its effect is rapid in onset, initially cytostatic with potential for reversibility if removed early enough (Figs. 4.13 – 4.16). Ultrafiltration experiments suggest that it is <500D in size though it appears heavier, 6,400kD on the HPLC column (Figs. 4.8 – 4.10). It is heat stable with an apparent sensitivity to Thymidine Phosphorylase but not to proteolytic enzymes (Figs. 4.19 – 4.21). Putative ISFs that meet these criteria have been investigated but have not yielded favourable results. Clearly the differing immunological response profiles seen with JBS and CarB tumours cannot be explained by ISF production. Two postgraduate students in our unit are endeavouring to isolate and identify a human oesophageal squamous cancer (OC-2)-derived ISF that has very similar properties to the ISF I have described here. To avoid duplication of work it was felt that the prudent approach was to wait until they had established/defined the methodology necessary to identify their ISF and to then apply that methodology to stored JBS and CarB crude and fractionated supernatants.

It is tempting, but dangerous, to speculate as to the final nature of the, probably sole/common ISF. The data in the lab from people working of human oesophageal tumour-derived ISF seems to suggest that we are pursuing the same substance. It will be very exciting when the nature of the JBS and CarB ISF is defined, as we can then look for evidence of it *in vivo*. We will be able to look for it in the serum and urine of tumour-bearing mice and correlate the levels with tumour burden and with the lymphocyte function data previously described. It should even be possible to generate monoclonal antibodies to it (conjugated to a hapten if necessary) and look for it immunohistochemically in tumour sections (already stored). If the ISF proves to be a short peptide as S. Nolans work seems to be suggesting or a nucleotide analogue as my work and that of F. Kenny would suggest, it should also be possible to either isolate it or more probably to manufacture it for further *in vitro* analysis of its mechanism of action. Given its speed of onset it would be very interesting to examine its effects on lymphocytes using confocal microscopy. Once defined, methods of counteracting/neutralising it may be devised and tested in the aforementioned, better-defined animal models. The possibility that it is the same ISF as that being investigated in the human oesophageal squamous cancer cell line is particularly exciting as it would immediately translate all of the above work into applications in human malignant disease. One could look for it in blood/serum and body secretions, notably urine, faeces and saliva of patients with cancer and normal controls. It offers the tantalising prospect of a simple dip-stick test for the presence of malignant disease and, because its production is dependant on the amount of tumour, for monitoring response to therapy/recurrence. Given that tumours are already known to produce a wide array of immunosuppressive substances elimination or

neutralisation of the effects of this ISF *in vivo* may unmask the suppressive effect of another entity (again S.Nolan's work seems to be pointing to 2 similar sized synergistic ISF; personal communication).

When tumours of mixed antigenicity are induced in mice, both cell lines, even the strongly antigenic SV3T3 cell line, which is normally incapable of inducing a tumour in immunocompetant mice, are initially capable of growing (Figs. 5.4 – 5.6). However with progressive tumour growth the strongly antigenic cells are selectively eliminated leaving the weakly antigenic cells to flourish. This corresponds with the presence of an inflammatory infiltrate (Fig. 5.8). There is indirect evidence that the growth of the weakly antigenic cells is unimpeded by the presence of an inflammatory infiltrate and the disappearance of the strongly antigenic cells.

Definitive evidence for the elimination of SV3T3 cells rather than any down regulated expression of large T antigen would come from analysis of DNA preparations from mixed CarB/SV3T3 tumours. A gene search with selection of suitable primers has been undertaken and would form the basis of research project and thesis for another investigator. That work could be preceded by the application of stereological methodology to accurately define relative proportions of each cell line in the mixed tumours. Finally proof of an immunological basis for the elimination of the strongly antigenic cells would come from establishing mixed tumours in a SCID mouse model which could then be immunologically reconstructed, enabling early assessment of immune

infiltration perhaps even immunohistochemically co-localising the expression of large-T antigen and inflammatory cell markers on tumour sections.

In the penultimate chapter, the failure to induce a DTH response despite immunising with tumour cells in adjuvant provides *in vivo* evidence that feeding mice tumour antigens prior to immunisation with the same antigens inhibits the development of an immune response in an antigen specific manner (Figs. 6.8 + 6.9). Of greater clinical significance feeding mice tumour cells prior to the induction of subcutaneous tumours facilitates a statistically significant enhancement of tumour growth (Fig. 6.6). Despite the differences in immunological response profiles detailed in Chapter 3 feeding of both JBS and CarB tumours were capable of potently inhibiting the DTH responses but characteristically the effect of CarB feeding on growth of CarB tumours was not as marked as that of JBS feeds on JBS tumour growth. This again is probably reflective of the weaker antigenicity of CarB relative to JBS.

This is as yet a largely unexplored area of tumour immunology with only one previous report on a related subject. Thus the description and definition of this response is in itself significant. Its relevance to clinical oncology however is more elusive apart from the obvious conclusion that removing the primary lesion would be of benefit is reducing ongoing tolerance. Further work in this field would follow that done for tolerance to simple less complex antigens and attempt to define the nature of the induced tolerising cell and the mechanism of tolerance induction as breaking this would be of clinical significance. Removing the primary tumour entirely is not always feasible in patients with foregut

malignancies however if any potential oral tolerance could be overcome it may be possible to put the foregut tumours on a similar survival curve as patients with distal GI tract malignancy. This would represent a significant improvement in the survival of patients with gastric and oesophageal malignancies.

The mixed tumour data provides very depressing evidence for people intent on immunotherapeutic elimination of malignant disease, as it implies that the antigenic heterogeneity observed in most tumours may limit therapeutic efficacy of antibody, T-cell and LAK cell therapies. Remember that 99.9% eradication of a $1cm^3$ tumour still leaves 10^6 cells to proliferate, thus providing a large base for the further generation of biological diversity. Though the implications of the mixed tumour data are largely disheartening, every cloud has a silver lining. The data also show that immune cells are capable of effectively reaching all areas of a vascularised tumour.

The remarkable success that has been achieved to-date in terms of improved survival of patients with malignant disease has been as much due to serendipity as to design. Whilst it is well recognised that most of the progress in Science/Medicine occurs through sudden and fortuitous leaps e.g. the discovery of penicillin, it is through painstaking often-tedious work that the mechanisms are elucidated, the effects refined and augmented. Oncology has had its' share of the former such as deLisle's pus, the discovery of IL-2, the myelosuppressive effect of Mustard Gas seen in World War I which have each of them spawned novel approaches to tumour therapy. Our greater understanding of the fields of tumour cell biology and immunology are leading to more directed and specific

drug and immune therapies. All of the work presented in this thesis has an impact on potential immunotherapeutic strategies.

In order to win the war that Richard Nixon declared on cancer we must first know our enemy (Sporn 1996). By defining the immune response to developing and progressive tumours we are able to identify those cellular elements that have failed to do their job and also established whether that loss of function is permanent or reversible. By better understanding the nature of the malignant cells we can identify strengths to be overcome (such as the elaboration of ISFs) and weaknesses to exploit (novel antigen expression, rapid cell turnover). The development of tumour vaccination demonstrates exquisitely how all of this knowledge interrelates and can be used successfully. Unlike vaccination against infectious diseases cancer vaccines are not used to induce prophylactic immunity, though there is no reason why in the long-term we will not be vaccinating against the more common tumour antigens such as p53, certain HSV epitopes or a newly discovered and seemingly universal tumour antigen, Survivin, which is expressed by most human neoplasms but not in normal differentiated tissues (Andersen and Thor 2002). Survivin makes a particularly good choice as its down-regulation or loss of expression severely inhibits the growth potential of the tumour cell and so too the emergence of antigen-loss variants. Rather cancer vaccines are used to enhance the appropriate cellular or humoral immune responses. It seems unlikely, however, that vaccines will be able to restore immunocompetance to the host without first overcoming tumour-induced immunosuppression. Hence the need to identify tumour-derived ISFs as these can then be incorporated into the vaccination strategy. This has been

demonstrated very successfully by the use of polyvalent vaccines for melanoma of the kind described by Donald Morton's group (Morton and Ravindranath 1996). Vaccination with their melanoma cell vaccine (MCV) as well as immunising against six different melanoma antigens also immunised against gangliosides, which are known to be shed by melanoma cells and to be immunosuppressive.

With immunosuppression eliminated the vaccine must next stimulate CTLs. When presenting an antigen for vaccination knowledge of antigen processing and lymphocyte activation is invaluable. When compared with native P815 Mastocytoma cells, plasma membrane vesicles derived from P815 cells and engrafted with co-stimulatory molecules B7.1 and CD40 and the immunostimulatory cytokine IL-2 induced significantly more protection against subsequent challenges with P815 cells (van Broekhoven and Altin 2002). The antigen can be presented as a pure soluble antigen such as purified ganglioside GM2 or recombinant MAGE-1 protein (Livingston 1993). Given the emergence of antigen-loss variants immunising with a polyvalent vaccine, such as HLA-depleted shed tumour surface antigens, makes immunological sense (Bystryn, Oratz et al. 1992). Other sources of polyvalent vaccines that have been used include cell membrane vesicles described above, tumour cell lysates and increasingly whole allogeneic tumour cells such as Morton's MCV or the cells used in chapter 6 to sensitise to DTH responses. It appears that whole cell vaccines are better able to sensitise the immune response than univalent or polyvalent soluble antigens (Morton and Ravindranath 1996).

Many techniques have been used to augment the immunogenicity of these vaccines, such as the generation of tumour cell lysates from vaccinia or Newcastle virus-infected cells (Savage, Rossen et al. 1986; Berthier-Vergnes, Portoukalian et al. 1994). Others have used immunostimulant mixtures of oil and bacterial cell wall products not unlike the Freunds adjuvant that was used in chapter 6. Melacine (Ribi Immunochemical Research Inc., Montana) is a commercially available vaccine composed of melanoma cell lysate combined with DETOX – a mixture of non-toxic lipidA, derived from *Salmonella minnesota*, cell-wall skeletons of *Mycobacterium phlei*, squalene oil and Tween-80 – that has been used in clinical trials (Elliott, McLeod et al. 1992). Immunogenicity has also been enhanced by binding the antigen source to a hapten such as Dinitrophenol (DNP) (Berd, Maguire Jr. et al. 1990).

One of the most interesting advances in tumour vaccination in recent years has arisen out of an awareness of antigen presentation. Dendritic cells present antigen to the host immune system in an appropriate form together with the necessary co-stimulators and minimal toxicity. The most common arrangement is to use dendritic cells that have been pulsed with tumour cell lysate. These vaccines have been shown to be safe, non-toxic and capable of inducing tumour-cell specific $CD8^+$ CTLs (Schnurr, Galambos et al. 2001; Chang, Redman et al. 2002; Santin, Bellone et al. 2002). Other methods of inducing dendritic cell expression of tumour antigens have been explored including transfection with cDNA that encodes the tumour antigen (Shibagaki and Udey 2002). This approach is hampered by the need to know the antigen to which we are immunising and also is a univalent vaccine that would permit the emergence of

immune escape variants. To overcome this dendritic cells have been transfected with total tumour mRNA (Nair, Morse et al. 2002). Both of these approaches can be hindered by difficulty with the transfection process. To avoid this others are developing vaccines based on electrofusion of tumour cells to dendritic cells (Orentas, Schauer et al. 2001).

The final therapeutic approach to the management of malignant disease will be a combination of all therapies. Immunotherapy alone is unlikely to be successful due to the continuing elaboration of tumour-derived ISFs and the persistent tumour burden acting as a sump for the immunotherapeutic agents (Eaton, Perry et al. 2002). Removing he tumour will also provide allogeneic tumour cells for use in a vaccine. Additionally chemotherapeutic agents have been successfully combined with immunotherapeutic approaches without the latter being impaired by the former (Baars, Claessen et al. 2002)

We have slowly come to the realisation that cancer is not a single disease entity but rather a disease process. If this thesis proves anything it is that for every question posed more questions than answers are generated. Our attempts at reductionist understanding and use of simplistic concepts were undermined by the complexity of the disease. However this is no longer the case and with solid evidence of immune recognition of tumour cells behind us it is time to press on with investigation of increasingly more complex models and systems. Some of this work can proceed using cell culture techniques or computer modelling but clearly more complex models require the use of *in vivo* systems. This is not possible in humans as the induction of cancer is unethical and undisturbed

observation of the natural history of pre-malignant lesions or developing tumours unlikely. Prior to their application in humans most investigators would wish to test any novel interventional strategy in a relevant animal model. The critical aspect here is to ensure that as far as possible the model used is relevant and appropriate (Hewitt 1982; Hewitt 1983). Whilst Hewitt would have us believe that the only relevant animal tumour model is to use spontaneously arising animal tumours, not every one agrees (Herberman 1983; Herberman 1983). Though not spontaneously arising both of the tumours employed in this study have the potential to be good models as they are weakly antigenic and weakly immunogenic, properties they share with many human tumours. All of the work presented in this thesis is of relevance to the field of tumour immunotherpay. From the documentation of different immunological responses to two different tumours we can conclude that the immune response to the tumours we are hoping to treat must be known if we are to enhance/augment it to overcome those tumours. The effects of tumour-derived ISFs have to be overcome if Immunotherapy is to succeed. The importance of polyvalent vaccination to avoid the emergence of antigen-loss variants is demonstrated by the mixted tumour data. The work on oral tolerance demonstrates that we can approach the systemic immune response to tumours via other routes and should encourage further cross-polination with mucosal immunologists. Despite the current climate of hostility to animal research I believe further investment in studies of these tumours is warranted.

'There is an awe in this view of neoplasia, with its unfathomable but predictable complexity, having been originally formed as a simple, focal cellular proliferation in response to singularly disparate stimuli. From such a simple beginning there evolves a progressively disorganized aberrant life form in a parent life form that maintains its order under the fixed laws of organismal existence. The progressive forms of disorganised and aberrant life are similar in quite dissimilar species of organized life and, therefore aberrant life must be governed by biological principles that are similar in the disparate species.'

'Within the biological principles governing the coming into being, the passing, and the evolution of the serial forms of neoplastic life, lie the clues and guides to the altering of and the controlling of aberrant life: the directions to the altering and controlling of cancer.' (Clark Jr. 1995).

"A man's reach should exceed his grasp, or what's a heaven for."

Browning.

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Appendix : Stock Solutions

DAPI

(Used to stain DNA as in the detection of Mycoplasma contamination. Hoechst 33258 is a suitable alternative.)

Dissolve 5mg of 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI, Sigma) in 50mls of phosphate buffered saline to make a stock solution. Dilute this 1/100 with methanol to make the working solution.

ConA

A stock solution, with a concentration of 1mg/ml, of Concavalin A in PBS is prepared. To generate the working solution 2mls of foetal calf serum (FCS) is added to 7.95 mls of SFM together with 50μls of Con A stock solution to yield a final solution of 20% FCS with 5μgm/ml of Con A. When added to the microtiter plate wells the final solution is composed of 10% FCS and 2.5μgm.ml of ConA. This amount of Con A generates sufficient lymphocyte stimulation.

IL-2

Interleukin-2 (Sigma, No. I-4517) was reconstituted with sterile distilled deionised water as per the manufacturer's instructions, to yiled a stock solution with 26,700 units of activity per ml. To produce the working solution 2mls of foetal calf serum (FCS) is added to 7.25 mls of SFM together with 750μls of rIL-2 to yield a final solution of 20% FCS with 2000units/ml of Il-2. When added to the microtiter plate wells the final solution is composed of 10% FCS and 1000units/ml of IL-2. For preliminary experiments this amount of IL-2 generates sufficient lymphocyte stimulation

7.5% TCA

7.5gms of Trichloroacetic acid are reconstituted in distilled deionised water and cooled at –20°C until used.

Tris-Buffer Saline (TBS)

To produce 1l of TBS, 6.06gm Trizma.HCl, 1.39gm Trizma.Base, 8.75gm NaCl and 0.375gm KCl are placed in a glass beaker and 950mls of water added. When

the solutes have dissolved the pH is adjusted using either HCl or NaOH to a ph = 8.2. The final volume is increased to 1000mls with distilled water.

APES coated slides

A 2% (2gm/dl) solution of 2-aminopropyltriethoxysilane (APES) in absolute alcohol is prepared. Slides are immersed in this solution for 10 seconds, withdrawn and excess APES removed by gentle shaking. This is repeated in two consecutive baths of absolute alcohol for 10 secs each and a final 10 sec wash with distilled water for secs. They are allowed to dry overnight at 37°C.

APAAP Substrate

In a dry glass tube 2mg Naphtol AS-MX phosphate (Sigma, N4875)is dissolved in 0.2ml Dimethylformamide (DMF). This is diluted to 10mls with 9.8ml 0.1M Tris Buffer (pH 8.2) and 10ml 1M Levamisol added to block endogenous alkaline phosphatase activity.

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Thank's Car.